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Volume 16 (2); June 2026

Research Paper

Anti-inflammatory and Subacute Safety Effects of Pomegranate (*Punica granatum*) Rind Extract in Recurrent Carrageenan-Induced Inflammation in BALB/c Mice

Jonas TNK, Waihenya RW, and Kariuki DW.

World Vet. J. 16(2): 312-323, 2026; pii:S232245682600030-16

DOI: https://dx.doi.org/10.54203/scil.2026.wvj30



ABSTRACT: Pomegranate (*Punica granatum*) contains a high level of polyphenols and has anti-inflammatory properties. However, there is limited available data on the safety and effectiveness of pomegranate use during prolonged inflammatory conditions. The present study aimed to evaluate the anti-inflammatory properties and subacute toxicity profile of a 70% ethanolic extract of *Punica granatum* (*P. granatum*) rind in carrageenan-induced mice. A total of 30 male mice, aged five weeks and weighing 22-23 grams, were randomly assigned to five groups of six mice each. The study included a negative control group that received normal saline, a positive control group that received meloxicam at 2 mg/kg, and three treatment groups that received *P. granatum* rind extract at doses of 50, 100, and 200 mg/kg orally over 28 days. Inflammation was induced once weekly in all experimental groups by 1% carrageenan injection into the right hind paw, after which the edema was measured with a digital vernier caliper. The toxicity of the pomegranate extract was assessed using hematological and biochemical profiles of hepatic and renal parameters. Tissue samples from the heart, spleen, kidney, lungs, and liver were collected on day 29. To assess the inflammation status, concentrations of tumor necrosis factor-alpha (TNF-α) and IL-10 were quantified using enzyme-linked immunosorbent assay. The *P. granatum* rind extract significantly reduced paw edema without mortality or observable toxicity, compared to the negative control group. Mice maintained their normal feeding habits, and body weight increased normally across all groups. Hematological parameters, hepatic, and renal markers were within the normal physiological range across all groups. Histopathological analysis revealed no abnormalities in extract-treated groups. Extract-treated groups demonstrated a dose-dependent elevation of IL-10, peaking at 200 mg/kg, and a reduction in TNF-α level across all doses, with the highest suppression observed at 100 mg/kg compared to the negative control. Daily oral administration of *P. granatum* rind extract for 28 days exhibited dose-dependent anti-inflammatory effects, as evidenced by reduced paw edema and modulation of cytokines. The absence of systemic toxicity across all tested doses suggested that *P. granatum* rind extract can be a safe therapeutic agent for treating inflammatory conditions.

Keywords: Anti-inflammatory, Biochemical marker, Carrageenan, Cytokine, Hematological parameter, Inflammation, *Punica granatum*

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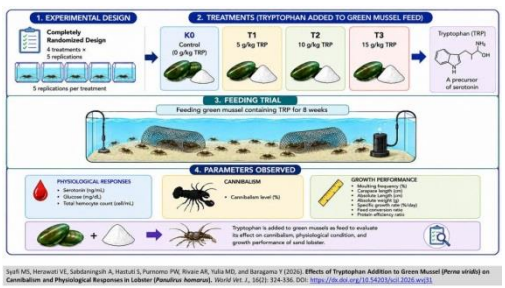
Research Paper

Effects of Tryptophan Addition to Green Mussel (*Perna viridis*) on Cannibalism and Physiological Responses in Lobster (*Panulirus homarus*)

Syafi MS, Herawati VE, Sabdaningsih A, Hastuti S, Purnomo PW, Rivaie AR, Yulia MD, and Baragama Y.

World Vet. J. 16(2): 324-336, 2026;
pii:S232245682600031-16

DOI: https://dx.doi.org/10.54203/scil.2026.wvj31



ABSTRACT: The increasing demand for sand lobsters requires higher aquaculture productivity. However, sand lobster production is still constrained by high mortality due to cannibalism, a behavior in which individuals attack and consume conspecifics. One approach to suppress cannibalism is through supplementation of the amino acid tryptophan as a precursor to serotonin, which plays a role in regulating stress and aggressiveness. The

present study aimed to determine the effectiveness and optimal formulation of tryptophan in green mussel feed on cannibalism, survival, and growth rate in sand lobsters. The study used a completely randomized design with four treatments and five replications. The treatments consisted of adding tryptophan to green mussels used as feed, including a control without tryptophan (K0), and supplementation at doses of 5 g/kg (T1), 10 g/kg (T2), and 15 g/kg (T3). Observed parameters included physiological responses, cannibalism levels, and growth. The results revealed that T3 treatment was most effective in increasing serotonin levels (89.45 ± 3.069 ng/mL) and survival rate (55.29 ± 5.26 %), and reducing cannibalism (7.06 ± 2.63 %), which caused lower glucose (14.19 ± 3.206 mg/dL) and total hemocyte count (81.00 ± 4.243 cell/mL) levels. However, the highest dose (T3; 15 g/kg) was less effective in enhancing growth performance, indicating that this level of supplementation was not optimal compared to the lower doses. In contrast, T1 (5 g/kg) resulted in relatively higher growth performance compared to the other treatments, as indicated by moulting frequency (1.40 ± 0.23%), carapace length (1.25 ± 0.15 cm), length increase (1.70 ± 0.12 cm), absolute weight (1.14 ± 0.35 g), specific growth rate (3.13 ± 0.46 %/day), feed conversion ratio (5.04 ± 0.93), and protein efficiency ratio (1.42 ± 0.18). However, in T1 (5 g/kg), serotonin (70.66 ± 4.165 ng/mL), survival rate (42.35 ± 4.92%), cannibalism (38.82 ± 3.22%), glucose (16.26 ± 3.352 mg/dL), and total hemocyte count (110.50 ± 9.192 cell/mL) were not as optimal as those observed in T3. Tryptophan supplementation revealed potential to enhance physiological condition, survival, and growth under the experimental conditions.
Keywords: Cannibalism, Growth, Lobster, Physiological, Tryptophan

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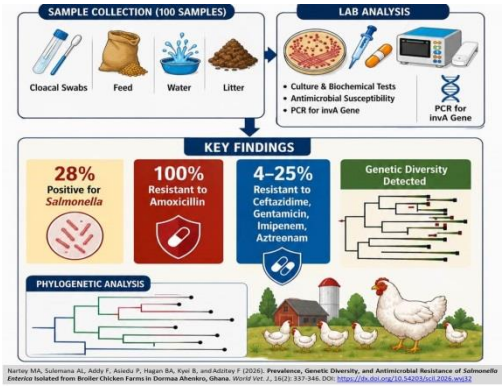
Research Paper

Prevalence, Genetic Diversity, and Antimicrobial Resistance of *Salmonella Enterica* Isolated from Broiler Chicken Farms in Dormaa Ahenkro, Ghana

Nartey MA, Sulemana AL, Addy F, Asiedu P, Hagan BA, Kyei B, and Adzitey F.

World Vet. J. 16(2): 337-346, 2026; pii:S232245682600032-16

DOI: https://dx.doi.org/10.54203/scil.2026.wvj32

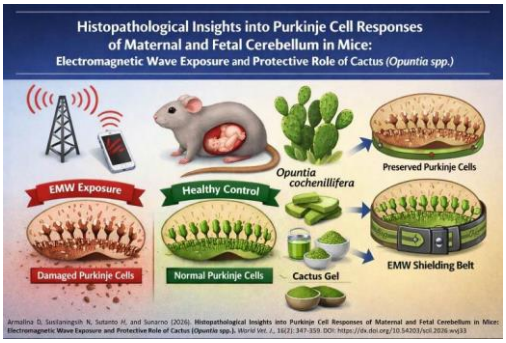


ABSTRACT: *Salmonella enterica* (*S. enterica*) remains one of the most important bacterial agents linked to foodborne illness, particularly in regions with expanding poultry production. The present study aimed to investigate the occurrence, antimicrobial resistance, and genetic characteristics of *S. enterica* isolated from broiler chicken farms in Dormaa Ahenkro, Ghana. One hundred samples (cloacal swabs, feed, water, and litter) were collected from five farms and examined through culture, biochemical tests, antimicrobial susceptibility assays, and molecular confirmation of the *invasion gene A* (*invA*). Twenty-eight isolates (28%) were confirmed as *Salmonella*, all of which exhibited resistance to amoxicillin. Resistance to the antibiotics Ceftazidime, Gentamicin, Imipenem, and Aztreonam tested ranged from 4-25%. Nineteen isolates amplified the *invA* gene, and sixteen out of 19 submitted samples were successfully sequenced and confirmed as *S. enterica* with 93.85-99.80% identity. Phylogenetic and haplotype analyses demonstrated substantial genetic diversity, suggesting localized evolutionary divergence. The present findings highlight the presence of multidrug-resistant *Salmonella* within broiler production systems, emphasizing the need for improved biosecurity and responsible antimicrobial use to safeguard public health. Broiler chicken farms serve as an essential food source in many Ghanaian communities, yet they can harbour bacteria that pose risks to consumers. It was observed that *Salmonella* contamination in birds and their surroundings in Dormaa Ahenkro. Notably, *Salmonella* strains seen in these studies were also resistant to commonly used antibiotics, underscoring the need for stronger hygiene practices, prudent antibiotic use, and consistent monitoring to limit the spread of drug-resistant strains from farms to the wider population. Improving control measures will enhance food safety and support the sustainability of poultry production in Ghana.
Keywords: Antimicrobial resistance, Broiler chicken, Molecular characterization, *Salmonella*, One Health

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Research Paper

Histopathological Insights into Purkinje Cell Responses of Maternal and Fetal Cerebellum in Mice: Electromagnetic Wave Exposure and Protective Role of Cactus (*Opuntia* spp.)



Armalina D, Susilaningsih N, Sutanto H, and Sunarno.

World Vet. J. 16(2): 347-359, 2026; pii:S232245682600033-16

DOI: https://dx.doi.org/10.54203/scil.2026.wvj33

ABSTRACT: The rapid expansion of wireless technologies has significantly increased human exposure to electromagnetic fields (EMFs), raising concerns about potential effects on reproductive and developmental health. *Opuntia cochenillifera* (*O. cochenillifera*), a cactus rich in mucilage and mineral oxides, has potential as a bio-based material for reducing the intensity of electromagnetic waves (EMWs) through dielectric and magnetic interactions. The present study aimed to evaluate the effects of EMW exposure on Purkinje cell morphology in maternal and fetal cerebellum and to assess the protective potential of distinct *O. cochenillifera* formulations. A total of 42 pregnant BALB/c mice were randomly divided into six groups, including healthy mice as the positive control group, EMW-exposed mice as the negative control group, and four treatment groups. The treatment groups were subjected to EMW exposure alongside the administration of either fresh cactus, dried cactus gel, or powdered cactus (3 g each). The EMW exposure was administered at a specific absorption rate of 1.74 W/kg throughout gestation. Cerebellar tissues were collected on day 20 for histological analysis and characterized by X-ray fluorescence and Fourier-transform infrared spectroscopy. Histological findings demonstrated that EMW exposure disrupted Purkinje cell alignment and decreased Purkinje cell counts in the negative control group. In contrast, all treatment groups demonstrated preserved cellular structure and morphology comparable to that of the healthy mice. Quantitative analysis confirmed significantly higher cell counts in treated groups than in the negative control group. The current findings indicated that EMW exposure adversely affected cerebellar development, while *O. cochenillifera* exhibited protective effects, supporting its potential as a natural EMF-attenuating material.
Keywords: Cactus, Cerebellum, Electromagnetic wave, Histopathology, Purkinje cell

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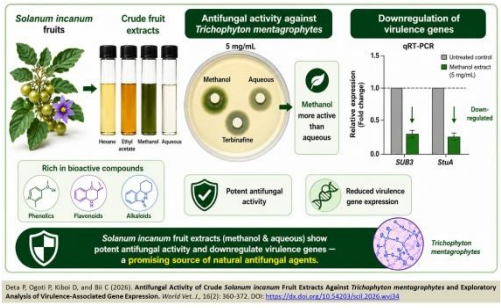
Research Paper

Antifungal Activity of Crude Solanum incanum Fruit Extracts Against *Trichophyton mentagrophytes* and Exploratory Analysis of Virulence-Associated Gene Expression

Deta P, Ogoti P, Kiboi D, and Bii C.

World Vet. J. 16(2): 360-372, 2026; pii:S232245682600034-16

DOI: https://dx.doi.org/10.54203/scil.2026.wvj34



ABSTRACT: Dermatophyte infections in humans and animals are a significant public health concern, and increasing resistance to antifungal medicines highlights the need to explore alternative therapeutic agents. The present study aimed to preliminarily assess the *in vitro* antifungal activity of crude extracts from *Solanum incanum* (*S. incanum*) fruit against *Trichophyton mentagrophytes (T. mentagrophytes)*. *Solanum incanum* fruits were extracted with hexane, ethyl acetate, methanol, and water. The levels of phenolic, flavonoid, and alkaloid compounds were measured, and antifungal activity was assessed using disc diffusion, minimum inhibitory concentration (MIC), and minimum fungicidal concentration (MFC) assays. Methanol and aqueous extracts exhibited the highest phenolic contents (~19 mg Gallic Acid Equivalents/g extract) and demonstrated significant antifungal activity, producing inhibition zones of 14.46 mm and 8.57 mm, respectively, at a concentration of 5 mg/mL. However, terbinafine demonstrated marginally greater inhibitory activity (15.02 mm) at a lower concentration of 0.1 mg/mL. The present findings indicated that using methanol and aqueous extracts of *S. incanum* fruit was associated with lower transcript levels of the targeted virulence-related genes (*SUB3* and *StuA*). The current findings provided preliminary evidence regarding the antifungal activity of *S. incanum* fruit extract against *T. mentagrophytes* at a concentration of 5 mg/mL, indicating its potential as an antifungal agent.
Keywords: Antifungal activity, Bioactive compound, Dermatophyte, Gene expression, Medicinal plant

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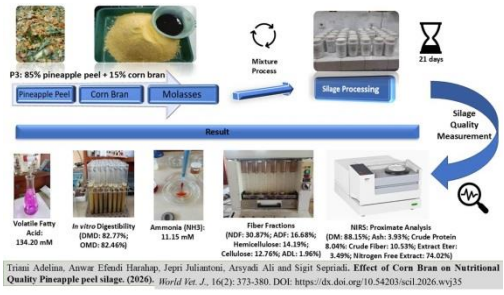
Research Paper

Effects of Corn Bran on the Nutritional Quality of Pineapple Peel Silage

Adelina T, Harahap AE, Juliantoni J, Ali A, and Sepriadi S.

World Vet. J. 16(2): 373-380, 2026; pii:S232245682600035-16

DOI: https://dx.doi.org/10.54203/scil.2026.wvj35



ABSTRACT: Combining pineapple peel waste with a specific level of corn bran produces high-quality silage that meets the nutritional needs of ruminants. The present study aimed to evaluate the effectiveness of incorporating corn bran in improving the physical properties and nutritional quality of pineapple peel silage for ruminant feeding in tropical regions. The silage treatment was conducted using a completely randomized design with four treatment groups and five replications. The experimental diets consisted of four levels of pineapple peel and corn bran, including 100% of pineapple

peel with no bran (P0), 95% of pineapple peel with 5% of bran (P1), 90% of pineapple peel with 10% of bran (P2), and 85% of pineapple peel with 15% of bran (P3). Each treatment group received 5% of molasses as an additive. Silage quality was analyzed using near-infrared spectroscopy. To evaluate rumen fermentation and digestibility, the *in vitro* Conway method was utilized, employing rumen fluid obtained from fistulated Holstein-Friesian bulls. Pineapple peel silage containing different corn starches exhibited a higher pH than the control group. Group P3 significantly produced higher crude protein (CP) and nitrogen-free extract (NFE) levels than the other treatment groups. Increasing the amount of corn bran in group P3 increased ammonia (NH3) production by 11.15 mM and total volatile fatty acids (VFA) by 134.20 mM compared to the control group. Group P3 exhibited the highest digestibility coefficients, with dry matter and organic matter values reaching 82.77% and 82.46%, respectively. The feed mixture comprising 85% pineapple peel and 15% corn bran is recommended for ruminants in tropical regions due to its superior overall nutritional quality. This feed mixture elevated CP, NFE, NH3 production, total VFA, dry matter, and organic matter.
Keywords: Corn bran, Digestibility, Rumen, Tropical region

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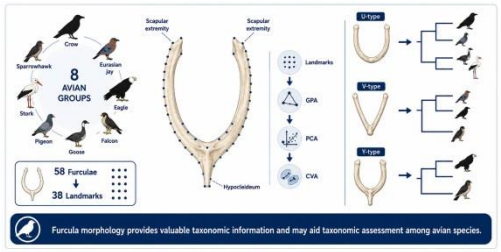
Research Paper

Shape Variations of Furcula in Different Avian Species

Karaavcı FA, Demiraslan Y, and Genç B.

World Vet. J. 16(2): 381-390, 2026; pii:S232245682600036-16

DOI: https://dx.doi.org/10.54203/scil.2026.wvj36



ABSTRACT: The furcula is a distinctive bone in avian species formed by the fusion of the left and right clavicles into a single structure. By acting as a spring during flight, the furcula adjusts the distance between the wing and the shoulder joint, contributing significantly to avian flight. The present study aimed to analyze furcular shapes across several bird species and investigate how these differences influence avian taxonomy. A total of 58 furculae collected from wild adult birds found dead or that died during treatment between 2023 and 2024 were examined. Samples consisted of seven crows, eight Eurasian jays, two eagles, one falcon, 19 pigeons, two storks, 10 sparrowhawks, and nine geese. Furculae were dissected and morphologically examined for overall shape, hypocleideum morphology, and the presence of pneumatic foramina. Morphological examination revealed that the furcula was V-shaped in the crow, Eurasian jay, goose, and pigeon, U-shaped in the eagle, falcon, and sparrowhawk, and Y-shaped in the stork. Geometric morphometric analysis was performed with 38 landmarks. Geometric morphometric analysis revealed that shape variation was primarily concentrated at the scapular ends of the furcula and secondarily in the hypocleideum region. Crow and Eurasian jay samples exhibited relatively similar shape patterns, whereas significant differences were detected among species groups in the canonical variate analysis. The current findings demonstrated species-specific variation in furcular morphology and provided valuable information for avian taxonomy and the functional interpretation of flight-related skeletal adaptations.
Keywords: Avian, Furcula, Geometric morphometry, Morphology, Shape

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Research Paper

Effects of Javanese Ginseng (*Talinum paniculatum*) Leaf Decoction on Immunohistochemical Expression and Superoxide Dismutase Levels in Diabetic Rats

Suarsana IN, Kardena IM, Suriani NL, Artika IM, and Swastini DA.

World Vet. J. 16(2): 391-401, 2026;
pii:S232245682600037-16

DOI: https://dx.doi.org/10.54203/scil.2026.wvj37



ABSTRACT: Java Ginseng has long been used by Indonesian communities as a medicinal plant and is traditionally consumed as an alternative therapy, even in managing Diabetes Mellitus (DM). The present study aimed to evaluate the effects of the decoction of Javanese ginseng leaf simplicia (DJGLS) on blood glucose levels, serum superoxide dismutase (SOD), the levels and expression intensity of SOD, and pancreatic histopathology in DM rats. Twenty adult male rats were randomly assigned to four groups. The normal control group (GJ-0, n = 5), the DM group (GJ-1, n = 5) received a placebo, and the DM groups (GJ-2, n = 5 and GJ-3, n = 5) were administered a DJGLS (100 g/100 mL, w/v powder simplicia), at volume of 0.5 mL and 1 mL/100 g body weight, respectively for 21 consecutive days. Blood glucose levels were measured using a glucometer. Serum and pancreatic SOD levels were determined using an ELISA kit. Pancreatic histopathology was evaluated using hematoxylin and eosin staining, while pancreatic SOD expression was assessed by immunohistochemical staining. In addition, phytochemical screening was performed on the Javanese ginseng leaf simplicia powder. The present study demonstrated that the phytochemical

analysis of Java Ginseng leaf simplicia powder revealed the presence of alkaloids, flavonoids, saponins, tannins, and steroids. DJGLS treatment reduced blood glucose levels and serum SOD levels in diabetic rats, with a significant reduction observed in the GJ-3 group compared with the GJ-1. Administration of DJGLS in the GJ-2 and GJ-3 groups significantly increased pancreatic SOD levels compared with the GJ-1. The DJGLS treatment in the GJ-2 and GJ-3 groups demonstrated restorative effects, as evidenced by the recovery of the Islets of Langerhans area and increased intensity of pancreatic SOD expression compared with the GJ-1. In conclusion, DJGLS treatment was able to reduce blood glucose levels and serum SOD levels, although these values had not fully returned to normal conditions, and it increased pancreatic SOD levels. The DJGLS indicated potential for promoting recovery of the pancreatic Islets of Langerhans area.
Keywords: Decoction, Diabetes, Immunohistochemistry, Javanese ginseng, Superoxide dismutase

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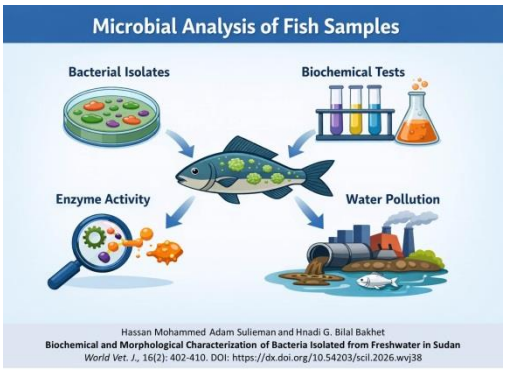
Research Paper

Biochemical and Morphological Characterization of Bacteria Isolated from Freshwater in Sudan

Sulieman HMA and Bakhet HGAB.

World Vet. J. 16(2): 402-410, 2026; pii:S232245682600038-16

DOI: https://dx.doi.org/10.54203/scil.2026.wvj38

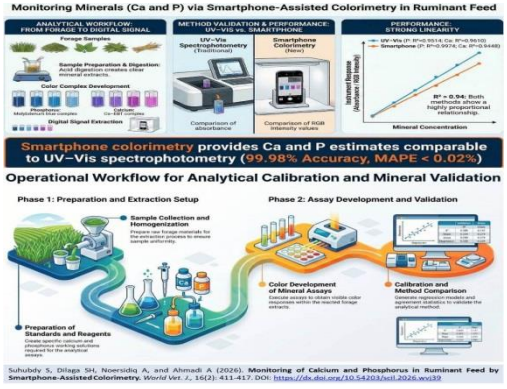


ABSTRACT: The microbial quality of freshwater fish is a crucial indicator of both aquatic environmental health and food safety. The present study aimed to isolate and characterize bacteria from three common freshwater fish species, including *Oreochromis niloticus*, *Clarias gariepinus*, and *Synodontis alberti*, collected from three sites in Khartoum, Sudan. Six fish of each species were collected from each sampling location where the species were present. Total viable bacterial counts in fish gill and intestinal tissues obtained from Green Belt Sewage, Jebel Aulia fish landing, and Al Mourda fish market ranged from 1.2×10^4 to 3.9×10^5 CFU/g, with higher bacterial loads generally observed in intestinal tissues compared to gills. The highest bacterial load (3.9×10^5 CFU/g) was recorded in the intestine of *Clarias* species collected from Green Belt Sewage. The current results demonstrated a predominance of Gram-negative, rod-shaped bacteria in gill and intestinal tissue samples across all sampling sites. All isolates were catalase-positive and capable of fermenting glucose, indicating facultative anaerobic metabolism. Oxidase activity of bacterial isolates differed by location; fish samples from Al Murda Fish Market had the highest number of oxidase-positive isolates, suggesting the potential presence of *Aeromonas* and *Pseudomonas* species. Urease activity was predominantly observed in isolates of fish samples collected from Al Murda and Jebel Aulia, suggesting a greater risk of fish spoilage or pathogenicity. Notably, isolates from the Green Belt Sewage fish samples comprised Gram-positive coccus, potentially identified as *Staphylococcus* spp., underscoring the likelihood of anthropogenic contamination. The present results indicated that fish obtained from all sampling locations generally exhibited similar microbial communities. However, the differences in enzyme activity across fish from all sites likely reflected variations in environmental factors, sanitation practices, and potential public health risks.
Keywords: Bacterial isolate, Biochemical characterization, Enzyme activity, Fish, Microbial count

[Full text-PDF] [Crossref Metadata] [Scopus] [Export from ePrint]

Research Paper

Monitoring of Calcium and Phosphorus in Ruminant Feed by Smartphone-Assisted Colorimetry



Suhubdy S, Dilaga SH, Noersidiq A, and Ahmadi A.

World Vet. J. 16(2): 411-417, 2026;
pii:S232245682600039-16

DOI: https://dx.doi.org/10.54203/scil.2026.wvj39

ABSTRACT: Routine laboratory analysis for ruminant feed minerals is often unavailable in a field setting; therefore, rapid mineral monitoring is necessary. The present study aimed to evaluate a smartphone-assisted colorimetry method for estimating calcium and phosphorus in forage extracts and compare its performance with UV-Visible spectrophotometry. Standard solutions ranging from 1 to 5 ppm were prepared for smartphone-assisted colorimetry and UV-Visible spectrophotometry, and reagent-treated extracts from four forage species, including *Pennisetum purpureum* cv. Mott, *Pennisetum purpupoides*, *Leucaena leucocephala*, and *Sesbania grandiflora* were measured by a Genesys 10S spectrophotometer and by smartphone imaging under controlled lighting. Linear regression was used to develop calibration models, and method agreement was assessed using Pearson's r and mean absolute percentage error. Both methods indicated strong linearity for calcium and phosphorus. For the smartphone

method, the coefficient of determination ranged from 0.9448 to 0.9974, compared to 0.9514 to 0.9610 for the UV-Vis method, with absolute differences between the two methods remaining below 0.5%. Smartphone-assisted colorimetry and UV-Visible spectrophotometry produced closely comparable estimates for each tested forage sample for the tested forages. Smartphone-assisted colorimetry provided accurate estimates of calcium and phosphorus in forage extracts, comparable to those produced by spectrophotometry, and demonstrated potential as a low-cost tool for routine screening of ruminant feed minerals**.**

Keywords: Calcium, Colorimetry, Forage, Nutrition, Phosphorus, Ruminant feed, Smartphone

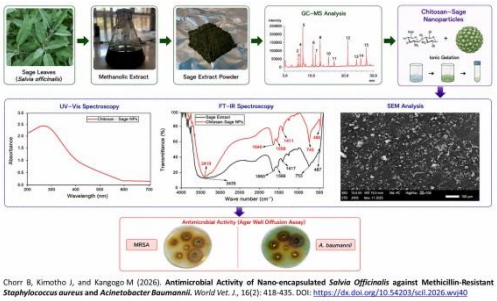
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Research Paper

Antimicrobial Activity of Nano-encapsulated *Salvia Officinalis* against Methicillin-Resistant *Staphylococcus aureus* and *Acinetobacter Baumannii*

Chorr B, Kimotho J, and Kangogo M.

World Vet. J. 16(2): 418-435, 2026; pii:S232245682600040-16
DOI: https://dx.doi.org/10.54203/scil.2026.wvj40



ABSTRACT: Antimicrobial resistance represents a global health burden requiring innovative therapeutic strategies. The antibacterial properties of *Salvia officinalis* (*S. officinalis*) are attributed to its bioactive compounds. The present study aimed to investigate the antimicrobial activity of nano-encapsulated *S. officinalis* leaf extract against methicillin-resistant *Staphylococcus aureus* (MRSA) and *Acinetobacter baumannii* (*A. baumannii*). The crude extract of *S. officinalis* was subjected to methanolic extraction, and the resulting methanolic fraction was analyzed through phytochemical screening. Phytocompounds were identified using gas chromatography-mass spectrometry (GC-MS), and their potential mechanisms of action were evaluated *in silico*. The *S. officinalis* extract was nano-formulated using chitosan and characterized by scanning electron microscopy (SEM), Fourier transform infrared spectroscopy, and UV-visible spectroscopy. Antibacterial activity of the nano-formulation against MRSA and *A. baumannii* was assessed using agar well diffusion, broth microdilution, and minimum bactericidal concentration analysis. Qualitative phytochemical analysis revealed the presence of flavonoids, alkaloids, tannins, saponins, and terpenoids. The GC-MS analysis demonstrated 15 peaks, representing 14 distinct compounds, with one compound (3,7,11,15-tetramethyl-2-hexadecen-1-ol) eluting at two retention times. Chitosan nanoparticle formation was confirmed by an absorption peak near 290 nm. The functional groups of chitosan nanoparticles included hydroxyl (-OH) and amide (-NH_2) groups, along with C–H, C–N, C–O, and P=O stretching. The SEM imaging exhibited relatively homogeneous, well-dispersed, spherical nanoparticles. The antimicrobial efficacy of *S. officinalis* chitosan nanoparticles was concentration-dependent, reaching maximum inhibition zones of 21 ± 0.5 mm against MRSA and 17 ± 1.5 mm against *A. baumannii* at 500 µg/mL. Additionally, susceptibility testing revealed minimum inhibitory concentrations of 62.5 µg/mL and 125 µg/mL, and minimum bactericidal concentrations of 125 µg/mL and 500 µg/mL against MRSA and *A. baumannii*, respectively. Molecular docking analysis indicated that phytocompounds within the methanolic extract exhibited binding affinity toward penicillin-binding protein 2a (PBP2a) and the carbapenem-associated outer membrane protein (CarO). The interactions between the phytochemicals and protein targets indicated that binding to catalytic sites could inhibit the function of these resistance-associated proteins, potentially reducing antibiotic degradation. The biosynthesized *S. officinalis* chitosan nanoparticles exhibited strong antimicrobial activity against multidrug-resistant bacterial isolates, supporting their potential as antimicrobial agents.

Keywords: *Acinetobacter baumannii*, Antimicrobial resistance, Methicillin-resistant *Staphylococcus aureus*, Nanoparticle, *Salvia officinalis*

[Full text-PDF] [Crossref Metadata] [Scopus] [Export from ePrint]

Research Paper

Molecular Investigation and Risk Assessment of Neonatal Calf Diarrhea in Morocco: Emphasis on Rotavirus and Coronavirus Infections



Choukri I, Touil N, Zouagui Z, Rhazzar Z, El Ouadi H, Belouad EM, Fassi Fihri O, and El Berbri I.

World Vet. J. 16(2): 436-448, 2026;
pii:S232245682600041-16

DOI: https://dx.doi.org/10.54203/scil.2026.wvj41

ABSTRACT: Neonatal calf diarrhea represents a major health and economic burden in Moroccan cattle farming and remains a leading cause of calf mortality. It is commonly associated with infectious agents such

as rotavirus, bovine coronavirus, *Escherichia coli*, and *Cryptosporidium parvum*, and is often aggravated by dehydration and electrolyte imbalances. The present study aimed to update the epidemiological profile of viral pathogens involved in neonatal calf diarrhea in Morocco, with particular emphasis on rotavirus group A (RVA) and bovine coronavirus (BCoV). A total of 889 newborn calves from 20 farms located in five Moroccan regions (Casablanca-Settat, Rabat-Salé-Kenitra, Souss-Massa, Fes-Meknes, and Beni Mellal-Khenifra) were included in this cross-sectional observational study. Among them, 130 rectal swabs were collected from diarrheic calves between November 2022 and March 2023 and analyzed using reverse transcription quantitative PCR (RT-qPCR). The results indicated that 34% of diarrheic calves tested positive for RVA and 62% for BCoV, with a co-infection rate of 24%. Infection rates were influenced by several factors, including calf age, geographic region, dam vaccination status, calving rank, production system, and farm size. RVA infection peaked in calves aged 8–14 days, whereas BCoV was most prevalent in calves aged 21–29 days. Notably, RVA positivity was higher in calves born to vaccinated dams (40%) compared to those from unvaccinated dams (20%), while BCoV infection was more frequently detected in calves from heifers than from multiparous cows. Higher infection rates in calves were also observed in intensive farming systems. Overall, these findings confirmed the major role of rotavirus and bovine coronavirus in neonatal calf diarrhea in Morocco and suggest that the limited effectiveness of vaccination may be influenced by herd management, colostrum transfer, and biosecurity practices. The potential mismatch between vaccine and circulating strains highlights the need for continuous molecular surveillance.
Keywords: Bovine coronavirus, Calf, Neonatal diarrhea, Rotavirus A, Risk factor

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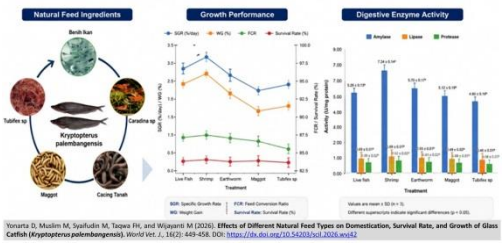
Research Paper

Effects of Different Natural Feed Types on Domestication, Survival Rate, and Growth of Glass Catfish (*Kryptopterus palembangensis*)

Yonarta D, Muslim M, Syaifudin M, Taqwa FH, and Wijayanti M.

World Vet. J. 16(2): 449-458, 2026; pii:S232245682600042-16

DOI: https://dx.doi.org/10.54203/scil.2026.wvj42



ABSTRACT: Intensive, uncontrolled fishing has primarily reduced glass catfish (*Kryptopterus palembangensis*) populations in Indonesian waters, especially in Sumatra, leading to a steady decline in natural populations through continuous overexploitation. The present study aimed to evaluate the effects of different natural feeds on the growth performance and survival of glass catfish during the domestication phase (process of adapting wild fish to aquaculture conditions using appropriate natural feed types). A total of 150 glass catfish, with an average initial length of 14.62 ± 0.53 cm and an average initial weight of 15.58 ± 0.73 g, were subjected to the present study, using a completely randomized design with five treatment groups and three replications. Each experimental unit contained 10 fish, resulting in 30 fish per treatment. The treatment groups consisted of different natural feed types, namely small live fish (P1), shrimp (P2), earthworms (P3), maggots (P4), and *Tubifex* sp. (P5). Growth performance, survival rate, and digestive enzyme activity of glass catfish were observed after 30 days of rearing. The present results indicated that Group P2 significantly improved the growth performance of glass catfish, including absolute weight (3.61 ± 0.16 g), absolute length (2.85 ± 0.11 cm), specific growth rate (0.85 ± 0.03% day^{-1}), and daily growth rate (0.12 ± 0.01 g day^{-1}) compared to other groups. Additionally, Group P2 exhibited the highest survival rate at 100% and the highest digestive enzyme activities, with amylase at 7.24 ± 0.14 U mL^{-1}, lipase at 0.37 ± 0.01 U mL^{-1}, and protease at 0.69 ± 0.01 U mL^{-1}. The second-best performance was observed in Group P1, followed by P3, P5, and P4. Shrimp was identified as the most effective natural feed for improving the growth performance and survival rate of glass catfish.
Keywords: Digestive enzyme, Domestication, Glass catfish, Growth, Survival rate

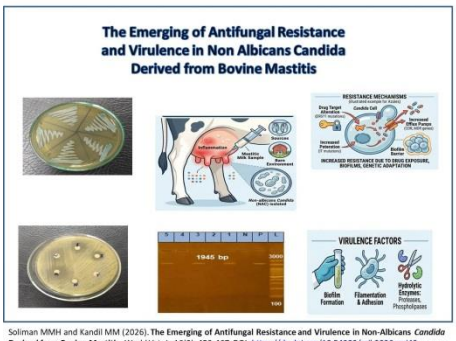
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Research Paper

The Emerging of Antifungal Resistance and Virulence in Non-Albicans *Candida* Derived from Bovine Mastitis

Soliman MMH and Kandil MM.

World Vet. J. 16(2): 459-467, 2026; pii:S232245682600043-16
DOI: https://dx.doi.org/10.54203/scil.2026.wvj43



ABSTRACT: Mastitis represents a considerable economic burden on the dairy industry, adversely affecting milk yield and quality. In mycotic mastitis, *Candida* (*C.*) species are frequently responsible for opportunistic fungal infections in cows. The present study aimed to isolate *C. tropicalis* from aseptically collected milk samples from 60 dairy cows (average age 4.2

years) that tested positive for the California mastitis test (CMT). Mycotic etiology was confirmed through direct microscopy (KOH wet mount), culture on Sabouraud dextrose agar, and biochemical identification (API 20C AUX system). Antifungal sensitivity testing was conducted employing the broth microdilution method in accordance with CLSI M27-A3 guidelines. Additionally, molecular detection of antifungal resistance genes (*ERG3* and *CDR1*) and virulence genes (*SAPT3*, *ALS2*, and *LIP1*) was carried out using polymerase chain reaction (PCR). A total of five *C. tropicalis* isolates were obtained from 60 mastitis milk samples, yielding a prevalence of 8.3%. Antifungal susceptibility profiles demonstrated that all tested isolates were sensitive to itraconazole, nystatin, and ketoconazole. However, two of five isolates (40%) were resistant to fluconazole, all five isolates (100%) were resistant to terbinafine, and three of five isolates (60%) were resistant to voriconazole. Virulence genes (*SAPT3*, *ALS2*, and *LIP1*) were identified in 100%, 40%, and 60% of the five examined isolates, respectively. Meanwhile, antifungal resistance genes (*CDR1* and *ERG3*) were found in 100% and 80% of the isolates, respectively. The present study underscored the prevalence of multidrug-resistant *C. tropicalis* in bovine mycotic mastitis, emphasizing the need for routine antifungal susceptibility testing and molecular screening in dairy farms.

Keywords: Antifungal resistance, *Candida tropicalis*, Mastitis, Resistance gene, Virulence gene

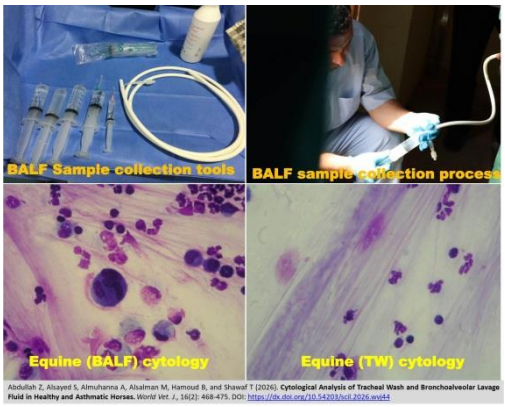
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Research Paper

Cytological Analysis of Tracheal Wash and Bronchoalveolar Lavage Fluid in Healthy and Asthmatic Horses

Abdullah Z, Alsayed S, Almuhanna A, Alsalman M, Hamoud B, and Shawaf T.

World Vet. J. 16(2): 468-475, 2026; pii:S232245682600044-16
DOI: https://dx.doi.org/10.54203/scil.2026.wvj44



ABSTRACT: Although numerous recent studies have explored the cytology of equine asthma, its cytological profile in Arabian horses is still insufficiently described. This study compared the differential cell counts in tracheal lavage (TW) and bronchial lavage fluid (BALF) samples between healthy and asthmatic Arabian horses in Saudi Arabia. Seventeen horses (5 healthy, 12 asthmatic), with an average age of 7 ± 5.5 years, and an average weight of 325 ± 120 kg, enrolled in this study to collect and analyze tracheal wash (TW) or bronchoalveolar lavage fluid (BALF) samples. The affected horses were taken from the respiratory cases admitted to the veterinary teaching hospital of Faisal University, while the healthy horses were taken from the horse research center in Faisal University (Saudi Arabia). Differential counts were performed on TW and BALF samples using microscopic examination after staining the samples with Diff Quick stain for evaluation of macrophages, lymphocytes, neutrophils, mast cells, eosinophils, and epithelial cells. A significant increase in neutrophils was observed in the affected group in both TW and BALF, compared to the control group. In contrast, a marked decrease in the percentage of lymphocytes was observed in asthmatic BALF, while macrophage proportions decreased in affected and control groups. TW eosinophils were elevated in asthmatic horses compared to healthy horses, though BALF eosinophil counts did not differ between healthy and asthmatic horses. The percentage of epithelial cells was decreased in TW but modestly increased in the BALF of asthmatic horses. No change in the number of mast cells was observed in any of the groups, as it was low. These findings confirmed that equine asthma in Arabian horses enrolled in this study is characterized by pronounced neutrophilic inflammation with concomitant lymphocytic and macrophagic depletion, particularly in the BALF samples.

Keywords: Bronchoalveolar, Cytology, Horse, Respiratory, Tracheal, Wash

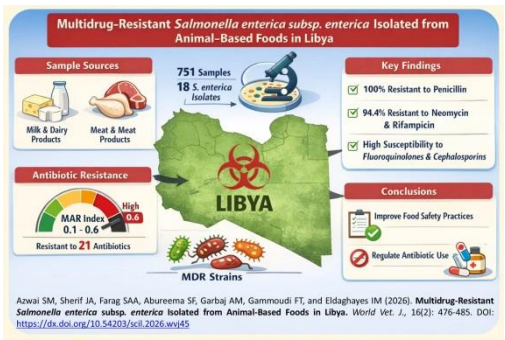
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Research Paper

Multidrug-Resistant *Salmonella enterica* subsp. *enterica* Isolated from Animal-Based Foods in Libya

Azwai SM, Sherif JA, Farag SAA, Abureema SF, Garbaj AM, Gammoudi FT, and Eldaghayes IM.

World Vet. J. 16(2): 476-485, 2026;
pii:S232245682600045-16
DOI: https://dx.doi.org/10.54203/scil.2026.wvj45



ABSTRACT: The emergence of *Salmonella enterica* subsp. *enterica* in food represents a significant public health concern worldwide, particularly in developing countries such as Libya. Addressing antimicrobial resistance in *Salmonella enterica* subsp. *enterica* is essential, particularly where food hygiene and antimicrobial regulations are weak. The present study aimed to evaluate the occurrence and antimicrobial susceptibility profile of *Salmonella enterica* subsp. *enterica* isolated

from different animal-derived food products collected at retail markets in Libya. In the current study, 18 *Salmonella enterica* subsp. *enterica* isolates were obtained from 751 food samples, including milk, dairy products, meat, and meat products, collected randomly from retail markets in Libya. The isolates were tested for antimicrobial susceptibility, and their antibiotic resistance profiles were evaluated against 33 antibiotics representing 12 different classes. Antimicrobial susceptibility testing demonstrated that all isolates were 100% resistant to penicillin, with high resistance rates to neomycin and rifampicin (94.4%). However, high susceptibility rates were observed for chloramphenicol, nitrofurantoin, sulfamethoxazole/trimethoprim, and norfloxacin (88.9%). The multiple antibiotic resistance (MAR) index varied from 0.1 to 0.6, with the maximum MAR value (0.6) recorded in isolates exhibiting resistance to 21 antibiotics. The current findings underscore the necessity to enhance food safety protocols, as multidrug resistance was prevalent among the isolates, particularly to penicillin, neomycin, and rifampicin. Therefore, the utilization of antimicrobial agents in both veterinary and human settings should be strictly regulated to control the emerging threat of multidrug-resistant strains. Additionally, the present results suggested that fluoroquinolones and cephalosporins remain among the most effective antimicrobial agents and may be considered for the treatment of salmonellosis. The detection of multidrug-resistant *Salmonella enterica* subsp. *enterica* in animal-derived foods in Libya represented a significant public health concern, underscoring the critical need for enhanced surveillance, stricter antimicrobial use regulations, and improved food safety measures.
Keywords: Foodborne pathogen, Meat, Milk, Multidrug resistance, *Salmonella enterica* subsp. *enterica*

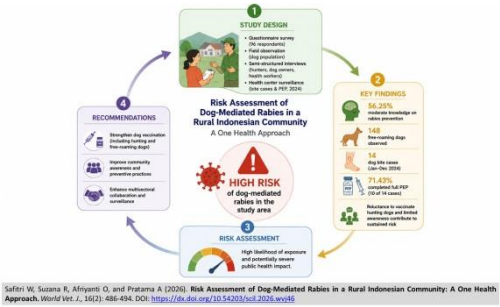
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Research Paper

Risk Assessment of Dog-Mediated Rabies in a Rural Indonesian Community: A One Health Approach

Safitri W, Suzana R, Afriyanti O, and Pratama A.

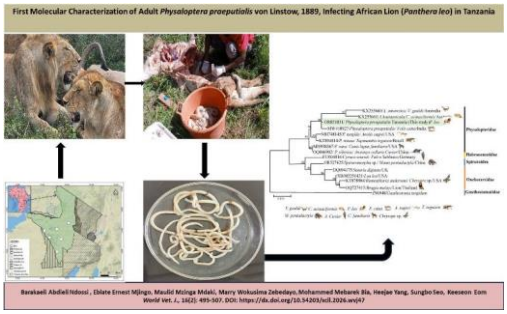
World Vet. J. 16(2): 486-494, 2026; pii:S232245682600046-16
DOI: https://dx.doi.org/10.54203/scil.2026.wvj46



ABSTRACT: Rabies remains a fatal zoonotic disease that continues to cause thousands of human deaths annually, particularly in regions where dog-mediated transmission persists. In Indonesia, rabies remains endemic in different provinces, and rural communities with frequent human-dog interactions may face an increased risk of exposure. The present study aimed to assess the risk of dog-mediated rabies transmission in a rural Indonesian community using a One Health perspective. A cross-sectional descriptive study was conducted in Sirukam Village, Solok District, West Sumatra, Indonesia, in August 2025. Data were collected through structured questionnaires administered to 96 respondents, field observations of dog populations, and semi-structured interviews with hunters, dog owners, and local health workers. Secondary data on bite incidents and post-exposure prophylaxis (PEP) completion were obtained from the local public health center. The results revealed that 56.25% of respondents had moderate knowledge regarding rabies prevention, while preventive practices, including wound washing and vaccination adherence, were inconsistent. A total of 14 dog bite cases were reported during January-December 2024. Only 10 of the 14 exposed individuals (71.43%) completed the full PEP vaccination schedule, indicating suboptimal adherence. Field observations revealed 148 free-roaming dogs in the study area, indicating frequent opportunities for human-dog interactions. Qualitative interviews with selected participants suggested that reluctance to vaccinate hunting dogs and limited awareness of rabies prevention may contribute to sustained transmission risks. However, the qualitative findings from interviews with selected participants should be interpreted cautiously due to the limited number of informants. Based on a qualitative risk assessment combining the likelihood of exposure and the potential public health impact using a semi-quantitative scoring approach, rabies was categorized as a high-risk health threat in the study area. The results highlight the importance of strengthening dog vaccination programs, improving community awareness, and enhancing multisectoral collaboration to reduce rabies transmission in rural endemic settings.
Keywords: Dog bite, Rabies, Risk assessment, Vaccination, Zoonotic disease
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Research Paper

First Molecular Characterization of Adult *Physaloptera praeputialis* von Linstow, 1889, Infecting African Lion (*Panthera leo*) in Tanzania



Ndossi BA, Mjingo EE, Mdaki MM, Zebedayo MW, Bia MM, Yang H, and Eom K.

World Vet. J. 16(2): 495-507, 2026;
pii:S232245682600047-16

DOI: https://dx.doi.org/10.54203/scil.2026.wvj47

ABSTRACT: Physalopterid nematodes are common gastric parasites of

carnivorous mammals, yet their diversity, host associations, and evolutionary relationships in African wildlife remain poorly understood. The present study aimed to investigate the adult *Physaloptera* specimens recovered from African lions (*Panthera leo*) in the Serengeti National Park in the Mara region, Tanzania, using morphological and molecular techniques. Seven non-captive lions comprising three males and four females were opportunistically necropsied between 2023 and 2025. The sampled lions included two subadults (4-5 years), three prime adults (6-9 years), and two older individuals (10-14 years). A total of 157 adult spirurid nematodes were recovered from three infected hosts with infection intensities ranging from 48 to 57 worms per host. Morphometric characterizations, such as cephalic collarette structures, marked unequal spicules, well-developed caudal alae in males, and an anterior positioned vulva in females, together with typical eggs, were consistent with *Physaloptera praeputialis*. Nuclear *18S rRNA* sequences showed high similarity of 98-99.5% to *P. praeputialis* reference sequences. In contrast, *mitochondrial cytochrome c oxidase subunit I* (*Cox1*) sequences formed a distinct lineage, exhibiting approximately 12% divergence from available *P. praeputialis* sequences retrieved from the GenBank database at the time of analysis. A molecularly characterized record of adult *Physaloptera* from an African lion in Tanzania is presented, revealing previously unrecognized genetic diversity among African physalopterids. Findings highlight the role of apex predators as indicator hosts in trophic parasite transmission and emphasize the importance of multilocus approaches for resolving spirurid taxonomy, parasite biogeography, and host–parasite associations in wildlife ecosystems.

Keywords: African lion, Molecular characterization, Parasitic nematode, Phylogenetic, Serengeti ecosystem

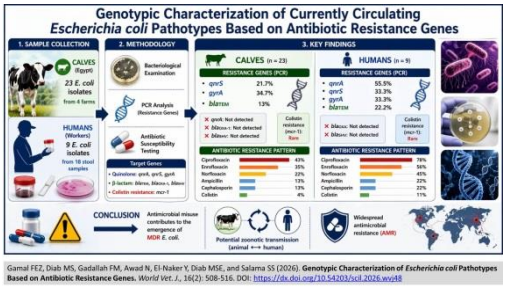
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Research Paper

Genotypic Characterization of *Escherichia coli* Pathotypes Based on Antibiotic Resistance Genes

Gamal FEZ, Diab MS, Gadallah FM, Awad N, El-Naker Y, Diab MSE, and Salama SS.

World Vet. J. 16(2): 508-516, 2026; pii:S232245682600048-16
DOI: https://dx.doi.org/10.54203/scil.2026.wvj48



ABSTRACT: Diarrhea represents a major health challenge in young calves and is a leading cause of mortality, arising from infectious and non-infectious factors. The present study aimed to investigate antibiotic-resistant, multi-virulent diarrheagenic *Escherichia coli* (*E. coli*) strains isolated from human and animal samples. The current study included 23 *E. coli* strains obtained from calves across four different farms in Egypt, as well as nine *E. coli* isolates derived from 18 stool samples from workers. These samples were transferred to the microbiological laboratory for bacteriological and molecular analysis utilizing polymerase chain reaction (PCR). Molecular analysis of the antibiotic resistance gene was performed using PCR, followed by antibiotic sensitivity testing against ciprofloxacin, enrofloxacin, norfloxacin, ampicillin, cephalosporin, and colistin. Among 23 *E. coli* isolates obtained from calves, the resistance genes, including *qnrS* (21.7%), *gyrA* (34.7%), and *blaTEM* (13%), were identified. Conversely, *qnrA*, *blaOXA-1*, and *blaSHV* were not detected. Only one isolate exhibited colistin resistance, which was uncommon. Human *E. coli* isolates demonstrated moderate prevalence of *qnrA* (55.5%), *qnrS* (33.3%), *gyrA* (33.3%), and *blaTEM* (22.2%), while *blaOXA* and *blaSHV* were not detected, and a colistin resistance isolate (*mcr-1*) was rare. The antibiotic sensitivity test of *E. coli* isolates from calves exhibited that 43% were resistant to ciprofloxacin and 35% to enrofloxacin. Meanwhile, 22% of isolates demonstrated resistance to norfloxacin. The β-lactam group revealed 13% resistance to ampicillin and cephalosporin. Only 4% of all isolates exhibited resistance to colistin. Antibiotic sensitivity test of *E. coli* isolates from humans revealed significant resistance patterns. Among the quinolones, 78% demonstrated resistance to ciprofloxacin, 56% to enrofloxacin, and 45% to norfloxacin. In contrast, only 22% of isolates were resistant to ampicillin and cephalosporin, while resistance to colistin was detected in just 11% of isolates. The present study confirmed that the misuse of different antibiotics is a significant factor in the development of antimicrobial resistance genes, especially in *E. coli* strains isolated from both humans and infected calves.

Keywords: Antibiotic sensitivity test, *Escherichia coli,* Multidrug resistance, PCR

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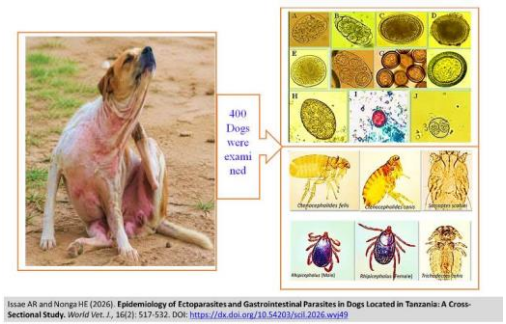
Research Paper

Epidemiology of Ectoparasites and Gastrointestinal Parasites in Dogs Located in Tanzania: A Cross-Sectional Study

Issae AR and Nonga HE.

World Vet. J. 16(2): 517-532, 2026;
pii:S232245682600049-16

DOI: https://dx.doi.org/10.54203/scil.2026.wvj49



ABSTRACT: Dogs, common companion animals, serve several cultural, social, and economic functions in society but can pose public health risks if not properly managed. The present study aimed to examine the epidemiology of canine parasites in the Mvomero district and the Morogoro municipality in Tanzania. A cross-sectional study was conducted in 2024 involving 400 randomly selected dogs, aged from 3 months to over 1 year, selected from households in the Mvomero district and the Morogoro municipality of Tanzania. The study population consisted predominantly of indigenous mongrels (n = 372), with a smaller proportion of crossbreeds (n = 28). Ectoparasites were collected through full-body examination, including the skin, ears, and interdigital spaces. Ticks, fleas, mites, and lice were manually removed using forceps and combing, with ticks carefully extracted to preserve their mouthparts. Fleas were collected using an acetone application followed by brushing onto white cloths. All samples were preserved in 70% ethanol and identified morphologically under a light microscope using standard identification keys. Fecal samples were collected from the study dogs and examined using flotation, sedimentation, direct saline/iodine wet mount, and modified Ziehl-Neelsen staining techniques. The overall prevalence of ectoparasites was 83.8%, while gastrointestinal parasites were detected in 76.8% of the dogs. Helminths included *Ancylostoma* spp. (60.5%), *Uncinaria stenocephala* (22.0%), *Toxocara canis* (11.5%), *Toxascaris leonina* (6.3%), *Ascaris* spp. (3.8%), *Taeniidae* (6.0%), and *Dipylidium caninum* (1.8%). Protozoa included *Cryptosporidium* spp. (15.5%), *Isospora* spp. (8.0%), *Cyclospora* spp. (4.3%), and *Entamoeba* spp. (3.0%). Dogs from Mvomero, Tanzania, had significantly higher parasite prevalence than those from Morogoro municipality, Tanzania. Risk factors for parasitic infection included dog age, geographic location, management practices, and lack of deworming. The current study highlighted an alarming burden of parasitic infestations among dogs in Morogoro, Tanzania, posing significant threats to animal health, public health, and animal welfare.
Keywords: Disease, Dog, Ectoparasite, Gastrointestinal parasite, Risk factor, Zoonoses

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Research Paper

Conjunctival Cytology and Bacterial Isolation from Arabian Horses with Conjunctivitis in Saudi Arabia

Al-mashabirah Z, Al-Shehab M, Alsalman M, Salouci M, Asvad M, Elbager K, and Shawaf T.

World Vet. J. 16(2): 533-540, 2026; pii:S232245682600050-16
DOI: https://dx.doi.org/10.54203/scil.2026.wvj50



ABSTRACT: Cytological and bacterial analyses are vital for diagnosing equine conjunctival diseases. These analyses facilitate accurate pathogen detection and enable targeted treatments. The present study aimed to describe and compare the conjunctival cytology and bacterial flora of clinically healthy Arabian horses and those with conjunctivitis in the Eastern Province of Saudi Arabia. Eighteen Arabian horses, including 5 healthy and 13 with conjunctivitis, were referred to King Faisal Hospital and examined. Healthy horses were distinguished from those with conjunctivitis through case history and an ocular examination. Conjunctival swabs were collected for cytological analysis and for aerobic bacterial culture and identification, with Diff-Quik staining. Differential cell counts were compared across the groups, and the findings were correlated with bacterial isolates. Biochemical identification of bacterial isolates was performed using conventional methods, including Gram staining and catalase and oxidase tests. Healthy horses exhibited a cytological profile primarily composed of superficial epithelial cells (59 ± 12.0%), with only a small number of inflammatory cells, including neutrophils and eosinophils. In contrast, horses with conjunctivitis exhibited a significant decrease in superficial epithelial cells (28 ± 17%) and a significant increase in basal cells (5 ± 3.5%). Conjunctival samples exhibited marked neutrophilia (32 ± 12.5%) and a significant increase in eosinophils (12 ± 9.5%) compared to healthy controls, indicating an inflammatory condition in the conjunctiva. Bacterial growth was found in 40% of healthy horse samples and in 92% of infected horse samples. *Staphylococcus aureus* was the most common isolate in infected horse samples. Gram-negative bacteria, including *Moraxella* sp., *Escherichia coli*, and *Klebsiella* sp., were isolated from 7 of 13 affected horses and were associated with a severe neutrophilic response (39 ± 6.5%) and significant epithelial disruption. Conjunctivitis in Arabian horses in the Eastern Province of Saudi Arabia was characterized by neutrophilic and eosinophilic inflammation with concurrent epithelial disruption.
Keywords: Bacteria, Conjunctivitis, Cytology, Equine, Ocular, Swab

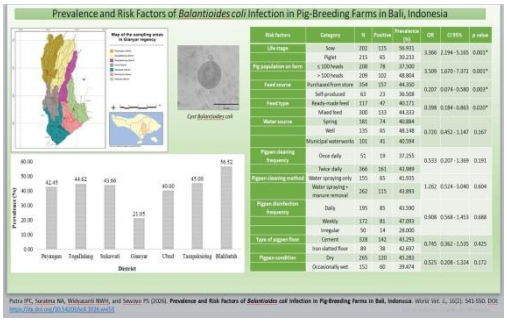
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Research Paper

Prevalence and Risk Factors of *Balantioides coli* Infection in Pig-Breeding Farms in Bali, Indonesia

Putra IPC, Suratma NA, Widyasanti NWH, and Sewoyo PS.

World Vet. J. 16(2): 541-550, 2026;
pii:S232245682600051-16
DOI: https://dx.doi.org/10.54203/scil.2026.wvj51



ABSTRACT: *Balantioides coli* (*Balantidium coli* [*B. coli*]) infection poses significant health and economic burdens in pigs, causing diarrhea, intestinal dysbiosis, and increased susceptibility to secondary infections. The present study aimed to assess the occurrence of balantidiasis and pinpoint related risk factors in pigs at breeding facilities in Gianyar Regency, Bali, Indonesia. A cross-sectional study with random sampling was conducted from August 2024 to February 2025. Fecal samples were collected from 15 to 35 pigs from 16 pig-breeding farms that utilized a semi-intensive rearing system. In total, 417 fecal samples were collected from pigs in seven districts, including 202 sows and 215 piglets. The samples were preserved in 3% potassium dichromate ($K_2Cr_2O_7$) and examined using the coprological sedimentation method. Morphological identification was based on the detection of *B. coli* cysts in fecal samples. Data on potential risk factors were evaluated using logistic regression analysis, which was conducted on data obtained from structured questionnaires and direct on-farm observations. The results indicated that 43.17% of pigs were infected with *B. coli*, with a higher prevalence in sows (56.95%) than in piglets (30.23%). Logistic regression analysis indicated that sows had a 3.366 times higher risk of infection than piglets. Farms with pig populations exceeding 100 heads also exhibited a higher risk of infection compared to farms housing 100 or fewer pigs. In contrast, farmers who purchased feed from commercial suppliers and those who used ready-made feed had a significantly lower risk of *B. coli* infection, reducing the incidence by 79.3% and 60.2%, respectively, compared to farmers who produced their own feed or used mixed feed. Factors of water source, pigpen condition, and pigpen cleaning practices were not significantly associated with infection. The prevalence of *B. coli* infection in pig-breeding farms in Gianyar Regency is notably high (43.17 %). The analysis indicated that life stage, herd size, feed source, and feed type are significantly associated with the risk of balantidiasis in pigs and should be prioritized in disease prevention and control strategies by farmers in the study region.
Keywords: Balantidiasis, *Balantidium coli*, Piglet, Protozoa, Sow

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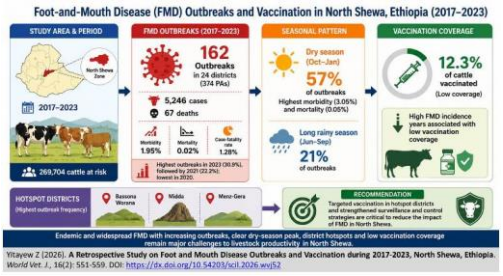
Research Paper

A Retrospective Study on Foot and Mouth Disease Outbreaks and Vaccination during 2017-2023, North Shewa, Ethiopia

Yitayew Z.

World Vet. J. 16(2): 551-559, 2026; pii:S232245682600052-16

DOI: https://dx.doi.org/10.54203/scil.2026.wvj52



ABSTRACT: Foot-and-mouth disease (FMD) is a highly contagious viral disease that reduces livestock productivity in Ethiopia. The present study aimed to assess the spatial and temporal patterns of FMD outbreaks and associated morbidity, mortality, case-fatality rates, and vaccination coverage in the North Shewa Zone from 2017 to 2023. Data were collected from the Disease Outbreak and Vaccination Activities Reports (DOVAR) of the North Shewa Zone Livestock Office and analyzed using descriptive statistics. A total of 162 outbreaks were recorded across 24 districts and 374 peasant associations (PAs) over the study period, resulting in an overall outbreak proportion of 43.4% (162/374 PAs), with considerable variation between years. The highest number of outbreaks was recorded in 2023 (30.9%), followed by 2021 (22.2%), while the lowest was in 2020. Overall, there were 5,246 recorded FMD cases and 67 deaths among 269,704 cattle at risk, yielding morbidity, mortality, and case-fatality rates of 1.95%, 0.02%, and 1.28%, respectively. Outbreak occurrence differed by season, peaking in the dry season (October to January) at 57% and reaching its lowest in the rainy season (June to September) at 21%. Morbidity and mortality were highest in the dry season (3.05% and 0.05%, respectively). Outbreaks and cases were unevenly distributed across districts, with BassonaWorana, Midda, and Menz-Gera reporting the highest outbreak frequencies, while Merhabet and Siyadeber exhibited relatively higher morbidity rates relative to their cattle populations. Over the seven years, vaccine coverage was limited, reaching only 12.3% of the at-risk population. Years with a high incidence of FMD were generally correlated with low vaccination coverage. Endemic FMD across the North Shewa Zone, along with increasing outbreaks, dry-season peaks, district hotspots, and inadequate vaccination coverage, highlighted FMD as a significant obstacle to livestock productivity. The results indicated the critical need for targeted vaccination programs and strengthened control strategies, particularly in high-risk districts.
Keywords: Epidemiology, Foot and mouth disease, Morbidity, Mortality, Outbreak, Vaccination

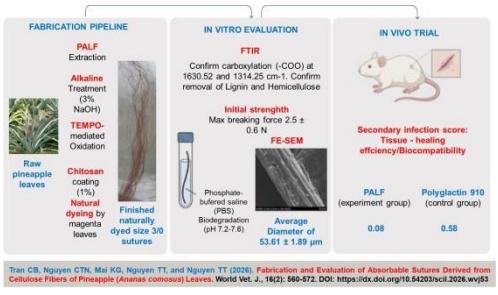
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Research Paper

Fabrication and Evaluation of Absorbable Sutures Derived from Cellulose Fibers of Pineapple (*Ananas comosus*) Leaves

Tran CB, Nguyen CTN, Mai KG, Nguyen TT, and Nguyen TT.

World Vet. J. 16(2): 560-572, 2026;



pii:S232245682600053-16

DOI: https://dx.doi.org/10.54203/scil.2026.wvj53

ABSTRACT: Developing sustainable and biodegradable veterinary biomaterials is crucial for addressing limitations of synthetic sutures and avoiding secondary suture removal. The present study aimed to develop a novel, eco-friendly suture derived from pineapple leaves as an alternative to synthetic surgical threads and to assess its functional and clinical potential. The present study fabricated absorbable veterinary sutures from pineapple leaf fibers (PALF) via alkaline treatment and 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO)-mediated oxidation. The purified fibers, characterized by an average diameter of 53.61 ± 1.89 µm were successfully isolated via alkaline treatment and TEMPO-mediated oxidation, dyed naturally with *Peristrophe bivalvis*, coated with 1% chitosan, and subsequently twisted into size 3/0 sutures. *In vitro* degradation in phosphate buffer solution over four weeks confirmed their biodegradability, as demonstrated by increased fragility and strand separation. For the *in vivo* trial, 24 male mice (20-24 grams) were housed at a density of three mice per cage throughout the cultivation periods of 7, 14, 28, and 45 days. The mice were divided into two groups, including those that underwent surgery and were treated with PALF sutures (experimental group) and those that were treated with Polyglactin 910 (control group). A 2-3 cm dorsal-to-abdominal skin incision was made to expose the subcutaneous tissue, followed by a 1-2 cm connective tissue incision to establish the wound model for testing PALF sutures. Specific parameters, including the degree of edema and erythema, localized temperature, and the presence of exudate, suppuration, secondary infection, and hemorrhage, were assessed for each group. Critical complications, including wound dehiscence or marginal necrosis, were recorded. The current results demonstrated that PALF sutures were biocompatible and followed a healing pathway similar to that of Polyglactin 910. The present results revealed that PALF sutures had significantly lower secondary infection scores (0.08 ± 0.28) than Polyglactin 910 (0.58 ± 0.90), indicating superior anti-infective property. All clinical inflammatory signs completely resolved by day 45. The TEMPO-oxidized PALF sutures were a viable, biocompatible, and sustainable alternative to absorbable veterinary sutures, demonstrating effective tissue healing and a significantly lower incidence of secondary infection.
Keywords: Absorbable suture, *Ananas comosus*, Cellulose, Pineapple leaf, Mice

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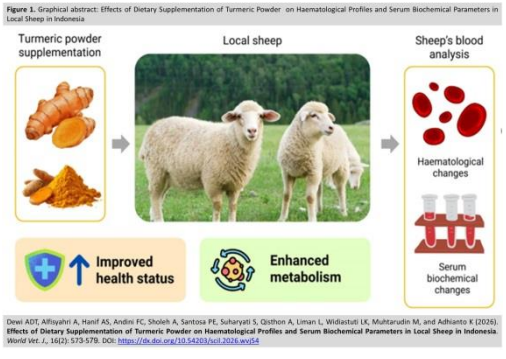
Research Paper

Effects of Dietary Supplementation of Turmeric Powder on Haematological Profiles and Serum Biochemical Parameters in Local Sheep in Indonesia

Dewi ADT, Alfisyahri A, Hanif AS, Andini FC, Sholeh A, Santosa PE, Suharyati S, Qisthon A, Liman L, Widiastuti LK, Muhtarudin M, and Adhianto K.

World Vet. J. 16(2): 573-579, 2026; pii:S232245682600054-16

DOI: https://dx.doi.org/10.54203/scil.2026.wvj54



ABSTRACT: Productivity and livestock health are significantly affected by nutritional strategies, especially natural feed additives with immunomodulatory and antioxidant properties. Turmeric (*Curcuma domestica*) is considered a sustainable, safe alternative to synthetic additives. The present study aimed to evaluate the haematological and serum biochemical parameters in local sheep following the inclusion of turmeric powder in their diet. The experiment involved 12 male local thin-tailed sheep (*Ovis aries*), approximately 1 year old, with an average body weight of 24 kilograms. The present study was carried out at a private farm in Pringsewu, Lampung, Indonesia, using a completely randomized design with three dietary treatments, including a basal diet without turmeric supplementation (P0), a basal diet supplemented with 2.5% dry matter (DM) of turmeric powder (P1), and a basal diet supplemented with 5% DM of turmeric powder (P2). Hemoglobin, packed cell volume (PCV), total plasma protein (TPP), blood glucose, and low-density lipoprotein (LDL) levels were assessed. Dietary supplementation with turmeric powder in Group P2 modulated haematological parameters, as indicated by increased hemoglobin (14.30 ± 0.48 g/dL) and PCV levels (43.43 ± 1.48%) compared to the control group. Serum biochemical parameters were affected in Group P2. Total plasma protein remained within the physiological range in all groups (6.50-6.80 g/dL), while blood glucose levels decreased as turmeric inclusion increased, with a level of 60.00 ± 6.27 mg/dL in Group P0 compared with groups P1 and P2. The LDL concentrations demonstrated a moderate increase across the treatment groups, reaching the highest level in Group P2 at 23.25 ± 5.05 mg/dL. Supplementing local sheep diets with 5% DM of *Curcuma domestica* powder influenced hemoglobin, PCV, glucose, and LDL.
Keywords: *Curcuma domestica*, Haematological parameter, Phytogenic feed additive, Serum biochemical parameter, Sheep

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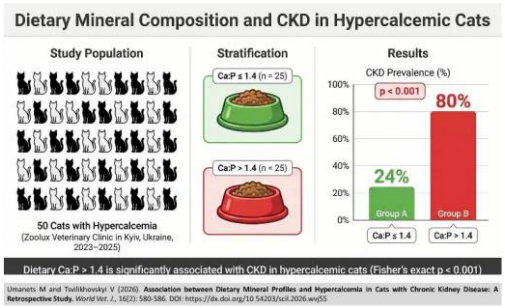
Research Paper

Association between Dietary Mineral Profiles and Hypercalcemia in Cats with Chronic Kidney Disease: A Retrospective Study

Umanets M and Tsvilikhovskyi V.

World Vet. J. 16(2): 580-586, 2026; pii:S232245682600055-16

DOI: https://dx.doi.org/10.54203/scil.2026.wvj55



ABSTRACT: Hypercalcemia is an important clinical problem in feline veterinary medicine due to its association with a wide range of pathological conditions, particularly chronic kidney disease (CKD). The present study aimed to characterize the clinical and biochemical characteristics of hypercalcemic cats and to investigate the association between the dietary calcium-to-phosphorus (Ca:P) ratio and CKD, urolithiasis, and total and ionized calcium levels. A retrospective analysis was conducted of 50 hypercalcemic domestic cats presented to the Veterinary Clinic in Kyiv, Ukraine, between 2023 and 2025. For each cat, data on age, breed, total calcium, and ionized calcium were collected. Additionally, serum phosphorus levels and any concurrent diseases were documented from medical records. Dietary composition was assessed based on manufacturer-reported analytical values, including calcium, phosphorus, the Ca:P ratio, and vitamin D content. Cats were divided into two groups based on their dietary Ca:P ratio; one with a ratio of 1.4 or less (≤ 1.4) and the other with a ratio greater than 1.4. (>1.4). The majority of cats (70%) were over six years old. The median total calcium concentration was 3.05 mmol/L, while the median ionized calcium concentration was 1.60 mmol/L. Elevated ionized calcium levels, despite normal total calcium levels, was observed in 16% of cases. Chronic kidney disease was observed in 60% of cats with hypercalcemia, while 18% had urolithiasis, mainly calcium oxalate. Additionally, 18% of the cats had both conditions simultaneously. Comparative analysis demonstrated that cats fed diets with Ca:P ratios > 1.4 had a significantly higher prevalence of CKD (20/25, 80%) than cats fed diets with Ca:P ratios ≤ 1.4 (6/25, 24%). However, total and ionized calcium concentrations did not differ significantly between the groups. The current findings highlighted the diagnostic value of total calcium, the importance of measuring ionized calcium, and suggested a possible association between high dietary Ca:P ratios and concurrent CKD in hypercalcemic cats.
Keywords: Calcium-to-phosphorus ratio, Chronic kidney disease, Hypercalcemia, Ionized calcium, Vitamin D

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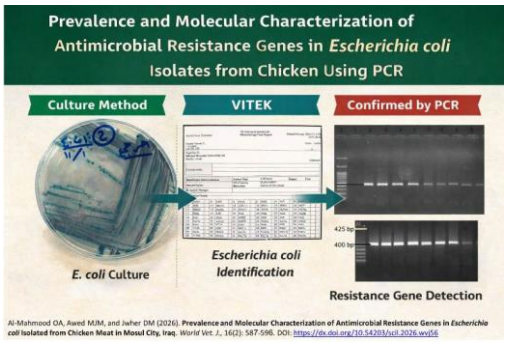
Research Paper

Prevalence and Molecular Characterization of Antimicrobial Resistance Genes in *Escherichia coli* Isolated from Chicken Meat in Mosul City, Iraq

Al-Mahmood OA, Awed MJM, and Jwher DM.

World Vet. J. 16(2): 587-596, 2026; pii:S232245682600056-16

DOI: https://dx.doi.org/10.54203/scil.2026.wvj56



ABSTRACT: Antimicrobial resistance (AMR) in poultry is a growing concern, driven by the widespread use of antibiotics in farming, which can promote the development of resistant bacteria. Resistant pathogens in the food chain can spread AMR and impair the treatment of infectious diseases. The present study aimed to examine the incidence of *Escherichia coli* (*E. coli*) and the presence of AMR genes in chicken meat samples collected from different markets in Mosul City, Iraq. Of 120 samples, 27 isolates (22.5%) tested positive for *E. coli*, identified by chromogenic agar, the VITEK® system, and polymerase chain reaction (PCR) targeting the *uidA* gene. All isolates (27/27; 100%) were confirmed positive for *uidA* detection. The positive isolates were evaluated for five AMR genes, including *dihydrofolate reductase A* (*dfrA*), *β-lactamase* TEM (*bla*TEM), *aminoglycoside acetyltransferase* (*aac*), *DNA gyrase subunit A* (*gyrA*), and *DNA gyrase subunit B* (*gyrB*). The current findings indicated that 66.7% of the isolates carried at least one resistance gene, and 14.8% carried more than one. The most commonly detected gene was *dfrA* (37.0%), followed by *bla*TEM (29.6%) and *aac* (14.8%). The *gyrA* gene was found in one isolate (3.7%); however, the *gyrB* gene was not detected. Identifying resistance genes causing resistance to commonly used antibiotics, such as trimethoprim, β-lactams, and aminoglycosides, represented a significant public health concern. The current findings can help identify and reduce the spread of AMR across the food supply chain in Iraq.
Keywords: Antimicrobial resistance gene, Chicken meat, *Escherichia coli*, Polymerase chain reaction, Public health

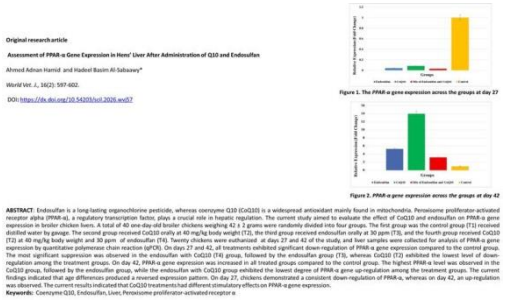
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Research Paper

Assessment of *PPAR-α* Gene Expression in Hens' Liver After Administration of Q10 and Endosulfan

Hamid AA and Al-Sabaawy HB.

World Vet. J. 16(2): 597-602, 2026; pii:S232245682600057-16
DOI: https://dx.doi.org/10.54203/scil.2026.wvj57



ABSTRACT: Endosulfan is a long-lasting organochlorine pesticide, whereas coenzyme Q10 (CoQ10) is a widespread antioxidant mainly found in mitochondria. *Peroxisome proliferator-activated receptor alpha* (*PPAR-α*), a regulatory transcription factor, plays a crucial role in hepatic regulation. The current study aimed to evaluate the effect of CoQ10 and endosulfan on *PPAR-α* gene expression in broiler chicken livers. A total of 40 one-day-old broiler chickens weighing 42 ± 2 grams were randomly divided into four groups. The first group served as the control and received distilled water by gavage (T1). The second group received CoQ10 orally at 40 mg/kg body weight (T2), the third group received endosulfan orally at 30 ppm (T3), and the fourth group received the combination of CoQ10 at 40 mg/kg body weight and 30 ppm of endosulfan (T4). On days 27 and 42 of the study, 20 chickens were euthanized, and liver samples were collected for analysis of *PPAR-α* gene expression by quantitative polymerase chain reaction (qPCR). On days 27 and 42, all treatments exhibited significant downregulation of *PPAR-α* gene expression compared to the control group. The most significant suppression was observed in the endosulfan with CoQ10 (T4) group, followed by the endosulfan group (T3), whereas CoQ10 (T2) exhibited the lowest level of downregulation among the treatment groups. On day 42, all treatment groups exhibited elevated *PPAR-α* gene expression compared to the control group. The highest *PPAR-α* expression level was observed in Group T2, followed by Group T3, while Group T4 demonstrated the lowest degree of upregulation among the treatment groups. The current findings indicated an age-dependent reversal pattern in *PPAR-α* expression. On day 27, chickens demonstrated a consistent downregulation of *PPAR-α*, whereas on day 42, an upregulation was observed. The current results indicated that CoQ10 administration produced differential stimulatory effects on *PPAR-α* gene expression.
Keywords: Coenzyme Q10, Endosulfan, Liver, *PPAR-α*

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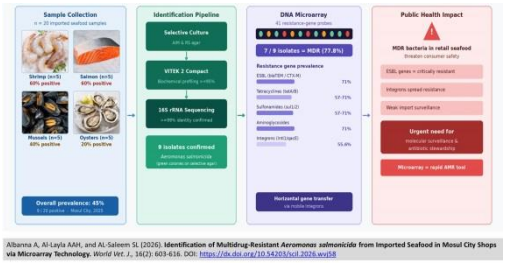
Research Paper

Identification of Multidrug-Resistant *Aeromonas salmonicida* from Imported Seafood in Mosul City Shops via Microarray Technology

Albanna A, Al-Layla AAH, and AL-Saleem SL.

World Vet. J. 16(2): 603-616, 2026; pii:S232245682600058-16

DOI: https://dx.doi.org/10.54203/scil.2026.wvj58



ABSTRACT: The widespread emergence of multidrug-resistant (MDR) pathogens in imported seafood is an increasing public health concern. The present study aimed to detect *Aeromonas salmonicida* (*A. salmonicida*) in seafood samples and to assess the antibiotic resistance gene profiles of the isolated bacteria using conventional polymerase chain reaction (PCR) and DNA microarray analysis. Twenty samples consisting of shrimp, mussels, oysters, and salmon (five of each) were collected from shops in Mosul city, Iraq. The biochemical characteristics of the strains were determined using the VITEK 2 compact system, and their identities were confirmed by *16S rRNA* gene sequencing, which showed ≥99% identity to database sequences. Nine samples tested positive for *A. salmonicida*, with a prevalence of 45 percentage, with shrimp and salmon each accounting for three isolates (60%), mussels for two (40%), and oysters for one (20%). Microarray testing identified seven isolates as MDR, whereas two strains had no resistance genes and were antibiotic-sensitive. The current results revealed the absence of genes encoding extended-spectrum β-lactamases (ESBLs), including *bla*SHV, *bla*CTX-M, *bla*FOX, *bla*PER, *bla*VEB, and *bla*CMY, in all MDR strains. Tetracycline resistance was observed in 28.6% (2/7) of MDR isolates, with the *tetA* gene present, whereas the *tetB* gene was completely absent from all MDR isolates. Concerning sulfonamide resistance, all MDR isolates were found to carry sul1, whereas none carried sul2. The *aphA* and *aadA1* genes were detected in all seven MDR strains, while the *aac(6')-Ib* gene was found in only three of the seven MDR isolates (42.9%). Additionally, each of these strains carried *intI1* and *qacEΔ1* genes. The widespread occurrence of MDR *A. salmonicida* carrying diverse resistance genes in imported seafood presented a significant public health risk.

Keywords: *Aeromonas salmonicida,* Multidrug-resistant, Microarray, Public health, Seafood

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Research Paper

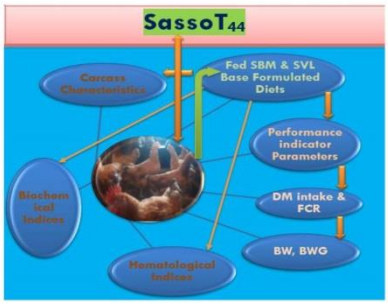
Hematological and Biochemical Indices and Performance of Sasso T44 Chicken Fed on Sweet Lupine-Based Formulated Feed

Tiruneh BB, Chekol YM, Limenih BA, and Yilkal S.

World Vet. J. 16(2): 617-630, 2026; pii:S232245682600059-16

DOI: https://dx.doi.org/10.54203/scil.2026.wvj59

ABSTRACT: Protein sources are essential components of poultry diets, with Soybean meal (SBM) being the most widely used due to its high protein content and balanced amino acid profile. However, its increasing cost and limited availability have encouraged the search for alternative plant protein sources such as sweet lupine (*lupinus angustifolius*). Dietary protein plays a crucial role in growth, performance, and blood profile parameters in determining the overall effectiveness of dietary treatments. Hematological and biochemical indices are valuable indicators of the physiological condition, metabolic activity, and health status of poultry. The current study aimed to evaluate the effect of replacing SBM with sweet vitabor lupine (SVL) in Sasso T_{44} dual-purpose chicken diets on growth performance and blood hematological and biochemical indices. A total of 180 chickens with five dietary treatments (Ts) were used. SBM was replaced by SVL at 0% (T1, control), 15% (T2), 25% (T3), 30% (T4), and 50% (T5). Each treatment had three replicates of 12 chickens and was fed for 90 days. Blood hematological parameters and biochemical indices of experimental chickens were measured on day 90. Results revealed that increasing SVL inclusion significantly affected the hematological, biochemical, and growth performance of experimental chickens. Live weight, carcass weight, and dressing percentage were significantly improved in T5 chickens compared to T1, T2, T3, and T4, indicating enhanced production performance with SVL inclusion. However, feed conversion efficiency remained statistically unchanged across treatments. Compared with the control diet (T1), chickens fed the highest SVL level (T5) had higher red blood cell counts and hemoglobin concentrations; however, despite the hematocrit value (36.18%) remaining within the normal standard reference range (25-45%), it was significantly lower. Diets T4 and T5 also resulted in higher total protein and lower uric acid and creatinine levels relative to the control, while glucose and triglycerides were unaffected; however, cholesterol was higher in T5. Overall, the results of the present study suggest that SVL may serve as a potential alternative plant protein source to partially replace SBM in diets of Sasso T_{44} dual-purpose chickens, with no observable negative effect on growth performance and almost all hematological and biochemical parameters.



Tiruneh BB, Chekol YM, Limenih BA, and Yilkal S (2026). **Hematological and Biochemical Indices and Performance of Sasso T_{44} Chicken Fed on Sweet Lupine-Based Formulated Feed.** *World Vet. J.*, 16(2): 617-630. DOI: https://dx.doi.org/10.54203/scil.2026.wvj59

Keywords: Biochemical indices, Hematological parameter, Growth performance, Sasso T_{44} chicken, Soybean meal, Sweet lupine

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Research Paper

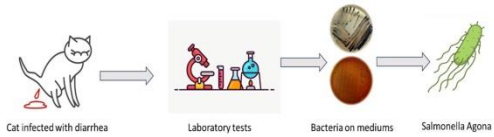
Molecular Detection of Virulence Genes of Salmonella Agona Isolated from Diarrheic Cats in Al-Anbar Province, Iraq

Ismail OA and Abdullah AH.

World Vet. J. 16(2): 631-639, 2026; pii:S232245682600060-16

DOI: https://dx.doi.org/10.54203/scil.2026.wvj60

ABSTRACT: *Salmonella* infections are a major cause of foodborne infection in both humans and animals. Some serotypes are capable of inducing severe clinical manifestations, including gastroenteritis, septicemia, and abortion. The present study aimed to identify, isolate, and detect *Salmonella* Agona in cats with diarrhea in Al-Anbar Governorate, Iraq, and to evaluate the antimicrobial resistance profiles of the recovered isolates. Between November 2024 and May 2025, 100 fecal samples were collected from cats of different breeds presenting with diarrhea at veterinary clinics in Iraq. These samples were processed using established culture methods, including pre-enrichment in buffered peptone water followed by selective enrichment in tetrathionate broth. The enriched samples were then cultured on selective and differential media, including xylose lysine deoxycholate (XLD) agar and *Salmonella*-Shigella (SS) agar. For isolates identification, biochemical analyses, including catalase, indole, methyl red, Simmons citrate, oxidase, urease, lactose fermentation, triple sugar iron test, and phenylalanine, were performed, and the automated VITEK®2 test was used to confirm the results. The polymerase chain reaction targeted the *invA* gene to identify *Salmonella* species, followed by sequence analysis. Eventually, additional virulence genes were identified, including *Salmonella* enterotoxin (*stn*) and *Salmonella* outer protein E (*sopE*). The current results demonstrated that 4%



Ismail OA and Abdullah AH (2026). **Molecular Detection of Virulence Genes of *Salmonella* Agona Isolated from Diarrheic Cats in Al-Anbar Province, Iraq.** *World Vet. J.*, 16(2): 631-639. DOI: https://dx.doi.org/10.54203/scil.2026.wvj60

of the samples (4 out of 100) were positive for *Salmonella* Agona. Antibiotic susceptibility testing against 16 antimicrobial agents demonstrated that the isolates were resistant to eight antibiotics (50%), namely amoxicillin, streptomycin, cefotaxime, oxytetracycline, doxycycline, tetracycline, trimethoprim-sulfamethoxazole, and gentamicin. The virulence gene results indicated that 2 out of 4 isolates (50%) possessed the *sopE* gene, whereas only 1 isolate (25%) carried the *stn* gene. The current findings indicate that *Salmonella* Agona was not prevalent among cats presenting with diarrhea; however, the existence of highly virulent, multidrug-resistant strains represented a vital public health concern for both humans and animals.
Keywords: Feline, *invA* gene, Polymerase chain reaction, *Salmonella* Agona, Virulence gene

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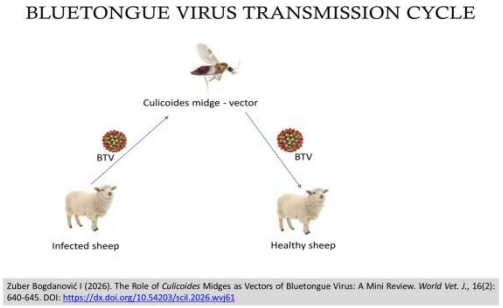
Review

The Role of *Culicoides* Midges as Vectors of Bluetongue Virus: A Mini Review

Zuber Bogdanović I.

World Vet. J. 16(2): 640-645, 2026; pii:S232245682600061-16

DOI: https://dx.doi.org/10.54203/scil.2026.wvj61



ABSTRACT: *Culicoides* biting midges (Ceratopogonidae) are the primary biological vectors of Bluetongue virus (BTV). The BTV is an orbivirus that causes substantial economic losses in livestock worldwide. Although over 50 arboviruses have been isolated from *Culicoides* species, BTV remains the most economically impactful disease. The present study aimed to evaluate the vector-virus association, including vector competence, transmission dynamics, and environmental factors influencing the spread of BTV. Vector competence in *Culicoides* is determined by midgut infection and dissemination barriers, which vary significantly among species. Transmission efficiency is influenced by temperature, extrinsic incubation period, and host factors, with cattle serving as long-term reservoirs and sheep exhibiting greater disease severity. Understanding vector-virus associations is essential for predicting outbreaks and developing effective control methods, especially as climate change promotes vector spread.
Keywords: Arbovirus, Bluetongue virus, *Culicoides*, Transmission, Vector competence

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Review

Single Nucleotide Polymorphisms of the Growth Hormone Gene and Their Association with Growth Traits in Sheep: A Systematic Review

Salka K, Nurgiartiningsih VMA, As A, Akram S, Novianti I, Marjuki M, and Suyadi S.

World Vet. J. 16(2): 646-659, 2026; pii:S232245682600062-16

DOI: https://dx.doi.org/10.54203/scil.2026.wvj62

ABSTRACT: Growth hormone (GH) is synthesized and secreted by the pituitary gland. It is regulated by the *GH* gene, located on chromosome 11 in sheep, and is crucial for regulating postnatal growth and development in sheep. Single-nucleotide polymorphisms (SNPs) in the *GH* gene are associated with substantial differences in weight and body size in sheep. This study aimed to systematically evaluate SNPs of the *GH* gene and their association with growth parameters such as weaning weight (WW), yearling weight (YW), average daily gain (ADG), and linear body conformation in sheep. A total of 71 studies were extracted from January 2011 to March 2026. The results identified 25 distinct SNPs (with 15 SNPs reported on exon 5), of which five SNPs, A781G, A1544G, C1765A, T1772A, and G1769C, were detected in more than one breed. Eighteen SNPs were found to be significantly associated with growth traits; these associations were not uniform, as individual SNPs were linked to single or multiple traits, including body weight, birth weight, pre-weaning gain, weaning weight, 6-month weight, daily live weight gain, yearling weight, and morphometric traits. In conclusion, polymorphism at the *GH* gene influences growth traits in sheep, and several SNPs such as C1776G, A1544G, A1678G, A1558G, C1765A, G1550A, T1772A, G1769C, G1756C, 476G>A, 480 G>A, 55G>A, G871A, G1383A, A1509G, A781G, C408G and T735A associated with growth traits are promising candidate markers after validation in larger breed-specific and trait-specific validation.
Keywords: Breeding program, Growth hormone gene, Marker-assisted selection, Sheep growth, Single nucleotide polymorphism

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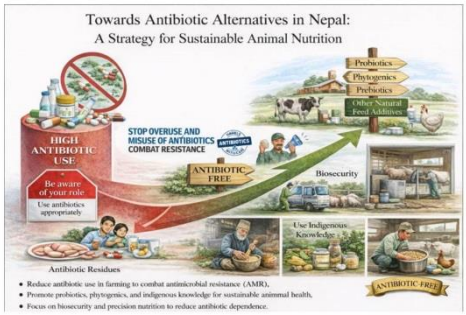
Review

Use of Antibiotic Alternatives in Nepal: A Strategy for Sustainable Animal Nutrition

Acharya A and Pandey U.

World Vet. J. 16(2): 660-670, 2026; pii:S232245682600063-16

DOI: https://dx.doi.org/10.54203/scil.2026.wvj63



Acharya A and Pandey U (2026). **Use of Antibiotic Alternatives in Nepal: A Strategy for Sustainable Animal Nutrition**. *World Vet. J.*, 16(2): 660-670. DOI: https://dx.doi.org/10.54203/scil.2026.wvj63

ABSTRACT: The routine use of antibiotics in animal nutrition has historically improved growth and productivity. However, the continued and often indiscriminate use of these compounds has accelerated the emergence of antimicrobial resistance (AMR), posing serious risks to animal health, food safety, and public health. Nearly three-quarters of antimicrobials are consumed by livestock, allowing resistant pathogens to cross animal, human, and environmental boundaries and undermining the concept of One Health. The present study aimed to assess sustainable feed-based alternatives to antibiotics and their effectiveness in enhancing animal health in the Nepalese context. Based on global evidence and national experiences, the review synthesizes the roles of probiotics, prebiotics, phytobiotics, organic acids, enzymes, precision feeding, and complementary management practices in maintaining animal performance while reducing reliance on antibiotics. Nepal presents both urgency and opportunity for this transition, as livestock production remains largely smallholder-based, regulatory enforcement is uneven, and antimicrobial residues continue to be detected in animal-derived foods. At the same time, increasing awareness, ethno-veterinary knowledge, emerging feed innovations, and recent policy commitments under the national AMR action framework signal positive momentum. The review suggests that replacing antibiotics with alternatives should not be viewed as a simple substitution but rather as a system-level transformation integrating nutrition, biosecurity, vaccination, farmer capacity building, and governance within a One Health framework. Strengthening locally adaptable, cost-effective solutions through field-based research and extension will be critical for sustaining productivity while contributing to national and global efforts to mitigate AMR.
Keywords: Antimicrobial resistance, Feed additive, Sustainable livestock production, One Health

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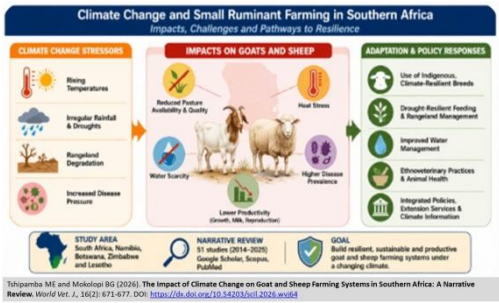
Review

The Impact of Climate Change on Goat and Sheep Farming Systems in Southern Africa: A Narrative Review

Tshipamba ME and Mokolopi BG.

World Vet. J. 16(2): 671-677, 2026; pii:S232245682600064-16

DOI: https://dx.doi.org/10.54203/scil.2026.wvj64



Tshipamba ME and Mokolopi BG (2026). **The Impact of Climate Change on Goat and Sheep Farming Systems in Southern Africa: A Narrative Review.** *World Vet. J.*, 16(2): 671-677. DOI: https://dx.doi.org/10.54203/scil.2026.wvj64

ABSTRACT: Smallholder goat and sheep farming is a critical component of rural livelihoods in Southern Africa. Climate change has, however, aggravated the situations of drought, heat stress, and disease burdens, which threaten the productivity of small ruminants. The present review aimed to determine the multidimensional effects of climate change on goat and sheep farming in Southern Africa, to analyze existing adaptation measures, and identify policy measures to build resilience. The current review used Google Scholar, Scopus, and PubMed to cover peer-reviewed articles and grey literature published between 2014 and 2025. Based on the inclusion criteria, 102 articles were initially screened, and 51 articles were considered for the narrative review. The review focused on studies conducted in South Africa, Namibia, Botswana, Zimbabwe, and Lesotho. The results indicated a strong relationship between climate variability and decreased ruminant productivity, driven by pasture degradation, water shortages, heat stress, and limited access to veterinary services in Southern African countries. Additionally, the results showed that adaptive strategies include the use of indigenous breeds, drought-resistant feeding, and ethnoveterinary practices to cope with climate variations. The present narrative review highlights the importance of adaptive strategies and integrated policies in Southern African countries for sustaining the development of small ruminant farming amid climate variability.
Keywords: Climate change, Heat stress, Rangeland management, Small ruminant

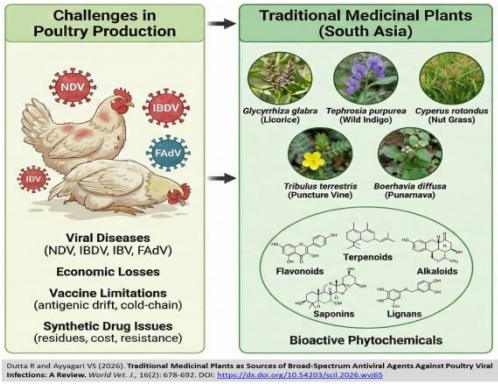
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Review

Traditional Medicinal Plants as Sources of Broad-Spectrum Antiviral Agents Against Poultry Viral Infections: A Review

Dutta R and Ayyagari VS.

World Vet. J. 16(2): 678-692, 2026; pii:S232245682600065-16
DOI: https://dx.doi.org/10.54203/scil.2026.wvj65



ABSTRACT: Across Asia, poultry farming plays a critical role in ensuring food security and supporting rural communities, delivering affordable protein to billions of people. Sustainable poultry production in Asia, especially in India, has been consistently affected by viral diseases. Conventional interventions, including vaccination and biosecurity protocols, often remain inadequate against viral infections, require specific storage conditions, elicit suboptimal immune responses, and pose economic challenges and limitations. In poultry, synthetic antiviral medications are often unsuitable due to their high cost, adverse effects, and concerns about residual compounds. Plant-based remedies emerge not as direct replacements but as valuable alternatives to consider for developing broad-spectrum antiviral solutions. The present study aimed to evaluate the antiviral potential of South Asian ethnomedicinal plants against Newcastle disease virus, infectious bursal disease virus, infectious bronchitis virus, and fowl adenovirus. Compounds such as flavonoids, terpenoids, alkaloids, saponins, and lignans were targeted for their multitarget activities, including inhibition of viral entry and polymerases, immunomodulation, and antioxidant properties. Different studies on *Glycyrrhiza glabra*, *Tephrosia purpurea*, *Cyperus rotundus*, *Tribulus terrestris*, *Boerhavia diffusa*, *Azadirachta indica*, *Andrographis paniculata*, and *Ocimum sanctum* reported promising therapeutic potential against poultry viral diseases. Available *in vitro* studies demonstrated strong antiviral activity across multiple phytochemical classes. Nevertheless, *in vivo* validation and implementation at the field level remain substantially limited. The current findings confirmed the strong potential of ethnomedicinal plants as sustainable, multi-target antivirals for poultry health.
Keywords: Flavonoid, Fowl adenovirus, Infectious bronchitis virus, Infectious bursal disease virus, Saponin, Terpenoid

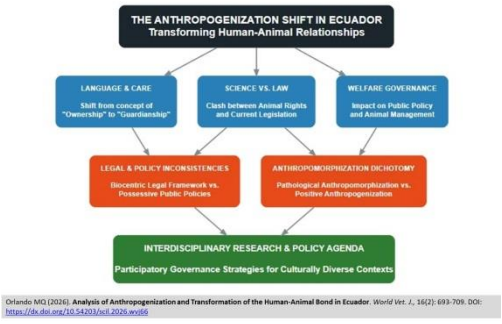
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Review

Analysis of Anthropogenization and Transformation of the Human-Animal Bond in Ecuador

Orlando MQ.

World Vet. J. 16(2): 693-709, 2026; pii:S232245682600066-16
DOI: https://dx.doi.org/10.54203/scil.2026.wvj66



ABSTRACT: The human-animal bond is currently experiencing a profound paradigm shift globally. The present study addresses the growing transformation of human–animal relationships in contemporary societies. The current study aimed to offer a critical narrative review of the anthropogenization of companion animals in Ecuador, understood as the process through which dogs, cats, and other domestic species acquire social, normative, and moral meanings that transform interspecies coexistence. Based on an interdisciplinary review of academic databases, regulatory documents, and institutional reports, the study integrates evidence from cognitive ethology, human–animal psychology, law, and urban sociology. Three central axes are analyzed. The first axis examined the impact of language, particularly ownership versus guardianship, on care practices. The second axis explores the tension between scientific evidence on animal agency and current regulations. The third axis evaluates the implications of anthropogenization for animal-welfare governance in diverse territorial contexts. The analysis identified contradictions between the biocentric constitutional framework and secondary legislation that retains possessive registers. The findings also revealed the simultaneous emergence of pathological anthropomorphization and positive anthropogenization processes. In addition, significant empirical gaps were identified for the design of coherent public policies. The study proposes an interdisciplinary research agenda and presents recommendations aimed at aligning terminology, education, and governance, with emphasis on territorial strategies and participatory approaches. Overall, the manuscript provides a conceptual framework to guide applied studies and regulatory reforms in countries characterized by sociocultural heterogeneity, such as Ecuador.
Keywords: Animal ethic, Animal right, Animal abuse, Anthropogenization, Education, Guardianship

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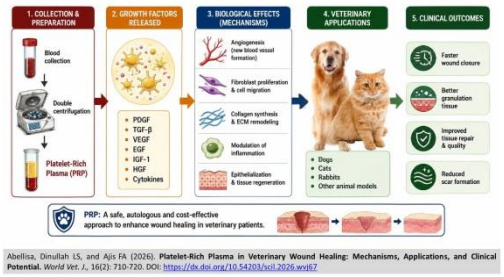
Review

Platelet-Rich Plasma in Veterinary Wound Healing: Mechanisms, Applications, and Clinical Potential

Abellisa, Dinullah LS, and Ajis FA.

World Vet. J. 16(2): 710-720, 2026; pii:S232245682600067-16

DOI: https://dx.doi.org/10.54203/scil.2026.wvj67



ABSTRACT: Wound healing is a significant challenge in both human and veterinary medicine, particularly in cases involving trauma, infection, or chronic tissue damage. Platelet-rich plasma (PRP), a product derived from the patient's own blood and enriched with growth factors and cytokines, has gained interest for its potential to promote regeneration and enhance healing. The present study aimed to evaluate current evidence on the application of PRP and its derivatives in wound healing, with a specific focus on veterinary animal models, and to identify key mechanisms, therapeutic outcomes, and existing knowledge gaps. While the present study highlighted veterinary applications, the included studies also consisted of rodent models, in vitro experiments, and human cell lines, providing broader mechanistic insights. A structured narrative review was conducted using 25 peer-reviewed articles published between 2024 and 2025. The selected studies included controlled trials, in vitro experiments, systematic reviews, and concept papers on PRP, platelet-rich fibrin, plasma rich in growth factors, and emerging formulations such as induced pluripotent stem cell-derived platelets and protein-rich PRP matrices. Key data related to study design, subjects, intervention types, outcomes, and theoretical frameworks were extracted and analyzed. All studies confirmed the positive role of PRP in promoting angiogenesis, collagen formation, epithelialization, and inflammation modulation. Evidence from veterinary models, including feline trauma cases and animal-derived PRP, supported the clinical applicability of PRP in wound healing. Several studies have demonstrated that the PRP approach is a valuable and effective treatment method in clinical practice, applicable to patients and animals. The PRP proved to be an efficacious intervention for cutaneous wounds, including surgical wounds, traumatic injuries, and chronic ulcers. However, differences in PRP preparation methods and outcome assessment criteria hinder direct comparisons across studies. Recent findings indicated new mechanisms involving telocytes and the regulation of vascular genes. It has been indicated that PRP and its derivatives are promising biological therapies for improving wound healing in veterinary practice.
Keywords: Growth factor, Platelet-rich plasma, Tissue repair, Veterinary medicine, Wound healing

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Review

Potential of Tropical Feed Resources for Inclusion in Commercial Livestock Diet: A Mini Review

Demlie M, Beyero N, and Kefyalew A.

World Vet. J. 16(2): 721-732, 2026; pii:S232245682600068-16

DOI: https://dx.doi.org/10.54203/scil.2026.wvj68



ABSTRACT: The demand for locally accessible, reasonably priced, and environmentally resilient alternative feed resources has increased due to rapid population growth, climate variability, land degradation, and growing competition for land between human food and animal feed. To address this problem, the utilization of emerging feed resources in the tropics is important. Therefore, the objective of this review was to identify emerging topical feed resources, nutritional potential, limitations, and integration techniques for commercial livestock production. Trees and shrubs, improved legumes and grasses such as lablab and alfalfa, insect-based proteins, aquatic feeds, root and tuber crops, agro-industrial by-products, fruit and vegetable wastes, and fermented or biotechnologically enhanced feeds are just a few of the many emerging feeds that were found to be valuable options. Emerging feeds partly fill the gap in feed supply, reduce competition for food between humans and animals, lower feed costs, and contribute to self-sufficiency in nutrients from locally available feed sources. Despite the high potential of emerging feeds, their utilization remains limited due to several interrelated constraints, including the presence of anti-nutritional factors, high fiber content and low digestibility, variable and inconsistent nutrient composition, poor palatability, high moisture content, rapid spoilage, and seasonal availability. Additional barriers include inadequate processing and preservation technologies, lack of infrastructure and equipment, limited farmer knowledge and technical skills, weak extension services, and insufficient research-based feed standards. Consequently, these factors result in inconsistent feed quality and reduced economic returns for commercial livestock producers. The review highlighted that appropriate processing techniques such as drying, chopping, grinding, ensiling,

fermentation, urea or ammonia treatment, heat treatment, enzyme supplementation, and mineral fortification are required to utilize emerging feeds. In conclusion, emerging feed resources represent a viable pathway toward sustainable, cost-effective, and climate-resilient livestock production in the tropics. Strengthening studies, improving processing and preservation technologies, enhancing extension services, and building farmer capacity are essential to unlock their full potential and ensure their successful adoption in commercial livestock feeding systems.
Keywords: Diet, Emerging feed, Integration strategy, Livestock, Nutritional factor

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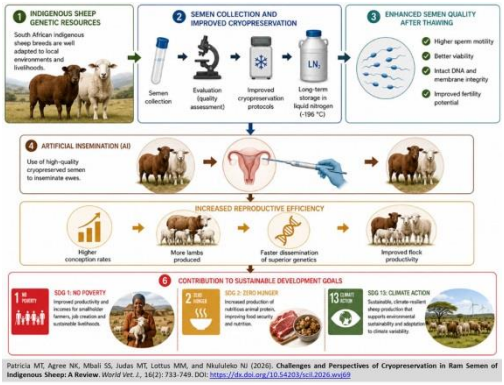
Review

Challenges and Perspectives of Cryopreservation in Ram Semen of Indigenous Sheep: A Review

Patricia MT, Agree NK, Mbali SS, Judas MT, Lottus MM, and Nkululeko NJ.

World Vet. J. 16(2): 733-749, 2026; pii:S232245682600069-16

DOI: https://dx.doi.org/10.54203/scil.2026.wvj69



ABSTRACT: Artificial insemination (AI) is a widely used technique to enhance genetic diversity and breeding efficiency in livestock such as cattle, sheep, goats, and pigs. Sheep are important in developing the livelihoods of poor farmers by reducing poverty and promoting sustainable agriculture and food security. Therefore, the present study aimed to explore the challenges and perspectives associated with cryopreserving ram semen to conserve indigenous sheep ecotypes. Indigenous sheep populations are vital for meat production in challenging environmental conditions, where feed is limited and land is scarce. However, concerns have emerged about the decline in indigenous sheep populations, mainly due to the growth of composite breed production. Composite breeds, which are preferred for their faster growth rates and larger body size, reduce the capacity of indigenous sheep to survive in environments affected by climate change. This decline highlighted the importance of assisted reproductive technologies, such as AI and semen cryopreservation, in preserving the genetic resources of indigenous sheep before they become extinct. While AI and semen cryopreservation have been successful in livestock species such as cattle and pigs for genetic preservation, results in sheep have been less successful. Ram semen quality and fertility are often compromised by the freezing and thawing process. Ram spermatozoa produce high levels of reactive oxygen species (ROS) and are sensitive to cold shock, leading to oxidative stress, cellular damage, and conception rates of 30-50%. Adding antioxidants, such as polyunsaturated fatty acids and plant extracts, to semen extenders can reduce ROS production and mitigate oxidative damage, thereby improving sperm quality and functionality during cryopreservation. Despite these advances, optimizing freezing and thawing protocols and addressing knowledge gaps are necessary to enhance semen preservation outcomes.
Keywords: Antioxidant, Artificial insemination, Extender, Nanoparticle, Sheep, Semen cryopreservation

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Review

Artificial Intelligence Applications in Small Animal Medicine: The Current Status, Key Challenges, and Future Prospects with Special Emphasis on Blockchain Innovation

Shiri F, Shahinzadeh A, Heidari A, Safaie A, Ghanbari S, Safari N, Atazadeh AH, and Simab PA.

World Vet. J. 16(2): 750-764, 2026; pii:S232245682600070-16

DOI: https://dx.doi.org/10.54203/scil.2026.wvj70



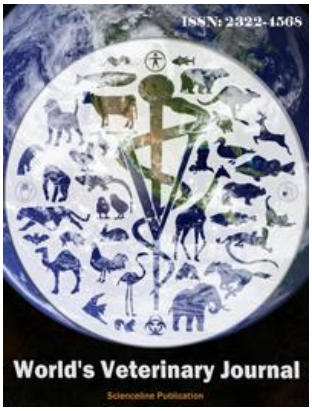
ABSTRACT: Artificial intelligence (AI) and machine learning are rapidly transforming companion animal veterinary medicine. The present narrative review examined current applications, challenges, and future directions for AI in the care of dogs, cats, and other companion animals. The current study is based on a structured literature search across PubMed/MEDLINE, Scopus, and IEEE Xplore from January 2015 to April 2026, with clearly defined inclusion and exclusion criteria to ensure transparency and reproducibility. In diagnostic imaging, computer vision algorithms improve the speed and accuracy of interpreting radiographs, ultrasound, and dermatoscopic images, helping detect conditions such as hip dislocation, osteoarthritis, foreign bodies, skin lesions, and parasites. In digital pathology, AI aids in identifying abnormal cells from blood and histology slides, supporting diagnoses such as lymphoma and anemia. Wearable devices with AI algorithms continuously monitor physiological data, including activity, sleep, and early signs of pain. Natural language processing (NLP) extracts information from electronic health records to facilitate epidemiological studies. Despite this promise, several challenges remain. Technical hurdles include the

need for large, high-quality annotated datasets, which are harder and more costly to obtain than in human medicine, and the high genetic diversity among dog and cat breeds, which makes it difficult to develop generalizable algorithms. Ethical and legal issues involve liability for diagnostic errors, data privacy, and algorithmic bias, requiring new regulatory frameworks. Practical barriers include high costs, integration into clinical workflows, and the need to train veterinarians to effectively use and critically evaluate AI outputs. Future progress depends on close collaboration among AI specialists, veterinarians, biologists, and industry. Priorities include developing cost-effective, user-friendly tools; creating internationally shared, standardized databases; and establishing ethical and professional guidelines. Blockchain may serve as a trust layer for recording and verifying data access events, rather than as a primary repository for large-scale medical data. Ultimately, AI is expected to act as a powerful assistant, not a replacement for veterinary expertise, enabling earlier diagnoses, personalized treatments, improved preventive care, and a better quality of life for companion animals and their owners.
Keywords: Artificial intelligence, Companion animal, Diagnostic imaging, Machine learning, Veterinary medicine

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Anti-inflammatory and Subacute Safety Effects of Pomegranate (*Punica granatum)* Rind Extract in Recurrent Carrageenan-Induced Inflammation in BALB/c Mice

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ABSTRACT

Pomegranate (*Punica granatum*) contains a high level of polyphenols and has anti-inflammatory properties. However, there is limited available data on the safety and effectiveness of pomegranate use during prolonged inflammatory conditions. The present study aimed to evaluate the anti-inflammatory properties and subacute toxicity profile of a 70% ethanolic extract of *Punica granatum* (*P. granatum*) rind in carrageenan-induced mice. A total of 30 male mice, aged five weeks and weighing 22-23 grams, were randomly assigned to five groups of six mice each. The study included a negative control group that received normal saline, a positive control group that received meloxicam at 2 mg/kg, and three treatment groups that received *P. granatum* rind extract at doses of 50, 100, and 200 mg/kg orally over 28 days. Inflammation was induced once weekly in all experimental groups by 1% carrageenan injection into the right hind paw, after which the edema was measured with a digital vernier caliper. The toxicity of the pomegranate extract was assessed using hematological and biochemical profiles of hepatic and renal parameters. Tissue samples from the heart, spleen, kidney, lungs, and liver were collected on day 29. To assess the inflammation status, concentrations of tumor necrosis factor-alpha (TNF-α) and IL-10 were quantified using enzyme-linked immunosorbent assay. The *P. granatum* rind extract significantly reduced paw edema without mortality or observable toxicity, compared to the negative control group. Mice maintained their normal feeding habits, and body weight increased normally across all groups. Hematological parameters, hepatic, and renal markers were within the normal physiological range across all groups. Histopathological analysis revealed no abnormalities in extract-treated groups. Extract-treated groups demonstrated a dose-dependent elevation of IL-10, peaking at 200 mg/kg, and a reduction in TNF-α level across all doses, with the highest suppression observed at 100 mg/kg compared to the negative control. Daily oral administration of *P. granatum* rind extract for 28 days exhibited dose-dependent anti-inflammatory effects, as evidenced by reduced paw edema and modulation of cytokines. The absence of systemic toxicity across all tested doses suggested that *P. granatum* rind extract can be a safe therapeutic agent for treating inflammatory conditions.

Keywords: Anti-inflammatory, Biochemical marker, Carrageenan, Cytokine, Hematological parameter, Inflammation, *Punica granatum*

INTRODUCTION

Inflammation is a complex biological response that occurs in reaction to infection, chemical irritation, tissue damage, or immune system dysregulation. While acute inflammation is essential for healing, incomplete healing can lead to persistent tissue damage, which may contribute to chronic metabolic diseases, including type 2 diabetes and cardiovascular disease (Medzhitov, 2008; Chen et al., 2018). Chronic inflammation primarily involves the sustained release of inflammatory mediators, including pro-inflammatory cytokines, reactive oxygen species, and inducible enzymes such as cyclooxygenase-2 (COX-2; Mittal et al., 2014). Collectively, inflammatory mediators exacerbate tissue damage, thereby contributing to disease progression. Conventional anti-inflammatory therapies, including nonsteroidal anti-inflammatory drugs (NSAIDs), are effective in controlling inflammation; however, their long-term use is limited by adverse effects, necessitating the search for safer alternatives (Bindu et al., 2020; Schjerning et al., 2020; Sohail et al., 2023).

Natural products are popular as alternatives or complementary options for addressing inflammation. *Punica granatum* (pomegranate) has been extensively documented in the literature for its anti-inflammatory and antioxidant properties. These properties are primarily attributed to its abundant polyphenolic content, which includes ellagitannins, flavonoids, and the hydrolyzable tannin punicalagin (Lansky and Newman, 2007; Viuda-Martos et al., 2010; Aviram and Rosenblat, 2012). The rind of the fruit, which is often discarded as a by-product of juice production, is the tissue with the highest polyphenol content. The rind contains higher concentrations of punicalagins, ellagic acid, and other bioactive ellagitannins than the seeds (Ismail et al., 2012; Kandylis and Kokkinomagoulos, 2020; Singh et al., 2023). Findings

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from *in vitro* and *in vivo* studies have demonstrated that compounds derived from *Punica granatum* (*P. granatum*) rind can effectively regulate the expression and activity of key inflammatory mediators and pathways associated with oxidative stress (Ismail et al., 2012; Singh et al., 2023). Preclinical studies indicated that polyphenols derived from pomegranate peel can influence inflammatory and oxidative stress pathways, although the effects may vary depending on extract preparation, dosage, and study design (Singh et al., 2023).

Carrageenan-induced paw edema is a well-established model for evaluating anti-inflammatory agents in rodents, characterized by a biphasic inflammatory response, with the early release of histamine, serotonin, and bradykinin, followed by sustained production of cytokines and prostaglandins (Posadas et al., 2004; Woode et al., 2022). Repeated weekly administration of carrageenan extends the acute model to simulate recurrent inflammatory conditions, thereby offering a clinically relevant model for assessing sustained anti-inflammatory efficacy (Perrot et al., 1999).

There is considerable evidence supporting the anti-inflammatory effects of *P. granatum*; however, most studies have focused on short-term or acute outcomes. There was limited evidence concerning the long-term safety profile and biological efficacy of *P. granatum* during multiple dosing in chronic inflammation. The lack of data on subacute conditions limited the use of *P. granatum* in treating inflammation and antioxidant therapy. A comprehensive evaluation of inflammatory and toxic markers of *P. granatum* is required to determine whether ongoing administration of pomegranate extract is effective and safe. The present study aimed to evaluate the anti-inflammatory effects and the subacute safety of *P. granatum* rind ethanolic extract in mice using a recurrent carrageenan-induced inflammation model.

MATERIALS AND METHODS

Ethical approval

All the procedures used in the present study were reviewed and approved by the Mount Kenya University Institution Scientific Review Committee (ISER), Kenya (Ref: MKU/ISERC/5682). The present study was conducted in compliance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. All possible measures were taken to minimize animal suffering and distress during experimental procedures.

Punica granatum rind extract preparation

Fresh pomegranate fruits were collected from Karson Farm, Nyeri County, Kenya. Nine fruits were thoroughly rinsed with distilled water, the rinds were isolated and air-dried in the shade for three days, then freeze-dried for 48 hours. The dried rind powder (100 g) was extracted with 1 liter of 70% (v/v) aqueous ethanol at a 1:10 (w/v) solvent-to-solid ratio at 22°C ± 2 for 48 hours under continuous shaking (Sultana et al., 2009). The extract was filtered through Whatman filter paper, concentrated by rotary evaporation, and oven-dried, yielding 20 g of dried extract from 100 g of the initial powder (20% w/w extraction yield).

Animals and study design

A total of 30 male BALB/c mice, five weeks old, weighing from 22 to 23 g, were purchased from the Small Animal Facility for Research and Innovation (SAFARI), JKUAT, Kenya, and used as inflammatory disease models due to their well-characterized immune response and extensive use (Malm Tillgren et al., 2023). The mice were acclimatized in their cages for one week before the study began. The mice were maintained in clean plastic cages with a stainless-steel top grill that contained proper aeration and a 12-hour light/dark cycle (Larrosa et al., 2010). The cages were cleaned weekly to maintain a clean environment. The mice were provided with pelleted feed (Unga Feeds Limited, Nairobi, Kenya) and unrestricted access to water (Karwasra et al., 2019).

Pomegranate rind extract administration

For the subacute toxicity investigation, five groups, each with six mice, were designed. The first treatment group received 50 mg/kg of *P. granatum* rind extract, the second treatment group received 100 mg/kg of *P. granatum* rind extract, and the third treatment group received 200 mg/kg of *P. granatum* rind extract, according to the study of Patel et al. (2008) and Bhandary et al. (2013). Based on the study by Dudhgaonkar et al. (2006), meloxicam at 2 mg/kg was administered orally as the positive control, while normal saline at 10 mL/kg served as the negative control. Inflammation was induced once weekly for four weeks (28 days) using 1% carrageenan by injection into the right hind paw (Winter et al., 1962) to maintain a persistent inflammatory condition (Perrot et al., 1999). Paw swelling was measured hourly after carrageenan injection, for four hours, and at 4 hours post-treatment using a digital Vernier caliper. The reduction of edema percentage was calculated using Formula 1.

Edema reduction (%) = [(Negative control edema − Treated group edema) ÷ Negative control edema] × 100 (Formula 1)

where NC is the negative control group mean edema, T is the treated group mean edema, and edema is the mean paw thickness at 5:00 PM minus the mean paw thickness at 9:00 AM. Treatment was administered daily by oral gavage.

Clinical observations

The mice were observed throughout the 28-day study for any signs of toxicity, including ataxia, diarrhea, lacrimation, ptosis, tremors, convulsions, and coma (Parasuraman, 2011). The mice were monitored four hours after daily oral treatment. The body weights of the mice were measured at the beginning of the study and thereafter weekly to track the changes in body weight. Food and water intake were measured daily by weighing the remaining feed and recording the residual water volume per group.

Sample collection

On day 29, mice were humanely euthanized with carbon dioxide asphyxiation. Blood samples (1.8-1.9 mL) were collected via cardiac puncture into micro tubes containing ethylenediaminetetraacetic acid (EDTA) for hematological assessment and into plain tubes for biochemical analyses. Vital organs, including the spleen, lungs, liver, kidneys, and heart, were subjected to macroscopic evaluation. The organs were subsequently excised and fixed in 10% formalin for histopathological examination.

Histopathology

The excised organs were fixed in 10% formalin. A sample from each organ was dehydrated through a series of isopropanol dilutions (up to 100%), then cleared in xylene. The tissues were infiltrated with molten paraffin wax, then embedded and allowed to solidify on the cooling plate. The embedded blocks were sectioned at a thickness of 2 µm using a microtome. The tissue sections were stained with hematoxylin and eosin (H&E) following standard laboratory procedures (Fischer et al., 2008). The prepared slides were subsequently examined using a light microscope (Optika, Italy) fitted with a SwiftCam digital camera (Model SC-2003, Swift Optical Instruments, USA) at magnifications of 100x and 400x.

Hematological investigation

Whole blood samples (0.2-0.4 mL) were collected into micro EDTA tubes to prevent coagulation (Lippi et al., 2005) and analyzed using a Mindray hematology analyzer (BC-10, China). With 50 µL of blood sample, red blood cell count (RBC), hemoglobin concentration (HGB), red cell distribution width coefficient of variation, mean corpuscular hemoglobin (MCH), hematocrit (HCT), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular volume (MCV), red cell distribution width (coefficient of variation), and red cell distribution width standard deviation were assessed. The white blood cell (WBC) and platelet (PLT) parameters analyzed included total WBC count, lymphocyte count (LYMP), granulocyte count (GRAN), mid-sized cell count (MID), the respective percentages of LYMP%, MID%, and GRAN%, platelet count (PLT), platelet distribution width (PDW), mean platelet volume (MPV), and PCT. Reference ranges for interpreting hematological parameters were set by the Mindray BC-10 hematology analyzer (Mindray, China) and established during factory calibration for laboratory mice.

Biochemical and ELISA analyses

The blood (1.5 mL) was collected into plain tubes, left to clot at room temperature for 30 minutes, and thereafter centrifuged at 3,500 rpm for 10 minutes to obtain serum (Diehl et al., 2001; Parasuraman et al., 2010). The sera were used for subacute toxicity, biochemical, and immunohistochemical assays and were analyzed using the Architect biochemistry automated analyzer (model c16000, Abbott, USA). The levels of albumin, alanine aminotransferase (ALT), potassium, bilirubin (total), gamma glutamyl transferase, protein (total, albumin, and globulin), direct bilirubin, chloride ion, Alkaline phosphatase, sodium, aspartate aminotransferase (AST), calcium, creatinine (serum, fluid), blood urea nitrogen, for liver and kidney function were evaluated. The ELISA was used to assess tumor necrosis factor-alpha (TNF-α) levels using the Mouse TNF-α Sandwich ELISA kit (Proteintech, USA; catalog no: KE10002), and interleukin-10 (IL-10) levels were assessed using the Mouse IL-10 Sandwich ELISA kit (Proteintech, USA; catalog no: KE10103). Reference ranges for interpreting serum biochemical parameters were predefined by the Abbott Architect c16000 chemistry analyzer (Abbott Laboratories, Abbott Park, USA), established through factory calibration for laboratory mice.

Statistical analysis

Statistical analysis of the collected data was performed using GraphPad Prism software version 8.02. (263). All data collected, including hematological analysis, biochemistry, ELISA, body weights, and food and water consumption, were analyzed as mean ± standard error (SEM). Analysis of variance (ANOVA) was used, followed by Dunnett's multiple

comparisons test, to determine statistically significant differences between the control and treatment groups. The significance level was set at a p-value less than 5% ($p < 0.05$).

RESULTS

Water and feed intake

There were no significant differences in water and feed consumption between the treated groups and the control group ($p > 0.05$; Figure 1).

Body weight gain

All experimental groups exhibited a normal, gradual increase in body weight over the 28-day study period, with no statistically significant differences observed between any treatment group and the negative control group ($p > 0.05$; Figure 2). At baseline, the mean body weights were 22.8 g, 22.5 g, 22.7 g, 22.3 g, and 22.2 g for the negative control, 50 mg/kg, 100 mg/kg, 200 mg/kg, and positive control groups, respectively. By day 28, the mean body weights were 25.4 g, 25.9 g, 25.5 g, 24.9 g, and 25.0 g for the negative control, 50 mg/kg, 100 mg/kg, 200 mg/kg, and positive control groups, respectively.

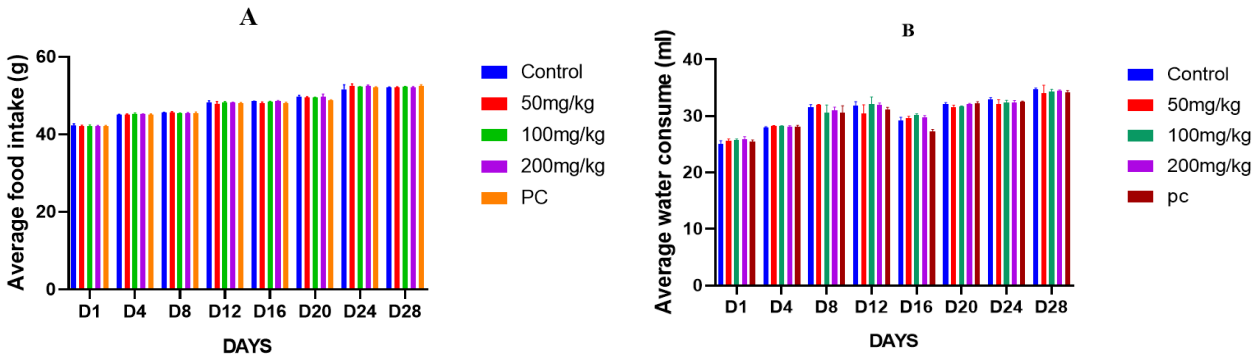


Figure 1. Effects of pomegranate rind extract on feed and water intake during 28 days under a subacute condition. There was no significant difference between the negative control and all groups ($p < 0.05$). **A:** Effects of pomegranate rind extract on the average feed intake. **B:** Effects of Pomegranate rind extract on the average water intake. Control: Negative control group, experimental groups: 50 mg/kg, 100 mg/kg, and 200 mg/kg of the pomegranate rind extract, PC: Positive control group.

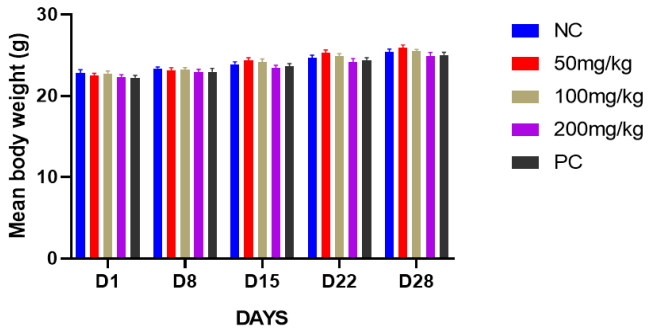


Figure 2. Mean body weight changes of mice subjected to different dosages of the pomegranate rind extract during 28 days under a subacute condition. There was no significant difference between the negative control and all the groups ($p < 0.05$). NC: Negative control group, experimental groups: 50 mg/kg, 100 mg/kg, and 200 mg/kg of the pomegranate rind extract, PC: Positive control group.

Hematological parameters

The results regarding the extract's impact on hematological parameters are presented in Table 1. Daily oral intake of pomegranate rind extract did not result in significant differences in most of the evaluated parameters ($p > 0.05$). However, a significant increase in red cell distribution width standard deviation (RDW-SD) was observed in mice treated with 200 mg/kg of pomegranate rind extract compared with the negative control (0.0384).

Biochemical parameters

Levels of liver and kidney function markers after treatment with 50 mg/kg, 100 mg/kg, and 200 mg/kg of the pomegranate rind extract are presented in Table 2. There were no significant differences between the treatment groups and the negative control group ($p > 0.05$). All hepatic, renal, and electrolyte markers remained within established physiological reference ranges for laboratory mice.

Table 1. The hematological results of mice treated with pomegranate rind extract for 28 days

Parameters	Control	50 mg/kg	100 mg/kg	200 mg/kg	PC	P-value
White blood cell count (10^9/L)	6.56 ± 1.22[a]	6.73 ± 0.52[a]	7.46 ± 0.68[a]	6.63 ± 0.42[a]	8.20 ± 0.74[a]	0.2392
Lymphocyte count (10^9/L)	2.96 ± 0.77[a]	3.30 ± 1.20[a]	1.43 ± 0.49[a]	2.20 ± 0.41[a]	2.20 ± 0.24[a]	0.1540
Absolute mid-range cell count (10^9/L)	0.73 ± 0.24[a]	0.40 ± 0.08[a]	0.56 ± 0.23[a]	0.40 ± 0.28[a]	0.43 ± 0.16[a]	0.5169
Absolute granulocyte count (10^9/L)	6.00 ± 0.35[a]	3.86 ± 1.24[a]	3.36 ± 0.97[a]	3.23 ± 0.69[a]	5.80 ± 0.73[a]	0.0974
Lymphocyte percentage (%)	36.80 ± 1.76[a]	34.33 ± 1.24[a]	43.66 ± 2.05[a]	43.66 ± 2.04[a]	35.66 ± 6.23[a]	0.1404
Mid-range cells (%)	8.46 ± 0.94[a]	7.50 ± 1.39[a]	6.80 ± 1.33[a]	7.03 ± 1.81[a]	7.10 ± 0.85[a]	0.7251
Granulocytes (%)	52.80 ± 2.56[a]	55.30 ± 3.85[a]	52.56 ± 3.36[a]	62.03 ± 3.95[a]	61.96 ± 2.04[a]	0.0575
Red blood cell count (10^{12}/L)	3.83 ± 0.35[a]	4.53 ± 0.73[a]	4.83 ± 0.24[a]	4.53 ± 0.57[a]	2.36 ± 0.16[b]	0.0023
Hemoglobin (g/dL)	16.83 ± 0.44[a]	15.10 ± 0.61[a]	14.86 ± 1.12[a]	16.60 ± 0.22[a]	13.26 ± 1.15[b]	0.0007
Hematocrit (%)	46.00 ± 4.06[a]	39.90 ± 0.71[a]	41.00 ± 2.40[a]	45.03 ± 0.87[a]	41.90 ± 1.68[a]	0.1012
Mean corpuscular volume (fl)	81.26 ± 0.89[a]	83.36 ± 0.79[a]	84.46 ± 3.24[a]	80.53 ± 2.12[a]	68.83 ± 1.27[b]	< 0.0001
Mean corpuscular hemoglobin (pg)	26.76 ± 0.59[a]	27.83 ± 0.41[a]	28.40 ± 0.10[a]	28.43 ± 0.43[a]	26.36 ± 2.17[a]	0.2363
MCHC (g/dL)	35.16 ± 0.71[a]	36.73 ± 0.85[a]	36.13 ± 0.63[a]	36.26 ± 0.72[a]	36.50 ± 0.35[a]	0.2770
RDW-CV (%)	12.46 ± 0.89[a]	14.10 ± 0.66[a]	13.66 ± 0.41[a]	12.33 ± 1.20[a]	13.73 ± 0.41[a]	0.1391
RDW-SD (fl)	40.73 ± 0.97[a]	42.96 ± 0.94[a]	44.26 ± 0.82[a]	45.53 ± 2.06[b]	45.10 ± 1.79[b]	0.0384
Platelet count (10^9/L)	400.66 ± 1.69[a]	410.66 ± 3.68[a]	415.00 ± 4.08[a]	396.00 ± 3.00[a]	432.66 ± 13.91[a]	0.0035
Mean platelet volume (fl)	8.76 ± 0.41[a]	8.93 ± 0.33[a]	10.30 ± 0.45[a]	9.86 ± 0.25[a]	7.60 ± 1.77[a]	0.5427
Platelet distribution width (fl)	16.90 ± 1.65[a]	15.43 ± 0.32[a]	15.30 ± 0.43[a]	16.00 ± 0.77[a]	15.53 ± 0.32[a]	0.3681
Platelet large cell ratio (%)	32.60 ± 2.57[a]	30.36 ± 2.02[a]	30.96 ± 1.56[a]	34.53 ± 0.57[a]	31.43 ± 1.96[a]	0.2576

MCHC: Mean corpuscular hemoglobin concentration, RDW-CV: Red cell distribution width (coefficient of variation), RDW-SD: Red cell distribution width (standard deviation). PC: Positive control. Values are expressed as mean ± SEM. [ab] Different superscript letters in a row indicate significant differences ($p < 0.05$).

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Table 2. Biochemical results of the mice treated with pomegranate rind extract for 28 days

Parameters	Control	50mg/kg	100mg/kg	200mg/kg	PC	P-value
Alkaline phosphatase (U/L)	70.33 ± 1.69[a]	73.66 ± 1.69[a]	73.66 ± 1.69[a]	65.33 ± 2.05[a]	72.00 ± 2.80[b]	0.0002
Alanine aminotransferase (U/L)	43.33 ± 1.24[a]	41.66 ± 1.69[a]	41.23 ± 0.89[a]	42.33 ± 1.69[a]	40.66 ± 5.90[b]	0.0276
AST (U/L)	35.66 ± 0.94[a]	32.66 ± 1.24[a]	33.00 ± 0.81[a]	33.66 ± 1.25[a]	35.00 ± 0.81[b]	0.0011
GGT (U/L)	48.00 ± 0.81[a]	45.66 ± 2.49[a]	51.00 ± 0.81[a]	49.66 ± 1.69[a]	46.00 ± 0.81[a]	0.0206
Albumin (g/L)	42.40 ± 1.42[a]	42.33 ± 1.24[a]	43.66 ± 1.69[a]	42.93 ± 1.35[a]	43.23 ± 1.67[a]	0.8805
Total protein (g/L)	77.56 ± 1.23[a]	79.80 ± 3.82[a]	74.56 ± 1.03[a]	76.23 ± 0.65[a]	76.46 ± 1.13[a]	0.1757
Urea (mmol/L)	7.10 ± 0.71[a]	7.70 ± 0.57[a]	7.40 ± 0.69[a]	7.26 ± 0.20[a]	7.13 ± 0.16[a]	0.7870
Creatinine (mmol/L)	100.00 ± 2.16[a]	99.66 ± 2.49[a]	101.00 ± 2.16[a]	101.00 ± 1.63[a]	103.00 ± 0.81[a]	0.5024
Sodium (mmol/L)	150.33 ± 1.24[a]	147.33 ± 1.69[a]	147.00 ± 0.81[a]	147.00 ± 0.81[a]	148.33 ± 0.94[a]	0.0723
Potassium (mmol/L)	3.90 ± 0.08[a]	3.90 ± 0.08[a]	3.86 ± 0.12[a]	3.83 ± 0.12[a]	4.06 ± 0.26[a]	0.6001
Chloride (mmol/L)	103.33 ± 8.99[a]	101.33 ± 6.18[a]	98.33 ± 2.86[a]	99.66 ± 2.49[a]	129.66 ± 3.29[b]	0.0009
Total bilirubin (mmol/L)	17.53 ± 0.49[a]	17.30 ± 0.37[a]	17.73 ± 0.46[a]	18.10 ± 0.16[a]	17.53 ± 0.44[a]	0.4146
BILD (mmol/L)	< 1.80	< 1.80	< 1.80	< 1.80	< 1.80	

AST: Aspartate aminotransferase, GGT: Gamma-glutamyl transferase, BILD: Direct bilirubin (conjugated bilirubin), PC: Positive control. The values are expressed as mean ± SEM (n = 6). [ab]Different superscript letters in a row indicate significant differences from the negative control ($p < 0.05$).

Paw edema reduction

Carrageenan injection produced a marked increase in paw thickness across all groups at 4 hours post-injection. Following treatment administration, paw thickness at 4 hours post-treatment was presented as a percentage of edema reduction relative to the negative control (Table 3).

Tumor necrosis factor alpha levels

Serum TNF-α concentrations differed significantly across the experimental groups ($p < 0.0001$; Figure 3). The negative control group recorded the highest concentration of TNF-α at 410 pg/ml. The treatment group administered 50 mg/kg of pomegranate rind extract recorded a TNF-α level of 327.3 pg/mL, whereas the 100 and 200 mg/kg groups recorded 132.3 and 235.7 pg/mL, respectively. In addition, the level of TNF-α in the positive control group was 139.7 pg/mL. All the treatment groups and the positive control group exhibited significantly lower levels of TNF-α than the negative control ($p < 0.05$; Figure 3).

Interleukin-10 levels

All extract-treated groups demonstrated significantly higher IL-10 levels than the negative control group ($p < 0.05$), with the 50 mg/kg group at 134.7 pg/mL, the 100 mg/kg group at 258.0 pg/mL, and the 200 mg/kg group at 367.3 pg/mL. Furthermore, the IL-10 level in the positive control group was measured at 120.0 pg/mL (Figure 4).

Macroscopic evaluation

Macroscopic evaluation revealed that organs from the extract-treated groups and the positive control group exhibited no structural alteration. No observable differences among organs were detected in mice receiving 50 mg/kg, 100 mg/kg, or 200 mg/kg of pomegranate rind extract compared with the negative control group.

Histopathological parameters

Histological examination of the spleen, kidneys, heart, lungs, and liver revealed preserved structural organization in the negative control and extract-treated groups, with no pathological alterations (Figure 5). The positive control group revealed mild centrilobular hepatocyte vacuolation in the liver and mild tubular epithelial cell vacuolation in the kidneys.

Table 3. Percentage edema reduction at 4 hours post-treatment during four weeks of weekly carrageenan induction in mice treated with *Punica granatum* rind extract

Treatment group	Week 1	Week 2	Week 3	Week 4
Negative control	0.0%	0.0%	0.0%	0.0%
PRE 50 mg/kg	25.3%	37.8%	40.2%	42.5%
PRE 100 mg/kg	51.9%	58.5%	61.0%	67.5%
PRE 200 mg/kg	73.4%	75.6%	84.1%	86.2%
PC	84.8%	89.0%	90.2%	98.8

PRE: *Punica granatum* rind extract, PC: Positive control

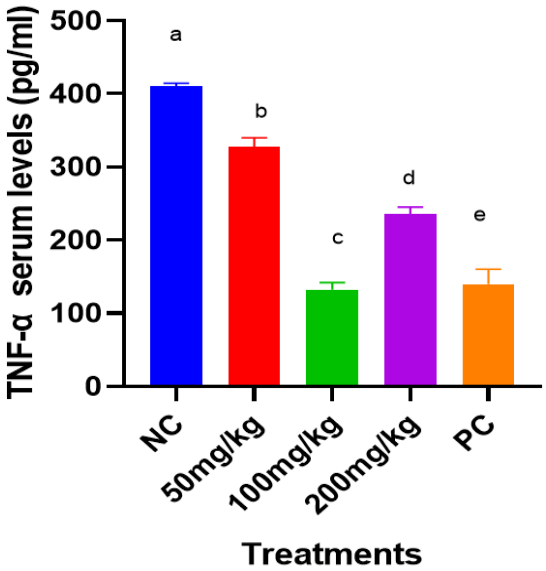


Figure 3. Serum TNF-α concentrations in mice following 28 days of oral administration of *Punica granatum* rind extract. Values are expressed as mean ± SEM (n = 6). [abc] Different superscript letters above bars indicate statistically significant differences from the negative control ($p < 0.0001$). NC: Negative control, PC: Positive control.

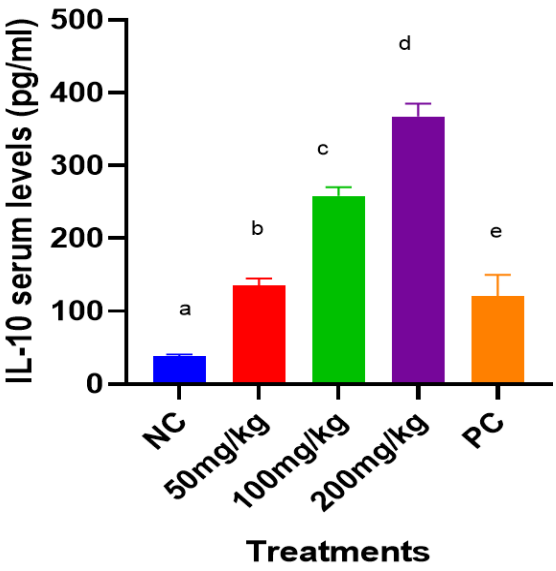


Figure 4. Serum IL-10 concentrations in mice following 28 days of oral administration of *Punica granatum* rind extract. Values are expressed as mean ± SEM (n = 6). [abcd] Different superscript letters above bars indicate statistically significant differences from the negative control ($p < 0.0001$). NC: Negative control, PC: Positive control.

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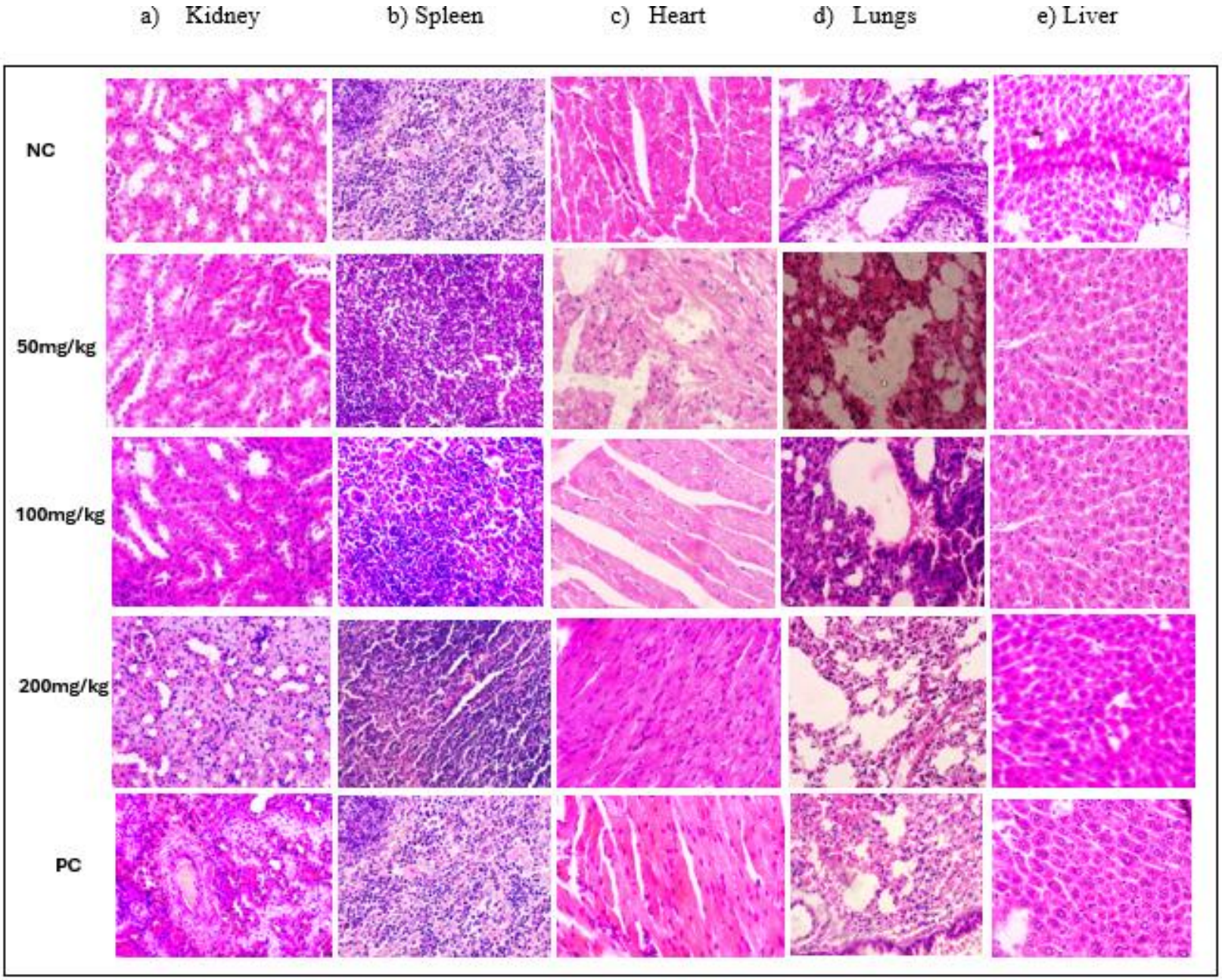


Figure 5. Microscopic evaluation of vital organs in mice that received *Punica granatum* rind extract collected on day 29. **a:** Renal sections from the negative control and extract-treated groups displayed normal cortical and medullary architecture with intact glomerular and tubular structures. The positive control group exhibited mild tubular epithelial cell vacuolation, **b:** Splenic sections across all groups showed preserved follicular architecture with distinct red and white pulp regions and no observable abnormalities, **c:** Cardiac tissue sections across all experimental groups demonstrated intact myocardial fibers with no evidence of cellular disruption or inflammatory infiltration, **d:** Pulmonary sections revealed a well maintained alveolar architecture with no structural compromise observed in any group, **e:** Hepatic sections from the negative control and all extract-treated groups showed normal lobular organization with well-preserved hepatocytes. Mild centrilobular hepatocyte vacuolation was noted in the positive control group (H&E, magnification 400x).

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DISCUSSION

According to the current findings, exposure to *P. granatum* extract did not result in any signs of systemic toxicity, and the extract was well tolerated throughout the study (Widyarini et al., 2023). Posadas et al. (2004) demonstrated that carrageenan injection into the rodent paw produced a biphasic response, characterized by the early release of mediators followed by prolonged cellular infiltration. Multiple administrations of carrageenan prolong the inflammatory response, resulting in a recurrent inflammatory condition that affects physiological functions and tissue integrity in animal models (Komisarska et al., 2024). Evaluating the safety profile of therapeutic agents during chronic inflammatory conditions provides a more clinically relevant assessment than studies conducted on healthy subjects without inflammatory stress. An increase in paw thickness indicated immune cell infiltration along with the buildup of inflammatory exudates at the injection site. The observed increase in the paw thickness following carrageenan administration in the present study was consistent with the characteristic inflammatory response of the carrageenan-induced paw edema model in rodents (Posadas et al., 2004). In the current study, post-carrageenan injection and paw thickness increased progressively across all the groups, confirming inflammation induction. Paw edema reduction was dose-dependent across all four weeks, with each week showing more reduction than the previous week. Peak edema was recorded at 4 hours post-induction, consistent with the cellular phase of the carrageenan inflammatory response, which occurs between the third- and fifth hours post-injection (Widyarini et al., 2023). The progressive improvement in edema reduction suggested a cumulative anti-inflammatory effect with repeated *P. granatum* rind extract administration. The current findings were consistent with those of Karwasra et al. (2019), who also observed a dose-dependent decrease in carrageenan-induced inflammation after pomegranate supplementation. Meloxicam (2 mg/kg) produced the greatest edema reduction, reaching up to 98.8% by week four. Although 200 mg/kg of pomegranate rind extract did not have the efficacy of meloxicam, its dose-dependent cumulative anti-inflammatory effects, along with its favorable safety profile, indicated that pomegranate rind extract could be a potential alternative therapy. The mild anti-inflammatory effects observed at lower doses align with the bioavailability of the polyphenols, indicating that higher concentrations are necessary for greater inflammation suppression (Singh et al., 2023).

Daily administration of pomegranate rind extract exhibited no fatalities or morbidity related to toxicity during the entire study period. To assess the extract's toxicity, changes in physical appearance, feeding behavior, behavioral shifts such as lethargy or aggression, and gastrointestinal disturbances should be observed (Parasuraman, 2011); none of the signs were observed in any groups treated with pomegranate extract during the present study. The current findings indicated that daily administration of the pomegranate ethanol extract did not affect appetite or cause weight loss. The results of the current study are consistent with previous subacute toxicity studies of *P. granatum* peel extract in Swiss albino mice (Bassiri Jahromi et al., 2015) and with the study by Vale et al. (2020) in mice receiving hydroethanolic peel extract at doses up to 2000 mg/kg. The lack of notable differences in feed and water intake in the group that received meloxicam at 2 mg/kg aligns with the well-documented minimal dose-dependent gastrointestinal effects of NSAIDs at the specified dose of meloxicam (Botta et al., 2001; Richardson et al., 2022). Meloxicam is known to be associated with gastrointestinal disturbances at high doses (Botta et al., 2001; Richardson et al., 2022). Under subacute conditions in the present study, there were no substantial changes in feeding habits. Gastrointestinal toxicity associated with meloxicam administration in mice, including intestinal lesions and gastric ulcerations, has been reported at higher doses of 15-20 mg/kg (Botta et al., 2001; Richardson et al., 2022). The meloxicam dose administered in the present study was insufficient to induce gastrointestinal disturbances under subacute conditions. The treated mice maintained their normal feeding behaviors, and their body weights increased consistently, comparable to the control group. The findings of the current study suggested that prolonged exposure to pomegranate rind extract did not adversely affect normal growth, alter feeding behavior, or induce physiological changes that could compromise the overall health of Balb/c mice. Furthermore, macroscopic examination of the liver, spleen, heart, lungs, and kidneys from extract-treated mice demonstrated no visible abnormalities compared to the control group.

Histopathological evaluation supported the safety profile of the pomegranate rind extract, as no structural alterations were observed in the mice's tissues. Normal tissue structure was preserved across all treated groups, indicating that, under the subacute experimental conditions of the present study, the extract did not induce toxicity. The findings of the current study are particularly relevant to sustained inflammatory stress, in which failure to resolve chronic inflammation is often associated with tissue damage and organ injury (Medzhitov, 2008; Komisarska et al., 2024). Mild centrilobular hepatocyte vacuolation, indicating intracellular accumulation, was observed in the liver of the positive control group. In the kidney, mild vacuolation of tubular epithelial cells was observed. The histopathological observations were supported by liver and kidney biochemical markers in the present study.

The kidneys and liver are key organs for detoxifying the body, as they metabolize harmful substances and facilitate their removal (Wurihan et al., 2022). Consequently, assessing serum-based, organ-specific biochemical markers associated with the kidneys is essential for determining potential toxicity (Wurihan et al., 2022). Elevated serum levels of hepatic enzymes, particularly AST and ALT, along with renal waste products such as urea and creatinine, may indicate

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hepatic or renal tissue damage or necrosis, resulting in their release into the circulatory system. Therefore, increased levels of hepatic and renal markers are commonly used as indicators of hepatotoxicity or nephrotoxicity (Jegnie et al., 2023; Adeyele et al., 2024). In the present study, serum concentrations of ALP, AST, ALT, GGT, urea, creatinine, and electrolytes remained within established physiological reference ranges across all extract-treated groups, indicating that oral administration of *P. granatum* rind extract did not adversely affect hepatic or renal function in BALB/c mice. The findings of the present study are consistent with previous subacute toxicity investigations of pomegranate peel extract, in which renal biochemical markers remained within physiological reference ranges following oral administration, thereby supporting the safety of the pomegranate-derived extract for hepatic and renal health (Bassiri Jahromi et al., 2015; Vale et al., 2020). The TNF-α is a strong pro-inflammatory cytokine, involved in the recruitment of leucocytes and tissue injury (Mittal et al., 2014). In the present study, TNF-α was notably reduced in all extract-treated groups compared with the negative control group. The strongest suppression in TNF-α level was observed at a dosage of 100 mg/kg of pomegranate rind extract (132.3 pg/mL), which was similar to that of the meloxicam group (139.7 pg/mL). The IL-10, a cytokine involved in anti-inflammatory responses and tissue repair, increased in a dose-dependent manner across all extract-treated groups, surpassing the level observed in the meloxicam group. The contrasting regulation of TNF-α and IL-10 indicated a coordinated immunomodulatory effect of the pomegranate rind extract. Similar cytokine modulation patterns have been reported in mice administered pomegranate peel extract. Manickam et al. (2022) demonstrated that daily oral administration of pomegranate peel extract at 200 mg/kg for 12 weeks in Apoe$^{-/-}$ mice reduced TNF-α and increased IL-10, accompanied by decreased atherosclerotic plaque necrosis and improved collagen content in lesions. Similarly, Karwasra et al. (2019) found that pomegranate supplementation at 50, 100, and 200 mg/kg in experimental rheumatoid arthritis decreased inflammation and improved joint function by inhibiting NF-κB signaling. However, the observed nonlinear dose response in the present study, in which 100 mg/kg of pomegranate rind extract resulted in greater TNF-α suppression than 200 mg/kg, which was contrasted with the dose-dependent reduction reported in previous studies. The variations observed in these studies could be due to differences in the animal model, extract preparation, or the impact of recurrent inflammation on cytokine dynamics. Balanced cytokine regulation is essential for resolving inflammation. In chronic inflammatory models, such as sustained carrageenan exposure, cytokine imbalance can cause progressive tissue damage (Posadas et al., 2004).

Hematological parameters were assessed as part of the subacute toxicity evaluation, as changes in blood indices provide measurable evidence of potential systemic toxicity following repeated compound exposure (Jegnie et al., 2023). In the present study, hematological results demonstrated a notable difference in RDW-SD between the control and 200 mg/kg group. All values remained within established physiological reference ranges with no signs of systemic toxicity, indicating that these differences were not biologically adverse. The positive control group similarly exhibited significant differences in erythrocyte parameters, including RBC and hemoglobin. Carrageenan initiates a strong inflammatory response, resulting in increased pro-inflammatory cytokines and alterations in hematopoiesis (Posadas et al., 2004). Inflammatory stress alone was sufficient to alter blood parameters, including white blood cell counts, platelet counts, and red blood cell indices, without any direct toxic effects of the administered extract. Therefore, the notable differences could suggest an ongoing inflammatory condition rather than a direct toxic effect of the extract.

Toxicological interpretation should consider both the biological context and the observed change, rather than relying solely on statistical significance, when determining adverse effects (Organisation for Economic Co-operation and Development, 2002; EFSA Scientific Committee et al., 2017). Parameters within normal physiological limits are generally regarded as non-adverse, especially when no histopathological or biochemical abnormalities are present (Organization for Economic Co-operation and Development, 2002). As demonstrated in the present study, the absence of tissue damage and maintenance of normal body weight in all extract-treated groups supported that the extract was well tolerated under the subacute conditions.

CONCLUSION

The current findings demonstrated that repeated oral administration of pomegranate rind extract over 28 days produced dose-dependent anti-inflammatory effects. A progressive decrease in paw edema, along with suppression of TNF-α and an increase in IL-10, occurred in a dose-dependent manner. There were no mortalities, clinical signs of toxicity, treatment-associated histopathological changes, or adverse effects on hepatic and renal function, which suggested that the extract was well tolerated throughout the study period. The current findings supported the potential of *P. granatum* rind extract as a safe and effective therapeutic candidate for inflammatory conditions. Although the present study was conducted in male mice over a 28-day period, long-term toxicity studies including both sexes, as well as investigations of the underlying molecular mechanisms, are necessary to establish a comprehensive safety and efficacy profile for broader therapeutic application.

DECLARATIONS

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Authors' contributions

Twahafifwa Nghuuluka Kanemo Jonas was responsible for developing the study methodology, overseeing data curation, and preparing the original manuscript draft. Rebecca Wanjiku Waihenya and Daniel Wainaina Kariuki contributed to the critical revision and editing of the manuscript. All authors have read and approved the final edition of the manuscript before publication in the present journal.

Availability of data and materials

All data produced and examined in this study are included in the manuscript, with no supplementary files. Requests for additional data generated in this study should be directed to the corresponding author.

Competing interests

The authors declared no conflicts of interest.

Ethical considerations

The authors confirmed that the present study is original, has not been previously submitted for publication or under review elsewhere, and adheres to ethical standards regarding data integrity and the avoidance of plagiarism. The authors also affirmed that no artificial intelligence tools were used in the writing and preparation of the manuscript.

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Effects of Tryptophan Addition to Green Mussel (*Perna viridis*) on Cannibalism and Physiological Responses in Lobster (*Panulirus homarus*)

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ABSTRACT

The increasing demand for sand lobsters requires higher aquaculture productivity. However, sand lobster production is still constrained by high mortality due to cannibalism, a behavior in which individuals attack and consume conspecifics. One approach to suppress cannibalism is through supplementation of the amino acid tryptophan as a precursor to serotonin, which plays a role in regulating stress and aggressiveness. The present study aimed to determine the effectiveness and optimal formulation of tryptophan in green mussel feed on cannibalism, survival, and growth rate in sand lobsters. The study used a completely randomized design with four treatments and five replications. The treatments consisted of adding tryptophan to green mussels used as feed, including a control without tryptophan (K0), and supplementation at doses of 5 g/kg (T1), 10 g/kg (T2), and 15 g/kg (T3). Observed parameters included physiological responses, cannibalism levels, and growth. The results revealed that T3 treatment was most effective in increasing serotonin levels (89.45 ± 3.069 ng/mL) and survival rate (55.29 ± 5.26 %), and reducing cannibalism (7.06 ± 2.63 %), which caused lower glucose (14.19 ± 3.206 mg/dL) and total hemocyte count (81.00 ± 4.243 cell/mL) levels. However, the highest dose (T3; 15 g/kg) was less effective in enhancing growth performance, indicating that this level of supplementation was not optimal compared to the lower doses. In contrast, T1 (5 g/kg) resulted in relatively higher growth performance compared to the other treatments, as indicated by moulting frequency (1.40 ± 0.23%), carapace length (1.25 ± 0.15 cm), length increase (1.70 ± 0.12 cm), absolute weight (1.14 ± 0.35 g), specific growth rate (3.13 ± 0.46 %/day), feed conversion ratio (5.04 ± 0.93), and protein efficiency ratio (1.42 ± 0.18). However, in T1 (5 g/kg), serotonin (70.66 ± 4.165 ng/mL), survival rate (42.35 ± 4.92%), cannibalism (38.82 ± 3.22%), glucose (16.26 ± 3.352 mg/dL), and total hemocyte count (110.50 ± 9.192 cell/mL) were not as optimal as those observed in T3. Tryptophan supplementation revealed potential to enhance physiological condition, survival, and growth under the experimental conditions.

Keywords: Cannibalism, Growth, Lobster, Physiological, Tryptophan

INTRODUCTION

The sand lobster (*Panulirus homarus*) is a high-value food commodity whose global demand increases annually (Efrizal et al., 2025). Sand lobster is a leading fishery product that contributes significantly to national exports and the income of farmers and coastal communities in Indonesia (Zuhriyah et al., 2025). Currently, sand lobster is one of the most widely cultivated lobster species (Petersen et al., 2013; Kintani et al., 2020), with national production reaching 8,279 tons and a production value of IDR 1.94 trillion (PDSPKP, 2023). The application of sustainable cultivation technology supported by optimal feed management has the potential to increase sand lobster cultivation productivity in coastal areas (Effendi et al., 2025). However, lobster cultivation still faces different complex challenges, particularly related to physiological, behavioral, and environmental aspects, which directly impact lobster health and productivity (Sarkar et al., 2026). One of the main problems in lobster cultivation is the high mortality of seeds in the early stages of life, which has been reported to range from 96.0 to 99.4% (Subagio et al., 2021).

The high mortality of lobster larvae in the early stages of life is closely related to several management issues, including limited food availability, which triggers cannibalism among individuals (Kelly et al., 2023). Cannibalism is a crucial issue in lobster cultivation, particularly during the moulting phase, which leaves lobsters physiologically highly vulnerable to attack (Codabaccus et al., 2025). Furthermore, nutritional deficiencies, high population densities, and limited shelter also contribute to cannibalism (Ma et al., 2021; Kelly et al., 2023). Physiological stress can also result from degraded water quality, leading to higher mortality and slower growth (Spencer et al., 2025; Sarkar et al., 2026).

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Therefore, the development of effective technology and managerial approaches to suppress cannibalism and stress is needed, one of which is through the use of the amino acid tryptophan in feed formulations, which has the potential to play a role in suppressing stress responses and aggressive behavior in lobsters (Feng et al., 2024; Debnath et al., 2024).

Tryptophan is an essential amino acid that acts as the main precursor of serotonin (5-hydroxytryptamine, 5-HT), a neurotransmitter that has an important function in modulating physiological responses, stress, and aggressive behavior in crustaceans, including lobsters (Rathore and Sarkar, 2021; Dinu et al., 2022). Through the neuroendocrine signaling pathway, tryptophan influences the synthesis and secretion of serotonin, which is regulated by neuroendocrine cells sensitive to environmental signals, thereby regulating the function of the central and peripheral nervous systems and maintaining organism homeostasis (Liu et al., 2020). Enrichment of tryptophan in the feed enables lobsters to adapt better to stressful conditions, especially during critical phases such as moulting, which is often followed by increased aggression and cannibalism (Leopoldo et al., 2010). Additionally, tryptophan enrichment in the feed can suppress cannibalism and promote growth in freshwater lobsters (Trisnasari et al., 2020), vannamei shrimp (Rachmawati et al., 2021), and mangrove crabs (Suharyanto and Yudhistira, 2012). These findings highlight the need for further investigation into the effectiveness of dietary tryptophan supplementation in sand lobsters for enhancing growth and reducing cannibalism.

Green mussels (*Perna viridis*) are the primary natural feed commonly used in lobster cultivation due to their high availability and their rich in nutritional and bioactive compounds (Jusadi et al., 2020; Rejeki et al., 2021). Green mussels contain high levels of protein, lipids, and essential fatty acids, thus playing a vital role in supporting lobster growth and health (Chakraborty et al., 2016). Different studies have reported that lobsters fed green mussels exhibited better growth performance, indicated by increased carapace length, body length, and total weight compared to those fed trash fish or pellets (Arumugam et al., 2020; Rivaie et al., 2023). The addition of the amino acid tryptophan to green mussels is expected to address the mortality due to stress and cannibalism, especially during the critical phase of lobster cultivation. The present study aimed to determine the effects and optimal formulation of tryptophan in green mussels on cannibalism, survival rate, and growth parameters in sand lobsters.

MATERIALS AND METHODS

Ethical approval

The present study was approved by the Health Research Ethics Committee, Faculty of Medicine, Diponegoro University, Indonesia (No. 297/EC/KEPK/FK-UNDIP/X/2025). All procedures were conducted in accordance with established laboratory and field guidelines. Experimental animals were observed daily, and feeding was administered according to the respective treatment groups.

Study place and time

The study was conducted from October to November 2025 at the Fish Health and Environment Laboratory, Lampung Marine Aquaculture Center, and Central Research and Clinical Diagnostic Laboratory, Healthy Animals, Malang, East Java, Indonesia.

Study design

The study used an experimental method to evaluate the effectiveness of tryptophan-enriched green mussel feed at different doses over a 60-day period. The experiment was designed using a completely randomized design consisting of four treatments with five replications, including a control without tryptophan supplementation (K0) and tryptophan supplementation in green mussels at doses of 5 g/kg (T1), 10 g/kg (T2), and 15 g/kg (T3) of feed.

Sand lobster seed sample

The sand lobsters used in the present study were in the puerulus stage, with an average total length of 2.2-2.4 cm and an average weight of 0.2-0.4 g. The pueruli were obtained from fishermen or collectors in the Pesisir Barat Regency, Lampung Province, Indonesia, and a total of 340 individuals were selected, all healthy and unblemished. The animals were distributed into experimental units at a density of 17 individuals per container. For transportation, the pueruli were packed in plastic bags filled with seawater, equipped with two sheets of black netting (0.5 cm mesh size), supplemented with pure oxygen, and tightly sealed. The bags were then placed in Styrofoam boxes containing ice wrapped in paper to maintain temperature stability, then closed, wrapped in plastic, and labeled according to lobster seed packaging standards (BSNI, 2024).

Preparation of the study container

The present study used 20 square plastic containers measuring 70 cm × 45 cm × 35 cm, operated under a flow-through water system. Each container was filled to an operational water depth of approximately 25 cm, resulting in a total water volume of about 70-80 L per container. Each unit was equipped with two aeration points for oxygen supply and six outlet holes (1 cm in diameter), with three outlets on each side, which served as overflow drainage to maintain a stable water level and continuous water exchange. The inflow water rate was maintained at 3 L min^{-1}. Additionally, each

container was provided with two netting shelters inserted into ½-inch polyvinyl chloride (PVC) pipes to provide refuge for the pueruli.

Test feed preparation

The test feed was prepared according to the method of Suharyanto and Yudhistira (2012) using green mussel (*Perna viridis*) meat as the main ingredient. The green mussels were opened using a knife, then the meat was removed and roughly chopped using a blender. 30 g of green mussel meat was prepared for each treatment. Next, the amino acid tryptophan was added at the treatment dosages reported in previous studies, namely 5 g/kg (Leopoldo et al., 2010), 10 g/kg (Trisnasari et al., 2020), and 15 g/kg (Ardina, 2021). Progol was added at 0.15 g as an adhesive and 2 mL of distilled water as a diluent. All test ingredients were stirred until homogeneous and then mixed evenly with the green mussel meat. The prepared feed was placed in plastic bags, labeled according to the tryptophan dosage, and stored in a refrigerator at approximately 4˚C until use.

Distribution and maintenance of sand lobster seeds

Sand lobster puerulus seeds were stocked into plastic containers at a density of 55 per m², equivalent to 17 per container. Before the test feed, the lobster seeds were acclimatized for 7 days by feeding them green mussels without added tryptophan to aid environmental adaptation, reduce stress, and accustom them to the feed. After acclimatization, the pueruli were reared for 60 days and fed green mussel meat enriched with tryptophan according to the treatment dosage. The daily feeding rate was set at 30% of biomass (body weight day^{-1}; BBPBL, 2022), which was divided into three feeding times: 30% of the daily ration at 07:00, 20% at 12:00, and 50% at 17:00 Western Indonesia Time (WIB), considering the higher nocturnal feeding activity of lobsters. Uneaten feed was siphoned out prior to the next feeding to maintain water quality. During the rearing period, approximately 20% of the population (four individuals per container) was sampled every two weeks.

Physiological parameters of lobster

Total hemocyte count

Total hemocyte count (THC) is commonly used as an indicator of stress in crustaceans. In this study, THC in lobster hemolymph was measured on days 0 and 60 of the culture period. Hemolymph samples were collected using a 1 mL syringe pre-rinsed with 10% (w/v) citric acid solution, which functions as an anticoagulant to prevent hemocyte aggregation and clotting, and were drawn from the base of the walking legs near the abdomen (Domínguez-Borbor et al., 2018). A drop of hemolymph was then loaded onto a hemocytometer (Neubauer chamber; chamber depth 0.1 mm), covered with a coverslip, and observed under a light microscope at 400× magnification. Hemocyte counts were performed in five large squares of the counting chamber, corresponding to a known volume, and THC values were calculated using the following formula by Abdollahi-Arpanahi et al. (2018).

$$\text{THC} = \frac{\sum \text{Total hemocyte counted}}{\text{Volume Box}} \text{ x Dilution factor}$$

Hemolymph glucose levels

Hemolymph samples were collected with a syringe rinsed with anticoagulant, drawn from the base of the walking leg near the abdomen, and transferred to microtubes. Samples were incubated for 10-20 minutes at 20-25˚C before analysis. Hemolymph glucose levels were determined by the glucose oxidase-peroxidase (GOD-PAP) method (Barham and Trinder, 1972). Briefly, 10 µL of hemolymph was mixed with 1000 µL of reagent solution containing glucose oxidase, peroxidase, phenol, and 4-aminoantipyrine. A glucose standard (100 mg dL^{-1}) was prepared and processed identically. Reaction mixtures were incubated at 37˚C for 10 minutes, and absorbance was measured at 500 nm with a spectrophotometer (PerkinElmer, USA). Glucose concentration was calculated by the following formula through comparing the sample absorbance with that of the standard (Barham and Trinder, 1972).

KG [mg/dl] = 100×ΔASTDΔAsample

Hemolymph glucose level (KG) was calculated as the ratio of the sample absorbance (ΔA_sample) to the standard solution absorbance (ΔA_STD), multiplied by 100 to express the result in mg/dL. The absorbance values were obtained using a spectrophotometer at the specified wavelength, where ΔA_sample represents the absorbance of the hemolymph sample, and ΔA_STD represents the absorbance of the glucose standard solution.

Serotonin content

Serotonin testing was performed to evaluate lobster aggression, with higher serotonin levels associated with reduced aggression (Esmaeili et al., 2025). Observations were made by taking hemolymph samples on day 0 (1 sample before

treatment) and day 60 (4 samples after treatment). Serotonin levels were measured using an Enzyme-Linked Immunosorbent Assay (ELISA) method and expressed in ng/mL to assess the relationship between stress and changes in serotonin levels in each treatment.

Cannibalism level

Observation of cannibalism levels was conducted using Closed Circuit Television (CCTV) installed above the experimental containers, with recordings taken before and after feeding at 5-hour intervals during the first 14 days of rearing. Cannibalism events were identified based on direct visual evidence of aggressive interactions leading to the attack and consumption of conspecifics. Mortality was classified as cannibalism when individuals indicated clear physical damage, such as missing body parts (such as the abdomen and appendages) or bite marks, or when predation events were directly recorded by CCTV. In contrast, mortality without visible signs of injury or predation was classified as non-cannibalism-related death. The level of lobster cannibalism was calculated using the formula below of Hseu et al. (2003).

$$K=\frac{KA-KS-KBK}{KA}\times 100\%$$

The cannibalism rate (K) was calculated as the percentage of individuals lost due to cannibalism relative to the initial number of individuals (KA). In this formula, KA represents the initial number of lobsters at the beginning of the experiment, KS represents the number of surviving individuals at the end of the observation period, and KBK represents mortality not caused by cannibalism. Thus, the value of K reflects the proportion of mortality specifically attributed to cannibalistic behavior.

Moulting

Moulting is a physiological process in crustaceans involving the periodic shedding of the exoskeleton (exuviae) to allow growth and development. The moulting frequency of lobsters was calculated based on the following formula described by Handayani and Syahputra (2018).

$$MFq = \frac{X_{molt}}{N_{tot}}$$

Moulting frequency (MFq) was calculated as the ratio of the total number of moulting events (X_{molt}) to the total number of individuals observed during the study period (N_{tot}). X_{molt} represents the cumulative number of moulting events recorded, while N_{tot} is the total number of lobsters maintained throughout the experimental period. The result is expressed as the average number of moulting events per individual.

Lobster growth parameters

Absolute length growth

The absolute length growth of sand lobsters during the study was calculated using the formula of Effendie (1997).

$L = L_t - L_0$

Absolute length increase (L) was calculated as the difference between the final length (L_t) and the initial length (L_o) of the lobster during the culture period. The parameter L_t represents the length of the lobster at the end of the experiment, while L_o represents the length at the beginning of the experiment. The result is expressed in centimeters (cm).

Absolute weight gain

The absolute weight growth of sand lobsters during the study was calculated using the formula of Effendie (1997).

$W = W_t - W_0$

where W is the absolute weight gain (g), W_t is the average final weight (g), and W_0 is the average initial weight (g). The parameter represents the total increase in body weight during the experimental period.

Kerapas length

Carapace length was measured from the tip of the rostrum to the posterior margin of the carapace using a digital caliper, following Baskoro et al. (2019). Carapace length increment was calculated as the difference between final and initial measurements ($CL = CL_t - CL_o$). The initial carapace length of the lobsters at the beginning of the experiment ranged from 2.2 to 2.4 cm.

Specific growth rate

The specific growth rate is the daily percentage increase in lobster weight. The specific growth rate was calculated using the formula of Wu et al. (2024).

SGR (% day 1) = 100 × (ln (FBW) - ln (IBW)) / day

Specific growth rate (SGR) was calculated as the natural logarithmic difference between the final body weight (FBW) and the initial body weight (IBW) over the experimental period, expressed as a percentage per day (% day^{-1}). FBW represents the average body weight of lobsters at the end of the rearing period, while IBW represents the average body weight at the beginning of the experiment.

Survival rate

Survival rate (SR) is the ratio of the total number of lobsters that survive until the end of cultivation to the number of lobsters at the start of stocking. The survival rate was calculated using the formula of Santoso et al. (2024).

$$SR = \left[\frac{Nt}{No}\right] x\ 100\%$$

The survival rate was calculated as the percentage of individuals remaining at the end of the experiment relative to the initial number stocked. In this calculation, Nt represents the number of lobster seeds at the end of the study, while N_0 represents the number of seeds at the beginning of the study. The result is expressed as a percentage (%).

Feed conversion rate

The feed conversion ratio (FCR) of sand lobsters during maintenance was calculated using the formula of Zonneveld et al. (1991).

$$FCR = F / (Wt - Wo)$$

Feed conversion rate (FCR) was calculated as the ratio between the total feed provided (F) and the weight gain of lobsters during the study period. In this calculation, F represents the total amount of feed given (g), W_t represents the total weight of lobsters at the end of the study (g), and W_0 represents the total weight of lobsters at the beginning of the study (g).

Protein efficiency ratio

The protein efficiency ratio (PER) of sand lobsters during the rearing period was calculated based on Jafri (1999). Protein intake was determined by measuring the total feed consumption, calculated as the difference between the amount of feed offered and the uneaten feed collected after each feeding. The uneaten feed was carefully collected, dried, and weighed to obtain accurate estimates of consumption. The amount of protein intake was then calculated by multiplying the actual feed consumption by the crude protein content of the feed, as determined through proximate analysis. PER was subsequently calculated as the ratio between weight gain and total protein intake.

$$PER = Wg / P$$

Protein efficiency ratio (PER) was calculated as the ratio between total weight gain (Wg) and protein intake (P). In this calculation, Wg represents the total absolute weight gain of lobsters during the rearing period (g), while P represents the total amount of protein consumed (g), which was estimated from feed intake multiplied by the crude protein content of the feed.

Water quality

Water quality measurements were conducted throughout the maintenance period. Several parameters were measured, including temperature, pH, dissolved oxygen (DO), salinity, nitrite, and nitrate. The methods and water quality parameters measured during the study are shown in Table 1.

Table 1. Water quality parameters measured during the study

Parameter	Unit	Methods/tools	Measurement time
Temperature	˚C	Thermometer	once a week
pH	-	pH meter	once a week
Dissolved oxygen	mg/L	Dissolved oxygen meter	once a week
Salinity	Ppt	Refractometer	once a week
Nitrite	mg/L	Spectrophotometer	once a week
Ammonia	mg/L	Spectrophotometer	once a week

Data analysis

Data on total hemocyte count (THC), haemolymph glucose levels, serotonin, cannibalism levels, moulting frequency, carapace length, absolute length, absolute weight, specific growth rate, feed conversion ratio, survival rate, protein efficiency, and water quality during maintenance were tabulated using Microsoft Excel and statistically analyzed using IBM SPSS Statistics (version 25). A least significant difference (LSD) test was performed when significant differences among treatments were detected.

RESULTS

Physiological parameters

The results of the physiological response test revealed that all observed parameters had relatively similar initial values in all treatments (week 0), indicating that the lobsters' initial physiological state was homogeneous. The initial THC value was recorded at 203 ± 70.74 cells/mL, hemolymph glucose levels were 19.48 ± 1.075 mg/dL, and serotonin levels were 100.35 ± 11.667 ng/mL. The uniformity of these initial values indicated that the lobsters had not yet experienced the effects of the feed treatment and remained in a normal physiological state.

After feeding tryptophan-enriched green mussels for eight weeks of maintenance, different changes occurred in each physiological parameter. THC values experienced a significant decrease ($p < 0.05$) in all treatments compared to the initial conditions. The lowest THC values were found in the T3 and control treatments, at 81.00 ± 4.243 cells/mL and 91.50 ± 4.950 cells/mL, respectively, followed by the T1 treatment at 110.50 ± 9.192 cells/mL, while the highest value was found in T2 at 129.00 ± 7.071 cells/mL.

Contrary to THC parameters, hemolymph glucose levels did not differ significantly between treatments ($p > 0.05$), ranging from 14.05 ± 0.276 mg/dL to 20.54 ± 3.465 mg/dL. The THC results indicated that tryptophan supplementation does not directly affect energy metabolism, and lobsters are still able to maintain their physiological balance. Meanwhile, serotonin levels differed significantly among treatments ($p < 0.05$), with the lowest value observed in the control (56.37 ± 5.812 ng/mL), followed by T1 (70.66 ± 4.165 ng/mL). Treatments T2 (90.09 ± 6.463 ng/mL) and T3 (89.45 ± 3.069 ng/mL) were not significantly different from each other ($p > 0.05$), but both were significantly higher than the control and T1 treatments ($p < 0.05$). The results of the physiological parameters of the lobsters after treatment are shown in Table 2.

Table 2. Physiological parameters of lobsters before and after treatment of adding tryptophan to green mussel feed during the 60-day rearing period.

Parameter	Treatment	Observation time	
		Initial of maintenance (Week 0)	End of maintenance (Week 8)
Total hemocyt count (cell/mL)	K0	203 ± 70.74	91.50 ± 4.950[a]
	T1		110.50 ± 9.192[b]
	T2		129.00 ± 7.071[c]
	T3		81.00 ± 4.243[a]
Glucose (mg/dL)	K0	19.48 ± 1.075	14.05 ± 0.276[a]
	T1		16.26 ± 3.352[a]
	T2		20.54 ± 3.465[a]
	T3		14.19 ± 3.206[a]
Serotonin (ng/mL)	K0	100.35 ± 11.667	56.37 ± 5.812[a]
	T1		70.66 ± 4.165[b]
	T2		90.09 ± 6.463[c]
	T3		89.45 ± 3.069[c]

K0**:** Control (green mussels without tryptophan supplementation), T1**:** 5 g/kg, T2**:** 10 g/kg, T3**:** 15 g/kg

Cannibalism level

The results indicated significant differences in cannibalism rates among treatments ($p < 0.05$). The highest cannibalism rate was observed in the control treatment (47.06 ± 4.16%), followed by T1 (38.82 ± 3.22%) and T2 (34.12 ± 4.92%), which were not significantly different from each other ($p < 0.05$). The lowest cannibalism rate was recorded in T3 (7.06 ± 2.63%). A comparison of cannibalism rates among treatments is presented in Figure 1.

Moulting frequency

The moulting frequency of sand lobsters fed green mussels enriched with tryptophan revealed significant differences among treatments ($p < 0.05$). The highest moulting frequency was observed in T1 (1.40 ± 0.23 times per individual), followed by the control treatment (K0; 0.98 ± 0.30 times per individual). Lower moulting frequencies were recorded in T3 (0.64 ± 0.03 times per individual) and T2 (0.60 ± 0.06 times per individual). The moulting frequency of sand lobsters fed green mussels enriched with tryptophan is shown in Figure 2.

Carapace length

The results indicated that enrichment of green mussel feed with tryptophan had a significant effect on the carapace length of sand lobsters ($p < 0.05$). The highest carapace length values were observed in T1 (1.25 ± 0.15) and T2 (1.23 ± 0.11), which were not significantly different from each other ($p > 0.05$). Lower values were recorded in the control treatment (K0; 0.95 ± 0.13) and T3 (0.86 ± 0.15), which were also not significantly different from each other ($p > 0.05$). The results of carapace length growth measurements are presented in Figure 3.

Absolute length

The results indicated that enrichment of green mussel feed with tryptophan had a significant effect on the absolute length of sand lobsters ($p < 0.05$). The highest absolute length values were observed in T1 (1.70 ± 0.12) and T2 (1.70 ± 0.06), which were not significantly different ($p > 0.05$). Lower values were recorded in the control treatment (K0; 1.48 ± 0.13) and T3 (1.35 ± 0.17), which were also not significantly different from each other ($p > 0.05$). The results of absolute length measurements of sand lobsters are presented in Figure 4.

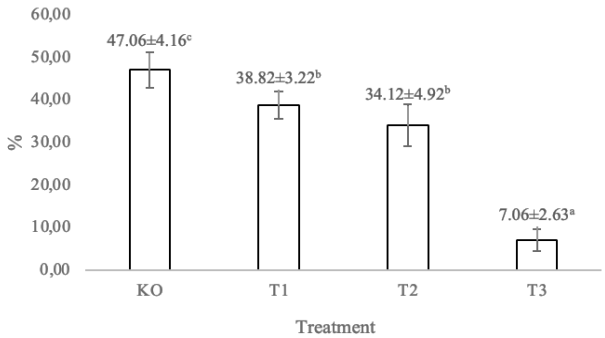


Figure 1. Cannibalism levels of sand lobsters during the 60-day rearing period fed with tryptophan-enriched green mussels at different doses. Treatments consisted of K0 (control, without tryptophan), T1 (5 g/kg), T2 (10 g/kg), and T3 (15 g/kg).

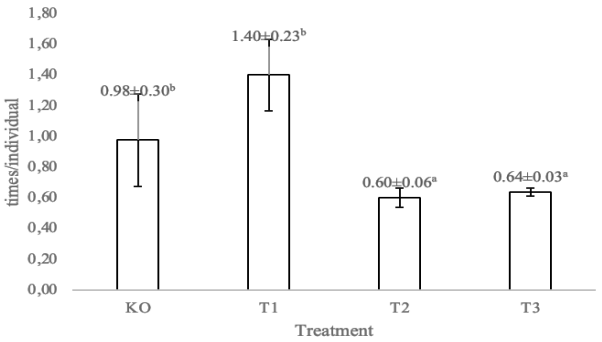


Figure 2. Moulting frequency of sand lobsters during the 60-day rearing period fed with tryptophan-enriched green mussels at different doses. Treatments consisted of K0 (control, without tryptophan), T1 (5 g/kg), T2 (10 g/kg), and T3 (15 g/kg).

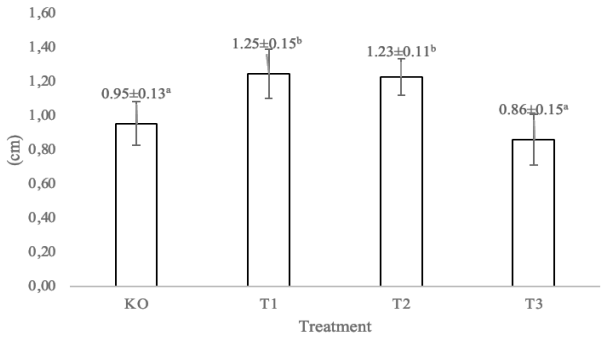


Figure 3. Carapace length of sand lobsters during the 60-day rearing period fed with tryptophan-enriched green mussels at different doses. Treatments consisted of K0 (control, without tryptophan), T1 (5 g/kg), T2 (10 g/kg), and T3 (15 g/kg).

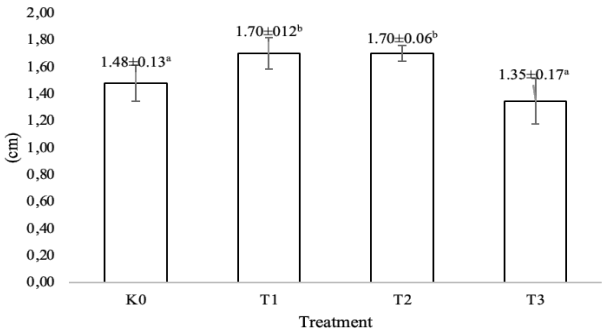


Figure 4. Absolute length of sand lobsters during the 60-day rearing period fed with tryptophan-enriched green mussels at different doses. Treatments consisted of K0 (control, without tryptophan), T1 (5 g/kg), T2 (10 g/kg), and T3 (15 g/kg).

Absolute weight

The results indicated that enrichment of green mussel feed with tryptophan had a significant effect on the absolute weight of sand lobsters ($p < 0.05$). The highest absolute weight values were observed in T1 (1.14 ± 0.35) and T2 (1.02 ± 0.15), which were not significantly different ($p > 0.05$). Lower values were recorded in the control treatment (K0; 0.68 ± 0.12) and T3 (0.60 ± 0.10), which were also not significantly different from each other ($p > 0.05$). The results of absolute weight measurements of sand lobsters are presented in Figure 5.

Specific growth rate

The results of the study revealed that enriching green mussel feed with tryptophan resulted in different specific growth rates among treatments. T1 (3.13 ± 0.46) and T2 (3.02 ± 0.22) were not significantly different from each other ($p > 0.05$), and both revealed higher values compared to the control (K0; 2.43 ± 0.26) and T3 (2.27 ± 0.23), which were also not significantly different from each other ($p > 0.05$). The results of specific growth rate are presented in Figure 6.

Feed conversion ratio

The results of the study indicated that enriching green mussel feed with tryptophan significantly improved the feed conversion ratio of sand lobsters ($p < 0.05$). The lowest feed conversion ratio values were observed in T1 (5.04 ± 0.93) and T2 (5.65 ± 0.54), which were not significantly different from each other ($p > 0.05$). The next highest values were recorded in the control treatment (K0; 6.43 ± 1.00), while the highest value was observed in T3 (7.69 ± 0.91). The feed conversion ratio values for sand lobsters are presented in Figure 7.

Protein efficiency ratio

The results indicated that enriching green mussel feed with tryptophan significantly affected the protein efficiency ratio (PER) of sand lobsters ($p < 0.05$). The highest PER value was observed in T1 (1.42 ± 0.18), which was significantly different from T3 (0.95 ± 0.08) ($p < 0.05$), but not significantly different from the control treatment (K0; 1.33 ± 0.20) ($p > 0.05$). The PER in T2 (1.17 ± 0.11) was not significantly different from K0 or T1 ($p > 0.05$), but was significantly higher than T3 ($p < 0.05$). Overall, T3 revealed the lowest PER value and differed significantly from the higher treatments ($p < 0.05$). The protein efficiency ratio values of sand lobsters are presented in Figure 8.

Survival rate

The results revealed that enriching green mussel feed with tryptophan significantly increased the survival rate of sand lobsters ($p < 0.05$). The highest survival rate values were observed in T3 (55.29 ± 5.26) and T2 (52.94 ± 7.50), which were not significantly different from each other ($p > 0.05$). These were followed by T1 (42.35 ± 4.92), while the lowest value was recorded in the control treatment (K0; 28.24 ± 7.67). The survival rate values of sand lobsters are presented in Figure 9.

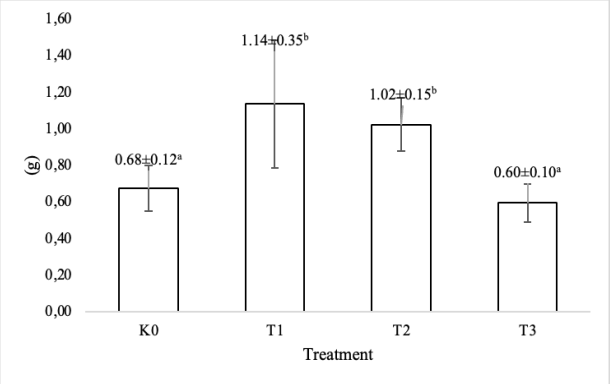


Figure 5. Absolute weight of sand lobsters during the 60-day rearing period fed with tryptophan-enriched green mussels at different doses. Treatments consisted of K0 (control, without tryptophan), T1 (5 g/kg), T2 (10 g/kg), and T3 (15 g/kg).

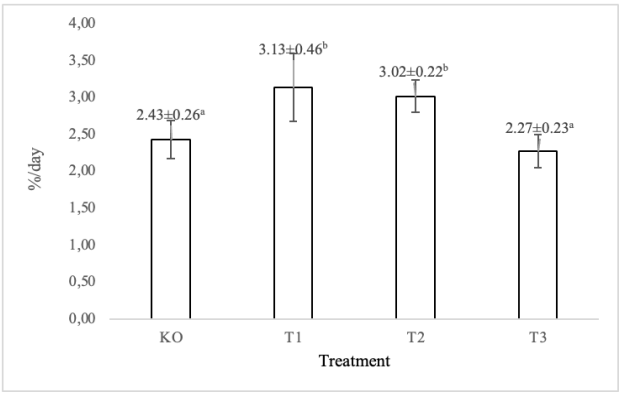


Figure 6. Specific growth rate of sand lobsters during the 60-day rearing period fed with tryptophan-enriched green mussels at different doses. Treatments consisted of K0 (control, without tryptophan), T1 (5 g/kg), T2 (10 g/kg), and T3 (15 g/kg).

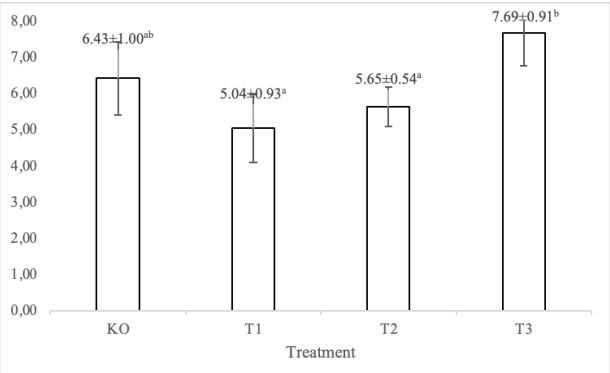


Figure 7. Feed conversion ratio values of sand lobsters during the 60-day rearing period fed with tryptophan-enriched green mussels at different doses. Treatments consisted of K0 (control, without tryptophan), T1 (5 g/kg), T2 (10 g/kg), and T3 (15 g/kg).

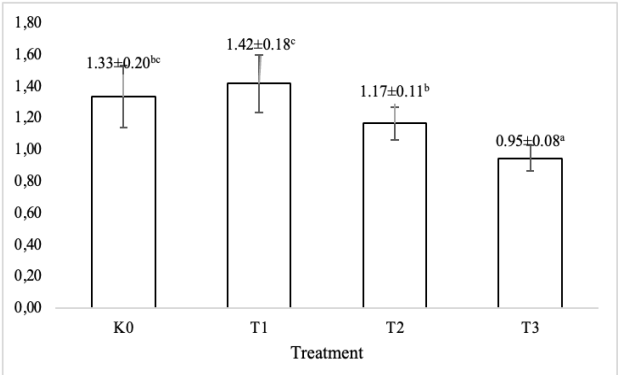


Figure 8. Protein efficiency ratio value of sand lobster during the 60-day rearing period fed with tryptophan-enriched green mussels at different doses. Treatments consisted of K0 (control, without tryptophan), T1 (5 g/kg), T2 (10 g/kg), and T3 (15 g/kg).

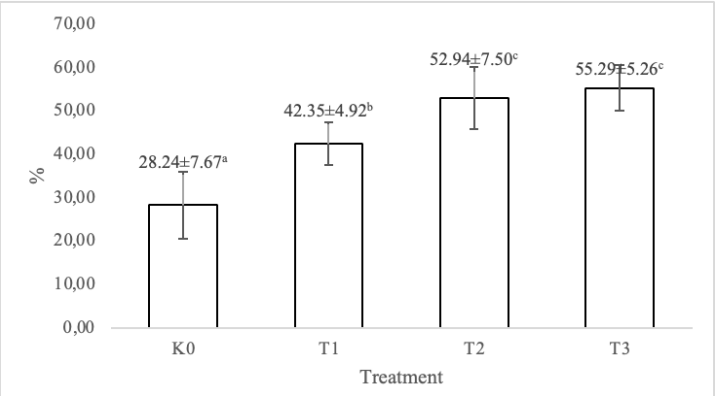


Figure 9. Survival rate of sand lobsters during the 60-day rearing period fed with tryptophan-enriched green mussels at different doses. Treatments consisted of K0 (control, without tryptophan), T1 (5 g/kg), T2 (10 g/kg), and T3 (15 g/kg).

Water quality

Water quality during the maintenance period ranged within values that remained optimal for sand lobster cultivation. The measured parameters included a temperature of 29.43-29.54˚C, pH 7.66-8.01, salinity 31.40-31.60 ppt, dissolved oxygen (DO) 5.98-6.07 mg/L, ammonia 0.11-0.12 mg/L, and nitrite 0.08 mg/L. The complete results of the water quality measurements are shown in Table 3.

Table 3. Average water quality parameters during the 60-day rearing period of sand lobsters

Parameter \ Treatment	Unit	K0	T1	T2	T3
Temperature	˚C	29.43 ± 0.315	29.45 ± 0.277	29.47 ± 0.286	29.54 ± 0.367
Dissolved Oxygen	(mg/L)	6.07 ± 0.075	6.02 ± 0.076	5.98 ± 0.087	6.02 ± 0.058
Salinity	(ppt)	31.40 ± 0.894	31.60 ± 0.894	31.40 ± 0.894	31.40 ± 0.894
pH	-	7.96 ± 0.084	7.96 ± 0.069	7.66 ± 0.708	8.01 ± 0.113
Ammonia	(mg/L)	0.12 ± 0.005	0.11 ± 0.002	0.12 ± 0.003	0.12 ± 0.003
Nitrite	(mg/L)	0.08 ± 0.001	0.08 ± 0.002	0.08 ± 0.002	0.08 ± 0.001

K0: Control (green mussels without tryptophan supplementation), T1: 5 g/kg, T2: 10 g/kg, T3: 15 g/kg

DISCUSSION

Physiological stress responses in crustaceans, particularly lobsters, are generally evaluated using biochemical and immunological parameters, such as hemolymph glucose levels and Total hemocyte count (THC) values , during culture (Djai et al., 2017). The results of the present study indicated that there was no significant increase in hemolymph glucose levels between treatments at either the beginning or end of culture, suggesting that the tryptophan content in green mussels does not directly affect hemolymph glucose metabolism. Consistent increases in glucose levels are usually indicators of environmental or physiological stress in crustaceans (Conneely and Coates, 2021; Turnbull et al., 2024), so the stability of glucose levels in the present study indicated that the lobsters were under relatively controlled environmental conditions. On the other hand, THC values at the beginning and end of culture revealed a significant decrease, which is thought to reflect the process of cellular immune system adjustment during culture in response to tryptophan supplementation (Mayall et al., 2021). THC is an important parameter in evaluating crustacean health because changes in hemocyte count are closely related to immune responses, physiological adaptation, and stress exposure (Ooi et al., 2019; Baladrat et al., 2022). Overall, the combination of stable hemolymph glucose levels and changes in THC indicates that lobsters fed a diet supplemented with the amino acid tryptophan are not under severe physiological stress, as stress generally results in an increase in L-lactate followed by an increase in glucose, and changes in THC are significant predictors of stress in crustaceans (Aji et al., 2019; Conneely and Coates, 2024). Furthermore, these results suggest that tryptophan supplementation in green mussel feed may primarily function through serotonergic pathways that regulate behavior, thereby reducing aggression and cannibalism, rather than directly affecting primary energy metabolism (Cao et al., 2025).

Serotonin levels in all tryptophan-supplemented treatments were relatively high, ranging from 70.66-90.09 ng/mL, and were higher than those observed in the control treatment. The increase in serotonin levels was accompanied by a reduction in cannibalism rates in sand lobsters during the study period, with tryptophan-supplemented treatments showing lower cannibalism rates (7.06-47.06%) than the control. According to Trisnasari et al. (2020) and Cao et al. (2025), tryptophan is an essential amino acid that has been shown to reduce cannibalism rates. Furthermore, tryptophan is considered a major precursor in serotonin synthesis, a process that occurs primarily in the nervous system, including

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the brain, through a conserved metabolic pathway across species, including crustaceans (Roth et al., 2021; Moon et al., 2022). Increased serotonin not only plays a role in neurotransmission and the control of aggressive behavior but also contributes to the regulation of animal metabolism and physiological responses, as well as to the modulation of stress responses and cannibalism in aquatic organisms (Höglund et al., 2019; Maffei, 2021). The use of tryptophan in suppressing cannibalism rates has also been reported in a study by Ardina (2021) which indicated that the addition of 1.5% tryptophan to commercial whiteleg shrimp feed was able to suppress cannibalism by up to 2.50%, and in a study by Trisnasari et al. (2020) which reported that 1% tryptophan supplementation in artificial feed can increase growth while reducing cannibalism rates in freshwater lobsters by 10.00 ± 7.07%.

The present study revealed that the addition of tryptophan to green mussels at a dose of 5 g/kg (T1) resulted in a relatively high moulting frequency, reaching 1.40 ± 0.23 times/individual, indicating an acceleration of the sand lobster growth process during the rearing period. A high moulting frequency is known to be positively correlated with crustacean growth, as the moulting process allows for an increase in body size after the formation of a new exoskeleton; the more frequent the moulting, the greater the opportunity for increased biomass (Zhang et al., 2021; Ding et al., 2024). Hormones and metabolic regulation that control moulting are also strongly influenced by nutritional status and the availability of essential amino acids, which play a role in the synthesis of structural proteins and the formation of new tissue after moulting (Shyamal et al., 2018). The results of the present study are in line with the findings reported by Shyamal et al. (2018), where the 5 g/kg (T1) dose treatment indicated higher specific growth rate, carapace length, absolute weight gain, and absolute length gain compared to other treatments. Tryptophan, as an essential amino acid, is also known to influence feeding behavior and energy utilization efficiency, thus supporting overall crustacean growth (Mohammady et al., 2025). Furthermore, the lower FCR and higher protein efficiency observed in the tryptophan-supplemented treatment (T3) indicated that tryptophan supplementation increases nutrient utilization efficiency, allowing more feed protein to be allocated to growth rather than metabolic activity or stress responses (Agustiana et al., 2022; Mohammady et al., 2025).

The survival rate values for all tryptophan-enriched green mussel feed treatments were higher than those for the control, with the highest value, 55.29%, observed in the T3 treatment. The high value is attributed to the fact that high mortality rates generally occur during the puerulus phase of lobster seed rearing, reaching 96.0-99.4% (Subagio et al., 2021). The high survival rate in the tryptophan-supplemented treatment is related to a decrease in cannibalism rates during rearing, where lobster mortality in the tryptophan treatment tended to be normal compared to the control, which was largely caused by cannibalism. These findings are supported by studies by Kelly et al. (2023) and Rivaie et al. (2023), which state that cannibalism is the main cause of low survival in the post-puerulus phase and that this behavior becomes more dominant when lobsters experience physiological stress that can weaken the immune system and trigger aggressiveness (Conneely and Coates, 2024). Tryptophan, an essential amino acid, plays a role in serotonin synthesis, which suppresses stress responses, reduces aggressive behavior and cannibalism, and maintains the physiological balance of lobsters, allowing for more efficient energy utilization for body maintenance and environmental adaptation (Esmaeili et al., 2025). Furthermore, although the survival rate and lobster growth were relatively high during the maintenance period, the measured ammonia concentrations (0.11-0.12 mg/L) were higher than the optimal level for sand lobster reported by Prama and Kurniaji (2022), which is < 0.01 mg/L. According to Prama and Kurniaji (2022), optimal water quality parameters for sand lobster cultivation include a temperature of 25-30˚C, salinity of 32-36 ppt, pH of 7.5-8.5, dissolved oxygen > 5 mg/L, ammonia < 0.01 mg/L, and nitrite < 0.1 mg/L. The water quality conditions, particularly ammonia levels, were not fully optimal and may have induced suboptimal or mild stress in the culture system. Therefore, the relatively high survival rate values observed in the present study are likely influenced not only by water quality conditions but also by other factors, including tryptophan supplementation, which may have contributed to stress mitigation and improved physiological performance of sand lobsters (Cao et al., 2025).

CONCLUSION

Based on the present results, dietary tryptophan supplementation in green mussel feed affected the physiological responses and growth performance of sand lobsters, with variations observed among the tested doses. The highest dose (15 g/kg; T3) was associated with higher serotonin levels, survival rate, and lower cannibalism, but tended to result in reduced growth performance. In contrast, the lower dose (5 g/kg; T1) indicated higher values in several growth parameters, including carapace length, absolute length, weight gain, specific growth rate, feed conversion ratio (FCR), and protein efficiency ratio (PER), while exhibiting relatively lower effects on physiological responses. These results suggest a possible trade-off between physiological responses and growth performance in relation to tryptophan dosage; however, this interpretation should be considered cautiously in relation to the statistical outcomes presented in the main text. The study is limited by the range of doses tested and the absence of long-term observations; therefore, further

research is recommended to evaluate a broader dosage range and to assess long-term effects on growth, health status, and stress physiology in sand lobsters.

DECLARATIONS

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Authors' contributions

Muhamad Sabar Syafi conducted data collection and prepared the original manuscript. Meci Desi Yulia and Yos Baragama were involved in the research implementation and data collection. Vivi Endar Herawati and Aninditia Sabdaningsih contributed to data analysis, administration, and conceptualization. Sri Hastuti, Pujiono W. Purnomo, and Arief Rahman Rivaie contributed to data interpretation, drafting, and manuscript preparation. All authors have read and approved the final version of the manuscript.

Availability of data

All relevant data generated during the study are included in this published article and are available upon reasonable request from the corresponding author.

Competing interests

The authors declare no conflict of interest.

Ethical considerations

All authors have carefully reviewed the manuscript to ensure there are no ethical concerns, including plagiarism, research misconduct, data fabrication or falsification, and redundant publication. The authors confirmed that no AI tools were used during the preparation of the present study.

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Prevalence, Genetic Diversity, and Antimicrobial Resistance of *Salmonella Enterica* Isolated from Broiler Chicken Farms in Dormaa Ahenkro, Ghana

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ABSTRACT

Salmonella enterica (*S. enterica*) remains one of the most important bacterial agents linked to foodborne illness, particularly in regions with expanding poultry production. The present study aimed to investigate the occurrence, antimicrobial resistance, and genetic characteristics of *S. enterica* isolated from broiler chicken farms in Dormaa Ahenkro, Ghana. One hundred samples (cloacal swabs, feed, water, and litter) were collected from five farms and examined through culture, biochemical tests, antimicrobial susceptibility assays, and molecular confirmation of the *invasion gene A* (*invA*). Twenty-eight isolates (28%) were confirmed as *Salmonella*, all of which exhibited resistance to amoxicillin. Resistance to the antibiotics Ceftazidime, Gentamicin, Imipenem, and Aztreonam tested ranged from 4-25%. Nineteen isolates amplified the *invA* gene, and sixteen out of 19 submitted samples were successfully sequenced and confirmed as *S. enterica* with 93.85-99.80% identity. Phylogenetic and haplotype analyses demonstrated substantial genetic diversity, suggesting localized evolutionary divergence. The present findings highlight the presence of multidrug-resistant *Salmonella* within broiler production systems, emphasizing the need for improved biosecurity and responsible antimicrobial use to safeguard public health. Broiler chicken farms serve as an essential food source in many Ghanaian communities, yet they can harbour bacteria that pose risks to consumers. It was observed that *Salmonella* contamination in birds and their surroundings in Dormaa Ahenkro. Notably, *Salmonella* strains seen in these studies were also resistant to commonly used antibiotics, underscoring the need for stronger hygiene practices, prudent antibiotic use, and consistent monitoring to limit the spread of drug-resistant strains from farms to the wider population. Improving control measures will enhance food safety and support the sustainability of poultry production in Ghana.

Keywords: Antimicrobial resistance, Broiler chicken, Molecular characterization, *Salmonella*, One Health

INTRODUCTION

Poultry production continues to play a central role in food security across Africa, contributing 2.5 million metric tons annually and supporting rural livelihoods (Mottet and Tempio, 2017; FAO, 2023). However, the sector faces significant threats from foodborne pathogens such as *Salmonella enterica* (*S. enterica*), a leading cause of gastrointestinal disease globally that contaminates poultry through feed, litter, drinking water, housing environments, and bird-to-bird transmission (Kulkarni et al., 2019).

Globally, non-typhoidal *S. enterica* causes 93.8 million human infections and 155,000 deaths annually, with poultry serving as the primary transmission vehicle (Ramtahal et al., 2022). Different poultry-related serovars make broiler farms a very important place to watch (EFSA, 2019; CDC, 2022). While many countries, such as the UK, Germany, and the United States, have established monitoring systems, Sub-Saharan Africa, including Ghana, remains data-deficient on non-typhoidal *Salmonella* prevalence and antimicrobial resistance (AMR) in poultry systems (Andoh et al., 2016; FAO, 2021a).

Dormaa Ahenkro, a major poultry-producing area in Ghana's Brono Region, lacks comprehensive baseline data on broiler farm contamination. The insufficient data on *S. enterica* strains hinder effective control measures and pose a threat to public health (Thye et al., 2025). The present study aimed to address these critical gaps by investigating *S. enterica* prevalence, AMR profiles, and genetic characteristics in Dormaa Ahenkro broiler farms, providing essential evidence for targeted farm-level interventions and national One Health strategies.

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MATERIALS AND METHODS

Ethical approval

Ethical clearance was obtained from the Research and Ethics Committee of the School of Agriculture and Technology, University of Energy and Natural Resources, Sunyani, Ghana. The study design and protocol were approved with the following certificate number, SOAT/ECE/015/2025.

Study area and sample collection

A cross-sectional study was conducted from April to August 2025 across five broiler farms purposively selected, targeting active producers representing typical area operations in Dormaa Ahenkro, Bono Region, Ghana. According to the 2020 population and housing census, 1.8 million poultry birds were reared by 200 registered farmers (Veterinary Service Department, Dormaa Ahenkro, 2020). A total of 100 samples comprising 25 each of cloacal swabs, feed, water, and litter were collected using sterile cotton swabs for cloaca (inserted 2-3 cm, rotated, placed in Amies transport medium), sterile bags for feed/litter (100g composite), and sterile bottles for water (100 ml). All were sealed and labelled with farm ID/sample type/date, transported in a cooler at 4-8˚C, and processed within 24 hours for isolation of *Salmonella* on Xylose Lysine Deoxycholate (XLD) agar.

Culturing, isolation, and identification of *Salmonella*

To identify *S. enterica* in cloacal swab, feed, litter, and water samples, standard microbiological culture followed by biochemical confirmation (ISO 6579-1:2017) was employed. *Salmonella* isolation and identification involved pre-enrichment in Buffered Peptone Water (BPW), followed by selective enrichment in Modified Semi-solid Rappaport Vassiliadis broth at 42˚C for 24 hours. *S. Enteritidis* ATCC 13076 served as the positive control. Ten mls of BPW was used for cloacal swabs, and 25 g of all other samples were inoculated into 225 mL of BPW and incubated at 37˚C for 18-24 hours. Selective plating was performed on XLD agar to isolate presumptive *Salmonella* colonies, with the positives appearing as red or pink colonies, typically with black centers. Colonies were then subjected to biochemical confirmation using Triple Sugar Iron (TSI) agar (red slant/yellow butt ± gas/H_2S after 18-24 hours), citrate utilization (positive), and indole test (blue color change on Simmons agar; Andoh et al., 2016). All media were purchased from Oxoid Limited, Basingstoke, UK.

Antimicrobial susceptibility testing

The Kirby-Bauer disk diffusion method (Bauer et al., 1966) was used to assess susceptibility to five antibiotic classes: Penicillin (Amoxicillin [AML 10 µg]), carbapenems (Imipenem [IMP 10 µg]), monobactams (Aztreonam [ATM 30 µg]), cephalosporins (Ceftazidime [CAZ 30 µg]), and aminoglycosides (Gentamicin [CN 30 µg]). Antibiotic susceptibility testing utilized commercially purchased disks from UK OXOID (Thermo Scientific, Basingstoke, UK). Isolates were prepared to a 0.5 McFarland turbidity standard and tested on Mueller-Hinton agar (Oxoid, UK). Zones of inhibition were interpreted using European Committee on Antimicrobial Susceptibility Testing 2025 (EUCAST) clinical breakpoints, and isolates resistant to at least three antibiotic classes were classified as multidrug-resistant (Magiorakos et al., 2012). A cotton swab was used to spread the standardized *Salmonella* suspension evenly across the entire surface of the Mueller-Hinton agar in three different directions, creating an even bacterial lawn. After allowing the plates to dry for 10 minutes, antibiotic discs were then gently placed on the surface of the inoculated agar plates using sterile forceps. The inoculated agar plates were left undisturbed to dry at room temperature for approximately 10 minutes. Following this, antibiotic discs were aseptically positioned on the agar surface with sterile forceps, ensuring gentle and precise placement (Ogodo et al., 2022). It was also ensured that enough and even spacing was considered to prevent the antibiotic disc from overlapping its zone of inhibition. After which, the plates were incubated at 37˚C for 16-24 hours. Results were interpreted according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints and classified into the following categories, including Resistant and Susceptible.

Measurement of inhibition zones

The diameter of each inhibition zone was measured using a calibrated ruler and recorded in millimeters. Antimicrobial susceptibility outcomes were assessed in accordance with EUCAST 2025 interpretive criteria and stratified into three distinct response groups. Isolates classified as Susceptible (S) are expected to respond to the tested antimicrobial agent when administered at conventional therapeutic dosages. The Intermediate (I) category reflects situations in which treatment efficacy can be achieved by increasing drug exposure, either through dose adjustment or improved penetration at the site of infection. Isolates designated as Resistant (R) are not expected to respond to therapy, as effective antimicrobial activity cannot be achieved within safe and clinically attainable concentration ranges. All

susceptibility profiles were recorded for individual isolates, and isolates indicating resistance to three or more antibiotic classes were considered multidrug-resistant (MDR).

Molecular confirmation and characterization

Extraction of Genomic DNA was performed from purified isolates using the crude lysate boiling method (Adzitey et al., 2025), in which a colony was lysed in 30 µL molecular-grade water at 99°C. PCR targeting the *invasion* gene A (*invA*; 285 bp) was performed in a 25 µL reaction mixture which contained a 13.75 µL of nuclease-free water, 2.5 µL of 10x PCR buffer, 1.5 µL of 25 mM $MgCl_2$, 1.0 Taq polymerase (5 U/µL), 3.75 Template DNA lysate 0.5 µL of 10 mM dNTPs, 1.0 µL each of forward primers (5'-GTGAAATTATCGCCACGTTCGGGCAA-3') and reverse primers (5'-TCATCGCACCGTCAAAGACC-3') targeting the *invA* gene (Adzitey et al., 2025). PCR cycling conditions included initial denaturation at 94°C for 5 minutes, followed by 40 cycles of denaturation at 94°C for 30 seconds, annealing at 64°C for 30 seconds, and extension at 72°C for 30 seconds. Amplicons were visualized by electrophoresis on a 1% agarose gel (CSL-AG500, Cleaver Scientific Ltd, UK) stained with ethidium bromide (Sigma-Aldrich, USA; 0.5 µg/mL), using a 100 bp DNA ladder as the molecular size marker, and imaged under UV transillumination (Bio-Rad, USA). Additionally, amplicons were subjected to Sanger sequencing using an Applied Biosystems 3500 Genetic Analyzer thermocycler (Thermo Fisher Scientific, USA), and the resulting sequences were edited, aligned, and compared with those in the National Centre for Biotechnology Information (NCBI) database for species confirmation.

Data, sequence, and phylogenetic analyses

Descriptive statistics represented in tables and graphs were inferred using Microsoft Office Suite 2021. Cleaned sequences were analysed using Molecular Evolutionary Genetics Analysis (MEGA; Kumar et al., 2018) for phylogenetic reconstruction, and Population Analysis with Reticulate Trees (POPART) and DNA Sequence Polymorphism (DNAsP; Leigh and Bryant, 2015) were applied to determine haplotype variation, nucleotide diversity, and mutation patterns. Neutrality tests, including Tajima's D, Fu's Fs, Fu and Li's D*, and Fu and Li's F, were calculated using DnaSP version 6 (Rozas et al., 2017) to assess signatures of population expansion, selection, or demographic history. BLAST searches from NCBI were performed to determine relatedness to global strains.

RESULTS

Prevalence

Out of 100 samples examined, 28% were verified as *Salmonella* positive. All sample sources recorded contamination (Figure 1), with feed recording the highest prevalence at 36% (9/25), followed by cloacal swabs at 28% (7/25). Litter and water revealed lower prevalence rates of 24% each (6/25). Despite the observed differences, prevalence among the sample sources was not statistically significant ($p > 0.05$).

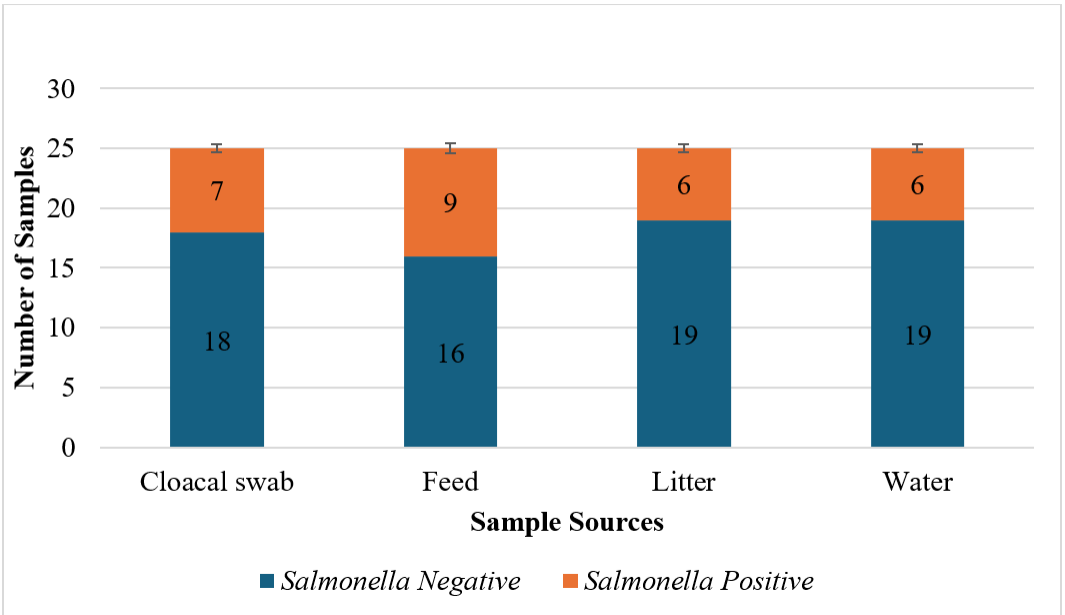


Figure 1. Occurrences of *Salmonella enterica* isolated from litter, cloacal swabs, drinking water, and feed samples in broiler chicken farms located in Dormaa Ahenkro from April to September 2025

Antimicrobial resistance profiles

All 28 *Salmonella* isolates indicated 100% resistance to amoxicillin (Table 1), with breakpoints defined in Table 2 (EUCAST, 2025). Resistance rates to ceftazidime, gentamicin, imipenem, and aztreonam ranged from 4% to 25%. Isolate BW05 (water sample, Farm B) was resistant to all tested antibiotic classes and was identified as an MDR isolate.

Table 1. Antibiogram profile of *Salmonella enterica* isolated from Dormaa Ahenkro broiler chicken farms from April to September 2025

Antibiotic name	Samples type	Resistant	Sensitive
Ceftazidime	Feed, cloacal swabs, litter, and water	1 (4%)	27 (96%)
Gentamicin	Feed, cloacal swabs, litter, and water	2 (8%)	26 (92%)
Amoxicillin	Feed, cloacal swabs, litter, and water	28 (100%)	0 (0%)
Imipenem	Feed, cloacal swabs, litter, and water	4 (16%)	24 (84%)
Aztreonam	Feed, cloacal swabs, litter, and water	2 (8%)	26 (92%)

Table 2. Antibiotic breakpoints with disk diffusion test based on EUCAST 2025

Antibiotic	Susceptible (≥ mm)	Intermediate (mm)	Resistance (≤ mm)
Ceftazidime	18	15-17	≤14
Gentamicin	15	13-14	≤12
Amoxicillin	18	14-17	≤13
Imipenem	23	20-22	≤19
Aztreonam	21	17-20	≤16

Table 3. Antibiotic resistance profile of *Salmonella enterica* samples isolated from broiler chicken farms in Dormaa Ahenkro from April to September 2025

Sample ID	Ceftazidime	Gentamicin	Amoxicillin	Imipenem	Aztreonam
A-FE04	28 mm (S)	29 mm (S)	11 mm (R)	29 mm (S)	33 mm (S)
B-FS05	30 mm (S)	29 mm (S)	6 mm (R)	26 mm (S)	37 mm (R)
B-W05	12 mm (R)	16 mm (S)	6 mm (R)	10 mm (R)	18 mm (R)
C-LT01	29 mm (S)	26 mm (S)	12 mm (R)	15 mm (R)	35 mm (S)
C-LT03	29 mm (S)	22 mm (S)	6 mm (R)	26 mm (S)	36 mm (S)
C-LT04	30 mm (S)	25 mm (S)	6 mm (R)	24 mm (S)	33 mm (S)
C-W01	25 mm (S)	26 mm (S)	16 mm (R)	24 mm (S)	34 mm (S)
C-W04	25 mm (S)	24 mm (S)	6 mm (R)	24 mm (S)	32 mm (S)
C-FE02	22 mm (S)	15 mm (S)	6 mm (R)	15 mm (R)	16 mm (R)
D-W01	24 mm (S)	25 mm (S)	16 mm (R)	27 mm (S)	30 mm (S)
D-W02	28 mm (S)	26 mm (S)	11 mm (R)	28 mm (S)	33 mm (S)
D-FE02	25 mm (S)	25 mm (S)	16 mm (R)	32 mm (S)	31 mm (S)
D-FE03	26 mm (S)	28 mm (S)	13 mm (R)	34 mm (S)	37 mm (S)
D-FE04	26 mm (S)	24 mm (S)	16 mm (R)	33 mm (S)	36 mm (S)
D-FE05	32 mm (S)	30 mm (S)	16 mm (R)	28 mm (S)	38 mm (S)
D-LT02	27 mm (S)	22 mm (S)	6 mm (R)	18 mm (I)	36 mm (S)
D-FS02	25 mm (S)	22 mm (S)	12 mm (R)	24 mm (S)	36 mm (S)
D-FS03	27 mm (S)	26 mm (S)	17 mm (R)	33 mm (S)	35 mm (S)
D-FS04	27 mm (S)	22 mm (S)	6 mm (R)	24 mm (S)	36 mm (S)
D-FS05	26 mm (S)	28 mm (S)	12 mm (R)	28 mm (S)	36 mm (S)
E-FE02	26 mm (S)	34 mm (S)	18 mm (R)	26 mm (S)	35 mm (S)
E-FE03	28 mm (S)	30 mm (S)	18 mm (R)	26 mm (S)	32 mm (S)
E-W01	28 mm (S)	27 mm (S)	17 mm (R)	32 mm (S)	34 mm (S)
E-W02	29 mm (S)	28 mm (S)	6 mm (R)	31 mm (S)	36 mm (S)
E-LT02	26 mm (S)	20 mm (S)	6 mm (R)	22 mm (S)	35 mm (S)
E-LT03	26 mm (S)	26 mm (S)	17 mm (R)	32 mm (S)	32 mm (S)
E-FE04	24 mm (S)	27 mm (S)	16 mm (R)	29 mm (S)	33 mm (S)
E-FS01	25 mm (S)	24 mm (S)	13 mm (R)	28 mm (S)	34 mm (S)

FE: Feed, CS: Cloacal swabs, LT: Litter, W: Water, S: Susceptible (standard dosing), I: Susceptible with increased exposure, R: Resistant, A, B, C, D, and E represent the farms. Results were interpreted using EUCAST 2025 breakpoints for disk diffusion (mm inhibition zones).

PCR, molecular confirmation, and characterization of *Salmonella enterica*

A uniform fragmentation length of 285 bp was produced using the partial *invA* gene primers. Nineteen isolates were PCR-positive for the *invA* gene. Sixteen isolates were successfully sequenced, which yielded a clear, readable 285 bp band of the target *invA* gene. While the three remaining isolates failed to produce clear bands on gel electrophoresis and were discarded. The Nucleotide BLAST algorithm from NCBI identified all 16 isolates as *Salmonella enterica subsp*. Comparing them to earlier data in the GenBank, with a 93.85 to 99.80% identification rate (Table 3). The identified isolates were AFE04, CLT03, CLT04, CW04, DFE03, DW02, DFS04, DFE05, DLT02, DW01, DFE0, EFE02, EFE03, EW02, ELT03, and EFE04. BLAST analysis indicated that the sequences shared high similarity with *Salmonella* isolates from Egypt, Nigeria, Zambia, and France.

Haplotype analysis of *Salmonella enterica's InvA* gene (285 bp) across 16 sequences (Figure 2) identified 19 segregating (polymorphic) sites (S = 6.7%), of which 14 were singleton mutations. A total of 25 mutations (Eta) were detected, yielding 15 haplotypes with high haplotype diversity (Hd = 0.959 ± 0.00129) and nucleotide diversity (π = 0.043). Average pairwise nucleotide differences were k = 12.3. Neutrality tests indicated a Tajima's D value of -1.92870, which was statistically significant ($P < 0.05$), indicating an excess of low-frequency polymorphisms probably caused by recent population growth or purifying selection. Fu's Fs statistic was 2.404, while Fu and Li's D (-1.87464) and F (-2.20404) statistics were negative but not statistically significant ($P > 0.05$), pointing to a similar trend of excess rare alleles, but without strong statistical support.

Table 4. *Salmonella enterica* subsp. *enterica* GenBank deposits compared to the sequences of bacteria in the present study

Sample ID	Identical to	GenBank accession number	Country	Identity (%)	Host
A-FE04	*Salmonella*	PQ720689	Egypt	98.80	Poultry
C-LT03	*Salmonella*	PQ720689	Egypt	98.80	Poultry
C-LT04	*Salmonella*	MZ683411	Nigeria	98.80	Poultry
C-W04	*Salmonella*	MF678530	Egypt	93.85	Poultry
D-FE03	*Salmonella*	MT478121	Egypt	93.85	Poultry
D-W02	*Salmonella*	KJ718884	Egypt	99.21	Poultry
D-FS04	*Salmonella*	PQ720689	Egypt	98.80	Poultry
D-FE05	*Salmonella*	PP921862	Zambia	99.21	Poultry
D-LT02	*Salmonella*	MT478121	Egypt	98.79	Poultry
D-W01	*Salmonella*	MT478121	Egypt	99.60	Poultry
D-FE04	*Salmonella*	KX524162	Egypt	96.28	Poultry
E-FE02	*Salmonella*	KX524162	Egypt	94.05	Poultry
E-FE03	*Salmonella*	MT478121	Egypt	97.98	Poultry
E-W02	*Salmonella*	MT478121	Egypt	98.39	Poultry
E-LT03	*Salmonella*	KX524156	Egypt	98.00	Poultry
E-FE04	*Salmonella*	KX524162	Egypt	98.02	Poultry

FE: Feed, CS: Cloacal swabs, LT: Litter, W: Water, A, B, C, D, and E represent the sample sources.

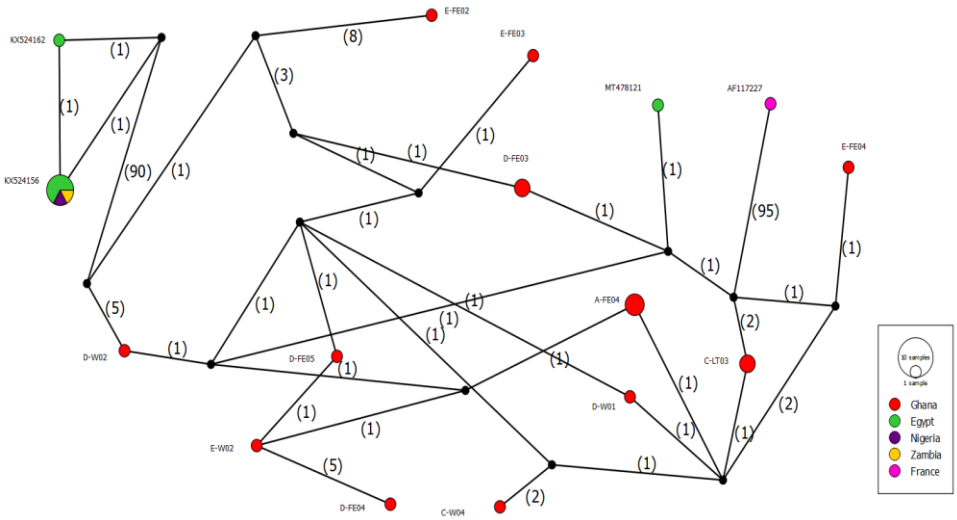


Figure 2. Haplotype network of *Salmonella enterica* based on the *invasion gene A* gene sequences (~285bp) isolated from broiler chicken farms in Dormaa Ahenkro from April to September 2025

Phylogenetic analysis based on the partial *invasion gene A*

Phylogenetic analysis of *S. enterica* conducted in MEGA X (Kumar et al., 2018) revealed a Ghanaian clade encompassing all 16 local *S. enterica* isolates, with strong support (bootstrap = 100 at root node) and differentiating them from reference sequences from Egypt, Nigeria, Zambia, and France (Figure 3). In the Ghanaian isolates, sub-lineages like DFE03, DLT02, DW02, and EW02 formed a group with robust support (bootstrap = 78). On the other hand, other internal branches had less support (bootstraps = 50-72). Egyptian and Nigerian genomes formed distinct clusters (bootstrap = 92); the Zambian isolate formed a distinctive lineage (bootstrap = 88); and the French isolate served as the basal reference.

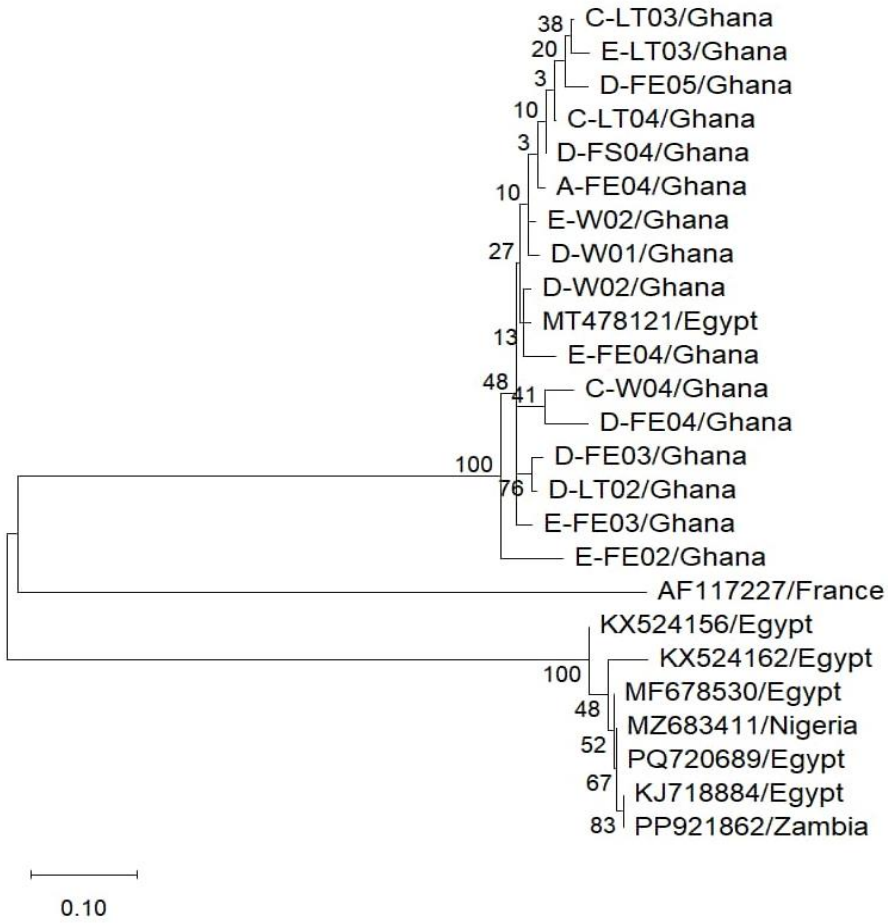


Figure 3. Maximum-likelihood phylogenetic tree of *Salmonella enterica* isolated from broiler chicken farms (April-September 2025) based on partial *invasion* gene A sequences from GenBank and the present study. The phylogenetic tree was constructed in MEGA 12 using the Tamura-Nei model with 100 bootstrap replications

DISCUSSION

Salmonella was identified in 28% of broiler chicken farms in the present study from Dormaa Ahenkro. Isolates were confirmed through molecular characterization using the *invA* gene, which verified the identity of *S. enterica* in 19 out of 28 presumptive isolates (67.9%), which is consistent with previous work in which phenotypic identification was confirmed molecularly through the *invA* gene and aligns with studies that document false positives from biochemical and serological methods due to cross-reactivity and non-specific agglutination (Grimont and Weill, 2007; Foley et al., 2013; El-Baz et al., 2017). The observed 28% farm-level prevalence is consistent with similar findings from other regions within Ghana. For example, a 25% farm-level prevalence of *Salmonella* was reported in Accra, Ghana (Andoh et al., 2016), along with a 44% overall prevalence in commercial poultry and a 6-10% prevalence in the environment on Ashanti-region farms (Andoh et al., 2021). Another study in the Accra Plains, Ghana, found that 32.4% of poultry environmental samples were contaminated with *Salmonella* and highlighted that farming conditions and biosecurity practices are key factors driving contamination (Adzitey et al., 2025). *Salmonella* detected in food-producing animals serves as a direct reservoir for zoonotic transmission, posing a considerable risk to food safety and public health in

Dormaa Ahenkro and surrounding communities (Feasey et al., 2012). The current study confirmed the presence of *Salmonella. enterica* in broiler chicken farms and their surrounding environment in Dormaa Ahenkro Municipality, Ghana, detecting the *invA* gene with 28% farm-level prevalence consistent with regional studies in Ghana and West Africa (Andoh et al., 2016; FAO, 2021a;b; Baah, 2023). The isolates exhibited complete resistance to amoxicillin, with no susceptible strains detected, which is consistent with reports of excessive β-lactam use in Ghanaian poultry production. The high MDR prevalence observed is consistent with WHO and FAO recommendations for antibiotic stewardship (FAO, 2021a; WHO, 2021; Ferreira et al., 2022). The identification of extensively drug-resistant (XDR) isolate B-W05 (resistant to ≥ 4 antibiotic classes) suggests potential zoonotic transmission risks and limited treatment options, consistent with the need for improved biosecurity and surveillance in broiler farms (WHO, 2021). Furthermore, the combination of PCR and partial *invA* gene sequencing enabled robust bacteriological identification of *S. enterica* in feed, water, litter, and cloacal swabs collected from broiler chicken farms in Dormaa Ahenkro, Ghana. It is important to note that the *invA* gene is a single, highly conserved virulence gene involved in invasion across *Salmonella* serovars and is not expected to reflect the full range of whole-genome diversity (Rahn et al., 1992; Nhung et al., 2022). The high haplotype and nucleotide diversity within *invA* likely result from micro-epidemiological variation among closely related strains rather than deep phylogenetic divergence across serotypes.

Overall, the phylogenetic tree supports regional structuring and diversification of *S. enterica* populations in Ghana, with Ghanaian isolates forming distinct clusters rather than mixing closely with isolates from other African countries or Europe (Feasey et al., 2012). The observed genotypic clustering indicates regional diversification of *S. enterica* lineages in sub-Saharan Africa. The findings from the present study suggest that local transmission and farm-level dynamics may be more influential than international spread in shaping the current *S. enterica* population in Ghanaian poultry. The Centers for Disease Control and Prevention (CDC, 2011) and Bugarel et al. (2017) have reported that *S. enterica* ranks among the most concerning foodborne pathogens due to its frequent association with multidrug resistance. In Ghana, *S. enterica* has been isolated from human clinical cases, including invasive bloodstream infections in children and invasive nontyphoidal *Salmonella* (iNTS) disease involving *S. Enteritidis* and *S. Typhimurium*, indicating circulation in the human population and emphasizing the zoonotic threat posed by poultry-associated serovars (Feasey et al., 2012; Ablordey et al., 2018; Park, 2019). However, due to the limited number and diversity of isolates in the present study, it is currently not possible to confirm that *S. Enteritidis* is the most common serovar in Ghana. *S. Enteritidis* has also been reported in other African countries, including Nigeria and Zambia, where it has been detected in poultry and associated with human infections (Akinyemi et al., 2021), underscoring the transboundary nature of *Salmonella* and antimicrobial resistance challenges in the region. The One Health framework offers a valuable approach for interpreting the present findings on *Salmonella* and antimicrobial resistance in poultry (WHO, 2021; FAO, 2021b). The discovery of zoonotic *S. enterica* in Ghanaian broiler farms, coupled with near-universal resistance to widely used antibiotics such as amoxicillin (100%), highlights the interconnectedness of human, animal, and environmental health (OIE, 2020; WHO, 2021; FAO, 2021b). Multidrug-resistant *Salmonella* in poultry products can reach consumers through contaminated meat and eggs. Previous studies in Ghana have linked poultry-derived *S. Enteritidis* and other non-typhoidal serovars to a substantial proportion of reported salmonellosis and invasive bloodstream infections, although the exact attribution fraction remains to be determined and would benefit from dedicated source-attribution and One Health genomic studies (Feasey et al., 2012; Ablordey et al., 2018; Park, 2019). On-farm antimicrobial use, farm hygiene, and environmental contamination are likely to contribute to the emergence and spread of resistant *Salmonella* strains that threaten both animal productivity and human food safety (Andoh et al., 2016; FAO, 2021b; WHO, 2021) Within this context, the One Health operational framework encourages countries to coordinate surveillance and control measures across veterinary, public-health, and environmental sectors to prevent and contain zoonotic infections, manage antimicrobial resistance, and strengthen food-safety systems (OIE, 2020; FAO, 2021b; WHO, 2021; Mumford, 2023). Integrating laboratory-based AMR monitoring in poultry, routine carcass and environmental sampling along the value chain, and clinical reporting of human salmonellosis would allow earlier detection of resistant strains such as those identified in the present study and support evidence-based regulation of antimicrobial use (FAO, 2021b; WHO, 2021).

CONCLUSION

The present study found *Salmonella enterica* in 28% of samples from broiler farms in Dormaa Ahenkro, with all 28 isolates exhibiting complete resistance to amoxicillin; isolate B-W05 also revealed resistance to all four antibiotics tested, qualifying as multidrug-resistant (MDR). Limited funding prevented whole-genome sequencing for serovar identification, traditional antisera serotyping, and expanding sampling beyond 100 purposively selected samples across five farms. The *invA* PCR (285 bp) confirmed species identity but did not provide serovar or full genomic details. Future adequately funded research should prioritize Whole Genome Sequencing for serotyping, resistance profiling, and source

attribution across +500 samples from Ghana's major poultry regions, integrating human clinical isolates for One Health transmission studies. Routine surveillance, strict antimicrobial stewardship, farmer biosecurity training, and combined molecular-traditional serotyping will control environmental contamination and reduce zoonotic risks along the poultry value chain. Note that population growth, globalization of trade, ecological change, and advances in technology make such integrated approaches increasingly essential for improving public health at local and global levels. It is recommended that a larger study with broader sampling from multiple sources, such as farms, processing plants, markets, and human cases, would be necessary to draw definitive conclusions.

DECLARATIONS

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Authors' contributions

Moses Addo Nartey has contributed to conceptualization, data curation, methodology, writing, original draft, validation, and supervision. Abdul Lateef Sulemana contributed to investigation, methodology, visualization, writing, original draft, and software procedures. Francis Addy contributed to resources, writing, editing, and formal analysis. Peter Asiedu contributed to visualization, writing, and editing. Bernard Ato Hagan contributed to data curation, resources, and project administration. Bismark Kyei contributed to writing, and editing. Frederick Adzitey contributed to resources, writing, editing, and supervision. All authors have read and approved the final edition of the manuscript.

Availability of data and materials

The data to support the present study's findings are available upon reasonable request to the corresponding author.

Conflicts of interests

The authors declared no conflict of interest.

Ethical considerations

The authors have checked the ethical considerations, including issues such as plagiarism, consent for publication, misconduct, data fabrication and/or falsification, as well as double publication and/or submission redundancy. The authors confirm that no AI tools were used in conducting and preparing this study.

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Histopathological Insights into Purkinje Cell Responses of Maternal and Fetal Cerebellum in Mice: Electromagnetic Wave Exposure and Protective Role of Cactus (*Opuntia* spp.)

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ABSTRACT

The rapid expansion of wireless technologies has significantly increased human exposure to electromagnetic fields (EMFs), raising concerns about potential effects on reproductive and developmental health. *Opuntia cochenillifera* (*O. cochenillifera*), a cactus rich in mucilage and mineral oxides, has potential as a bio-based material for reducing the intensity of electromagnetic waves (EMWs) through dielectric and magnetic interactions. The present study aimed to evaluate the effects of EMW exposure on Purkinje cell morphology in maternal and fetal cerebellum and to assess the protective potential of distinct *O. cochenillifera* formulations. A total of 42 pregnant BALB/c mice were randomly divided into six groups, including healthy mice as the positive control group, EMW-exposed mice as the negative control group, and four treatment groups. The treatment groups were subjected to EMW exposure alongside the administration of either fresh cactus, dried cactus gel, or powdered cactus (3 g each). The EMW exposure was administered at a specific absorption rate of 1.74 W/kg throughout gestation. Cerebellar tissues were collected on day 20 for histological analysis and characterized by X-ray fluorescence and Fourier-transform infrared spectroscopy. Histological findings demonstrated that EMW exposure disrupted Purkinje cell alignment and decreased Purkinje cell counts in the negative control group. In contrast, all treatment groups demonstrated preserved cellular structure and morphology comparable to that of the healthy mice. Quantitative analysis confirmed significantly higher cell counts in treated groups than in the negative control group. The current findings indicated that EMW exposure adversely affected cerebellar development, while *O. cochenillifera* exhibited protective effects, supporting its potential as a natural EMF-attenuating material.

Keywords: Cactus, Cerebellum, Electromagnetic wave, Histopathology, Purkinje cell

INTRODUCTION

In recent years, exposure to electromagnetic fields (EMF) has increased significantly due to the widespread use of technological devices and telecommunications networks (Ikinci et al., 2019). While mobile phones, Wi-Fi routers, and wireless systems have improved connectivity, the increasing use of these technologies has raised concerns about their potential biological effects. Previous studies have reported oxidative stress, DNA damage, and other harmful biological responses following EMF exposure in experimental models (Jagetia, 2022; Panagopoulos et al., 2025). However, multiple systematic reviews of human and epidemiological data found limited or inconsistent evidence for adverse physiological effects from Wi-Fi or mobile-phone exposure (Bodewein et al., 2022; Dongus et al., 2022). Regarding reproductive health, considerable uncertainty exists. Despite some studies suggesting that EMF exposure could lead to adverse outcomes such as preterm birth, fetal demise, low birth weight, and congenital anomalies, the epidemiological results are not consistent (Kim et al., 2021). Therefore, the World Health Organization (WHO) International EMF Project has designated reproductive outcomes, oxidative stress, cognitive impairment, and cancer risk as priority areas for investigation (Verbeek et al., 2021). Regulatory authorities, such as the International Commission on Non-Ionizing Radiation Protection (ICNIRP, 2020) and the U.S. Federal Communications Commission (FCC, 2025), have established safety guidelines for mobile devices; however, not all devices consistently adhere to these guidelines. Furthermore, the WHO has identified electromagnetic radiation as one of the most rapidly expanding environmental threats worldwide (Davies et al., 1995).

Microwave radiation, a subset of EMF with frequencies ranging from 300 MHz to 300 GHz, is widely employed in telecommunications, industry, and healthcare (Lahiri, 2023). Numerous studies have documented adverse biological effects of microwave radiation, including structural and functional alterations in the nervous, cardiovascular, reproductive, and endocrine systems (Pall, 2016). In experimental models, pregnant mice exposed to 2.45 GHz electromagnetic waves (EMW) exhibited oxidative stress and morphological disruptions at the placenta-embryo implantation site, resulting in embryo resorption (Shahin et al., 2013). Additionally, epidemiological findings indicated

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potential associations between prolonged maternal EMF exposure and outcomes like miscarriage, preterm labor, reduced fetal growth, and congenital malformations (Abdul-Al et al., 2022). More evidence indicates that EMF affects overall physiology and brain development (Bodin et al., 2025). Animal studies have demonstrated that extended EMF exposure leads to cortical thinning, neuron loss, and alterations in brain structure (Akakin et al., 2021; Arslan et al., 2022; Eskandani and Zibaii, 2024). At the molecular level, EMF is linked to oxidative stress, mitochondrial dysfunction, and disrupted calcium signaling, all of which compromise neuronal health (Saliev et al., 2019; Lai, 2021; Leach et al., 2025). These disturbances particularly affect the cerebellum, which is crucial for motor coordination and cognitive functions (Cordelli et al., 2023; Mehmet et al., 2024). Previous studies demonstrated that exposure to 900 MHz EMF leads to Purkinje cell degeneration and oxidative imbalance, although antioxidant treatments such as luteolin can mitigate these effects (Arslan et al., 2022). Additionally, a neurobehavioral investigation reported that EMF exposure led to impaired balance and coordination, which may be partially explained by damage to cerebellar neurons (Ganjalikhan-hakemi et al., 2023).

The challenges associated with electromagnetic radiation have prompted the development of materials designed to absorb or block such radiation (Rong et al., 2025). Traditional absorbers, such as conductive materials, dielectric ceramics, and magnetic composites, often encounter challenges including impedance mismatch, high density, and limited biocompatibility (Ren et al., 2025). Consequently, natural plant-based biomaterials have garnered interest due to sustainability, cost-effectiveness, and multifunctional properties (Shi et al., 2025). Agricultural by-products, including porous carbon from coconut shells, *Agave atrovirens*, and *Opuntia ficus-indica* powders, have demonstrated considerable potential for microwave absorption (Simón et al., 2019; Kumar and Kuanr, 2024). Specifically, measurements of reflection, transmission, and absorption parameters between 1.7-2.6 GHz indicated that *Opuntia ficus-indica* acts as a high-loss dielectric material. The presence of a fibrous multilayered internal structure and high-water content promotes greater absorption and lower transmission (Lee et al., 2024; Alves et al., 2025), whereas the observed increase in reflection of the Opuntia material suggests a relatively high dielectric constant. These combined properties support that *Opuntia* can function effectively as a natural electromagnetic shielding material (Erdem, 2026). Based on waveguide measurements, *Opuntia ficus-indica* functioned as a moderate microwave-absorbing material, showing an absorption coefficient near 0.23 in the 8-13 GHz range, which indicated potential applicability of *Opuntia ficus-indica* as a natural electromagnetic shielding material (Simón et al., 2019).

The mechanisms by which Opuntia interacts with EMF, particularly the susceptibility of the fetal cerebellum, remain poorly understood. Therefore, the present study aimed to evaluate the effects of fresh, dried, gel, and powder forms of *Opuntia cochenillifera* (*O. cochenillifera*) as natural dielectric materials in reducing EMFs in pregnant mice, utilizing morphological and spectroscopic analyses to elucidate their interactions with EMW and assess their potential as environmentally friendly shielding materials.

MATERIALS AND METHODS

Ethical approval

Ethical approval for all animal procedures was obtained from the Medical and Health Research Ethics Commission of Diponegoro University, Indonesia (No. 116/EC-H/KEPK/FK-UNDIP/IX/2023). Experiments were conducted in accordance with the institution's guidelines and internationally recognized standards for the care and use of laboratory animals, and reporting follows the ARRIVE recommendations where applicable. Throughout the study, animal welfare was prioritized by minimizing pain, stress, and discomfort. All procedures were carried out by trained personnel to ensure proper handling and reduce distress. Human endpoints and strategies to minimize distress were applied throughout. In addition, the study adhered to the principles of the 3Rs (Replacement, Reduction, and Refinement), and the minimum number of animals necessary to achieve the scientific objectives was used.

Animals

A total of 56 BALB/c mice were used in the present study, consisting of 42 females and 14 males, all aged 2-3 months and weighing 25-30 g. The mice were acclimated to the laboratory environment for seven days, with environmental conditions maintained at 24 ± 2°C, 50-60% humidity, and a 12-hour light/dark cycle. Animals had free access to water and food pellets. Following acclimatization, mating was conducted overnight (12 hours) starting at 6:00 PM, with females and males housed together at a 3:1 female-to-male ratio. The next morning, vaginal plugs and swabs were inspected to confirm mating. Males were immediately removed after confirmation, and only the pregnant females were retained for the study. The pregnant females were then randomly assigned to six experimental groups, with seven mice in each group. The treatment groups consisted of healthy mice serving as the positive control, mice exposed to EMW as the negative control, and four EMW-exposed groups fitted with belts containing fresh, dried gel, or powdered cactus, respectively. Successful breeding was confirmed at gestational days 10 (G10) and 13 (G13), evidenced by a 3-4 g

increase in female body weight. Weight changes were used to monitor pregnancy status, with weight gain indicating successful gestation and sudden loss suggesting prenatal complications or miscarriage (Neres et al., 2008). The pregnant mice were housed in 45 × 45 × 25 cm polypropylene cages, with absorbent wood chip bedding replaced three times daily to maintain clean and comfortable living conditions. Cage positions were rotated regularly to minimize environmental variation.

Experimental design and treatment procedures

The study comprised one healthy control group (HCG) containing mice that were not exposed to EMW radiation, a negative control group (NCG), and four experimental groups (E1-E4). The NCG was exposed to EMW without any protective intervention, whereas the experimental groups (E1-E4) were each fitted with abdominal belts containing different forms of *O. cochenillifera* to mitigate the effects of EMW. Mice in the first experimental group received a belt with fresh cactus sliced into small fragments (E1), mice in the second experimental group received a belt containing sun-dried cactus processed into a coarse powder (E2), in the third experimental group, mice received a belt containing a cactus gel extract prepared by macerating cactus in 70% ethanol and formulated into a gel (E3), and mice in the fourth experimental group received a belt containing cactus powder obtained by dehydrating the gel extract used in E3 into a fine powder (F4). All treatment groups were exposed to continuous EMW radiation from a cellular phone operating at 900 MHz and 2.45 GHz Wi-Fi, with a whole-body specific absorption rate (SAR) of 1.74 W/kg, for 24 hours daily during gestational days 1–19. Each belt was constructed from a sterilized plastic bag filled with one of the cactus preparations (fresh, dried, gel, or powdered extract) and shaped to fit the abdominal contour of the mice. Before application, the abdominal fur was removed to ensure direct skin-to-skin contact. The belt was securely attached using a waterproof adhesive plaster, ensuring uniform positioning and uninterrupted skin contact throughout EMW exposure. The belts were examined daily and replaced as needed to ensure proper hygiene and secure connections.

Belt composition and application

Each therapeutic belt comprised a waterproof adhesive patch measuring 15 × 3 cm, including a 2 × 1 cm central capsule constructed from a modified paper clip. The belt contained the cactus formulation corresponding to each treatment group. The cactus formulations were not administered trans-dermally or orally, and there was no direct biochemical contact with the cutaneous cells or systemic antioxidant mechanisms. The cactus served as a physical interface engineered to reflect or absorb incoming EMWs, providing a non-invasive protective mechanism.

Sample collection

On gestational day 20, euthanasia was performed via intraperitoneal injection of ketamine. Euthanasia was performed via intraperitoneal injection of ketamine (Ket-A-100, injection 100 mg/mL, Indonesia) at a dose of 120 mg/kg body weight, which is commonly used in rodents to induce deep anesthesia and achieve humane euthanasia. The selected dose is within the established range of 85-200 mg/kg for ketamine in mice. Mice were observed for loss of pedal reflex to confirm adequate anesthesia before dissection (Jamal et al., 2008; Hohlbaum et al., 2018). Following confirmation of death, the pregnancy was terminated, and the maternal and fetal cerebellar tissues were carefully dissected. The samples were immediately fixed in 10% neutral buffered formalin for 24-48 hours. After fixation, tissues underwent established paraffin-processing, including graded ethanol dehydration (70%, 80%, 90%, 95%, 100%), xylene clearing, and embedding in paraffin blocks. Paraffin blocks were sectioned at a thickness of 4-5 µm using a rotary microtome and mounted on glass slides. The slides were then subjected to Hematoxylin-Eosin (H&E) staining, consisting of hematoxylin nuclear staining, differentiation, bluing, eosin counterstaining, dehydration, clearing, and coverslip mounting. Histological preparations were examined under a light microscope for structural assessment in accordance with histopathology protocols (Ghoneim and Arafat, 2016; Javaeed et al., 2021).

X-ray Fluorescence analysis

The elemental composition of the *O. cochenillifera* preparations was determined using an energy-dispersive X-ray fluorescence spectrometer (EDX-7000, Shimadzu Corporation, Japan). Samples weighing 0.5-1 g were dried to constant weight, homogenized, and pressed into pellets before analysis. Measurements were carried out at 50 kV and 1 mA with a 200-second acquisition time, and the spectra were analyzed using the manufacturer's energy-dispersive X-ray fluorescence (EDXRF) software package (Santo et al., 2025).

Fourier transform infrared spectroscopy analysis

Functional group characterization was performed using a Fourier transform infrared spectrometer equipped with a diamond attenuated total reflectance (ATR) accessory (IRSpirit ATR, Shimadzu Corporation, Japan). Spectra were collected from 4000-400 cm^{-1} with a resolution of 4 cm^{-1} and 32 scans per sample. Baseline correction and normalization were performed using the built-in LabSolutions IR software (Gieroba et al., 2023).

Statistical analysis

All statistical analyses were performed using SPSS version 26.0. Data normality was assessed using the Shapiro-Wilk test, which indicated a non-normal distribution for cerebellar tissue examinations in both maternal and fetal samples. Therefore, nonparametric statistical methods were employed for group comparisons. The Kruskal-Wallis test was used to compare across groups, followed by post-hoc pairwise comparisons using the Mann-Whitney U test. The present results were presented as mean ± standard deviation (SD), with statistical significance defined as a p-value less than 5% ($p < 0.05$).

RESULTS AND DISCUSSION

The HCG exhibited Purkinje cells arranged in a continuous monolayer at the junction of the molecular and granular layers, characterized by large euchromatic nuclei and intact morphology. In contrast, the NCG revealed disorganized Purkinje cell alignment, reduced cellularity, and degenerative features, including pyknotic nuclei and areas of focal loss.

In the treatment groups (E1-E4), Purkinje cells remained detectable as a distinct monolayer, with only minimal degenerative changes compared to the NCG. Group E1 exhibited a well-preserved morphology, closely resembling HCG. Groups E2-E4 demonstrated a well-defined Purkinje cell layer characterized by decreased yet recognizable cellular alignment, and overall histological preservation was greater than that observed in the NCG.

The descriptive results indicated that the HCG and treatment groups (E1-E4) exhibited relatively comparable Purkinje cell size and count, with mean values ranging from 1.39-1.40 µm for cell size and 9.4-9.6 cells for cell count. In contrast, the NCG consistently demonstrated reduced values, with an average Purkinje cell size of 1.13 ± 0.22 µm and a cell count of 4.80 ± 2.00. A similar trend was observed in fetal Purkinje cell measurements, with NCG values remaining significantly lower than those in the HCG and E1–E4 groups (Table 1). Significant overall differences were observed across all six groups (HCG, NCG, E1, E2, E3, and E4) for Purkinje cell size and count, as well as for fetal parameters ($p < 0.05$). The NCG demonstrated statistically significant differences compared to the HCG and all treatment groups for all parameters ($p < 0.05$). However, no statistically significant differences were found among the experimental groups (E1-E4), nor between the HCG and any of the experimental groups ($p > 0.05$). The current findings suggested that the experimental interventions effectively maintained Purkinje cell morphology at levels comparable to HCG, whereas NCG consistently exhibited impairment across all parameters.

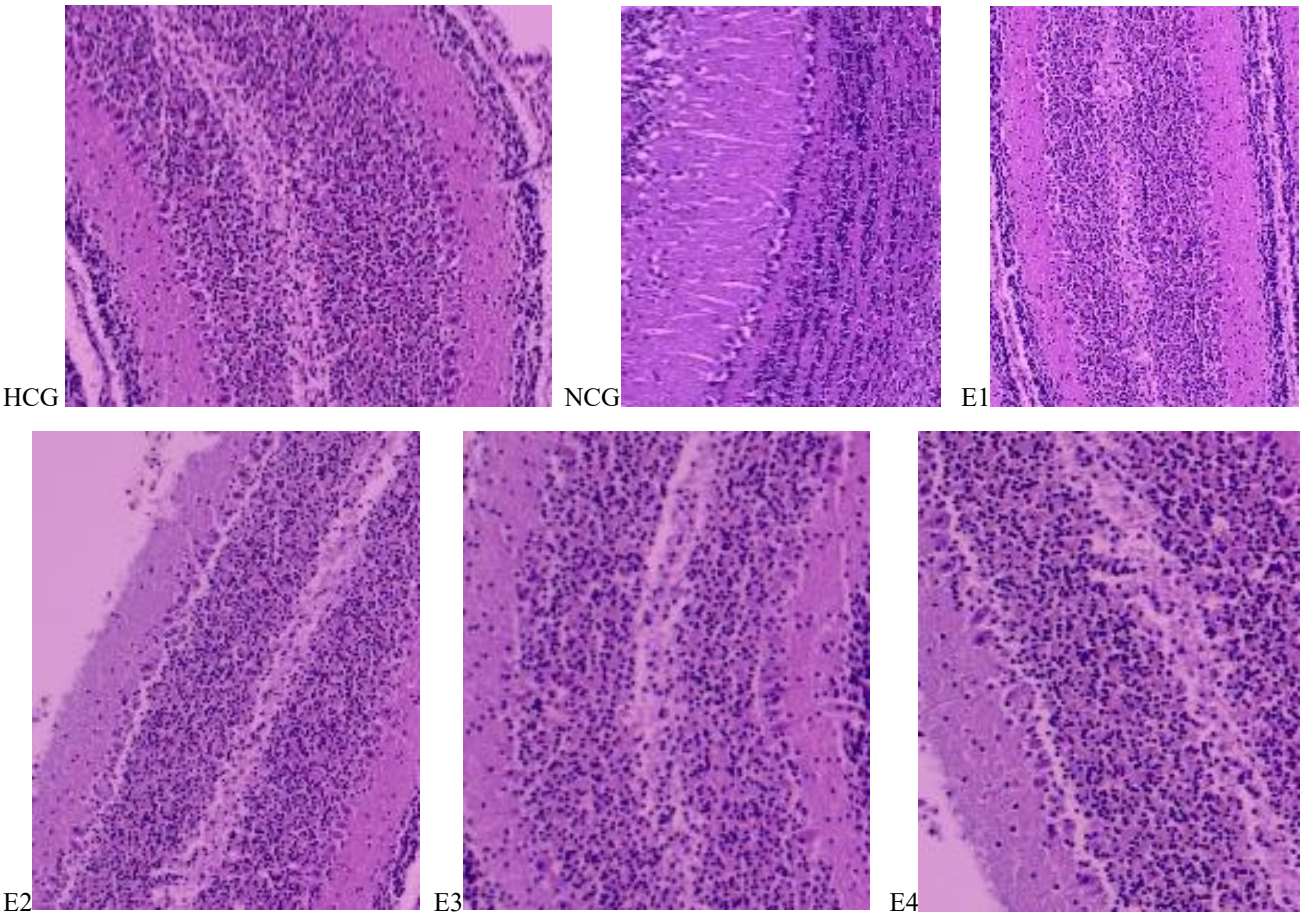


Figure 1. Mice fetal cerebellar Purkinje cell. HCG: Health control group, NGC: Negative control group, E1: Fresh cactus belt (raw cactus), E2: Desiccated cactus belt, E3: Cactus extract gel belt, E4: Cactus extract powder belt (dehydrated extract). H&E, 400×.

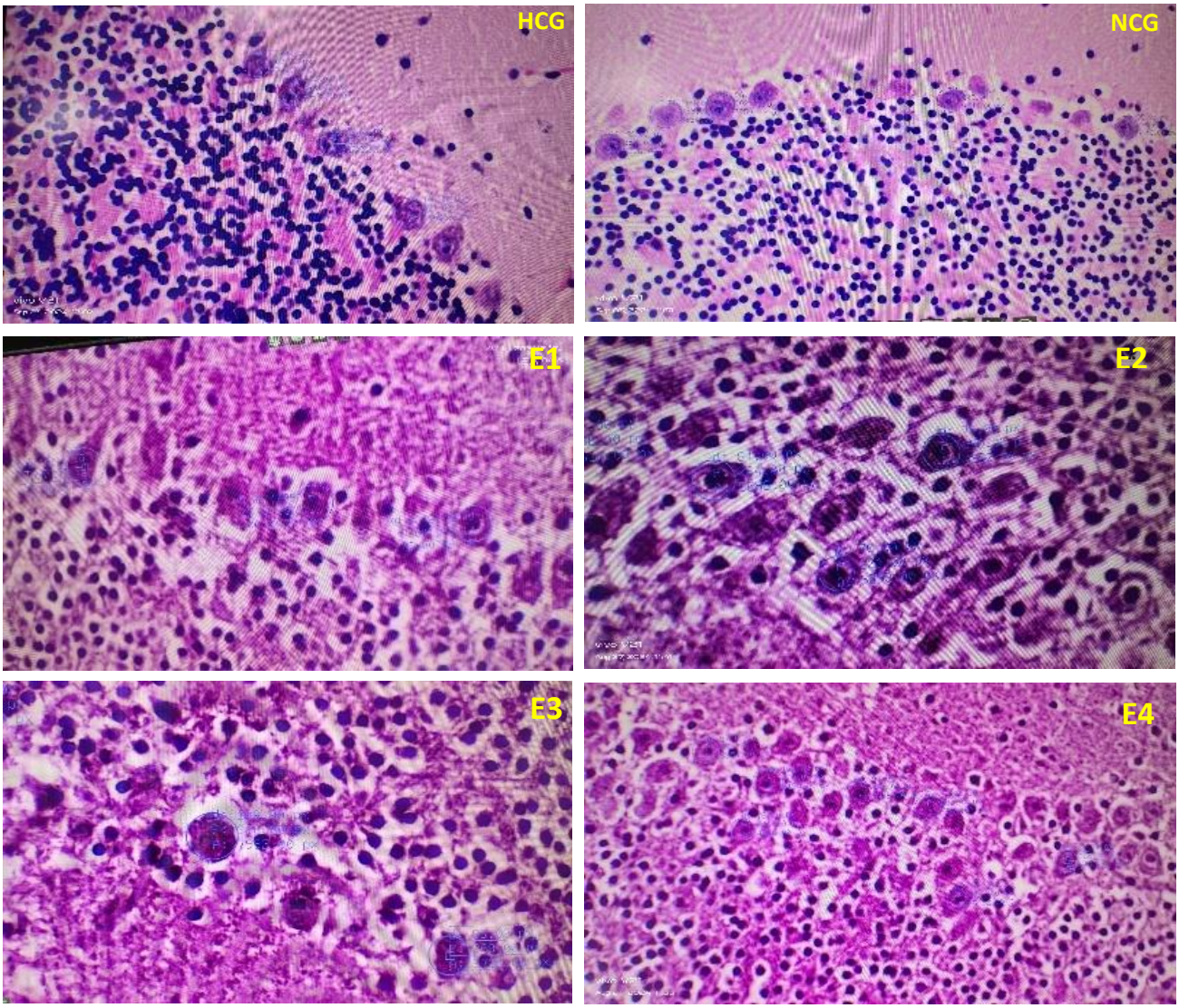


Figure 2. Maternal cerebellar Purkinje cells of Female 2-3 months old mice. HCG: Health control group, NGC: Negative control group, black arrow: Reduced number of cells, E1: Fresh cactus belt (raw cactus), E2: Desiccated cactus belt, E3: Cactus extract gel belt, E4: Cactus extract powder belt (dehydrated extract). H&E, ×400.

Table 1. Effects of cactus-based interventions on maternal and fetal Purkinje cell size and count across experimental groups

Group	Maternal Purkinje Size (µm)	Maternal Purkinje Count	Fetal Purkinje Size (µm)	Fetal Purkinje Count	P value
HCG	1.39 ± 0.20[a]	9.60 ± 1.80[a]	1.37 ± 0.22[a]	9.40 ± 2.10[a]	<0.000
NCG	1.13 ± 0.22[b]	4.80 ± 2.00[b]	1.09 ± 0.19[b]	4.60 ± 1.80[b]	-
E1	1.40 ± 0.23[a]	9.50 ± 1.70[a]	1.38 ± 0.24[a]	9.50 ± 1.90[a]	<0.000
E2	1.40 ± 0.21[a]	9.40 ± 1.80[a]	1.39 ± 0.23[a]	9.60 ± 1.70[a]	<0.000
E3	1.39 ± 0.22[a]	9.60 ± 1.90[a]	1.37 ± 0.20[a]	9.50 ± 2.00[a]	<0.000
E4	1.39 ± 0.19[a]	9.50 ± 1.80[a]	1.38 ± 0.21[a]	9.40 ± 1.80[a]	<0.000

HCG: Healthy control group, NCG: Negative control group, E1: Fresh cactus belt (raw cactus), E2: Desiccated cactus Belt, E3: Cactus extract gel belt, E4: Cactus extract powder belt. P values were calculated relative to the negative control group (NCG). Data are expressed as mean ± standard deviation (SD). [ab]Means significant differences between groups are indicated by different superscript letters within the same column, as determined by Mann–Whitney post hoc tests following Kruskal–Wallis analysis ($p < 0.05$).

Mineral composition and Fourier transform infrared spectroscopy

The results of the X-ray fluorescence demonstrated notable differences in mineral compositions among the raw cactus, dried cactus, cactus extract gel, and cactus extract powder preparations (Table 2). Dried cactus demonstrated the highest calcium oxide (CaO) concentration of 1.90%, whereas the cactus extract gel was significantly enriched in

potassium oxide (K_2O, 8.45%) and chloride (Cl, 5.43%). Magnesium oxide (MgO) was significantly elevated in the cactus extract gel (1.49%) compared to the other forms of cactus. Trace elements such as iron (III) oxide (Fe_2O_3), silver oxide (Ag_2O), and iodine were mostly identified in the raw cactus, whereas bromine (Br) was exclusively present in the cactus extract gel and powder.

Fourier-transform infrared (FTIR) spectroscopy revealed essential functional groups in all samples (Table 3). Raw cactus spectra revealed significant hydroxyl stretching (3305 cm^{-1}), carbonyl amide (1636 cm^{-1}), and ether/polysaccharide peaks, indicative of elevated water and carbohydrate contents. Dried cactus exhibited preserved hydroxyl and aromatic ring signals and heightened ester/protein-associated peaks. The cactus extract gel demonstrated pronounced carbonyl stretching (1722 cm^{-1}), suggestive of esters or carboxylic acids, and aromatic and amine functionalities. The cactus extract powder exhibited pronounced hydroxyl and phenolic signals, intricate polysaccharide bands, and substituted aromatic ring patterns. The current results indicated that drying increased the CaO and K_2O. Simultaneously, extraction, especially in the gel form, significantly enriched K_2O, Cl, and MgO, along with notable functional groups associated with aromatic compounds and polysaccharides. The variability in biological activities among the cactus forms might be attributed to the differences in their chemical and structural characteristics.

Table 2. Mineral contents of cactus preparations determined by X-ray fluorescence

Mineral	Raw cactus	Dried cactus	Cactus extract gel	Cactus extract powder
CaO	0.288%	1.90%	0.05%	0.12%
MgO	0.0728%	0.353%	1.49%	0.0953%
K_2O	0.0553%	1.37%	8.45%	0.764%
Cl	0.0683%	0.245%	5.43%	0.349%
Trace elements	Fe_2O_3, Ag_2O, I, RuO_2, TeO_2, ZrO_2/Nb_2O_5	Fe_2O_3, P_2O_5, SO_3, SrO	Br, P_2O_5, SO_3, Rb_2O	P_2O_5, SO_3, Br, Rb_2O

CaO: Calcium oxide, MgO: Magnesium oxide, K_2O: Potassium oxide, Cl: Chloride, Fe_2O_3: Iron (III) oxide, Ag_2O: Silver oxide, I: Iodine, RuO_2: Ruthenium oxide, TeO_2: Tellurium oxide, ZrO_2: Zirconium oxide, Nb_2O_5: Niobium oxide, P_2O_5: Phosphorus pentoxide, SO_3: Sulfur trioxide, SrO: Strontium oxide, Br: Bromine, Rb_2O: Rubidium oxide

Table 3. Representative Fourier transform infrared spectroscopy bands and assigned functional groups observed across cactus-derived samples

Sample	Main peaks (cm^{-1})	Key functional group
Raw cactus	3305 (O–H), 2110 (Alkyne/N=C=O), 1636 (C=O amide), 1018 (C–O–C)	Hydroxyl, amide, ether/polysaccharide, reactive triple bond
Dried cactus	3265 and 2917 (O–H, C–H), 1606 (C=C aromatic), 1316 and 1033 (C–O/C–N ester)	Hydroxyl, aromatic ring, ester/protein
Cactus extract gel	3217 (O–H), 1722 (C=O ester), 1584 (C=C aromatic/N–H), 1047 (C–O)	Hydroxyl, ester/carboxylic acid, aromatic/amine, carbohydrate
Cactus extract powder	3324 (O–H), 1593 (C=C aromatic/amide), 1030–1068 (C–O), 749 and 670 (Aromatic sub.)	Hydroxyl, phenolic, polysaccharide, substituted aromatic rings

Impacts of electromagnetic waves on maternal and fetal Purkinje cells

The present study demonstrated that Purkinje cell size and count in the HCG and experimental groups (E1-E4) were maintained at comparable levels. In contrast, the NCG exhibited significantly reduced values and distinct histological degeneration ($p < 0.05$). The present findings indicated that exposure to EMFs without protective measures compromised cerebellar integrity, whereas controlled interventions were able to mitigate this damage.

Purkinje cells, which serve as the primary neurons of the cerebellar cortex, exhibited high susceptibility to oxidative stress because of elevated metabolic demands, complex dendritic architecture, and mitochondrial dependence (Altunkaynak et al., 2016; Manolaras et al., 2023; Chui et al., 2024). Increased reactive oxygen species (ROS) can lead to mitochondrial malfunction, lipid peroxidation, protein oxidation, and DNA damage, ultimately inducing apoptosis through caspase activation and jeopardizing neuronal survival (Chen et al., 2003; Liu et al., 2025; Toader and Covache-busuioc, 2025). The impairment of ion channel function and synaptic communication has been implicated as a contributing factor, further disrupting Purkinje cell physiology and heightening the risk of degeneration (Orfali et al., 2024). Furthermore, oxidative stress can compromise ion channel function by inducing oxidative modifications in protein channels, which alter the gating properties of voltage-gated and ligand-gated ion channels, further disrupting neuronal physiology and enhancing vulnerability to degeneration (Georgiou and Margaritis, 2021; Orfali et al., 2024).

Previous studies examining the effects of 900-MHz EMFs on the cerebellum using histopathological and immunohistochemical techniques reported the presence of pyknotic nuclei in Purkinje cells and granular layer neurons, along with a marked reduction in cytoplasmic content in the EMF-exposed groups (Bas et al., 2022). Depending on the frequency, intensity, and duration of exposure, EMWs can produce diverse cellular effects. Ion channel impairment occurs through a mechanism in which non-ionizing EMFs disturb the electrochemical balance across cellular membranes, modify cation flux, and impair voltage-gated ion channel function. Such disturbances may increase oxidative stress through the activation of membrane-associated enzymes such as NADPH oxidase (NOX), resulting in the overproduction of ROS, compromised cellular function, and even DNA damage, which is implicated in carcinogenesis (Georgiou and Margaritis, 2021; Panagopoulos and Karabarbounis, 2021). Several studies have indicated that EMF-induced disruption of ion channels was associated with the generation of ROS and oxidative stress via NOX activation, resulting in subsequent cellular damage, including DNA strand breaks (Georgiou and Margaritis, 2021). The specific function of NOX in facilitating oxidative stress during EMF exposure continues to be a primary focus of investigations (Georgiou and Margaritis, 2021).

Cellular stress response

Exposure to non-ionizing EMF activates a cellular stress response, a protective mechanism designed to preserve cellular integrity (Lai and Levitt, 2024). The cellular response involves a series of molecular and cellular alterations, such as cell cycle arrest and activation of repair mechanisms to manage macromolecular damage (Meyer et al., 2024). This cellular reaction is not specific to EMF but is a general reaction to several stressors and can lead to repair or, in cases of severe damage, cell death (Haidar et al., 2025). The stress response follows a non-linear pattern dependent on dose and timing and may influence several health outcomes, such as cancer risk and nerve regeneration (Lai and Levitt, 2024). These approaches revealed the complex nature of EMWs' interactions with biological systems (Meyer et al., 2024). Biological responses to EMF were substantially affected by the parameters of exposure. While excessive or improperly regulated exposure may lead to oxidative stress and cellular damage, carefully controlled EMF application has been associated with adaptive or therapeutic effects, such as enhanced tissue repair and increased bacterial susceptibility to antibiotics (Haidar et al., 2025).

The cerebellar alterations observed in the present study are consistent with the cellular stress response described in the literature. Exposure to non-ionizing EMFs triggered a conserved cellular defense response characterized by cell-cycle arrest, enhanced DNA and protein repair pathways, and, in instances where damage surpassed repair capacity, apoptotic cell death (Lai and Levitt, 2024). The observed mechanistic pattern aligned with the structural disruptions noted in maternal and fetal cerebella following EMF exposure, suggesting that cerebellar tissue experienced a transition toward maladaptive stress responses.

Oxidative stress has been widely identified as a central mediator of EMF-induced cellular injury in neural tissues. Numerous *in vitro* and *in vivo* studies have demonstrated that EMF exposure increased ROS, disrupted mitochondrial function, and promoted oxidative DNA and protein damage (Saliev et al., 2019; Schuermann and Mevissen, 2021). The findings of reduced Purkinje cell counts and altered cerebellar morphology in fetal mice, alongside corresponding changes in EMF-exposed dams, were related to these mechanisms. The cerebellum, particularly Purkinje neurons, is highly susceptible to oxidative imbalance, which aligns with the ROS-driven injury observed in the present study (Schuermann and Mevissen, 2021).

According to multiple studies, EMF biological effects are strongly influenced by exposure dose, duration, developmental stage, and the inherent vulnerability of neural tissue (Tian et al., 2022; Lai and Levitt, 2024). These findings are consistent with the more pronounced alterations observed in fetal cerebellar tissue in the present study, as the developing brain exhibits immature antioxidant defenses and a higher proliferative index, making it especially vulnerable to EMF-induced oxidative stress and stress-response activation. Consequently, although EMF exposure may result in adaptive or neutral outcomes, the prenatal cerebellum is far more likely to experience growth impairment or structural disruption under the same exposure conditions (Tian et al., 2022).

Potential electromagnetic wave attenuation and mineral composition

Iron oxide (Fe_2O_3) is widely known as an effective microwave absorber owing to its dual contribution to magnetic and dielectric loss mechanisms (Akinay et al., 2023; Ghosh et al., 2023). Incorporating α-Fe_2O_3 nanostructures into composites has indicated delivering reflection loss values greater than −30 dB within the gigahertz range, largely due to interfacial polarization and magnetic resonance effects (Mensah et al. 2022; Zheng et al., 2022). The notable EMW absorption capacity of Fe_2O_3-based materials in engineered composites, attributed to combined magnetic and dielectric loss, suggested that even low concentrations of Fe_2O_3 particles within biological matrices may contribute to partial wave attenuation before deep tissue penetration (Lesbayev et al., 2025; Lodi et al., 2025).

In contrast, CaO and MgO function predominantly as dielectric agents, enhancing polarization under alternating EMFs. The MgO-based ceramics and glass-ceramics are widely used for dielectric applications in microwave and radiofrequency systems (Hu et al., 2021). The current results demonstrated that MgO or CaO/MgO mixtures could serve as dielectric buffers in composite matrices (Cabeza et al., 2002). The ability of such oxide particles to attenuate incident EMFs when incorporated into biological tissues has not yet been experimentally validated, necessitating focus on *in vivo* investigations (Lodi et al., 2025; Reghunath et al., 2025a). The observed polarization causes dipole alignment and energy loss, converting electromagnetic energy into localized heat at the microstructural level (Muradyan et al., 2025). Under these conditions, dried cactus preparations demonstrated the highest combined CaO and MgO content, suggesting a strong dielectric contribution to EMW attenuation.

Potassium oxide (K_2O), which was relatively abundant in the cactus gel, may help modulate the local dielectric properties of the hydrated matrix. Absorption efficiency in engineered EMW-absorbing materials relies heavily on precise control of permittivity and impedance matching with free space, typically achieved through dielectric and/or magnetic loss mechanisms (Gabriel et al., 1996a; Ruscica et al., 2024). Any involvement of K_2O-rich biological matrices in modulating impedance and attenuating EMW remains theoretical, as no direct *in vivo* data are currently available (Akinay et al., 2023; Chen et al., 2024). The combination of K_2O-rich domains and organic components facilitates dielectric heterogeneity, resulting in enhanced multiple scattering and efficient attenuation of EMW transmission (Reghunath et al., 2025b).

The presence of chloride ions (Cl^-) in hydrated matrices can influence ionic conductivity, dielectric response, and microstructural organization, thereby modulating the interaction of these materials with alternating EMFs. Studies have documented this effect in inorganic composites like chloride-exposed cement pastes, where Cl^- alters impedance and dielectric behavior. However, whether similar effects happen in biological gcenchenels remains unknown and has not been proven (Hu et al., 2016).

Chloride ions increase ionic conductivity in hydrogels, a useful property for sensors (Ji et al., 2025). In cement materials, chloride and moisture changes can alter electrical properties and create irregularities that scatter, reflect, or reduce EMWs (Hu et al., 2019, 2020; Elseady et al., 2023; Mizobuchi et al., 2026). Cl^- affects ion distribution and interfacial conductivity in anti-corrosion coatings, thereby altering wave propagation through the material (Zhang et al., 2021; Tzaneva and Kostov, 2023). These mineral oxides and salts contribute to three principal electromagnetic attenuation mechanisms. Iron oxide (Fe_2O_3) predominantly promoted magnetic loss through the hysteresis mechanism and eddy-current dissipation, while dielectric oxides such as CaO, MgO, and K_2O enhanced dielectric loss via dipole polarization and relaxation under alternating EMFs (Zhang et al., 2022; 2025). Additionally, components such as K_2O and Cl^- can induce impedance mismatch and microstructural heterogeneity in hydrated matrices, leading to multiple scattering and reflection that further reduce EMW transmission (Liang et al., 2021; Katheriya et al., 2025).

Interaction of dielectrics with organic functional groups

The FTIR spectroscopy analysis provided insight by identifying organic functional groups capable of interacting with EMW. All cactus samples displayed pronounced O–H stretching bands between 3200 and 3500 cm^{-1}, indicating the existence of hydroxyl-rich compounds, such as phenolics and polysaccharides. These functional groups demonstrated high polarity, allowing interaction with alternating electric fields in the gigahertz range via mechanisms such as dipole rotation and dielectric heating, as reported by Gabriel et al. (1996b), Soria et al. (2021), and Wang et al. (2023).

Cactus gel and powder extracts exhibited significant C=O stretching bands at approximately 1720 cm^{-1}, indicative of esters and carboxylic acids. Carboxyl groups improved hydrophilicity and surface polarity, yet recent findings indicated that their effectiveness in reducing EMWs may rely on their interactions with conductive elements. In hydrated matrices, these groups may still promote the dissipation of microwave energy via intermolecular collisions (Płowaś-Korus and Buchner, 2021). The C-O stretching region (1030-1068 cm^{-1}) observed in all samples indicated the presence of polysaccharides capable of forming hydrogen-bonded networks. These networks, especially when ionically crosslinked with Ca^{2+} or Mg^{2+} derived from the mineral component, can create inhomogeneous dielectric domains within the polymer matrix. These domains enhanced electromagnetic dispersion and reduced the penetration depth in bio-based composites (Prashanth et al., 2024). The aromatic C=C vibrations detected in the dried cactus and gel extract during the present study indicated the presence of phenolic compounds that may participate in π-electron resonance interactions with EMEs. These interactions may contribute slightly to overall EMEs reduction, despite being less efficient than ionic or polar processes.

Coordinated neuroprotective effects on the maternal and fetal cerebellum

The preservation of cerebellar profiles in the cactus-treated groups was likely attributable to the synergistic action of mineral- and organic-based attenuation mechanisms. Mineral oxides act as inorganic shielding agents, contributing to

stable dielectric and magnetic losses, whereas organic polar groups enable dynamic dipole-based dissipation. This dual-mode attenuation reduces the net EMW energy reaching the cerebellum, thereby minimizing microstructural disruption. From a biological perspective, reduced EMW exposure may result in preserved Purkinje cell morphology, decreased neuronal apoptosis, and maintained organization of the cerebellar layers. Although inflammatory and oxidative stress biomarkers were not directly measured, histological assessments of the present study supported the neuroprotective effect. Differences among the cactus preparations may stem from their composition. Dried cactus is rich in CaO and MgO, exhibiting strong dielectric loss potential. Cactus gel extract had very high levels of K_2O and Cl, which enhance scattering and impedance mismatch. Cactus powder extract contained a balanced mix of minerals and organic matter, offering stable shielding. Raw cactus had lower oxide levels but higher water content, which might have promoted dielectric heating and scattering through water dipoles. Although substantial differences were observed in the XRF and FTIR profiles among the four *O. cochenillifera* formulations (raw, dried, gel extract, powder), no considerable differences were detected in the cerebellar outcomes of maternal and fetal mice. The current findings indicated that electromagnetic shielding was influenced less by specific mineral levels and more by collective physicochemical features such as bound water content, ionic composition, and polysaccharide matrix structures (Simón et al., 2019). According to foundational studies on dielectric properties, water dipole polarization and ionic conduction are the primary factors influencing the dielectric behavior of biological and hydrated plant materials, whereas differences in mineral composition have only a limited effect on radiofrequency (RF) reduction (Meenu et al., 2025).

Microwave absorption studies on plant-based materials such as *Opuntia* have revealed that cladodes exhibited high absorption coefficients, a characteristic attributed to their hydrated polysaccharide framework, even when chemical composition differs (Gupta et al., 2024). Consequently, all four cactus formulations probably provided sufficient dielectric shielding to prevent RF exposure from reaching biologically damaging levels. The neuroprotective effects were similar across all cactus formulations, despite differences in their XRF/FTIR profiles.

CONCLUSION

All four forms of *O. cochenillifera*, including raw, dried, gel extract, and powder, demonstrated comparable neuroprotective effects. Each cactus preparation, regardless of its differing XRF and FTIR profiles, provided robust dielectric shielding. This protection effectively prevented EMW-induced cerebellar damage and maintained the morphology and number of maternal and fetal Purkinje cells, achieving levels similar to those of healthy mice. The current findings indicated that the observed neuroprotective effects stem from common physicochemical properties shared across the cactus preparations, rather than from any single form. Therefore, cactus-derived materials show potential as effective, bio-based agents for protecting the nervous system from EMW exposure during pregnancy. This approach offered a sustainable and safer alternative to conventional synthetic composites, with particular relevance for maternal health due to the heightened vulnerability of pregnant individuals to EMW exposure. Future studies should focus on long-term neurodevelopmental investigations on maternal and fetal cerebellar structures, mechanistic investigations clarifying oxidative stress and signaling pathways underlying EMW-induced cerebellar alterations, and optimization of cactus extract formulations to maximize neuroprotective efficacy during pregnancy.

DECLARATIONS

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Authors' contributions

Desy Armalina was responsible for the conceptualization and design of the study, coordination and administration of the project, preparation of *O. cochenillifera* materials (including fresh, dried, gel, and powdered forms), oversight of animal scheduling and logistical arrangements, supervision of the *in vivo* phase, and served as the corresponding author while also contributing to the writing of the original draft. Heri Sutanto contributed to the methodology for EMW exposure, including the setup and calibration of the EMW system and exposure parameter instrumentation, performed FTIR and XRF characterization of cactus preparations, and was involved in data curation, visualization, as well as writing, reviewing, and editing of the present study. Sunarno conducted animal handling and randomization, executed the exposure sessions, performed dissection and collection of maternal and fetal cerebella, carried out histology processing and Purkinje-cell morphometry, completed fetal anthropometric assessments, and contributed to data curation, formal analysis, and writing, review, and editing of the manuscript. Neni Susilaningsih supervised the histopathological assessments, interpreted cerebellar findings, integrated mechanistic context related to dielectric and magnetic interactions and mineral-oxide content with experimental results, provided analytical guidance, and performed critical revision of the manuscript, wrote, reviewed, and edited the present study. All authors verified the underlying

data, contributed to data interpretation, agreed to be accountable for all aspects of the study, and approved the final edition of the manuscript.

Availability of the data and materials

All data generated or analyzed during the present study are included in the published article. No additional datasets are available.

Competing interests

The authors have not declared any conflict of interest.

Ethical considerations

The authors confirm that this manuscript is original and not derived from previously published works. An artificial intelligence tools (Grammarly) was only used for language improvement.

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Antifungal Activity of Crude *Solanum incanum* Fruit Extracts Against *Trichophyton mentagrophytes* and Exploratory Analysis of Virulence-Associated Gene Expression

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ABSTRACT

Dermatophyte infections in humans and animals are a significant public health concern, and increasing resistance to antifungal medicines highlights the need to explore alternative therapeutic agents. The present study aimed to preliminarily assess the *in vitro* antifungal activity of crude extracts from *Solanum incanum* (*S. incanum*) fruit against *Trichophyton mentagrophytes (T. mentagrophytes)*. *Solanum incanum* fruits were extracted with hexane, ethyl acetate, methanol, and water. The levels of phenolic, flavonoid, and alkaloid compounds were measured, and antifungal activity was assessed using disc diffusion, minimum inhibitory concentration (MIC), and minimum fungicidal concentration (MFC) assays. Methanol and aqueous extracts exhibited the highest phenolic contents (~19 mg Gallic Acid Equivalents/g extract) and demonstrated significant antifungal activity, producing inhibition zones of 14.46 mm and 8.57 mm, respectively, at a concentration of 5 mg/mL. However, terbinafine demonstrated marginally greater inhibitory activity (15.02 mm) at a lower concentration of 0.1 mg/mL. The present findings indicated that using methanol and aqueous extracts of *S. incanum* fruit was associated with lower transcript levels of the targeted virulence-related genes (*SUB3* and *StuA*). The current findings provided preliminary evidence regarding the antifungal activity of *S. incanum* fruit extract against *T. mentagrophytes* at a concentration of 5 mg/mL, indicating its potential as an antifungal agent.

Keywords: Antifungal activity, Bioactive compound, Dermatophyte, Gene expression, Medicinal plant

INTRODUCTION

Over the past twenty years, there has been a notable increase in both superficial and systemic fungal infections, many of which pose significant public health concerns (Richardson, 2022). The increase is particularly noted among individuals with compromised immune systems, including those living with Human Immunodeficiency Virus (HIV), patients undergoing cancer chemotherapy, and individuals receiving extended courses of antibiotic treatment (Bongomin et al., 2017). Dermatophytes, a type of filamentous fungus, are the main cause of superficial mycoses and colonize keratinized tissues such as the skin, nails, and hair (Weitzman and Summerbell, 1995). Dermatophyte infections are generally limited to the skin; however, dissemination to other keratinized or extra-cutaneous tissues may occur in immunocompromised individuals, resulting in more severe clinical manifestations and substantial health complications (Bongomin et al., 2017).

Trichophyton mentagrophytes (*T. mentagrophytes*) is a zoophilic dermatophyte and a major causative agent of dermatophytosis (ringworm) in a wide range of animal species (Paryuni et al., 2020). *Trichophyton mentagrophytes* frequently infects companion animals such as dogs and cats, as well as livestock, including cattle, sheep, and goats, and several rodent species (Paryuni et al., 2020). In veterinary medicine, dermatophytosis represented a significant health and economic burden (Kappes et al., 2023). In livestock, infections can result in reduced weight gain, decreased productivity, and damage to hides and wool, thereby lowering their market value (Kappes et al., 2023). Furthermore, the disease increases expenses for treatment, control efforts, and farm management (Kappes et al., 2023). Notably, *T. mentagrophytes* is zoonotic and can be transmitted from animals to humans, posing occupational risks to farmers, veterinarians, and animal handlers (Plangsiri et al., 2025). The management of dermatophytosis in animals remains challenging due to the limited availability of licensed veterinary antifungal medications, the emergence of antifungal resistance, and concerns associated with drug residues and withdrawal periods in food-producing animals (Frymus et al., 2013).

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Fungal skin infections affect approximately 25% of the world's population, making them some of the most common infectious diseases in humans. *Trichophyton* species are the leading cause of dermatophytosis, accounting for about 70% of cases (Bongomin et al., 2017). Although antifungal agents such as azoles and allylamines are available, their use is often limited by resistance, toxicity, high costs, and limited access in low-resource settings (Perlin et al., 2017). These limitations highlight the urgent need for safer, more affordable, and more effective antifungal agents. Traditional medicine plays a vital role in healthcare systems across Africa, with around 80% of the population relying on herbal medicines for their well-being (WHO AFRO, 2003). *Solanum incanum* (*S. incanum*), commonly known as Sodom's apple, plays a crucial role in African ethnomedicine for treating several diseases, including skin infections such as ringworm, dermatitis, and wounds (Kokwaro, 2009; De Wet et al., 2013). Several studies have demonstrated that extracts of *S. incanum* exhibited antibacterial, analgesic, and anti-inflammatory activities, as reported in experimental studies evaluating its pharmacological properties (Sbhatu and Abraha, 2020; Musyimi et al., 2021). However, despite comprehensive ethnobotanical documentation, the antifungal efficacy of *S. incanum* against dermatophytes, particularly those caused by *T. mentagrophytes*, remains inadequately investigated.

Previous qualitative phytochemical studies of *S. incanum* have consistently identified several major classes of secondary metabolites, such as flavonoids, alkaloids, tannins, and saponins (Mshimba, 2014). The complex phytochemical profile of *S. incanum* suggested potential pharmacological properties that may explain its traditional therapeutic uses.

Understanding the mechanism of antifungal action is essential for scientific and clinical advancement. The pathogenicity of *Trichophyton* spp. is linked to increased expression of several virulence genes, including secreted proteases such as *subtilisin 3* (*SUB3*), which facilitate host tissue degradation and invasion (Martínez-Rossi et al., 2018). In addition, transcriptional regulators such as *StuA* play a key role in stress tolerance, fungal growth, and virulence regulation (Gauthier et al., 2010; Lang et al., 2020). Previous studies have demonstrated that natural compounds can modulate fungal gene expression (Silva et al., 2016). For instance, eugenol, an antifungal compound derived from plants, notably downregulated the SUB3 gene in *T. rubrum*, with effects comparable to those observed with terbinafine treatment (Silva et al., 2016). The assessment of gene expression profiles provides a molecular approach to evaluating the ability of plant extracts to disrupt fungal virulence pathways and elucidating the mechanisms of antifungal action (Ball et al., 2020). The present study aimed to assess the antifungal properties of *S. incanum* extracts against *T. mentagrophytes* and to investigate their potential effects on virulence-related genes.

MATERIALS AND METHODS

Ethical approval

The study was conducted in accordance with the ethical principles and approved procedures of the Institutional Scientific and Ethical Review Committee at Jomo Kenyatta University of Agriculture and Technology (JKUAT), Kenya. Ethical approval for the study was obtained from the committee before the study began under the approval number JKU/ISERC/02316/1907. All experimental procedures were performed in compliance with the approved institutional guidelines and ethical standards.

Solanum incanum collection

A total of 4 kg of whole *S. incanum* plants were collected from the Mungetho agricultural area of Juja Farm in Kiambu County, Kenya, for identification, and approximately 2 kg of yellow fruits were used for extraction. The verification of *S. incanum* plants was conducted at the Botany Laboratory of JKUAT, Kenya. A deposit of *S. incanum* plants has been made in the herbarium under accession number PSD-JKUATBH/001.2/2025.

Plant extracts preparation

The fruits of *S. incanum* were washed with distilled water and air-dried at room temperature (25°C) for nine days. The yellow outer pericarp was removed, and the seeds were retained for extraction. The dried fruits of *S. incanum* were ground into powder using an electric grinder. About 100 g of this powdered material was extracted sequentially with solvents of increasing polarity, hexane, ethyl acetate, and methanol, to obtain different fractions from the same plant source. In addition, an aqueous extract was prepared independently to mimic traditional usage and to evaluate water-soluble bioactive compounds not fully represented in organic solvent fractions. The powdered substance was immersed in 1000 mL of hexane inside a conical flask sealed with a rubber stopper and left for 72 hours away from direct sunlight, with occasional shaking. Then, sterile filter paper was used to filter the mixture, and a rotary vacuum evaporator was used to concentrate the filtrate at 42°C. The residue was re-immersed in 1000 mL of ethyl acetate and left in the dark for five days, then filtered and concentrated at 42°C. The remaining residue was re-soaked in 1000 mL of methanol for 72

hours under the same conditions, then filtered and concentrated at 65°C under reduced pressure to prevent damage to thermolabile compounds. For the aqueous extraction procedure, 100 g of the powder was suspended in 1000 mL of distilled water, heated at 50°C for a duration of 20 minutes, then cooled, and subsequently kept at 25°C with agitation for a period of 24 hours. Following filtration, the extract was stored at −80°C for 72 hours prior to lyophilization (freeze-drying; Raghuvanshi and Gupta, 2023). The dried *S. incanum* extracts were weighed, recorded, and stored at 4°C until further analysis. The extraction yield (%) was calculated using Formula 1.

$$\text{Extraction yield (\%)} = \frac{\text{Weight of dried extract}}{\text{Weight of dried plant material}} \times 100 \quad \text{(Formula 1)}$$

Dermatophytes strain isolation

Trichophyton mentagrophytes strain was obtained from the Kenya Medical Research Institute (KEMRI) Mycology Laboratory's culture collection in Nairobi, Kenya, and was initially isolated from an infected patient in 2013. Before experimentation, the isolate was subcultured under standard laboratory conditions, and its identity was reconfirmed by microscopic examination with lactophenol cotton blue staining. Morphological features were examined with an light microscope (Olympus, Japan) to verify characteristic fungal structures before use in experiments. The isolate was subsequently subcultured on Sabouraud dextrose agar (SDA) following standard mycological procedures (De Hoog et al., 2020). Inoculated plates were incubated at 30°C for six days under controlled laboratory conditions, with regular monitoring of fungal growth.

Quantitative screening of flavonoid, alkaloid, and phenolic content

Total flavonoid content

A quercetin standard stock solution (1 mg/mL) was prepared by dissolving 10 mg of quercetin in 10 mL of methanol. This stock solution was then diluted with distilled water to produce working standard solutions at concentrations of 0, 10, 20, 30, and 40 µg/mL. A calibration curve was established by plotting absorbance at 510 nm against quercetin concentration. Total flavonoid levels in the *S. incanum* extracts, including hexane, ethyl acetate, methanol, and aqueous fruit extracts, were measured using the aluminum chloride colorimetric method (Chang et al., 2002). For each extract (1 mg/mL), 1 mL was mixed with 4 mL of distilled water and 0.3 mL of 5% $NaNO_2$. After five minutes, 0.3 mL of 10% $AlCl_3$ was added, and the mixture sat for an additional six minutes. Then, 2 mL of 1 M NaOH was added, and the total volume was adjusted to 10 mL with distilled water. Absorbance was measured at 510 nm using a UV-Visible spectrophotometer (Shimadzu UV-1800, Shimadzu Corporation, Kyoto, Japan). A reagent blank containing all assay reagents except the extract, which was replaced with the corresponding solvent, was employed to calibrate the spectrophotometer and rectify background absorbance. The total flavonoid content was determined from the quercetin calibration curve as depicted in Formula 2.

$$\text{TFC} = \frac{C \times V}{m} \quad \text{(Formula 2)}$$

Where C indicated the concentration of flavonoids from the calibration curve (as quercetin equivalent), V was the volume of extract used in the assay (mL), and m represented the mass of extract used (g), expressed as mg Quercetin Equivalents (QE)/g extract.

Total alkaloid content

The acid-base precipitation method was used to estimate the total alkaloid concentrations in *S. incanum* fruit extracts. Briefly, 0.1 g of the extract was placed in a 250 mL beaker, and 200 mL of 10% acetic acid in ethanol was added. The mixture was covered and allowed to stand at room temperature (25°C) for four hours (Harborne, 1998). The solution was then filtered, and the filtrate was concentrated in a water bath maintained at 50°C until the volume was reduced to one-quarter of its original volume. Concentrated ammonium hydroxide was then added dropwise to the concentrated extract until complete precipitation was achieved. The mixture was left undisturbed to allow the precipitate to settle completely. The precipitated alkaloids were then collected by filtration, rinsed with dilute ammonium hydroxide, dried to a constant weight, and weighed. A reagent blank was prepared following the same procedure, but without the plant extract. The alkaloid mass was calculated according to Formula 3.

$$m_{alkaloid} = m_{total} - m_{container} - m_{blank} \quad \text{(Formula 3)}$$

Where m total represented the mass of the container with all residues (g), m container was the mass of the empty container (g) used to hold the extract, and m blank represented the mass of residues arising from solvent impurities (g).

Total alkaloid content (TAC) was calculated using Formula 4, based on an initial extract mass of 0.1 g.

$$\text{TAC (\%)} = \frac{\text{Mass of alkaloid}}{\text{Initial extract mass}} \times 100 \quad \text{(Formula 4)}$$

Alkaloid content was expressed as a percentage (%) of the dry extract based on the gravimetric determination method, whereas total flavonoid content was expressed in mg/g using a standard calibration curve.

Total phenolic content

Total phenolic concentrations of the four different extracts were measured using the Folin-Ciocalteu colorimetric method (Ainsworth and Gillespie, 2007). A gallic acid stock solution (1 mg/mL) was prepared by dissolving 10 mg of gallic acid in 10 mL of distilled water. Working standard solutions (0, 10, 20, 30, 40, 50 µg/mL) were created through serial dilution of this stock. During the assay, 0.5 mL of each extract (1 mg/mL) or standard solution was added to a test tube, then mixed with 2.5 mL of diluted Folin-Ciocalteu reagent. The mixture was vortexed thoroughly and incubated at 25°C for 10 minutes. Then, 2 mL of 7.5% sodium carbonate (Na_2CO_3) solution (Sigma-Aldrich, St. Louis, MO, USA) was added, vortexed, and incubated in the dark at room temperature (25°C) for 60 minutes. Absorbance was measured at 760 nm using a UV–Visible spectrophotometer (Shimadzu UV-1800, Shimadzu Corporation, Kyoto, Japan) against a reagent blank containing all assay reagents except the plant extract, which was replaced with the corresponding solvent. The working reagent was prepared by diluting the Folin-Ciocalteu reagent 1:1 with distilled water, and a 7.5% (w/v) sodium carbonate solution was prepared by dissolving 7.5 g of Na_2CO_3 in 100 mL of water, yielding a total reaction volume of 10 mL, corresponding to 0.5 mg of extract per assay. Total phenolic content (TPC) was determined using the gallic acid calibration curve, according to Formula 5.

$$TPC = \frac{C \times V}{m} \quad \text{(Formula 5)}$$

Where C was the concentration from the calibration curve (as gallic acid equivalent), V represented the volume of extract used (mL), and m indicated the mass of extract used (g), and was expressed as mg gallic acid equivalent per gram of extract (mg GAE/g extract).

***In vitro* antifungal activity of *Solanum incanum* fruit extracts**

A stock solution of 1000 mg/mL was prepared in 100% Dimethyl sulfoxide (DMSO) with vigorous vortexing to ensure maximum dissolution. Working solutions (5 mg/mL) were prepared by diluting the stock with Sabouraud Dextrose Broth (SDB), ensuring that the final DMSO concentration was below 1% (v/v). No visible precipitation was observed at the working concentrations used in the assays. Conidia were collected using sterile saline containing 0.05% Tween 80, filtered to remove hyphal fragments, and adjusted to approximately 1×10^6 conidia/mL with a hemocytometer, following CLSI (2008) guidelines. Sterile SDA plates were inoculated by evenly swabbing the standardized suspension to create a uniform lawn. Sterile Whatman No. 1 filter paper discs (6 mm diameter) were soaked with 10 µL of plant fruit extracts and then dried aseptically (CLSI, 2008). The discs were placed on the inoculated agar with sterile forceps. Discs containing terbinafine served as positive controls, while discs containing 1% DMSO and distilled water served as negative controls. Plates were incubated at 30°C for 5 days, and the inhibition zone diameters were measured in millimeters (mm) with a digital caliper.

Minimum inhibitory concentration

The minimum inhibitory concentration (MIC) of the methanolic and aqueous fruit extracts was ascertained utilizing the broth microdilution technique (CLSI, 2008). Two-fold serial dilutions of each extract were prepared in sterile SDB, starting at 10 mg/mL and extending through 10 successive dilutions. Each well of a sterile 96-well microtiter plate contained a final volume of 200 µL, consisting of the plant extract, SDB, and fungal inoculum. Control wells included SDB with fungal inoculum only for growth assessment, while sterile control wells contained SDB without inoculum. Terbinafine was incorporated as a positive control under identical conditions. The fungal inoculum was standardized to a final concentration of 1×10^5 conidia/mL in each well. The plates were incubated at 30°C for five days and monitored daily for fungal growth. The MIC was defined as the lowest concentration of the extract required to prevent visible fungal growth after five days, relative to the control. The assay was carried out in three independent experiments, each performed in triplicate, and the results were expressed in mg/mL.

Minimum fungicidal concentration

To determine the minimum fungicidal concentration (MFC), 10 µL aliquots were aseptically taken from wells that exhibited no visible growth in the MIC assay at different concentrations, from growth control wells on control plates, and from sterile control wells, and then subcultured onto SDA plates. The plates were then incubated at 30°C for five days and monitored daily for fungal growth. The MFC was defined as the lowest extract concentration that exhibited no visible colony growth on agar plates after five days of incubation (Scorzoni et al., 2007). The assay was conducted in three independent experiments, each performed in triplicate, and the results were reported in mg/mL.

Culture conditions and exposure to *Solanum incanum* extracts

Trichophyton mentagrophytes inoculum was prepared as a conidial suspension adjusted to 1×10^6 conidia/mL using a hemocytometer (Neubauer improved chamber, Marienfeld-Superior, Germany). The 5 mL fungal suspension was

incubated at 30°C with gentle shaking (120 rpm) for seven hours to facilitate conidial germination and the development of metabolically active hyphae before treatment. Cultures were then treated with *S. incanum* extracts at their respective MICs (Cowen et al., 2015) and incubated at 30°C for an additional 12 hours, providing a biologically relevant period to evaluate the expression of genes related to growth and virulence (Balouiri et al., 2016). Untreated cultures maintained under identical conditions served as negative controls.

RNA extraction and quality assessment

Total RNA was extracted from 60 mg of fungal mycelia using the Illustra RNAspin Mini RNA Isolation Kit (GE Healthcare, Little Chalfont, United Kingdom), according to the manufacturer's instructions. Each experimental condition consisted of three independent biological replicates. An on-column DNase I digestion step was included to eliminate genomic DNA contamination. The RNA concentration and purity were assessed using UV spectrophotometry (NanoDrop, Thermo Fisher Scientific, USA), with acceptable A260/A280 ratios ranging between 1.8 and 2.1. The RNA integrity was confirmed by electrophoresis on a 1.2% denaturing agarose gel, which revealed distinct 28S and 18S rRNA bands with minimal degradation. Only pure, high-quality RNA samples were used for subsequent analysis.

Quantitative real-time PCR

Gene expression analysis was conducted on two virulence-associated genes (*SUB3* and *StuA*) as well as two reference genes (*β-tubulin* and *GAPDH*). The complementary DNA (cDNA) was synthesized from 1 µg of total RNA within a 20 µL reaction volume, utilizing the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Vilnius, Lithuania) in accordance with the manufacturer's instructions. Quantitative real-time PCR (qRT-PCR) was carried out in a total volume of 20 µL, which included 10 µL of SYBR Taq ReadyMix (Sigma-Aldrich, USA), 0.5 µM of each primer, 4 µL of cDNA template, and 5 µL of nuclease-free water. Amplifications were conducted on an Mx3300P QPCR System (Agilent Technologies, Stratagene, USA) under the following conditions. The initial denaturation at 95°C for one minute was followed by 40 cycles at 95°C for 15 seconds and at 60°C for 60 seconds. A melt curve analysis (60-95°C) was performed at the end of each run to confirm amplification specificity (Bustin et al., 2009). No-template controls (NTC) and no-reverse transcription controls (no-RT) were included to verify the absence of contamination and genomic DNA. Untreated *T. mentagrophytes* cultures were used as the calibrator condition. Each sample was analyzed with three biological replicates, each with three technical replicates. Primer efficiency (90-110%) was validated using standard curves before analysis (Liu et al., 2023). Delta cycle threshold (ΔCt) values were calculated for each biological replicate by subtracting the geometric mean cycle threshold (Ct) of the reference genes (*GAPDH* and *β-tubulin*) from the Ct of the target gene. Delta Delta Ct (ΔΔCt) values were then calculated relative to the untreated control group. Relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$, with normalization performed using the geometric mean of *β-tubulin* and *GAPDH* Ct values, following validation of their expression stability (Livak and Schmittgen, 2001).

Table 1. Primers used for qRT-PCR in the present study

Gene	Sequence	Size (bp)	Reference number
SUB3	F: 5′- TCATGAAGGACGGTGTCACT -3′ R: 5′- ACGCTTAGCCTTGTTGAGGT -3′	104	KF_146901.1
StuA	F: 5'- CGTCCTGAAACCAGCGAAGA -3' R: 5'- ACCCATGTCACGATGCCATA -3'	98	XM_003010308.1
GAPDH	F: 5'- TCATCTCTGCTCCCTCTGCT -3' R: 5'-GACGGTGGTCATCAAACCCT-3'	174	MW_959142.1
β-tubulin	F: 5'- CGACCAAGACCCTTCCCTTC -3' R: 5'-GACTGACCGAAGACGACGTT -3'	168	MG_251832.1

Primer design and gene selection were based on previous studies on dermatophyte virulence genes (Achterman and White, 2012).

Statistical analysis

All experimental data were analyzed using GraphPad Prism software (version 11.0; GraphPad Software Inc., USA). All experiments were performed in triplicate, and the present results were reported as Mean ± SD. Statistical significance was analyzed using ordinary one-way and two-way ANOVA, with the Geisser-Greenhouse correction followed by Tukey's post hoc test. Differences were considered statistically significant at a p-value less than 0.05 ($p < 0.05$).

RESULTS

Yield of *Solanum incanum* fruit extracts

The amount of *S. incanum* fruit extracts differed significantly according to the extraction solvent used (p = 0.0196) as shown in Figure 1. The aqueous extract exhibited the highest extraction yield at 4.74 ± 0.24%, followed by the methanol extract at 4.21 ± 0.12%. In comparison, n-hexane and ethyl acetate extracts yielded lower amounts, at 3.39 ± 0.19% and 3.19 ± 0.09%, respectively (Table 2).

Total flavonoids, alkaloids, and phenolic contents estimation

The quantitative phytochemical analysis of *S. incanum* fruit extracts demonstrated differences in total flavonoid concentrations (TFC), TAC, and TPC. For TFC, the quercetin standard calibration curve was used, and for TPC, the gallic acid standard calibration curve was used. The quercetin standard calibration curve indicated a linear relationship between quercetin concentration (0-40 µg/mL) and absorbance measured at 510 nm. The regression equation was y = 0.00554x, with a correlation coefficient (R^2) of 0.99659 (Figure 2). The gallic acid standard calibration curve demonstrated the linear relationship between gallic acid concentration (0-50 µg/mL) and absorbance measured at 760 nm. The regression equation was y = 0.00570x + 0.001713, with a correlation coefficient of 0.99990 (Figure 3).

The TAC of *S. incanum* fruit extracts differed according to the extraction solvent used (p = 0.004). The highest alkaloid content was recorded in the hexane fruit extract (HFE) at 2.7 ± 0.1%, followed by the ethyl acetate fruit extract (EAFE) at 2.2 ± 0.1%. The methanolic fruit extract (MFE) demonstrated a lower alkaloid content of 1.4 ± 0.2%, while the aqueous fruit extract (AFE) recorded the lowest value at 1.2 ± 0.4% (Table 3).

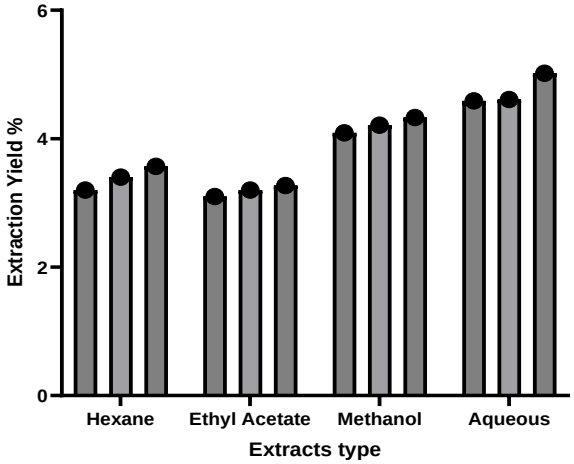


Figure 1. The yields of the *Solanum incanum* fruit extracts from three independent experiments

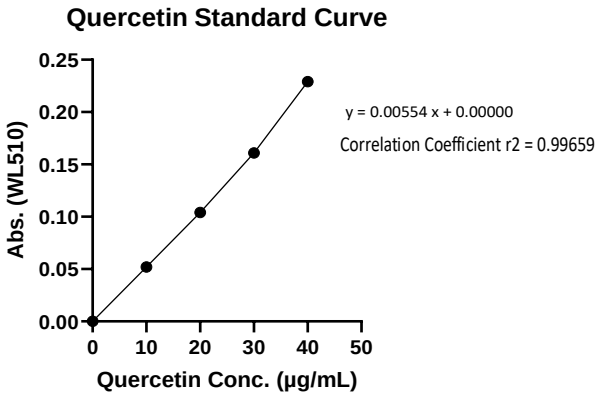


Figure 2. Quercetin standard calibration curve used for total flavonoid content estimation. Abs: Absorbance, Conc: Concentration, WL: Wavelength

Table 2. Extraction yield of the *Solanum incanum* fruit extracts

Extraction solvent	Weight of plant material (g)	Solvent volume	Dry extract (g) Mean ± SD	Yield (%)
n-Hexane	100	1L	3.39 ± 0.19[b]	3.39[b]
Ethyl Acetate	100	1L	3.19 ± 0.09[b]	3.19[b]
Methanol	100	1L	4.21 ± 0.12[a]	4.21[a]
Aqueous	100	1L DW	4.74 ± 0.24[a]	4.74[a]

DW: Distilled water; n-Hexane: Normal hexane. Values are presented as mean ± SD; Measurements were performed in triplicate. There was a statistically significant difference among extraction solvents (p = 0.0196). [ab] Different superscript letters within the same column indicate statistically significant differences at p < 0.05.

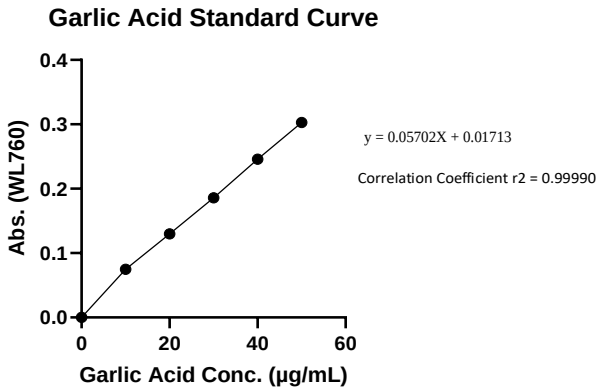


Figure 3. Gallic acid standard calibration curve used for total phenolic content estimation. Abs: Absorbance, Conc: Concentration, WL: Wavelength

Table 3. Total alkaloid content of *Solanum incanum* fruit extracts

Extract Type	Initial extract mass (g)	Total mass (g)	Container mass (g)	Blank residue (g)	Alkaloid mass (g)	TAC (%)
MFE	0.1	1.1024	1.0900	0.0110	0.0014	1.4 ± 0.2^{b}
AFE	0.1	1.1022	1.0900	0.0110	0.0012	1.2 ± 0.4^{b}
EAFE	0.1	1.1032	1.0900	0.0110	0.0022	2.2 ± 0.1^{a}
HFE	0.1	1.1037	1.0900	0.0110	0.0027	2.7 ± 0.1^{a}

MFE: Methanol fruit extract, AFE: Aqueous fruit extract, HFE: Hexane fruit extract, EAFE: Ethyl acetate fruit extract, TAC: Total alkaloid content. Extracts sharing the same superscript letter are not significantly different. Superscript a denotes the highest alkaloid content group (HFE and EAFE), while superscript b denotes the lower alkaloid content group (MFE and AFE). [a,b] Mean different superscript letters within the same column indicate statistically significant differences among extraction solvents ($p = 0.004$).

Phytochemical content of *Solanum incanum* fruit extracts across different solvent extraction methods

The phytochemical content of *S. incanum* fruit extracts differed significantly depending on the extraction solvent. The EAFE and HFE extracts exhibited significantly higher TFC and TAC levels compared to MFE and AFE extracts ($p = 0.0035$). In contrast, the MFE and AFE extracts demonstrated significantly higher TPC than the EAFE and HFE extracts ($p = 0.0035$; Table 4). No significant differences were observed between MFE and AFE, or between EAFE and HFE, for each parameter ($p = 0.193$).

Table 4. Total flavonoids, alkaloids, and phenolic contents of *Solanum incanum* fruit extracts

Type of extract	TFC (mg QE/g extract)	TAC (%)	TPC (mg GAE/g extract)
MFE	1.72 ± 0.24^{b}	1.4 ± 0.20^{b}	19.65 ± 0.30^{a}
AFE	1.88 ± 0.07^{b}	1.2 ± 0.40^{b}	19.39 ± 0.09^{a}
EAFE	3.03 ± 0.07^{a}	2.2 ± 0.12^{a}	2.52 ± 0.03^{b}
HFE	3.43 ± 0.44^{a}	2.7 ± 0.11^{a}	2.58 ± 0.05^{b}

TFC: Total flavonoid content, expressed as mg quercetin equivalent per gram of extract (mg QE/g extract). TPC: Total phenolic content, expressed as mg gallic acid equivalent per gram of extract (mg GAE/g extract). TAC: Total alkannin content. Values are presented as mean ± SD; measurements were performed in triplicate. Comparisons were made within each column (TFC, TAC, and TPC) across different extracts. [a,b] Mean different superscript letters within the same column indicate statistically significant differences at $p < 0.05$.

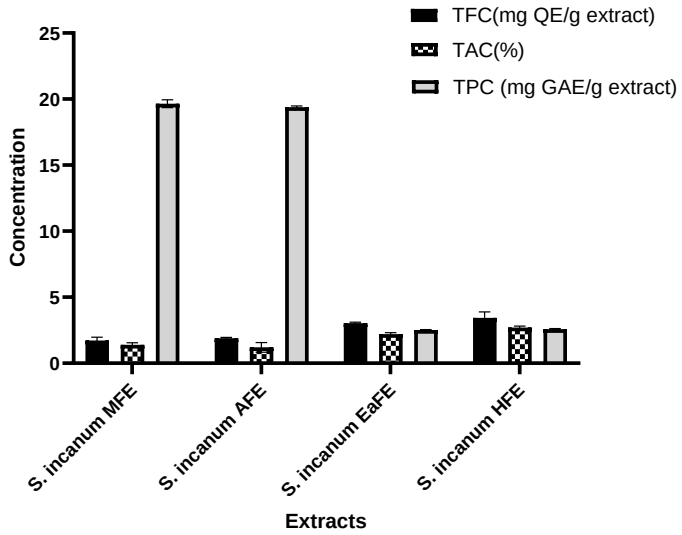


Figure 4. Flavonoid, alkaloid, and phenolic contents of four *Solanum incanum* fruit extracts. TFC: Total flavonoid content (mg QE/g extract), TAC: Total alkaloid content (%), TPC: Total phenolic content (mg GAE/g extract). MFE: Methanol fruit extract, AFE: Aqueous fruit extract, HFE: Hexane fruit extract, EAFE: Ethyl acetate fruit extract.

***In vitro* antifungal activity of *Solanum incanum* fruit extracts**

The MFE exhibited the highest inhibition zone of 14.43 ± 0.17 mm at a concentration of 5 mg/mL (equivalent to 50 µg/disc), followed by the AFE with an inhibition zone of 8.57 ± 0.18 mm. The HFE and EAFE demonstrated significantly lower inhibition zones of 4.54 ± 0.24 mm and 3.96 ± 0.25 mm, respectively ($p = 0.0033$; Table 5). The positive control, terbinafine, demonstrated the highest antifungal activity (15.02 ± 0.10 mm) at a lower concentration of 0.1 mg/mL (1 µg/disc). No inhibition zone was observed with 1% DMSO, confirming its lack of antifungal activity. All treatments differed significantly from one another ($p = 0.0033$; Table 5).

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Table 5. Inhibition zone of *Solanum incanum* fruit extracts against *Trichophyton mentagrophytes*

Treatment	Concentration	Inhibition zone (mm)			
		T1	T2	T3	Mean ± SD
MFE	5 mg/ml	14.23	14.49	14.57	14.43 ± 0.17[a]
AFE	5 mg/mL	8.38	8.61	8.74	8.57 ± 0.18[b]
HFE	5 mg/mL	4.32	4.50	4.80	4.54 ± 0.24[c]
EAFE	5 mg/mL	3.76	3.88	4.24	3.96 ± 0.25[d]
Terbinafine	0.1 mg /mL	14.92	15.02	15.12	15.02 ± 0.1[e]
DMSO 1%	--	0	0	0	0[f]

T1, T2, and T3 represent three independent treatments for each extract. MFE: Methanol fruit extract, AFE: Aqueous fruit extract, HFE: Hexane fruit extract, EAFE: Ethyl acetate fruit extract. DMSO 1%: Dimethyl sulfoxide used at a final concentration of 1% (v/v) as a solvent control. Values are expressed as Mean ± SD (n = 3). [a,b,c,d,e,f] Means within the same column followed by different superscript letters are significantly different at $p < 0.05$.

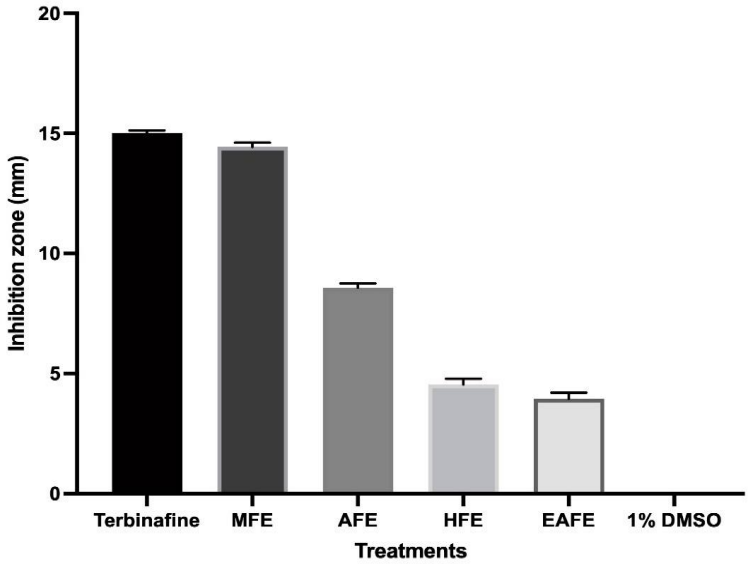


Figure 5. The antifungal activity of the *Solanum incanum* fruit extracts against *Trichophyton mentagrophytes*. MFE: Methanol fruit extract, AFE: Aqueous fruit extract, HFE: Hexane fruit extract, EAFE: Ethyl acetate fruit extract. DMSO 1%: Dimethyl sulfoxide used at a final concentration of 1% (v/v) as a solvent control.

The minimum inhibitory concentration of *Solanum incanum* methanolic and aqueous fruit extracts

The MIC values of *S. incanum* fruit extracts against *T. mentagrophytes* are presented in Table 6. The MFE completely inhibited fungal growth at concentrations ranging from 10 to 0.312 mg/mL, with an MIC of 0.312 mg/mL. The AFE completely inhibited fungal growth at concentrations from 10 to 0.625 mg/mL, corresponding to an MIC of 0.625 mg/mL. The MIC of MFE was one two-fold dilution lower than that of AFE. All triplicates demonstrated consistent MIC values after five days of incubation, indicating complete agreement among them. No visible fungal growth was observed in wells at or above the MIC values, while growth appeared in the control wells and was absent in the sterile control wells. The reference antifungal agent, terbinafine, demonstrated complete inhibition at a concentration of 0.0125 mg/mL.

The minimum fungicidal concentration of *Solanum incanum* methanolic and aqueous fruit extracts

The MFC values of the *S. incanum* fruit extracts are summarized in Table 6. Subculturing from wells exhibiting no visible growth confirmed the complete fungicidal activity of the MFE against. *Trichophyton mentagrophytes* at concentrations ranging from 10 to 0.625 mg/mL, with an MFC of 0.625 mg/mL. The AFE exhibited fungicidal activity between 10 and 1.25 mg/mL, corresponding to an MFC of 1.25 mg/mL. The MFC of MFE was one two-fold dilution lower than that of the AFE, indicating stronger fungicidal potency under the present study conditions. No colony growth was observed on the subculture plates at or above the respective MFC values after five days of incubation. The reference antifungal agent, terbinafine, demonstrated complete fungicidal activity at a much lower concentration (MFC = 0.025 mg/mL). Although both extracts demonstrated fungicidal activity against *T. mentagrophytes*, they were less potent than terbinafine because fungicidal effects were achieved only at higher concentrations. Fungal growth appeared in the growth control plates, but did not occur in the sterile control plates. The current results were consistent across all triplicates.

Table 6. The minimum inhibitory concentration and the minimum fungicidal concentration of *Solanum incanum* methanolic and aqueous fruit extracts after five days of incubation

Extracts	MIC (mg/mL)	MFC (mg/mL)	Ratio
MFE	0.312[b]	0.625[b]	2
AFE	0.625[c]	1.25[c]	2
Terbinafine	0.0125[a]	0.025[a]	2

MFE: Methanol fruit extract, AFE: Aqueous fruit extract. An MFC/MIC ratio <4 indicates a fungicidal effect. [abc] Mean values with different superscript letters within the same column indicate differences in antifungal activity based on MIC and MFC magnitude. Lower values correspond to higher antifungal potency. Results were identical across triplicates.

Expression level of *SUB3* and *StuA*

Expression of *SUB3* and *StuA* was normalized using *GAPDH* and *β-tubulin* as reference genes. Minor differences in Ct values were observed between untreated and treated samples and were presented as Mean ± SD (Table 7). Treatment with MFE and AFE reduced transcript levels compared with the untreated group. For *SUB3*, the Ct value increased from 28.88 in the untreated group to 35.64 in the MFE group and 34.56 in the AFE group, respectively (Table 8). The higher Ct values observed in both treated groups suggested a decrease in transcript level compared to the untreated control, with fold changes of 0.09 for MFE and 0.14 for AFE. Similarly, the expression of *StuA* exhibited an increase in Ct values from 29.08 in the untreated group to 33.50 (MFE) and 32.14 (AFE), with corresponding calculated fold changes of 0.48 and 0.86, respectively. Statistical analysis of ΔCt values revealed a significant difference among treatment groups ($p = 0.0002$). The current results demonstrated that MFE and AFE treatments differed significantly from the untreated group ($p < 0.05$), with MFE demonstrating a greater reduction in the gene expression levels of *SUB3* and *StuA* than AFE.

Table 7. Cycle threshold values of the reference genes following treatment with methanolic and aqueous fruit extracts of *Solanum incanum*

Reference genes	Untreated (Ct)	MFE (Ct)	AFE (Ct)
GAPDH	29.21 ± 0.77	32.23 ± 1.04	31.39 ± 0.67
β-tubulin	27.70 ± 1.18	31.32 ± 1.13	31.19 ± 0.79

Untreated: Fungal inoculum without any treatment, MFE: Methanol fruit extract of *Solanum incanum*, AFE: Aqueous fruit extract of *Solanum incanum*. Ct: Cycle threshold (unitless value representing the number of amplification cycles required for the fluorescent signal to cross the detection threshold).

Table 8. Cycle threshold values and *SUB3* and *StuA* gene expression in *Trichophyton mentagrophytes* following treatment with methanolic and aqueous fruit extracts of *Solanum incanum*

Target genes	Untreated (Ct)	MFE (Ct)	AFE (Ct)
SUB3	28.88 ± 0.16[c]	35.64 ± 0.57[a]	34.56 ± 0.51[b]
StuA	29.08 ± 0.13[c]	33.50 ± 0.52[a]	32.14 ±0.34[b]

Ct: Cycle threshold; higher Ct values indicate lower gene expression levels. MFE: Methanol fruit extract, AFE: Aqueous fruit extract. Values are expressed as Mean ± SD (n = 3). [abc]Mean within the same row followed by different superscript letters are significantly different at $p < 0.05$.

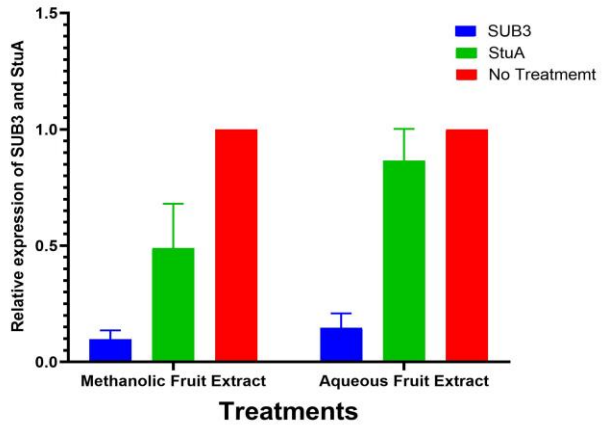


Figure 6. Relative expression of *SUB3* and *StuA* in *Trichophyton mentagrophytes* following treatment with methanolic and aqueous fruit extracts of *Solanum incanum*. Fold change values < 1 indicate downregulation of gene expression.

DISCUSSION

Differences in extraction yield among solvents indicated that polar solvents extracted a higher percentage of hydrophilic compounds. The higher yield from distilled water might be due to its ability to extract polar substances; however, unlike

the sequential solvent fractions, the aqueous extract was obtained from a single extraction. Similar findings have been reported by Do et al. (2014) for medicinal plants, indicating that aqueous and methanol solvents yielded higher extraction amounts due to the abundance of polar secondary metabolites in plant materials.

The results of the present study indicated that solvent polarity played a key role in affecting the efficiency of phytochemical extraction (Azwanida, 2015). Phytochemical quantitative screening indicated that methanol and aqueous extracts possessed elevated concentrations of phenolic compounds, while ethyl acetate and hexane extracts exhibited comparatively larger quantities of flavonoid and alkaloid compounds. The observed pattern demonstrated that plant secondary metabolites exhibit different solubilities across solvent polarities. Phenolic compounds exhibited greater solubility in polar solvents owing to their hydroxyl functional groups, which facilitate interactions with polar extraction solvents (Dai and Mumper, 2010). Several medicinal plants, such as *Azadirachta indica*, *Ocimum sanctum*, and *Moringa oleifera*, indicated solvent-dependent phytochemical differences (Lee et al., 2024; Ranjan, 2024). Methanol extracts often contain higher levels of phenolic compounds, which enhance antibacterial activity (Sasidharan et al., 2011).

The methanolic and aqueous fruit extracts exhibited notable antifungal activity against *T. mentagrophytes*; however, these extracts were evaluated at a considerably higher concentration (50 µg/disc) than the positive control (1 µg/disc), thereby indicating lower relative potency. The discrepancy may be attributed to the fact that crude plant extracts comprise a mixture of compounds with diverse bioactivities, whereas the established medicines consist of a purified compound with a specific antifungal mechanism (Cowan, 1999). The MFE of *S. incanum* had an MIC of 0.312 mg/mL and an MFC of 0.625 mg/mL, whereas the AFE had an MIC of 0.625 mg/mL and an MFC of 1.25 mg/mL. For both extracts (MFE and AFE), the MFC/MIC ratio was 2, which indicated fungicidal activity (MFC/MIC ≤ 4; CLSI, 2008). The current findings demonstrated that *S. incanum* extracts not only inhibited the growth of *T. mentagrophytes* but also eliminated fungal cells. The present study reported an MIC of 0.312 mg/mL for the MFE, indicating notable antifungal activity compared to the previous studies. Several plant extracts, such as *S. hispidum*, demonstrated activity against Trichophyton spp. (Mendoza-León et al., 2022), displaying moderate antifungal efficacy with MIC values of 1 mg/mL (≥1) or higher, depending on variables such as plant species, extraction solvent, and assay conditions. For instance, *Azadirachta indica* leaf extracts exhibited MIC values ranging from 0.5 to 2 mg/mL against *T. rubrum* (Noites et al., 2023). The observed MIC value of 0.312 mg/mL for MFE in the current study indicated comparatively strong antifungal activity compared with other crude plant extracts. While some highly active plant-derived fractions or purified compounds can achieve lower MIC values (< 0.1 mg/mL), this level of potency is generally not observed in crude extracts (Newman and Cragg, 2020). The antifungal efficacy observed in the MFE in the present study aligned with previous findings for taxa within the genus *Solanum*. The methanolic extracts of leaves and fruits of *Artemisia afra* exhibited inhibition zones of approximately 18 mm at a concentration of 50 mg/mL and MIC values ranging from 0.5 to 2 mg/mL against dermatophytes such as *T. rubrum* and *Epidermophyton floccosum* (Afolayan and Meyer, 1997). The differences observed between methanol and the other fruit extracts indicated that solvent-dependent extraction affected antifungal activity against *T. mentagrophytes*, probably due to the larger inhibitory zone recorded in the methanolic extract (Do et al., 2014).

The current findings indicated that treating *T. mentagrophytes* with *S. incanum* fruit extracts reduced the expression of the virulence-related genes (*SUB3* and *StuA*). The methanolic extract caused a greater decrease than the aqueous extract. The notable downregulation of the *SUB3* and *StuA* genes by the MFE corresponded to its antifungal activity observed in MIC/MFC assays. The more pronounced decrease in *SUB3* expression compared to *StuA* may indicate differential sensitivity of these genes to exposure to the extract. Since *SUB3* is linked to extracellular proteolytic activity, which plays a key role in dermatophyte colonization, its suppression could substantially reduce pathogenicity (Bitencourt et al., 2021). The reduced response of *StuA* may indicate a partial, not complete, disruption of the regulatory pathways that control fungal development and virulence. However, transcriptional alterations alone are insufficient to confirm functional suppression of the associated biological processes (Deshpande et al., 2025). The relatively minor alteration observed in *StuA* indicated that not all fungal regulatory genes respond uniformly to the extract treatment. Additionally, upstream signaling pathways such as the cAMP–PKA and MAPK, as well as other transcription factors, might exhibit different sensitivities to the extract (Bitencourt et al., 2021). The selective modulation aligned with the findings of Cushnie and Lamb (2011), indicating that phytochemicals such as flavonoids (quercetin, kaempferol), phenolic acids (gallic acid, caffeic acid), and alkaloids specifically inhibited virulence genes such as *SAP*, *ALS*, and *HWP1* in pathogenic fungi, without impacting their broader regulatory systems. Despite the lack of compound identification in the present study, the observed transcriptional changes suggested that phytochemicals mediated antifungal activity through mechanisms other than direct growth inhibition.

Importantly, the present findings have implications for animal health and veterinary medicine. *Trichophyton mentagrophytes* is a zoonotic pathogen that can be transmitted from animals to humans, particularly among farmers and veterinarians (Plangsiri et al., 2025). The antifungal and gene-modulating effects observed in the present study indicate

that *S. incanum* fruit extracts could serve as a potential alternative or supplement for treating dermatophytosis caused by *T. mentagrophytes* in veterinary practice. The use of plant-based antifungal agents may offer advantages, including affordability, accessibility, and reduced likelihood of resistance development (Gómez-Gaviria et al., 2025).

Despite the promising antifungal activity observed, the potential toxicity of *S. incanum* should be considered. *Solanum incanum* is known to contain bioactive steroidal glycoalkaloids such as solanine and solamargine, which have been associated with toxic effects including gastrointestinal irritation, neurological disturbances, and cytotoxicity at elevated concentrations (Al Sinani and Eltayeb, 2017). These compounds may limit the safe therapeutic use of crude extracts when not properly regulated. Therefore, comprehensive toxicological assessments, such as dose-response studies, safety evaluations, and determination of therapeutic indices, are crucial before clinical or veterinary application. Furthermore, isolating and characterizing specific active compounds can help identify fractions with strong antifungal activity while exhibiting lower toxicity, thereby enhancing the safety profile of potential treatments. The current results of the present study indicated an association between exposure to *S. incanum* extract and altered expression of selected genes linked to fungal pathogenicity, rather than to fungicidal activity alone.

CONCLUSION

The present study demonstrated that solvent polarity significantly influences the extraction yield, phytochemical composition, and antifungal activity of *S. incanum* fruit extracts. Methanol fruit extract demonstrated the highest efficiency and notable activity against *T. mentagrophytes*, along with reduced expression of virulence genes (*SUB3* and *StuA*). Although less potent than terbinafine, *S. incanum* demonstrated potential as an antifungal agent. Nevertheless, the constraints included reliance on a single strain, a limited selection of gene targets, and an *in vitro* study design. Further studies should explore additional species, *in vivo* efficacy, and isolate active compounds to clarify mechanisms and therapeutic potential.

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Authors' contributions

Pita Deta conducted the experiments, collected and analyzed the data, and drafted the manuscript. Peter Ogoti, Daniel Kiboi, and Cristine Bii supervised the study, observed the experiments, and critically reviewed the scientific content of the manuscript. All authors reviewed and approved the final edition of the manuscript before publication.

Availability of data and materials

The datasets produced and examined in the current study are accessible from the corresponding author upon reasonable request.

Competing interests

The authors declared that they have no competing interests.

Ethical considerations

The authors declare that QuillBot was used solely for grammar checking and plagiarism assessment during manuscript preparation. No AI tools were used for data generation, analysis, or preparation of the present study. All scientific content, results, and conclusions were developed and verified by the authors.

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Effects of Corn Bran on the Nutritional Quality of Pineapple Peel Silage

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ABSTRACT

Combining pineapple peel waste with a specific level of corn bran produces high-quality silage that meets the nutritional needs of ruminants. The present study aimed to evaluate the effectiveness of incorporating corn bran in improving the physical properties and nutritional quality of pineapple peel silage for ruminant feeding in tropical regions. The silage treatment was conducted using a completely randomized design with four treatment groups and five replications. The experimental diets consisted of four levels of pineapple peel and corn bran, including 100% of pineapple peel with no bran (P0), 95% of pineapple peel with 5% of bran (P1), 90% of pineapple peel with 10% of bran (P2), and 85% of pineapple peel with 15% of bran (P3). Each treatment group received 5% of molasses as an additive. Silage quality was analyzed using near-infrared spectroscopy. To evaluate rumen fermentation and digestibility, the *in vitro* Conway method was utilized, employing rumen fluid obtained from fistulated Holstein-Friesian bulls. Pineapple peel silage containing different corn starches exhibited a higher pH than the control group. Group P3 significantly produced higher crude protein (CP) and nitrogen-free extract (NFE) levels than the other treatment groups. Increasing the amount of corn bran in group P3 increased ammonia (NH3) production by 11.15 mM and total volatile fatty acids (VFA) by 134.20 mM compared to the control group. Group P3 exhibited the highest digestibility coefficients, with dry matter and organic matter values reaching 82.77% and 82.46%, respectively. The feed mixture comprising 85% pineapple peel and 15% corn bran is recommended for ruminants in tropical regions due to its superior overall nutritional quality. This feed mixture elevated CP, NFE, NH3 production, total VFA, dry matter, and organic matter.

Keywords: Corn bran, Digestibility, Rumen, Tropical region

INTRODUCTION

Providing ruminant feed, particularly in tropical countries, commonly encounters different complex issues such as scarcity and poor nutritional quality. Natural grass is commonly used on grazing land to lower production costs, offering an economical and efficient energy source (Dillon et al., 2005; Gyamfi et al., 2025). During the rainy season, grass is abundant; however, in the dry season, its availability declines because forage land, which provides fiber, is often converted into agricultural land (Teferedegne, 2000; Herrmann et al., 2020; Woldemariam, 2021). Consequently, implementing a forage development strategy is essential, as ruminant diets depend on natural grasses (Alemayehu et al., 2017; Billen et al., 2018). Since pasture grass often fails to meet nutritional standards, farmers regularly supplement it with different concentrates, resulting in higher feed costs and reduced efficiency (Al-Jassim et al., 1997). This condition persistently results in financial losses in the livestock industry. Consequently, it is important to investigate innovative feed sources derived from alternative agricultural waste materials, including pineapple peel waste. Pineapple plants are widely available in Indonesia, producing significant quantities of peel waste.

Pineapple peel waste exhibited a favorable nutritional profile, characterized particularly by its high glucose content. Pineapple waste yields approximately 34 g/L of glucose, 12 g/L of fructose, and 10 g/L of sucrose (Ferone et al., 2019). According to Sukruansuwan and Napathorn (2018), pineapple peel waste specifically contains 22.9% cellulose, 13.9% hemicellulose, and 5.1% lignin. To preserve the nutritional quality of pineapple peel waste as animal feed, it should be processed into silage with corn bran and molasses. Silage is a preservation method based on fermentation that decreases pH and generates a distinctive odor (Kung et al., 2013; Bai et al., 2023). The success of the fermentation process within the silo is highly reliant on anaerobic conditions, the availability of water-soluble carbohydrates (WSC), and an optimal dry matter content to facilitate the proliferation of lactic acid bacteria (LAB) and capable of decreasing pH (Weinberg and Muck, 1996; Xu et al., 2022). Corn bran and molasses serve as non-structural carbohydrate sources that provide high energy levels for LAB metabolism (Ferraretto et al., 2013). Non-structural carbohydrates, such as the starch found in

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plant cells, are abundant in corn bran, whereas molasses typically contains 50% sucrose (Nikodinovic et al., 2013). Rumen microbes favor these carbohydrates for their rapid effects on rumen pH (Russell and Wilson, 1996; Shi et al., 2025). Rumen digestibility is measured by the adequacy of dry matter (DM), organic matter (OM), and fermentation value. Ultimately, a well-balanced nutritional profile in silage ensures proper levels of DM and OM, improving feed digestibility and boosting overall rumen function. Higher rumen digestibility coefficients suggest that the feed is readily fermentable, enabling optimal metabolite utilization to support animal growth and production. The present study aimed to thoroughly assess the physical characteristics, nutritional profile, fiber fractions, rumen fermentation parameters, and in vitro digestibility of pineapple peel waste silage supplemented with corn bran for ruminant feed.

MATERIALS AND METHODS

Ethical approval

This experiment adhered to research ethics guidelines for livestock, in accordance with the Animal Science and Health regulations outlined in Government Law No. 41/2014 of the Republic of Indonesia.

Silage preparation and treatments

The silage production process began with the physical processing of pineapple peel waste. The peels were diced into 3-5 cm segments and allowed to wilt for 18 hours to reduce their moisture content. The silage ingredients were then prepared using a completely randomized design comprising four treatments and five replications. The treatment groups consisted of 100% of pineapple peel with no bran (P0), 95% of pineapple peel with 5% of corn bran (P1), 90% of pineapple peel with 10% of corn bran (P2), and 85% of pineapple peel with 15% of corn bran (P3), with all treatments supplemented with 5% of molasses. Once the ingredients were thoroughly combined, they were transferred to a 10-liter silo, compacted, and sealed securely to preserve the anaerobic environment. The mixture was subsequently fermented for 21 days. After fermentation, the silo was opened, and the silage was assessed for color, texture, odor, and mold (Ali et al., 2023). Subsequent analyses were performed to quantify crude protein (%), crude fiber (%), ether extract (%), and nitrogen-free extract (NFE %) using near-infrared spectroscopy (NIRS; Despal et al., 2020). The content of neutral detergent fiber (NDF), acid detergent fiber (ADF), lignocellulose, cellulose, and hemicellulose was evaluated using fiber fractionation methodology (Vansoest, 1963). Additionally, OM, DM, dry matter digestibility (DMD), organic matter digestibility (OMD), ammonia (NH_3) production, and total volatile fatty acids (VFA) were measured through *in vitro* analysis (Tilley and Terry, 1963).

Physical quality of silage

A panel of 50 untrained assessors evaluated the physical quality of the silage, considering texture, color, odor, and mold presence. The assessment followed the scoring system and indicators adapted from Marselinus et al. (2019), in which odor was categorized from foul (1.0-1.9) to slightly sour (4.0-4.9), and mold growth was rated from extensive (1.0-1.9) to none (4.0-4.9). Color quality ranged from black (1.0-1.9) to the ideal golden yellow (4.0-4.9), while texture was assessed from very coarse (1.0-1.9) to soft and easily separable (4.0-4.9). Additionally, silage quality was determined by pH values classified as excellent (3.2-4.2), good (4.2-4.5), or poor (> 4.5).

Chemical composition and fiber fraction measurement

The NIRS analysis employed a Buchi NIRFlex N500, Butchi Company (Switzerland), equipped with dense cells. Spectral data were collected from dry samples that had been ground and placed in a Petri dish, which was then placed in a Petri dish holder. The samples were irradiated with near-infrared radiation in the wavelength range of 4,000 to 10,000 cm. The irradiation process was repeated three times to generate three spectra for each sample. These chemical structures served as indicators of quality parameters, including protein, fiber, carbohydrates, fat, DM, and moisture content, as well as NDF, ADF, hemicellulose, and lignin (Vansoest, 1963; Despal et al., 2020).

Fermentation rumen characteristic

Ammonia production and total VFA were measured *in vitro* using the method developed by Tilley and Terry (1963). Rumen fermentation and digestibility were assessed using a two-stage *in vitro* technique with rumen fluid obtained from fistulated Frisian Holstein bulls aged 2-3 years and averaging 320 ± 60.08 kg, a reliable source maintained at the Dairy Animal Nutrition Laboratory, Bogor Agricultural University, Indonesia. Fermentability analysis was performed in 100 mL fermentation tubes. A 0.5 g sample was combined in each tube with 40 mL of preheated McDougall's buffer from Sigma Aldrich Limited Company (Scotland) and 10 mL of rumen fluid. The contents of rumen liquid were aerated with CO_2 gas for 30 seconds to establish anaerobic conditions, then sealed with a ventilated rubber stopper and incubated in a

shaking water bath at 39°C for four hours. After fermentation, the tubes were centrifuged at 3500 g for 15 minutes, and the supernatant was collected for analysis of NH_3 and total VFA. The NH_3 levels were measured using the Conway method (Department of Dairy Science, 1966), while total VFA was determined by steam distillation. The digestibility analysis comprised two stages. The initial stage followed the fermentation process, which lasted for 48 hours. After 48 hours, the tubes were centrifuged once more at 3500 g for 15 minutes, and the resultant pellet was collected. This residue was subsequently combined with 40 mL of HCl-pepsin solution and incubated aerobically in a water bath maintained at 39°C for an additional 48 hours.

***In vitro* nutrient digestibility**

After 48 hours of enzymatic processing, the residue was filtered, dried, and weighed to determine the DM residue. The OM content of the residue was determined by incineration in a muffle furnace at 600°C for 6 hours. The DM and OM digestibility coefficients were calculated using the following formulas (Tilley and Terry, 1963).

$$DMD = \frac{(\text{sample weight} \times DM) - (\text{residue weight} \times DM - \text{blank weight} \times DM) \times 100\%}{(\text{Sample weight} \times DM)} \quad \text{(Formula 1)}$$

$$OMD = \frac{(\text{sample weight} \times OM) - (\text{residue weight} \times OM - \text{blank weight} \times OM) \times 100\%}{(\text{Sample weight} \times OM)} \quad \text{(Formula 2)}$$

Statistical analysis

Data were analyzed with one-way ANOVA in SPSS version 20.0 (IBM Corp., USA). When significant differences among treatments were observed, Duncan's multiple-range test was used for mean separation. The threshold for statistical significance was $p < 0.05$, and all results were expressed as mean ± standard deviation (SD).

RESULTS AND DISCUSSION

Physical quality of silage

The physical properties of pineapple peel silage supplemented with different levels of corn bran are presented in Table 1. The silage condition was classified as good. Including up to 15% corn bran did not significantly change the silage's color ($p > 0.05$). The silage retained its characteristic color, ranging from brownish-yellow to golden-yellow, with average color scores of 3.77 to 4.00, primarily brownish-yellow. This color alteration from the original golden-yellow to brownish-yellow was probably due to the addition of diverse carbohydrate sources and the 21-day fermentation process. Similarly, Anas et al. (2024) reported that corn straw silage mixed with different legumes resulted in brown and yellowish-brown colors. Furthermore, color alterations were influenced by temperature during the ensiling process (Sukri et al., 2023). The initial phase of ensiling involved aerobic respiration, which persisted until the plant sugars and remaining oxygen supply were depleted. During the initial phase of the silage process, sugars were metabolized into CO_2 and water, generating heat and increasing the temperature (Martens et al., 2024). Sio et al. (2022) reported that the color of rice straw silage remained yellowish-green after adding molasses and cattle rumen fluid, similar to the result.

Regarding odor, the treatment groups significantly affected silage odor, with Group P3 recording the highest score (3.68; $p < 0.05$). Odor scores ranged from 3.60 to 3.78, indicating noticeable differences due to added ingredients, such as corn bran. The sour odor observed in this study was due to fermentation, in which active anaerobic bacteria converted sugars into organic acids. Lactic acid bacteria inoculants could alter the odor by changing microbial communities of the alfalfa silage (Zhang et al. 2021). Similarly, the texture of the pineapple peel silage was significantly affected by the inclusion of different carbohydrate sources ($p < 0.05$). The texture scores ranged from 3.64 to 3.89, indicating a soft, cohesive consistency that was hard to separate, reflecting superior silage quality. Group P2 demonstrated the highest texture value, probably due to the high moisture and carbohydrate content naturally present in pineapple peel waste.

Finally, the treatment groups significantly affected silage pH, with average values ranging from 2.77 to 2.91 ($p < 0.05$). This pH result reflected the positive impacts of carbohydrate-based ingredients on the fermentation process, as the easily soluble carbohydrates in pineapple peels served as an excellent substrate for lactic acid bacteria. The silage achieved an optimal pH level, presumably attributable to a 21-day fermentation period that facilitated bacterial proliferation. A higher concentration of lactic acid produced during fermentation effectively lowers the pH, thereby inhibiting the proliferation of spoilage bacteria and extending the shelf life of the feed (Yu et al., 2023). In the present study, the observed pH level was lower than the range of 3.77-4.04 reported by Ramirez et al. (2020) for sunflower silage with different additives. Furthermore, the pH levels observed in the current study were lower than those reported by Ferreira et al. (2022) for mixed Tanzanian grass and babassu fruit by-product silage used as feed for dairy cattle, which ranged from 5.08 to 5.26.

Chemical composition of silage

The chemical composition of pineapple peel silage supplemented with corn bran is presented in Table 2. Incorporating corn bran at concentrations up to 15% did not significantly affect the DM content of the pineapple peel silage, with no substantial increase observed at the 5%, 10%, or 15% levels ($p > 0.05$). Any minor fluctuations in DM content were primarily driven by respiration prior to the establishment of anaerobic conditions. The inclusion of corn bran significantly increased the crude protein content relative to the control group ($p < 0.05$). Group P0 exhibited the lowest crude protein content (6.84%), attributable to the absence of corn bran, which likely reduced microbial activity and, consequently, microbial protein synthesis. Conversely, an increase in the bacterial population enhanced the contribution of bacterial amino acids to the total protein content. Notably, the crude protein values observed in the present study exceed those reported by Kizilşimşek et al. (2020), who found average values ranging from 6.16% to 7.48% in corn silage intercropped with legumes. The ether extract content of the silage was affected by the treatments, with the highest value recorded in Group P1 at 3.97% and the lowest in Group P0 at 3.43% ($p < 0.05$). The crude fiber content was significantly affected by the addition of corn bran. Group P0 exhibited the highest crude fiber level at 23.71%, whereas Group P3 recorded the lowest crude fiber level at 10.53%. The increased levels of crude fiber in Group P0 were due to restricted energy availability, which limited microbial activity required for fiber decomposition (Li et al., 2026). Finally, the addition of corn bran significantly increased NFE levels in the P3 group compared to the other treatments ($p < 0.05$). The highest NFE level was observed in Group P3 at 74.02%, while the lowest NFE level was observed in Group P0 at 53.86%. The NFE levels observed in the present study were higher than the 40.34% to 45.04% reported by Sriagtula et al. (2026), who used sorghum silage with indigofera and molasses.

Table 1. Physical value of pineapple peel waste silage by the addition of different levels of corn starch

Parameter	Treatment	Mean ± S.D
Color	P0	3.92 ± 0.29
	P1	3.96 ± 0.08
	P2	3.99 ± 0.13
	P3	3.72 ± 0.18
Odor	P0	3.64 ± 0.13^{a}
	P1	3.77 ± 0.06^{b}
	P2	3.65 ± 0.06^{a}
	P3	3.68 ± 0,08^{ab}
Texture	P0	3.73 ± 0.08^{b}
	P1	3.77 ± 0.10^{b}
	P2	3.89 ± 0.09^{c}
	P3	3.65 ± 0.06^{a}
Mold	P0	4.41 ± 0.05^{c}
	P1	4.32 ± 0.05^{b}
	P2	4.28 ± 0.03^{ab}
	P3	4.26 ± 0.05^{a}
pH	P0	2.76 ± 0.03^{a}
	P1	2.84 ± 0.02^{b}
	P2	2.90 ± 0.02^{c}
	P3	2.86 ± 0.07^{bc}

P0: 100% pineapple peel; P1: 95 % pineapple peel + 5 % corn bran; P2: 90% pineapple peel + 10% corn bran; P3: 85 % pineapple peel + 15 % corn bran; ^{abcd} Different superscript letters in the same column indicate significant differences ($p < 0.05$); S.D: Standard deviation

Table 2. Chemical value of pineapple peel waste silage by the addition of corn starch

Parameter (% DM)	Treatment	Mean ± S.D
Dry Matter	P0	88.15 ± 0.62
	P1	88.10 ± 0.51
	P2	88.54 ± 0.50
	P3	88.15 ± 0,55
Ash	P0	12.17 ± 0.93^{c}
	P1	8.25 ± 1.27^{b}
	P2	6.72 ± 0.88^{b}
	P3	3.93 ± 1.45^{a}
Crude Protein	P0	6.84 ± 0.80^{a}
	P1	8.72 ± 0.99^{b}
	P2	8.47 ± 0.78^{b}
	P3	8.04 ± 0.96^{b}
Eter Extract	P0	3.43 ± 0.53^{a}
	P1	3.97 ± 0.35^{b}
	P2	3.95 ± 0.22^{b}
	P3	3.49 ± 0.20^{ab}
Crude Fiber	P0	23.71 ± 1.36^{d}
	P1	17.50 ± 3.17^{c}
	P2	14.00 ± 1.14^{b}
	P3	10.53 ± 2.46^{a}
NFE	P0	53.86 ± 0.97^{a}
	P1	6155 ± 3.09^{b}
	P2	66.85 ± 0.97^{c}
	P3	74.02 ± 3.84^{d}

P0: 100% pineapple peel; P1: 95 % pineapple peel + 5 % corn bran; P2: 90% pineapple peel + 10% corn bran; P3: 85% pineapple peel + 15% corn bran; ^{abcd} Different superscript letters in the same column indicate significant differences ($p < 0.05$); NFE: Nitrogen free extract

Fiber fraction of silage

Table 3 presents the fiber fractions of pineapple peel waste silage supplemented with corn bran. The analysis of these fiber components was conducted to evaluate the nutritional impact of different levels of corn bran inclusion on silage quality. Corn bran supplementation significantly reduced the fiber content of the silage ($p < 0.05$). Specifically, NDF decreased from 54.14% in Group P0 to 30.87% in Group P3 ($P < 0.05$). Similarly, the ADF content was highest in

P0 (29.48%) and lowest in P3 (16.68%). The decrease in these NDF and ADF fractions, which consist of lignin, cellulose, and hemicellulose, was accelerated by the addition of corn bran. Higher levels of corn bran supplementation enhanced microbial activity during ensiling, facilitating the degradation of these structural components. Consequently, the silage was more easily digestible, leading to higher digestibility coefficients. Furthermore, the treatments significantly affected the hemicellulose and cellulose contents ($p < 0.05$). The highest hemicellulose value was observed in Group P0 at 24.66%, whereas the lowest value (14.19%) was observed in Group P3. Similarly, the control group had the highest cellulose content at 24.16%, while Group P3 had the lowest cellulose content at 12.76%.

The NDF values observed in the present study (30.87%-54.14%) were similar to those reported by Kaplan and Akcura (2021) for mixed bean and corn silage, which ranged from 39.36% to 51.20%. Furthermore, the lignin content in the present study was lower than the 13.76%-14.91% noted by Tekin and Kara (2020) in tomato herb silage supplemented with molasses and barley.

Table 3. Fiber fraction of pineapple peel waste silage with added levels of corn starch

Parameter (% DM)	Treatment	Mean ± S.D
NDF	P0	54.14 ± 2.55^{c}
	P1	42.05 ± 1.31^{b}
	P2	41.38 ± 5.25^{b}
	P3	30.87 ± 6.15^{a}
ADF	P0	29.48 ± 2.02^{c}
	P1	21 97 ± 1.37^{b}
	P2	20.81 ± 2.35^{b}
	P3	16.68 ± 1.34^{a}
Hemiselulosa	P0	24.66 ± 2.80^{b}
	P1	20.08 ± 2.17^{b}
	P2	20.57 ± 3.64^{b}
	P3	14.19 ± 4.83^{a}
Lignin (ADL)	P0	3.87 ± 1.57^{b}
	P1	4.41 ± 1.06^{b}
	P2	4.35 ± 0.95^{b}
	P3	1.96 ± 0.03^{a}
Selulosa	P0	24.16 ± 1.27^{b}
	P1	17.57 ± 0.33^{b}
	P2	15.48 ± 1.88^{b}
	P3	12.76 ± 1.29^{a}

P0: 100% pineapple peel; P1: 95 % pineapple peel + 5 % corn bran; P2: 90% pineapple peel + 10% corn bran; P3: 85 % pineapple peel + 15 % corn bran; ^{abcd} Different superscript letters in the same column indicate significant differences ($P < 0.05$). S.D: Standard deviation; NDF: Neutral detergent fiber; ADF: Acid detergent fiber

Table 4. Rumen fermentability and *in vitro* digestibility of pineapple peel waste silage

Parameters	Treatment	*Mean ± S.D*
NH_3 Production (mM)	P0	7.67 ± 1.00^{a}
	P1	10.91 ± 1.29^{b}
	P2	10.86 ± 1.77^{b}
	P3	11.15 ± 1.09^{b}
VFA Total (mM)	P0	79.62 ± 9.67^{a}
	P1	105.32 ± 17.24^{b}
	P2	132.94 ± 21.47^{c}
	P3	134.20 ± 9.13^{c}
DMD (%)	P0	71.09 ± 1.23^{a}
	P1	72.69 ± 1.73^{a}
	P2	80.66 ± 2.72^{b}
	P3	82.77 ± 1.58^{b}
OMD (%)	P0	70.35 ± 1.20^{a}
	P1	71.72 ± 1.71^{a}
	P2	80.15 ± 2.91^{b}
	P3	82.46 ± 1.58^{b}
Rumen pH	P0	6.92 ± 0,01^{b}
	P1	6.88 ± 0,01^{c}
	P2	6.83 ± 0,02^{a}
	P3	6.82 ± 0,01^{a}

P0: 100% pineapple peel; P1: 95 % pineapple peel + 5 % corn bran; P2: 90% pineapple peel + 10% corn bran; P3: 85 % pineapple peel + 15 % corn bran; ^{abcd} Different superscript letters in the same column indicate significant differences ($P < 0.05$); DMD: Dry matter digestibility; OMD: Organic matter digestibility; S.D: Standard deviation; NH_3: Ammonia; VFA: Volatile fatty acids

Rumen fermentation and *in vitro* digestibility of silage

The rumen fermentation characteristics and *in vitro* digestibility of pineapple peel silage supplemented with corn bran are presented in Table 4. The treatments significantly increased NH_3 and total VFA production in pineapple peel silage ($p < 0.05$). The highest concentrations were observed in Group P3, with values of 11.15 mM for NH_3 and 134.20 mM for total VFA. Conversely, the lowest NH_3 and total VFA production were observed in the control group at 7.67 mM and 79.62 mM, respectively. The increased NH_3 production observed in the supplemented groups was attributed to the protein content in the corn bran concentrate, which was rapidly degraded by rumen microorganisms into ammonia. The NH_3 concentrations observed in the current study were higher than those reported by Htet et al. (2021) for corn straw silage with soybean bran (8.00-10.60 mM), but lower than the 10.50-12.88 mM reported by Tresia et al. (2024) in corn

straw silage. Similarly, adding 15% of corn bran increased total VFA production by supplying easily fermentable carbohydrates that rumen microbes efficiently converted into end products such as lactic, acetic, and propionic acids. However, the total VFA concentrations in the current study remained lower than those reported by Kielb et al. (2021), who observed values ranging from 190.8 mM to 361.0 mM in sorghum, corn, and grass silages.

Furthermore, corn bran supplementation significantly influenced the DMD and OMD of the silage ($p < 0.05$). The highest DMD was observed in Group P3 at 82.77%, while the lowest value was recorded in the control group at 71.09%. A similar trend was observed for OMD, which peaked at 82.46% in Group P3 and was lowest in Group P0 at 70.35%. This superior digestibility observed in the 15% corn bran P3 group was predominantly attributed to its reduced crude fiber content. Elevated fiber levels led to thicker cell walls, which hindered microbial penetration and, consequently, decreased digestibility. Increased corn bran levels enhanced LAB growth and increased acid production. Conversely, the lower OMD in Group P0 was due to the absence of corn bran, which limited degradation during fermentation. Since OM is a major component of DM, its digestibility values indicated a strong correlation. Notably, the DMD values observed in the present study were higher than the 58.26%-66.14% range reported by Gul (2023) for Caramba silage supplemented with wheat bran and molasses.

CONCLUSION

The silage composed of 85% pineapple peel and 15% corn bran yielded the most favorable overall outcomes. This specific ratio successfully maintained the physical quality of the silage while improving its chemical profile by increasing crude protein and NFE content and reducing crude fiber. Furthermore, 85% of pineapple peel and 15% of corn bran effectively reduced the structural fiber fractions, including NDF, ADF, ADL, hemicellulose, and cellulose. As a result of the substantial reduction in structural fibers, this optimized silage formulation was anticipated to enhance rumen fermentation and increase *in vitro* digestibility. Finally, further studies involving *in vivo* feeding trials on ruminants are recommended to comprehensively assess the palatability, feed intake, and actual growth performance of this formulated pineapple peel silage

DECLARATIONS

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Authors' contributions

Triani Adelina conducted the literature review, experimental investigations, and exploratory examinations and drafted the manuscript. Anwar Efendi Harahap supervised all experimental steps and the preparation of the manuscript and verified the results. Jepri Juliantoni, Arsyadi Ali, and Sigit Sepriadi supervised all experimental procedures and the development of the manuscript. All authors have reviewed and approved the final edition of the manuscript.

Availability of data and materials

The datasets generated and/or analyzed during this research are available upon reasonable request from the corresponding author.

Competing interests

The authors have not declared any conflict of interest.

Ethical considerations

The authors affirm that the manuscript is original, has not been published elsewhere, and that the most recent version has been verified prior to publication. Additionally, the authors declare that no AI tools were utilized in the preparation and composition of this article.

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Shape Variations of Furcula in Different Avian Species

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ABSTRACT

The furcula is a distinctive bone in avian species formed by the fusion of the left and right clavicles into a single structure. By acting as a spring during flight, the furcula adjusts the distance between the wing and the shoulder joint, contributing significantly to avian flight. The present study aimed to analyze furcular shapes across several bird species and investigate how these differences influence avian taxonomy. A total of 58 furculae collected from wild adult birds found dead or that died during treatment between 2023 and 2024 were examined. Samples consisted of seven crows, eight Eurasian jays, two eagles, one falcon, 19 pigeons, two storks, 10 sparrowhawks, and nine geese. Furculae were dissected and morphologically examined for overall shape, hypocleideum morphology, and the presence of pneumatic foramina. Morphological examination revealed that the furcula was V-shaped in the crow, Eurasian jay, goose, and pigeon, U-shaped in the eagle, falcon, and sparrowhawk, and Y-shaped in the stork. Geometric morphometric analysis was performed with 38 landmarks. Geometric morphometric analysis revealed that shape variation was primarily concentrated at the scapular ends of the furcula and secondarily in the hypocleideum region. Crow and Eurasian jay samples exhibited relatively similar shape patterns, whereas significant differences were detected among species groups in the canonical variate analysis. The current findings demonstrated species-specific variation in furcular morphology and provided valuable information for avian taxonomy and the functional interpretation of flight-related skeletal adaptations.

Keywords: Avian, Furcula, Geometric morphometry, Morphology, Shape

INTRODUCTION

The pectoral girdle (cingulum membri thoracici) exhibits morphological variation among bird species (Vickaryous and Hall, 2010). The furcula is a distinctive avian bone formed by the fusion of the left and right clavicles into a single structure (Vickaryous and Hall, 2010). The furcula is situated in a mid-ventral position within the avian pectoral apparatus and plays an important role in the structural integration of the shoulder girdle. The hypocleideum serves as an attachment site for pectoral musculature, while the furcula contributes to shoulder and wing movements through its associations with the pectoral girdle elements (Vickaryous and Hall, 2010; Pollard et al., 2017). During flight, the sternum transfers the force to the thorax, allowing the body to withstand the wing flapping (Beaufrère, 2009). The furcula adjusts the distance between the wing and the shoulder joint by acting as a spring, thereby playing an important role in flight (Maina, 1996). In birds, the furcula may exhibit morphological differences in shape depending on the ability to fly (König, 2016).

Several studies have described V-, Y-, and U-shaped furculae based on their morphological characteristics (Parvez et al., 2016; Keneisenuo Choudhary et al., 2020; Sridevi et al., 2020). A ventral projection known as the hypocleideum is located at the junction of the left and right clavicles (Sharma et al., 2022). Additionally, the presence of pneumatic foramina at the proximal ends of the furcula differs among bird species (Höfling and Alvarenga, 2001; John et al., 2014; Keneisenuo Choudhary et al., 2020). In some flightless birds, the clavicles may be absent, preventing the formation of a furcula (König, 2016). Traditional morphometry may be insufficient for accurately evaluating shape variations. As a result, geometric morphometric approaches have gained widespread use in studies investigating biological shape variation (Gündemir et al., 2023; Demiraslan et al., 2023; Gürbüz et al., 2024). Unlike traditional morphometric methods, geometric morphometric analyses evaluate shape independently of size and preserve the geometric relationships among anatomical landmarks, allowing more reliable comparisons of morphological variation among specimens (O'Higgins, 2000). This landmark-based technique enabled the quantitative assessment of morphological differences and provided detailed information on shape variation among structures (Viscosi and Cardini, 2012; Zelditch et al., 2012). By overlaying landmark coordinates in a Cartesian coordinate system, geometric morphometric analysis can detect shape similarities and differences both within and across groups. Landmarks are defined as anatomically corresponding points found in all samples and maintaining the same relative position (Slice, 2007).

Recent studies investigating morphological variation, particularly in zoological sciences, have demonstrated the value and broad applicability of geometric morphometric methods. These approaches have been successfully applied to a

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wide range of vertebrate taxa and anatomical structures, including os coxae morphology in horses (Gündemir et al., 2020), otolith morphology in fish species (Dörtbudak et al., 2022), cranial shape variation in sheep breeds (Demircioğlu et al., 2024), and functional shape analyses of the avian furcula in relation to flight mechanics and locomotor adaptations (Close and Rayfield, 2012). Although the furcula has been morphologically described in numerous avian species (Parvez et al., 2016; Keneisenuo Choudhary et al., 2020; Sridevi et al., 2020), most previous studies have focused on anatomical descriptions of individual species. In contrast, geometric morphometric analyses have been applied to investigate morphological variation in both avian and mammalian skeletal structures, including the carina sterni in birds (Gündemir et al., 2023), the occipital region and foramen magnum in mammals (Demiraslan et al., 2023), and the orbit in small ruminants (Gürbüz et al., 2024). However, comparative studies evaluating variation in furcular shape among avian taxa using geometric morphometric methods remain limited. Therefore, evaluating furcular morphology alongside geometric morphometric shape variation across a broad range of bird species may contribute to a greater understanding of avian diversity and the potential taxonomic significance of the furcula. The present study aimed to compare furcular morphology and shape variation among bird species belonging to different orders and families using morphological examination and geometric morphometric analysis, and to evaluate whether these characteristics reflect taxonomic relationships and may contribute to avian taxonomy.

MATERIALS AND METHODS

Ethical approval

All applicable ethical standards were followed during the conduct of the present study. The samples used in the present study were obtained in accordance with the relevant ethical approvals and legal permissions. Ethical approval was granted by the Bingöl University Local Ethics Committee for Animal Experiments (HADYEK; Approval No. E-85680299-020-169619; 08.08.2024). No animals were euthanized specifically for this study.

Samples

The samples used in the present study were obtained from the Bingöl Provincial Branch Directorate of Nature Conservation and National Parks, Bingöl, Türkiye, and transferred to the Department of Anatomy, Faculty of Veterinary Medicine, Bingöl University, Bingöl, Türkiye. The samples consisted of birds that had been found dead in the wild or had died during treatment; no animals were euthanized specifically for the present investigation. Therefore, sample sizes differed among species, and larger sample sizes were unavailable for some taxa. Species availability within the collection, representation of different taxonomic groups, and the presence of sufficiently preserved furculae suitable for morphological and geometric morphometric analyses were considered during sample selection. Bird species representing different avian orders and families were selected to enable a comparative assessment of furcular morphology and shape variation. Only samples with intact furculae and preserved anatomical integrity, showing no visible fractures, deformation, or postmortem damage that could influence morphological assessment or landmark digitization, were included in the present study. A total of 58 furculae obtained from 58 individual adult birds were examined, including seven crows (*Corvus corone*), eight Eurasian jays (*Garrulus glandarius*), two eagles (*Aquila chrysaetos*), one falcon (*Falco peregrinus*), nine geese (*Anser cygnoides domesticus*), nineteen pigeons (*Columba livia*), two storks (*Ciconia ciconia*), and ten sparrowhawks (*Accipiter nisus*). All samples were considered adults based on the degree of skeletal ossification and fusion observed in the examined bones. However, sex information was not available for the samples.

Morphology, imaging, and digitization

Birds that were found dead in the wild or died during veterinary treatment prior to inclusion in the study were transferred to the Department of Anatomy, Faculty of Veterinary Medicine, Bingöl University (Bingöl, Türkiye) and subsequently subjected to maceration. The use of naturally deceased specimens has been widely accepted in osteological, zooarchaeological, and avian postmortem investigations (Reitz and Wing, 2008; Garcês et al., 2018; 2020). Following morphological examination, each furcula was positioned in a standardized cranial view and photographed using a smartphone camera (Samsung Galaxy S22 Ultra, South Korea). A thin-plate spline (TPS) file was generated from the digital images using TPSUtil (Version 1.79; Rohlf, 2019), and a total of 38 landmarks, including six homologous landmarks, were digitized on the cranial-view digital images using TPSDig2 (Version 2.64; Rohlf, 2018). Homologous landmarks were determined as the lateral corner of the right facies articularis acrocoracoidea (LM1), the medial corner of the right facies articularis acrocoracoidea (LM38), the medial corner of the left facies articularis acrocoracoidea (LM19), the lateral corner of the left facies articularis acrocoracoidea (LM20), the caudal end of the junction of the clavicles (LM10), and the cranial end of the junction of the clavicles (LM29). The x and y Cartesian coordinates of the digitized landmarks on the cranial-view images were obtained for subsequent geometric morphometric analyses. The naming of

anatomical structures followed the guidelines provided by Nomina Anatomica Avium (Baumel et al., 1993).

Statistical analysis

Differences in size, position, and orientation among samples were removed using general Procrustes analysis (Slice, 2007). The Procrustes-aligned coordinates were subsequently subjected to principal components analysis (PCA) to identify the principal axes of shape variation among the furculae. The first principal component (PC1) and second principal component (PC2) were used to assess the major patterns of morphological variation and visualize shape changes along these axes. The effect of size on shape variation (allometry) was evaluated using multivariate regression of shape variables on centroid size. The significance of the regression was assessed using permutation tests (10,000 permutations). Canonical variate analysis (CVA) was performed to assess shape differences among groups and to evaluate patterns of group separation. Statistical significance was assessed using permutation tests (10,000 permutations), and group differences were evaluated based on Mahalanobis distances, Procrustes distances, and Pillai's trace statistics. A significance level of p-value less than 5% ($p < 0.05$) was adopted for all statistical analyses. All geometric morphometric analyses were conducted using MorphoJ software (Klingenberg, 2011). Because some species were represented by a limited number of samples, particularly the falcon (n = 1), eagle (n = 2), and stork (n = 2), the results for these taxa were interpreted with caution. Nevertheless, these samples were retained in the analyses to broaden the comparative framework and represent the available taxonomic diversity within the study material.

RESULTS

Morphological findings

In all analyzed species, the right and left clavicles converged ventrally and formed the furcula. The furcula was V-shaped in pigeons, Y-shaped in storks, and U-shaped in crows, Eurasian jays, eagles, falcons, geese, and sparrowhawks (Figure 1). The hypocleideum, located at the fusion of the right and left clavicles, was not very prominent in geese. The hypocleideum was located caudally in eagles, sparrowhawks, and falcons, and ventrally as a simple protrusion in pigeons. Crows and Eurasian jays had a prominent caudally directed hypocleideum. In storks, the proximal half of the clavicles was medio-laterally flattened, whereas the distal half was cylindrical (Figure 2). In pigeons, the right and left clavicles were rod-shaped. The clavicles were medio-laterally flattened at all levels in all species except storks. In storks, the proximal half of the clavicles was medio-laterally flattened, whereas the distal half was cylindrical (Figure 1).

Pneumatic foramina were located on the proximal extremity of both clavicles of the furcula in different numbers and sizes, laterally in crows, Eurasian jays, falcons, eagles, geese, and sparrowhawks, and medially in storks. Pneumatic foramina were not found in pigeons. Pneumatic foramina were recorded during direct anatomical examination; however, because their visualization required high magnification, they were not clearly demonstrable in the figures without compromising the overall anatomical context of the furcula.

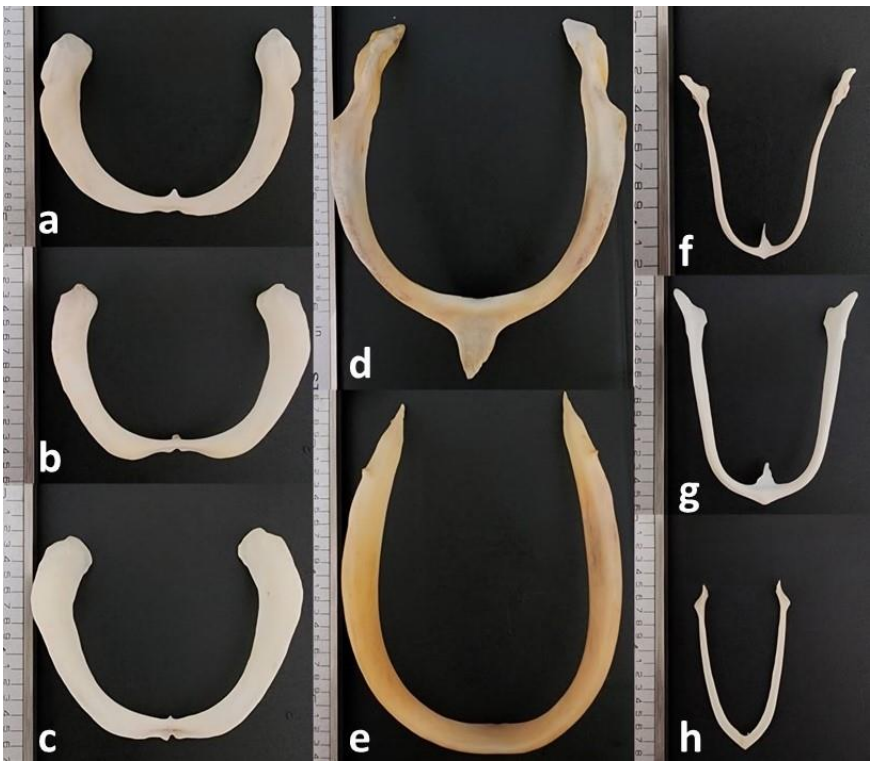


Figure 1. Cranial view of the furculae in different bird species. **a**: Sparrowhawk, **b**: Falcon, **c**: Eagle, **d**: Stork, **e**: Goose, **f**: Eurasian jay, **g**: Crow, **h**: Pigeon

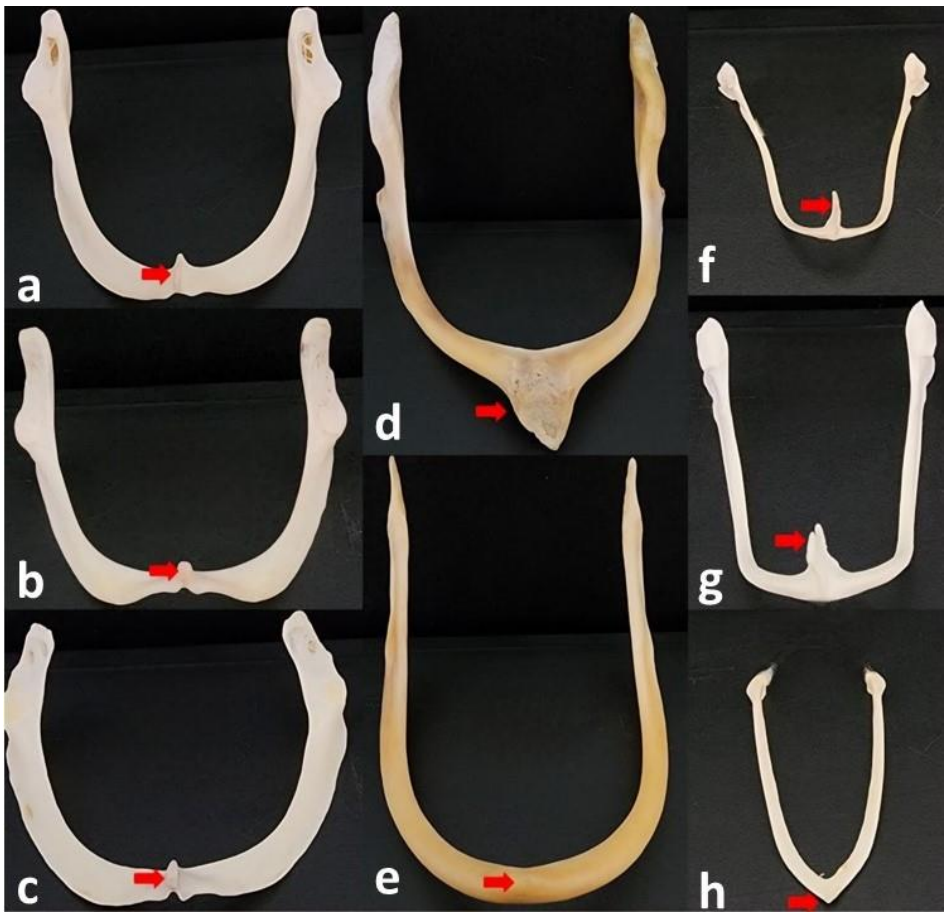


Figure 2. Caudal view of the furculae showing variation in the morphology of the hypocleideum among different bird species. **a**: Sparrowhawk, **b**: Falcon, **c**: Eagle, **d**: Stork, **e**: Goose, **f**: Eurasian jay, **g**: Crow, **h**: Pigeon. The tip of the red arrow indicates the hypocleideum.

Geometric morphometry analysis

Regression analysis demonstrated that the centroid size of the furcula accounted for 8.6525% of the variation in furcula shape ($p = 0.0011$). Since the allometric effect was statistically significant, residuals obtained from regressing shape variables on centroid size were extracted from the Procrustes coordinates and used in all subsequent analyses. In the present study, 57 principal components were calculated for the furcula. The PC1 accounted for 62.885% of the total shape variation, whereas the first two principal components (PC1+PC2) accounted for 82.230% of the total shape variation. The PC1 primarily reflected shape variation at the scapular ends of the furcula, whereas PC2 was mainly associated with variation in the hypocleideum region. The distribution of samples along PC1 and PC2 was illustrated in Figure 3. Along PC1, crow and Eurasian jay samples demonstrated a closely overlapping distribution pattern, whereas pigeon and sparrowhawk samples occupied more distinct regions of the morphospace. Falcon samples were positioned intermediate between the overlapping crow-Eurasian jay cluster and the more distinct pigeon and sparrowhawk groups. The distribution of the remaining species indicated different degrees of separation and overlap among groups (Figure 3). Figure 4 illustrates the landmarks contributing most strongly to shape variation along PC1 and PC2. The greatest shape variation was observed at the scapular ends of the furcula, followed by pronounced variation in the hypocleideum among species.

The data obtained from canonical variate analysis are presented in Table 1. A total of seven canonical variables (CV1-CV2) were calculated. The first CV1 accounted for 56.286% of the morphological variation and primarily reflected interspecific differences in corpus curvature, scapular end orientation, and hypocleideum shape (Figure 5). Pairwise comparisons based on Procrustes distances indicated significant differences in furcula shape among most species pairs, particularly between crow and pigeon, crow and sparrowhawk, Eurasian jay and goose, and pigeon and sparrowhawk ($p < 0.05$; Table 1). No significant differences were detected between crow and falcon, Eurasian jay and falcon, eagle and falcon, eagle and stork, falcon and goose, falcon and pigeon, falcon and stork, or falcon and sparrowhawk ($p > 0.05$; Table 1). The average furcula shape for each species is shown in Figure 5. In the crow and Eurasian jay, the corpus exhibited a medial slope, while the scapular tips were directed laterally. The ventral prominence of the hypocleideum was less pronounced in eagles, falcons, and sparrowhawks, but more prominent in crows, Eurasian jays, geese, pigeons, and storks.

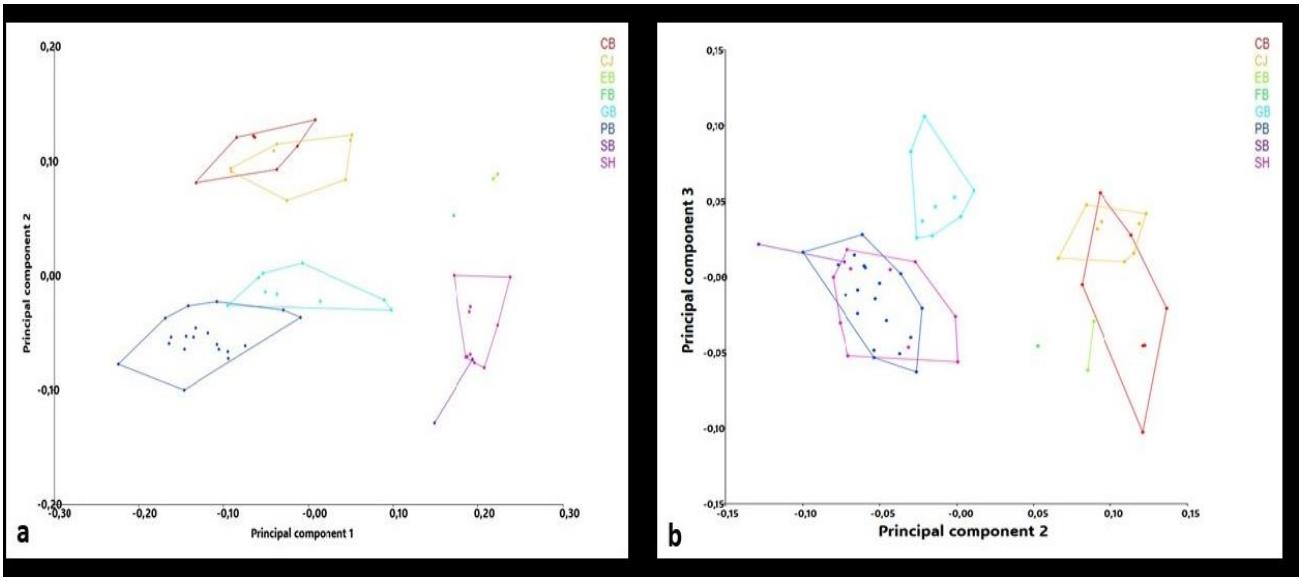


Figure 3. Principal component analysis (PCA) scatter plot showing the distribution of furcula shape variation among the examined bird species. **a:** Scatter plot of principal component 1 (PC1) compared to PC2, **b:** Scatter plot of PC2 compared to PC3. The principal components represent the major axes of shape variation in the dataset. Species located closer together exhibit more similar furcula morphologies, whereas those positioned farther apart display greater morphological differences. CB: Crow, CJ: Eurasian jay, EB: Eagle, FB: Falcon, GB: Goose, PB: Pigeon, SB: Stork, SH: Sparrowhawk.

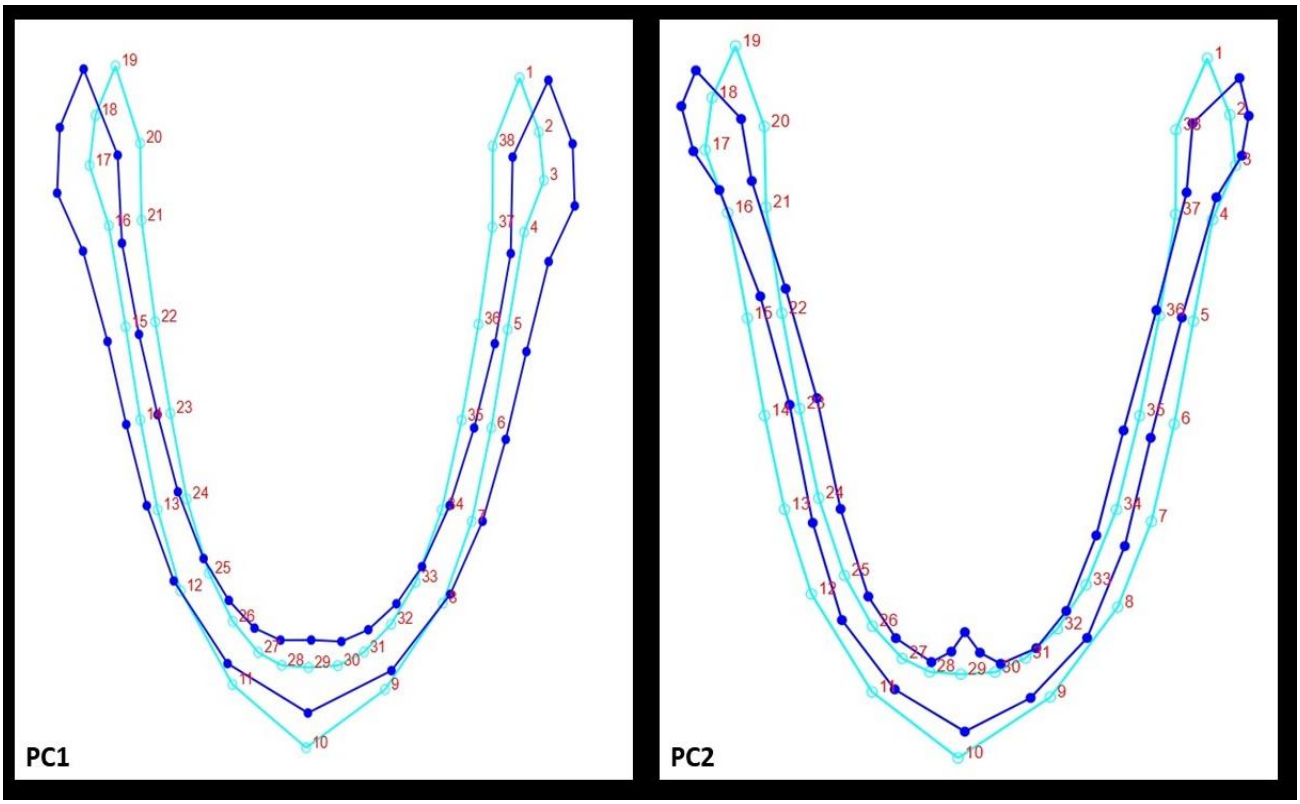


Figure 4. Wireframe graphical representation of furcula shape variation along principal component 1 (PC1) and principal component 2 (PC2). Dark blue represents the mean furcula shape, whereas light blue represents the shape configuration associated with variation along the respective principal component.

Table 1. Pairwise comparisons of furcula shape among the examined bird species based on Mahalanobis distances, Procrustes distances, and Pillai's trace statistics

Species	Statistic	CB	CJ	EB	FB	GB	PB	SB	SH
CB	MD	—	20.4583	50.0932	46.8808	38.6032	34.6839	47.2864	51.2434
	Pillai's P-value	—	0.4495	0.9512	0.9346	0.1919	0.9992	0.9919	0.0556
	PD	—	0.0658	0.2914	0.2493	0.1955	0.1756	0.3393	0.2779
	P-value	—	0.0461	0.0287	0.1230	0.0001	< 0.0001	0.0260	< 0.0001
CJ	MD	20.4583	—	53.3087	52.1876	39.2409	29.7080	46.8456	50.3137
	Pillai's P-value	0.4495	—	0.2999	0.8567	0.7799	0.8388	0.9612	0.7665
	PD	0.0658	—	0.2610	0.2275	0.1963	0.1994	0.3361	0.2620
	P-value	0.0461	—	0.0221	0.0705	< 0.0001	< 0.0001	0.0094	< 0.0001
EB	MD	50.0932	53.3087	—	22.2200	60.1618	61.7644	58.4511	37.2841
	Pillai's P-value	0.9512	0.2999	—	0.6607	0.4914	0.0591	0.3347	0.1219
	PD	0.2914	0.2610	—	0.0855	0.2982	0.3755	0.2491	0.1384
	P-value	0.0287	0.0221	—	0.3290	0.0193	0.0056	0.3347	0.0124
FB	MD	46.8808	52.1876	22.2200	—	59.9713	58.3066	56.2191	34.9491
	Pillai's P-value	0.9346	0.8567	0.6607	—	0.3229	0.0012	0.3280	0.7987
	PD	0.2493	0.2275	0.0855	—	0.2639	0.3245	0.2388	0.1224
	P-value	0.1230	0.0705	0.3290	—	0.0968	0.0509	0.3280	0.0625
GB	MD	38.6032	39.2409	60.1618	59.9713	—	26.9442	34.7629	56.9223
	Pillai's P-value	0.1919	0.7799	0.4914	0.3229	—	0.2324	0.6123	0.8617
	PD	0.1955	0.1963	0.2982	0.2639	—	0.1720	0.2232	0.2503
	P-value	0.0001	< 0.0001	0.0193	0.0968	—	< 0.0001	0.0085	< 0.0001
PB	MD	34.6839	29.7080	61.7644	58.3066	26.9442	—	32.3790	53.5977
	Pillai's P-value	0.9992	0.8388	0.0591	0.0012	0.2324	—	0.8519	0.9993
	PD	0.1756	0.1994	0.3755	0.3245	0.1720	—	0.3300	0.3180
	P-value	< 0.0001	< 0.0001	0.0056	0.0509	< 0.0001	—	0.0045	< 0.0001
SB	MD	47.2864	46.8456	58.4511	56.2191	34.7629	32.3790	—	46.3074
	Pillai's P-value	0.9919	0.9612	0.3347	0.3280	0.6123	0.8519	—	0.5037
	PD	0.3393	0.3361	0.2491	0.2388	0.2232	0.3300	—	0.1941
	P-value	0.0260	0.0094	0.3347	0.3280	0.0085	0.0045	—	0.0117
SH	MD	51.2434	50.3137	37.2841	34.9491	56.9223	53.5977	46.3074	—
	Pillai's P-value	0.0556	0.7665	0.1219	0.7987	0.8617	0.9993	0.5037	—
	PD	0.2779	0.2620	0.1384	0.1224	0.2503	0.3180	0.1941	—
	P-value	< 0.0001	< 0.0001	0.0124	0.0625	< 0.0001	< 0.0001	0.0117	—

CB: Crow, CJ: Eurasian jay, EB: Eagle, FB: Falcon, GB: Goose, PB: Pigeon, SB: Stork, and SH: Sparrowhawk, MD: Mahalanobis distance, Pillai's P-value: P-value calculated for Pillai's trace from permutation tests (10,000 rounds), PD: Procrustes distance, P-value: P-value calculated for Procrustes distance from permutation tests (10,000 rounds). The MD and PD values indicated significant pairwise differences between species in rows and columns at $p < 0.05$. Diagonal cells were not applicable because a species cannot be compared with itself.

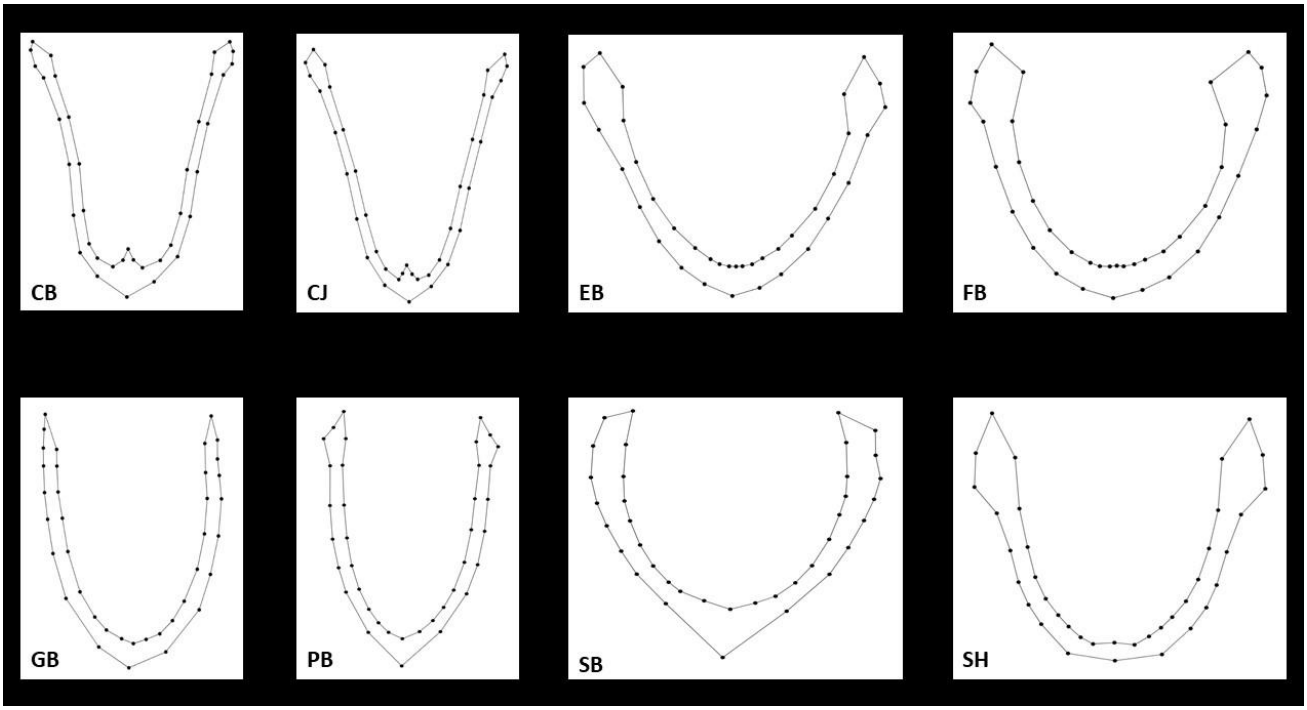


Figure 5. Mean furcula shapes of the examined bird species. CB: Crow, CJ: Eurasian jay, EB: Eagle, FB: Falcon, GB: Goose, PB: Pigeon, SB: Stork, SH: Sparrowhawk.

DISCUSSION

In the present study, the furcula exhibited notable shape variation among the bird species examined, with these differences especially evident at the scapular ends and the hypocleideum. Notably, the ventral prominence of the hypocleideum was limited in eagles, falcons, and sparrowhawks, which are prey birds. The observed variation in furcula shape may be associated with differences in hunting behavior, feeding ecology, and flight characteristics among species. Similar relationships between furcula morphology and functional or flight-related adaptations have previously been reported in birds (Bailey and DeMont, 1991; Hui, 2002). Likewise, the present study identified notable variation in the scapular ends and hypocleideum among species, supporting the view that furcula morphology may reflect functional and ecological differences among birds.

Previous studies have reported both V- and U-shaped furculae in pigeons. While Sridevi et al. (2020) described a V-shaped furcula, Parvez et al. (2016) reported a U-shaped configuration in pigeons. In the current study, all pigeon samples exhibited a V-shaped furcula, supporting the observations of Sridevi et al. (2020) and indicating potential variation in population, breed, or sampling across studies. A U-shaped furcula has been reported in geese (Getty, 1975), small Indian kites (Sharma et al., 2022), green-winged macaws (Indu et al., 2012), brown wood owls and crested serpent eagles (Keneisenuo Choudhary et al., 2020), crows (Sridevi et al., 2020), common hawk-cuckoo (Supriya et al., 2019), and pariah kites (Tomar et al., 2010). Similarly, the furcula was U-shaped in crows, Eurasian jays, eagles, sparrowhawks, geese, and falcons in the present study. The U-shaped furcula observed in geese was consistent with the report of Getty (1975), whereas the findings in crows were consistent with those of Sridevi et al. (2020). Comparable U-shaped furculae have been reported in green-winged macaws and peahens (Indu et al., 2012), crested serpent eagles and brown wood owls (Keneisenuo Choudhary et al., 2020), and small Indian kites and domestic fowl (Sharma et al., 2022). In the sparrowhawk, unlike the other bird species, the ventral margin of the furcula exhibited a distinct notch. The furculae of the eagle, sparrowhawk, and falcon were very similar to each other and exhibited a broad U-shaped configuration. In contrast, the furcula of the crow and Eurasian jay exhibited a narrower U-shaped configuration.

Höfling and Alvarenga (2001) reported the absence of the hypocleideum in birds belonging to the families Picidae, Indicatoridae, Galbulidae, and Coraciformes. Similarly, the hypocleideum was reported to be absent in the brown wood owl (Keneisenuo Choudhary et al., 2020) and Indian horned owl (Sridevi et al., 2020), absent or rudimentary in the small Indian kite (Sharma et al., 2022), and rudimentary in ducks (Rajani et al., 2018). In agreement with these findings, the hypocleideum was rudimentary in geese in the present study. In pigeons, Sridevi et al. (2020) reported the absence of the hypocleideum, whereas Jayachitra and Iniyah (2021) and Parvez et al. (2016) described an underdeveloped hypocleideum in domestic pigeons (*Columba livia*). In the present study, the hypocleideum was identified as a simple protrusion in pigeons, confirming the structure reported by Jayachitra and Iniyah (2021) and Parvez et al. (2016), though it differed from the findings of Sridevi et al. (2020). Keneisenuo Choudhary et al. (2020) reported the presence of a hypocleideum in the crested serpent eagle. Similarly, a simple hypocleideal protrusion was observed in eagles, sparrowhawks, and falcons in the present study. Sharma et al. (2022) reported a well-developed hypocleideum in domestic fowl (*Gallus domesticus*), whereas Jayachitra and Iniyah (2021) described a flattened plate-like hypocleideum. In the present study, a prominent caudally directed hypocleideum was observed in crows and Eurasian jays, whereas the hypocleideum in storks was flattened in the cranio-caudal direction and prominently elongated ventrally. Höfling and Alvarenga (2001) reported the presence of pneumatic foramina at the omal extremity of both clavicles in Ramphastidae (*Ramphastos tucanus*), Picidae (except the genus *Picumnus*, such as *Picoides major*), Indicatoridae (*Indicator indicator*), and Passeriformes (*Corvus brachyrhynchus*). Similarly, Keneisenuo Choudhary et al. (2020) reported lateral pneumatic foramina in the clavicle of the crested serpent eagle, whereas John et al. (2014) described them in crows. The absence of pneumatic foramina in pigeons, as observed in the current study, aligns with the findings of John et al. (2014), who reported that pneumatic foramina were absent from the clavicles of pigeons and owls, while such foramina were present on the lateral surface of the clavicles in crows. In the present study, pneumatic foramina with different numbers and sizes were observed on the lateral surface of the clavicle in crows, Eurasian jays, eagles, falcons, geese, and sparrowhawks. The occurrence of lateral pneumatic foramina in crows and eagles was consistent with the findings of John et al. (2014) and Keneisenuo Choudhary et al. (2020), respectively. In contrast, pneumatic foramina were observed on the medial surface of the clavicle in storks, a feature not reported in the cited studies.

CONCLUSION

Geometric morphometric analysis revealed notable interspecific differences in furcula morphology among the bird species examined. The observed shape variation was mainly associated with the scapular extremities and the hypocleideum region, which contributed substantially to species differentiation. Similarities in furcula morphology were

observed among some closely related taxa, although clear separation among all taxonomic groups was not evident. The current findings indicated that furcula morphology can provide valuable information for comparative anatomy, avian taxonomy, and functional interpretations of flight biomechanics. One limitation of the present study was the relatively small sample size for some species, particularly eagle, falcon, and stork specimens. Future studies involving larger sample sizes and a broader range of avian taxa are needed to further validate and expand the present findings.

DECLARATIONS

Authors' contributions

Funda Aksünger Karaavcı and Yasin Demiraslan participated in the design of this study, Funda Aksünger Karaavcı and Betül Genç prepared the furcula samples and performed the morphological analyses. Yasin Demiraslan performed the geometric morphometric analyses. Funda Aksünger Karaavcı and Yasin Demiraslan wrote the manuscript. All authors have read and confirmed the final edition of the manuscript for publication.

Availability of data and materials

The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Competing interests

The authors affirmed that there are no competing interests.

Ethical considerations

The author declares that this manuscript is an original study, has not been published previously, and is not under consideration for publication elsewhere. The authors further confirmed that the manuscript is free from plagiarism and complies with accepted ethical and publication standards. Artificial intelligence (Grammarly) was used solely to assist with English language editing, and ChatGPT (OpenAI) was used for the initial preparation of the graphical abstract. The graphical abstract revised text of the manuscript was subsequently reviewed and manually modified by the authors. No artificial intelligence tools were used in the study design, data collection, data analysis, interpretation of results, or formulation of scientific conclusions. All scientific content, analyses, interpretations, and conclusions were developed and verified by the authors, who take full responsibility for the content of the manuscript and graphical abstract.

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Effects of Javanese Ginseng (*Talinum paniculatum*) Leaf Decoction on Immunohistochemical Expression and Superoxide Dismutase Levels in Diabetic Rats

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ABSTRACT

Java Ginseng has long been used by Indonesian communities as a medicinal plant and is traditionally consumed as an alternative therapy, even in managing Diabetes Mellitus (DM). The present study aimed to evaluate the effects of the decoction of Javanese ginseng leaf simplicia (DJGLS) on blood glucose levels, serum superoxide dismutase (SOD), the levels and expression intensity of SOD, and pancreatic histopathology in DM rats. Twenty adult male rats were randomly assigned to four groups. The normal control group (GJ-0, n = 5), the DM group (GJ-1, n = 5) received a placebo, and the DM groups (GJ-2, n = 5 and GJ-3, n = 5) were administered a DJGLS (100 g/100 mL, w/v powder simplicia), at volume of 0.5 mL and 1 mL/100 g body weight, respectively for 21 consecutive days. Blood glucose levels were measured using a glucometer. Serum and pancreatic SOD levels were determined using an ELISA kit. Pancreatic histopathology was evaluated using hematoxylin and eosin staining, while pancreatic SOD expression was assessed by immunohistochemical staining. In addition, phytochemical screening was performed on the Javanese ginseng leaf simplicia powder. The present study demonstrated that the phytochemical analysis of Java Ginseng leaf simplicia powder revealed the presence of alkaloids, flavonoids, saponins, tannins, and steroids. DJGLS treatment reduced blood glucose levels and serum SOD levels in diabetic rats, with a significant reduction observed in the GJ-3 group compared with the GJ-1. Administration of DJGLS in the GJ-2 and GJ-3 groups significantly increased pancreatic SOD levels compared with the GJ-1. The DJGLS treatment in the GJ-2 and GJ-3 groups demonstrated restorative effects, as evidenced by the recovery of the Islets of Langerhans area and increased intensity of pancreatic SOD expression compared with the GJ-1. In conclusion, DJGLS treatment was able to reduce blood glucose levels and serum SOD levels, although these values had not fully returned to normal conditions, and it increased pancreatic SOD levels. The DJGLS indicated potential for promoting recovery of the pancreatic Islets of Langerhans area.

Keywords: Decoction, Diabetes, Immunohistochemistry, Javanese ginseng, Superoxide dismutase

INTRODUCTION

A report by the World Health Organization (WHO, 2024) states that in 2022, the prevalence of diabetes among adults aged ≥ 18 years reached approximately 14%, doubling from 7% in 1990. Furthermore, about 59% of individuals aged ≥ 30 years living with diabetes had not received pharmacological therapy, indicating a substantial gap in disease control and management. Diabetes imposes a substantial burden on healthcare systems, with global economic costs estimated to exceed USD 850 billion annually (Parker et al., 2024).

Diabetes mellitus (DM) is a metabolic disorder characterized by hyperglycemia and has become a major global health concern (IDF, 2025). Hyperglycemia triggers oxidative stress due to excessive free radical production through mitochondrial, protein kinase, and hexosamine pathways (Chen et al., 2025). Oxidative stress contributes to the progression of diabetes and organ damage, including the pancreas, by inducing β-cell necrosis, apoptosis, and the release of proinflammatory cytokines (An et al., 2022). The high susceptibility of β-cells to oxidative stress is also associated with reduced activity of antioxidant enzymes such as superoxide dismutase (SOD), catalase, and glutathione peroxidase (Gerber and Rutter, 2017). Therefore, controlling hyperglycemia by reducing blood glucose levels is crucial to prevent complications.

If hyperglycemia is not adequately controlled, it increases the risk of broader complications, including microvascular disorders such as retinopathy, nephropathy, and neuropathy, as well as macrovascular complications involving atherosclerotic, cardiovascular, cerebrovascular, and peripheral vascular diseases (Tan et al., 2022; IDF, 2025).

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According to Tan et al. (2022), hyperglycemia leads to excessive accumulation of free radicals in cells, resulting in reduced levels of SOD, catalase (CAT), and glutathione peroxidase (GPx).

Traditional medicine has long been utilized and continues to play an important role as an alternative option in healthcare therapy (Yakubu et al., 2020). According to the World Health Organization, approximately 80% of the global population continues to rely on herbal medicine for healthcare needs, particularly in low- and middle-income countries where access to conventional medical services is limited (Badhon, 2023), with the majority of traditional herbal practices originate from well-established medical traditions in China, India, and different regions of Africa (Latif and Nawaz, 2026). The widespread use of traditional medicine is largely attributed to its strong cultural acceptance, its perceived compatibility with the body's physiological conditions, and its relatively lower risk of adverse effects compared with modern pharmaceuticals (Tran et al., 2020).

More than 400 plant species with hypoglycemic activity have been reported (Patel et al., 2012). Nevertheless, the exploration of natural product-based antidiabetic agents remains relevant due to the therapeutic potential of their phytoconstituents in diabetes mellitus management. One such plant is Javanese ginseng (*Talinum paniculatum*), its root infusion has been reported to exhibit hypoglycemic effects in diabetic rats (Jenie and Indraswari, 2021).

Javanese ginseng (*Talinum paniculatum*) has long been empirically utilized by Indonesian communities as a medicinal plant (Susilo et al., 2024). In traditional healing practices, plant parts such as the leaves, roots, and stems are commonly prepared by boiling, and the resulting decoction is consumed for therapeutic purposes. *Talinum paniculatum*, known in Indonesia as Javanese Ginseng, shares characteristics with other well-known traditional medicinal plants, such as Panax Ginseng from East Asia (Oluba et al., 2019). Different studies have reported that Javanese Ginseng is rich in phytochemicals, including flavonoids, tannins, triterpenes, saponins, polyphenols, and polysaccharides (Aini and Susilo, 2023; Suriani et al., 2024). Furthermore, the Javanese ginseng plant also contains important sterol compounds such as campesterol, stigmasterol, and sitosterol as its main bioactive components (Tolouei et al., 2019). Pharmacologically, Javanese Ginseng is known to possess different biological activities, including antioxidant properties (Souto et al., 2021), free radical scavenging and anticancer activities (Aini and Susilo, 2023), potential in treating cardiovascular disorders, and strong antibacterial properties against *Candida albicans*, *Escherichia coli*, and *Staphylococcus aureus* (Tolouei et al., 2019). The root of Javanese ginseng (*Talinum panuculatum*) has also been reported to have hypoglycemic effects (Jenie and Indraswari, 2021). Therefore, a study on the use of Javanese ginseng (*Talinum paniculatum*), particularly its leaf decoction, is important to conduct in order to provide information on its hypoglycemic effects, as well as its influence on SOD levels and the histopathology of pancreatic tissue in diabetic rats. The present study aimed to evaluate the effects of a decoction of Javanese ginseng leaf simplicia (DJGLS) on blood glucose levels, SOD levels, and expression in the serum and pancreas of DM rats

MATERIALS AND METHODS

Ethical approval

All experimental procedures used in the study required approval from the Animal Research Ethics Committee, Faculty of Veterinary Medicine, Udayana University, Bali, Indonesia, which evaluated and approved the animal experiments. (Number: B/179/UN14.2.9/PT.01.04/2025)

Preparation of Javanese ginseng decoction

The preparation of decoction of Javanese ginseng leaf simplicia (DJGLS) was adapted from the method described by Jenie and Indraswari (2021). Fresh leaves of *Talinum paniculatum* (Javanese ginseng) were obtained from Dewandaru Flora Organic Farm, Tabanan Regency, Bali, Indonesia. The fresh green leaves were sorted and air-dried under morning sunlight between 08:00 and 10:00 until completely dried. The dried leaves were then ground using a blender (Type HR 2115, Philips, Indonesia) to obtain simplicia powder. A total of 100 g of the simplicia powder was mixed with 400 mL of distilled water and heated until the temperature reached 90˚C, then maintained for 10 minutes. The resulting solution was filtered through a clean cotton cloth into a volumetric flask. Distilled water was subsequently added to the flask to obtain a final volume of 100 mL, resulting in a preparation equivalent to 100 g/100 mL (w/v) of simplicia powder. The mentioned preparation served as the stock solution of simplicia powder for administration to the experimental rats.

Phytochemical test

A total of 25 g of Javanese ginseng leaf powder was mixed with 500 mL of 80% ethanol and stirred gently, then allowed to stand for 24 hours, placed at room temperature, and wrapped with aluminum foil. The mixture was subsequently filtered using a clean cotton cloth to obtain the filtrate. The filtrate was then concentrated using a vacuum rotary evaporator (IKA RV 10, Germany) at 50˚C until a thick extract was obtained. The resulting extract was subjected

to phytochemical screening to identify the presence of alkaloids, flavonoids, saponins, tannins, and steroids, following a method adapted from Nortjie et al. (2022).

Experimental preparation and treatment

Twenty adult male white rats of the Sprague-Dawley strain, weighing 135-140 g, were obtained from the Bio-Mice and Rat experimental animal breeding facility in Denpasar, Bali, Indonesia. The rats were housed individually in cage boxes (45 × 30 × 20 cm). The animal room was maintained at a temperature of 23-25˚C. During a one-week acclimatization period, the rats were provided with pellet-form feed (Br II, Japfa Comfeed Tbk, Indonesia) and drinking water *ad libitum*. After the acclimatization period, the rats were randomly divided into four groups, including the GJ-0 group (n = 5), which served as the normal control, while the other three groups were DM rats, namely GJ-1 (n = 5), GJ-2 (n = 5), and GJ-3 (n = 5). Diabetes mellitus in rats was induced by a single dose of 1% streptozotocin (STZ, Sigma-Aldrich Inc., USA) solution (40 mg/kg body weight) dissolved in 50 mM sodium citrate buffer (pH 4.5; Wu et al., 2021), via intraperitoneal injection. Four days after injection, the rats were fasted for 12 hours, and blood glucose levels were measured using a glucometer (GlucoDr.™AGM-2100, allmedicus, Korea). Rats were considered diabetic when their blood glucose levels exceeded 200 mg/dL, according to Furman (2021). Afterward, rats in the GJ-0 and GJ-1 groups were administered a placebo (distilled water) at a volume of 1 mL/100 g body weight. Diabetic rats in the GJ-2 and GJ-3 groups were administered a decoction of Javanese ginseng leaves simplicia (DJGLS), prepared at a dose of 100 g/100 mL (w/v) with a volume of 0.5 mL and 1 mL per 100 g body weight per day, respectively. The treatments were given orally using gastric gavage for 21 consecutive days.

Sample collection

On day 22, the rats were deeply anesthetized with ketamine 40 mg/kg body weight and xylazine 5 mg/kg body weight (veterinary use only, Troy Laboratories PTY LTD, NSW, Australia) administered intramuscularly into the thigh muscle. Following confirmation of anesthesia, euthanasia was performed by cervical dislocation in accordance with the AVMA Guidelines for the Euthanasia of Animals (AVMA, 2020).

Blood samples were collected from the orbital vein using a hematocrit capillary tube and transferred into tubes without anticoagulant to obtain serum. The pancreas was carefully removed and divided into two portions. One portion was fixed in 10% neutral buffered formalin for the preparation of histopathological and immunohistochemical staining slides. The remaining portion was placed in a clean container and stored in a refrigerator at 4˚C conditions for the determination of superoxide dismutase levels using an ELISA kit (Bioassay Technology Laboratory, Cat. No. E0168Ra, Zhejiang, China). ELISA analysis was performed on the same day (within a single day). Samples were collected in the morning, and the analysis was conducted in the afternoon. While awaiting preparation for the analysis, the samples were stored in a refrigerator.

Determination of blood glucose level

Blood glucose levels were determined using the blood glucose monitoring system GlucoDr.™AGM-2100 (allmedicus, Korea) according to Jendrike et al. (2017). Blood glucose measurements were performed on day 4 in both normal rats and STZ-injected rats. The data obtained from the measurement of glucose levels were used as the baseline blood glucose levels and were designated as day 0 for both normal and diabetic (DM) rats. Blood glucose levels were measured again at the end of the treatment period (day 22). Before measurement, the rats were fasted overnight (12 h) without access to food and water.

Determination of superoxide dismutase levels

The SOD levels were determined using an ELISA Kit (rat Superoxidase dismutase, Bioassay Technology Laboratory, China) using the procedure listed on the kit. The pancreatic tissue was rinsed in PBS (pH 7.4) to thoroughly remove excess blood and weighed prior to homogenization. The pancreas was minced and then placed into a 1.5 mL microtube containing PBS (pH 7.4). The sample was kept on ice in a beaker glass and subsequently sonicated for 1 minute using an ultrasonic homogenizer (Ultrasonicator, Tefic Biotech Co., Limited, China). The homogenate was centrifuged (Thermo scientific™ Multifuge, USA) at 2000-3000 rpm for approximately 20 minutes, and the supernatant was collected and transferred into an Eppendorf tube for immediate analysis. The plate was pre-coated with rat SOD antibody. The SOD present in the sample was then added and bound to the antibody coating the wells. Afterward, biotinylated rat SOD antibody was added and bound to the SOD in the sample. Streptavidin-HRP was then added and bound to the biotinylated SOD antibody. After incubation, unbound streptavidin-HRP was removed by washing. Substrate solution was then added, and color development was proportional to the amount of rat SOD in the sample. The

reaction was stopped by adding an acid stop solution, and absorbance was measured at 450 nm with an ELISA reader (Thermo Scientific Multiskan GO, Japan).

Histopathological staining

Histopathological staining was performed using hematoxylin and eosin (H&E) staining adapted from the method of Suarsana et al. (2020), which was originally applied to liver and kidney tissues. In the present study, pancreatic tissue was fixed in 10% neutral buffered formalin for 24h in room temperature, followed by dehydration, washing, paraffin embedding, and sectioning. Thin sections (4 µm) were floated on warm water, flattened, mounted onto glass slides, and allowed to dry. The sections were then stained with H&E solutions, dehydrated, rinsed, and mounted with a cover glass. Each slide was examined in five different fields of view. Observations were performed under a light microscope at 400x magnification equipped with a camera (Olympus CX33 microscope, Japan).

Immunohistochemical staining

The immunohistochemical analysis was performed using a procedure adapted from Magaki et al (2019). Five prepared slides from each group were subjected to immunohistochemical staining. The procedure was carried out as follows. Pancreatic tissues embedded in paraffin were sectioned at a thickness of 4 µm and mounted on silane-coated glass slides. The sections were then deparaffinized and rehydrated using standard protocols, followed by antigen retrieval. Endogenous peroxidase activity was blocked by immersing the slides in 3% hydrogen peroxide solution. To reduce nonspecific background staining, the sections were incubated with bovine serum albumin as a blocking serum. Subsequently, 200 µL of diluted primary antibody (1:200 dilution; SOD sc-101523; Santa Cruz Biotechnology, Oregon, USA) was applied to each slide and incubated at room temperature for 80 minutes. The slides were then incubated with a species-specific secondary antibody (Histofine Simple Stain MAX PO, Nichirei Biosciences Inc., Japan) for 15 minutes. After incubation, the slides were washed three times with 0.1% TBS-Tween solution. Chromogen development was performed by incubating the sections for 5 minutes, followed by rinsing in three changes of deionized water until the wash solution became clear. The sections were then counterstained with hematoxylin for 30 seconds and rinsed with several changes of distilled water. Subsequently, the slides were dehydrated, cleared in three changes of xylene, and mounted with a coverslip. Finally, the stained sections were examined under a light microscope (Olympus CX33 microscope, Japan).

Statistical analysis

The present study used a completely randomized design. Serum and pancreatic SOD levels were analyzed using One-Way ANOVA (Analysis of Variance). The significance level was set at 0.05, and to determine differences among treatment means, Duncan's Multiple Range Test was subsequently applied. While H&E staining and immunohistochemistry results were analyzed descriptively. The analysis was performed using SPSS version 26 software.

RESULTS

Phytochemical test

Phytochemical tests of Javanese ginseng (*Talinum paniculatum*) extract revealed the presence of different bioactive compounds. Qualitative test results indicated positive reactions for all tested parameters, namely alkaloid, flavonoid, saponin, tannin, and steroid (Table 1).

Blood glucose analysis

Blood glucose levels in rats differed markedly among the treatment groups. The normal group (GJ-0) revealed a mean blood glucose level of 119.6 mg/dL, whereas the diabetic group (GJ-1) of 400 mg/dL. At the end of the treatment period, the GJ-2 group exhibited a blood glucose level of 317.0 mg/dL, representing a reduction of approximately 42.1%, whereas the GJ-3 group indicated a level of 234.2 mg/dL, corresponding to a 51.4% decrease. Despite these reductions, blood glucose levels in both treatment groups remained higher than those observed in the normal control group (Table 2).

Serum analysis and pancreatic superoxide dismutase levels

The results of the analysis of SOD levels in the serum and SOD in the pancreas of diabetic rats treated with Javanese ginseng (*Talinum paniculatum*) extract are presented in Table 2. Serum SOD levels indicated in the GJ-0 treatment group were lower and significantly different ($P < 0.05$) compared to the GJ-1, GJ-2, and GJ-3 groups. The serum SOD level in the GJ-2 group (diabetic rats) treated with DJGLS at 50 g/100 mL was lower, but not significantly different ($P > 0.05$) compared to the GJ-1 group (diabetic rats). Meanwhile, in the GJ-3 group (diabetic rats) treated with DJGLS at 100 g/100 mL, the serum SOD level was lower and significantly different ($P < 0.05$) compared to the GJ-1 group (diabetic rats; Table 3).

Measurements of pancreatic SOD enzyme levels indicated that the diabetic group (GJ-1) had the lowest pancreatic SOD levels, significantly different ($P < 0.05$) compared to the other groups, indicating significant oxidative stress due to hyperglycemia. Conversely, DJGLS treatment in the GJ-2 and GJ-3 groups significantly increased ($P < 0.05$) pancreatic SOD levels compared to the GJ-1 group, but the levels were not significantly different ($P > 0.05$) compared to the normal control group (GJ-0). The increase in pancreatic SOD levels indicated that administration of DJGLS is quite effective in improving the endogenous antioxidant activity of SOD in the pancreatic tissue of diabetic rats, although the levels have not yet completely reached normal levels (Table 3).

Table 1. The phytochemical tests on Javanese ginseng extract ingredients

Parameter	Test type	Test results
Alkaloid	Wagner test	Positive
	Dragendorff's test	Positive
	Mayer's test	Positive
Flavonoid	Zinc and Magnesium test	Positive
Saponin	Foam saponin test	Positive
Tanin	Lead acetate test	Positive
Steroid	Lieberman test	Positive

Table 2. Blood glucose level in experimental rats of the Sprague-Dawley strain for 21 days of treatment

Groups	Adaptation period	Post STZ injection day 4 (baseline on day 0)**	After intervention (day 22)	Changes (%; from day 0-22)
GJ-0	109.8 ± 6.42^{a}	112.2 ± 4.71^{a}	119.6 ± 5.37^{a}	6.6 %
GJ-1	100.4 ± 3.36^{a}	502.2 ± 31.59^{b}	400 ± 22.47^{b}	20.4 %
GJ-2	102.6 ± 5.94^{a}	488.6 ± 46.90^{b}	317 ± 27.56^{bc}	42.1 %
GJ-3	103.4 ± 5.77^{a}	481.0 ± 58.01^{b}	234.2 ± 15.61^{c}	51.4 %

[a,b,c] Different superscript letters in the same column indicate significant differences between treatments ($p < 0.05$). GJ-0: Normal control group, GJ-1: Diabetic group, GJ-2 and GJ-3: Diabetic group treated with decoction of Javanese ginseng leaf simplicia (DJGLS; 100g/100 mL) of 0.5 mL and 1 mL per 100 g body weight, respectively**. Post-STZ injection day 4 (used as the baseline on day 0 of the Javanese ginseng leaf decoction treatment). STZ: Streptozotocin.

Table 3. Average superoxide dismutase levels in serum and pancreas of diabetic rats of the Sprague-Dawley strain for 21 days of treatment

Parameter / Group	SOD serum		SOD pancreas	
	Concentration (ng/mL)*	Changes (%)	Concentration (ng/mL)*	Changes (%)
GJ-0	1.893 ± 0.254^{a}	-	2.404 ± 0.573^{b}	-
GJ-1	3.115 ± 0.326^{b}	64.5%	0.716 ± 0.284^{a}	(70.2%)
GJ-2	2.803 ± 0.408^{bc}	48.1%	2.031 ± 0.364^{b}	(15.5%)
GJ-3	2.476 ± 0.331^{c}	30.8%	2.215 ± 0.338^{b}	(7.9%)

[a,b,c] Different superscript letters in the same column indicate significant differences between treatments ($p < 0.05$). GJ-0: Normal control group, GJ-1: Diabetic group. GJ-2 and GJ-3: Diabetic group treated with decoction of Javanese ginseng leaf simplicia (DJGLS; 100g/100 mL) of 0.5 mL and 1 mL per 100 g body weight, respectively. SOD: Superoxide dismutase. The data is presented as mean ± standard deviation.

Histopathological staining with hematoxylin and eosin

In H&E staining of the pancreas of the normal group (GJ-0), the microstructure of the pancreatic lobule is maintained, and the exocrine acini cells are neatly arranged with dense basophilic cell nuclei and eosinophilic cytoplasm. The islets of Langerhans appeared with relatively clear boundaries, compact islet cells, round/ovoid nuclei, and homogeneous nuclear cell distribution. H&E staining in GJ-1 indicated histopathological changes, the size of the islets of Langerhans was reduced (shrinking) with more fragile and heterogeneous islet cells; some beta cells indicated pyknotic nuclei and necrosis. H&E staining in GJ-2 indicated partial improvement in some islets of Langerhans, indicating recovery of the relative size and density of the islet cells, appearing more compact compared to the diabetic group (GJ-1). Signs of necrosis are reduced but not completely eliminated. H&E staining in GJ-3 indicated more marked improvement; the islets of Langerhans are clearer with cells indicating more intact nuclei and fewer pyknotic cells (Figure 1).

Superoxide dismutase immunohistochemical staining of the pancreas

The SOD immunohistochemical staining in GJ-0 indicated a strong positive reaction (intense brown color) to SOD expression, especially in the islet cells of Langerhans. The distribution of brown color appeared homogeneous in the cytoplasm of β cells. In some sections, there was a loss of positive staining. Immunohistochemical results in the GJ-2 group indicated a moderate increase in brown intensity compared to the diabetic group (GJ-1). Brown color appeared more pronounced in the cytoplasm of islet cells, although not as homogeneous as in a normal pancreas. Immunohistochemical results in the GJ-3 group indicated a fairly strong positive reaction (quite dark brown) that almost approached the condition of a normal pancreas. The distribution of color was more evenly distributed throughout the islet area (Figure 2).

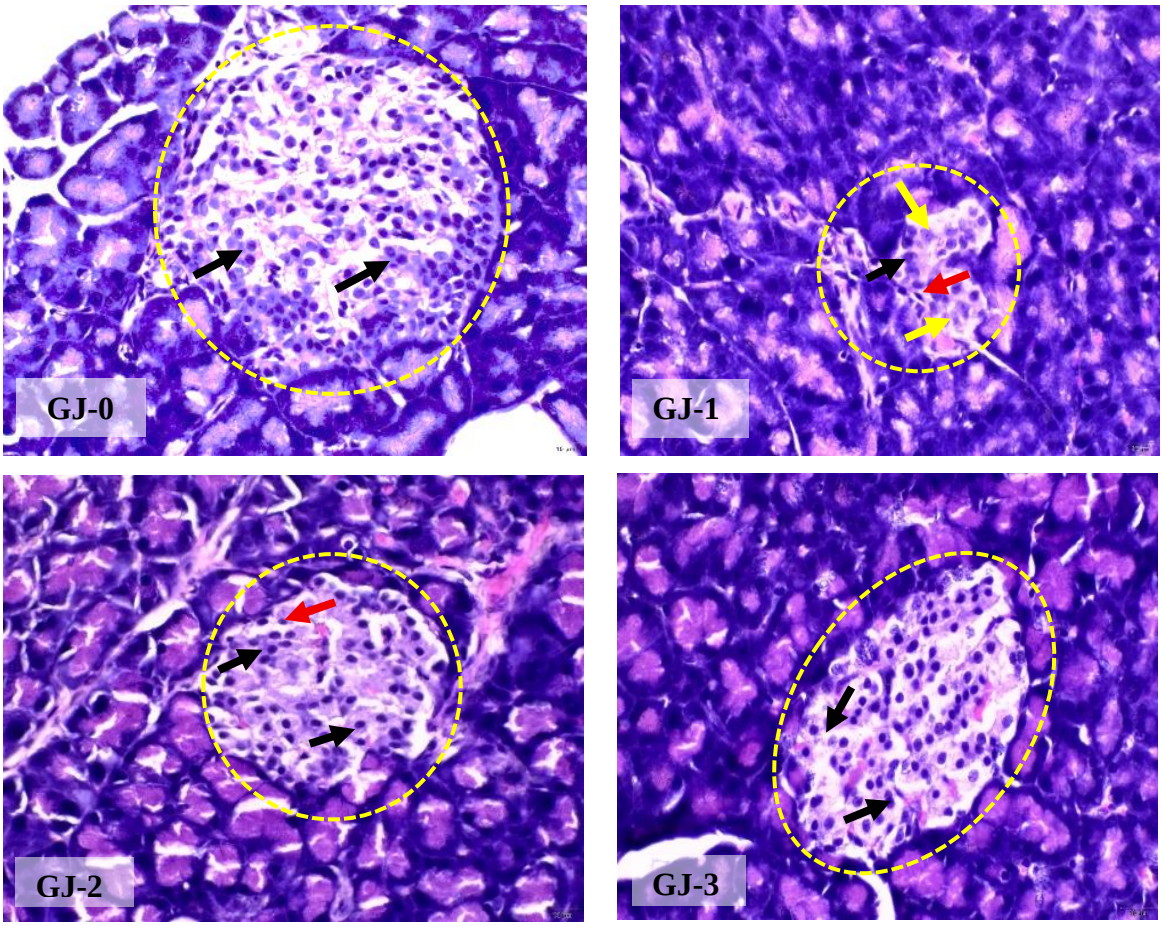


Figure 1. The H&E staining of pancreatic tissue of diabetic rats of the Sprague-Dawley strain after 21 days of treatment. **GJ-0**: Normal control group, **GJ-1**: Diabetic group, **GJ-2 and GJ-3**: Diabetic group treated with decoction of Javanese ginseng leaf simplicia (DJGLS; 100g/100 mL) of 0.5 mL and 1 mL per 100 g body weight, respectively. Islet cells are indicated by black arrows, necrosis is indicated by yellow arrows, pyknotic cell nuclei are indicated by red arrows, and yellow circles indicate islets of Langerhans. The scale bar is 10 µm, and the magnification is 400x.

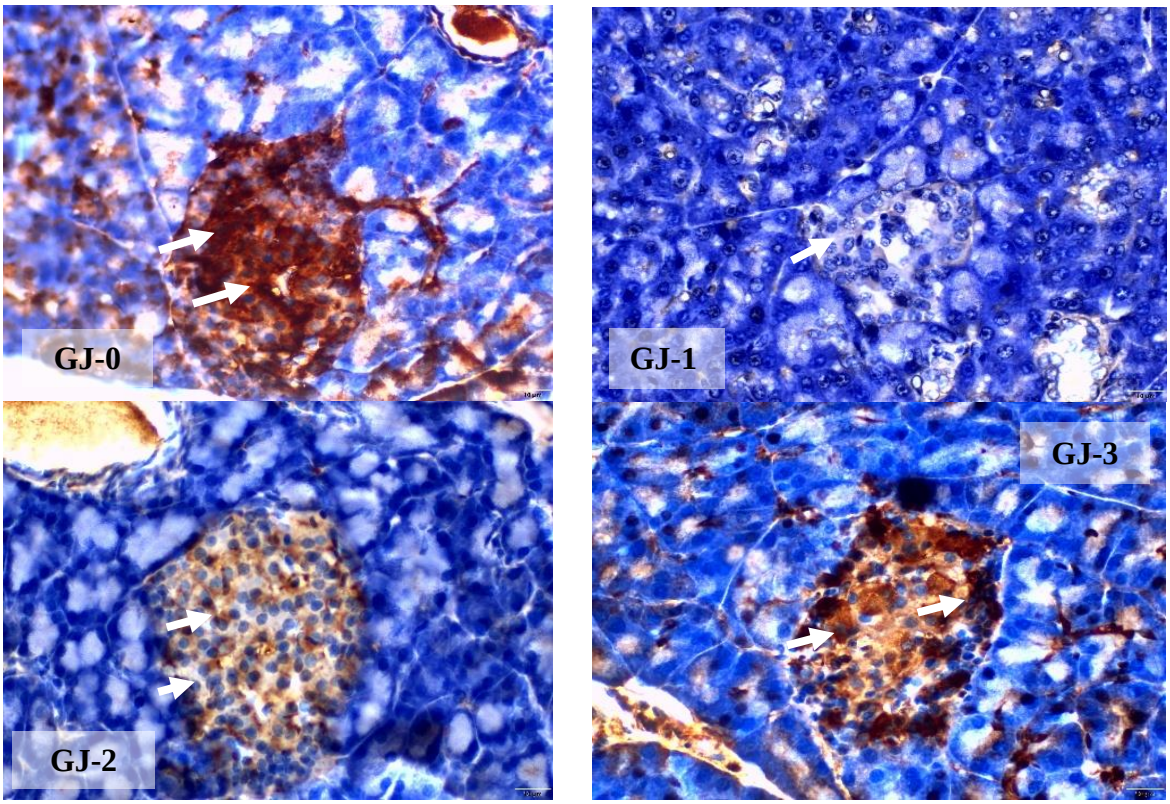


Figure 2. The Superoxide dismutase immunohistochemical staining of the pancreas in diabetic rats of the Sprague-Dawley strain for 21 days of treatment. **GJ-0**: Normal control group, **GJ-1**: Diabetic group, **GJ-2** and **GJ-3**: Diabetic group treated with decoction of Javanese ginseng leaf simplicia (DJGLS; 100g/100 mL) of 0.5 mL and 1 mL per 100 g body weight, respectively. The scale bar is 10 µm, and the magnification is 400x.

DISCUSSION

Medicinal plants are important sources of bioactive compounds and have long been widely utilized in traditional medicine. It is estimated that 70-90% of the global population relies on herbal remedies for healthcare, particularly in developing countries with high levels of biodiversity (Tran et al., 2020). The *Talinum paniculatum*, commonly known in Indonesia as Javanese ginseng, shares similarities with other well-known traditional medicinal plants, such as *Panax ginseng* in East Asia (Zheng et al., 2017; Park et al., 2021). In Indonesian traditional practices, different parts of the plant, including the leaves, roots, and bark, are commonly boiled and consumed as herbal preparations to treat several ailments (Susilo et al., 2024).

In this study, a decoction of Javanese ginseng (*Talinum paniculatum*) leaves was administered to streptozotocin (STZ)-induced diabetic rat models. Following STZ induction, rats in the GJ-1, GJ-2, and GJ-3 groups exhibited mean blood glucose levels exceeding 200 mg/dL, confirming the diabetic condition (Furman, 2021). Hyperglycemia in diabetic rats is generally defined by blood glucose levels ≥200 mg/dL. A study conducted by Qinna and Badwan (2015) reported that a single STZ dose of 80 mg/kg body weight can elevate blood glucose levels to 451-750 mg/dL. In the present study, rats injected with a single STZ dose of 40 mg/kg body weight indicated blood glucose levels ≥ 200 mg/dL by day 4. The mean glucose levels were 502.2 ± 31.59 mg/dL in the positive control group (GJ-1), 488.6 ± 46.90 mg/dL in the GJ-2 group, and 481.0 ± 58.01 mg/dL in the GJ-3 group. Blood glucose levels at the end of the treatment, particularly in the GJ-1 group (diabetic rats given a placebo, distilled water), decreased by 20.4% (from 502.2 to 400 mg/dL). In view of this, the decrease in blood glucose levels in diabetic rats (GJ-1 group) may be attributed to physiological adaptation (compensatory mechanisms).

The possibility of natural recovery cannot be completely excluded, since the untreated diabetic group (GJ-1) also indicated a 20.4% reduction in blood glucose levels during the 21-day study period. However, the reductions observed in the treatment groups, namely GJ-2 (42.1%) and especially GJ-3 (51.4%), were substantially greater than those in the untreated diabetic control group. The finding suggested that DJGLS treatment exerted an additional antihyperglycemic effect beyond the natural recovery process. Furthermore, the tendency for greater glucose reduction with increasing doses also supports the assumption that the decrease in blood glucose levels was associated with the therapeutic effect of DJGLS treatment rather than being solely due to natural recovery.

The reduction in blood glucose levels in diabetic rats may be attributed to physiological adaptation (body compensation) and the presence of compensatory β-cell mass expansion, primarily achieved through increased regeneration and proliferation of β-cells (Ackerman and Gannon, 2007). According to Kataoka et al. (2013), β-cell damage caused by streptozotocin (STZ) or alloxan is not always complete; β-cell mass gradually undergoes regeneration, allowing partial recovery of insulin function to reduce blood glucose levels, although glucose tolerance remains impaired. According to Gojani et al. (2024), β-cell regeneration is the process of restoring or increasing the number of functional pancreatic β-cells that produce insulin. The mentioned process can occur through natural physiological mechanisms involving two main pathways, including β-cell proliferation, in which existing β-cells divide to increase their number, and neogenesis from progenitor cells, in which pancreatic stem or progenitor cells differentiate into new β-cells (Gojani et al. 2024).

The study conducted by Jenie and Indraswari (2021) reported the use of Javanese ginseng root infusion given to alloxan-induced diabetic rats with a dose of 2% w/v solution with a volume of 1.8 mL/200 g body weight for 14 days, which significantly reduced blood glucose levels compared with untreated diabetic rats. Phytochemical analysis of the ethanol extracts of *Talinum paniculatum* leaves and roots identified 16 active compounds in the leaves and 17 in the roots, including phytol (a diterpenoid), stigmasterol (a steroid), and tocopherol (Susilo et al., 2024). The biological activity has been reported in international literature, and found that stigmasterol has been reported to possess antidiabetic properties (Wang et al., 2017), and tocopherol is also well known for its antioxidant effects (Liu et al., 2022). A study by Aini and Susilo (2023) on the bark extract of Javanese ginseng (*Talinum paniculatum*) identified 20 active compounds. The bark extract demonstrated significant anti-inflammatory activity and exhibited antioxidant potential.

Javanese ginseng (*Talinum paniculatum*) shared several characteristics with other well-known traditional medicinal plants, such as *Panax ginseng*, commonly known as Korean red ginseng (Zheng et al., 2017). Korean red ginseng has long been used in traditional medicine to help prevent and manage different diseases, including diabetes, cancer, inflammatory conditions, nervous system disorders, cardiovascular diseases, and hyperlipidemia (Park et al., 2021).

A plant from the same genus, *Talinum triangulare*, has been reported to exhibit hypoglycemic effects (Oluba et al., 2019). In Oluba et al. (2019) study, streptozotocin (STZ)-induced diabetic rats were administered a flavonoid extract of *Talinum triangulare* leaves at a dose of 10 mg/kg body weight for 21 days. Results presented in that study suggest that administration of a flavonoid extract of *Talinum triangulare* leaves for 21 days normalized STZ-induced hyperglycemia

and its associated dyslipidemia by a mechanism involving inhibition of α-amylase and HMG-CoA reductase activities, respectively, in rats.

Different studies have reported that ginseng contains a variety of bioactive compounds, including flavonoids, tannins, triterpenes, saponins, polyphenols, and polysaccharides (Aini and Susilo, 2023; Suriani et al., 2024), as well as important sterol compounds such as campesterol, stigmasterol, and sitosterol (Tolouei et al., 2019). Based on international literature, saponins have been reported to make an important contribution due to their hypoglycemic effects, including enhancing insulin secretion and improving insulin receptor sensitivity (Al-Ishaq et al., 2019). Tannins act as protein-binding compounds that can slow intestinal glucose absorption (Jomova et al., 2025). Steroids have been reported to possess anti-inflammatory and immunomodulatory properties (Yin et al., 2024). In addition, flavonoids are known to inhibit the enzymes α-glucosidase and α-amylase, thereby reducing intestinal glucose absorption and stimulating insulin secretion from pancreatic β-cells (Wu et al., 2025).

Different studies have reported that hyperglycemia in diabetes can trigger the excessive formation of free radicals, leading to cellular damage and increased activity of endogenous antioxidant enzymes as the body attempts to neutralize oxidative stress (Maritim et al., 2003; Giacco and Brownlee, 2010). Javanese ginseng may help reduce oxidative stress through its bioactive compounds, such as saponins, flavonoids, and polyphenols, which are capable of scavenging and neutralizing free radicals (Rizqia et al., 2023; Nyandoro et al., 2025). In addition, *Talinum paniculatum* has been reported to possess antioxidant properties (Souto et al., 2021) and free radical-scavenging activity (Liao et al., 2015). A study conducted by Attele et al. (1999) reported that the use of *Panax ginseng* can improve antioxidant status and reduce biomarkers of oxidative stress in diabetic rat models.

Hyperglycemia in diabetes is known to induce oxidative stress through the excessive production of ROS (González et al., 2023). Hyperglycemia in diabetes also increased levels of pro-inflammatory cytokines and chemokines, enhanced infiltration of immune cells, including macrophages, inflammatory cells, and granulocytes, and the development of local inflammation (Böni-Schnetzler and Meier, 2019). Persistent hyperglycemia exerts toxic effects on pancreatic β-cells, leading to apoptosis or necrosis (Kim et al., 2005) and ultimately resulting in a reduction in β-cell mass and impaired β-cell function (Costes et al., 2021). A study by Yin et al. (2024) reported that ginseng extract promotes pancreatic β-cell regeneration and protects β-cells from inflammatory damage, thereby helping to maintain β-cell mass and function and supporting the repair of pancreatic islet injury. In addition, Jenie and Indraswari (2021) demonstrated the hypoglycemic effect of Javanese ginseng in diabetic rats, which may reduce hyperglycemia as a major source of free radical generation. Furthermore, Riyana et al. (2022) reported that Javanese ginseng root extract increased SOD activity and reduced malondialdehyde levels in rats subjected to oxidative stress.

CONCLUSION

The results of this study indicated that administration of a decoction of Javanese ginseng leaf simplicia (DJGLS) for 21 days in diabetic rats reduced blood glucose and serum SOD levels, although these values had not fully returned to normal. Furthermore, the DJGLS indicated potential protective effects against damage to pancreatic β-cells in the islets of Langerhans and increased the intensity of SOD expression in the islets of Langerhans, although the increase had not yet reached the level observed in the normal control group. The present study utilized a decoction of Javanese ginseng leaf simplicia. It is recommended that future studies perform extraction using different solvents, followed by analysis of the active compound constituents present. In addition, pancreatic beta-cell damage should be quantified to obtain more accurate results in drawing conclusions regarding the protective effects against beta-cell damage, as well as measuring color intensity using quantitative image analysis or a scoring system. Furthermore, the mechanism of action of the active compounds in the decoction of Javanese ginseng leaf simplicia requires further investigation. Improvements in serum SOD levels, pancreatic SOD levels, and pancreatic histopathology following DJGLS administration suggest the presence of an oxidative protective mechanism that may help prevent cellular damage. However, the specific mechanisms by which the bioactive components of DJGLS influence serum SOD levels, pancreatic SOD levels, and SOD expression in pancreatic tissue remain unclear and require further investigation

DECLARATIONS

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Authors' contributions

I Nyoman Suarsana, contributed to the design of the experiment, supervision, treatment, data analysis, and drafting of the manuscript. I Made Kardena, conducted the data collection, data analysis, and drafting of the article. Ni Luh Suriani and Dewa Ayu Swastini contributed to the creation and analysis of extracts, the drafting of the manuscript, and the drafting of the article. I Made Artika, supervised and validated the manuscript and article. All authors have read and approved the final edition of the manuscript.

Availability of data and materials

All data underlying the findings of the present study are presented within the article, and no supplementary datasets are provided.

Competing interests

The authors have not declared any conflict of interest.

Ethical considerations

The authors confirm that the present manuscript is original, free from plagiarism, and has not been published elsewhere. The authors also stated that no AI tools were used during the writing of this manuscript. All authors have reviewed the original content of the manuscript.

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Biochemical and Morphological Characterization of Bacteria Isolated from Freshwater in Sudan

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ABSTRACT

The microbial quality of freshwater fish is a crucial indicator of both aquatic environmental health and food safety. The present study aimed to isolate and characterize bacteria from three common freshwater fish species, including *Oreochromis niloticus*, *Clarias gariepinus*, and *Synodontis alberti*, collected from three sites in Khartoum, Sudan. Six fish of each species were collected from each sampling location where the species were present. Total viable bacterial counts in fish gill and intestinal tissues obtained from Green Belt Sewage, Jebel Aulia fish landing, and Al Mourda fish market ranged from 1.2×10^4 to 3.9×10^5 CFU/g, with higher bacterial loads generally observed in intestinal tissues compared to gills. The highest bacterial load (3.9×10^5 CFU/g) was recorded in the intestine of *Clarias* species collected from Green Belt Sewage. The current results demonstrated a predominance of Gram-negative, rod-shaped bacteria in gill and intestinal tissue samples across all sampling sites. All isolates were catalase-positive and capable of fermenting glucose, indicating facultative anaerobic metabolism. Oxidase activity of bacterial isolates differed by location; fish samples from Al Murda Fish Market had the highest number of oxidase-positive isolates, suggesting the potential presence of *Aeromonas* and *Pseudomonas* species. Urease activity was predominantly observed in isolates of fish samples collected from Al Murda and Jebel Aulia, suggesting a greater risk of fish spoilage or pathogenicity. Notably, isolates from the Green Belt Sewage fish samples comprised Gram-positive coccus, potentially identified as *Staphylococcus* spp., underscoring the likelihood of anthropogenic contamination. The present results indicated that fish obtained from all sampling locations generally exhibited similar microbial communities. However, the differences in enzyme activity across fish from all sites likely reflected variations in environmental factors, sanitation practices, and potential public health risks.

Keywords: Bacterial isolate, Biochemical characterization, Enzyme activity, Fish, Microbial count

INTRODUCTION

Freshwater and marine fish are vital in the human diet, especially in developing countries, where they provide an accessible, affordable source of high-quality animal protein, essential amino acids, vitamins, and micronutrients. Additionally, freshwater and marine fish support global markets and contribute to the nation's economy (Thilsted et al., 2016). In Sudan, species such as *Oreochromis niloticus* (Nile tilapia), *Clarias gariepinus* (African catfish), and *Synodontis albertis* (squeaker catfish) are widely consumed and constitute a major part of the national inland fisheries, especially in the Nile River and its branches. However, microbial safety and fish quality can be compromised by exposure to contaminated water, unhygienic post-harvest handling, and poor storage conditions (Chichester and Graham, 2013; Elhadi, 2014; Deivansigamani, 2026).

Fish harbor diverse microbiota on their skin, gills, and gastrointestinal tracts, including both commensal and opportunistic bacteria such as *Micrococcus* spp., *Bacillus* spp., *Aeromonas* spp., *Pseudomonas* spp., *Vibrio* spp., and *Enterobacteriaceae* members (Chichester and Graham, 2013). While many of these microorganisms are harmless or beneficial, others, such as *Escherichia coli* (*E. coli*), *Salmonella* spp., *Vibrio cholerae*, and *A. hydrophila* (*A. hydrophila*), can serve as indicators of environmental pollution or as potential pathogens to humans and fish (Austin and Austin, 2012). The composition of bacteria associated with fish can vary depending on the fish species, water quality, and geographic location (Zhang et al., 2022). Commonly reported genera include *Aeromonas*, *Pseudomonas*, *Enterobacter*, *Klebsiella*, and *Staphylococcus*, some of which are linked to spoilage, fish diseases, or infections (Janda and Abbott, 2010). Previous studies have highlighted the microbial risks associated with fish harvested or sold in local markets in Africa and the Middle East (Al-Harbi and Uddin, 2005; Oniong et al., 2018). For instance, Omeji et al. (2022) reported the presence of *A. hydrophila* and *Pseudomonas* spp. in Nile tilapia and catfish from Egyptian and Saudi Arabian waters. InSudan, although limited studies have been conducted, preliminary assessments have suggested microbial contamination in fish sold in Khartoum markets (Elhadi, 2014). Biochemical characterization of bacterial isolates is a crucial approach for identifying and understanding the metabolic functions of fish-associated microbes

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(Newaj-Fyzul et al., 2008). Evaluation methods such as Gram staining, catalase, oxidase, glucose fermentation, urease activity, and nitrate reduction offer important insights into the taxonomy and possible pathogenicity of bacterial isolates (Newaj-Fyzul et al., 2008). Furthermore, the biochemical profiles assist in distinguishing between environmental and clinically relevant strains. The current study aimed to isolate and biochemically characterize bacteria isolated from the gills and intestines of three common freshwater fish species, including *Oreochromis niloticus* (Nile tilapia), *Clarias gariepinus* (African catfish), and *Synodontis albertis*, collected from three different locations in South and West Khartoum, Sudan.

MATERIALS AND METHODS

Ethics approval

The present study did not involve any live experimental manipulation of fish. All samples were purchased from local fish markets after harvest for commercial purposes, and all procedures adhered to national and institutional guidelines for ethical handling and sample processing.

Study location and design

A cross-sectional study was conducted using fish samples collected from three sites in South and West Khartoum, Sudan, including Green Belt Sewage, Jebel Aulia Fish Landing, and Al Murda Fish Market. These sites were selected due to their importance in local fish marketing and human consumption. A total of 36 fish samples representing three species (*Clarias gariepinus*, *Oreochromis niloticus*, and *Synodontis alberti)* were collected from three different locations. A total of six fish per species were collected at each sampling location where that species was present. However, *Oreochromis niloticus* was not available in the Green Belt Pond at the time of sampling; therefore, this species was collected only from the remaining two locations. Consequently, the total number of fish samples collected for the study was 36. Each fish sample was considered an independent biological replicate. Tissue samples from the gills and intestine were analyzed from each specimen to assess microbial contamination associated with external exposure and colonization of the digestive tract. The experimental design consisted of two primary factors, including the sampling location and fish species. The present study involved a comparative analysis of microbial load across different species and collection sites.

Sample collection

Immediately after purchase, fish samples were aseptically placed into sterile polyethylene bags and transported in insulated containers filled with ice to maintain a temperature of approximately 4°C, thereby reducing microbial growth and ensuring sample integrity. Standard cold chain procedures were maintained during transportation. All samples were transported to the Central Veterinary Laboratories and Research Center in Soba, Khartoum, and processed within four hours of collection to ensure microbial stability. In the laboratory, fish samples were aseptically dissected with sterile tools. Gill tissues and intestinal contents were removed separately and homogenized in sterile phosphate-buffered saline (PBS, pH 7.2) at a ratio of 1:10 (w/v). Serial tenfold dilutions were then prepared from each homogenate using sterile diluent by transferring one mL of homogenate into nine mL of sterile normal saline, yielding successive decimal dilutions (10^{-1} to 10^{-6}) for subsequent microbiological analysis.

Microbiological procedures

Total viable bacterial counts were determined using the drop plate technique following standard microbiological methods (Newaj-Fyzul et al., 2008). Nutrient agar plates were dried in an incubator and inverted for two hours prior to use. Each plate was divided into eight equal segments using a glass marking pencil and labeled according to the corresponding dilution. One gram of the sample was homogenized in ten mL of sterile normal saline to create the initial inoculum. From this solution, one mL was transferred into nine mL of sterile normal saline, and the process was repeated to produce a series of decimal dilutions up to 10^{-9}. The tubes were thoroughly mixed to ensure proper homogenization. From each dilution, 0.02 mL aliquots were inoculated onto the labeled segments of nutrient agar plates using a sterile micropipette. The inoculated plates were allowed to absorb the inoculum and then incubated at 37°C for 24 hours under aerobic conditions. Following incubation, colonies appearing in each segment were counted. The average colony count from three plates for each dilution was calculated, and the results were expressed as colony-forming units per gram (CFU/g) of sample.

Biochemical characterization

Pure bacterial isolates were chosen for biochemical characterization according to distinct colony morphology observed after primary cultivation. From each sample, 3-6 representative colonies exhibiting differences in size, shape,

color, and texture were carefully selected and purified by subculturing to obtain single, well-isolated colonies. The purified isolates were then subjected to a series of standard biochemical tests for preliminary identification, following the protocols described by Newaj-Fyzul et al. (2008). Catalase activity was determined by adding 3% hydrogen peroxide to bacterial smears, where bubble formation indicated a positive reaction. Oxidase activity was assessed using oxidase strips; a purple color indicated a positive result. Glucose fermentation was evaluated using phenol red glucose broth to detect acid and/or gas production. Oxidative and fermentative metabolic activities were distinguished utilizing Hugh and Leifson's oxidation-fermentation (O/F) test. Nitrate reduction was assessed by adding sulfanilic acid and α-naphthylamine to nitrate broth cultures; a color change indicated a positive result. Urease activity was determined using Christensen's urea agar, where a color change indicated ammonia production. Motility was examined by stab inoculation into motility test medium and observing the growth pattern. Arginine dihydrolase activity was tested using Moeller's decarboxylase medium supplemented with arginine. Indole production was determined by incubating isolates in tryptone broth, then adding Kovac's reagent; a red layer indicated a positive reaction. The Voges-Proskauer (VP) test was performed using Barritt's reagents to detect acetoin production. Quality control for biochemical tests was ensured by using established positive and negative control bacterial strains. Control organisms with established biochemical reactions were tested alongside the isolates to confirm the accuracy of the test procedures and reagents. All biochemical tests, including catalase, oxidase, indole production, citrate utilization, urease activity, nitrate reduction, motility, and O/F tests, were performed according to established microbiological procedures, and test results were interpreted only when control reactions exhibited expected results (Newaj-Fyzul et al., 2008).

Data analysis

All results were analyzed in SPSS version 20 using descriptive statistics to determine the prevalence and percentage of pathogens.

RESULTS

The highest viable microbial count was recorded in the intestine of *Clarias gariepinus* from the Green Belt Sewage site (3.9×10^5 CFU/g; Tables 4, Figure 2), suggesting significant microbial contamination likely associated with sewage influence. In all sampling locations and across all fish species, intestinal samples consistently demonstrated higher bacterial loads than gill samples. *Oreochromis niloticus* and *Synodontis alberti* generally exhibited lower viable microbial counts than *Clarias gariepinus* across the study locations (Table 4, Figure 5). At Al Murda Fish Market, the predominant bacteria isolated from *Oreochromis niloticus* were *Pseudomonas* spp., while *Proteus* spp. were commonly found in *Clarias gariepinus* and *Synodontis alberti* (Table 4, Figure 2). The isolates obtained from fish collected in the Green Belt Sewage site were *Aerococcus* spp., *Staphylococcus* spp., and *Bacillus mycoides* (Tables 1,4). In samples from the Jebel Aulia fish landing, *E. coli* was the dominant species found in *Oreochromis* spp. and *Clarias* spp., whereas *Synodontis* spp. was associated with *Staphylococcus* spp. Fish samples collected from Green Belt Sewage exhibited the most diverse and abundant bacterial populations, indicating contamination from untreated domestic or industrial waste (Tables 1 and 4, Figures 2 and 4). Fish samples collected from Al Murda fish market demonstrated post-harvest bacterial contamination, whereas samples collected from Jebel Aulia fish landing indicated the presence of *E. coli*, suggesting fecal contamination (Tables 2, 3, and 4, Figures 2 and 4).

Table 1. Biochemical characteristics of bacteria isolated from fish in Green Belt Sewage, South Khartoum, Sudan

Sample	Shape and gram reaction	Catalase	Oxidase	Glucose	Oxidation fermentation	Nitrate reduction	Arginine	Urea	Motility	Indole	V.P
Clarias gariepinus (intestine)	G +ve Cocci	+	-	+	F	+	-	-	-	-	-
Clarias gariepinus (gill)	G –ve Rod	+	-	+	F	+	-	-	+	-	-
Syuodontis alberti (intestine)	G –ve Rod	+	-	+	F	+	-	-	+	-	-
Syuodontis alberti (gill)	G –ve Rod	+	-	+	F	+	-	-	+	-	-

G –ve: Gram-negative, + / –: Positive/Negative result, F: Fermentative metabolism, V.P: Voges-Proskauer test

Table 2. Biochemical characteristics of bacteria isolated from fish species in Al Mourda Fish Market, West of Khartoum, Sudan

Test Sample	Shape and gram reaction	Catalase	Oxidase	Glucose	Oxidation fermentation	Nitrate reduction	Arginine	Urea	Motility	Indole	V.P
Orechromis niloticus (intestine)	G –ve Rod	+	+	+	F	+	+	-	+	-	-
Orechromis niloticus (gill)	G –ve Rod	+	+	+	F	+	+	-	+	-	-
Orechromis niloticus (intestine)	G –ve Rod	+	+	+	F	+	+	-	+	-	-
Orechromis niloticus (gill)	G –ve Rod	+	+	+	F	+	+	-	+	-	-
Clarias gariepinus (intestine)	G –ve Rod	+	+	+	F	+	+	-	+	-	-
Clarias gariepinus (Gill)	G –ve Rod	+	+	+	F	+	+	-	+	-	-
Syuodontis alberti (intestine)	G –ve Rod	+	-	+	F	+	-	+	+	-	-
Syuodontis alberti (gill)	G –ve Rod	+	-	+	F	+	-	+	+	-	-

G –ve: Gram-negative, + / –: Positive/Negative result, F: Fermentative metabolism, V.P: Voges-Proskauer test

Table 3. Biochemical characteristics of bacteria isolated from different fish species in Jebel Aulia fish Landing, South of Khartoum, Sudan

Test sample	Shape and gram reaction	Catalase	Oxidase	Glucose	Oxidation fermentation	Nitrate reduction	Arginine	Urea	Motility	Indole	V.P
Orechromis niloticus (intestine)	G –ve Rod	+	-	+	F	+	-	+	+	-	-
Orechromis niloticus (gill)	G –ve Rod	+	-	+	F	+	-	+	+	-	-
Clarias gariepinus (intestine)	G –ve Rod	+	-	+	F	+	-	+	+	-	-
Clarias gariepinus (Gill)	G –ve Rod	+	-	+	F	+	-	+	+	-	-
Syuodontis alberti (intestine)	G –ve Rod	+	-	+	F	-	-	+	+	-	-
Syuodontis alberti (gill)	G –ve Rod	+	-	+	F	+	-	+	+	-	-

G –ve: Gram-negative, + / –: Positive/Negative result, F: Fermentative metabolism, V.P: Voges-Proskauer test

Table 4. Viable microbial counts and bacterial isolates from different organs of the studied fish species and locations in Khartoum, Sudan

Location	Fish species	Organ	Viable count (CFU/g)	Isolated bacteria
Al Mourda fish market	*Oreochromis niloticus*	Intestine	3.35×10^4	*Pseudomonas*
	Oreochromis niloticus	Gill	9.65×10^3	*Pseudomonas*
	Syuodontis alberti	Intestine	3.2×10^4	*Proteus*
	Syuodontis alberti	Gill	1.5×10^4	*Proteus*
	Clarias gariepinus	Intestine	1.5×10^4	*Proteus*
	Clarias gariepinus	Gill	1.3×10^4	*Proteus*
Green Belt Sewage	*Syuodontis alberti*	Intestine	3.7×10^4	*Bacillus mycoides*
	Syuodontis alberti	Gill	1.8×10^4	*Bacillus mycoides*
	Clarias gariepinus	Intestine	3.9×10^5	*Aerococcus*
	Clarias gariepinus	Gill	1.5×10^4	*Staphylococcus*
Jebel Aulia fish landing	*Oreochromis niloticus*	Intestine	1.9×10^4	*Escherichia coli*
	Oreochromis niloticus	Gill	1.3×10^4	*Escherichia coli*
	Syuodontis alberti	Intestine	3.2×10^4	*Staphylococcus*
	Syuodontis alberti	Gill	1.4×10^4	*Staphylococcus*
	Clarias gariepinus	Intestine	3.9×10^4	*Escherichia coli*
	Clarias gariepinus	Gill	1.2×10^4	*Escherichia coli*

Viable microbial counts are expressed as colony-forming units per milliliter (CFU/g).

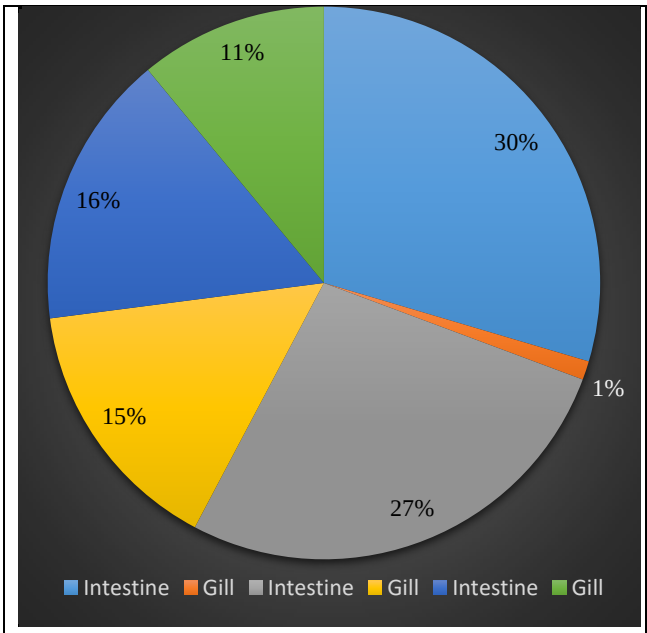


Figure 1. Viable bacterial counts in the gills and intestines of *Oreochromis niloticus* collected from West and South Khartoum, Sudan. Results were expressed as percentage colony-forming units per gram (CFU/g) of the original sample.

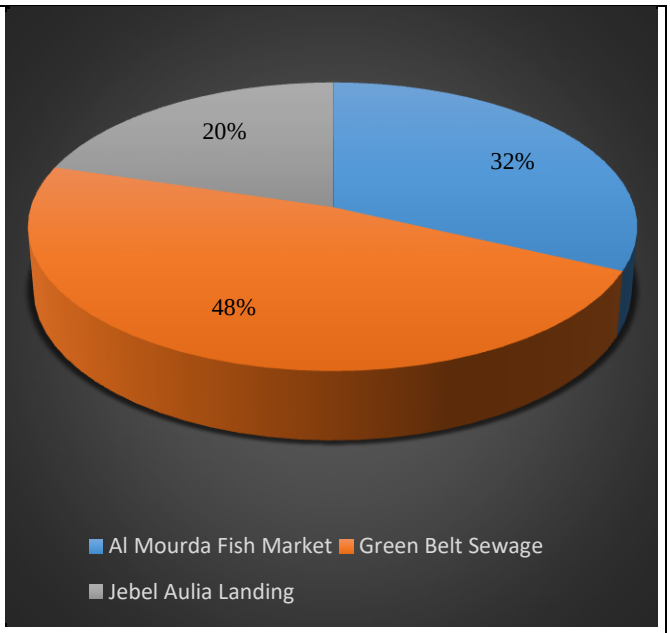


Figure 2. The viable bacterial counts in West and South Khartoum, Sudan. Results were expressed as percentage colony-forming units per gram (CFU/g) of the original sample.

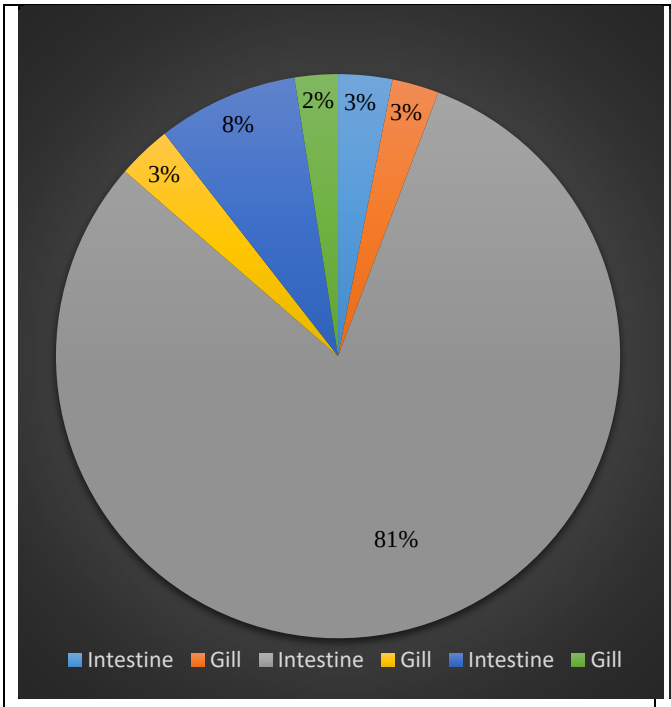


Figure 3. Viable bacterial counts in the gills and intestines of *Clarias* spp. collected from West and South Khartoum, Sudan. Results were expressed as percentage colony-forming units per gram (CFU/g) of the original sample.

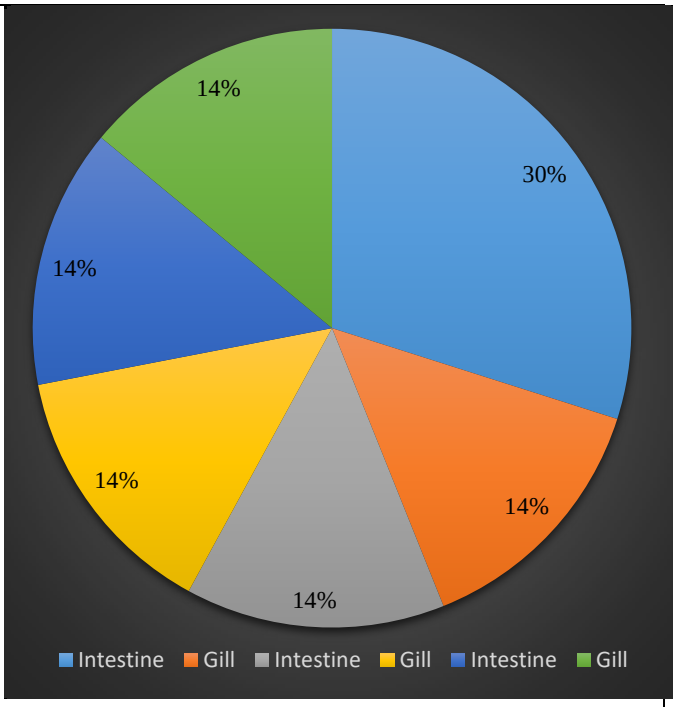


Figure 4. Viable bacterial counts in the gills and intestines of *Synodontis* spp. collected from West and South Khartoum, Sudan. Results were expressed as percentage colony-forming units per gram (CFU/g) of the original sample.

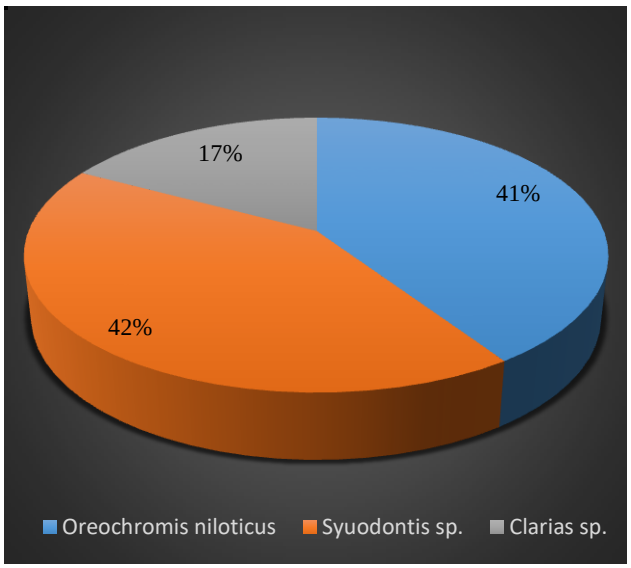


Figure 5. The viable bacterial counts of the gill and intestine fish species collected from West and South Khartoum, Sudan. Results were expressed as percentage colony-forming units per gram (CFU/g) of the original sample.

DISCUSSION

The bacterial loads observed in the present study were within the range commonly reported for freshwater fish in both natural and polluted environments. Previous studies indicated that bacterial counts in fish intestines typically ranged from 10^3 to 10^7 CFU/g, reflecting the naturally dense microbial community in the intestinal tract (Olugbojo and Ayoola, 2015; Austin and Austin, 2016). The guidelines of the International Commission on Microbiological Specifications for Foods stated that microbial counts below 10^6 CFU/g in fresh fish are generally considered acceptable, depending on fish species and conditions (Oliva-Teles, 2012). Similarly, reports by the Food and Agriculture Organization (FAO, 2018) indicated that bacterial loads on the skin and gills of freshly caught fish commonly ranged from 10^2 to 10^5 CFU/g, whereas intestinal microbial counts were higher due to the normal microbiota.

Austin and Austin (2016) and Omeji et al. (2022) reported comparable bacterial loads in freshwater fish from polluted waters, with total viable microbial counts ranging from 10^4 to 10^6 CFU/g, particularly in bottom-feeding species such as *Clarias gariepinus*. In contrast, *Oreochromis niloticus* and *Synodontis alberti* exhibited comparatively lower bacterial loads, potentially attributable to differences in feeding habits, habitat preferences, and environmental exposure. The present findings were consistent with those of Austin and Austin (2012) and Mohanta and Goel (2014), who reported *Enterobacteriaceae*, *Aeromonadaceae*, and *Pseudomonadaceae* as the predominant bacterial families associated with freshwater fish across all sampling sites and fish species. The predominant morphology of bacteria associated with fish was Gram-negative rods. The present results were consistent with those of Omeji et al. (2022), who reported a high prevalence of Gram-negative bacteria in Nile tilapia and catfish within aquatic environments in sub-Saharan Africa. The predominance of these bacteria indicated persistent exposure to water contaminated with fecal material or organic waste, thereby promoting the proliferation of facultative anaerobic bacteria, including *Aeromonas* spp., *Pseudomonas* spp., *Enterobacter* spp., and *Klebsiella* spp. The intestine of *Clarias gariepinus* collected from the Green Belt sewage was the only sample that yielded Gram-positive cocci, possibly *Staphylococcus* spp., suggesting contamination from external sources, such as human handling or contaminated sediments (Olugbojo et al., 2015). All isolates were catalase positive, reflecting their aerobic or facultative anaerobic characteristics. However, the oxidase test was used to differentiate the isolates. Fish samples collected from the Al Murda fish market exhibited multiple oxidase-positive isolates, particularly from *Oreochromis niloticus* and *Clarias gariepinus*, indicating the possible presence of *Aeromonas hydrophila* or *Pseudomonas* spp. These microorganisms are known to colonize the gill and intestinal mucosa of fish and act as opportunistic pathogens (Janda and Abbott, 2010). Fish samples from Jebel Aulia fish landing and Green Belt Sewage demonstrated primarily oxidase-negative isolates, suggesting dominance of *Enterobacteriaceae* spp. such as *Enterobacter*, *Proteus*, or *Citrobacter* spp. These patterns reflected differences in microbial ecology across different locations, potentially attributable to water quality, market sanitation, and post-harvest handling, as reported by Janda and Abbott (2010). Glucose fermentation by the isolates was detected in the O/F test, confirming that the bacteria were facultative anaerobic bacteria typically found in aquatic environments and in fish intestines. These bacteria proliferate in environments rich in organic matter, indicating that the nutrient-enriched waters are likely contaminated by agricultural activities (Olugbojo et al., 2015).

Urease-positive bacteria were prevalent, especially in fish samples obtained from the Al Murda Fish Market and Jebel Aulia fish landing. Urease activity is often correlated with pathogenicity, facilitating bacterial survival in acidic environments such as the fish gut or infected tissues (Al-Harbi and Uddin, 2005). Bacteria such as *Proteus mirabilis*, *Klebsiella pneumoniae*, and *Aeromonas* spp. are known for their urease production and have been associated with fish spoilage and disease (Ludwig et al., 2015). Nitrate reduction was observed in the majority of isolates, thereby confirming their capacity for anaerobic respiration, a characteristic frequently associated with enteric and environmental bacteria, as documented by Mahmoud et al. (2022). Nitrate-negative isolates were observed in intestinal samples from *Synodontis alberti* collected at Jebel Aulia, potentially indicating the presence of bacterial strains with reduced metabolic diversity or experiencing environmental stress, consistent with findings reported by Ali (2022). Most isolates were negative for arginine utilization, except those of *Synodontis alberti* obtained from Al Murda fish market, indicating bacterial diversity within this species. Additionally, all isolates were negative for indole and VP tests, indicating that Enterobacter cloacae and other typical enteric bacteria were not predominant, or that the isolates were non-pathogenic environmental strains (Veljović et al., 2015). All isolates were motile, consistent with facultative or free-living aquatic bacteria, capable of colonizing different fish tissues. Motility, a pathogenicity trait of *Aeromonas hydrophila*, enhances its ability to invade host tissues and colonize mucosal surfaces (Semwal et al., 2023).

The fish microbiota across all sites generally had a similar structure, primarily consisting of Gram-negative, catalase-positive, fermentative bacteria. Fish samples collected from Al Murda fish market exhibited the most biochemically active bacteria (oxidase-positive, urease-positive), indicating that market conditions might promote the growth of opportunistic fish pathogens. Jebel Aulia Landing indicated more uniform oxidase-negative profiles, suggesting possible dominance of *Enterobacteriaceae* and lower environmental diversity. The detection of urease and nitrate-reducing bacteria in all sites posed potential public health risks through zoonotic transmission, particularly due to poor hygiene, inadequate fish storage, or consumption of undercooked fish, as reported by Omeji et al. (2022).

Despite geographic variation among sampling locations, bacterial isolates from all sites exhibited similar phenotypic characteristics, including Gram-negative rod morphology, catalase positivity, and fermentative metabolism. This similarity might be attributed to comparable environmental conditions across the aquatic systems in Khartoum, Sudan, particularly water temperature, organic matter content, and nutrient availability, which are known to influence microbial community structure. Aquatic environments receiving organic inputs and sewage contamination often support the proliferation of Gram-negative bacteria such as *Aeromonas*, *Pseudomonas*, *Enterobacter*, and *E. coli*, which are commonly associated with fish and aquatic habitats. Therefore, the similarity in bacterial characteristics across locations

indicated that environmental factors, rather than geographical distance alone, played a major role in shaping microbial populations in these aquatic ecosystems. Similar findings were reported by Zhang et al. (2022), who observed that environmental conditions and water quality parameters notably influenced the microbial communities associated with fish and aquatic environments. Fish samples collected from the Green Belt sewage exhibited the highest biochemical diversity, specifically Gram-positive cocci. Bacterial isolates obtained from fish samples at Al Murda Fish Market exhibited the highest biochemical activity, as evidenced by positive oxidase and urease tests. This indicated a greater potential for pathogenicity or spoilage, potentially due to inadequate hygiene practices or suboptimal fish-handling conditions. Jebel Aulia fish landing isolates were more uniform and oxidase-negative, suggesting the dominance of enteric-type bacteria (Austin and Austin, 2016).

CONCLUSION

According to the present findings, the microbial communities associated with fish were broadly similar across all sites; however, site-specific differences, particularly in enzyme activity, indicated distinct environmental conditions, hygiene practices, and associated public health risks. Continuous monitoring of bacterial communities in commercially well-known fish species is essential to ensure food safety and aquatic health. Nevertheless, the present study is limited by its reliance on culture-dependent techniques and a relatively restricted sample size, which may underestimate the full diversity of microbial communities and overlook non-culturable taxa.

DECLARATIONS

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Authors' contributions

Data analysis was conducted by Hassan Mohammed Adam Sulieman and Hnadi Gism Alla Bilal Bakhet, who assisted with the study design and scheduling the experiments. Additionally, samples were collected from different regions by Hnadi Gim, Alla Bilal, and Bakhet following the review of the data analysis. All authors have reviewed and approved the final edition of the manuscript prior to its publication in this journal.

Availability of the data and materials

The paper contains the original data used in this investigation, which may be obtained from the corresponding author upon reasonable request.

Competing interests

The authors declare that there are no conflicts of interest.

Ethical considerations

The authors stated that this is an original study, authored collectively by all contributors, and submitted to the journal for the first time. The authors confirmed that no AI tools were utilized in the preparation and writing the present study.

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Monitoring of Calcium and Phosphorus in Ruminant Feed by Smartphone-Assisted Colorimetry

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ABSTRACT

Routine laboratory analysis for ruminant feed minerals is often unavailable in a field setting; therefore, rapid mineral monitoring is necessary. The present study aimed to evaluate a smartphone-assisted colorimetry method for estimating calcium and phosphorus in forage extracts and compare its performance with UV-Visible spectrophotometry. Standard solutions ranging from 1 to 5 ppm were prepared for smartphone-assisted colorimetry and UV-Visible spectrophotometry, and reagent-treated extracts from four forage species, including *Pennisetum purpureum* cv. Mott, *Pennisetum purpupoides*, *Leucaena leucocephala*, and *Sesbania grandiflora* were measured by a Genesys 10S spectrophotometer and by smartphone imaging under controlled lighting. Linear regression was used to develop calibration models, and method agreement was assessed using Pearson's r and mean absolute percentage error. Both methods indicated strong linearity for calcium and phosphorus. For the smartphone method, the coefficient of determination ranged from 0.9448 to 0.9974, compared to 0.9514 to 0.9610 for the UV-Vis method, with absolute differences between the two methods remaining below 0.5%. Smartphone-assisted colorimetry and UV-Visible spectrophotometry produced closely comparable estimates for each tested forage sample for the tested forages. Smartphone-assisted colorimetry provided accurate estimates of calcium and phosphorus in forage extracts, comparable to those produced by spectrophotometry, and demonstrated potential as a low-cost tool for routine screening of ruminant feed minerals**.**

Keywords: Calcium, Colorimetry, Forage, Nutrition, Phosphorus, Ruminant feed, Smartphone

INTRODUCTION

Ruminant production systems, which depend on nutritionally balanced diets, contribute substantially to food security and rural livelihoods. Among dietary components, minerals are essential for skeletal development, enzyme-mediated reactions, osmotic balance, and reproductive performance. The mineral composition of forages and other feed ingredients can differ with botanical source, soil conditions, fertilizer input, processing, and season, which complicates accurate ration formulation (Hawu et al., 2022; Fanelli et al., 2023; Pérez-Reverón et al., 2024). Without appropriate monitoring of mineral supply, deficiencies or excesses can occur, potentially reducing animal performance and increasing nutrient losses (Wasson et al., 2022; Šaljić et al., 2024).

While conventional analytical techniques such as atomic absorption spectrometry and inductively coupled plasma methods remain reliable, their high cost, dependence on laboratory facilities, and limited accessibility make these techniques impractical for routine farm-level screening (Jamarun et al., 2020). Additionally, wet-chemical methods are beneficial, although they require careful handling and can be time-consuming when processing a high volume of samples. To address these limitations, digital image analysis has been explored as an alternative analytical approach in agricultural samples. Smartphone-assisted colorimetry relies on a mineral-specific reaction to generate a visible color response that can be quantified from red-green-blue image analysis under controlled illumination. Within an appropriate linear range, this optical response can be related to analyte concentration, following a principle comparable to the Beer-Lambert law used in spectrophotometric analysis (Yanti et al., 2021; Ünlü et al., 2022). Smartphone-based assays are well-suited for field use because a portable device can handle image capture, data storage, and numerical processing at relatively low cost. Recent studies indicated that calibrated digital imaging can support quantitative evaluation of agricultural and feed samples when acquisition conditions are carefully managed (Ünlü et al., 2022; Batista and Campos, 2024). The portable nature of these assays may shorten the time required for mineral screening in ruminant feeding systems and facilitate faster supplementation decisions in regions with limited access to centralized laboratories (Sapkota et al., 2019; Poulsen et al., 2021; Ponnampalam et al., 2024).

Many available applications focus on single analytes, simplified matrices, or tightly controlled laboratory

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conditions, whereas practical feed samples are chemically diverse and can react differently during extraction and color development (Khan et al., 2020; Lucio et al., 2020; Uushona et al., 2021). Differences in device response, calibration strategy, and ambient light can influence comparability among studies. Therefore, direct validation against a reference method remains essential before smartphone imaging can be recommended for routine mineral screening. The present study aimed to compare smartphone-assisted colorimetry with UV-Visible spectrophotometry for determining calcium and phosphorus in forage extracts and to evaluate the reliability of smartphone image-based regression models for routine feed screening.

MATERIALS AND METHODS

Ethical approval

The present study was conducted in accordance with the guidelines of Mataram and Mandalika University of Education, Mataram, Indonesia.

Study design

The present study was s a comparative analytical validation study. The target analytes were calcium and phosphorus in forage extracts, and the performance of smartphone-assisted colorimetry was compared directly with UV-Visible spectrophotometry as the reference method under the same experimental conditions (Yanti et al., 2021; Batista and Campos, 2024). The evaluation of calcium and phosphorus in forage extracts focused on calibration linearity, correlation, and agreement between methods for standard solutions and forage samples.

Materials

The laboratory equipment included a UV-Visible spectrophotometer (Genesys 10S, Thermo Scientific, USA), a smartphone equipped with a digital camera, beakers, graduated cylinders, funnels, filter papers, sieves, micropipettes, volumetric flasks, and an analytical balance. During image acquisition, the smartphone was fixed on a stand, and measurements were conducted under controlled illumination to minimize variation among images (Uushona et al., 2021; Yanti et al., 2021).

Reagents and standards

Analytical-grade reagents included distilled water, nitric acid, ammonium molybdate, tin (II) chloride, Eriochrome black T, and ammonium chloride–ammonium hydroxide buffer (pH 10.0). Stock standards of calcium and phosphorus (each at 100 ppm) were prepared and diluted to 1-5 ppm to establish calibration curves within the tested calibration range (Pérez-Reverón et al., 2024; Lambert, 2025).

Sample preparation

The experiment was conducted at the Animal Science Laboratory of the University of Mataram and the Chemistry Laboratory of Mandalika University of Education, Indonesia. Four forage materials used in the comparison were *Pennisetum purpureum cv. Mott, Pennisetum purpupoides*, *Leucaena leucocephala, and Sesbania grandiflora* that were obtained from a local forage shop. The samples were homogenized, finely ground, and sieved before analysis. Representative subsamples were weighed, digested with 5% nitric acid to release soluble minerals, and filtered through Whatman filter paper to obtain purified extracts for colorimetric measurement.

Solution preparation

A 5% nitric acid solution was prepared by dilution with distilled water. Calcium and phosphorus stock solutions (100 ppm) were serially diluted to 1-5 ppm, and fresh color-developing reagents were prepared on the day of analysis. Calibration curves were constructed using standard solutions, whereas method performance under sample conditions was assessed using the forage extracts.

Measurement procedures

Absorbance of the reacted solutions was measured at the appropriate wavelength for each mineral using the Genesys 10S. In parallel, images of the same reacted solutions were captured under fixed distance, focus, ISO, and lighting conditions. Phosphorus was determined using the ammonium molybdate-tin (II) chloride reaction, which produced a blue complex, whereas calcium was determined using Eriochrome Black T. After color stabilization, Red Green Blue (RGB) values were extracted from the images and used as the digital response variable (Grünberg et al., 2019; Uushona et al., 2021).

Quality control and validation

All measurements were performed in triplicate, and analytical blanks were included to assess contamination and baseline shift. Calibration was repeated across analytical sessions, and color reference charts were used during smartphone imaging to reduce the effect of lighting and sensor variation. Validation of the smartphone-assisted procedure was based on direct comparison with UV-Visible spectrophotometry under the same experimental conditions (Yanti et al., 2021; Batista and Campos, 2024). Accordingly, the smartphone-assisted colorimetric method was evaluated as a practical screening approach for calcium and phosphorus in forage extracts under established laboratory conditions.

Calibration and data analysis

Calibration curves were constructed by plotting concentration (ppm) against absorbance (UV-Visible) and against the RGB-derived response (smartphone). Linear regression equations were generated for calcium and phosphorus, where X represents the instrumental response and Y the estimated concentration (ppm). The regression equations followed the general formula $Y = mX + c$, where Y was mineral concentration (ppm), X was the measured optical response, m was the slope, and c was the intercept. Model performance was evaluated using the coefficient of determination (R^2), Pearson correlation coefficient (r), and mean absolute percentage error (MAPE), using the following Formula. Data processing and graph preparation were performed in Microsoft Excel (Ciaccheri et al., 2023).

$$MAPE = (1/n) \times \Sigma \mid (observed_i - predicted_i)/observed_i \mid \times 100$$

where n is the number of paired observations included in the calculation, $observed_i$ is the reference concentration measured by UV-Visible spectrophotometry, and $predicted_i$ is the concentration estimated using the smartphone-assisted colorimetric method**.**

RESULTS

Calibration models for calcium and phosphorus

Calibration models demonstrated a linear relationship between instrumental response and concentration for both analytes. For calcium, the UV-Visible equation was $Y = 228.41X - 0.0174$ with $R^2 = 0.961$ and $r = 0.980$, whereas the smartphone equation was $Y = 212.28X - 0.0615$ with $R^2 = 0.9448$ and $r = 0.972$. For phosphorus, the UV-Visible equation was $Y = 524.34X - 0.0442$ with $R^2 = 0.9514$ and $r = 0.975$, while the smartphone equation was $Y = 145.94x - 0.0986$ with $R^2 = 0.9974$ and $r = 0.999$. In these calibration equations, Y represents the estimated concentration of calcium or phosphorus expressed in ppm, whereas X represents the instrumental response, namely absorbance for UV-Visible spectrophotometry and the RGB-derived response for smartphone-assisted colorimetry. The R^2 denotes the coefficient of determination, which indicates the proportion of concentration variation explained by the linear regression model, while r denotes the Pearson correlation coefficient, which reflects the strength of the linear relationship between instrumental response and concentration. The present findings indicated strong linearity for both methods within the tested concentration range.

Goodness-of-fit summary

Across both analytes, the UV-Visible calibration models yielded mean R^2 and r values of 0.9562 and 0.9775, respectively, indicating that the fitted models accounted for most of the variation in the calibration data.

Method agreement

Agreement between the two methods was close for both analytes. Mean absolute differences were 0.012% for calcium and 0.007% for phosphorus, indicating minimal deviation between smartphone-based estimates and UV-Visible measurements.

Sample-level results

At the sample level, smartphone-derived concentrations were close to UV-Visible values for all tested forages. For calcium in *Pennisetum purpupoides*, the smartphone and UV-Visible methods yielded concentrations of 3.7936 and 3.7984 ppm, respectively. In *Leucaena leucocephala*, the corresponding values were 3.0238 and 3.0233 ppm for smartphone and UV-Visible methods, respectively. For phosphorus, the smartphone and UV-Visible methods yielded values of 3.1585 and 3.1579 ppm in *Pennisetum purpureum* cv. Mott and 3.3450 and 3.3333 ppm for *Sesbania grandiflora*, respectively. Table 1 presents the regression equations and goodness-of-fit statistics for calcium and phosphorus. Figures 1 and 2 illustrate the linear calibration relationships obtained for the two analytes by the present methods.

Table 1. Regression models for calcium and phosphorus determined by UV-Visible spectrophotometry and smartphone imaging using estimated calcium concentration (Y) from the instrumental response (X)

No	Mineral Elements	UV-Visible spectrophotometry	Digital image (Smartphone)
1	Calcium	Y = 228.41X – 0.0174; R^2 = 0.961; r = 0.980	Y = 212.28X – 0.0615; R^2 = 0.9448; r = 0.972
2	Phosphorus	Y = 524.34X – 0.0442; R^2 = 0.9514; r = 0.975	Y = 145.94 – 0.0986; R^2 = 0.9974; r = 0.999

Y: The estimated concentration of calcium or phosphorus expressed in ppm, X: The instrumental response, R^2: The coefficient of determination, r: The Pearson correlation coefficient

R^2 measures the proportion of variance in the dependent variable (Y) that was explained by the independent variable(s) (X). The R^2 Value ranges from 0 to 1 (or 0% to 100%). r measures the strength and direction of the linear relationship between two variables. In the regression equations, X represents the instrumental response, namely absorbance for UV-Visible spectrophotometry or the RGB-derived response for smartphone imaging, while Y represents the estimated mineral concentration expressed in ppm. R^2 represents the coefficient of determination, with values closer to 1 indicating better model fit, whereas r represents the Pearson correlation coefficient, with values closer to +1 indicating a stronger positive linear relationship between X and Y.

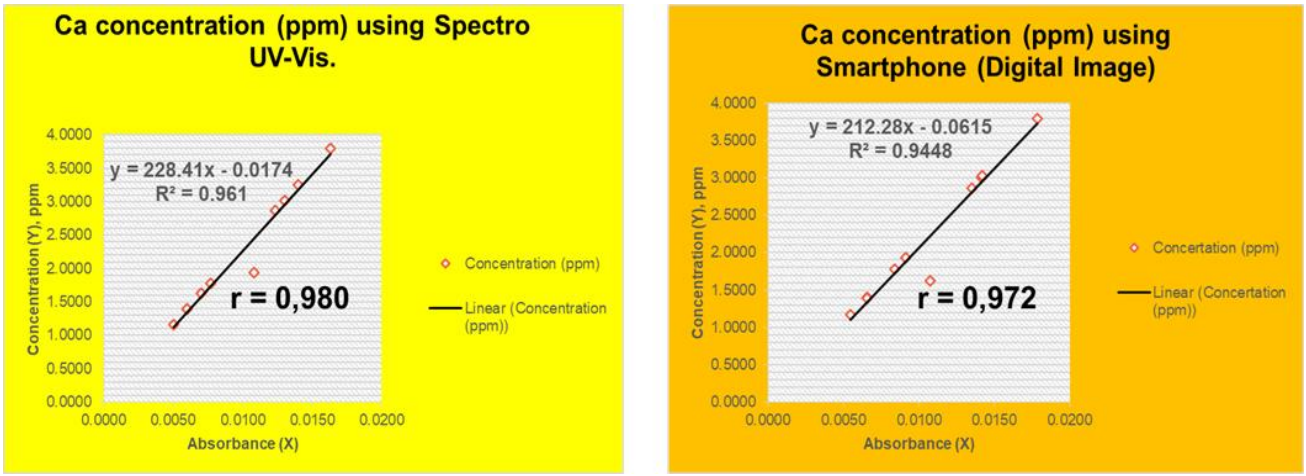


Figure 1. Regression equation used to estimate calcium concentration (Y, ppm) from the instrumental response (X) in ruminant feed extracts

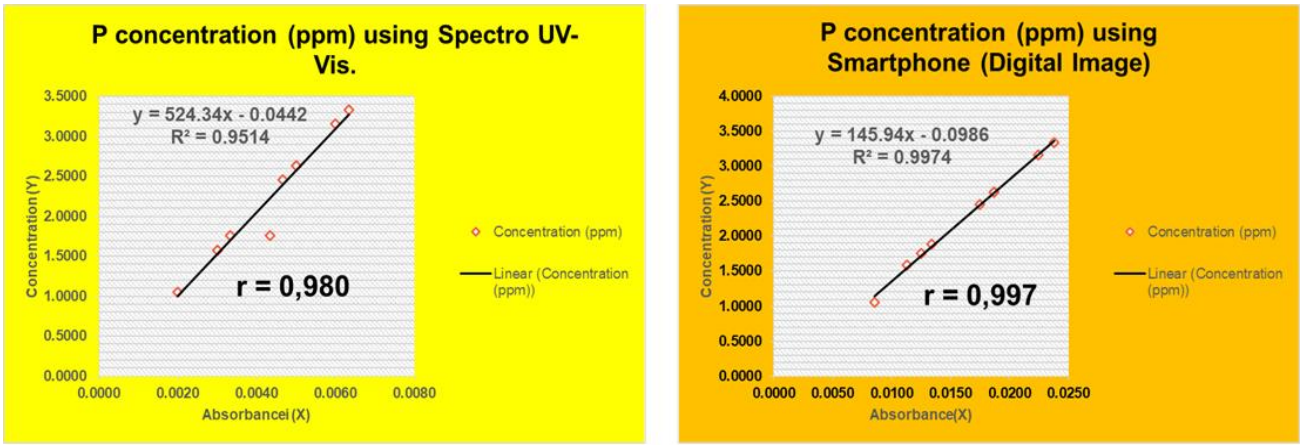


Figure 2. Regression equation used to estimate phosphorus concentration (Y, ppm) from the instrumental response (X) in ruminant feed extracts

DISCUSSION

Model performance and predictive strength

The calibration results indicated that smartphone-assisted colorimetry can generate quantitative responses that closely track those obtained by UV-Visible spectrophotometry. The high R^2 and r values observed for both analytes were consistent with the findings of Yanti et al. (2021) and Batista and Campos (2024), who reported that controlled digital imaging can support analytical measurements in agricultural matrices. The present findings suggested that under

established conditions, smartphone imaging was sufficiently sensitive for screening calcium and phosphorus in forage extracts.

Interpretation of correlations and calibration

The present study found that smartphone-derived RGB responses were strongly associated with calcium and phosphorus concentrations in forage extracts, indicating that smartphone-assisted colorimetry could provide calibration performance comparable to UV-Visible spectrophotometry under controlled laboratory conditions. This proportional relationship was consistent with the Beer-Lambert principle, which established a linear relationship between optical response and analyte concentration over an appropriate concentration range (Madzingira et al., 2021).

Agreement with feed-matrix evaluation

The close agreement between methods across four forage matrices indicated that the smartphone approach was not limited to a single plant source. These findings were important because forage composition can differ widely among species and processing conditions, potentially affecting extraction behavior and color development (Hawu et al., 2022; Fanelli et al., 2023; Fuentes et al., 2025). Thus, the present results supported the use of matrix-specific calibration as a practical strategy for routine feed evaluation.

Analytical principles

Both UV-Visible spectrophotometry and smartphone-assisted colorimetry depended on the conversion of chemical reactions into measurable optical signals. While UV-Visible spectrophotometry measured absorbance directly, the smartphone method captured color intensity that was transformed into a comparable response variable under standardized image conditions. Similar photometric approaches have been applied in recent digital image-based analyses for agricultural samples (Uushona et al., 2021; Batista and Campos, 2024).

Reaction schemes

The color reactions used in the present study supported the observed linear response. Phosphorus was determined by forming a molybdenum blue complex after reaction with ammonium molybdate and tin (II) chloride, whereas calcium was detected after color development with Eriochrome Black T. The distinct, concentration-dependent color responses produced by both reactions made these reactions suitable for detection by spectrophotometry and smartphone imaging. This finding was consistent with previous studies demonstrating that the molybdenum blue reaction, whose signal intensity is proportional to orthophosphate concentration, served as a reliable basis for phosphate determination and can be successfully adapted to smartphone-based colorimetric platforms. Consistent with previous studies, Eriochrome Black T is an established metallochromic reagent for alkaline-earth ions, such as calcium, and its color response has been reliably quantified in both spectrophotometric and smartphone-based digital-image methods. Therefore, the present results aligned with previous evidence demonstrating that visually measurable chromogenic reactions can produce robust analytical signals in both conventional optical instruments and image-based detection systems (Fiske et al., 1925; Fan et al., 2021; Graña-Dosantos et al., 2026).

Limitations and future prospects

From a practical perspective, the smartphone-assisted colorimetric method can serve as a rapid can serve as a rapid, portable alternative for preliminary mineral screening in settings where conventional instrumentation is unavailable. In the context of feed management, such accessibility is important because rapid access to calcium and phosphorus levels allows producers to adjust rations and optimize mineral supplementation in ruminant systems (Poulsen et al., 2021; Ponnampalam et al., 2024; Šaljić et al., 2024). Further validation across additional feed ingredients and wider field conditions is needed before routine large-scale application. From a practical perspective, the smartphone-assisted colorimetric method can serve as a rapid, portable alternative for preliminary mineral screening in settings where conventional instrumentation is unavailable. In the context of feed management, such accessibility is important because rapid access to calcium and phosphorus concentrations allows producers to adjust rations and optimize mineral supplementation in ruminant systems (Poulsen et al., 2021; Šaljić et al., 2024; Ponnampalam et al., 2024).

CONCLUSION

Smartphone-assisted colorimetry provided calibration models for calcium and phosphorus that were closely comparable to those obtained by UV-Visible spectrophotometry. The forages selected in the present study, including *Pennisetum purpureum* cv. Mott, *Pennisetum purpupoides*, *Leucaena leucocephala*, and *Sesbania grandiflora* were selected for their

suitability to tropical and subtropical climates, their nutritional quality, and their resilience in resource-limited settings. With the established conditions of the present study, smartphone-assisted colorimetry indicated strong linearity and minimal deviation from reference measurements, confirming its suitability as a low-cost screening tool for forage mineral analysis. The present study had several limitations. Validation was restricted to two minerals and conducted under controlled imaging conditions; therefore, broader application could be affected by feed matrix complexity, extraction efficiency, and environmental lighting variation. Future studies should validate smartphone-assisted colorimetry across several feed ingredients and mineral fractions, evaluate performance under diverse field conditions, and develop more detailed device standardization protocols to ensure reliable on-farm use translation.

DECLARATIONS

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Availability of data and materials

The datasets generated during the current study are available from the corresponding author on reasonable request.

Authors' contributions

Suhubdy designed the study, supervised the article, and drafted the manuscript. Syamsul Hidayat Dilaga and Azhary Noersidiq conducted laboratory analysis and data collection. Ahmadi contributed to analytical procedures, data interpretation, and manuscript revision. All authors read and approved the final edition of the manuscript.

Competing interests

The authors declared that they have no competing interests.

Ethical considerations

The authors confirmed that the manuscript is original and has been checked for plagiarism, duplication, and other ethical considerations in accordance with the journal's principles. The manuscript has not been published previously and is not under consideration elsewhere. No AI tools were used to conduct the present study.

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Antimicrobial Activity of Nano-encapsulated *Salvia Officinalis* against Methicillin-Resistant *Staphylococcus aureus* and *Acinetobacter Baumannii*

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ABSTRACT

Antimicrobial resistance represents a global health burden requiring innovative therapeutic strategies. The antibacterial properties of *Salvia officinalis* (*S. officinalis*) are attributed to its bioactive compounds. The present study aimed to investigate the antimicrobial activity of nano-encapsulated *S. officinalis* leaf extract against methicillin-resistant *Staphylococcus aureus* (MRSA) and *Acinetobacter baumannii* (*A. baumannii*). The crude extract of *S. officinalis* was subjected to methanolic extraction, and the resulting methanolic fraction was analyzed through phytochemical screening. Phytocompounds were identified using gas chromatography-mass spectrometry (GC-MS), and their potential mechanisms of action were evaluated *in silico*. The *S. officinalis* extract was nano-formulated using chitosan and characterized by scanning electron microscopy (SEM), Fourier transform infrared spectroscopy, and UV-visible spectroscopy. Antibacterial activity of the nano-formulation against MRSA and *A. baumannii* was assessed using agar well diffusion, broth microdilution, and minimum bactericidal concentration analysis. Qualitative phytochemical analysis revealed the presence of flavonoids, alkaloids, tannins, saponins, and terpenoids. The GC-MS analysis demonstrated 15 peaks, representing 14 distinct compounds, with one compound (3,7,11,15-tetramethyl-2-hexadecen-1-ol) eluting at two retention times. Chitosan nanoparticle formation was confirmed by an absorption peak near 290 nm. The functional groups of chitosan nanoparticles included hydroxyl (-OH) and amide (-NH_2) groups, along with C–H, C–N, C–O, and P=O stretching. The SEM imaging exhibited relatively homogeneous, well-dispersed, spherical nanoparticles. The antimicrobial efficacy of *S. officinalis* chitosan nanoparticles was concentration-dependent, reaching maximum inhibition zones of 21 ± 0.5 mm against MRSA and 17 ± 1.5 mm against *A. baumannii* at 500 µg/mL. Additionally, susceptibility testing revealed minimum inhibitory concentrations of 62.5 µg/mL and 125 µg/mL, and minimum bactericidal concentrations of 125 µg/mL and 500 µg/mL against MRSA and *A. baumannii*, respectively. Molecular docking analysis indicated that phytocompounds within the methanolic extract exhibited binding affinity toward penicillin-binding protein 2a (PBP2a) and the carbapenem-associated outer membrane protein (CarO). The interactions between the phytochemicals and protein targets indicated that binding to catalytic sites could inhibit the function of these resistance-associated proteins, potentially reducing antibiotic degradation. The biosynthesized *S. officinalis* chitosan nanoparticles exhibited strong antimicrobial activity against multidrug-resistant bacterial isolates, supporting their potential as antimicrobial agents.

Keywords: *Acinetobacter baumannii*, Antimicrobial resistance, Methicillin-resistant *Staphylococcus aureus*, Nanoparticle, *Salvia officinalis*

INTRODUCTION

Antimicrobial resistance (AMR) represents a significant global health challenge, largely attributed to the misuse and overuse of antibiotics across human and veterinary medicine, leading to the emergence of resistant microorganisms (Ahmed et al., 2024). Globally, it is estimated that direct deaths attributed to AMR exceeded 1.2 million in 2019, and without sufficient preventive measures, this number was expected to rise to almost 10 million deaths by 2050 (Salam et al., 2023).

The rapid emergence of antimicrobial drug-resistant bacteria is a growing global concern (WHO, 2022). Methicillin-resistant *Staphylococcus aureus* (MRSA) is a well-characterized strain that exhibits reduced susceptibility to β-lactam antibiotics, including methicillin and related compounds, and has become a prevalent cause of community-acquired infections (Ali et al., 2017). The MRSA is a significant source of serious infections in humans and animals, and it is a major public health threat (Omoshaba et al., 2020). *Staphylococcus*-related infections, particularly MRSA, predominantly affect immunocompromised individuals (Obanda et al., 2022). *Acinetobacter baumannii* (*A. baumannii*) is a clinically important opportunistic pathogen frequently associated with hospital-acquired infections globally and

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notable for its multidrug resistance, posing a major health concern (Castillo-Ramírez et al., 2025). The World Health Organization has ranked *A. baumannii* as the top-priority bacterial pathogen urgently requiring new antibiotics (WHO Bacterial Priority Pathogens List, 2024).

Salvia officinalis (*S. officinalis*) is a perennial aromatic shrub classified within the Lamiaceae family (Ezema et al., 2024). Although *S. officinalis* is native to the Mediterranean basin and regions of the Middle East, its cultivation has expanded globally due to its medicinal and culinary significance (Ghorbani and Esmaeilizadeh, 2017). In traditional medicine, *S. officinalis* has been utilized for treating a wide range of ailments, including neurological disorders such as seizures and tremors, gastrointestinal conditions such as ulcers and diarrhea, metabolic disorders including hyperglycemia, as well as inflammatory diseases such as gout and rheumatism (Ghorbani and Esmaeilizadeh, 2017). Additionally, sage (*S. officinalis*) is recognized for its diverse pharmacological properties, such as sedative, antimicrobial, antioxidant, antitumor, and antihypertensive effects (Ghorbani and Esmaeilizadeh, 2017). Sage has been applied in the management of different conditions, including excessive sweating, cardiovascular diseases, chronic bronchitis, asthma, renal dysfunction, liver cirrhosis, dysmenorrhea, insomnia, infantile colic, and dyspepsia (Ezema et al., 2024).

Nanotechnology is significantly transforming modern pharmacognosy (Devi et al., 2026). Nanotechnology offers a novel and promising approach to improve medicinal plant systems (Devi et al., 2026). Nanotechnology has transformed herbal medicine by improving the bioavailability and effectiveness of phytocompounds (Kathole et al., 2025). Advancements in plant-based medicine have demonstrated that herbal remedies hold significant potential for treating currently incurable disorders (Tiwari et al., 2024). Among the most notable attributes of herbal medicines employing nanotechnology-based delivery systems is the ability to transform compounds with limited solubility, poor absorption, and instability into promising medicines (Dewi et al., 2022). Nanoparticles (NPs) are materials with dimensions ranging from 1 to 100 nm and play a crucial role in biosensing technologies, particularly in enhancing signal transduction, sensitivity, and specificity when used in electrode modifications (Esfandiari et al., 2025). Metal NPs such as gold and silver are widely applied in medicine due to their antimicrobial, anticancer, and drug delivery capabilities, enabling targeted therapy and improved therapeutic efficiency (Rahdari et al., 2023). Nanotechnology is changing medical diagnostics, treatments, and disease prevention, using powerful, innovative tools (Patel et al., 2024). Ongoing investigations and collaboration among many fields could lead to greater treatment outcomes, lower healthcare costs, and new generations of customized and accurate medicines (Kazi et al., 2025). Thus, the present study aimed to evaluate the antimicrobial activity of nano-encapsulated *S. officinalis* against MRSA and *A. baumannii*.

MATERIALS AND METHODS

Ethical approval

Ethical approval for the present study was obtained from Mount Kenya University, Kenya, with the approval number 4180. All procedures were conducted in accordance with the ethical standards of the institutional biosafety and laboratory guidelines.

Plants materials

Fresh leaves of *S. officinalis* were harvested during the dry season in Nyeri County, Kenya. The identification and authentication of the plant material was carried out at the Jomo Kenyatta University of Agriculture and Technology (JKUAT), Kenya, herbarium by a taxonomist and was assigned the voucher specimen number BC-JKUATBH/001.28/07/2025.

Pre-extraction and extraction procedures

The leaves were thoroughly rinsed with running tap water and subsequently air-dried under shade at room temperature (25°C) for 21 days. The dried leaves were ground into a fine powder using an electric blender (Model: IBL30U1, Itel, China) supplemented with a mortar and pestle to ensure uniform particle size. The powdered leaves were stored in an air-tight, light-resistant container until extraction. For extraction, 50 g of powdered leaves was soaked in 1 L of methanol in a conical flask and macerated with continuous shaking in a shaker incubator (GYROMAXTM 727, United States) at 140 rpm and room temperature (25°C) for 72 hours. Following extraction, the mixture was sequentially filtered through sieve cloth and Whatman Number 1 filter paper (Sterilin, Teddington, Middlesex, England) to remove particulate matter. The filtrate was concentrated on a rotary evaporator (Labtech DAIHAN, VP30, EV11, South Korea) at 65°C, 175 mbar reduced pressure, and 120 rpm. The resulting semi-solid extract was further dried in a hot-air oven at 37°C for 24 hours to a constant weight. The percentage yield of the crude extract was calculated and recorded as 10.17%, using Formula 1. Finally, the dried extract was stored in sterile Falcon tubes at 4°C until further use.

$$\text{Percentage (\%) yield} = \frac{Weight\ of\ the\ dried\ extract\ (g)}{Initial\ weight\ of\ the\ dried\ plant\ material\ (g)} \times 100 \qquad \text{(Formula 1)}$$

Phytochemical screening

Qualitative phytochemical screening procedures for different classes of secondary metabolites of the crude extract were conducted using established procedures employed by Yahaya et al. (2020), Noumi et al. (2023), and Rao et al. (2023) to analyze terpenoids, flavonoids, saponins, alkaloids, tannins, and phenols.

Terpenoids

Approximately 0.5 g of the crude extract was dissolved in 2 mL of chloroform (Sisco Research Laboratories Pvt. Ltd, India). Concentrated sulfuric acid (Loba Chemie Pvt. Ltd, India) was then carefully introduced along the wall of the test tube to form a distinct lower layer. The reddish-brown appearance was interpreted as evidence of terpenoid compounds.

Flavonoids

Approximately 0.5 g of the extract was reacted with small fragments of magnesium ribbon (FINAR Ltd, India), followed by gradual addition of concentrated hydrochloric acid (Finar Ltd, India). After five minutes of incubation, the appearance of a pink-to-magenta color indicated the presence of flavonoids.

Saponins

About 0.5 g of the extract was mixed with 2 mL of distilled water in a test tube and shaken vigorously. The formation and persistence of froth for about 10 minutes was indicative of saponins.

Alkaloids

Approximately 0.5 g of the extract was dissolved in dilute HCl (Finar Ltd, India) and filtered to obtain a clear solution. Wagner's reagent (Sigma-Aldrich, Germany) was then added to the filtrate solution, and the formation of a brownish or reddish precipitate confirmed the presence of alkaloids.

Tannins

About 0.5 g of the extract was heated with 10 mL of water and subsequently filtered. A few drops of 0.1% ferric chloride solution (Sisco Research Laboratories Pvt. Ltd, India) were added to the filtrate, and the appearance of blue-black or green-black color was interpreted as a positive result for tannins.

Phenols

About 3-4 drops of ferric chloride solution were added to 0.5 g of the extract. The appearance of a bluish-black color indicated the presence of phenolic compounds.

Gas chromatography mass-spectrometry

The chemical composition of the crude extract was analyzed using gas chromatography-mass spectrometry (GC-MS), following the procedure previously described by Konappa et al. (2020). The analysis was performed using a GC-MS system (Shimadzu GCMS-QP2010 SE, Japan) equipped with a low polarity BPX5 capillary column (30 m × 0.25 mm internal diameter, 0.25 µm film thickness). High-purity helium gas (99.99%) served as the carrier gas and was maintained at a constant flow rate of 1 mL/min. Ionization was carried out using the electron impact (EI) mode at 70 eV, with a scan interval of 0.2 seconds over a mass range of 40-600 m/z. A 1 µL sample volume was injected into the system, with the injector temperature set to 250°C. The column oven temperature program was initiated at 50°C and held for two minutes, then increased at 10°C/min to 200°C. The temperature was then raised to 280°C and maintained for 3 min to ensure complete elution of the compounds. Identification of the phytochemical components was accomplished by comparing the obtained mass spectra and chromatographic parameters, including retention times and peak features, with those of standard reference compounds available in the National Institute of Standards and Technology (NIST) spectral database.

Nanoencapsulation of *Salvia officinalis*

Sage-chitosan NPs were synthesized using an ionic gelation technique, according to the method reported by Portia et al. (2024). A 2% (w/v) chitosan was dissolved in 1% (v/v) aqueous acetic acid (Loba Chemie Pvt. Ltd, India) to obtain a 2% (w/v) polymer solution. The pH of the solution was subsequently adjusted to 4.8 using 1 N sodium hydroxide to optimize solubility. The resulting mixture was maintained under continuous magnetic stirring at 25°C for 24 hours for complete dissolution and homogeneity of the polymeric solution. A 40 mL portion of the chitosan solution was then mixed with Tween 80 (Griffchem, India) as a surfactant and stirred at 45°C for two hours to achieve a uniform solution. Methanolic extract of *S. officinalis* was added gradually at different weights (0, 0.2, 0.4, 0.6, 0.8, and 1 g) and stirred at 45°C for 30 minutes. Subsequently, 0.5% (w/v) tripolyphosphate (Griffchem, India) was added dropwise to the mixture under continuous stirring for 40 minutes to facilitate nanoparticle formation. The formed NPs were then recovered by centrifugation (ThermoFisher, Germany) at 4000 rpm for 30 minutes at 25°C, then washed several times with distilled water to remove unbound materials and excess reagent. To obtain a suspension, the NPs pellet was re-dispersed in 30 mL of distilled water after purification. The dispersion was then subjected to probe sonication using a probe sonicator (ThermoFisher, Germany) in an ice bath for four minutes at a pulsed cycle (2 seconds on, 1 second off) to enhance the uniformity. The resulting suspension was subsequently lyophilized at -65°C for 72 hours. The freeze-dried samples were collected in a sterile Falcon tube and stored at 4°C until further use.

Characterization of nanoparticles

The physicochemical properties of NPs, including size, shape, surface morphology, and surface functional groups, significantly influence their toxicity and biological activity (Samadian et al., 2020). Characterization of the NPs was carried out according to the methods described by Alkhathlan et al. (2020) and Daoudi et al. (2024). The optimal properties of the biosynthesized chitosan-sage NPs were evaluated using a UV-1800 UV-Visible spectrophotometer (Shimadzu, Kyoto, Japan) at a wavelength range of 200-700 nm using quartz cuvettes. Fourier transform infrared spectroscopy (FTIR) was conducted using the compact FTIR spectrometer ALPHA II (Bruker, Germany) over the spectral range of 4000-400 cm^{-1}. The FTIR analysis was performed to identify the functional groups present and to examine possible interactions between chitosan and the encapsulated phytocomponents. Surface morphology and structural characteristics were examined using scanning electron microscopy (SEM; JEOL 0860 0000 ALS [257], Japan), with a high accelerating voltage of 15 kV to obtain high-resolution images for detailed analysis.

Test organisms

The standard bacterial isolate of MRSA (ATCC 12493) and the clinical isolate of *A. baumannii* were obtained from Kenyatta University Referral Hospital, Nairobi, Kenya.

Determination of antimicrobial activity of biosynthesized chitosan-sage nanoparticles

Agar well diffusion method

Antimicrobial activity was evaluated using an agar well diffusion method as described by Enan et al. (2021) and Amou et al. (2025). Each Mueller-Hinton agar (MHA) plate (Oxoid Limited, United Kingdom) had six wells, representing different concentrations of the test sample and a negative control. Each Petri dish received 20 mL of sterile MHA, and evenly spaced wells (6 mm in diameter) were created around the margins using a sterilized cork borer. A Bunsen burner flame was employed during the process to ensure sterility. The bacterial test cultures were grown overnight in Mueller-Hinton broth and adjusted to a 0.5 McFarland standard using normal saline. A sterile cotton was immersed in the inoculum, excess liquid was gently drained, and the cotton was used to evenly inoculate the agar surface, which was then allowed to dry for 20 minutes. Aliquots (100 µL) of chitosan-sage NPs at concentrations of 500 µg/mL, 250 µg/mL, 125 µg/mL, 62.5 µg/mL, and 31.25 µg/mL were added into the wells. At the center of the plates, vancomycin and meropenem antibiotic discs were placed as positive controls for MRSA and *A. baumannii*, respectively (Saka et al., 2024). Each test was performed in triplicate and incubated at 37˚C for 18-24 hours. Zones of inhibition were measured using a ruler, and the three distinct readings from each plate were documented and analyzed to determine the mean and standard deviation (SD).

Minimum inhibitory concentration

In three replicates, 100 µL of Mueller-Hinton broth was dispensed into 96-well plates (Sterilin, Teddington, Middlesex, England), and 100 µL of each nano-formulated *S. officinalis* extract, the crude extract, and the positive controls were added in different wells of the first column of plates and serially diluted across the plates to obtain concentrations of 500, 250, 125, 62.5, and 31.25µg/mL. Then, 20 µL of the bacterial inoculum was added to all wells except those in the last column, which contained only broth to check sterility. Chitosan (2% w/v) and methanol were included as negative controls, whilst vancomycin and meropenem were employed as reference antibiotics for MRSA and *A. baumannii*, respectively. Following incubation at 37˚C for 18 hours, 20 µL of resazurin indicator (Glentham Life Sciences, United Kingdom) was added to each well, and the plates were further incubated at 37˚C for two hours. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of the test sample that inhibited visible bacterial growth, as evidenced by the persistence of the blue color of the resazurin indicator, similar to the negative control.

Minimum bactericidal concentration

According to Mah (2014), the minimum bactericidal concentration (MBC) was the lowest concentration of an antimicrobial agent required to reduce the initial bacterial population by 99.9% or higher, after 18 to 24 hours of incubation at 37˚C. After incubation at 37˚C for 18 hours, test samples from wells containing concentrations exceeding the MIC were aseptically streaked onto MHA plates (using a sterile wire loop) and evaluated. The samples with the minimal concentration and no bacterial growth during incubation were indicated as MBC.

***In silico* analysis**

Bioactive compounds of the methanolic extract of *S. officinalis* with antimicrobial potential, identified by GC-MS analysis, were selected as ligands. Their 2D structures were retrieved from the PubChem database. Three-dimensional structures of the target proteins, namely the crystal structure of penicillin-binding protein 2a (PBP2a) double clinical mutant N146K-E150K from MRSA (ID: 4CPK) and the crystal structure of *A. baumannii* carbapenem-associated outer membrane protein (CarO; ID: 4FUV), were retrieved from the Research Collaboratory for Structural Bioinformatics

(RCSB) Protein Data Bank (PDB) in PDB format. The canonical SMILES representations of selected bioactive compounds were obtained and used as input ligands for docking analysis. Both protein and ligand files were prepared using appropriate preprocessing steps, including the removal of co-crystallized water molecules and conversion to Protein Data Bank charge type (Pdbqt) format. The ligand structures were energy-minimized before docking. Molecular docking simulations were conducted using AutoDock Vina (version 1.2.0) to predict the best binding pose and binding affinities (Bugnon et al., 2024). The binding regions were defined by setting a grid box with dimensions of 20 × 20 × 20 Å, centered on the active site using appropriate XYZ coordinates, and an exhaustiveness value of 4 was applied to control the search efficiency. The resulting complexes were visualized using BIOVIA Discovery Studio 2025 client software. Binding energies less than 0 indicated favorable interactions, with lower energy values suggesting stronger binding affinity (Khan and Lee, 2022).

Statistical analysis

All experiments were conducted in triplicate, and the present results were expressed as mean ± standard deviation (SD). Statistical and FTIR data analyses were performed using Origin Pro Lab 2026 (Student Version) software.

RESULTS

Phytochemical screening

The qualitative phytochemical analysis of the methanolic crude extract of *S. officinalis* indicated a high abundance of phenols, tannins, alkaloids, and saponins, while flavonoids and terpenoids were detected in moderate amounts (Table 1).

Gas chromatography-mass spectrometry

The mass spectrometer identified the nature and structural properties of chemical compounds by analyzing eluted samples at distinct time points. Peaks corresponding to the retention times of the compounds were observed in the chromatogram (Figure 1). The GC-MS of the methanolic crude extract of *S. officinalis* revealed compounds belonging to hydrocarbons, ketones, esters, cyclic compounds, and fatty acids classes, expressed as percentage area (Area%; Table 2).

Table 1. Qualitative phytochemical screening of *Salvia officinalis* crude extracts

Secondary metabolites	Methanolic crude extract of *Salvia officinalis*
Phenols	+++
Flavonoids	++
Tannins	+++
Alkaloids	+++
Terpenoids	++
Saponins	+++

+++: Abundantly present, ++: Moderately present

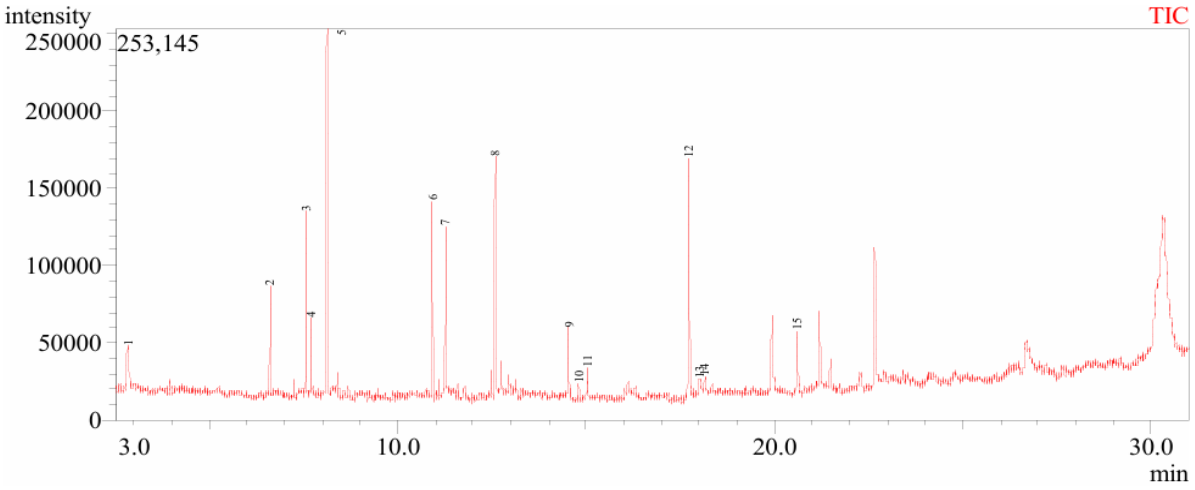


Figure 1. Gas chromatography-mass spectrometry of methanolic crude extract of *Salvia officinalis*

Table 2. Gas chromatography-mass spectroscopy analysis of methanolic crude extract of *Salvia officinalis*

Peaks	Retention time	Area (%)	Compounds names	Molecular weight (g/mol)	Molecular formula
1	2.839	4.87	2,2-Dimethoxybutane	118	$C_6H_{14}O_2$
2	6.595	4.71	Eucalyptol	154	$C_{10}H_{18}O$
3	7.558	7.93	Bicyclo[3.1.0]hexan-3-one,4-methyl-1-(1-methylethyl)	152	$C_{10}H_{16}O$
4	7.692	3.32	Thujone	152	$C_{10}H_{16}O$
5	8.116	16.56	Camphor	152	$C_{10}H_{16}O$
6	10.913	9.28	Caryophyllene	204	$C_{15}H_{24}$
7	11.263	7.66	1,4,7,-Cycloundecatriene, 1,5,9,9-tetramethyl-, Z,Z,Z-	204	$C_{15}H_{24}$
8	12.593	12.76	H-Cycloprop[e]azulene, decahydro-1,1,7-trimethyl-4-methylene	204	$C_{15}H_{24}$
9	14.535	3.87	Neophytadiene	278	$C_{20}H_{38}$
10	14.808	0.81	Phytol, acetate	338	$C_{22}H_{42}O_2$
11	15.028	2.28	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	296	$C_{20}H_{40}O$
12	17.730	18.36	Naphthalene, decahydro-1,1,4a-trimethyl-6-methylene-5-(3-methyl-2,4-pentadienyl)-,[4aS-(4a.alpha.,5.alpha.,8a.beta.)]-	272	$C_{20}H_{32}$
13	18.010	1.82	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	296	$C_{20}H_{40}O$
14	18.146	1.04	Humulane-1,6-dien-3-ol	222	$C_{15}H_{26}O$
15	20.598	4.75	4,5,6,7-Tetrahydroxy-1,8,8,9-tetramethyl-8,9-dihydrophenaleno[1,2-b]furan-3-one	342	$C_{19}H_{18}O_6$

Characterization of nanoparticles

Figure 2 exhibited the UV-visible absorption profile of *S. officinalis* chitosan NPs. A distinct absorption band centered at approximately 290 nm was observed in the spectrum, indicating the formation of the NPs. The FTIR analysis was employed to examine the chemical characteristics of the samples over a wavenumber range of 400-4000 cm^{-1}, enabling the identification of functional groups and assessment of potential interactions within the formulation. The absorption bands observed for chitosan-sage NPs and the crude extract are presented in Figure 3 and Table 3. Prominent peaks were detected at 3421 cm^{-1} in the NPs and at 3435 cm^{-1} in the crude extract, both representing strong broad O–H and NH_2 stretching, denoting hydroxyl and amide groups. Additionally, a medium broad peak at 1651 cm^{-1} was attributed to C=O (carbonyl) stretching (amide I group), and a weak broad peak at 1564 cm^{-1} corresponded to N–H stretching (amide II group). The band observed at 1412 cm^{-1} was associated with C-H bending vibrations of alkyl groups, and the distinct peak at 1257 cm^{-1} might have been attributed to C-N stretching vibrations corresponding to the amide III group. The prominent peak at 1125 cm^{-1} was indicative of P=O stretching vibrations, suggesting the presence of phosphate, while the absorption at 1024 cm^{-1} was assigned to C-O stretching vibrations of alcoholic groups. In the lower-wavenumber region, the band at 746 cm-1 might be related to C-Cl stretching in halogenated compounds, whereas the peak at 483 cm-1 was attributed to metal-oxygen (M-O) vibrations, indicating the presence of inorganic components.

The morphological features of the synthesized NPs were examined using SEM. The micrographs of the synthesized *S. officinalis* chitosan NPs demonstrated predominantly spherical particles with a relatively uniform distribution (Figure 4). Further analysis using ImageJ revealed that most of the NPs were in the 40-80 nm size range.

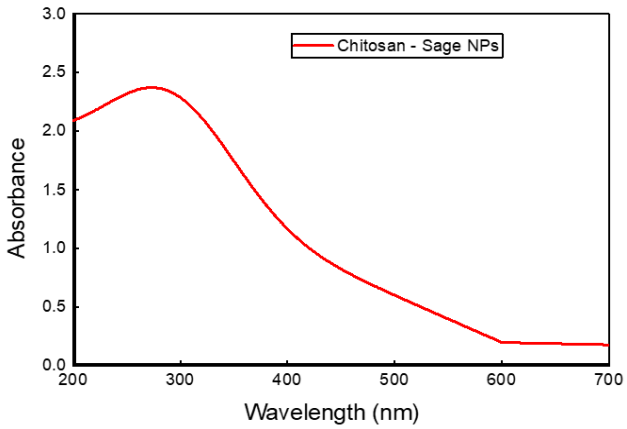


Figure 2. UV-Visible spectrum of biosynthesized chitosan-sage nanoparticles

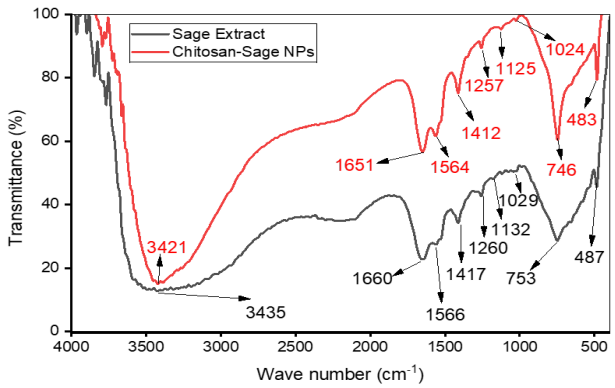


Figure 3. Fourier transform infrared spectra of biosynthesized chitosan-sage nanoparticles and sage crude extract

Table 3. Fourier transform infrared spectra of the functional group in chitosan-sage nanoparticles

Peak (cm^{-1})	Functional group
3421	O-H and NH_2 stretching / Hydroxyl and amide groups
1651	C=O Stretching / Amide I group
1564	N-H bending / Amide II group
1412	C-H bending / Alkyl group
1257	C-N stretching / Amide III group
1125	P=O stretching / Phosphate group
1024	C-O stretching / Alcoholic group
746	C-Cl stretching / Halo compound
483	M-O stretching / Metal-oxygen (Inorganic)

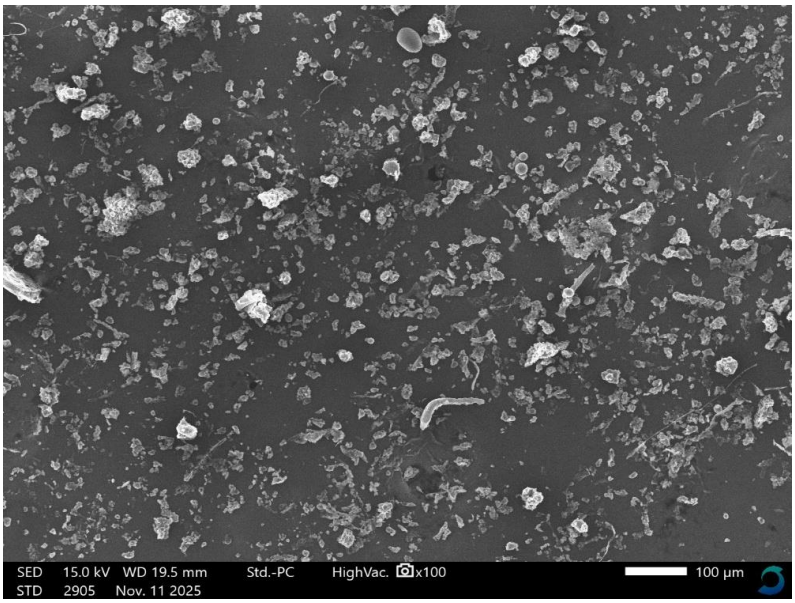


Figure 4. Scanning electron microscopy micrograph of biosynthesized chitosan-sage nanoparticle at 100x magnification

Antimicrobial activity of biosynthesized chitosan-sage nanoparticles

The antimicrobial potential of the biosynthesized *S. officinalis* chitosan NPs was evaluated using the agar well diffusion method (Table 4). The nano formulation demonstrated inhibitory effects against MRSA and *A. baumannii* across the tested concentrations, with greater inhibition observed at 500 µg/mL. At this concentration (500 µg/mL), clear zones of inhibition were recorded against MRSA and *A. baumannii,* indicating effective suppression of bacterial growth.

Minimum inhibitory concentration

The lowest concentrations required to prevent visible microbial growth were identified through MIC determination of chitosan-sage NPs. The NPs exhibited inhibitory activity at 62.5 µg/mL against MRSA and at 125 µg/mL against *A. baumannii*. In comparison, the MIC values for the crude extract were 250 and 500 µg/mL against MRSA and *A. baumannii,* respectively. Additionally, the positive controls had MICs of 25 µg/mL for vancomycin and 50 µg/mL for meropenem (Table 5).

Minimum bactericidal concentration

Chitosan-sage NPs inhibited the physical growth of MRSA at 125 µg/mL and *A. baumannii* at 500 µg/mL. The MBCs for the positive controls were 50 µg/mL for vancomycin and 100 µg/mL for meropenem (Table 6).

Table 4. Inhibition zones of chitosan-sage nanoparticles against methicillin-resistant *Staphylococcus aureus* and *Acinetobacter baumannii* following CLSI protocols

Bacterial organisms	*Salvia officinalis* nanoparticles (µg/mL)					Positive control	Negative control
	500	250	125	62.5	31.25	Vancomycin / meropenem	Methanol / normal saline
MRSA	21 ± 0.5	15 ± 1.0	12 ± 0.5	10 ± 0.5	0	17 ± 1.5	0
Acinetobacter baumannii	17 ± 1.5	13 ± 0.5	11 ± 1.0	9 ± 1.0	0	10 ± 1.5	0

CLSI: Clinical and laboratory standards institute, MRSA: Methicillin-resistant *Staphylococcus aureus*, NPs: Nanoparticles

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Table 5. Minimum inhibitory concentration of biosynthesized chitosan-sage nanoparticles

Bacterial organisms	*Salvia officinalis* nanoparticles (µg/mL)	Crude extract (µg/mL)	Standards antibiotics (µg/mL)	Negative controls (µg/mL)	
				Methanol	Chitosan
MRSA ATCC 12493	62.5	250	Vancomycin 25	Growth	Growth
Acinetobacter baumannii	125	500	Meropenem 50	Growth	Growth

MRSA: Methicillin-resistant *Staphylococcus aureus*

Table 6. Minimum bactericidal concentration of biosynthesized chitosan-sage nanoparticles

Bacterial organisms	*Salvia officinalis* nanoparticles (µg/mL)	Standards antibiotics (µg/mL)
MRSA	125	Vancomycin 50
Acinetobacter baumannii	500	Meropenem 100

MRSA: Methicillin-resistant *Staphylococcus aureus*

Molecular docking

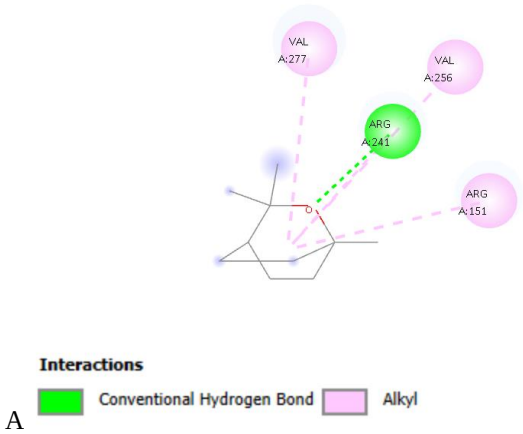
The GC-MS analysis of the methanolic crude extract of *S. officinalis* identified a total of 15 bioactive compounds (Table 2). The phytochemical compounds were subsequently evaluated for their potential activity against MRSA and *A. baumannii* as demonstrated in Table 7. Molecular docking simulations were performed using AutoDock Vina (version 1.2.0) to assess their binding affinities toward the target proteins. Caryophyllene exhibited the strongest binding affinity with values of -6.7 kcal/mol for PBP2a and -6.3 kcal/mol for CarO. The interactions with both targets were mostly hydrophobic. The decahydro-naphthalene derivative exhibited a binding affinity of -6.6 kcal/mol for PBP2a and -6.3 kcal/mol for CarO, closely following caryophyllene. The interactions with PBP2a involved hydrogen bonding, hydrophobic contacts, and ionic interactions, whereas those with CarO involved hydrogen and hydrophobic bondings. Thujone demonstrated similar binding affinities, with scores of -5.7 kcal/mol for PBP2a and -5.8 kcal/mol for CarO. Thujone's interactions with both targets included hydrogen bonding and hydrophobic interactions. Camphor exhibited binding affinities of -5.7 kcal/mol for PBP2a (via hydrogen bonds and hydrophobic interactions) and -5.5 kcal/mol for CarO (through hydrophobic interactions). Eucalyptol exhibited a binding affinity of -5.6 kcal/mol for PBP2a, including hydrogen bonding and hydrophobic interactions, and a relatively lesser affinity of -4.9 kcal/mol for CarO via hydrogen bonding. Neophytadiene had moderate affinities of -5.6 kcal/mol for PBP2a and -5.0 kcal/mol for CarO.

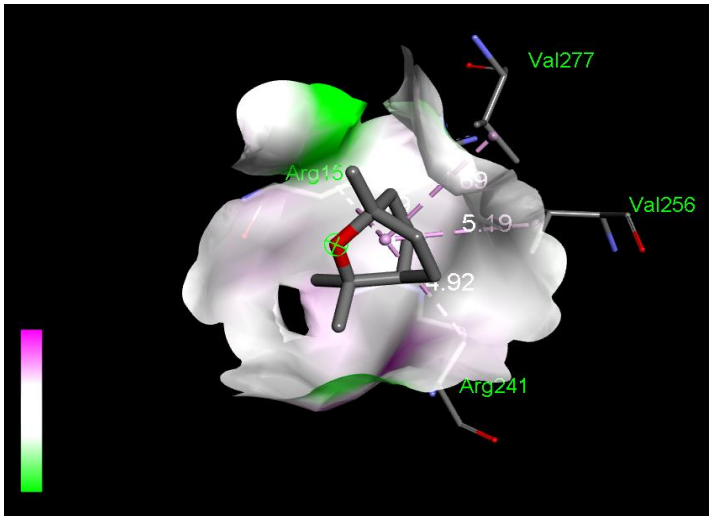
Table 7. Molecular docking of phytocompounds against methicillin-resistant *Staphylococcus* and *Acinetobacter baumannii*

Phytocompounds	Binding affinity (Kcal/mol)		Interactions	
	PBP2a (4CPK) MRSA	CarO (4FUV) *A. baumannii*	PBP2a (4CPK) MRSA	CarO (4FUV) *A. baumannii*
Eucalyptol	-5.6	-4.9	H-bond, Hydrophobic	H-bond
Thujone	-5.7	-5.8	H-bond, Hydrophobic	H-bond, Hydrophobic
Camphor	-5.7	-5.5	H-bonds, Hydrophobic	Hydrophobic
Caryophyllene	-6.7	-6.3	Hydrophobic	Hydrophobic
Neophytadiene	-5.6	-5.0	Hydrophobic	Hydrophobic
Naphthalene, decahydro-1,1,4a-trimethyl-6-methylene-5-(3-methyl-2,4-pentadienyl)	-6.6	-6.3	H-bond, Hydrophobic, and ionic interactions	H-bond, Hydrophobic

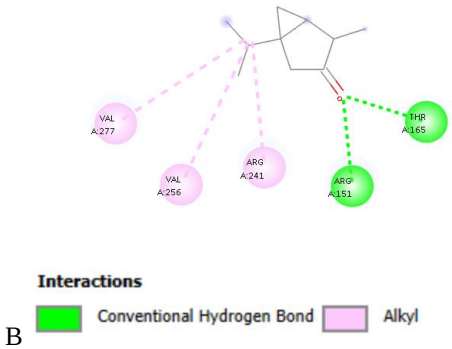
PBP2a: Penicillin binding protein 2a, CarO: Carbapenem-associated outer membrane protein, MRSA: Methicillin-resistant *Staphylococcus aureus, A. baumannii: Acinetobacter baumannii*

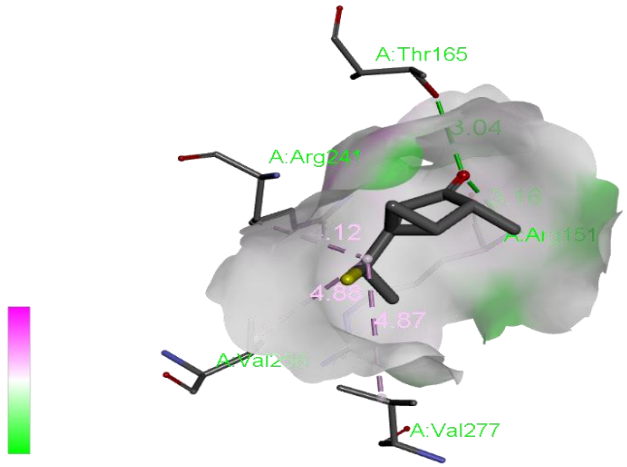
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Interactions
Conventional Hydrogen Bond
Alkyl

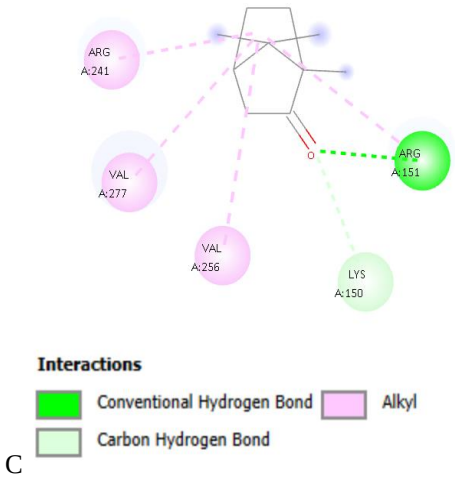

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Arg241

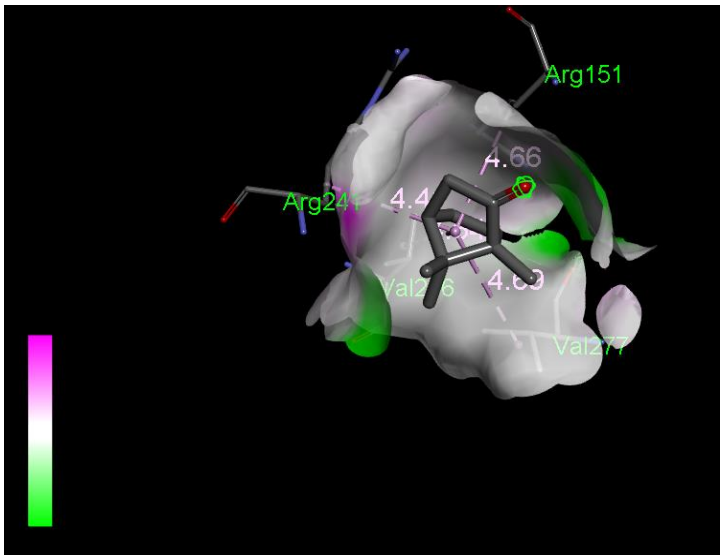
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Interactions
Conventional Hydrogen Bond
Alkyl


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C


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Interactions
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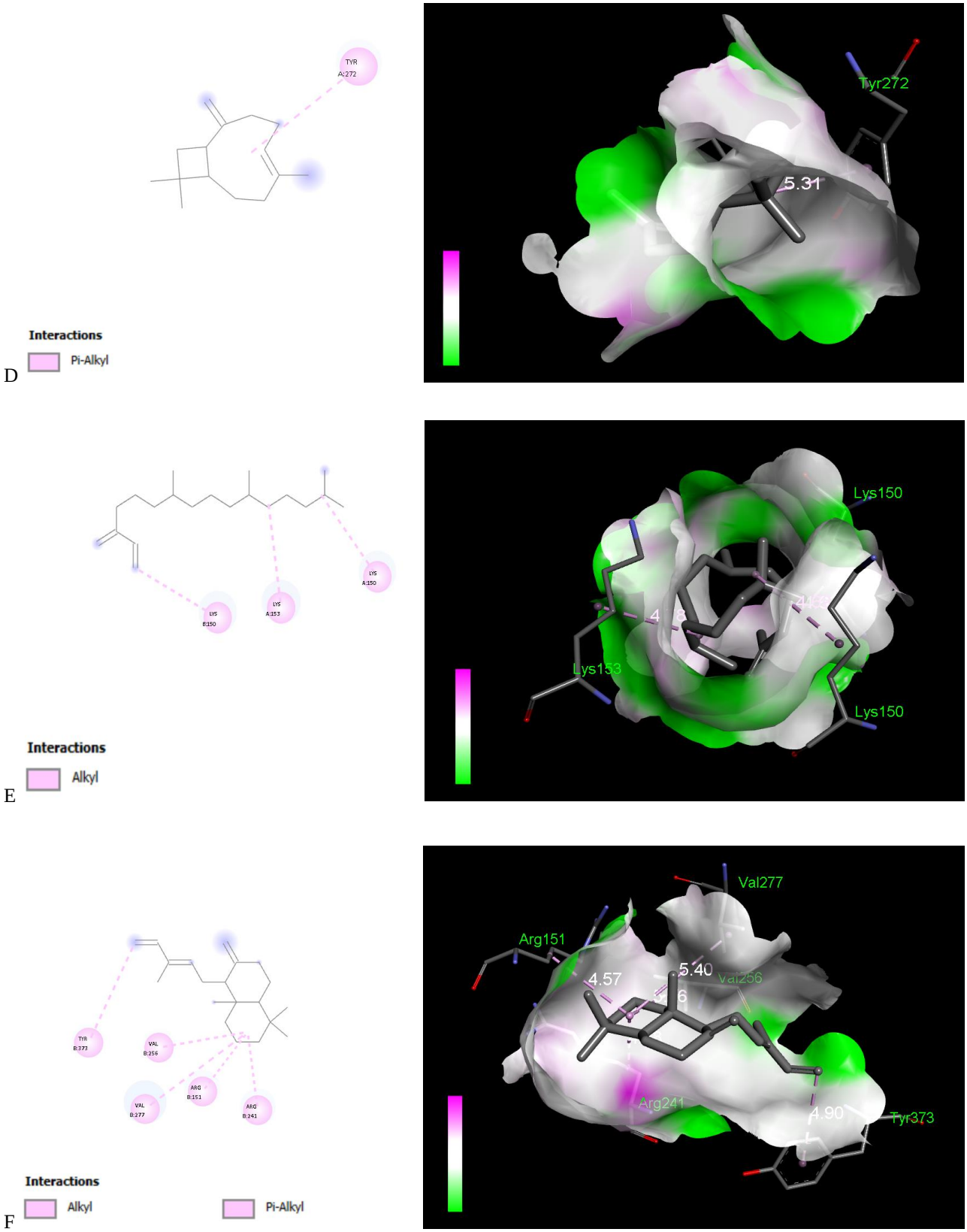
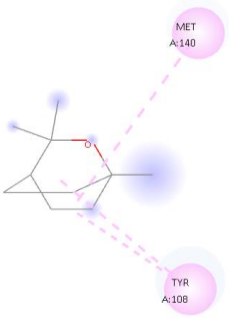
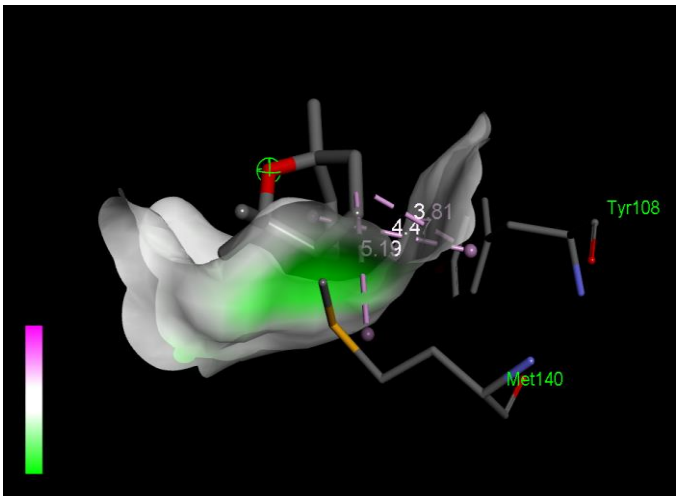


Figure 5. The 2D diagrams and 3D interaction profiles illustrating ligand binding for six compounds within the binding site of PBP2a. **Å:** Interaction distances, **A:** Eucalyptol, **B:** Thujone, **C:** Camphor, **D:** Caryophyllene, **E:** Neophytadiene, **F:** Naphthalene.

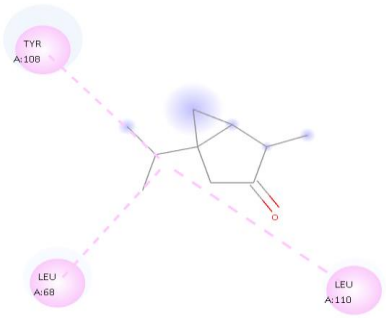
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TYR
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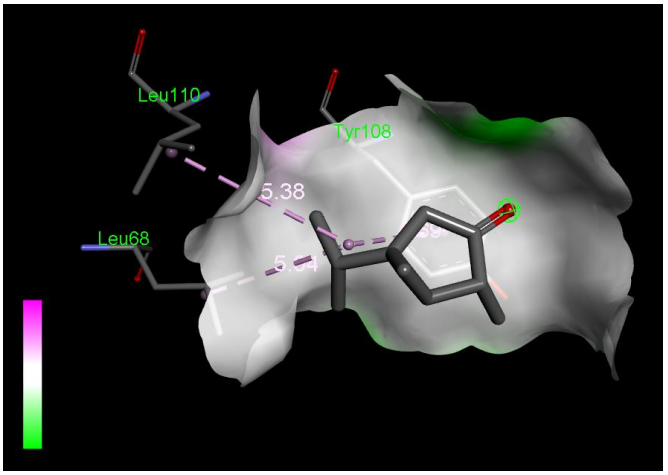

Interactions
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Pi-Alkyl


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Tyr108
Met140

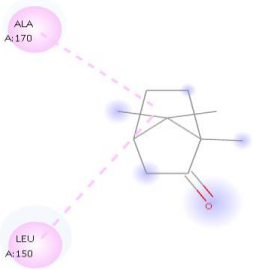
B


TYR
A:108
LEU
A:68
LEU
A:110

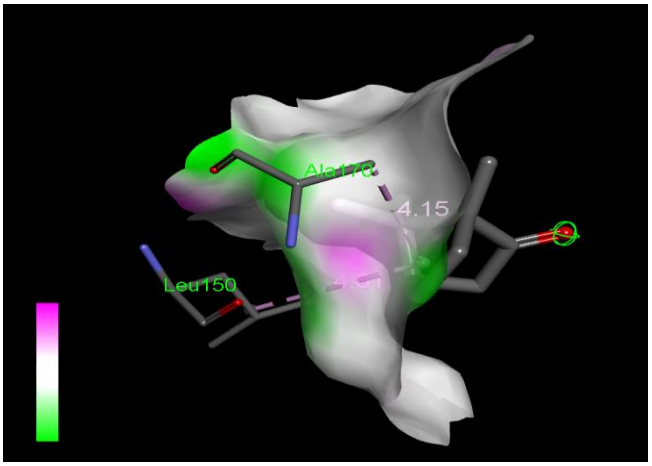

Interactions
Alkyl
Pi-Alkyl


Leu110
Tyr108
5.38
Leu68

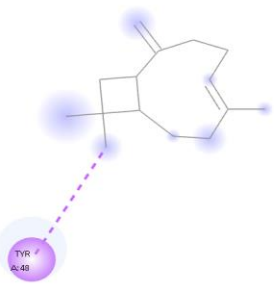
C


ALA
A:170
LEU
A:150

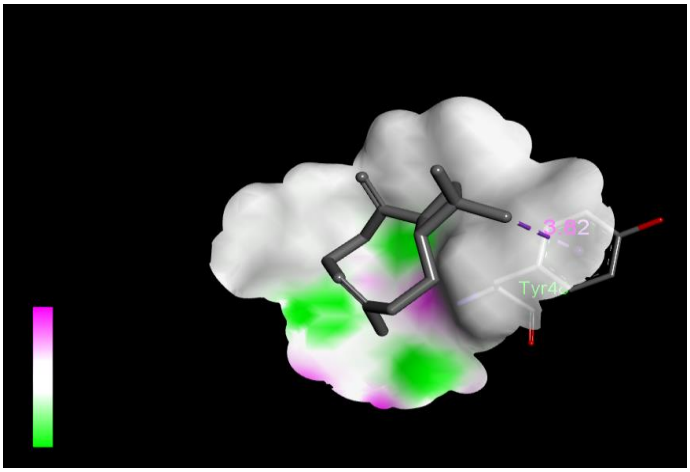

Interactions
Alkyl


4.15
Leu150

D


TYR
A:48


Interactions
Pi-Sigma



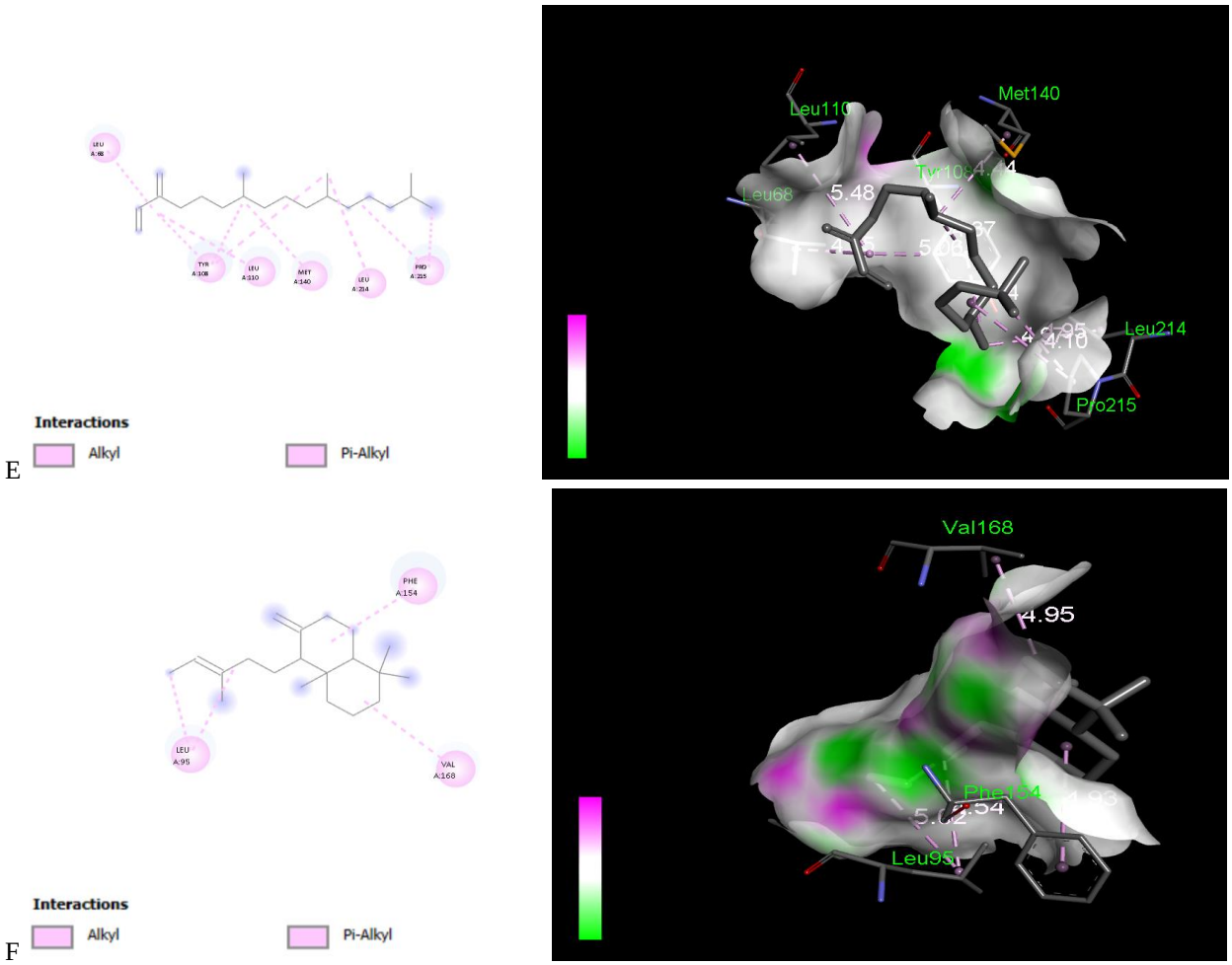


Figure 6. The 2D diagrams and 3D interaction profiles illustrating ligand binding for six compounds within the binding site of CarO. **Å:** Interaction distances, **A:** Eucalyptol, **B:** Thujone, **C:** Camphor, **D:** Caryophyllene, **E:** Neophytadiene, **F:** Naphthalene.

DISCUSSION

The qualitative phytochemical screening of *S. officinalis* crude methanolic extract revealed the presence of bioactive secondary metabolites, including phenols, flavonoids, tannins, alkaloids, terpenoids, and saponins. Such secondary metabolites are responsible for the medicinal activity of plants (Kumari et al., 2017). The presence of these metabolites may explain the observed antimicrobial activity and support the plant's traditional (folkloric) use in managing different diseases (Kanarek et al., 2025). This finding aligned with those of Khadse (2019) and Patel et al. (2024), who reported similar phytochemical profiles in *S. officinalis* leaf extract. The high concentration of these compounds' likely accounts for their potent antimicrobial activity, which was consistent with the findings of Amal and Wael (2016), who demonstrated that the biologically active polyphenolic components in *S. officinalis* extracts exhibit significant antimicrobial activity.

The GC-MS analysis of the *S. officinalis* methanolic extract revealed 15 peaks, representing the presence of 15 different compounds. The most abundant components included naphthalene (peak 12, 18.36%), camphor, thujone, eucalyptol, and neophytadiene. Previous studies have reported the antimicrobial properties of these compounds (Selim et al., 2022; He et al., 2026), further supporting the antimicrobial potential of *S. officinalis*. The existence of bioactive compounds supported the traditional medicinal applications of *S. officinalis* (Anjali et al., 2024).

The UV-visible spectroscopy was employed as a preliminary technique to confirm the NPs formation. The biosynthesized chitosan-sage NPs were analyzed across a wavelength range of 200-700 nm. A maximum absorbance peak was observed at 290 nm, which was consistent with previous reports on plant-mediated nano-formulations, where absorption bands in the range of 270-320 nm were typically attributed to phenolic-rich extracts and their involvement in NPs interaction or stabilization (Kolahalam et al., 2019; Jamzad and Kamari Bidkorpeh, 2020). These findings were similar to those of El-Naggar et al. (2022), who reported an absorbance peak at 295 nm, while El-Naggar et al. (2024) observed a peak at 285 nm for the chitosan standard. Furthermore, Dutta et al. (2023) reported an absorbance band for chitosan NPs at 330.25 nm.

FTIR analysis characterized the functional groups present in the biosynthesized chitosan NPs derived from *S. officinalis* leaf extracts. The FTIR technique provided insight into the interactions between chitosan and phytochemicals within the extract. Typical functional groups in chitosan NPs include amide (-NH_2), hydroxyl (-OH), and C–H, C–N, C–O, and P=O bonds (Boruah and Dutta, 2021). The broad absorption band observed between 3000 and 3500 cm^{-1}, corresponding to (-OH) and (-NH_2) groups, was considered a key indicator of the chitosan NPs formation (Parida et al., 2013). The present study recorded a peak at 3421 cm^{-1}, aligning with the findings of Krishnaveni and Ragunathan (2015), who observed a band at 3437 cm^{-1} in a chitosan standard sample attributed to strong dimeric O–H stretching. Similarly, Choudhary et al. (2017) reported a peak at 3424 cm^{-1} associated with a combined amide (-NH_2) and hydroxyl (-OH) groups. The peak at 1651 cm^{-1} was attributed to C=O stretching vibrations of the amide carbonyl group (Zhang et al., 2017). The band observed at 1564 cm^{-1} indicated the presence of a primary amine group, associated with the N-H bending vibration. Although Sathiyabama et al. (2024) reported a C-H stretching peak at 2927 cm^{-1} as a chitosan structure indicator, the present study identified C–H bonds in chitosan-sage NPs at 1412 cm^{-1}. The peak at 1125 cm^{-1}, corresponding to P=O stretching, confirmed ionic cross-linking between tripolyphosphate and chitosan, which was essential for nanoparticle formation (López-León et al., 2005). Similarly, Boruah and Dutta (2021) reported the same functional groups at closely matching wavenumbers. The C-O stretching vibration of the glycosidic linkage appeared at 1024 cm^{-1} in the present study, which was close to the peak at 1029 cm^{-1} reported by Sathiyabama et al. (2024). Additionally, minor peaks at 746 cm^{-1} and 483 cm^{-1} corresponded to lower frequency skeletal bending of the chitosan backbone (Zhang et al., 2017).

The SEM analysis revealed that the biosynthesized *S. officinalis* chitosan NPs were relatively homogeneous, with particle size well-dispersed and spherical in shape. These findings were consistent with those of Sharma et al. (2013), who reported that chitosan NPs typically exhibit spherical morphology with smooth surfaces. In the present study, particle sizes ranged from 40 to 80 nm, which was comparable to the range of sizes of 33.64 and 74.87 nm reported by Khanmohammadi et al. (2015) using field emission SEM.

The antimicrobial evaluation of the biosynthesized chitosan-sage NPs demonstrated susceptibility in MRSA (Gram-positive) and *A. baumannii* (Gram-negative), with MRSA exhibiting greater sensitivity than the crude extract of *S. officinalis*. A study conducted by Randhawa et al. (2018) on the methanolic crude extract of *S. officinalis* against the standard reference strains of MRSA and *A. baumannii* revealed the sensitivity of these organisms. Previous studies revealed the antimicrobial efficacy of the crude extract of *S. officinalis*; however, these studies utilized non-encapsulated extracts, which could be constrained by poor stability, rapid degradation, and the reduced bioavailability of active compounds (Rudhani, 2018; Mendes et al., 2020; Al-Qaysi and Al-Tulaibawi, 2022). In contrast, the present study employed a chitosan-based nano-formulation, which offered several advantages, including enhanced stability, improved solubility, and controlled release of phytocomponents. Furthermore, nanoscale delivery systems can facilitate the interaction with microbial cell membranes, potentially increasing antimicrobial efficacy (Chandrasekaran et al., 2020). Therefore, the nano-encapsulation approach may offer improved antimicrobial efficacy compared to the conventional crude extract applications, suggesting its potential as a promising strategy for enhancing phytochemical delivery and antibacterial activity. Karagözlü and Özeşer (2024) reported that silver NPs synthesized from *S. officinalis* indicated inhibition zones measuring 10.5, 10, 9, and 7 mm against *Escherichia coli*, *Pseudomonas aeruginosa*, *S. aureus*, and *Bacillus subtilis*, respectively. The biosynthesized chitosan-sage NPs demonstrated maximum antimicrobial activity at 500 µg/mL, with inhibition zones reaching 21 mm for MRSA and 17 mm for *A. baumannii*. Furthermore, the MIC of chitosan-sage NPs against MRSA and *A. baumannii* was 62.5 µg/mL and 125 µg/mL, respectively, while the MBC was 125 µg/mL for MRSA and 500 µg/mL for *A. baumannii*. Lower MIC and MBC values indicated greater susceptibility of the microorganism to the tested antimicrobial agent (Balouiri et al., 2016). Therefore, MRSA was more susceptible to chitosan-sage NPs compared to *A. baumannii*. The reduced susceptibility observed in *A. baumannii* relative to MRSA might be due to the presence of an outer membrane in Gram-negative bacteria, a barrier that limited the entry of antimicrobial agents (Lazar et al., 2023). Nonetheless, the activity observed against *A. baumannii* and MRSA suggested a broad-spectrum effect of the nano-formulation. The absence of antimicrobial activity in the negative controls confirmed that the antibacterial activity was attributable to the formulated NPs rather than the solvent system. Nano-chitosan exhibited greater efficacy than its bulk chitosan due to its unique physico-chemical characteristics, which facilitated penetration through cell walls and membranes (Naskar et al., 2019). Chitosan NPs have been widely recognized for broad-spectrum antimicrobial properties (Ke et al., 2021; Dutta et al., 2023; Dou et al., 2024).

The increasing global threat of multidrug-resistant bacteria, particularly MRSA and carbapenem-resistant *A. baumannii*, underscored the urgent need for novel antimicrobial strategies (Kar et al., 2023; Mohamed et al., 2025). Molecular docking served as a computational approach for the preliminary screening and identification of potential key compounds from different sources, including natural phytocompounds, by predicting their binding affinities and interaction mechanisms with specific bacterial targets (Kar et al., 2021; Tabassum et al., 2023; Shalini et al., 2024).

Molecular docking enables the systematic development of novel therapies, particularly targeting notable resistance factors, such as PBP2a in MRSA porin and CarO in *A. baumannii* (Rosado et al., 2025; Swain and Pan, 2025). The PBP2a is a major target for the development of new anti-MRSA treatment agents, including allosteric modification and direct inhibition (Sanapalli et al., 2025). *Acinetobacter baumannii* presented a notable challenge owing to its extensive drug resistance and biofilm-forming capacity, and porins such as CarO further reinforce its resistance profile (Tram et al., 2021; Lokhande et al., 2023). The current study examined the molecular docking of certain phytocompounds, including eucalyptol, thujone, camphor, caryophyllene, neophytadiene, and a derivative of decahydro-naphthalene against PBP2a of MRSA and CarO of *A. baumannii*, to evaluate their potential as antimicrobial agents. The binding affinities observed at -4.9 to -6.7 kcal/mol indicated moderate to strong interactions, suggesting that these compounds may contribute to antibacterial activity by targeting essential bacterial proteins. Notably, compounds such as caryophyllene and the naphthalene derivative exhibited relatively higher binding affinities, suggesting a strong potential to disrupt protein function. Previous studies have highlighted caryophyllene's role in preventing biofilm formation in *S. aureus* and its presence in plant essential oils with antibacterial properties (Setiawansyah and Gemantari, 2022; Sharma et al., 2025). Additionally, camphor, thujone, eucalyptol, and neophytadiene exhibited moderate binding affinities closely following that of caryophyllene and the naphthalene derivative (Juzer et al., 2022; Vinutha et al., 2025). Neophytadiene has been identified from different plant extracts and studied for its inhibitory effects on biofilm formation in *S. aureus* (Maheswari and Kalaiselvi, 2024; Pouline et al., 2024). The slightly lowered affinity of eucalyptol and neophytadiene for CarO could be related to the fewer polar interactions, emphasizing the need for specific interaction types to achieve effective binding (Noumi et al., 2023; Khatoon et al., 2024).

The PBP2a is a critical enzyme responsible for β-lactam resistance in MRSA, and its inhibition can disrupt peptidoglycan crosslinking, ultimately compromising bacterial cell wall integrity (Shidiki and Vyas, 2022). The observed interactions, including hydrogen bonding and hydrophobic interactions, were consistent with previous studies demonstrating that stable ligand-protein interactions enhanced the inhibitory effect by improving binding stability in the active or allosteric sites (Verma et al., 2022). Similarly, interactions with the CarO protein, which was involved in membrane permeability in *A. baumannii*, suggested that eucalyptol, thujone, camphor, caryophyllene, neophytadiene, and naphthalene derivatives may interfere with nutrient transport or membrane-associated processes, thereby contributing to antibacterial effects.

The predominance of hydrophobic interactions observed for most of the compounds, particularly terpenoids, such as caryophyllene and neophytadiene, could be related to their nonpolar nature, which improved binding within the hydrophobic regions of target proteins. Moreover, the presence of hydrogen bonding in some compounds, including eucalyptol and thujone, may further stabilize ligand-protein complexes, enhancing biological activity. Importantly, the binding energies observed in the present study fell within the range reported for other bioactive phytochemicals with proven antimicrobial activity, supporting their possible role as key compounds (Degfie et al., 2022).

CONCLUSION

In the present study, chitosan NPs were biosynthesized using *S. officinalis* leaf extracts as part of a nanomedicine approach to address the growing challenge of AMR. The prepared NPs exhibited notable antimicrobial activity against multidrug-resistant MRSA and *A. baumannii* strains. The improved performance observed for the chitosan-based nano-formulation, compared to the crude extract, might be attributed to enhanced surface reactivity, improved dispersion, and more efficient interaction with microbial cells. The present findings indicated that such nanoscale systems could serve as viable candidates for antimicrobial applications. However, the present study was limited by the absence of absorption, distribution, metabolism, excretion, and toxicity (ADMET) analysis, *in vivo* evaluation, and cytotoxicity assessment, as well as the lack of detailed investigation into the long-term stability of the NPs under physiological conditions. Therefore, further studies are required to establish the safety, therapeutic efficacy, and stability of chitosan-sage NPs before potential clinical application.

DECLARATION

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Authors' contributions

Bakary Chorr conducted the laboratory experiments, data analysis, and drafted the original manuscript. James Kimotho and Mourine Kangogo supervised, reviewed, and edited the study. All authors have read and agreed to publish the final edition of the manuscript.

Availability of data and materials

The data generated during the present study are available from the corresponding author upon reasonable request.

Competing interests

The authors have declared no conflict of interest.

Ethical considerations

All authors have checked and confirmed ethical issues, including plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy. The authors have not used any AI tools for writing or preparing the present manuscript.

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Molecular Investigation and Risk Assessment of Neonatal Calf Diarrhea in Morocco: Emphasis on Rotavirus and Coronavirus Infections

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ABSTRACT

Neonatal calf diarrhea represents a major health and economic burden in Moroccan cattle farming and remains a leading cause of calf mortality. It is commonly associated with infectious agents such as rotavirus, bovine coronavirus, *Escherichia coli*, and *Cryptosporidium parvum*, and is often aggravated by dehydration and electrolyte imbalances. The present study aimed to update the epidemiological profile of viral pathogens involved in neonatal calf diarrhea in Morocco, with particular emphasis on rotavirus group A (RVA) and bovine coronavirus (BCoV). A total of 889 newborn calves from 20 farms located in five Moroccan regions (Casablanca-Settat, Rabat-Salé-Kenitra, Souss-Massa, Fes-Meknes, and Beni Mellal-Khenifra) were included in this cross-sectional observational study. Among them, 130 rectal swabs were collected from diarrheic calves between November 2022 and March 2023 and analyzed using reverse transcription quantitative PCR (RT-qPCR). The results indicated that 34% of diarrheic calves tested positive for RVA and 62% for BCoV, with a co-infection rate of 24%. Infection rates were influenced by several factors, including calf age, geographic region, dam vaccination status, calving rank, production system, and farm size. RVA infection peaked in calves aged 8–14 days, whereas BCoV was most prevalent in calves aged 21–29 days. Notably, RVA positivity was higher in calves born to vaccinated dams (40%) compared to those from unvaccinated dams (20%), while BCoV infection was more frequently detected in calves from heifers than from multiparous cows. Higher infection rates in calves were also observed in intensive farming systems. Overall, these findings confirmed the major role of rotavirus and bovine coronavirus in neonatal calf diarrhea in Morocco and suggest that the limited effectiveness of vaccination may be influenced by herd management, colostrum transfer, and biosecurity practices. The potential mismatch between vaccine and circulating strains highlights the need for continuous molecular surveillance.

Keywords: Bovine coronavirus, Calf, Neonatal diarrhea, Rotavirus A, Risk factor

INTRODUCTION

Neonatal calf diarrhea syndrome is a major health problem for the cattle industry worldwide (Tajik et al., 2012; Guise, 2023). Diarrhea in newborn calves causes significant financial losses for breeders due to poor growth, decreased milk production in the first lactation, higher veterinary costs, and an increased risk of death before weaning (Abuelo et al., 2021). This syndrome has been linked to sickness behaviors in calves, such as prolonged lying periods and decreased milk intake, which indicate compromised health and welfare in cattle (Sutherland et al., 2018; Goharshahi et al., 2021). Neonatal diarrhea is one of the most severe diseases impacting calves less than one month of age worldwide (Schild et al., 2020), leading to high morbidity and mortality due to dehydration, acidosis, and electrolyte imbalance (Foster and Smith, 2009).

From an etiological perspective, infectious diarrhea in infants and animals was attributed solely to bacteria and protozoa until the development of advanced methods, such as electron microscopy and molecular biology (Mebus et al., 1969). In 1969, the first evidence of a viral cause of diarrhea in veterinary medicine was reported, following the electron-microscopic detection of a reovirus-like particle in the feces of a diarrheic calf. This pathogen was subsequently

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identified as rotavirus, and four years later, its role as an etiological agent in severe pediatric diarrhea was confirmed (Bishop et al., 1973; Brugere and Tessier, 2010). Over time, coronaviruses were established as additional enteric pathogens contributing to neonatal diarrhea in cattle worldwide, either independently or in combination with rotavirus (Erdoğan et al., 2003).

At the molecular level, rotavirus has a double-stranded RNA genome and is classified within the family *Reoviridae*, subfamily *Sedoreovirinae*, and genus rotavirus. The genus rotavirus is divided into nine officially recognized species, designated rotavirus A-J, based on their antigenic properties and genomic characteristics. Among these species, rotavirus A (RVA) is the most prevalent species among both humans and animals and is a leading cause of neonatal diarrhea in calves (Matthijnssens et al., 2011). Rotavirus A is consistently identified as the most common and epidemiologically important viral agent associated with neonatal calf diarrhea worldwide, especially in calves during their first weeks of life (Brunauer et al., 2021). Rotaviruses infect and destroy the villous epithelial cells of the small intestine, leading to nutrient malabsorption and diarrhea. In older calves and adult cows, infection is often subclinical; however, viral shedding increases significantly around calving, likely due to physiological stress and temporary immunosuppression. This shedding contaminates the environment and increases the risk of transmission to newborn calves, which are highly susceptible to infection. Once infected, diarrheic calves excrete large amounts of virus, becoming the primary source of viral transmission within the herd and sustaining the infection cycle on the farm (Naylor, 2009).

On the other hand, bovine coronavirus (BCoV) is a single-stranded RNA virus classified within the family *Coronaviridae*, subfamily Orthocoronavirinae, genus Betacoronavirus, subgenus Embecovirus, and species Betacoronavirus 1. The genus Betacoronavirus includes five subgenera and 14 species, among which are closely related bovine-like coronavirus (BCoV-like) variants found in wild ruminants (Decaro and Lorusso, 2020).

In calves, BCoV infect and destroy villous epithelial cells of the small intestine, causing villous atrophy. Furthermore, coronaviruses infect the epithelial cells of the large intestine, potentially resulting in colitis-associated signs, such as difficulty or increased exertion during defecation (straining). Similar to rotavirus, asymptomatic adult bovines may shed the BCoV and serve as an initial source of infection for neonatal calves. Once an outbreak occurs, calves that are clinically affected serve as the main sources of virus transmission (Naylor, 2009).

In Morocco, neonatal diarrhea has been identified as a primary cause of mortality within cattle farms since the initial epidemiological studies conducted in the 1980s. It has been associated with morbidity rates of up to 70% and mortality ranging from 10% to 15%. Approximately 70% of cases are attributed to RVA, *Escherichia coli*, and *Cryptosporidium parvum* (Fassi-Fihri et al., 1988; Ebode, 1989; Chhaibi, 2008). More recent investigations using rapid immunochromatographic tests reported a 10% prevalence of diarrhea in calves, with RVA detected in 37.2% of cases and BCoV in 25% (Zouagui et al., 2017). Despite the implementation of vaccination programs and improved hygiene practices, neonatal calf diarrhea has remained a persistent and significant challenge for cattle farms across Morocco from the 1980s to recent years (Fassi-Fihri et al., 1988; Ebode, 1989; El-Haous, 1990; Chhaibi, 2008; Zouagui et al., 2017). This sustained burden reflects ongoing concerns among breeders and field veterinarians, particularly regarding the continued occurrence of the disease despite control measures (Zouagui et al., 2017).

Therefore, the present study aimed to understand the mechanisms of neonatal calf diarrhea transmission and to identify key risk factors in Morocco, in order to support the development of more effective prevention strategies. To achieve this, reverse transcription quantitative PCR (RT-qPCR) was employed to update epidemiological data, focusing on the positivity rates and distribution of RVA and BCoV according to geographic region, production system, calf age, sex, dam vaccination status, and calving rank.

MATERIALS AND METHODS

Ethical approval

The Ethics Committee authorized this study concerning Animal Sciences, Animal Health, and Veterinary Public Health at the Hassan II Agronomic and Veterinary Institute in Rabat, Morocco, in accordance with approval code CESASPV 2025 A10.

Study time and location

The present cross-sectional study was conducted between November 2022 and March 2023 and involved 20 cattle farms distributed across five major livestock-producing regions of Morocco. These regions encompass Casablanca-Settat, Rabat-Salé-Kenitra, Souss-Massa, Fes-Meknes, and Beni Mellal-Khenifra (Figure 1).

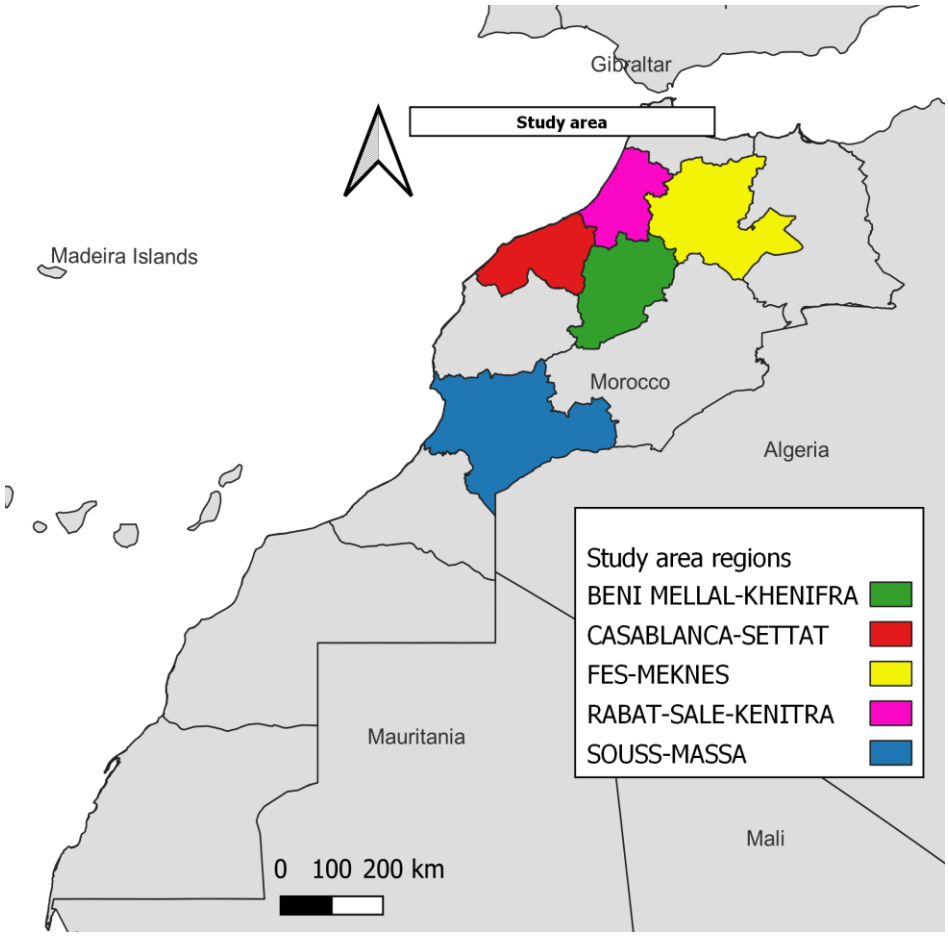


Figure 1. Geographic distribution of the five Moroccan regions involved in the study. Map produced by the author using Geographic Information System, based on administrative boundaries from the High Commission for Planning, Morocco.

Samples

A total of 889 newborn calves were clinically examined using convenience sampling. Among them, 130 animals presenting diarrhea were selected, and rectal swabs were collected between November 2022 and March 2023. Samples were immediately placed in an insulated cooler and transported at 4°C. Upon arrival at the laboratory, they were stored at -80°C until analysis. The calves included in the study were Holstein and crossbred dairy breeds, with an average birth weight ranging from 20 to 45 kg. At the time of sampling, detailed information was recorded for each individual, including the total number of calves, number of diarrheic cases, age, sex, and farming system. These data were collected through structured interviews with farm personnel and technicians. Additional information on the dams, including vaccination status and calving rank (primiparous or multiparous), was also obtained.

Molecular assays

RNA extraction

Each rectal swab was suspended in 1–2 mL of sterile phosphate-buffered saline (PBS) to produce a uniform sample while maintaining swab integrity. Viral RNA was manually isolated using the NucleoSpin® RNA Virus kit (Macherey-Nagel, Germany) in accordance with the manufacturer's protocol.

Identification and detection of pathogens by RT-qPCR

For rotavirus Group A detection, an initial denaturation step was required due to the double-stranded nature of its RNA genome. Rotavirus RNA was amplified by RT-qPCR using an in-house assay based on the PCL COVID-19 Speedy RT-PCR master mixture, originally developed for SARS-CoV-2 detection. This procedure was carried out using the following primers (Xie et al., 2015), which targeted the VP6 gene of group A rotavirus (forward primer: TCATTTCAATTGATGAGGCCACC; reverse primer: ATTCAATTCTAAGCGTGAGTCCTAC), along with a HEX-labeled probe (AATATGACACCAGCGGTAGCGGC). Thermal cycling conditions started with an initial denaturation step at 95°C for 3 minutes, followed by reverse transcription at 50°C for 15 minutes, a second denaturation at 95°C for 2 minutes, and 40 amplification cycles consisting of 95°C for 15 seconds and 60°C for 30 seconds. Samples were considered positive when a sigmoidal amplification curve was observed in the HEX fluorescence channel with a cycle

threshold (Ct) value of 40 or less (≤ 40), following the manufacturer's guidelines. For BCoV detection, the same master mix was used in combination with primers and probe described by Cho et al. (2010). The forward primer was CTAGTAACCAGGCTGATGTCAATACC, and the reverse primer was GGCGGAAACCTAGTCGGAATA, and a FAM/MGB-labeled probe CGGCTGACATTCTCGATC was used. The thermal profile for reverse transcription and amplification included 50°C for 5 minutes, followed by 95°C for 20 seconds, then 40 cycles of 95°C for 5 seconds and 60°C for 30 seconds. Samples were considered positive for BCoV when a sigmoidal amplification curve appeared in the FAM/MGB fluorescence channel with a Ct value of ≤ 40, following the manufacturer's instructions.

Statistical analysis

Statistical analyses were performed using RStudio software (version 2023). Descriptive statistics were used to summarize the distribution of bovine rotavirus A (RVA) and bovine coronavirus (BCoV) positivity according to the studied factors, including sex, age, geographic region, farming system, dam's vaccination status, and calving rank. Associations between categorical variables and virus positivity were assessed using Pearson's chi-square (χ^2) test. When the expected cell counts were less than five, Fisher's exact test was used instead. A p-value < 0.05 was considered statistically significant.

RESULTS

Among the 889 calves examined, 130 (14.6%) presented with diarrhea. RT-qPCR analysis of these samples indicated that 34% (44/130) tested positive for RVA, while 62% (80/130) tested positive for BCoV. Notably, 24% (31/130) of the diarrheic calves were co-infected with both viruses (Graph 1). Multiple factors were assessed for their potential association with RVA and BCoV detection, such as calf sex, region, age, dam vaccination status, calving rank, and the production system.

Variation by sex

The present results indicated that calf sex had no significant effect on RVA or BCoV positivity rates ($p > 0.05$; Graph 2).

Regional variation

PCR testing revealed that the Rabat region had the highest RVA positivity rate (58%), whereas the Fez region showed the highest BCoV positivity rate (81%, Figure 2). Pearson's chi-square test showed that BCoV positivity differed significantly among geographic regions ($\chi^2 = 11.148$, $p < 0.05$), whereas no significant regional differences were observed for RVA positivity.

Variation by calf's age

The current findings indicate that the 8-14-day age group had the highest RVA infection rate, at 42%. The infection remained at high levels until approximately 30 days of age. However, BCoV prevalence was highest in the 21-29-day age group, reaching 75% (Graph 3). The current results indicated that these differences in both RVA and BCoV by calf age were not statistically significant ($p > 0.05$).

Variation by dams' vaccination status

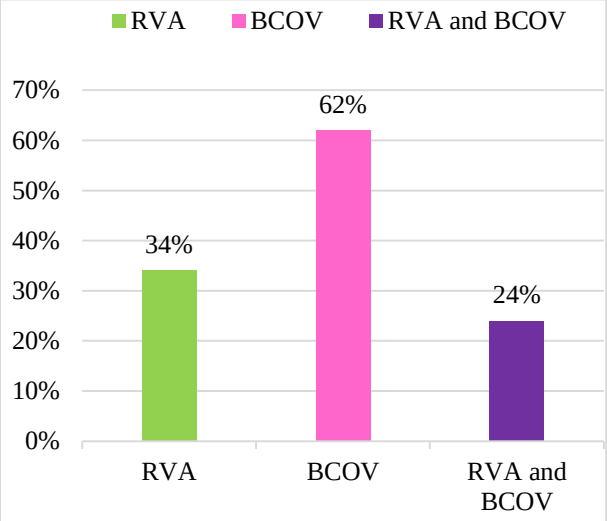
Graph 4 illustrates the positivity rates for RVA and BCoV by maternal vaccination status. Regarding RVA infection, calves born to vaccinated dams had a higher positivity rate (40%) than those born to unvaccinated dams (20%; $p < 0.05$). For BCoV infection, the positivity rate was marginally higher in calves born to vaccinated dams (64%) than in their unvaccinated counterparts (60%), although this difference was not statistically significant ($p > 0.05$).

Variation by calving rank

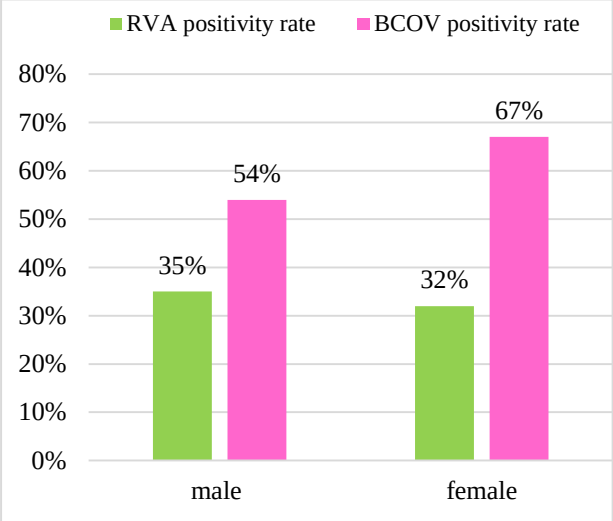
No significant difference was observed in RVA positivity between calves born to heifers and multiparous dams ($p > 0.05$). However, calves from heifers exhibited a significantly higher BCoV positivity rate compared with those from multiparous cows (Graph 5).

Variation by farming system

RT-qPCR analysis indicated that calves from industrial farms exhibited a significantly higher infection rate than those from small- or medium-sized farms ($p < 0.05$; Graph 6).



Graph 1. Positivity rates of rotavirus group A (RVA) and bovine coronavirus (BCoV) in diarrheic calves in Morocco from November 2022 to March 2023



Graph 2. Variation of rotavirus group A (RVA) and Bovine Coronavirus (BCoV) positivity rates by calf's sex in Morocco from November 2022 to March 2023

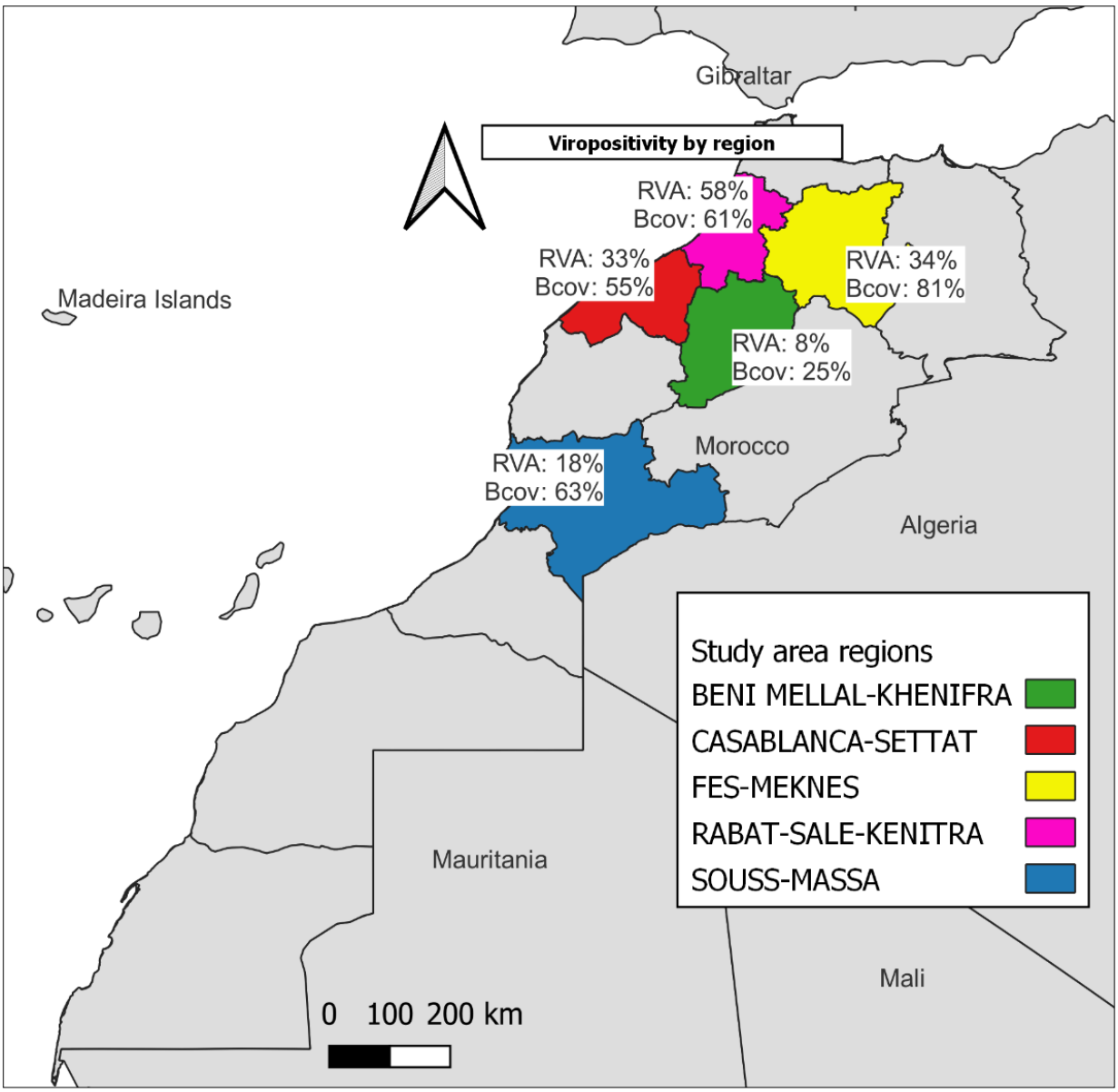
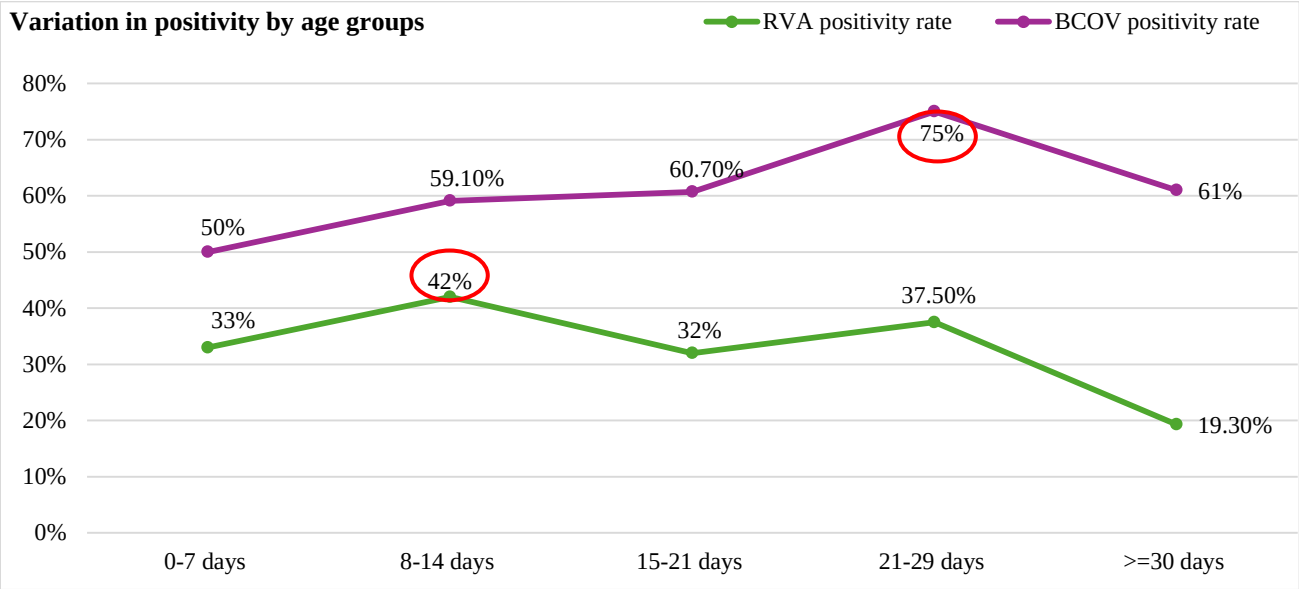
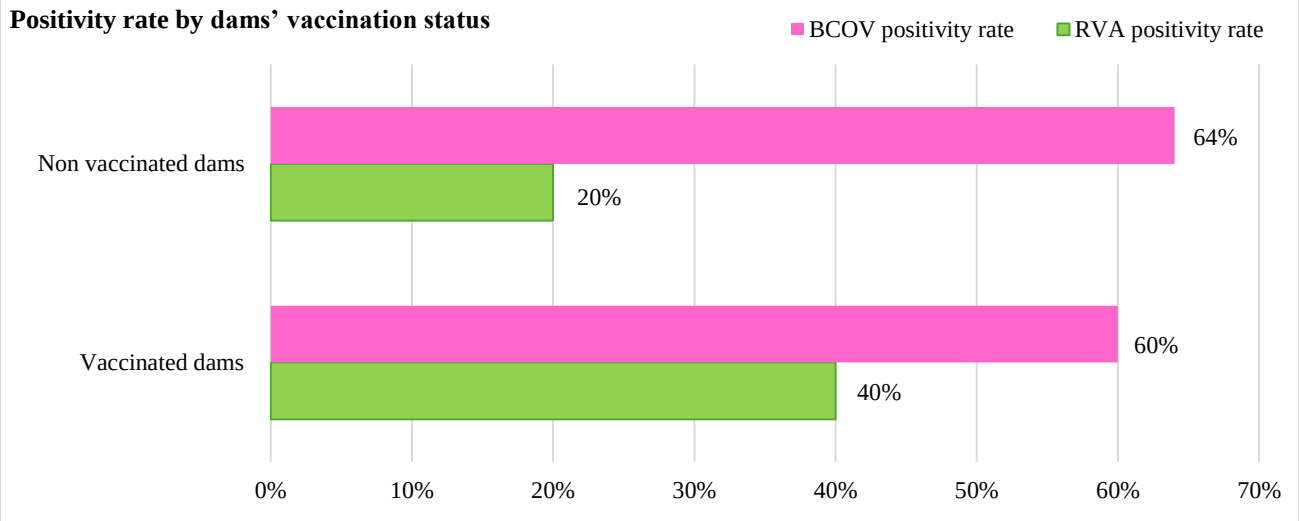


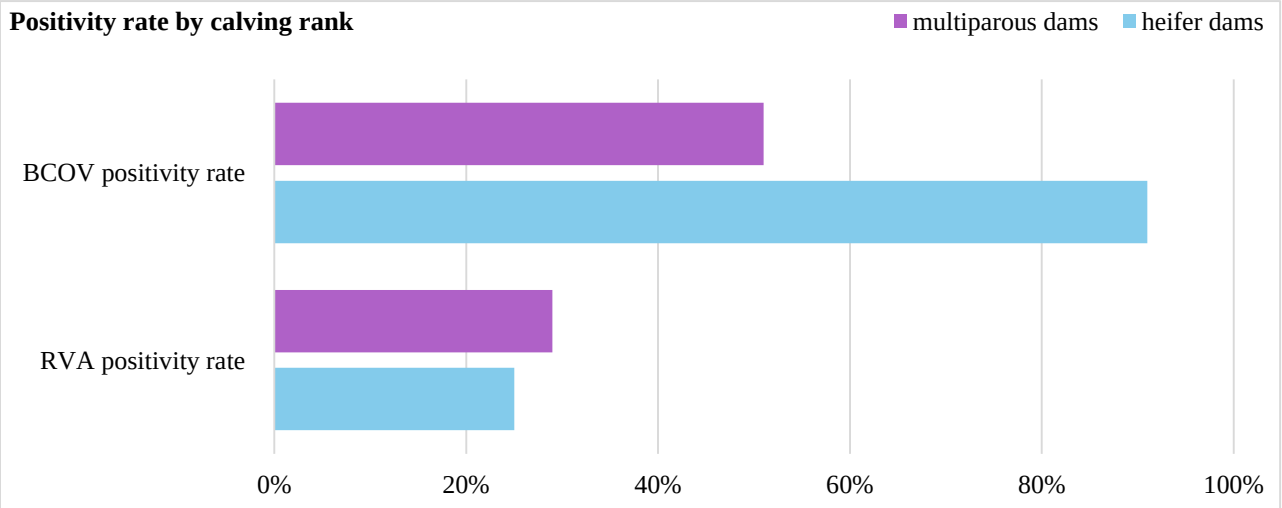
Figure 2. Variation of Rotavirus group A (RVA) and Bovine Coronavirus (BCoV) positivity rates according to regions in Morocco from November 2022 to March 2023



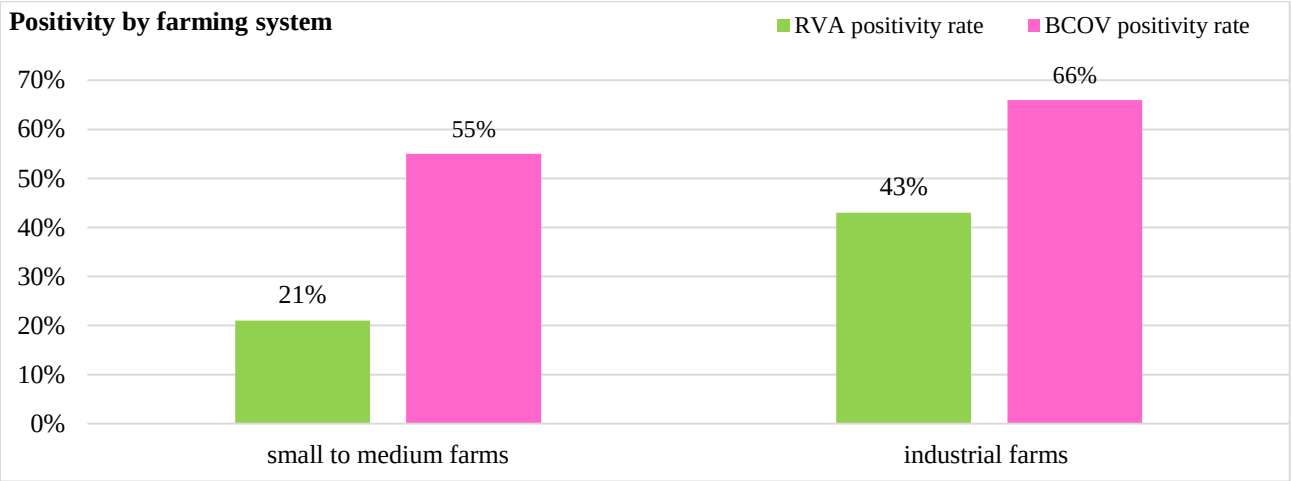
Graph 3. Variation of rotavirus group A (RVA) and Bovine Coronavirus (BCoV) positivity rates by calf's age in Morocco from November 2022 to March 2023



Graph 4. Variation of rotavirus group A and Bovine Coronavirus positivity rates according to dams' vaccination status in Morocco between November 2022 and March 2023



Graph 5. Variation of rotavirus group A and Bovine Coronavirus positivity rates according to the calving rank in Morocco between November 2022 and March 2023



Graph 6. Variation in rotavirus group A and bovine coronavirus positivity rates according to farming system in Morocco between November 2022 and March 2023

DISCUSSION

Neonatal calf diarrhea is a complex syndrome that impacts cattle farms, affecting both productivity and animal welfare. Neonatal calf diarrhea is recognized as the leading cause of illness and death in newborn calves on dairy farms worldwide (Urie et al., 2018; Caffarena et al., 2021). The health of calves in their early days is crucial for the success of any dairy operation. High rates of diarrhea-related illnesses result in substantial economic losses attributable to increased mortality rates and treatment costs (Rocha et al., 2019). Investigations into neonatal calf diarrhea in Morocco predominantly date back to studies conducted during the 1980s and 1990s (Fassi-Fihri et al., 1988). Only one recent study, employing a rapid immunochromatographic test, was conducted in 2017 (Zouagui et al., 2017). Consequently, the existing epidemiological data on this syndrome in Moroccan livestock remained outdated, despite the ongoing incidence of neonatal diarrhea in the country, underscoring the importance of conducting the present study.

Diarrhea prevalence in the study area

The current study identified a prevalence of neonatal diarrhea at 14.6% among Moroccan calves, exceeding the 10% rate reported by Zouagui et al. (2017). This prevalence was considerably lower than the high rates recorded in the 1980s and 1990s, which were 75% and 74.1%, respectively (Fassi-Fihri et al., 1988; El-Haous, 1990). The prevalence of neonatal calf diarrhea differed considerably across countries. For instance, rates of 30%, 37.5%, and 33.9% have been reported in Kuwait (Majeed and Alazemi, 2023), Korea (Chae et al., 2021), and Ethiopia (Umer Seid et al., 2020), respectively. In Canada, morbidity rates of neonatal calf diarrhea ranged from 20% to 40% (Bongers et al., 2022). In Morocco, the incidence of neonatal calf diarrhea has declined markedly from over 75% in the 1980s and 1990s to approximately 10% in 2017. This decline could be due to improvements in calf health, advances in husbandry practices, increased farmer awareness of hygiene protocols, the implementation of vaccination programs, and greater colostrum management through early ingestion, freezing methods, and immunoglobulin measurement to ensure adequate immunity in newborn calves against the pathogens responsible for neonatal diarrhea (Zouagui et al., 2017). Despite these control measures, neonatal diarrhea is still a persistent and possibly increasing problem in Moroccan cattle farming. The persistent prevalence of neonatal diarrhea in calves (14%) highlighted the need for updated epidemiological monitoring and the use of advanced diagnostic tools.

Positivity rates

The RT-qPCR analysis revealed an RVA positivity rate of 34%, slightly lower than the 37.2% reported by Zouagui et al. (2017) but higher than the 29.9% reported by Fassi-Fihri et al. (1988). Notably, the RVA prevalence on Moroccan farms has remained relatively unchanged over the past years, despite improvements in breeding practices and vaccination programs. It should be noted that the diagnostic methods differed among studies; Zouagui et al. (2017) employed a rapid immunochromatographic test, whereas Fassi-Fihri et al. (1988) used the Enzyme-Linked Immunosorbent Assay. Comparable findings have been reported in Turkey at 30% (Mamak et al., 2023), whereas lower prevalence has been reported in China at 23.2% (Wang et al., 2023) and Algeria at 20.7% (Ammar et al., 2014). In contrast, BCoV indicated a markedly higher positivity rate in the present study (62%), exceeding the 25% reported by Zouagui et al. (2017). This rate of BCoV was considerably higher than those reported in China (11.8%; Wang et al., 2023), Turkey (8%; Mamak et

al., 2023), Algeria (20.7%; Ammar et al., 2014), and Kuwait (1%; Majeed and Alazemi, 2023). The present results indicated that in Morocco, the RVA positivity rate has slightly changed over time, whereas BCoV demonstrated a marked increase compared with previous Moroccan studies.

Positivity rate by sex

In the present study, the prevalence of RVA and BCoV infections did not notably differ between male and female calves. Similar results have been documented in previous studies conducted by Radhy (2015), Yavru et al. (2016), and Zaitoun et al. (2018). This lack of difference might be attributed to the comparable anatomical, physiological, and hormonal characteristics of calves at an early age, leading to similar susceptibility to infection (Yavru et al., 2016). Conversely, some studies have reported sex-related differences. In Algeria, Ammar et al. (2014) found higher infection rates in males (59.3%) than in females (40.7%) for RVA and BCoV. This finding was attributed to the higher birth weight of males, which increases the risk of dystocia and, consequently, impairs colostrum absorption, predisposing cattle to infection. In Ethiopia, Debelo et al. (2021) observed that male calves were more likely to be infected with RVA, which was consistent with the findings of Ammar et al. (2014). In addition, management practices may influence infection rates, as in some production systems, female calves receive greater health care and husbandry due to their higher economic value, which might explain the lower prevalence of infection observed in females (Chaudhary et al., 2013).

Positivity rate by region

The results of this study showed that the prevalence of RVA did not vary significantly between the regions studied. Similarly, in Turkey, the proportion of RVA-positive calves ranged from 9.7% in Basgedikler to 36.4% in Kumbetli, although these inter-locality differences were not statistically significant ($p > 0.05$) (Erdoğan et al., 2003). Such variations in positivity rates may be influenced by factors including farm hygiene, herd management practices, and regulations on animal movement (Asadi et al., 2015). In contrast, BCoV prevalence exhibited substantial regional variation, consistent with Asadi et al. (2015), who reported differences in BCoV prevalence across geographic locations, with prevalence varying between countries such as Turkey (1.96%; Okur-Gumusova et al., 2007), Costa Rica (9%; Pérez et al., 1998), and Switzerland (7–8%; Uhde et al., 2008), whereas a study in Egypt found no significant association between BCoV prevalence and geographic location (Elgioushy et al., 2025). According to Wang et al. (2023), farm location may influence calves' susceptibility to RVA and BCoV infections, with drier areas associated with higher infection rates, whereas wetter environments, particularly near rivers, were linked to a higher incidence of diarrhea caused by *Cryptosporidium parvum*. In Morocco, environmental conditions were relatively similar across the regions studied, suggesting that the observed differences in viral prevalence are more likely attributable to variations in hygiene practices and herd management rather than geographic or climatic factors.

Positivity rate by age

The current findings revealed that calves aged 8-14 days had the highest RVA infection rate (42%), whereas BCoV prevalence peaked in calves aged 21-29 days (75%). These results were consistent with the findings of Ammar et al. (2014), who reported that calves in their second (8-14 days) and fourth (22-30 days) weeks of life are most susceptible to RVA and BCoV, respectively. Similarly, Al-Robaiee and Farwachi (2013) in Iraq and Volkan et al. (2016) in Turkey detected RVA predominantly in calves aged 8-15 days. Broader studies by Zaitoun et al. (2018) and Berber et al. (2021) reported that RVA infections occurred mainly between 3-15 and 4-14 days, respectively, and BCoV infections occurred between 30-37 days and 4-30 days, respectively. In Morocco, Fassi-Fihri et al. (1988) reported a 29.7% incidence of RVA in diarrheic calves, with lower prevalence observed after 20 days of age. In 2017, Zouagui et al. (2017) observed that viral infections, such as RVA and BCoV, were most frequent during the first two weeks of life. The difference between the present results and those of previous Moroccan studies was most apparent during the first week of life, when infection rates were higher than those observed in the present study. This may be explained by the enhanced care provided to calves during the first week of life. After the first week, the decline in passive immunity, combined with limited natural resistance to these pathogens, may increase susceptibility to infection (Okur-Gumusova et al., 2007). Furthermore, the presence of BCoV infection in older calves (22-30 days) could be attributed to group housing practices for calves older than two weeks, rather than the use of individual stalls (Ammar et al., 2014).

Positivity rate by dams' vaccination

The RT-qPCR analysis indicated a notably higher RVA positivity rate in calves born to vaccinated dams (40%) compared to those from unvaccinated dams (20%). For BCoV infection, rates were almost similar between calves from vaccinated and unvaccinated dams at 60% and 64%, respectively. The current results are consistent with Zouagui et al.

(2017), who reported no considerable differences in viral prevalence between vaccinated and unvaccinated farms in Morocco. Similarly, a study conducted in Brazil by Rocha et al. (2017) compared two groups of herds with similar colostrum management practices. Rocha et al. (2017) reported that calves born to dams producing sufficient colostrum (4 liters at the first feeding) had a higher RVA positivity rate when the dams were vaccinated than when unvaccinated (3.92% versus 1.11%). In addition, the clinical appearance of feces was evaluated in both groups, and the proportions of normal and diarrheic samples were nearly identical (27.4% versus 27.3%; Rocha et al., 2017). The higher RVA positivity rate observed in calves born to vaccinated dams indicated that maternal vaccination alone may not be sufficient to prevent viral infection under field conditions (Weaver et al., 2000; Dhama et al., 2009). This finding may reflect the complex interactions across the circulating viral strains, maternal immune response, colostrum management, and herd-level infection pressure. While vaccination is designed to enhance passive immunity, its effectiveness can be influenced by strain diversity and herd management, which may limit the expected reduction in viral circulation (Parreño et al., 2010).

It has been indicated that pre-calving vaccination programs should systematically include pregnant heifers, as they are often overlooked in practice. Excluding heifers from vaccination reduces the passive immunity transferred to their calves, thereby increasing the risk of neonatal diarrhea. Additionally, Viidu and Mõtus highlighted the critical role of feeding calves not only with colostrum but also with hyperimmune transition milk from vaccinated dams for at least the first two weeks of life. This milk, produced in the days after colostrum, retains high levels of antibodies induced by vaccination, providing persistent protection in the gastrointestinal tract. Implementing such feeding practices is crucial to maintain lactogenic immunity during the period when calves are most vulnerable to enteric infections. Bürki et al. (1986) demonstrated notable protective effects of dam vaccination, notably reducing clinical signs and the duration of RVA and coronavirus shedding in calves. Furthermore, Bürki et al. (1986) highlighted the importance of feeding calves' milk from their vaccinated dams for at least 14 days to ensure sustained lactogenic immunity.

These findings indicated that vaccination of pregnant cows in the final third of gestation could substantially reduce the clinical impact of RVA and coronavirus infections in calves. Nevertheless, the effectiveness of vaccination depends on multiple factors, including colostrum quality and hygiene management during the peripartum period. Several studies have reported different causes of vaccination failure. For instance, the absence of reduction in RVA infection despite vaccination might be linked to the virus's high mutation rate. Reassortment and rapid replication enable RVA to acquire new virulence traits that may not be protected against maternal vaccination (Thiry, 2007; Zouagui et al., 2017). Furthermore, management-related issues, such as improper storage, failure to maintain the cold chain, and administering the vaccine close to calving period, can compromise vaccine efficacy and effective immune responses (Quillet et al., 2006). Other factors may interfere with vaccine-induced protection against rotavirus infection, even when vaccination is applied. In Moroccan herds, failures of the maternal immune response following vaccination might be associated with nutrition, parasitism, or immunosuppressive conditions such as bovine viral diarrhea (BVD) or mycotoxicosis (Zouagui et al., 2017). Moreover, Fontaine (2018) reported that a mismatch between vaccine valence and the actual etiological agents responsible for neonatal enteritis was a key factor contributing to vaccination failure in cattle herds. These findings highlighted the importance of identifying circulating viral strains through molecular characterization and sequencing to ensure the relevance of vaccine strains under Moroccan field conditions.

Positivity rate by calving rank

Analysis of 39 calves with available breeding-rank data revealed a notably higher positivity rate of BCoV-infected calves born to heifers compared to those born to multiparous dams. This observation is consistent with the findings of Quillet et al. (2006), who reported that the frequency of BCoV infection decreased with increasing calving rank. In contrast, no association between RVA infection and calving rank was detected in either study. Regarding BCoV infection, the higher susceptibility of calves born to heifers may be related to lower antibody levels in first-lactation colostrum compared with those of multiparous cows, as colostral antibody levels typically increase from the third lactation (Guerin, 2009).

Positivity rate by type of farming system

The present findings indicated that calves raised on industrial farms were more frequently infected with RVA and BCoV than those raised on small farms. This finding might be due to higher animal density, which has been reported to increase the risk of diarrheal disease in calves (Erdoğan et al., 2003). High infectious pressure and a large proportion of susceptible animals in close contact can spread the viral infections. Both healthy adult cattle and calves can shed RVA in their feces, thereby contaminating the environment and increasing infectious pressure in crowded herds. Animals in larger herds or groups with close contact are more likely to be exposed to infectious pathogens and experience more stress. Thus, infection pressure, environmental contamination, stress-related immunosuppression, and overcrowding are

key factors that contribute to epidemics in herds where calves and their dams are closely housed (Torres et al., 1985; Wells et al., 1997; Erdoğan et al., 2003).

Positivity rates according to season

In addition to the risk factors examined, the present study was conducted during the optimal season for viral detection, from November to March (Berber et al., 2021). Hassine-Zaafrane (2011), Nour Mohammad Zadeh et al. (2012), and Chen et al. (2022) have reported higher RVA incidence during colder months, as low temperatures and relative humidity prolong viral survival (Brandt et al., 1982). Furthermore, colostral IgG levels in dairy cows are lower in winter compared to other seasons, reducing passive immunity in newborn calves (Gulliksen et al., 2008). Additionally, the prevalence of BCoV was affected by seasonal variations. Consistent with the present findings, Williams et al. (2007) observed a prevalence of 2% during summer and 9% in winter. The increased occurrence of BCoV-associated diarrhea in winter might be due to enhanced viral stability under colder environmental conditions (Radostits et al., 2007). Similarly, Zaitoun et al. (2018) observed that RVA and coronavirus infections in calves peaked during colder months, with lower infection rates recorded during warmer periods.

CONCLUSION

The present study showed that RVA remains a major causative agent of neonatal calf diarrhea, consistent with previous reports. Bovine coronavirus was also frequently detected and showed a higher positivity rate than RVA. The association between both RVA and BCoV and the examined risk factors was evaluated, including calf sex, region, age, dam vaccination status, calving rank, and farming system. The overall diarrhea prevalence (14.6%) confirmed that the syndrome continues to pose a major health and economic challenge to Moroccan cattle farming. The limited effectiveness of vaccination observed in this study indicated that protection against rotavirus and Coronavirus is influenced by multiple other factors, including herd management, colostrum feeding, and biosecurity measures. In addition, the possible mismatch between vaccine valence and circulating viral strains highlights the importance of ongoing molecular surveillance and sequencing of field isolates to ensure vaccine relevance. Altogether, these findings emphasize the need for integrated control strategies combining molecular diagnostics, adapted vaccination, and improved farm management to reduce the burden of neonatal diarrhea in Moroccan calves.

DECLARATIONS

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Authors' contributions

Imane Choukri conducted the fieldwork and laboratory experiments, analyzed the data, and wrote the manuscript. Nadia Touil and Ouafaa Fassi Fihri supervised the laboratory experiment and reviewed the manuscript. Zaid Zouagui supervised the fieldwork and data analysis. Zineb Rhazzar, Hanan El Ouadi, and El Mehdi Belouad participated in the laboratory experiment. Ikhlass El Berbri supervised both field and laboratory activities and contributed to manuscript writing. All authors read and approved the final edition of the manuscript.

Availability of data and materials

The data that support the findings of the present study are available from the corresponding author upon reasonable request.

Ethical considerations

The authors confirm that the manuscript was checked for plagiarism and duplicate submission and has not been submitted to any other journal. All authors reviewed and approved the final version of the manuscript before submission.

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Competing interests

The authors have not declared any conflict of interest.

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Effects of Different Natural Feed Types on Domestication, Survival Rate, and Growth of Glass Catfish (*Kryptopterus palembangensis*)

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ABSTRACT

Intensive, uncontrolled fishing has primarily reduced glass catfish (*Kryptopterus palembangensis*) populations in Indonesian waters, especially in Sumatra, leading to a steady decline in natural populations through continuous overexploitation. The present study aimed to evaluate the effects of different natural feeds on the growth performance and survival of glass catfish during the domestication phase (process of adapting wild fish to aquaculture conditions using appropriate natural feed types). A total of 150 glass catfish, with an average initial length of 14.62 ± 0.53 cm and an average initial weight of 15.58 ± 0.73 g, were subjected to the present study, using a completely randomized design with five treatment groups and three replications. Each experimental unit contained 10 fish, resulting in 30 fish per treatment. The treatment groups consisted of different natural feed types, namely small live fish (P1), shrimp (P2), earthworms (P3), maggots (P4), and *Tubifex* sp. (P5). Growth performance, survival rate, and digestive enzyme activity of glass catfish were observed after 30 days of rearing. The present results indicated that Group P2 significantly improved the growth performance of glass catfish, including absolute weight (3.61 ± 0.16 g), absolute length (2.85 ± 0.11 cm), specific growth rate (0.85 ± 0.03% day^{-1}), and daily growth rate (0.12 ± 0.01 g day^{-1}) compared to other groups. Additionally, Group P2 exhibited the highest survival rate at 100% and the highest digestive enzyme activities, with amylase at 7.24 ± 0.14 U mL^{-1}, lipase at 0.37 ± 0.01 U mL^{-1}, and protease at 0.69 ± 0.01 U mL^{-1}. The second-best performance was observed in Group P1, followed by P3, P5, and P4. Shrimp was identified as the most effective natural feed for improving the growth performance and survival rate of glass catfish.

Keywords: Digestive enzyme, Domestication, Glass catfish, Growth, Survival rate

INTRODUCTION

Glass catfish (*Kryptopterus palembangensis*) is a freshwater species native to Indonesia, commonly found in the Sumatra rivers, such as the Musi, Lematang, and Ogan (Yonarta et al., 2023a), as well as in the Kalimantan region (Fishbase, 2023; Yonarta et al., 2023b). The population of this species tends to increase during the rainy season, as it is closely associated with food availability, migration patterns, and water levels (Gumiri et al., 2018; Nopiri and Elvyra, 2018). Glass catfish has high economic value and is highly popular among local communities, resulting in intense and uncontrolled fishing activities (Pratiwi et al., 2023). Consequently, natural populations of glass catfish continue to decline. Data from the Ministry of Maritime Affairs and Fisheries (KKP, 2022) indicated that total glass catfish production in South Sumatra decreased markedly from 460,511.78 tons in 2019 to 274,048.54 tons in 2021. This decline in production indicated increasing pressure on natural populations and threatened the long-term survival of glass catfish in its natural habitat. One potential solution to address this issue is domestication, which is defined as adapting wild fish to controlled aquaculture conditions, including feeding management and environmental regulation, to achieve sustainable production outside their natural habitat.

Domestication is a strategic approach in fisheries management that aims to adapt wild fish species to live and reproduce in controlled cultivation environments (Yonarta et al., 2023a). Domestication is a lengthy process that demands careful planning across several aspects, including technical requirements, socio-economic readiness, availability of facilities, technology, and human resources. Furthermore, a thorough understanding of fish biology, such as feeding habits, growth patterns, and survival rates, is essential for successful domestication (Teletchea, 2015; Koniyo and Juliana, 2025). According to Zulfadhli et al. (2023), the fish domestication process comprises three main stages,

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including adaptation to the cultural environment to ensure survival, growth optimization, and reproductive stimulation for successful breeding. Several preliminary studies have been conducted on glass catfish domestication, including natural diet and feeding preferences through stomach content analysis and growth performance (Lukas and Minggawati, 2014), feeding habits to understand dietary behaviour (Lestari et al., 2021), genetic relationships via DNA analysis (Syaifudin et al., 2021), growth patterns to evaluate biological characteristics (Ahmadi, 2022), stocking density to optimize rearing conditions (Agusnimar and Rosyadi, 2013), and reproductive biology to support breeding strategies (Pratiwi et al., 2023). Another key factor influencing the success of domestication is the fish's ability to adapt to cultural conditions and utilize available natural feed sources (Palińska-Żarska et al., 2020). According to Dwitasari et al. (2017), glass catfish are classified as carnivores (meat eaters) that generally rely on animal-based natural feeds; therefore, selecting appropriate feed types is crucial to support their growth in culture conditions. Previous studies have demonstrated that different natural feeds can significantly influence fish growth, such as the use of earthworms for snakehead fry (Herlina, 2016), shrimp for pomfret (Taufiq et al., 2016), maggots for Sangkuriang catfish (Berampu et al., 2021), and silkworms for dumbo catfish fry (Mahendra et al., 2022). These findings indicated that feed type plays an important role in determining growth performance across fish species. However, information regarding the effects of different natural feed types on the growth and survival rate of glass catfish during the domestication process remains limited.

Therefore, the present study aimed to evaluate the effectiveness of different natural feeds in improving growth performance and survival rate of glass catfish, to develop appropriate feeding strategies for successful domestication.

MATERIALS AND METHODS

Ethical approval

The present study was conducted in accordance with the guidelines of the Faculty of Agriculture, Sriwijaya University, Palembang, South Sumatra, Indonesia. All experimental procedures were carried out in compliance with established standards for both laboratory and field experiments. The experimental animals were monitored daily through physical observations, and feeding was administered according to the respective treatment groups.

Location and time

The present study was conducted at the Basic Fisheries Laboratory, Aquaculture Laboratory, and Experimental Pond, Aquaculture Study Program, Fisheries Department, Faculty of Agriculture, Sriwijaya University, Indonesia, from April to June 2025.

Study design

A total of 150 glass catfish with an average initial length of 14.62 ± 0.53 cm and an average initial weight of 15.58 ± 0.73 g were obtained from local fishermen in the Pengumbuk River, Petaling Village, Musi Banyuasin Regency, South Sumatra, Indonesia. The study employed a completely randomized design with five treatment groups and three replications. Each experimental unit contained 10 fish, yielding a uniform stocking density across all treatment groups and a total of 30 fish per treatment. The treatment groups were designed according to the different types of natural feed provided to the glass catfish, including small live fish (P1), shrimp (*Caradina* sp.; P2), earthworms (P3), maggots (P4), and *Tubifex* sp. (P5).

Container preparation

The rearing container used in the present study was a plastic box measuring 35 × 60 × 35 cm^3 with a water volume of 60 L. Before utilization, the plastic box was washed and disinfected with potassium permanganate at a concentration of 2.5 mg L^{-1} (Agustini et al., 2020). Subsequently, the boxes were equipped with an aeration system for oxygen supply and labeled with a random treatment code. Each rearing container contained 10 glass catfish. The fish were stocked in the morning and acclimatized for seven days to adapt to the new environment. After the acclimatization period, initial length and weight were recorded, and water quality parameters for fish rearing were measured.

Fish rearing

Glass catfish were reared for 30 days and fed according to the respective treatments, which included earthworms (30% of crude protein), maggots (50% of crude protein), shrimp (20% of crude protein), *Tubifex* sp. (50% of crude protein), and small live fish (50% of crude protein). The metabolizable energy of each feed was estimated based on crude protein content, following previous studies on nutrient utilization in glass catfish (*Kryptopterus lais*; Rosyadi et al., 2025). An at-satiation feeding regime was applied, with three times per day at 08:00, 12:00, and 16:00 Western Indonesian Time (WIB).

Growth parameters

The absolute weight growth of glass catfish during 30 days of rearing was calculated using Effendie's (2002) formula (Formula 1).

$W = Wt\text{-}W_0$ (Formula 1)

Where W represented the absolute weight gain (g), Wt represented the final fish weight at the end of the rearing period (g), and W0 represented the initial fish weight at the beginning of the rearing period (g). The absolute length growth of glass catfish during 30 days of rearing was calculated using the following formula (Formula 2).

$L = L_t\text{-}L_0$ (Formula 2)

Where L represented the absolute length growth (cm), Lt represented the final length of fish at the end of the rearing period (cm), and L0 represented the initial length of fish at the beginning of the rearing period (cm). The daily growth rate (DGR) of glass catfish during 30 days of rearing was calculated using Formula 3 (Zonneveld et al., 1991).

$$DGR = \frac{Wt-Wo}{t} \times 100 \quad \text{(Formula 3)}$$

Where DGR represented the daily growth rate (%/day), Wt represented the final average weight (g), Wo represented the initial average weight (g), and t represented the rearing time (days). The specific growth rate (SGR) of glass catfish during 30 days of rearing was calculated using Formula 4 (Zonneveld et al., 1991).

$$SGR = \frac{ln\,(Wt) - ln(Wo)}{t} \times 100\% \quad \text{(Formula 4)}$$

Where SGR represented the specific growth rate (%), Wt represented the average weight of fish on the final day of rearing (g), Wo represented the average weight of fish on the initial day of rearing (g), and t represented the rearing time (s).

Survival rate

The survival rate of glass catfish was calculated using Formula 5 (Effendie, 2002).

$$\text{Fish survival (\%)} = \frac{Nt}{N0} \times 100 \quad \text{(Formula 5)}$$

The number of surviving fish was determined by comparing the number of fish at the end of the rearing period (Nt) to the number at the beginning (N0), where Nt and N0 represented the number of fish on the final and initial days of rearing, respectively.

Digestive enzyme activity

The amylase, lipase, and protease activities were analyzed, highlighting the major digestive enzymes involved in carbohydrate, lipid, and protein digestion in fish. Enzyme activity assays were conducted at two sampling points, namely at the beginning of the experiment (day 0) and at the end of the rearing period (day 30). At each sampling time, three fish per treatment were randomly selected and anesthetized using clove oil (50 mg L^{-1}) before dissection. The fish were then humanely euthanized, and the digestive tract was carefully removed. Approximately 0.5-1.0 g of stomach and intestinal tissue was collected, pooled per replicate, and homogenized in cold phosphate buffer for enzyme analysis.

Amylase activity was determined following the method described by Worthington (1993). Briefly, 0.5 mL of enzyme extract was mixed with 1 mL of 1% starch solution and incubated at 95 °C for three minutes. The reaction was terminated by adding 0.5 mL of dinitrosalicylic acid (DNS) reagent (Merck, Germany), followed by further incubation at 95°C for five minutes. Absorbance was measured at 540 nm using a spectrophotometer (Shimadzu, Japan).

Lipase activity was measured according to the method described by Borlongan (1990). A mixture of 1.5 mL of olive oil substrate and 1 mL of 0.1 M Tris-HCl buffer (pH 8.0) was homogenized and incubated at 37°C for six hours. The reaction was stopped by adding 3 mL of 95% ethanol (Merck, Germany), and the released fatty acids were titrated with 0.01 N NaOH (Merck, Germany).

Protease activity was analyzed following the method of Bergmeyer et al. (1983). The reaction mixture contained 1 mL of 0.05 M phosphate buffer (pH 7) and 1 mL of 20 mg mL^{-1} casein solution (pH 7), which was incubated at 37°C for ten minutes. The reaction was terminated by adding 2 mL of 0.1 M trichloroacetic acid (TCA; Merck, Germany), followed by centrifugation at 3,500 rpm for ten minutes. The supernatant was then mixed with 5 mL of 0.4 M Na_2CO_3 (Merck, Germany) and 1 mL of Folin-Ciocalteu reagent (Merck, Germany), and incubated at 37 °C for 20 minutes. Absorbance was measured at 578 nm using a spectrophotometer (Shimadzu, Japan).

Water quality

Throughout the 30-day rearing period, water quality parameters, including temperature, pH, dissolved oxygen (DO), and ammonia, were monitored (Table 1). Temperature and pH were measured daily in the morning, afternoon, and evening. Dissolved oxygen and ammonia were measured at the beginning, middle, and end of cultivation.

Table 1. Water quality parameters measured during the study

Parameter	Unit	Methods/tools
Temperature	°C	Thermometer
pH	-	pH meter
DO	mg/L	DO meter
Ammonia	mg/L	Spectrophotometer

DO: Dissolved oxygen

Data analysis

The growth data obtained were processed using Microsoft Excel 2021 and IBM SPSS Statistics 25. Data were analyzed using one-way ANOVA at a significance level of p-value less than 5% ($p < 0.05$). Significant differences among treatments were further analyzed using Duncan's multiple range post hoc test.

RESULTS

Absolute weight

The present results indicated that providing different natural feeds significantly affected the absolute weight of glass catfish ($p < 0.05$; Figure 1). The highest absolute weight gain was observed in Group P2 (shrimp feed) at 3.61 ± 0.16 g, which differed significantly from the other four treatments ($p < 0.05$). Additionally, Group P1 (live fish feed) recorded an absolute weight of 3.22 ± 0.25 g, which was significantly higher than the other three treatments ($p < 0.05$). The remaining groups demonstrated progressively lower absolute weights, including 2.71 ± 0.36 g for Group P3, 2.44 ± 0.16 g for Group P5, and 2.28 ± 0.06 g for Group P4. The current results indicated that Group P2 had the greatest potential to increase the growth of glass catfish compared to other natural feed sources.

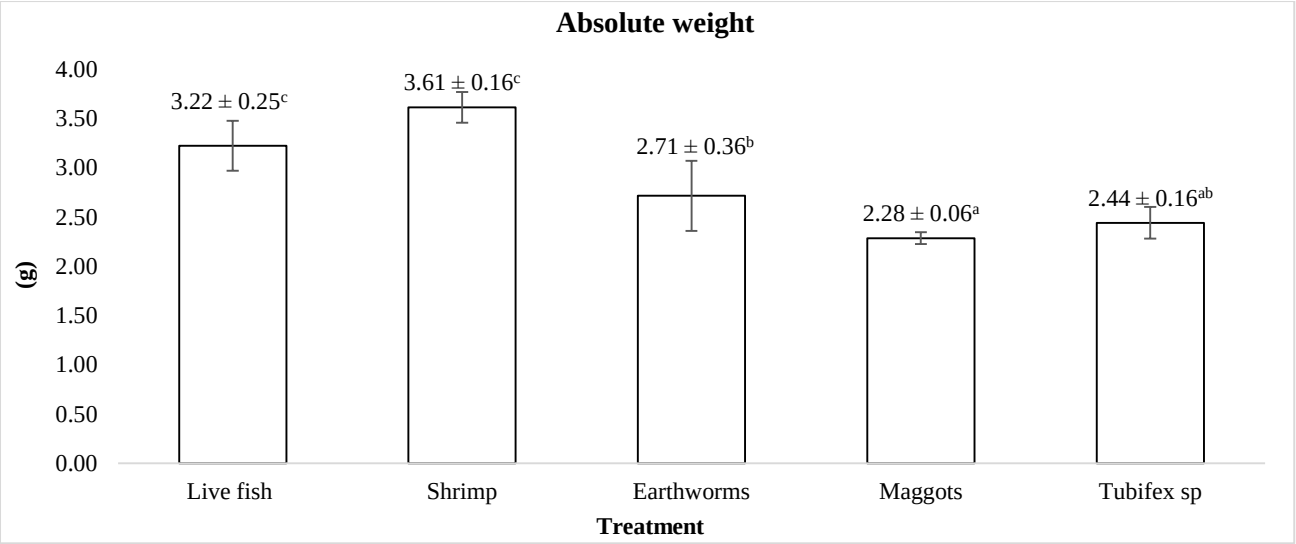


Figure 1. Absolute weight of glass catfish fed different natural feeds for 30 days. Data are expressed as mean ± standard deviation (SD). [a,b,c] The different superscript letters showed a significant difference among treatments ($p < 0.05$).

Absolute length

The current results demonstrated that providing different natural feeds significantly affected the absolute length of glass catfish ($p < 0.05$; Figure 2). Group P2 exhibited the highest absolute length (2.85 ± 0.11 cm), which was significantly different from the other four treatment groups ($p < 0.05$). Group P1 recorded the second-highest absolute length of 2.23 ± 0.20 cm, which was significantly different from the remaining treatment groups ($p < 0.05$). Meanwhile, Group P3 demonstrated the longest absolute length, 1.95 ± 0.16 cm, while the lowest values were recorded in groups P5 and P4, at 1.56 ± 0.13 cm and 1.40 ± 0.06 cm, respectively. These current results indicate that the type of natural feed significantly affected the absolute length of glass catfish, with shrimp and live fish feeds producing the greatest length increases during the rearing period.

Specific growth rate

The current results indicated that different natural feeds significantly affected the SGR of glass catfish during the rearing period ($p < 0.05$; Figure 3). Group P2 demonstrated the highest SGR (0.85 ± 0.03% day^{-1}), which was significantly different from the other treatment groups ($p < 0.05$). Group P1 indicated the second-highest SGR at 0.77 ±

0.05% day^{-1}, which differed significantly from other treatment groups ($p < 0.05$). The remaining groups exhibited progressively lower SGR values, including 0.65 ± 0.08% day^{-1} for Group P3, 0.60 ± 0.03% day^{-1} for Group P5, and 0.56 ± 0.01% day^{-1} for Group P4. The present results indicated that the type of natural feed influenced the SGR of glass catfish, with shrimp and live fish being the most effective feeds in promoting growth rate during the study.

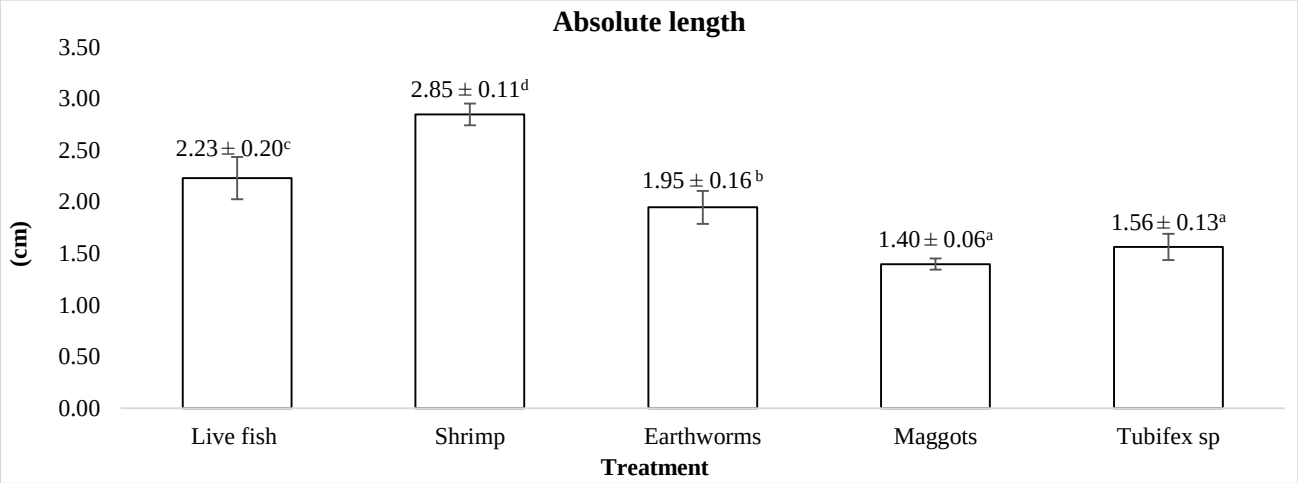


Figure 2. Absolute length of glass catfish fed different natural feeds for 30 days. Data are expressed as mean ± standard deviation (SD). [a,b,c] The different superscript letters showed a significant difference among treatments ($p < 0.05$).

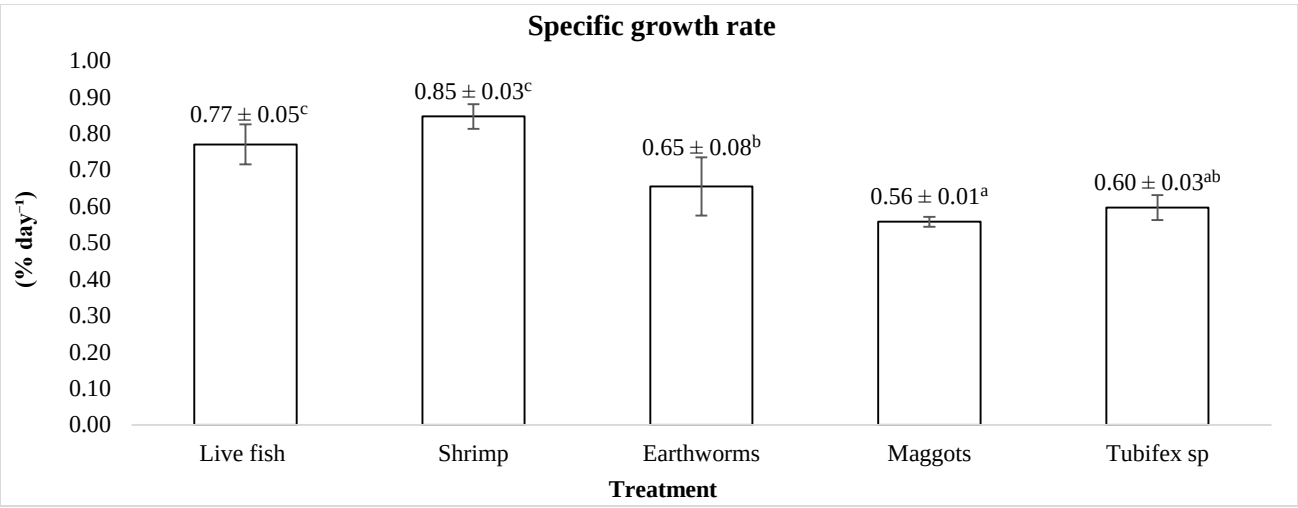


Figure 3. Specific growth rate of glass catfish fed different natural feeds for 30 days. Data are expressed as mean ± Standard deviation (SD). [a,b,c] The different superscript letters showed a significant difference among treatments ($p < 0.05$).

Daily growth rate

The current results indicated that different natural feeds significantly affected the DGR of glass catfish ($p < 0.05$; Figure 4). Group P2 demonstrated the highest DGR (0.12 ± 0.01 g day^{-1}), which was significantly different from the other four treatment groups ($p < 0.05$). Group P1 indicated the second-highest DGR at 0.11 ± 0.01 g day^{-1}, which differed significantly from other treatment groups ($p < 0.05$). Group P3 indicated a DGR of 0.09 ± 0.01 g day^{-1}, while the lowest values were recorded in groups P4 and P5, each at 0.08 ± 0.01 g day^{-1}. The present findings demonstrated that natural feed type significantly influenced the daily growth rate of glass catfish, as shrimp and live fish feeds yielded the greatest DGR over the 30-day rearing period.

Survival rate

The current findings indicated that different natural feeds had different effects on the survival rate of glass catfish (Figure 5). No significant differences in survival rate were observed among groups P1, P3, P4, and P5, with values ranging from 83.33 ± 5.77% to 96.67 ± 5.77% ($p > 0.05$). In contrast, Group P2 demonstrated the highest survival rate of 100 ± 0.00%, which was significantly different from the other treatment groups ($p < 0.05$). All treatments resulted in relatively high survival rates, indicating that the different types of natural feed were effective in supporting the survival of glass catfish during the rearing period.

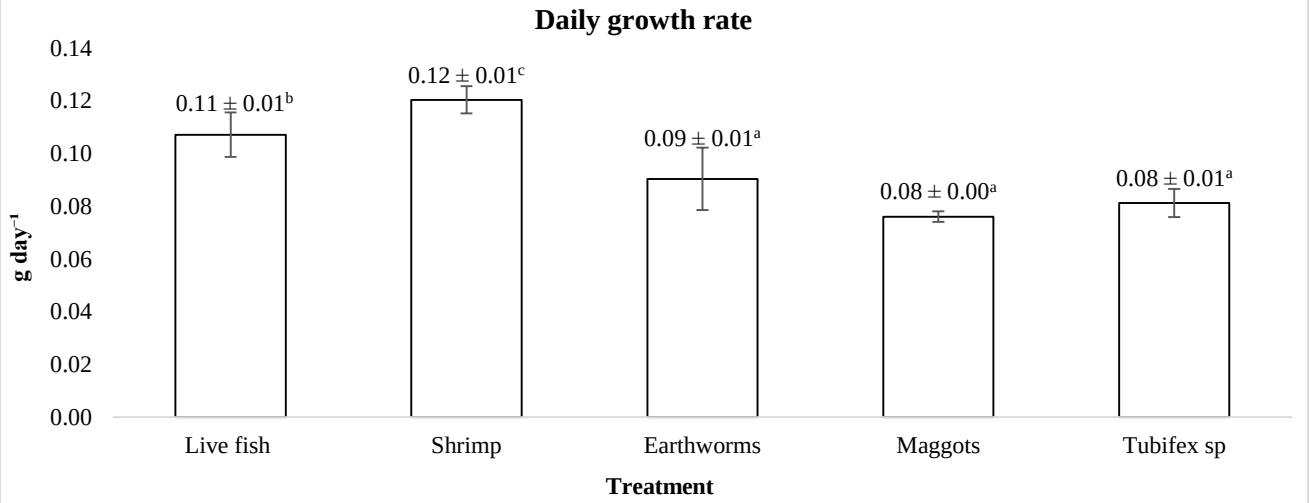


Figure 4. Daily growth rate of glass catfish fed different natural feeds for 30 days. Data are expressed as mean ± standard deviation (SD). [a,b,c] The different superscript letters showed a significant difference among treatments ($p < 0.05$).

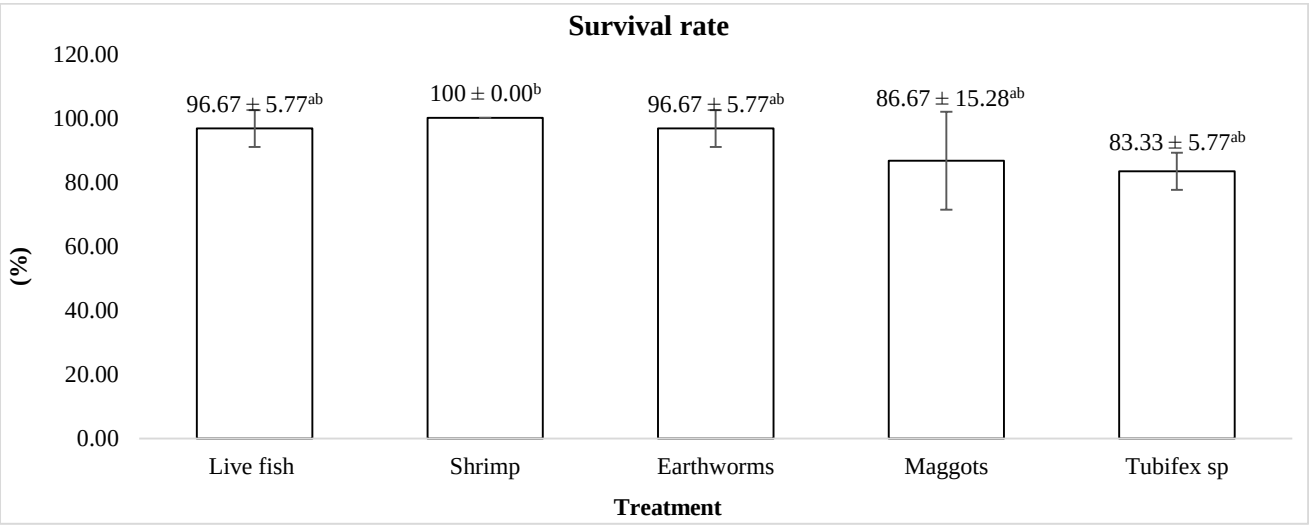


Figure 5. Survival rate of glass catfish fed different natural feeds for 30 days. Data are expressed as mean ± standard deviation (SD). [a,b] The different superscript letters showed a significant difference among treatments ($p < 0.05$).

Digestive enzyme activity

The current results indicated that the different natural feeds significantly affected the digestive enzyme activity of glass catfish, including amylase, lipase, and protease ($p < 0.05$; Figure 6). The highest digestive enzyme activities were consistently observed in Group P2, with amylase at 7.24 ± 0.14 U mL^{-1}, lipase at 0.37 ± 0.01 U mL^{-1}, and protease at 0.69 ± 0.01 U mL^{-1}, all of which differed significantly from the remaining treatment groups ($p < 0.05$). The second-highest amylase (5.70 ± 0.17 U mL^{-1}) and protease (0.55 ± 0.01 U mL^{-1}) activities were observed in Group P3, although its lipase activity (0.28 ± 0.01 U mL^{-1}) did not differ significantly compared with Group P1 ($p > 0.05$). Group P1 demonstrated relatively high enzyme activities, with values of 5.28 ± 0.15 U mL^{-1} for amylase, 0.28 ± 0.01 U mL^{-1} for lipase, and 0.50 ± 0.01 U mL^{-1} for protease. Meanwhile, Group P4 exhibited lower enzyme activities than groups P1, P2, P3, with values of 5.12 ± 0.19 U mL^{-1} for amylase, 0.26 ± 0.01 U mL^{-1} for lipase, and 0.49 ± 0.02 U mL^{-1} for protease, yet still higher than those of Group P5. The lowest enzyme activities were recorded in Group P5, for amylase (4.80 ± 0.16 U mL^{-1}), lipase (0.24 ± 0.01 U mL^{-1}), and protease (0.46 ± 0.01 U mL^{-1}). These findings indicated that shrimp feed was the most effective in stimulating digestive enzyme activity, which in turn improved digestive efficiency and promoted the growth of glass catfish.

Water quality

Based on water quality parameter measurements during the study, pH ranged from 5.43 to 5.75, water temperature from 29.33 to 29.63 °C, DO from 5.40 to 6.10 mg/L, and ammonia from 0.06 to 0.07 mg/L. These findings indicated relatively stable water quality conditions throughout the rearing period. The average values of water quality parameters during rearing are presented in Table 2.

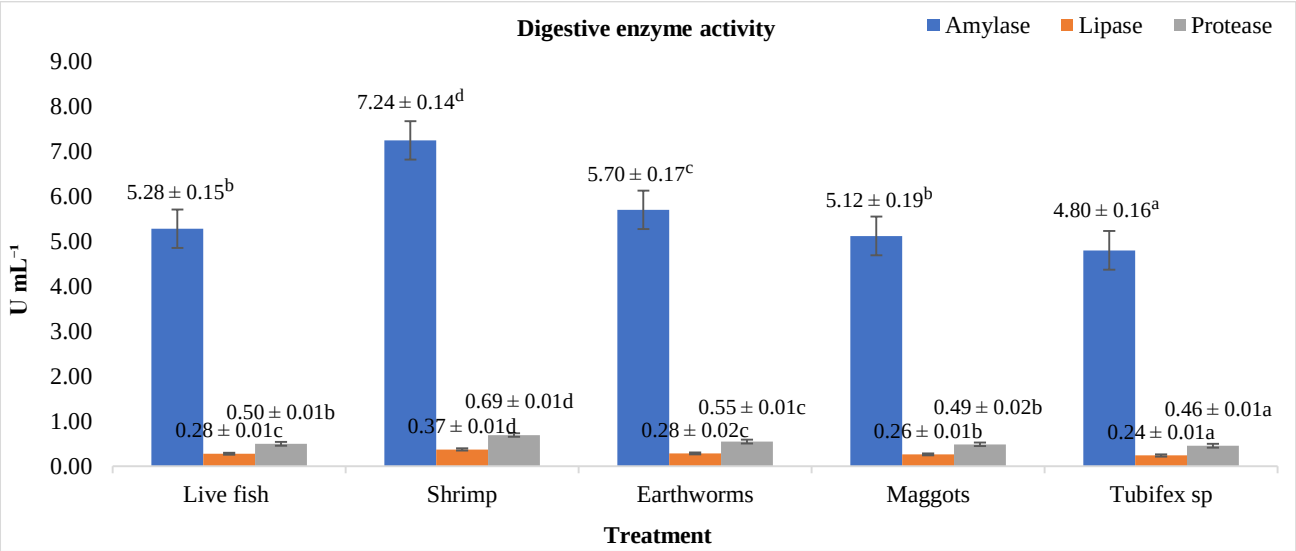


Figure 6. Digestive enzyme activities of amylase, lipase, and protease in glass catfish fed different natural feeds for 30 days. Data are expressed as mean ± standard deviation (SD). [a,b,c,d] The different superscript letters show a significant difference among treatments ($p < 0.05$). Enzyme activities are presented in U mL^{-1}.

Table 2. Average values of water quality parameters during the 30 days of rearing of glass catfish

Treatment	Average water quality parameters			
	pH	Temperature (°C)	DO (mg/L)	Ammonia (mg/L)
Live fish	5.74 ± 0.367	29.63 ± 0.462	6.00 ± 0.400	0.06 ± 0.003
Shrimp	5.52 ± 0.180	29.53 ± 0.451	6.10 ± 0.361	0.07 ± 0.004
Eartworm	5.55 ± 0.197	29.33 ± 0.493	5.53 ± 0.115	0.07 ± 0.013
Maggot	5.75 ± 0.006	29.50 ± 0.500	6.00 ± 0.529	0.07 ± 0.016
Tubifex sp.	5.43 ± 0.173	29.33 ± 0.493	5.40 ± 0.100	0.07 ± 0.021

Data are expressed as mean ± Standard deviation (SD), DO: Dissolved oxygen

DISCUSSION

Domestication is a crucial strategy for increasing fish production from natural waters by rearing wild species in controlled environments, particularly to improve their adaptability, growth performance, and survival under culture conditions (Yonarta et al., 2023b). Successful domestication depends on careful technical and socioeconomic planning, including the provision of facilities, appropriate technology, skilled human resources, and a thorough understanding of fish biology, such as feeding habits, growth performance, and survival. Feed management represented a critical aspect of the domestication process, involving the careful selection of feed type and its suitability during rearing. The feed used in domestication should align with the natural dietary preferences of the species in its habitat (Teletchea, 2015; Koniyo and Juliana, 2025). The carnivorous nature of glass catfish necessitates the use of high-protein natural feeds, such as live fish, shrimp, earthworms, maggots, and *Tubifex* sp. The effectiveness of feeding strategies in supporting domestication can be evaluated through improvements in growth performance, survival rate, and digestive enzyme activity, where growth performance reflects the efficiency of nutrient utilization, survival rate indicates the ability of fish to adapt to culture conditions, and digestive enzyme activity provides insight into the physiological capacity of fish to digest and absorb nutrients from the provided feed (Palińska-Żarska et al., 2020).

The use of different types of natural feed during the domestication of the glass catfish notably increased growth rates, as indicated by parameters such as absolute weight, absolute length, SGR, and DGR during the rearing period. Among the treatment groups, fish fed live shrimp exhibited the highest growth performance. Shrimp is one type of feed that is highly preferred by glass catfish, according to reports by Lukas and Minggawati (2014) and Evi and Rosana (2021). Insects, shrimp, and juvenile fish are the main food for glass catfish in their natural habitat. Treatment with live fish feed yielded favorable growth performance and was the second most effective treatment after shrimp feed in promoting growth in glass catfish. The effectiveness of these two feed types was attributable not only to their compatibility with the feeding habits of glass catfish but also to their live and active form, which naturally stimulated the fish's appetite (Assan et al., 2021). Furthermore, the high nutritional content of shrimp and small fish, including protein,

fat, vitamins, and minerals, was an important factor in supporting growth rates (Noreen et al., 2025). According to Koniyo and Juliana (2025), the availability of essential nutrients in the feed plays a crucial role in supporting fish growth and survival during domestication. Live feed provides highly digestible and readily available nutrients that enhance nutrient utilization efficiency and support optimal growth performance (Samat et al., 2020).

The type of natural feed remarkably influenced the survival rate of glass catfish, with the shrimp-fed group exhibiting the highest survival rate. Additionally, other treatment groups resulted in relatively high survival rates. The current findings indicated that the natural feeds used in the current experiment were generally suitable for maintaining fish survival under the rearing conditions. Live feed provides balanced, easily digestible nutrients that enhance growth, survival, and disease resistance (Samat et al., 2020). In addition, live feed is typically consumed directly by fish with minimal waste production, thereby reducing the accumulation of feed residues that may compromise water quality (Hossain et al., 2026). Fish survival and growth are influenced by external factors such as water quality and rearing systems, as well as internal factors including heredity, disease resistance, and the ability of fish to utilize the provided feed (Sumiarsih et al., 2022). Water quality during the rearing period remained within optimal ranges and fell within the recommended ranges for glass catfish aquaculture, including temperature of 25.20-32.00°C, pH of 4.70-7.90, DO of 2.60-8.00 mg/L, and ammonia of 0.010-2.000 mg/L (Yonarta et al., 2023a).

The shrimp-fed treatment group in the present study exhibited elevated digestive enzyme activities, including amylase, lipase, and protease, indicating an enhanced ability of glass catfish to digest and utilize the carbohydrates, lipids, and proteins in the shrimp diet, thereby supporting improved growth performance (Fang et al., 2019). In addition to shrimp, other natural feeds such as *Tubifex* sp., maggots, and earthworms demonstrated potential to support digestive processes, although their enzyme activity levels were comparatively lower, suggesting that their effectiveness depends on the fish's ability to efficiently digest and absorb their nutrient content. High digestive enzyme activity indicated a strong physiological ability in fish to hydrolyze and absorb nutrients, thereby supporting overall growth and development (Mohammadiazarm et al., 2023). In the present study, digestive enzyme activity, specifically amylase, lipase, and protease, served as a key biological indicator for evaluating the ability of glass catfish to utilize different nutrient components, including carbohydrates, lipids, and proteins. Thus, the evaluation of digestive enzyme activities was crucial for determining feed suitability and assessing the efficiency of nutrient utilization in supporting optimal growth (Hoseinifar et al., 2017). Additionally, differences in digestive enzyme activities are influenced by several factors, including physiological condition, temperature, pH, season, feeding habits, feed type, and nutrient composition (García-Meilán et al., 2023).

CONCLUSION

The domestication of glass catfish was evaluated using different natural feed sources, including live small fish, shrimp, *Tubifex* sp., maggots, and earthworms, by comparing the growth performance, survival rate, and digestive enzyme activity. Fish in the shrimp-fed group exhibited the highest overall performance among all treatment groups, achieving a mean absolute weight gain of 3.61 ± 0.16 g, an absolute length gain of 2.85 ± 0.11 cm, a specific growth rate of 0.85 ± 0.03% day^{-1}, and a daily growth rate of 0.12 ± 0.01 g day^{-1}, outperforming all other natural feed treatments. Additionally, the shrimp-fed group resulted in the highest survival rate (100%) and the greatest digestive enzyme activities, including amylase (7.24 ± 0.14), lipase (0.37 ± 0.01), and protease (0.69 ± 0.01), indicating more efficient nutrient digestion and utilization compared to the other diets. The current findings were achieved under stable, optimal water-quality conditions, including pH, temperature, dissolved oxygen, and ammonia, which supported fish physiological performance throughout the rearing period. Live shrimp proved to be the most effective feed among other natural feeds that were used for supporting the domestication of glass catfish. Further studies are recommended to assess long-term culture performance, feed conversion efficiency, and the potential of alternative live feeds such as *Tubifex* sp., maggots, and earthworms under different rearing conditions.

DECLARATIONS

Availability of data and materials

All relevant data generated during the study are included in this published article and are available upon reasonable request from the corresponding author.

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Authors' contributions

Danang Yonarta carried out data collection, prepared the original manuscript, and participated in research implementation. Muslim and Mochamad Syaifudin contributed to data analysis, administration, and conceptualization of the study. Ferdinand Hukama Taqwa and Marini Wijayanti contributed to data interpretation, drafting, and manuscript preparation. All authors have read and approved the final edition of the manuscript.

Competing interests

The authors declared no conflict of interest.

Ethical considerations

Ethical issues, including plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy, have been checked by all the authors. The authors confirm that no AI tools were used for preparing and conducting the present study.

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The Emerging of Antifungal Resistance and Virulence in Non-Albicans *Candida* Derived from Bovine Mastitis

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ABSTRACT

Mastitis represents a considerable economic burden on the dairy industry, adversely affecting milk yield and quality. In mycotic mastitis, *Candida* (*C.*) species are frequently responsible for opportunistic fungal infections in cows. The present study aimed to isolate *C. tropicalis* from aseptically collected milk samples from 60 dairy cows (average age 4.2 years) that tested positive for the California mastitis test (CMT). Mycotic etiology was confirmed through direct microscopy (KOH wet mount), culture on Sabouraud dextrose agar, and biochemical identification (API 20C AUX system). Antifungal sensitivity testing was conducted employing the broth microdilution method in accordance with CLSI M27-A3 guidelines. Additionally, molecular detection of antifungal resistance genes (*ERG3* and *CDR1*) and virulence genes (*SAPT3*, *ALS2*, and *LIP1*) was carried out using polymerase chain reaction (PCR). A total of five *C. tropicalis* isolates were obtained from 60 mastitis milk samples, yielding a prevalence of 8.3%. Antifungal susceptibility profiles demonstrated that all tested isolates were sensitive to itraconazole, nystatin, and ketoconazole. However, two of five isolates (40%) were resistant to fluconazole, all five isolates (100%) were resistant to terbinafine, and three of five isolates (60%) were resistant to voriconazole. Virulence genes (*SAPT3*, *ALS2*, and *LIP1*) were identified in 100%, 40%, and 60% of the five examined isolates, respectively. Meanwhile, antifungal resistance genes (*CDR1* and *ERG3*) were found in 100% and 80% of the isolates, respectively. The present study underscored the prevalence of multidrug-resistant *C. tropicalis* in bovine mycotic mastitis, emphasizing the need for routine antifungal susceptibility testing and molecular screening in dairy farms.

Keywords: Antifungal resistance, *Candida tropicalis*, Mastitis, Resistance gene, Virulence gene

INTRODUCTION

Bovine mastitis represents the most significant economic challenge for the global dairy industry, resulting in substantial losses from decreased milk production, discarded milk, and the premature culling of high-value livestock (Devanathan et al., 2024). While bacterial pathogens have traditionally been the primary focus, mycotic mastitis is increasingly recognized as a significant concern within veterinary medicine. Among fungal agents, species of the genus *Candida* (*C.*), specifically *C. tropicalis*, act as opportunistic pathogens that exploit the mammary gland's environment, particularly in cows with compromised immune systems or those experiencing a disrupted microbial balance due to prolonged antibacterial therapy (Dawoud et al., 2024; Schikora-Tamarit and Gabaldón, 2024). The consequences of mycotic mastitis are often more severe than those of bacterial infections due to irreversible damage to the udder tissue. The pathogenicity of *C. tropicalis* is driven by a robust set of virulence factors, including secreted aspartyl proteinases (SAPs), phospholipases, and lipases. These enzymes facilitate the degradation of mammary epithelial tissue, leading to chronic inflammation, udder induration, and permanent loss of secretory function (Zuza-Alves et al., 2017). Additionally, *C. tropicalis* forms extensive three-dimensional biofilms, dense microbial communities covered by an extracellular matrix. This matrix shields the fungi from host immune responses and antifungal agents, making the infection highly resistant to treatment (Cangui-Panchi et al., 2023; Yogendraiah et al., 2025).

The zoonotic potential of *Candida* species in dairy environments highlighted the need for effective treatment. The potential transmission of resistant strains between animals and human handlers contributes to a cycle of antimicrobial resistance (AMR), posing a significant public health threat (Devanathan et al., 2024). Historically, *C. albicans* was considered the predominant pathogenic yeast; however, recent clinical and veterinary studies have indicated a significant increase in isolation rates of non-albicans *Candida* (NAC) species (Sadeghi et al., 2018). *Candida tropicalis* has become one of the most important NAC species in clinical settings, ranking second after *C. albicans* in causing invasive and persistent infections (Jiang et al., 2024).

Management of these infections is severely limited by the increasing prevalence of antifungal resistance. Azoles, particularly fluconazole, are the preferred treatment option due to their safety and low cost (Paul et al., 2022). *Candida tropicalis* has demonstrated a strong ability to quickly develop acquired resistance against antifungal medicines. Unlike

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other species, *C. tropicalis* frequently exhibits cross-resistance, whereby exposure to a single azole agent confers resistance to subsequent azole derivatives, such as voriconazole (Wang et al., 2021). Recent studies have documented concerning resistance rates not only to azoles but also to amphotericin B and echinocandins, thereby limiting the therapeutic options available to veterinarians (Díaz-García et al., 2023; Dawoud et al., 2024; Schikora-Tamarit and Gabaldón, 2024). The necessity of the present study stemmed from the urgent need to understand the molecular factors driving resistance in the dairy industry. Identifying specific virulence and resistance genes in local bovine isolates improves the ability to predict clinical outcomes and facilitates the implementation of more targeted strategies for infection management. Therefore, the present study aimed to isolate *C. tropicalis* from bovine mastitis cases, characterize its antifungal susceptibility profiles, and utilize polymerase chain reaction (PCR) to detect key resistance genes (*ERG3* and *CDR1*) and virulence-associated genes (*SAP3*, *ALS2*, and *LIP1*).

MATERIALS AND METHODS

Ethical approval

The current investigation was conducted in accordance with the guidelines of the Department of Microbiology and Immunology, National Research Centre, Egypt. All procedures for milk sampling and animal handling were performed in accordance with institutional protocol.

Sampling and animals

A total of 60 milk samples were collected from 60 individual cows (one sample per animal) across three medium-scale dairy farms situated in Giza governorate, Egypt. The cows were primarily Holstein-Friesian crossbreeds, with an average age of 4.5 ± 1.2 years, with a mean body weight of 550±45 kg. Clinical mastitis was diagnosed through physical examination of the udder, noting symptoms such as swelling, heat, and pain, along with observable abnormalities in milk, including clots or discoloration. Subclinical mastitis was identified using the California mastitis test (CMT). Prior to sample collection, the teats were meticulously cleaned and disinfected with 70% ethyl alcohol, and the first three milk streams were discarded. Approximately 10 mL of mid-stream milk was collected aseptically into sterile screw-capped tubes, transported to the laboratory in an ice box at 4°C, and processed within 24 hours.

Fungal conventional isolation and identification

Each milk sample was streaked onto Sabouraud dextrose agar (SDA; Oxoid, UK) and incubated at 30°C for 48-72 hours. To differentiate *Candida* species, isolates were subcultured onto chromogenic *Candida* agar (CHROM agar; BD Diagnostics, USA). Pure colonies were identified based on morphology and conventional biochemical assays as described by Marinho et al. (2010).

Antifungal sensitivity testing

Antifungal susceptibility was determined according to the CLSI (2009) guidelines using the Kirby-Bauer disc diffusion method. Six antifungal agents were tested, including nystatin (100 IU), terbinafine (1 µg), itraconazole (8 µg), fluconazole (25 µg), ketoconazole (15 µg), and voriconazole (1 µg).

Molecular detection of antifungal resistance and virulence genes

Extracting DNA

Genomic DNA was extracted from five pure *C. tropicalis* isolates using the QIAamp DNA mini kit (Qiagen, Hilden, Germany). Briefly, 200 µL of the sample was incubated at 56°C for 10 minutes with 20 µL of Proteinase K and 200 µL of lysis buffer. After adding 200 µL of 100% ethanol, the lysate was washed and centrifuged. Nucleic acid was eluted in 100 µL of the provided elution buffer.

Primers

Primers from Bio Basic (Markham, ON, Canada) were used to detect resistance genes (*ERG3* and *CDR1*) and virulence genes (*SAP3*, *ALS2*, and *LIP1*). The primer sequences and detailed thermal cycling conditions, including denaturation, annealing, and extension, were adapted from Zhang et al. (2019), Paul et al. (2022), and Aboulela et al. (2023; Tables 1 and 2).

PCR amplification

Six µL of DNA template, one µL of each primer at 20 pmol, 4.5 µL of water, and 12.5 µL of EmeraldAmp Max PCR master mix (Takara, Japan) were combined to form a 25 µL reaction. The amplification reaction was performed using an Applied Biosystem 2720 thermal cycler.

Visualization of the PCR products

The PCR products were separated via electrophoresis on a 1.5% agarose gel (AppliChem GmbH, Darmstadt, Germany) in 1x Tris-Borate-Ethylenediaminetetraacetic acid (TBE) buffer. A consistent gradient of 5 Volts/cm was applied. Fragment sizes were determined using a GeneRuler 100 bp DNA Ladder (Thermo Fisher Scientific, Germany). Gels were photographed using the Alpha Innotech Gel Documentation System and subsequently analyzed with specialized software.

Table 1. Primers and PCR conditions of *ERG3* and *CDR1* resistance genes *in Candida tropicalis* isolated from mastitis cow's milk

Genes	Primers	Amplified fragment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
ERG3	ATTCCATTCACTATTGCCTGGTC AGTGCCTTATAACCACCAGTAGA	836 bp	94˚C 5 min	94˚C 30 sec	55˚C 40 sec	72˚C 50 sec	72˚C 10 min	Paul et al. (2022)
CDR1	TCGCCGTTTGCTGAAGAAGA GCAATCCCCAATTTCGATGGT	140 bp	94˚C 5 min	94˚C 30 sec	55˚C 30 sec	72˚C 30 sec	72˚C 7 min	Aboulela et al. (2023)

Min: Minute, Sec: Seconds

Table 2. Primers and PCR conditions of *SAPT3*, *ALS2*, and *L1P1* virulence genes *in Candida tropicalis* isolated from mastitis cow's milk

Genes	Primers	Amplified fragment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
SAPT3	AATTGGAATATAAATACGCT GGGGGTGAAACTACAATTTA	1325 bp	94˚C 5 min	94˚C 1 min	53˚C 1 min	72˚C 1.5 min	72˚C 12 min	Zhang et al. (2019)
ALS2	GGTAAAAGAAGTCGGATACGCAT CTACATCCGTTGCCATTACTATT	1214 bp	94˚C 5 min.	94˚C 1 min.	55˚C 1 min.	72˚C 1.5 min.	72˚C 12 min.	
L1P1	CCAAGGAGTCTATGGCTCAGTTA TAAGTGTAAAGTTGTCGGTGTTC	1945 bp	94˚C 5 min	94˚C 1 min	50˚C 1 min	72˚C 2 min	72˚C 12 min	

Min: Minute, Sec: Seconds

Statistical analysis

Data were analyzed using SPSS software (version 14.0). Descriptive statistics, including frequencies and percentages, were calculated to summarize the prevalence of *C. tropicalis* isolates, antifungal resistance patterns, and the distribution of virulence and resistance genes.

RESULTS AND DISCUSSION

In the present study, *C. tropicalis* was identified in 8.3% (5/60) of mastitis milk samples. These isolates formed creamy, shiny white colonies on SDA (Figure 1) and were verified as *C. tropicalis* by their distinct blue color on CHROM agar (Figure 2). The isolation rate observed in the present study aligns with the 10.1% reported by Seker (2010) in Turkey, but it markedly exceeds the 2% documented by Dworecka-Kaszak et al. (2012) in Poland. Conversely, higher prevalence rates have been reported by Eldesouky et al. (2016) at 19.5% in Egypt and 26.1% in Turkey (Erbas et al., 2017). These variations likely reflect differences in regional farming practices, humidity, and the hygiene protocols employed during milking. The emergence of *C. tropicalis* in dairy herds is often worsened by improper use of antibacterial agents. Although antibiotics effectively target bacteria, their indiscriminate use can disrupt the cow's natural mammary microbiota by eliminating beneficial bacterial communities. This microbial condition allows opportunistic, drug-resistant fungi such as *C. tropicalis* to proliferate and colonize the udder (Jiang et al., 2024). Furthermore, the present study suggested that prolonged antibiotic exposure may induce stress responses in fungal cells, potentially increasing their virulence and resilience against subsequent antifungal treatment (Devanathan et al., 2024). Antibiogram of *C. tropicalis* for six different antifungal agents (Figures 3-7; Table 3) demonstrated that *C. tropicalis* isolates were sensitive to itraconazole, nystatin, and ketoconazole. However, two of five isolates (40%) were resistant to fluconazole, all five isolates (100%) were resistant to terbinafine, and three of five isolates (60%) were resistant to voriconazole.

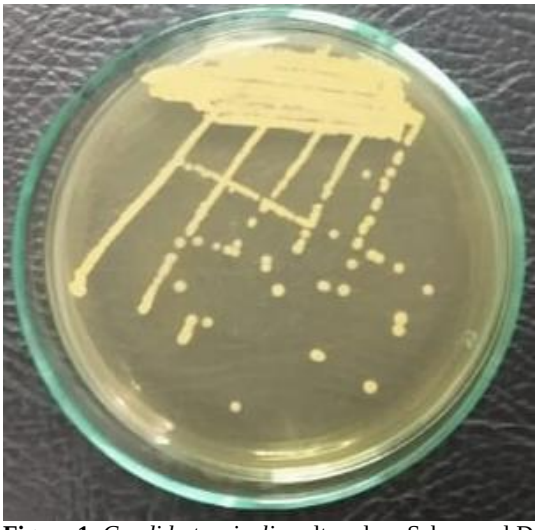


Figure 1. *Candida tropicalis* cultured on Sabouraud Dextrose Agar. The culture appears smooth, creamy-white, and convex colonies after incubation at 37°C for 48 hours.



Figure 2. Differential identification of *Candida tropicalis* on chromogenic *Candida* agar (CHROM agar). *Candida* medium, and the colonies appeared blue

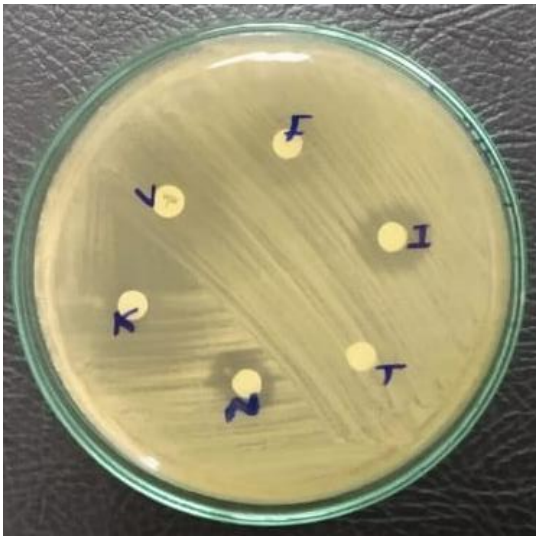


Figure 3. Antifungal susceptibility testing of *Candida tropicalis* using the disc diffusion method (Isolate 1). F: Fluconazole, I: Itraconazole, T: Terbinafine, N: Nystatin, K: Ketoconazole, and V: Voriconazole

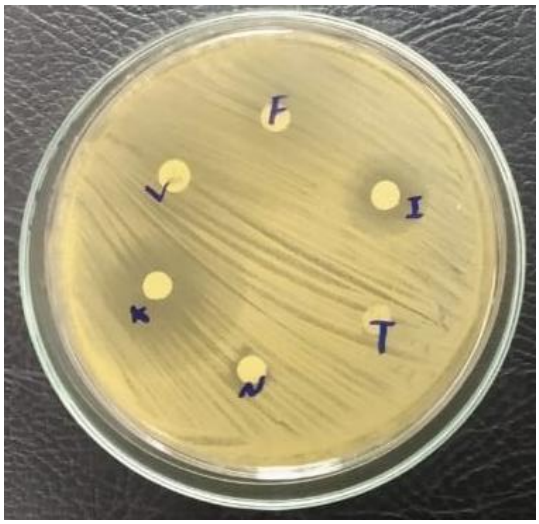


Figure 4. Antifungal susceptibility testing of *Candida tropicalis* using the disc diffusion method (Isolate 2). F: Fluconazole, I: Itraconazole, T: Terbinafine, N: Nystatin, K: Ketoconazole, and V: Voriconazole

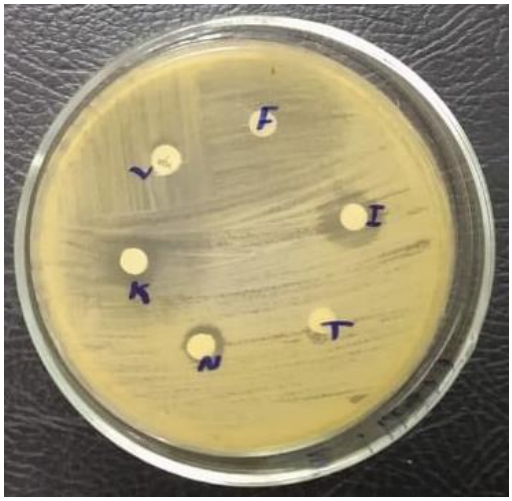


Figure 5. Antifungal susceptibility testing of *Candida tropicalis* using the disc diffusion method (Isolate 3). F: Fluconazole, I: Itraconazole, T: Terbinafine, N: Nystatin, K: Ketoconazole, and V: Voriconazole

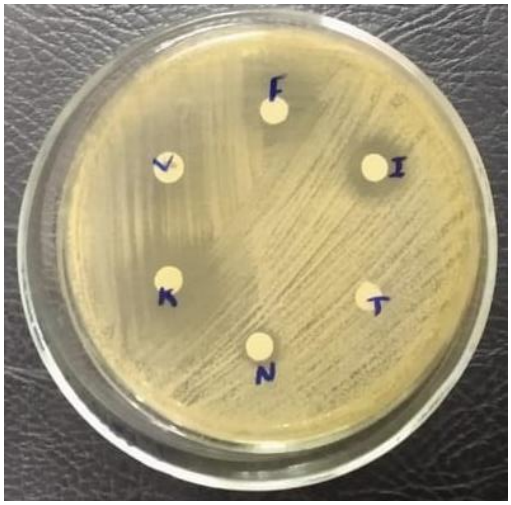


Figure 6. Antifungal susceptibility testing of *Candida tropicalis* using the disc diffusion method (Isolate 4). F: Fluconazole, I: Itraconazole, T: Terbinafine, N: Nystatin, K: Ketoconazole, and V: Voriconazole

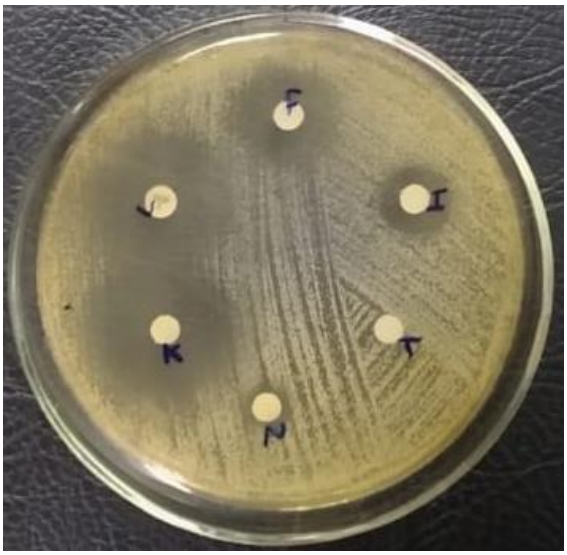


Figure 7. Antifungal susceptibility testing of *Candida tropicalis* using the disc diffusion method (Isolate 5). F: Fluconazole, I: Itraconazole, T: Terbinafine, N: Nystatin, K: Ketoconazole, and V: Voriconazole

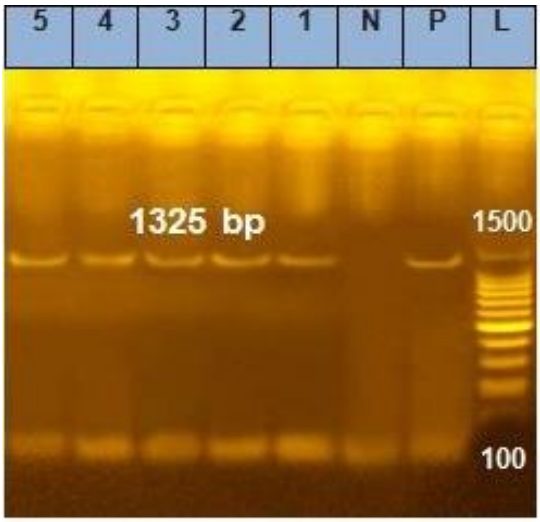


Figure 10. Agarose gel electrophoresis of the *SAP3* virulence gene (1325 bp) in *Candida tropicalis* isolates. Lane L: 100-1500 bp DNA ladder; Lane P: Positive control; Lane N: Negative control; Lanes 1, 2, 3, 4, 5: Positive isolates showing 100% prevalence of the *SAP3* gene.

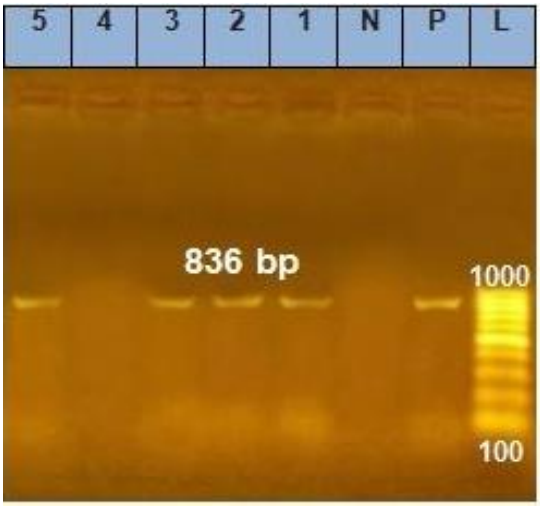


Figure 8. Agarose gel electrophoresis of the *ERG3* resistance gene (836 bp) in *Candida tropicalis* isolates. Lane L: 100-1000 bp DNA ladder, Lane P: Positive control, Lane N: Negative control, Lanes 1, 2, 3, 5: Positive isolates, Lane 4: Negative isolate.

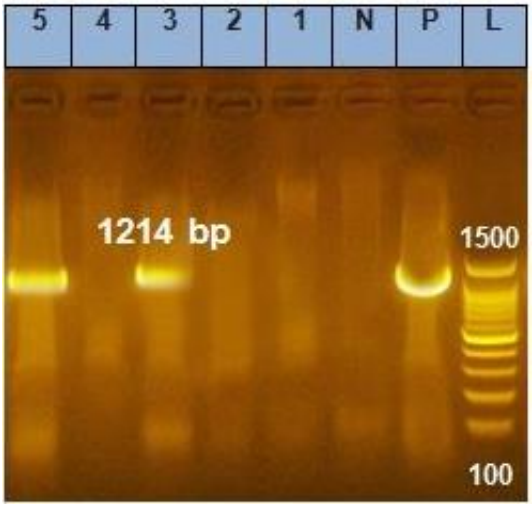


Figure 11. Agarose gel electrophoresis of the *ALS2* virulence gene (1214 bp) in *Candida tropicalis* isolates. Lane L: 100-1500 bp DNA ladder, Lane P: Positive control, Lane N: Negative control, Lanes 3, 5: Positive isolates, Lanes 1, 2, 4: Negative isolates.

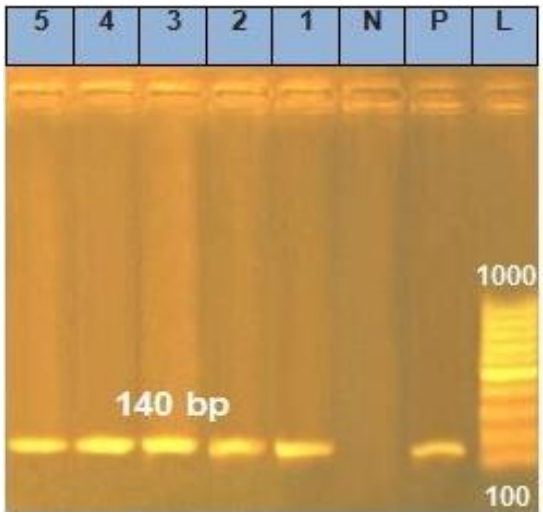


Figure 9. Agarose gel electrophoresis of the *CDR1* resistance gene (140 bp) in *Candida tropicalis* isolates. Lane L: 100-1000 bp DNA ladder; Lane P: Positive control, Lane N: Negative control, Lanes 1, 2, 3, 4, 5: All isolates positive for the LIP1 gene.

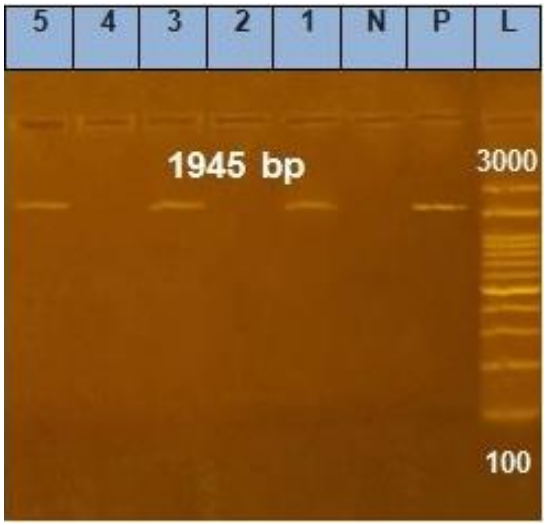


Figure 12. Agarose gel electrophoresis of the *L1P1* virulence gene (1945 bp) in *Candida tropicalis* isolates. Lane L: 100-3000 bp DNA ladder, Lane P: Positive control, Lane N: Negative control, Lanes 1, 3, 5: Positive isolates, Lanes 2, 4: Negative isolates.

Table 3. Antifungal sensitivity testing of *Candida tropicalis* isolated from mastitis cow's milk

C. troicalis isolates	Fluconazole	Itraconazole	Terbinafine	Nystatin	Ketoconazole	Voriconazole
Isolate (1)	12.3 ± 0.3	13.3 ± 0.3	R	9.3 ± 0.3	21.3 ± 0.3	16.3 ± 0.3
Isolate (2)	R	14.3 ± 0.3	R	7.7 ± 0.3	19 ± 0.6	R
Isolate (3)	R	14 ± 0.6	R	9.3 ± 0.3	12.3 ± 0.3	R
Isolate (4)	13.3 ± 0.3	15.3 ± 0.3	R	9.3 ± 0.3	19.3 ± 0.3	R
Isolate (5)	19.3 ± 0.3	15.3 ± 0.3	R	10 ± 0.0	26.3 ± 0.3	24 ± 0.6

The zones were measured in mm. R: Resistant, *C*: *Candida*

Table 4. Antifungal resistance genes and virulence genes of *Candida tropicalis* isolated from mastitis cow's milk

Sample	ERG3	CDR1	SAPT	ALS2	L1P1
1	+	+	+	-	+
2	+	+	+	-	-
3	+	+	+	+	+
4	-	+	+	-	-
5	+	+	+	+	+

+: Means the presence of this gene, -: Means the absence of this gene

The emergence of antifungal resistance in *C. tropicalis* results from multiple factors, including genetic changes and environmental influences (Paul et al., 2022). According to an investigation conducted in Turkey, Sonmez and Erbas (2017) reported that 25 *Candida* isolates demonstrated 100% resistance to miconazole, flucytosine, amphotericin B, and fluconazole. Additionally, Cândido et al. (2021) reported that *C. tropicalis* isolated from domestic and wild animals in Brazil demonstrated multidrug resistance, with 100% resistance to itraconazole but remaining susceptible to voriconazole. Antifungal resistance is mediated by point mutations in the *ERG11* gene and by increased expression of efflux pumps such as *CDR1* and *MDR1*, which help in avoiding common antifungal medicines. This resistance mainly stems from changes in the ergosterol biosynthesis pathway. Azole antifungal agents specifically target the enzyme lanosterol 14α-demethylase, which is encoded by the *ERG11* gene (Lee et al., 2021). In *C. tropicalis*, at least 31 distinct mutations in the *ERG11* gene have been identified; these structural alterations prevent antifungal drugs from binding to the target enzyme, making treatment ineffective (Lima et al., 2022).

Over the past decade, the resistance of *C. tropicalis* to fluconazole has been consistently observed across several nations and regions, with notably high resistance rates documented in Austria at 37.7% (Arendrup et al., 2023), Vietnam (18.8%; Ngo et al., 2023), India (21.1%; Lima et al., 2022), Singapore and Thailand (20%; Tan et al., 2016), and China (12.8%; Fan et al., 2019). In addition to fluconazole resistance, *C. tropicalis* has exhibited reduced susceptibility to other triazoles, such as itraconazole and voriconazole, across diverse geographic regions, including Brazil, Spain, and North America (Fernández-Ruiz et al., 2015; Chapman et al., 2017; Xisto et al., 2017). Notably, these resistance rates often align with those of fluconazole, reflecting the high prevalence rate of cross-resistance. This incident occurs because different azole medicines target the same enzyme (lanosterol 14α-demethylase enzyme); therefore, a single genetic mutation in the *ERG11* gene or the activation of multidrug efflux pumps (such as *CDR1*) can simultaneously make the fungus resistant to multiple medications within the same pharmacological class (Chong et al., 2012). In the current study, the molecular basis of this resistance was confirmed by the high prevalence of *CDR1* (100%) and *ERG3* (80%), as indicated in Figures 8 and 9 and Table 4. The *CDR1* gene encodes an Adenosine Triphosphate Binding Cassette (ABC) transporter that acts as an efflux pump, actively leading antifungal agents out of the fungal cell, lowering intracellular medicine levels to nonlethal levels (Prasad et al., 2019). Additionally, mutations or overexpression of *ERG3* (encoding sterol 5,6-desaturase) prevent the accumulation of toxic 14 alpha-methyl-3,6-diol, a byproduct of azole that typically results in fungal cell death. By altering this pathway, the fungus persists despite antifungal treatment (Paul et al., 2020).

Studies by Choi et al. (2016) and Rojas et al. (2023) have documented that *ERG11* and *ERG3* are the genes most frequently overexpressed in resistant isolates of *C. tropicalis*. This genetic adaptation typically occurs as a direct result of

prolonged and repeated exposure to azole antifungal agents, which apply selective pressure on the fungal population to overcome the drug's inhibitory effect on the ergosterol biosynthesis pathway. Although many studies have highlighted drug clearance, Jiang et al. (2013) found that efflux transporters may not be the main cause of azole resistance across all *C. tropicalis* populations. Jiang et al. (2013) suggested that alternative mechanisms play a more direct role in survival. These include modifications at the target site, such as mutations in the *ERG11* gene that decrease drug binding affinity, and the activation of bypass pathways in the sterol membrane, similar to those involving *ERG3* mutations. These mechanisms allow the fungus to preserve membrane integrity even when the main ergosterol pathway is inhibited.

In the present study, the virulence-associated genes *SAP3*, *ALS2*, and *LIP1* were detected in 100%, 40%, and 60% of the *C. tropicalis* isolates, respectively (Figures 10, 11, 12, and Table 4). These findings are consistent with the molecular pathogenesis models reported by Yu et al. (2016), who investigated the expression profiles of several virulence factors, including the secreted aspartyl proteinase family (*SAP1-4*), adhesins (*ALS1-2*), and lipases (*LIP1* and *LIP4*). Yu et al. (2016) demonstrated that the expression of the *ALS2* and *ALS3* genes, which encode critical cell-surface adhesins, was positively correlated with the extent of epithelial cell damage. This correlation highlighted an important stage in mycotic mastitis, indicating that *C. tropicalis* can adhere to the mammary epithelium, which is a crucial step in infection. Once adhered, the yeast can effectively utilize lytic enzymes, such as proteinases and lipases, to degrade host cell membranes. Thus, the high prevalence of *SAP3* (100%) and the presence of *ALS2* (40%) in the isolates during the present study indicated a strong genetic potential for both initial colonization and subsequent tissue destruction within the bovine udder.

CONCLUSION

The present study identified *C. tropicalis* as a significant opportunistic pathogen causing bovine mastitis, with an isolation rate of 8.3% in the examined dairy herds. Although isolates were sensitive to nystatin and ketoconazole, resistance rates to terbinafine (100%), voriconazole (60%), and fluconazole (40%) were high. The molecular analysis confirmed that this resistance was driven by the widespread presence of the *CDR1* efflux pump gene (100% of isolates) and the high prevalence of the *ERG3* gene (80%). Furthermore, the 100% detection rate for the *SAP3* virulence gene indicated that these isolates have a considerable capacity to degrade host tissue. The current results demonstrated that *C. tropicalis* was not merely a transient contaminant but a persistent pathogen with a strong genetic tendency for drug resistance and invading mammary tissue. Consequently, the current study suggested that routine veterinary diagnostics should shift from general fungal detection toward species-level identification and molecular resistance profiling. Future studies should focus on the transcriptomic profiles of *C. tropicalis* virulence genes during active infections and investigate the possibility of cross-transmission of these resistant strains between dairy handlers and livestock to enhance understanding of the zoonotic risks associated with mycotic mastitis.

DECLARATIONS

Authors' contributions

Mona Mohamed Hassan Soliman designed the study, while Mona Mohamed Hassan Soliman and Mai Mohamed Kandil conducted data collection, practical work, analysis of results, and the preparation of the manuscript. All authors have read and approved the final edition of the manuscript before publication.

Availability of data and materials

The data supporting the study's conclusions would be provided by the corresponding author upon request.

Competing interests

No conflicts of interest have been disclosed by the authors.

Ethical considerations

Each author has checked for ethical concerns, including plagiarism, consent to publish, misconduct, data fabrication and/or falsification, duplicate publication and/or submission, and redundancy. No AI tools were used to conduct or prepare the present study.

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Cytological Analysis of Tracheal Wash and Bronchoalveolar Lavage Fluid in Healthy and Asthmatic Horses

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ABSTRACT

Although numerous recent studies have explored the cytology of equine asthma, its cytological profile in Arabian horses is still insufficiently described. This study compared the differential cell counts in tracheal lavage (TW) and bronchial lavage fluid (BALF) samples between healthy and asthmatic Arabian horses in Saudi Arabia. Seventeen horses (5 healthy, 12 asthmatic), with an average age of 7 ± 5.5 years, and an average weight of 325 ± 120 kg, enrolled in this study to collect and analyze tracheal wash (TW) or bronchoalveolar lavage fluid (BALF) samples. The affected horses were taken from the respiratory cases admitted to the veterinary teaching hospital of Faisal University, while the healthy horses were taken from the horse research center in Faisal University (Saudi Arabia). Differential counts were performed on TW and BALF samples using microscopic examination after staining the samples with Diff Quick stain for evaluation of macrophages, lymphocytes, neutrophils, mast cells, eosinophils, and epithelial cells. A significant increase in neutrophils was observed in the affected group in both TW and BALF, compared to the control group. In contrast, a marked decrease in the percentage of lymphocytes was observed in asthmatic BALF, while macrophage proportions decreased in affected and control groups. TW eosinophils were elevated in asthmatic horses compared to healthy horses, though BALF eosinophil counts did not differ between healthy and asthmatic horses. The percentage of epithelial cells was decreased in TW but modestly increased in the BALF of asthmatic horses. No change in the number of mast cells was observed in any of the groups, as it was low. These findings confirmed that equine asthma in Arabian horses enrolled in this study is characterized by pronounced neutrophilic inflammation with concomitant lymphocytic and macrophagic depletion, particularly in the BALF samples.

Keywords: Bronchoalveolar, Cytology, Horse, Respiratory, Tracheal, Wash

INTRODUCTION

Equine asthma is a common disorder in horses, particularly in dusty and allergen-rich environments (Couetil et al., 2020; Diez de Castro and Fernandez-Molina, 2024). Equine asthma is a chronic, non-contagious inflammatory condition affecting the lower respiratory tract, impacting horses globally (Hermange et al., 2019; Lendl and Barton, 2024). Equine asthma causes a decline in respiratory function, leading to decreased athletic performance, high treatment costs, and the progression of many cases to a chronic form requiring ongoing veterinary care (Rossi et al., 2018; Sundman et al., 2020; Lendl et al., 2025).

Since its initial description as equine asthma (Lowell, 1964), it was later referred to as inflammatory airway disease, chronic obstructive pulmonary disease, and recurrent obstructive airway disease (Ainsworth et al., 2003; Gehlen et al., 2008; Couetil et al., 2020). The clinical manifestations of Equine asthma vary, ranging from mild respiratory allergies and inflammation to moderate inflammation and typical respiratory symptoms, culminating in severe symptoms accompanied by significant respiratory distress and hypoxia, as well as increased inflammation due to infiltration of inflammatory cells into the airways, mucus accumulation, and acute airway constriction resulting from contraction of the surrounding smooth muscles (Bosshard and Gerber, 2014; Lendl and Barton, 2024; Drespling et al., 2025).

Despite the clinical significance of this disease and its impact on performance, data detailing the cellular characteristics of equine asthma in Arabian horse breeds in Saudi Arabia, including those exposed to various respiratory allergens such as sand and date palm pollen, remain relatively scarce (Shawaf, 2019). Early diagnosis of respiratory infections is crucial for determining appropriate treatment to prevent disease progression and reduce the chances of recovery (Kozłowska et al., 2022). Accurate diagnosis of equine asthma relies on several methods, including clinical assessment, endoscopic examination of airway secretions, and cytological analysis of airway samples obtained via tracheal wash (TW) or bronchoalveolar lavage fluid (BALF, Wysocka and Kluciński, 2015; Morini et al., 2023;

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Drespling et al., 2025). Tracheal wash and BALF samples are valuable mirrors of different areas of the airway and can be used in combination with other methods, such as radiography or ultrasound, for the diagnosis of lower respiratory diseases (Wysocka and Kluciński, 2015; Rossi et al., 2018). Cytological examination of the lower airways is considered the gold standard for differentiating inflammatory subtypes and counting cells such as neutrophils, eosinophils, and mast cells, which reflect the severity of inflammation (Pickles et al., 2002a; Rossi et al., 2018). Tracheal wash sampling and analysis, as a less invasive and cost-effective procedure, complement this important approach to understanding the nature and extent of inflammatory changes in the airways of affected horses (Pickles et al., 2002b; Cullimore et al., 2018; Jocelyn et al., 2018). Dély et al. (2024) found that cytological examination of TW and BALF samples helps differentiate between healthy and asthmatic horses and the severity of the condition based on characteristic changes in the number and type of inflammatory cells. Horses with mild to moderate asthma showed an increase in neutrophils and mast cells in their BALF compared to healthy horses (Sage et al., 2024; Meiseberg et al., 2025), while in horses with severe asthma, a marked increase in neutrophils, often exceeding the established threshold values (≥ 20–25% neutrophils, characteristics of BALF cytological analysis), and exceeding 55% in BALF samples from affected horses was observed (Janssen et al., 2022; Lee et al., 2022).

Given the varying climatic conditions and diverse respiratory allergens in animals in Saudi Arabia (Shawaf, 2019; Shawaf et al., 2021), it is crucial to identify and compare the basic cytological characteristics of healthy and asthmatic horses. Therefore, the present study aimed to compare the analysis of TW and BALF cytology in healthy and asthmatic horses.

MATERIALS AND METHODS

Ethical approval

All experimental procedures used in this study, including TW and BALF sample collection, were approved by the Ethics Committee at King Faisal University, Saudi Arabia (Permission number KFU-REC / 2025-121).

Animals

A total of 17 Arabian horses (5 healthy controls and 12 affected with asthma) were included in the cytological analysis of TW and bronchoalveolar lavage fluid (BALF) between September 2025 and January 2026. Horses involved in this study were (7 stallions and 10 mares) of average aging (7 ± 5.5) and average weight (Kg, 325±120). Horses were fed a diet of hay and barley, while their training consisted of daily walking and cantering. The control group was selected randomly from animals maintained at the Horse Research Center, King Faisal University, Eastern Province, Saudi Arabia. Healthy horses selected based on physical examination (no fever or signs of abnormal respiratory signs like cough, nasal discharge, dyspnea, or abnormal lung sounds as well as normal white blood cell counts and biochemistry panel; Couetil et al., 2020). Horses with chronic respiratory symptoms were purposively selected from those brought to the Veterinary Teaching Hospital, College of Veterinary Medicine, King Faisal University. The horses were selected based on case history, clinical respiratory symptoms characteristic of the chronic form of the disease, such as coughing for more than three weeks, nasal discharges, abnormal breathing sounds, and the appearance of a heavy line on the abdomen (Gehlen et al., 2008). Horses with other diseases were excluded, and only those with chronic respiratory signs were included.

Sample collection

Samples were collected from horses without prior exercise in the standing position following mild sedation with Xylazine 2% (Rompun; Bayer Health Care, Germany) intravenously at a dose of 1 mg/kg body weight (Wiederkehr et al., 2021). The TW was taken during endoscopy, while BALF was collected immediately after endoscopy (Rossi et al., 2018). A twitch was used to restrain the horses when needed. A flexible endoscope (EVIS Olympus, OLYMPUS AUSTRIA GmbH., Vienna) with a 10 mm diameter and 140 cm long was used in endoscopy and for TW collection. The endoscope was passed via the nostril and advanced through to the mid-cervical trachea. 15 mL of sterile saline at room temperature was instilled through a plastic catheter (EQUIVET; 2.3 mm × 350 cm, Italy) to dilute mucous fluids and make them easier to withdraw. About 10 ml of tracheal fluid was immediately suckled back from the curvature of the distal trachea into a syringe. Samples were submitted for laboratory analysis (Veterinary teaching hospital lab, King Faisal University, Saudi Arabia) and were processed within 30 min of collection. Bronchoalveolar lavage fluid (BALF) collection was performed immediately following TW sampling. To prevent coughing while collecting BALF, 40 mL of 1% lidocaine (Lidocaine hydrochloride; Hikma, Canada) was infused as local anesthesia into the lower airway walls using a BALF catheter (EQUIVET B.A.L. catheter 240 cm, KRUUSE, Denmark, Rossi et al., 2018). The bronchoalveolar lavage fluid (BALF) tube was gently but securely wedged, as detected by resistance to further

advancement. The cuff was then gently inflated using a 10 ml syringe with 5-10 ml of air. This cuff assures a complete wedge to prevent the backflow of infused fluid. Prewarmed (37 ^{o}C) sterile physiologic saline solution, 0.9% NaCl (300-350 ml) was administered in one single volume in the distal airways and alveoli, followed by immediate aspiration. Syringes with the retrieved about 60% of infused fluid, which were immediately placed on ice and submitted to laboratory analysis within 30 min after collection (Shawaf, 2019).

Cytological analysis

To prepare the samples for differential cell counting, the TW and BALF samples were centrifuged at 1500 rpm for 15 minutes, and the supernatant was discarded. Smears of the cell precipitate were prepared on glass slides, air-dried, and stained with Diff Quick (Hemal Stain Co. Inc., Danbury, USA). Using an oil immersion lens at 1000x magnification and a standardized counting protocol (De Brauwer et al., 2002), the percentages of phagocytes, lymphocytes, neutrophils, mast cells, eosinophils, and epithelial cells were counted after 400 cells were counted using a microscope (Olympus BH2; Olympus Optical, Tokyo, Japan).

Statistical analysis

Differences between means were analyzed using one-way analysis of variance (ANOVA) with Bonferroni's post-hoc test for multiple comparisons using GraphPad Prism 7 statistical software. The D'Agostino-Pearson cross-test for normality was also used to assess normality. Differential cell counts were expressed as mean percentages ± standard deviation (SD) alongside the observed range. Differences between groups were considered statistically significant if the P-value was less than 0.05.

RESULTS

Marked differences in cellular profiles were observed both between healthy and asthmatic horses and between sampling techniques (TW versus BALF), concerning neutrophil, epithelial cell, macrophage, and lymphocyte distributions. The high percentage of neutrophils in horses with asthma compared to healthy horses in TW and BALF samples is considered one of the most important findings of this study. In TW samples, neutrophils constituted (57.29 ± 13.93%) of the total cell count in asthmatic horses, whereas healthy horses exhibited considerably lower values (22.11 ± 6.88%) with a significant difference ($p < 0.05$, Table 1 and Figure 3).

Similarly, in BALF, neutrophil percentages were increased in asthmatic horses (37.29 ± 13.32%) compared to their healthy horses (4.55 ± 2.06%) with a significant difference ($p < 0.05$, Table 1and Figure 4). In contrast, a decrease in macrophage percentages was observed in asthmatic horses in both TW and BALF samples compared to healthy horses. In TW, mean macrophage values decreased from 45.44 ± 13.24% in healthy horses to 15.29±7.44% in asthmatic horses ($p < 0.05$; Table 1 and Figure 3). This decline was consistent with BALF samples, where macrophage percentages decreased from 40.67 ± 6.01% in healthy animals to 29.57 ± 14.97% in affected horses, where the P value was recorded as $p < 0.05$ (Table 1 and Figure 4). A significant decrease in the percentage of lymphocytes was also observed in affected horses, particularly in BALF samples, compared to healthy horses. Healthy horses showed a lymphocyte percentage in BALF of 41.33 ± 6.42%, while the asthmatic horses exhibited BALF lymphocyte percentages of only 14.86 ± 6.09% ($p < 0.05$). In TW samples, lymphocyte percentages were also decreased in affected horses (6 ± 3.59%) compared to controls (9.44 ± 3.78%; $p < 0.05$), although this difference was less obvious than in BALF. In TW samples, epithelial cells were reduced in affected horses (10.93 ± 6.79%) compared to controls (18.44 ± 7.45%; $p < 0.05$).

Conversely, in BALF, epithelial cell percentages were increased in affected horses (10 ± 5.23%) compared to healthy horses (6.88 ± 3.22%). Eosinophil percentages were increased in TW samples from affected horses (10.21 ± 5.24%) compared to healthy controls (3.89 ± 2.42%, $p < 0.05$). However, BALF eosinophil percentage was nearly identical between healthy and affected groups (healthy horses: 6 ± 3.09%; asthmatic horses: 6.5 ± 3.57%, 0-12%) with no significant difference. Mast cell percentages recorded low values across both groups in TW and BALF samples (< 1.1% mean), with no significant differences.

Comparing the cell composition of TW and BALF in healthy horses revealed high proportions of macrophages, neutrophils, and epithelial cells in the tracheal lavage samples, while high proportions of lymphocytes and eosinophils were recorded in the alveolar lavage samples (Table 1; Figures 1 and 2). Similarly, affected horses showed higher values for neutrophils, eosinophils, and epithelial cells in TW, while elevated levels of phagocytes and lymphocytes were observed in BALF samples.

A notable finding was the absence of a difference in the percentage of epithelial cells among TW and BALF samples in affected horses, whereas a significant increase in epithelial cells was observed in TW samples compared to BALF samples in healthy horses (Figures 1 and 2).

Table 1. The differential cell counts (Mean ± SD) of tracheal wash and bronchoalveolar lavage fluid in healthy and asthmatic horses in Saudi Arabia

Cells	Tracheal wash (TW)			Bronchoalveolar Lavage Fluid (BALF)		
	Healthy Mean ± SD	Affected Mean ± SD	P value	Healthy Mean ± SD	Affected Mean ± SD	P value
Macrophages (%)	45.44 ± 13.24	15.29 ± 7.44**	0.006	40.67 ± 6.01	29.57 ± 14.97*	0.04
Lymphocytes (%)	9.44 ±3.78	6 ±3.59*	0.04	41.33 ± 6.42	14.86 ±6.09**	0.009
Neutrophils (%)	22.11± 6.88	57.29 ± 13.93**	0.008	4.55 ± 2.06	37.29 ± 13.32***	0.0009
Mast cells (%)	0.66 ± 1	0.42 ± 0.85	0.071	066 ± 0.71	1.07 ± 1.14	0.065
Eosinophils (%)	3.89 ± 2.42	10.21 ± 5.24*	0.045	6 ± 3.09	6.5 ± 3.57	0.076
Epithelial cells (%)	18.44 ± 7.45	10.93 ± 6.79*	0.03	6.88 ± 3.22	10 ± 5.23*	0.45

SD: Standard deviation; NS, Not significant. *p < .05; **p < .01; ***p < .001.

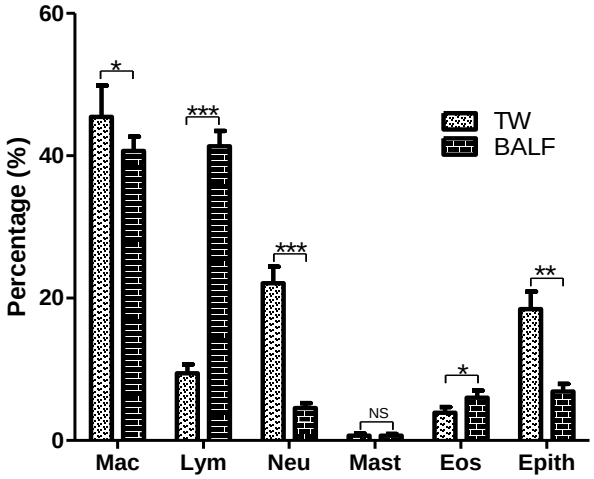


Figure 1. The differential cell counts (%) of tracheal wash (TW) and bronchoalveolar lavage fluid (BALF) from healthy horses. Mac: Macrophages, Lym: Lymphocytes, Neu: Neutrophils, Mast: Mast cells, Eos: Eosinophils, Epith: Epithelial cells, NS: not significant. *p < .05; **p < .01; ***p < .001.

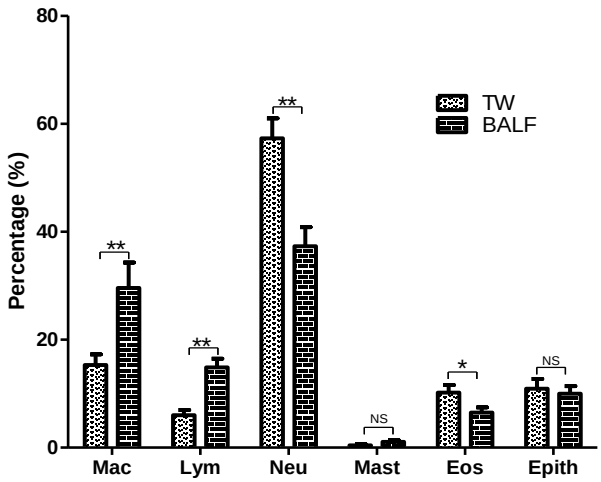


Figure 2. The differential cell counts (%) of tracheal wash (TW) and bronchoalveolar lavage fluid (BALF) from asthmatic horses. Mac: Macrophages, Lym: Lymphocytes, Neu: Neutrophils, Mast: Mast cells, Eos: Eosinophils, Epith: Epithelial cells, NS: not significant. *p < .05; **p < .01.

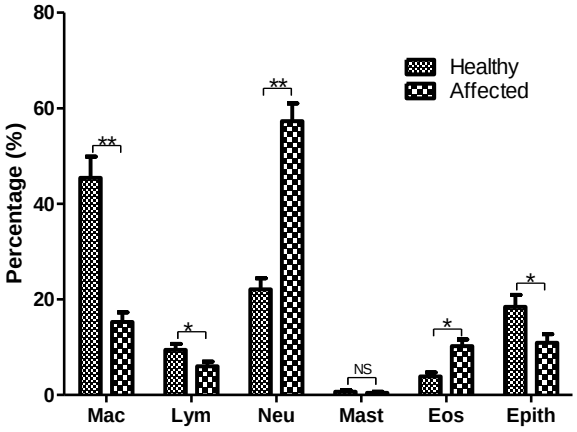


Figure 3. The differential cell counts (%) of tracheal wash (TW) from healthy and asthmatic horses. Mac: Macrophages, Lym: Lymphocytes, Neu: Neutrophils, Mast: Mast cells, Eos: Eosinophils, Epith: Epithelial cells, NS: not significant. *p < .05; **p < .01.

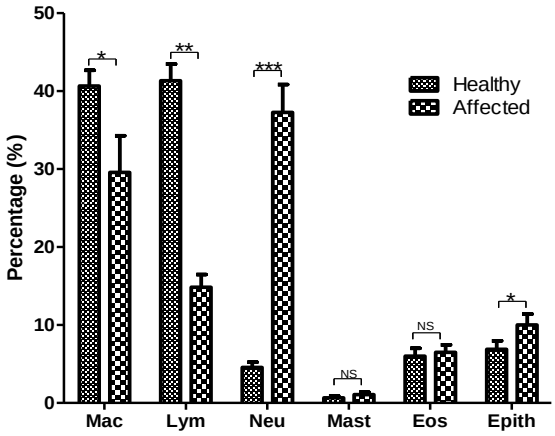


Figure 4. The differential cell counts (%) of bronchoalveolar lavage fluid (BALF) from healthy and asthmatic horses. Mac: Macrophages, Lym: Lymphocytes, Neu: Neutrophils, Mast: Mast cells, Eos: Eosinophils, Epith: Epithelial cells, NS: not significant. *p < .05; **p < .01; ***p < .001.

DISCUSSION

The results in this study showed similar values for differential cell counts in tracheal wash (TW) and bronchoalveolar lavage (BALF) of Arabian horses, with differences in some cells, which may be due to different allergens and diseases in dry and desert environments (Shawaf et al., 2021). Similar values for neutrophils were shown, which are very important in differentiating between healthy and affected horses that need treatment (Sage et al., 2020). However, the most significant finding in the present study was a marked increase in the number of neutrophils in both TW and BALF in asthmatic horses, exceeding the percentages established for healthy horses and equine asthma cases in previous studies (Rossi et al., 2018; Couetil et al., 2020).

A significant increase in neutrophil infiltration was observed in the tracheal space (57.29%) compared to the alveolar space (37.29%). These findings were previously documented by Rossi et al. (2018), who noted that the neutrophil count in tracheal fluid often exceeds that in TW from both healthy and asthmatic horses. A higher percentage of neutrophils could be attributed to the extrusive migration of neutrophils from distant sites of inflammation towards the trachea (Lendl et al., 2025) or to longer duration of neutrophil placement in the tracheal lumen (Uberti and Morán, 2018). The increased number of neutrophils in TW compared to BALF could also be explained by the persistent contamination resulting from the trachea's proximity to the pharynx, which serves as a food passageway (Shawaf et al., 2021).

It is important to note that findings in the current study that the mean neutrophil percentage in the BALF of asthmatic cohort (37.29%) significantly exceeded the diagnostic threshold of 10% for mild to moderate asthma and approached the 25% threshold typically cited for severe asthma (Janssen et al., 2022; Lee et al., 2022), suggesting that the affected horses included in this study predominantly represented moderate to severe cases. The concurrent decrease in the lymphocyte percentage in the BALF from 41.33% in healthy horses to 14.86% asthmatic horses indicates a marked increase in neutrophils, which affected the percentage of lymphocytes (Davis et al., 2019). This finding is consistent with that of Sage et al. (2024), who recently demonstrated, using single-cell transcription analysis, that severe equine asthma is characterized not only by a marked increase in neutrophils but also by a T-cell immune response that subsequently leads to a quantitative decrease in total lymphocyte recovery (Kang et al., 2022; Morini et al., 2023). Meiseberg et al. (2025) reported previously that the reason for the sharp decline in lymphocyte count in severe asthma may be due to lymphocyte death within the inflammatory microenvironment. In the present study, the percentage of macrophages decreased significantly in both TW and BALF of asthmatic horses, with the greatest decrease occurring in the TW. This decrease is likely attributable to the relative dilution effect of neutrophil infiltration, which was markedly elevated, consistent with the documented impaired phagocytic function of airway phagocytic cells in asthmatic horses (Hansen et al., 2020b; Wilson et al., 2020). The wide variation in macrophage percentages observed in the BALF of asthmatic horses may be attributed to differences in the type and stage of airway inflammation, leading to heterogeneity in the inflammatory phenotype (Lendl and Barton, 2024). The pattern of epithelial cell percentage changes in both TW and BALF in this study warrants particular attention. The reduced number of epithelial cells in TW of asthmatic horses may reflect ongoing debridement and regeneration of the epithelium due to chronic inflammation, a process previously described in acute equine asthma (Bosshard and Gerber, 2014). Alternatively, the reduction of epithelial cells in TW in affected horses may represent a relative attenuation of the high proportion of inflammatory cells, as noted in previous studies (Kang et al., 2022). Conversely, the slightly increased number of epithelial cells in BALF in asthmatic horses (from 6.88% to 10.00%) is surprising, but consistent with the findings reported previously (Drespling et al. 2025).

They hypothesized that the use of a plastic catheter for sampling, together with its potentially traumatic effect on the airway mucosa, could contribute to epithelial damage. This effect may be exacerbated by bronchial hyperresponsiveness and airway constriction during bronchoalveolar lavage catheter insertion in inflamed lungs. Consequently, increased epithelial disruption may occur, leading to the shedding and aspiration of epithelial cells into the cytological sample (Pickles et al., 2002a; Wysocka and Kluciński, 2015).

A marked increase in eosinophils was observed in the TW of asthmatic horses, while eosinophil counts in BALF remained similar between the two groups. This difference between TW and BALF samples from affected horses suggested that eosinophilic inflammation may be primarily concentrated in the large conducting airways rather than in the alveolar interstitial tissue (Couetil et al., 2020). The result of eosinophil counts in the current study supports the concept of individual phenotypic variability in equine asthma and the multi-factorial nature of equine asthma, as a subset of affected horses exhibit mixed granulomatous inflammation (Wysocka and Klucinski, 2015; Couetil et al., 2020). The lower values of eosinophils in BALF in affected horses in the present study differ from some European studies that have indicated eosinophil-predominant patterns of equine asthma (Hermange et al., 2019; Rasmussen et al., 2024), which may reflect differences related to geographical location, weather conditions, and asthma-associated allergens (Couetil et al., 2020).

The arid, sandy desert environment of Saudi Arabia, with its prevalence of date palm pollen and desert dust rather than mold spores and other pollen types common in temperate climates, may stimulate neutrophilic rather than eosinophilic airway responses (Shawaf, 2019). Mast cell proportions in the present study remained uniformly low in both TW and BALF from healthy and diseased animals. The absence of significant differences in mast cell counts between healthy and asthmatic horses may be attributed to the predominantly neutrophilic nature of inflammation in the affected animals. This type of inflammatory response is not typically associated with marked mast cell proliferation on the airway surfaces, as previously reported (Hansen et al., 2020a). Furthermore, the Diff-Quik staining method used may not be sufficient for staining mast cells (Pickles et al., 2002b).

CONCLUSION

The proportion of cellular changes in TW and BALF samples in asthmatic Arabian horses is similar to those studied in other breeds. TW and BALF analysis in this study also indicate some cytological differences from previous studies, which may be attributed to allergens and diseases in arid and desert environments. Further research is needed to study a larger sample size than this study and to investigate the impact of weather and seasons on the cellular composition of the airways in Arabian horses.

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Authors' contributions

Zakaria Abdullah, Abdulaziz Almuhanna, and Turke Shawaf did the examination; Saleh Alsayed, Mohammad Alsalman, Basel Hamoud, and Turke Shawaf did laboratory work. Zakaria Abdullah, Abdulaziz Almuhanna, and Turke Shawaf drafted the manuscript. The final manuscript was approved by all of the authors.

Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflict of interests

The authors have not declared any conflict of interest.

Ethical considerations

Ethical issues, including plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy have been checked by all the authors. The authors confirm that no AI tools were used in conducting and preparing this study.

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Multidrug-Resistant *Salmonella enterica* subsp. *enterica* Isolated from Animal-Based Foods in Libya

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ABSTRACT

The emergence of *Salmonella enterica* subsp. *enterica* in food represents a significant public health concern worldwide, particularly in developing countries such as Libya. Addressing antimicrobial resistance in *Salmonella enterica* subsp. *enterica* is essential, particularly where food hygiene and antimicrobial regulations are weak. The present study aimed to evaluate the occurrence and antimicrobial susceptibility profile of *Salmonella enterica* subsp. *enterica* isolated from different animal-derived food products collected at retail markets in Libya. In the current study, 18 *Salmonella enterica* subsp. *enterica* isolates were obtained from 751 food samples, including milk, dairy products, meat, and meat products, collected randomly from retail markets in Libya. The isolates were tested for antimicrobial susceptibility, and their antibiotic resistance profiles were evaluated against 33 antibiotics representing 12 different classes. Antimicrobial susceptibility testing demonstrated that all isolates were 100% resistant to penicillin, with high resistance rates to neomycin and rifampicin (94.4%). However, high susceptibility rates were observed for chloramphenicol, nitrofurantoin, sulfamethoxazole/trimethoprim, and norfloxacin (88.9%). The multiple antibiotic resistance (MAR) index varied from 0.1 to 0.6, with the maximum MAR value (0.6) recorded in isolates exhibiting resistance to 21 antibiotics. The current findings underscore the necessity to enhance food safety protocols, as multidrug resistance was prevalent among the isolates, particularly to penicillin, neomycin, and rifampicin. Therefore, the utilization of antimicrobial agents in both veterinary and human settings should be strictly regulated to control the emerging threat of multidrug-resistant strains. Additionally, the present results suggested that fluoroquinolones and cephalosporins remain among the most effective antimicrobial agents and may be considered for the treatment of salmonellosis. The detection of multidrug-resistant *Salmonella enterica* subsp. *enterica* in animal-derived foods in Libya represented a significant public health concern, underscoring the critical need for enhanced surveillance, stricter antimicrobial use regulations, and improved food safety measures.

Keywords: Foodborne pathogen, Meat, Milk, Multidrug resistance, *Salmonella enterica* subsp. *enterica*

INTRODUCTION

Foodborne diseases pose a major global public health challenge, mainly caused by consuming contaminated food, particularly products derived from animal sources. Foodborne illnesses are responsible for an estimated 600 million cases of sickness and approximately 420,000 fatalities annually worldwide (Temesgen et al., 2025). Approximately one-third of people in developed countries are affected annually (Asfaw Geresu et al., 2021). The incidence of foodborne diseases is considerably higher in developing countries due to poor hygiene practices, weak regulatory systems, limited resources, and low awareness of safe food handling practices (Wabeto et al., 2017; Bedassa et al., 2023). Most foodborne pathogens originate from animals and can be transmitted through animal-derived products such as milk, meat, cheese, and yogurt (Ejo et al., 2016; Gebeyehu et al., 2022). Although milk and dairy products are essential to human nutrition, raw milk may contain pathogenic microorganisms introduced by infected animals, handlers, or contaminated farm environments, posing a substantial risk to public health (Bereda et al., 2018; Geletu et al., 2022).

Pathogens originating from animal-derived foods, including *Escherichia coli*, *Staphylococcus aureus*, *Salmonella* species, and *Listeria monocytogenes*, represent one of the most significant threats to human health (Gebeyehu et al., 2022; Sherif et al., 2025). Among these pathogens, *Salmonella* species is one of the most common foodborne bacteria worldwide, causing substantial public health and economic burdens, particularly in developing countries (Carrasco et al., 2012; Asfaw Geresu et al., 2021; Garbaj et al., 2022). Multiple factors contribute to outbreaks of salmonellosis in humans, including the consumption of raw or improperly processed foods, cross-contamination during food preparation, inadequate food storage, poor personal hygiene practices, insufficient refrigeration, inadequate reheating of food, and extended time intervals between food preparation and consumption (Ejo et al., 2016).

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Salmonella comprises two species, *Salmonella bongori* and *Salmonella enterica* (*S. enterica*; Gebeyehu et al., 2022). Among *Salmonella* species, *S. enterica* is the most prevalent and widely distributed, with more than 2,600 identified serotypes. *Salmonella* species are broadly classified into typhoidal and non-typhoidal groups. Globally, *Salmonella enterica* serovars Typhimurium and Enteritidis account for nearly fifty percent of all human infections attributable to non-typhoidal *Salmonella* (Talukder et al., 2023). Most pathogenic and zoonotic *Salmonella* strains belong to *S. enterica* subsp. *enterica*, which causes salmonellosis in both animals and humans (Talukder et al., 2023).

In the past, antibiotics were regarded as highly effective agents for completely eradicating microbial infections. However, microorganisms have demonstrated the ability to survive and even proliferate in the presence of antibiotics, a phenomenon known as antimicrobial resistance (AMR), which has become a significant global concern (WHO, 2023). Currently, AMR in both humans and animals has reached alarming levels globally and was recognized as a major emerging threat to public health and food security (Geletu et al., 2022). Antibiotic-resistant *Salmonella* species originating from animal-derived foods have been increasingly reported, largely due to the misuse and overuse of antibiotics in livestock production (Abunna et al., 2017; Ketema et al., 2018). Antibiotic-resistant *Salmonella* species can be transmitted to humans via the food chain, thereby posing a substantial public health risk (Abunna, 2018). Many *Salmonella* isolates exhibited resistance to commonly used antimicrobial agents, particularly ampicillin, tetracycline, chloramphenicol, and trimethoprim-sulfamethoxazole. Therefore, multidrug-resistant (MDR) non-typhoidal *Salmonella* has become an increasing global concern (Winokur et al., 2000). In contrast, typhoid fever, caused by *Salmonella Typhi*, is typically treated with fluoroquinolones, third-generation cephalosporins, and macrolides (Adel et al., 2021; Asfaw Geresu et al., 2021; Kanat and Terzi Gulel, 2025).

Transmission of antibiotic-resistant pathogens to humans may occur through exposure to antibiotic residues in animal-derived foods. A recent study analyzing a variety of food samples derived from animal sources, human contact, and other environments reported a high prevalence of MDR to more than two antibiotics, particularly fluoroquinolones and third-generation cephalosporins, among *Salmonella* isolates (Asfaw Geresu et al., 2021). Recently, the emergence of antibiotic-resistant foodborne pathogens in the food chain has posed a serious risk of transmission to humans and may lead to higher infection rates. It is important to note that commonly encountered antibiotic-resistant bacteria, such as *Escherichia coli*, *Salmonella*, and *Staphylococcus aureus*, are likely to exert considerable pressure on healthcare systems globally (Naas et al., 2019; Geletu et al., 2022; Temesgen et al., 2025).

Antibiotic misuse is widespread in Libya, where medicines such as ampicillin, amoxicillin, tetracycline, and trimethoprim-sulfamethoxazole are readily available from private pharmacies without a prescription (Ghenghesh et al., 2013). A local study conducted in Tripoli, Libya, reported that 75% of *Salmonella* isolates obtained from children with diarrhea were MDR (Ali et al., 2005), while Rahouma (2011) reported that 63% of the strains were resistant to ciprofloxacin. Therefore, enhancing public awareness of *Salmonella* infections and conducting additional investigations on the prevalence and antimicrobial susceptibility of *S. enterica* in foods are essential. Hence, the present study aimed to determine the prevalence and antimicrobial susceptibility profile of *S. enterica* isolated from selected animal-origin foods in Libya.

MATERIALS AND METHODS

Salmonella isolates

Ethical approval was not required for the present study, as no live animals were involved. The *Salmonella enterica* subsp. *enterica* isolates used in the present study were obtained from previous investigations (Eshamah et al., 2020; Garbaj et al., 2022), in which a total of 751 food samples, including milk, dairy products, meat, and meat products, were collected from retail markets in Libya. In previous studies, *Salmonella* isolates were recovered, identified, and subsequently stored at -80˚C for further analysis. The present laboratory-based descriptive study employed a total of 18 *Salmonella enterica* subsp. *enterica* isolates, which were recovered and archived from a previous investigation. Of the 751 food samples examined, 131 consisted of milk and dairy products, including ricotta, massora, and raw cow's milk. The remaining 620 samples comprised meat and meat products, such as minced beef, beef sausage, chicken breast, chicken burger, camel liver, and cattle liver. Animal-origin food samples were randomly collected from retail markets in Tripoli, Sabha, Tobruk, and Regdalin, Libya. During the present experiment, the isolates were revived from -80˚C storage by taking a single cryobead from each cryovial and transferring it into 5 mL of peptone water. The cultures were subsequently incubated for 24 hours at 35-37˚C before further analysis.

Antimicrobial susceptibility testing

The antimicrobial susceptibility of the *Salmonella* isolates was determined using the Kirby-Bauer disk diffusion method (Antrim BT41, England) on Mueller-Hinton agar (MHA), following the guidelines of the Clinical and

Laboratory Standards Institute (CLSI). Briefly, the procedure involved growing a sterile nutrient broth culture of every isolate to the logarithmic phase and then normalizing the turbidity of the suspension to match a 0.5 McFarland standard. Following this standardization step, the bacterial suspension was spread evenly across the surface of MHA plates using a swabbing technique, then allowed to air-dry for approximately five minutes.

A total of 33 antibiotic disks representing 12 antimicrobial classes (Antrim Technology Park, Antrim BT41, England) were placed on the surface of the inoculated agar plates. The antibiotics tested included amoxicillin (AMX, 30 µg), ampicillin (AP, 30 µg), amoxicillin/clavulanate (AC, 20/10 µg), penicillin (P, 10 µg), methicillin (ME, 5 µg), piperacillin/tazobactam (PTM, 100/10 µg), cloxacillin (OB, 5 µg), ticarcillin/clavulanate (TCL, 75/10 µg), cefepime (CFP, 30 µg), cefoperazone (COZ, 75 µg), cefotaxime (CTX, 30 µg), ceftriaxone (CFT, 30 µg), cefoxitin (CFO, 30 µg), imipenem (IMP, 10 µg), meropenem (MER, 10 µg), ertapenem (ERT, 10 µg), neomycin (NEO, 10 µg), kanamycin (KAN, 30 µg), gentamicin (GEN, 10 µg), tobramycin (TOB, 10 µg), streptomycin (S, 10 µg), tetracycline (TET, 30 µg), doxycycline (DO, 30 µg), oxytetracycline (OT, 30 µg), levofloxacin (LEV, 5 µg), ciprofloxacin (CIP, 5 µg), polymyxin (POM, 300 units), chloramphenicol (CHL, 30 µg), sulfamethoxazole/trimethoprim (COT, 23.75/1.25 µg), aztreonam (AZT, 30 µg), nitrofurantoin (NIT, 300 µg), rifampicin (RIF, 5 µg), and norfloxacin (NOR, 10 µg). The plates were incubated at 37˚C for 24 hours, after which the inhibition zone diameters were measured and interpreted according to CLSI guidelines.

Multiple antibiotic resistance index

Multiple antibiotic resistance (MAR) values were determined and interpreted according to the method described by Hinton and Linton (1983) using the following formula.

MAR index = a/b

Where a represents the number of antibiotics against which an individual isolate is resistant, and b represents the total number of antibiotics tested. An A MAR index of 0.2 or higher (≥ 0.2) indicated that the isolate originated from a high-risk source where antibiotics were frequently used or misused.

Identification of multi-drug resistance, extensively drug-resistant, and pan-drug-resistant isolates

The isolates were categorized based on their resistance profiles as MDR, which was characterized by resistance to at least one antimicrobial agent across three or more distinct antimicrobial classes, extensively drug-resistant (XDR), defined by resistance to at least one antimicrobial agent in all but two or fewer antimicrobial classes, and pan drug-resistant (PDR), identified by resistance to all tested agents within all antimicrobial classes. The MDR, XDR, and PDR values for each isolate were determined according to the criteria established by Magiorakos et al. (2012) and WHO (2023).

RESULTS

Prevalence

Among the examined food samples, including raw milk, dairy products, meat, and meat products, 18 bacterial isolates were identified as *Salmonella enterica* subsp. *enterica* using conventional microbiological techniques and confirmed by partial 16S rDNA sequencing in the previous studies. Of these 18 isolates, the highest prevalence was observed in raw cow's milk, with 7 isolates (39.1%). Two isolates (11.1% each) were recovered from camel liver, cattle liver, and minced beef. The lowest prevalence of *Salmonella enterica* subsp. *enterica* (5.5%) was observed in chicken burger, beef sausage, chicken breast, massora, and ricotta.

Antimicrobial profiles

The antimicrobial susceptibility profiles of the confirmed *Salmonella enterica* subsp. *enterica* isolates are presented in Table 1. A total of 18 isolates were tested against 33 antimicrobial agents (Figure 1). All isolates exhibited complete resistance (100%) to penicillin. High resistance rates were observed for neomycin and rifampicin (94.4%), whereas resistance to aztreonam and tobramycin was low (5.6%). Among fluoroquinolones, levofloxacin and ciprofloxacin exhibited the lowest resistance rates (16.7%, 3/18), followed by norfloxacin (11.1%, 2/18). Within the cephalosporin class, cefoxitin was the most effective, with a susceptibility rate of 83.3% (15/18), while cefoperazone and cefepime indicated lower susceptibility rates of 61.1% and 38.9%, respectively. None of the isolates were resistant to imipenem, but 38.9% (7/18) exhibited resistance to meropenem. A notable proportion of isolates displayed intermediate resistance, particularly against cloxacillin (100%), polymyxin (83.3%), and kanamycin (66.7%). Overall, the highest susceptibility rates were observed for chloramphenicol, nitrofurantoin, sulfamethoxazole/trimethoprim, and norfloxacin, each demonstrating a susceptibility rate of 88.9%.

Table 1. Antibiotic susceptibility profiles of *Salmonella enterica* subsp. *enterica* from foods of animal origin

Categories	Antibiotics	Susceptible (%)	Intermediate (%)	Resistant (%)	MDR (%)
Carbapenems	Imipenem 10µg	12 (66.7)	6 (33.3)	0 (0)	0
	Meropenem 10 µg	1 (5.6)	10 (55.6)	7 (38.9)	38.9
	Ertapenem 10µg	10 (55.6)	5 (27.8)	3 (16.7)	16.7
Cephalosporins	Cefepime 30µg	7 (38.9)	11 (61.1)	0 (0)	0
	Cefoperazone 75 µg	11 (61.1)	10 (55.6)	3 (16.7)	16.7
	Cefotaxime 30 µg	6 (33.3)	11 (61.1)	1 (5.6)	5.6
	Ceftriaxone 30 µg	6 (33.3)	7 (38.9)	5 (27.8)	27.8
	Cefoxitin 30 µg	15 (83.3)	2 (11.1)	1 (5.6)	5.6
Penicillin	Amoxicillin 30 µg	13 (72.2)	1 (5.6)	4 (22.2)	22.2
	Ampicillin 30 µg	9 (50)	5 (27.8)	4 (22.2)	22.2
	Ticarcillin 75 µg + Clavulanate 10 µg	6 (33.3)	8 (44.4)	4 (22.2)	22.2
	Cloxacillin 5 µg	0 (0)	18 (100)	0 (0)	0
	Methicillin 5 µg	1 (5.6)	2 (11.1)	15 (83.3)	83.3
	Piperacillin 100 µg / Tazobactam 10 µg	1 (5.6)	13 (72.2)	4 (22.2)	22.2
	Amoxicillin clavulanate 20 µg/10 µg	12 (66.7)	1 (5.6)	5 (27.8)	27.8
	Penicillin 10 µg	0 (0)	0 (0)	18 (100)	100
Monobactum	Aztreonam 30 µg	14 (77.8)	3 (16.7)	1 (5.6)	5.6
Aminoglycosides	Neomycin 10 µg	0 (0)	1 (5.6)	17 (94.4)	94.4
	Kanamycin 30 µg	3 (16.7)	12 (66.7)	3 (16.7)	16.7
	Gentamicin 10 µg	13 (72.2)	3 (16.7)	2 (11.1)	11.1
	Tobramycin 10 µg	10 (55.6)	7 (38.9)	1 (5.6)	5.6
	Streptomycin 10 µg	1 (5.6)	9 (50)	7 (38.9)	38.9
Fluoroquinolones	Levofloxacin 5 µg	11 (61.1)	4 (22.2)	3 (16.7)	16.7
	Ciprofloxacin 5 µg	10 (55.6)	5 (27.8)	3 (16.7)	16.7
	Norfloxacin 10 µg	16 (88.9)	0 (0)	2 (11.1)	11.1
Tetracyclines	Oxytetracycline 30 µg	0 (0)	7 (38.9)	11 (61.1)	61.1
	Tetracycline 30 µg	8 (44.4)	0 (0)	10 (55.6)	55.6
	Doxycycline 30 µg	9 (50)	1 (5.6)	8 (44.4)	44.4
Sulfonamides	Sulfamethoxazole 23.75 µg / Trimethoprim 123.25 µg	16 (88.9)	0 (0)	2 (11.1)	11.1
Phenicols	Chloramphenicol 30 µg	16 (88.9)	0 (0)	2 (11.1)	11.1
Nitrofurantoin	Nitrofurantoin 300 µg	16 (88.9)	0 (0)	2 (11.1)	11.1
Rifampicin	Rifampicin 5 µg	1 (5.6)	0 (0)	17 (94.4)	94.4
Glycopeptides	Polymyxin 300 units	3 (16.7)	15 (83.3)	0 (0)	0

MDR: Multidrug-resistant

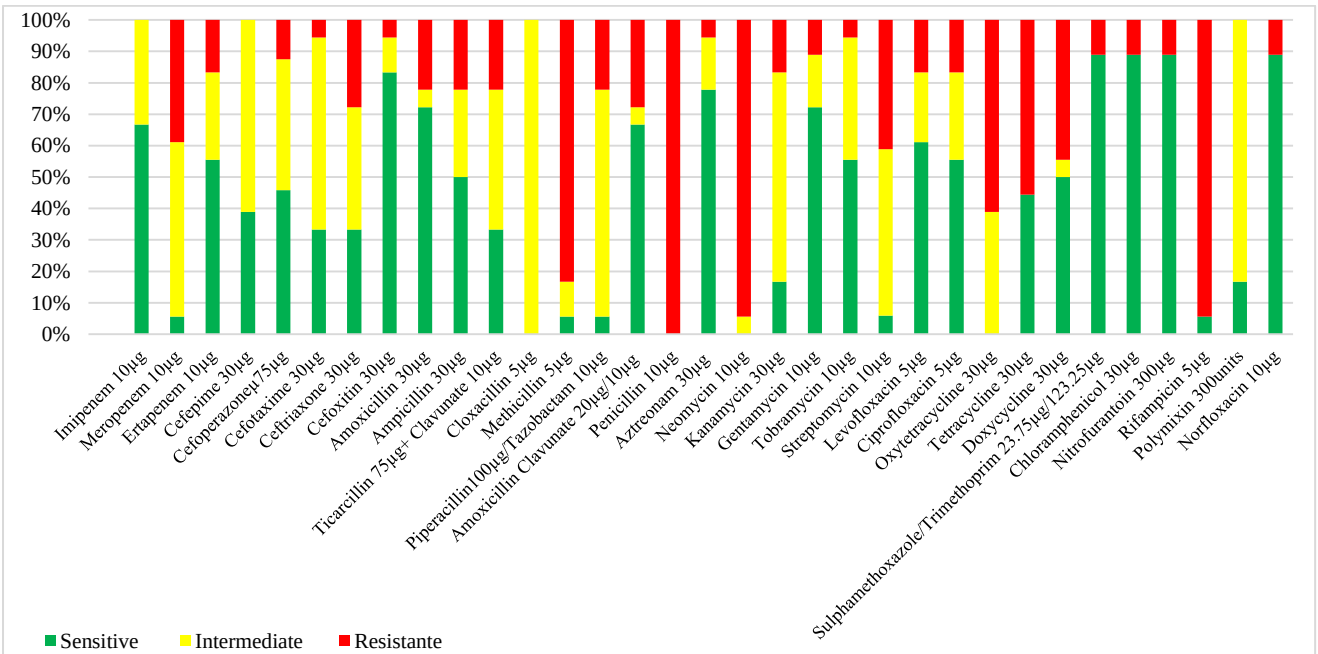


Figure 1. Antimicrobial susceptibility profiles of *Salmonella enterica* subsp. *enterica* isolated from foods of animal origin against 33 antimicrobial agents. All isolates exhibited different degrees of resistance, with the highest resistance observed against penicillin (100%).

Multiple antibiotic-resistant index

In the present study, the MAR index of *Salmonella enterica* subsp. *enterica* isolates ranged from 0.1 to 0.6. The highest MAR values were observed in isolates from chicken breast (S18, 0.6) and chicken burger (S34, 0.5), indicating significant exposure to multiple antibiotics. In contrast, isolates from raw cow's milk samples (S22, S38, S40, and S42) exhibited the lowest MAR indices (0.1; Table 2).

Multi-drug resistant patterns of *Salmonella enterica* subsp. *enterica*

All 18 tested isolates exhibited resistance to three or more antimicrobial classes, indicating MDR. Resistance patterns differed among the isolates, with numbers ranging from 4 to 9 antimicrobial classes (Table 2). The majority of isolates were MDR, reflecting extensive resistance across the tested samples. The highest level of resistance was observed in isolates S18 (from chicken breast) and S43 (from raw cow's milk), each resistant to nine antimicrobial classes. In contrast, isolates S22 and S42, both recovered from raw cow's milk, exhibited resistance to a minimum of four antimicrobial classes. No XDR or PDR isolates were identified in the current study.

Table 2. Antibiotic resistance profiles, multiple antibiotic resistance index, antibiotics class resistance in *Salmonella enterica* subsp. *enterica* isolated from foods of animal origin

Isolates no	Food sample	Resistant antibiotic	MAR index	Antimicrobial classes
S17	Minced beef	10	0.3	5
S18	Chicken breast	21	0.6	9
S19	Beef sausage	13	0.3	7
S20	Camel liver	10	0.3	6
S22	Raw cow's milk	4	0.1	4
S28	Camel liver	11	0.3	6
S29	Cattle liver	9	0.2	5
S30	Cattle liver	10	0.3	7
S34	Chicken burger	17	0.5	7
S38	Raw cow's milk	6	0.1	5
S40	Raw cow's milk	6	0.1	5
S41	Raw cow's milk	12	0.3	7
S42	Raw cow's milk	5	0.1	4
S43	Raw cow's milk	17	0.5	9
S92	Massora	7	0.2	5
S211	Minced beef	12	0.3	7
S307	Raw cow's milk	8	0.2	6
S308	Ricotta	7	0.2	6

MAR: Multiple antibiotic resistance

DISCUSSION

Salmonella enterica subsp. *enterica* is responsible for some of the most severe febrile infections, exhibiting high morbidity and mortality rates in developing countries (Roy et al., 2015). The prevalence of *S. enterica* subsp. *enterica* in foods of animal origin differed across countries (Elbediwi et al., 2021). In the present study, *S. enterica* subsp. *enterica* was detected as a microbial contaminant in milk, different dairy products, meat, and meat products. The highest prevalence was observed in raw cow's milk samples (39.1%). Similarly, Adzitey et al. (2025) reported the highest incidence of *S. enterica* in raw cow's milk at 38.0% in Ghana. In contrast, in Ethiopia, the prevalence of *Salmonella* in fresh cow's milk was reported to be lower, at 9.3% (Asefa et al., 2023). The variation in reported prevalence rates of *Salmonella enterica* in raw cow's milk in the current study, in Ghana (38.0%), and in Ethiopia (9.3%) may be attributed to differences in regional ecological conditions, sample size, animal husbandry practices, and milking hygiene (Adzitey et al., 2020). Consequently, the consumption of contaminated raw milk and its products posed a significant public health hazard in countries such as Libya, where many people consume raw milk without prior heat treatment.

According to the present findings, the incidence of *S. enterica* in ricotta and massora cheese samples was 5.5%. In contrast, Aragão et al. (2019) and Rebić et al. (2022) did not detect *S. enterica* in examined curd cheese samples. In the present study, the prevalence of *S. enterica* in chicken breast meat (5.5%) was lower than that reported by Regalado-Pineda et al. (2020), who reported a prevalence of 18.1% in chicken meat samples. Additionally, Khan et al. (2024) reported a notably higher prevalence of *S. enterica* in poultry (60%) than in other food types, including dairy products (33.3%), beef (30%), vegetables (26.7%), and fruits (20%). Although the prevalence of *S. enterica* in chicken breast

meat was comparatively low, the *Salmonella* isolate number S18 exhibited the highest antimicrobial resistance among all isolates in the present study, highlighting the potential risk posed by even low-prevalence sources.

In Libya, salmonellosis was not officially classified as an outbreak disease, and limited data were available to definitively assess the current state of food safety governance (Abuhlega, 2023). Nevertheless, different studies have investigated the occurrence of *Salmonella* in Libya (Garbaj et al., 2022; Asheg et al., 2023). A study conducted in Zliten, central Libya, reported that *Salmonella* was detected in approximately 13.6% of diarrheal cases among children, often in co-infection with other pathogens, and many of the identified strains exhibited resistance to commonly used antibiotics, including ampicillin, tetracycline, chloramphenicol, and trimethoprim-sulfamethoxazole (Ali et al., 2005). Additionally, a study in Tripoli found *Salmonella* in about 8% of food samples from bakeries, poultry outlets, and camel meat butcheries, with the highest prevalence observed in poultry product shops (Ibrahim et al., 2022).

In the present study, the antimicrobial susceptibility of *S. enterica* subsp. *enterica* isolates were evaluated against 33 antibiotics from 12 classes. All isolates exhibited different degrees of resistance, with the highest resistance observed against penicillin (100%, 18/18). Additionally, methicillin revealed a high resistance rate of 83.3%. The high resistance rates were consistent with those reported by Elshebrawy et al. (2022), who found that the highest resistance rates among *S. enterica* isolates recovered from frozen chicken carcasses in Egypt were to oxacillin (94.7%), penicillin (86.8%), and ampicillin (26.3%). The high resistance rates observed against penicillin in the present study may be attributed to its frequent use as a first-line treatment for bacterial infections in humans and animals, which can drive the development of microbial resistance (Shenoy et al., 2019). The susceptibility of isolates to amoxicillin and ampicillin was relatively high in the present study. The relatively high susceptibility of the isolates to amoxicillin and ampicillin observed in the present study is consistent with the results of Adzitey et al. (2020), who reported that 86.0% of *S. enterica* isolates from cow's milk and dairy products in Ghana were susceptible to ampicillin. Similarly, Moraes et al. (2024) found that 95.6% of *S. enterica* isolates from commercial laying hen farms in Brazil were susceptible to ampicillin. In contrast, different studies from different regions have reported higher levels of ampicillin resistance among foodborne *Salmonella* isolates, particularly in isolates from human samples (Mahmood et al., 2023; Kanat and Terzi Gulel, 2025). The differences in ampicillin resistance levels across the present study and previously reported studies likely reflected regional variations in antimicrobial usage and resistance patterns. The differences in ampicillin resistance levels between the present study and previously reported studies likely reflect regional variations in antimicrobial usage and resistance patterns. This variation in ampicillin resistance highlights the importance of conducting local antimicrobial-susceptibility testing of foodborne pathogens in Libya to inform food safety and public health programs.

Importantly, no *S. enterica* subsp. *enterica* isolates in the present study were fully resistant to imipenem; 66.7% were classified as susceptible, whereas 33.3% demonstrated intermediate susceptibility. The current finding serves as a cautionary indicator of potential treatment failures as antibiotic resistance continues to emerge. Correspondingly, Adzitey et al. (2025) reported that all *S. enterica* isolates in Ghana were susceptible to imipenem, and Kanat and Terzi Gulel (2025) found that all *Salmonella* spp. isolated from cow's milk and dairy products in Turkey were 100% susceptible to imipenem.

The current findings demonstrated variable resistance levels to different carbapenems, with *S. enterica* subsp. *enterica* isolates exhibiting resistance rates of 38.9% and 16.7% to meropenem and ertapenem, respectively. The observed resistance to meropenem contrasts with the findings of Listorti et al. (2022) in Italy, who reported that meropenem was the only antimicrobial agent to which no *Salmonella* strains exhibited resistance. The resistance to meropenem and ertapenem is concerning, as it indicates a potential decrease in the antibiotic efficacy. These findings are particularly concerning, as carbapenems are often the last treatment option for MDR infections (Adel et al., 2021). Moreover, 94.4% (17/18) of the isolates were highly resistant to neomycin. The current finding was contrasted with the findings of Kanat and Terzi Gulel (2025), who reported 100% susceptibility to neomycin among *S. enterica* isolates from cow's milk and dairy products in Turkey. Similarly, Mekonnen et al. (2022) in Ethiopia found that *S. enterica* isolates from fecal and cow's milk samples demonstrated a high susceptibility to the antimicrobial agents tested, including neomycin. The discrepancies in the results highlighted regional differences in antimicrobial use and resistance patterns, underscoring the need for localized surveillance of antibiotic resistance in foodborne pathogens.

Although the excessive use of aminoglycosides in veterinary and human medicine has led to a substantial rise in microbial resistance, these antibiotics remain useful for treating different bacterial infections (Wang et al., 2022). In the present study, low gentamicin resistance rates were observed (11.1%), consistent with a previous study in Egypt, which reported a resistance rate of 2.6% among *Salmonella* isolates (Elshebrawy et al., 2022). Notably, gentamicin plays an important role in routine monitoring systems for investigating resistance mechanisms in *Salmonella* species.

Fluoroquinolones and cephalosporins are considered first-line antimicrobials and are recommended for the treatment of salmonellosis. Consequently, resistance to these medicines has become a major public health concern (Li et al., 2020). However, the isolates examined in the present study did not exhibit resistance to fluoroquinolones or cephalosporins.

Among the fluoroquinolones, norfloxacin indicated the highest susceptibility rate (88.9%), followed by levofloxacin (61.1%) and ciprofloxacin (55.6%). The ciprofloxacin susceptibility rate in the present study (55.6%) was lower than that reported for *S. enterica* isolates from poultry carcasses in Brazil and Ghana, both of which demonstrated 100% susceptibility to ciprofloxacin (Panzenhagen et al., 2016; Adzitey et al., 2020). Conversely, the susceptibility rate observed in the current study was higher than that reported in India, where Mir et al. (2015) found a ciprofloxacin susceptibility rate of 46.87% in poultry-derived *S. enterica* isolates. Differences in ciprofloxacin susceptibility profiles might be attributed to variations in its use for therapeutic or prophylactic purposes across regions. Due to the increasing incidence of antimicrobial resistance, particularly against fluoroquinolones, cephalosporins have emerged as the primary treatments for *Salmonella* infections (Lamichhane et al., 2024). In the present study, *S. enterica* subsp. *enterica* isolates exhibited low resistance to cefotaxime (5.6%) and no resistance to cefepime (0%). In contrast, a study conducted in China reported higher resistance rates for cefotaxime (70.46%) and cefepime (58.72%) among *S. enterica* isolates (Ma et al., 2018).

All isolates in the present study were MDR (18/18, 100%), a level comparable to that reported by Ayichew et al. (2025), who found all *S. enterica* isolates to be MDR. In addition, a high prevalence of MDR *S. enterica* (50.9%) was reported in Poland (Wójcicki et al., 2022). However, the MDR rate observed in the present study was higher than that reported by Adzitey et al. (2025), who found that only 18.8% of *S. enterica* isolates were MDR. The MAR index of *S. enterica* subsp. *enterica* isolates in the present study varied, ranging from 0.1 to 0.6. Similarly, Adzitey et al. (2025) reported MAR indices ranging from 0.11 to 0.77 in *S. enterica* isolates. Furthermore, Durrani et al. (2024) observed MAR values ranging from 0.4 to 0.6, whereas previous studies reported that all tested *S. enterica* isolates had MAR indices greater than 0.2 (Mir et al., 2022; Fahmy et al., 2025). In contrast, Wójcicki et al. (2022) observed that the majority of *S. enterica* isolates demonstrated MAR indices below 0.3, with only a single isolate surpassing 0.5. The presence of isolates with MAR indices of 0.3, 0.5, and 0.6 in the current study highlighted the potential overuse or misuse of antibiotics and represented a significant public health concern, as such isolates may serve as reservoirs of multidrug resistance.

CONCLUSION

The presence of *Salmonella enterica* subsp. *enterica* in milk, dairy products, meat, and meat products indicated a potential public health concern in Libya, particularly because some dairy products are frequently consumed without adequate heat treatment. Contamination of foods derived from animal origin highlighted the risks associated with inadequate hygiene practices during processing, handling, and storage. The current findings revealed considerable antimicrobial resistance among the isolates examined, with all *S. enterica* strains exhibiting multidrug resistance. The presence of such multidrug-resistant phenotypes highlighted the potential spread of resistant foodborne pathogens through the food chain. Therefore, persistent surveillance of foodborne pathogens, enhanced hygienic practices during food production and distribution, proper heat treatment of dairy products, and the responsible use of antimicrobial agents are imperative to mitigate the risk of foodborne infections. Furthermore, consistent surveillance of antimicrobial susceptibility patterns of foodborne bacteria in Libya is essential to facilitate effective treatment strategies and enhance national food safety programs. The present study was limited by the relatively small number of *S. enterica* isolates and by its focus on foods collected from retail markets in Libya, which might not have fully reflected resistance patterns in other regions or across different food types. Furthermore, antimicrobial resistance profiles may fluctuate over time; consequently, the current results may not accurately predict future trends. Further studies with larger sample sizes, broader geographic coverage, and periodic monitoring are needed to confirm these findings and further understand the spread of multidrug-resistant strains.

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Authors' contributions

Salah Mohamed Azwai, Aboubaker Mohamed Garbaj, and Ibrahim Mohamed Eldaghayes were responsible for the conception and design of the study. Salah Mohamed Azwai, Aboubaker Mohamed Garrbaj, Jihan Ali Sherif, Samira Abd Farag, Salem Farhat Abureema, and Fatim Taher Gammoudi conducted the laboratory experiments and data collection. Salah Mohamed Azwai and Aboubaker Mohamed Garrbaj carried out the data analysis. The authors Salah Mohamed

Azwai, Aboubaker Mohamed Garrbaj, Ibrahim Mohamed Eldaghayes, Jihan ALi Sherif, Samira Abd Farag, Salem Farhat Abureema, and Fatim Taher Gammoudi contributed to the composition and revision of the manuscript. All authors reviewed and approved the final edition of the manuscript.

Availability of data and materials

All data produced during the study are relevant and included in this published article, and are accessible from the corresponding author upon reasonable request.

Competing interests

The authors declared that there is no conflict of interest.

Ethical considerations

All authors have reviewed ethical considerations, including plagiarism, consent to publish, misconduct, data fabrication and falsification, duplicate publication and submission, and redundancy. Furthermore, the authors did not utilize any artificial intelligence tools in data preparation, writing, or revision of the manuscript.

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Risk Assessment of Dog-Mediated Rabies in a Rural Indonesian Community: A One Health Approach

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ABSTRACT

Rabies remains a fatal zoonotic disease that continues to cause thousands of human deaths annually, particularly in regions where dog-mediated transmission persists. In Indonesia, rabies remains endemic in different provinces, and rural communities with frequent human-dog interactions may face an increased risk of exposure. The present study aimed to assess the risk of dog-mediated rabies transmission in a rural Indonesian community using a One Health perspective. A cross-sectional descriptive study was conducted in Sirukam Village, Solok District, West Sumatra, Indonesia, in August 2025. Data were collected through structured questionnaires administered to 96 respondents, field observations of dog populations, and semi-structured interviews with hunters, dog owners, and local health workers. Secondary data on bite incidents and post-exposure prophylaxis (PEP) completion were obtained from the local public health center. The results revealed that 56.25% of respondents had moderate knowledge regarding rabies prevention, while preventive practices, including wound washing and vaccination adherence, were inconsistent. A total of 14 dog bite cases were reported during January-December 2024. Only 10 of the 14 exposed individuals (71.43%) completed the full PEP vaccination schedule, indicating suboptimal adherence. Field observations revealed 148 free-roaming dogs in the study area, indicating frequent opportunities for human-dog interactions. Qualitative interviews with selected participants suggested that reluctance to vaccinate hunting dogs and limited awareness of rabies prevention may contribute to sustained transmission risks. However, the qualitative findings from interviews with selected participants should be interpreted cautiously due to the limited number of informants. Based on a qualitative risk assessment combining the likelihood of exposure and the potential public health impact using a semi-quantitative scoring approach, rabies was categorized as a high-risk health threat in the study area. The results highlight the importance of strengthening dog vaccination programs, improving community awareness, and enhancing multisectoral collaboration to reduce rabies transmission in rural endemic settings.

Keywords: Dog bite, Rabies, Risk assessment, Vaccination, Zoonotic disease

INTRODUCTION

Rabies is a lethal zoonotic disease caused by the rabies virus (RABV), which belongs to the genus Lyssavirus within the family Rhabdoviridae. Domestic dogs remain the main reservoir of the virus and are responsible for over 99% of human rabies infections worldwide (Hampson et al., 2015; WHO, 2023). Although rabies can be effectively prevented through mass dog vaccination and prompt administration of post-exposure prophylaxis (PEP), the disease still results in approximately 59,000 human deaths annually, with more than 95% of cases reported in Asia and Africa, where access to medical and veterinary services is often limited (WHO, 2023). Children younger than 15 years old account for a considerable proportion of rabies exposure incidents due to their frequent interaction with dogs, particularly in rural settings (Rehman et al., 2021). In Indonesia, rabies remains endemic in several provinces, including West Sumatra, where dog-mediated transmission continues to pose a public health concern. Surveillance reports indicate that animal bite cases remain a significant public health concern in Indonesia, with approximately 66,170 cases reported between January and July 2023 alone (WHO, 2023). Previous reports also estimate an average of 80,000-100,000 cases per year, highlighting the ongoing risk of rabies exposure in the country. At the regional level, West Sumatra reports hundred bite incidents annually, with 96 cases recorded during week 23 of 2024, highlighting the continued risk of rabies transmission in the area (Ministry of Health of the Republic of Indonesia, 2019; Ginuluh et al., 2024; Agricultural Quarantine Agency Class I Padang, 2024). In West Sumatra, wild boar hunting using dogs is a long-standing cultural practice that may increase rabies transmission risk due to frequent dog movement and interaction (Amanatin et al., 2019). Wild boar hunting practices in rural communities often involve the movement and aggregation of multiple dogs and hunters gathering from different areas, which may increase the likelihood of direct interactions involving bites or exposure to infected saliva, the primary routes of rabies transmission (WHO, 2023). In Sirukam, wild boar hunting practices involving the use of dogs are commonly observed (Amanatin et al., 2019), and previous studies have shown that vaccination coverage among free-roaming or hunting dogs in rural areas is often limited, increasing the risk of rabies transmission (Taylor et al., 2017; WHO, 2023). Limited vaccination coverage among hunting dogs may be influenced by

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socio-cultural beliefs and community perceptions regarding vaccination, including concerns that it may affect the physical performance of dogs used for hunting. In addition, the presence of free-roaming dogs used for hunting may increase exposure risk, although concerns regarding reduced performance after vaccination are not supported by scientific evidence (Widyastuti et al., 2015; Taylor et al., 2017). Despite the presence of multiple risk factors, including limited vaccination coverage, hunting practices involving dogs, and the presence of free-roaming dogs, no previous studies have specifically assessed rabies transmission risk in Sirukam village, Solok district, West Sumatra, Indonesia, where such factors are known to contribute to rabies transmission dynamics (Amanatin et al., 2019; Warembourg et al., 2021). National surveillance reports have documented over 80,000 animal bite cases annually in Indonesia, with vaccination coverage and PEP completion rates varying across regions, with vaccination coverage often reported below the recommended 70% threshold in several rural areas (Ministry of Health of the Republic of Indonesia, 2019; Ginuluh et al., 2024). Community practices such as hunting with dogs and allowing dogs to roam freely highlight the importance of understanding dog population dynamics and human behaviors that influence rabies exposure risk (Taylor et al., 2017; Amanatin et al., 2019; WHO, 2023).

However, specific data describing rabies transmission risk and dog population dynamics at the village level remain limited in many rural areas of West Sumatra, including Sirukam, as previous studies have mainly focused on knowledge and attitudes toward rabies rather than comprehensive risk assessments integrating human behavior, dog ecology, and environmental factors (Widyastuti et al., 2015; Taylor et al., 2017; WHO, 2023). The lack of comprehensive risk assessments integrating human behavior, dog ecology, and environmental factors highlights the need for a localized assessment of rabies transmission risk to support targeted prevention strategies (Taylor et al., 2017; WHO, 2023).

Global rabies elimination initiatives, including the Zero by 30 strategy, aim to eliminate human deaths caused by dog-mediated rabies by the year 2030 through coordinated actions such as mass dog vaccination, improved access to PEP, and strengthened surveillance systems (WHO, 2023). Achieving the global goal of eliminating human deaths caused by dog-mediated rabies by 2030 requires the implementation of a One Health approach, which promotes coordinated collaboration among human health, veterinary, and environmental sectors to address the interconnected drivers of rabies transmission (Cleaveland et al., 2014; WHO, 2023). In rural endemic settings, rabies risk is often shaped by multiple interacting factors, including dog population characteristics, human knowledge and preventive practices, patterns of human-dog interaction, and environmental conditions influencing dog movement and contact rates (Hampson et al., 2015; WHO, 2023). Cultural practices, including reluctance to vaccinate hunting dogs due to community perceptions regarding potential negative effects on their performance, as well as the common practice of allowing dogs to roam freely, may further influence rabies exposure risk (Widyastuti et al., 2015; Taylor et al., 2017). In addition, logistical barriers, including limited access to vaccination services, long distances to vaccination facilities, and inconsistent availability of vaccines, may reduce vaccination uptake and disease reporting (Hampson et al., 2015; Taylor et al., 2017; WHO, 2023). A comprehensive risk assessment is required to better understand rabies transmission dynamics in the study area, given the presence of multiple interacting factors, including hunting practices, free-roaming dog populations, limited vaccination coverage, socio-cultural beliefs, and logistical barriers (Cleaveland et al., 2014; Taylor et al., 2017). A One Health framework was employed to integrate data on human behavior (knowledge and practices), animal factors (dog population characteristics), and environmental context (interaction patterns and dog movement) to assess the risk of dog-mediated rabies in Sirukam, Solok District, West Sumatra, Indonesia. The present study aimed to assess the risk of dog-mediated rabies in a rural community of Sirukam, Solok District, West Sumatra, Indonesia.

MATERIALS AND METHODS

Ethical approval

Ethical approval for the study was obtained from the Ethics Committee of the State University of Padang (Approval No: 123/UNP/FK/EC/2025). Additional research permission was granted by the Solok District authorities. All participants provided written informed consent prior to data collection. No identifiable personal information was recorded, and all data were anonymized to ensure confidentiality. All participants were informed about the purpose of the study, the voluntary nature of participation, and their right to withdraw at any time without consequence. No personally identifiable information was recorded, and all data were anonymized to ensure confidentiality. Permission to conduct the study was also obtained from the relevant local authorities

Study area

The study was conducted in Sirukam Village, Solok District, West Sumatra Province, Indonesia, in August 2025. Sirukam consists of four sub-villages with an estimated population of approximately 5,654 inhabitants based on data from the (BPS-Statistics Solok Regency, 2024). Most residents are engaged in agriculture and traditional hunting activities. Primary healthcare services are provided by the Sirukam Public Health Center which serves as the main facility for rabies PEP. Veterinary services are available in Solok District, located approximately 15 km from the village, which may limit timely access to dog vaccination and rabies control measures. In addition, geographic and logistical

factors such as travel distance and transportation availability may influence access to both healthcare and veterinary services. The presence of free-roaming dogs and frequent human-dog interactions further increases the potential risk of rabies transmission in rural communities.

Study design

The current study employed a cross-sectional descriptive design conducted in August 2025 to assess the risk of dog-mediated rabies using a One Health perspective. The study integrated human, animal, and environmental factors influencing disease transmission (Destoumieux-Garzón et al., 2018; WHO, 2023). Data were collected using a structured questionnaire, field observations of dog populations, and semi-structured interviews with selected key informants. The questionnaire was developed based on previously published rabies knowledge, attitudes, and practices (KAP) studies and adapted to the local context (Sambo et al., 2014; Widyastuti et al., 2015). It was designed by the research team and reviewed by a public health officer from the Solok District Health Office with experience in rabies control. The One Health approach in the study considers the interactions between human behavior, dog population dynamics, and environmental conditions that may contribute to rabies transmission in rural communities. Quantitative data were obtained from 96 respondents selected through purposive sampling across four sub-villages in Sirukam. The number of respondents from each sub-village was proportionally determined based on the estimated number of households with frequent dog contact. Qualitative data were collected from three key informants selected based on their roles in the community, including a hunter, a community leader, and a public health officer. Data collection was conducted by the research team through face-to-face interviews using structured questionnaires and semi-structured interview guides. The sample size for the quantitative component was determined based on feasibility considerations, field accessibility, and the estimated population of households with regular dog exposure in the study area.

Participants and sampling

A total of 96 respondents were included in the study using a purposive sampling approach, consistent with methods used in previous rabies knowledge, attitudes, and practices (KAP) studies (Sambo et al., 2014; Widyastuti et al., 2015; WHO, 2023). The sample size was determined based on the estimated number of households with frequent dog contact in the study area and the feasibility of conducting face-to-face interviews within the study period. Purposive sampling was applied to ensure that participants had direct experience with dogs and potential exposure to rabies risk. Eligible participants included dog owners, hunters, farmers, and community members who frequently interacted with dogs in their daily activities. The distribution of respondents by occupation and dog ownership status is presented in Table 1. Inclusion criteria were adults aged 18 years or older who were willing to participate and able to complete the questionnaire. Individuals who declined participation or provided incomplete responses were excluded during the data cleaning process prior to analysis. Respondents were recruited with the assistance of local community leaders and village authorities who helped identify residents with regular contact with dogs in the four sub-villages of Sirukam. At the time of the study, participants were actively engaged in activities involving dogs, either through ownership or regular interaction. In addition to the questionnaire survey, qualitative data were obtained through semi-structured interviews with three key informants, including a hunter, a community leader, and a public health officer.

Table 1. Sociodemographic characteristics of respondents in Sirukam, Solok District, West Sumatra, Indonesia, August 2025

Variable	Category	Frequency (n)	Percentage
Gender	Male	61	63.50
	Female	35	36.50
Age group	< 20 Years	20	20.83
	21-30 Years	23	23.96
	31-40 Years	25	26.04
	> 40 Years	28	29.17
Education	No formal education	3	3.10
	Primary school	10	10.40
	Junior high school	13	13.50
	Senior high school	41	42.70
	University	25	30.20
Occupation	Farmer	13	13.54
	Hunter	23	23.96
	Housewife	13	13.54
	Trader	18	18.75
	Employee	16	16.67
	Others (students, laborers, drivers)	13	13.54
Dog ownership	Yes	41	43.75
	No	55	57.29
History of dog bites	Yes	13	13.54
	No	83	86.50

n: Number of respondents, %: Percentage of total respondents (N = 96). Occupation categories represent the main self-reported livelihood of respondents. The Others category includes respondents with occupations that did not fit into predefined categories and were reported in small proportions (each < 5% of the total sample), such as students, laborers, drivers, and other informal occupations.

Data collection

Structured questionnaires

A structured questionnaire was used to assess community knowledge, attitudes, and practices (KAP) related to rabies and dog bite risks. The full questionnaire is provided as Supplementary File 1, including both the English version for publication and the original Bahasa Indonesia version used during data collection. The questionnaire consisted of 25 items divided into five domains, including knowledge about rabies (5 items), perception of dog bite risk (5 items), attitudes toward bite management (5 items), perceptions of dog populations in the environment (5 items), and interactions with free-roaming dogs (5 items). The questionnaire was administered through face-to-face interviews to ensure clarity and completeness. Dog ownership status was self-reported by respondents during the household survey. Dog ownership was defined as respondents who reported owning at least one dog at the time of the survey. Items were measured using a four-point Likert scale ranging from 1 (strongly disagree) to 4 (strongly agree). The questionnaire was adapted from previously published rabies knowledge, attitudes, and practices (KAP) studies and international as well as national public health survey instruments, including guidelines from the World Health Organization (WHO), World Organization for Animal Health (WOAH), and national instruments from the Ministry of Health of the Republic of Indonesia (WHO/FAO/WOAH, 2018; Ministry of Health of the Republic of Indonesia, 2021). Prior to the main survey, the questionnaire was pilot-tested among 10 residents in a neighboring village to evaluate the clarity and relevance of the questions. Minor revisions were made based on participant feedback, particularly in the knowledge and attitudes sections, including clarification of question wording, simplification of technical terms, and adjustment of response options to improve clarity and comprehension. Content validity was assessed through expert review by a public health officer from the Solok District Health Office with experience in rabies control, while the pilot testing results were reviewed by the research team to identify unclear items and ensure clarity, relevance, and internal consistency of the questionnaire. Face-to-face interviews were conducted by trained personnel, including experienced researchers and a local public health officer involved in rabies control, to ensure clarity and completeness of responses. Data collection was conducted over a two-week period in August 2025. Responses were recorded manually using structured questionnaire forms and later entered into a digital database for analysis. Their familiarity with local conditions facilitated effective communication with respondents while maintaining standardized interview procedures to minimize interviewer bias. For scoring, each response was assigned a value from 1 to 4, resulting in a maximum possible total score of 100. Total scores were calculated by summing all item responses and were categorized into three levels, namely high (≥ 75% of the maximum score), moderate (50-74%), and low (< 50%). Each domain score was calculated by summing the responses to the items within that domain. Because each domain consisted of five items scored from 1 to 4, domain scores ranged from 5 to 20. For descriptive interpretation, knowledge scores were categorized as low (5-11), moderate (12-15), and high (16-20). Attitude scores ranged from 5 to 15 and were categorized as negative (6-10) and positive (11-15). Practice scores ranged from 5 to 15 and were categorized as poor (5-10) and good (11-15). All interviews were conducted in the local language to ensure that respondents clearly understood the questions. Negatively worded items were reverse-scored before calculating the total score, meaning that response values were inverted (with strongly agree = 1 and strongly disagree = 4) so that higher scores consistently reflected more favorable knowledge, attitudes, or practices. The score categorization thresholds were adapted from previously published rabies KAP studies and were used to facilitate interpretation of community knowledge, attitudes, and practices (Sambo et al., 2014; Monje et al., 2020; Rehman et al., 2021). Because the current study employed a descriptive design with purposive sampling, inferential statistical tests to examine associations between sociodemographic factors and KAP outcomes were not conducted. The study employed a descriptive design and did not include inferential statistical analysis to assess associations between variables.

Direct observation of dog populations

Free-roaming dogs were defined as dogs observed moving freely in public areas without physical restraint or direct human supervision. Observations focused on dog density, roaming behavior, and proximity to human settlements, which are important indicators of rabies transmission risk. Field observations were conducted to estimate the number and spatial distribution of free-roaming dogs across the four sub-villages of Sirukam during the study period in August 2025. Observations were performed during both morning and late afternoon periods, when dog activity is typically higher. Multiple observation points were used to reduce the likelihood of double-counting and to obtain a more representative estimate of dog presence in the study area. Repeated observations at different times of the day were conducted to reduce potential bias and improve the reliability of the dog population estimate. Observations were conducted by two trained observers over a two-week period, with each observation session lasting approximately 30-45 minutes. A structured observation guideline was applied, developed based on established international methodologies for surveying free-roaming dog populations, including direct observation along representative areas, repeated counts, and standardized counting protocols (WSPA, 2014; WOAH, 2022). To ensure consistency, both observers were trained prior to data

collection, and discrepancies between repeated observations were resolved through comparison and consensus between observers, considering observation time, location, and the potential for double-counting.

Semi-structured interviews

Semi-structured interviews were conducted with three key informants representing different stakeholder groups in the community. The key informants included one local hunter, one community leader, and one public health officer responsible for rabies control at the Sirukam Public Health Center. The interviews were conducted by members of the research team with experience in community-based data collection. Key points from the interviews were documented through detailed written notes during the discussions. To enhance accuracy, responses were clarified with participants during the interview process when needed.

The key informants were purposively selected based on their direct involvement in dog ownership practices, community decision-making, and zoonotic disease control in the study area. The interview guide was developed based on study objectives and relevant literature and was reviewed prior to data collection to ensure clarity and relevance. Secondary data on animal bite incidents, PEP completion, and vaccination records (2022-2024) obtained from the Sirukam Public Health Center, Solok District, West Sumatra, Indonesia, were used to complement field observations and to provide contextual information on rabies exposure and prevention practices in the study area.

Data analysis

Risk assessment was conducted using a semi-quantitative approach by evaluating two main components, namely the likelihood of exposure and the potential public health impact. Each component was assessed based on field observations, questionnaire data, and qualitative interviews. Scores were assigned using predefined criteria adapted from previous rabies risk assessment frameworks, and the overall risk level was determined by combining these components. The classification was conducted by the research team based on consensus.

Quantitative data were analyzed descriptively using frequency distributions and percentages to summarize respondents' knowledge, perceptions, and attitudes related to rabies risk. Quantitative data were organized and analyzed using Microsoft Excel (Microsoft Corporation, Redmond, WA, USA). Because the study employed a descriptive exploratory design with purposive sampling, inferential statistical tests were not applied. The descriptive exploratory design with purposive sampling is appropriate for studies aiming to describe patterns and identify potential risk factors rather than to establish causal relationships. Instead, the analysis focused on identifying patterns of community perceptions and behavioral risk factors associated with dog-mediated rabies exposure. Rabies risk was categorized using a qualitative risk matrix combining two parameters, namely probability of exposure and potential impact on public health. Each parameter was scored on a three-level scale (low, moderate, high) based on field observations, community practices, and reported bite incidents. The overall risk level was determined from the intersection of probability and impact categories in the matrix (Ginuluh et al., 2024). Qualitative data obtained from semi-structured interviews were analyzed thematically to identify recurring cultural and behavioral factors influencing rabies exposure and community risk perceptions. Given the small number of key informants, data saturation was considered achieved when no new themes emerged during analysis. Qualitative data were manually organized and coded by the research team to identify recurring patterns.

RESULTS

Sociodemographic characteristics

A total of 96 respondents participated in the study. Most respondents were male (63.50%). Respondents were distributed across four age groups, with the largest proportion aged over 40 years (29.17%), followed by 31-40 years (26.04%), 21-30 years (23.96%), and younger than 20 years (20.83%). In terms of occupation, hunters represented the largest group (23.96%), followed by traders (18.75%), employees (16.67%), farmers (13.54%), and housewives (13.54%). Dog ownership was reported by 43.75% of respondents, while 13.54% had experienced dog bites. Detailed sociodemographic characteristics are presented in Table 1.

Knowledge, attitudes, and practices regarding rabies

The assessment of KAP related to rabies prevention revealed varying levels among respondents. None of the participants was classified in the low knowledge category. Moderate knowledge was observed in 56.25% of respondents, while 43.75% demonstrated high knowledge. Regarding attitudes toward rabies prevention, 62 respondents (64.58%) indicated positive attitudes, while 34 respondents (35.42%) expressed negative attitudes. Preventive practices were less consistent; poor practices were observed in 55 respondents (57.29%), whereas 41 respondents (42.71%) demonstrated good preventive practices. Detailed KAP scores are shown in Table 2.

Table 2. Knowledge, attitudes, and practices regarding rabies among residents of Sirukam, Solok District, West Sumatra, Indonesia, August 2025

Domain	Category	Score interval	Frequency (n)	Percentage
Knowledge	Low	5 - 11	0	0.00
	Moderate	12 - 15	54	56.25
	High	16 - 20	42	43.75
Attitudes	Negative	6 - 10	34	35.42
	Positive	11 - 15	62	64.58
Practices	Poor	5 - 10	55	57.29
	Good	11 - 15	41	42.71
Total respondents (N)			96	100

Dog bite incidents and post-exposure prophylaxis

A total of 14 dog bite cases were recorded in Sirukam, Solok District, West Sumatra, Indonesia, during the 2024 surveillance period (January-December 2024). All bite victims initiated PEP by receiving the first dose of the anti-rabies vaccine (VAR1). Completion of the full vaccination schedule varied, with only 10 cases (71.43%) completing all recommended doses (VAR1-VAR3), while 4 cases (28.57%) did not complete the full vaccination series primarily due to missed follow-up doses and loss to follow-up before receiving subsequent vaccine doses. Incomplete vaccination occurred due to missed follow-up doses or loss to follow-up before receiving the third dose. Immediate wound washing was also not consistently reported among several bite cases. Detailed information on the biting dogs, including vaccination status and observation history, was not consistently available in the surveillance records. Due to the limited number of reported cases, seasonal patterns of dog bite incidents could not be reliably assessed. Data on dog bite incidents and PEP were collected retrospectively for the period January-December 2024 based on respondent recall and available local records during the 2025 survey. Monthly variations in bite incidents and vaccination completion are summarized in Table 3.

Table 3. Reported dog bite cases and post-exposure prophylaxis completion in Sirukam, Solok District, West Sumatra, Indonesia

Date (2024)	Number of bite cases	Wound washing	VAR dose 1	VAR dose 2	VAR dose 3	Notes
March	1	Yes	Yes	Yes	Yes	Completed on schedule
April	3	Yes (2/3)	Yes (3/3)	Yes (3/3)	Yes (2/3)	1 case incomplete (missed VAR3)
June	1	Yes	Yes	Yes	Yes	Completed
July	1	No	Yes	Yes	Yes	Wound not washed
August	2	Yes (1/2)	Yes (2/2)	Yes (2/2)	Yes (2/2)	1 case did not wash the wound
September	1	No	Yes	Yes	-	Lost to follow-up before VAR3
October	2	Yes (1/2)	Yes (2/2)	Yes (1/2)	-	1 case received VAR1 only
November	1	Yes	Yes	-	-	Only VAR1 received
December	1	Yes	Yes	Yes	Yes	Completed

VAR1: First dose of anti-rabies vaccine, VAR2: Second dose, VAR3: Third dose, PEP: Post-exposure prophylaxis. Data were collected from January to December 2024 (N = 14).

Dog population and human-dog interaction

Field observations recorded 148 free-roaming dogs distributed across four sub-villages in Sirukam, based on repeated direct observations conducted at multiple observation points. The highest number of dogs was observed in Kubang Duo sub-village, with 80 dogs (54.05%) of the total observed population recorded in the area. Based on an estimated human population of 5,654 individuals, the human-to-dog ratio was approximately 38:1. The presence of free-roaming dogs in residential areas indicated frequent opportunities for contact between humans and dogs in the study area. The estimation of dog numbers was based on direct field observations conducted across multiple observation points. Information on the vaccination status of the observed dogs was not available. Observations also indicated that many dogs were freely roaming in public and residential areas without restraint. Detailed dog population distribution across sub-villages is presented in Table 4.

Cultural and behavioral factors related to dog management and rabies risk

Semi-structured interviews with three key informants (one hunter, one public health officer, and one community leader) identified several cultural practices related to dog management that may influence rabies risk in the study area. Among interviewed respondents, the hunter reported reluctance to vaccinate hunting dogs due to concerns that

vaccination might reduce the dogs' stamina during hunting activities. The dog owner informant reported allowing dogs to roam freely for guarding farms and households. Free-roaming practices observed in residential areas may increase opportunities for human–dog contact in the community.

Table 4. Dog population and human-to-dog ratio in Sirukam Sub-villages, Solok District, West Sumatra, Indonesia, August 2025

Sub-village (Jorong)	Number of dogs	Human population*	Human: Dog ratio
Koto Tingga	18	565	1: 31
Kubang Duo	91	2262	1: 25
Lubuak Pulai	19	1131	1: 65
Gantiang	20	1696	1: 87
Total	148	5,654	1: 38

* Human population data; Source: BPS-Statistics Solok Regency (2024).

DISCUSSION

The study suggests that dog-mediated rabies may indicate a potential public health concern in Sirukam, based on observed patterns of community knowledge, preventive practices, and the presence of free-roaming dogs. The identification of 148 free-roaming dogs and a human-to-dog ratio of approximately 38:1 suggested frequent opportunities for human-dog interactions, which may facilitate rabies transmission. The observed variation in the number of free-roaming dogs across sub-villages may be influenced by differences in human population density, land use, and dog management practices, including the use of dogs for hunting and the tendency to allow dogs to roam freely. For example, sub-villages with higher numbers of dogs relative to the human population may reflect a greater reliance on dogs for hunting activities, whereas sub-villages with lower dog numbers may be associated with higher population density and more restricted dog ownership practices. Similar findings have been reported in rabies-endemic regions where free-roaming dog populations contribute to sustained exposure risks, including studies in Indonesia that documented the role of free-roaming dog populations and limited vaccination coverage in sustaining rabies transmission (Hampson et al, 2015; Subrata et al., 2020). Although most respondents demonstrated moderate to high knowledge about rabies, preventive practices remained inconsistent. The observed gap between knowledge levels and preventive practices suggested that knowledge alone may not be sufficient to ensure appropriate rabies prevention behaviors. The discrepancy between relatively high knowledge levels and inconsistent preventive practices has also been reported in previous studies, where higher levels of knowledge were not consistently accompanied by appropriate preventive practices (Rehman et al., 2021; Kardikayasa et al., 2023). In particular, incomplete wound washing or PEP was observed in 4 of 14 reported bite cases (29%), representing a substantial proportion of exposures. Inadequate wound management and incomplete vaccination are recognized risk factors for rabies transmission following dog bites (WHO, 2023). The concentration of free-roaming dogs in certain sub-villages highlighted the importance of dog population management in rabies control. Global rabies control programs emphasize that vaccinating at least 70% of the dog population is necessary to disrupt transmission cycles (Hampson et al., 2015; WHO, 2023). However, specific cultural beliefs and practical barriers may limit participation in dog vaccination programs in the study area. Qualitative findings from the study indicated that some hunters were reluctant to vaccinate their dogs due to concern that vaccination might reduce stamina or hunting performance. In addition, free-roaming practices reported by dog owners may complicate vaccination coverage and monitoring. The absence of respondents in the low knowledge category may reflect relatively good baseline awareness in the community or potential social desirability bias during data collection. The low proportion of respondents in the low knowledge category, which may reflect relatively good baseline awareness or potential social desirability bias during data collection, should be interpreted cautiously and may indicate the need for further validation of the questionnaire in similar settings. Similar barriers related to perceptions of dog health and utility have been reported in other rabies-endemic regions (Gebeyaw and Teshome, 2016; Rehman et al., 2021). Although the Ethiopian context differed from Indonesia, they highlight that perceptions of dog function may influence vaccination behavior in rural communities.

Addressing barriers to dog vaccination, including concerns about reduced hunting performance, free-roaming dog management practices, and limited participation in vaccination programs, may require targeted strategies such as community-based education involving local hunters, awareness programs emphasizing that vaccination does not impair dog performance, and improved access to veterinary services to facilitate routine vaccination. The rabies risk assessment was conducted based on several observed factors, including the occurrence of dog bite incidents, incomplete adherence to post-exposure prophylaxis, and the presence of free-roaming dogs within the community. Similar risk factors

contributing to rabies transmission have been reported in other endemic regions (Hampson et al., 2015; WHO, 2023). Similar epidemiological patterns have been reported in rabies-endemic countries, including Indonesia, Tanzania, and Ethiopia, where dog-mediated rabies continues to impose substantial public health and economic burdens (Hampson et al., 2015; WHO, 2023). A One Health approach has been widely recognized as essential for rabies control in endemic settings, as it facilitates coordination between veterinary services, public health authorities, and community stakeholders in addressing interconnected transmission drivers (Destoumieux-Garzón et al., 2018; WHO, 2023).

CONCLUSION

The study assessed factors associated with the risk of dog-mediated rabies transmission in Sirukam, West Sumatra, Indonesia. Rabies exposure risk in the study area is influenced by multiple interacting factors, including frequent human-dog interactions, the presence of a substantial free-roaming dog population, inconsistent wound management practices, and cultural perceptions influencing vaccination of hunting dogs. The combination of the identified risk factors highlights the importance of integrated approaches involving both veterinary and public health sectors to address rabies transmission. Rabies prevention in the study area may benefit from expanding community-based dog vaccination programs, increasing public awareness of immediate wound washing and timely post-exposure prophylaxis, and promoting responsible dog ownership practices, including limiting free-roaming and improving participation in vaccination programs. The study was limited by its cross-sectional design and the absence of direct data on the vaccination status of the observed dog population. In addition, potential associations between knowledge, attitudes, and practices, as well as between attitudes and dog ownership, were not analyzed, as such analyses were beyond the scope of this descriptive study. Future studies incorporating longitudinal surveillance and detailed assessment of dog vaccination coverage are needed to provide a more comprehensive understanding of rabies transmission risk in similar rural settings.

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Authors' contributions

Widia Safitri conceptualized the study, conducted data collection, performed data analysis, and drafted the manuscript. Rita Suzana contributed to the study design, supervised the research process, and reviewed the manuscript. Olivia Afriyanti contributed to data analysis and manuscript revision. Anggi Pratama assisted with data collection and data interpretation. All authors read and approved the final version of the manuscript.

Availability of data and materials

The datasets used and analyzed in the study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that there is no conflict of interest regarding the publication of the study.

Ethical consideration

The authors confirm that the current manuscript is original, has not been published previously, and is not under consideration for publication elsewhere. All sources have been appropriately cited, and the manuscript has been checked for plagiarism. Artificial intelligence (AI) tools (ChatGPT, OpenAI) were used for language editing and manuscript refinement. No AI tools were used for data analysis, data interpretation, or the generation of scientific conclusions. All scientific content remains the responsibility of the authors.

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First Molecular Characterization of Adult *Physaloptera praeputialis* von Linstow, 1889, Infecting African Lion (*Panthera leo*) in Tanzania

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ABSTRACT

Physalopterid nematodes are common gastric parasites of carnivorous mammals, yet their diversity, host associations, and evolutionary relationships in African wildlife remain poorly understood. The present study aimed to investigate the adult *Physaloptera* specimens recovered from African lions (*Panthera leo*) in the Serengeti National Park in the Mara region, Tanzania, using morphological and molecular techniques. Seven non-captive lions comprising three males and four females were opportunistically necropsied between 2023 and 2025. The sampled lions included two subadults (4-5 years), three prime adults (6-9 years), and two older individuals (10-14 years). A total of 157 adult spirurid nematodes were recovered from three infected hosts with infection intensities ranging from 48 to 57 worms per host. Morphometric characterizations, such as cephalic collarette structures, marked unequal spicules, well-developed caudal alae in males, and an anterior positioned vulva in females, together with typical eggs, were consistent with *Physaloptera praeputialis*. Nuclear *18S rRNA* sequences showed high similarity of 98-99.5% to *P. praeputialis* reference sequences. In contrast, *mitochondrial cytochrome c oxidase subunit I* (*Cox1*) sequences formed a distinct lineage, exhibiting approximately 12% divergence from available *P. praeputialis* sequences retrieved from the GenBank database at the time of analysis. A molecularly characterized record of adult *Physaloptera* from an African lion in Tanzania is presented, revealing previously unrecognized genetic diversity among African physalopterids. Findings highlight the role of apex predators as indicator hosts in trophic parasite transmission and emphasize the importance of multilocus approaches for resolving spirurid taxonomy, parasite biogeography, and host–parasite associations in wildlife ecosystems.

Keywords: African lion, Molecular characterization, Parasitic nematode, Phylogenetic, Serengeti ecosystem

INTRODUCTION

The genus *Physaloptera* Rudolphi, 1819 (Nematoda: *Spirurida*: Superfamily *Physalopteridae*) comprises gastric parasitic nematodes infecting a broad range of vertebrate hosts, including mammals, birds, reptiles, and amphibians (Mohamadain and Ammar, 2012). Members of the genus *Physaloptera* are prevalent in carnivorous mammals, where adult worms inhabit the stomach and attach firmly to the gastric mucosa using a well-developed cephalic collarette and paired pseudolabia (Maharana et al., 2021). Several species, including *Physaloptera rara*, *P. felidis*, *P. maxillaris*, *P. canis*, *P. dilatata*, *P. tumefaciens*, and *P. praeputialis*, have been reported from domestic to wild carnivores across Asia, North America, and Europe (Faria et al., 2019; Chen et al., 2023; Macedo et al., 2023).

Physaloptera species exhibit an indirect life cycle involving orthopteran and coleopteran insects, particularly cockroaches, beetles, crickets, and grasshoppers, as intermediate hosts in which infective third-stage larvae develop (Naem and Asadi, 2013). Rodents, reptiles, amphibians, and birds may serve as paratenic hosts (Kalyanasundaram et al., 2018). Carnivores acquire infection primarily through predation or accidental ingestion of infected intermediate or paratenic hosts (Maharana et al., 2021). Adult *Physaloptera* worms embed in the gastric mucosa of carnivorous definitive hosts and feed on host tissues and blood, often causing focal erosions, hemorrhages, and chronic gastritis (Alves et al., 2022).

Physaloptera infections in carnivorous definitive hosts, particularly domestic dogs and cats and occasionally wild carnivores, have been associated with vomiting, anorexia, weight loss, anemia, and, in severe cases, gastric ulceration and chronic gastritis (Alves et al., 2022; Chen et al., 2023). Despite the rarity of human infections, sporadic cases of physalopteriasis have been reported, suggesting a limited but recognized zoonotic potential (Mohamadain and Ammar,

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2012). Species-level identification within *Physaloptera* remains challenging and has traditionally relied on morphological features such as pseudolabia structure, the number and arrangement of male caudal papillae, spicules morphology, vulvar position, and cuticular ornamentation (Soulsby, 1965). However, morphological characteristics of *Physaloptera* species often overlap among congeners and may vary according to host species, developmental stage, or preservation quality (Pereira et al., 2012). As a result, historical records lack sufficient diagnostic resolution, leading to frequent misidentifications among closely related species (Chen et al., 2023).

In recent years, molecular approaches have been used in spirurid nematodes to clarify classical morphology and improve taxonomic resolution (Chen et al., 2023). Nuclear ribosomal genes such as *18S rRNA*, along with mitochondrial markers, particularly *cytochrome c oxidase subunit I* (*Cox1*), have proven valuable for reconstructing phylogenetic relationships, resolving cryptic diversity, and verifying species boundaries (Maharana et al., 2021; Chen et al., 2023). However, molecular information for physalopterid nematodes remains sparse, and available reference sequences are largely skewed towards isolates from domestic animals from Asia and Europe, with minimal representation from African wildlife (Ferroglio et al., 2009; Maharana et al., 2021).

In Tanzania, *Physaloptera praeputialis* (*P. praeputialis*) has been reported only once based on a coprological study, without genetic confirmation or morphological observation (Müller-Graf, 1995). To date, there are no published integrative morphological and molecular characterizations of adult *Physaloptera* species from wildlife hosts in Tanzania, despite the high diversity of carnivores and the ecological conditions favorable for trophic-transmitted parasites in the Serengeti ecosystem. Large felids, including African lions (*Panthera leo*), are apex predators that frequently consume insectivorous and small vertebrate prey that may act as paratenic hosts for *Physaloptera* spp. Apex predators represent valuable sentinel hosts for investigating the diversity, transmission ecology, and population structure of trophically transmitted helminths (Palomba et al., 2023). Identification of gastric nematodes in apex predators is essential for understanding host–parasite associations, parasite diversity, and the epidemiology of infections in wildlife populations (Moudgil et al., 2015). Characterizing these parasites contributes to improved knowledge of disease ecology and provides baseline information for wildlife health monitoring and conservation. Therefore, the present study aimed to provide the first integrative morphological and molecular characterization of adult *Physaloptera* specimens recovered from the African lions in the Serengeti ecosystem of Tanzania.

MATERIALS AND METHODS

Ethical approval

The objectives and procedures of the present study, which is part of an ongoing project, were reviewed and approved by the Joint Management Research Committee of the Tanzania Wildlife Research Institute. Additionally, research permits were granted by the Commission for Science and Technology, Tanzania (CST00001738-2025-2025-01459). Field collections of Physalopterid nematodes were conducted in the Serengeti National Park, following the necessary permissions to enter these protected areas. All nematodes were isolated exclusively from naturally dead African lions encountered during the study.

Study area and specimen collection

The present study was conducted in the Central and Eastern Serengeti National Park within the Serengeti ecosystem in northern Tanzania, between 2023 and 2025 (Figure 1). The area is characterized by semi-arid savannah with woodland mosaics, dominated by open grasslands, acacia woodlands, and riverine vegetation, and supports a large, well-studied population of African lions. The ecosystem experiences a bimodal rainfall pattern with annual precipitation ranging approximately from 500 to 1,200 mm. Mean annual temperatures range from 20°C to 27°C, with daytime maxima often exceeding 30°C during the dry season. Relative humidity varies seasonally, averaging about 40-60% during the dry season and increasing to 65-80% during the wet season, particularly in riverine and grassland habitats (Sinclair et al., 2015). These environmental conditions support diverse intermediate arthropod hosts and contribute to parasite transmission dynamics within the ecosystem. Lion carcasses were found through routine wildlife health surveillance and carcass monitoring programs jointly implemented by the Tanzania Wildlife Research Institute (TAWIRI) and Serengeti National Park veterinary and research units.

Sampling was opportunistic and non-invasive; no lions were euthanized or intentionally sacrificed during sample collection. A total of seven lion carcasses were recovered at different locations and time points within the study area. The carcasses comprised 3 males and 4 females, collected between 2023 and 2025 (2 in 2023, 3 in 2024, and 2 in 2025). All carcasses were classified as fresh to moderately decomposed within a post-mortem interval of ≤ 48-72 hours based on field assessment and were considered suitable for gastrointestinal parasitological examination. Age estimation was conducted by trained wildlife veterinarians and carnivore specialists during post-mortem examination using established

criteria based on dental eruption and wear patterns, combined with body condition assessment and cranial morphology (Whitman et al., 2007). Two lions were classified as subadults, approximately 4-5 years, three as prime adults of 6-9 years, and two older adult lions of about 10-14 years. Three lions had been observed intermittently for 2-3 weeks prior to death and showed signs of poor body condition, including marked emaciation and reduced mobility. However, no definitive cause of morbidity could be established prior to death, and the observed clinical deterioration was considered multifactorial and potentially associated with age-related decline, injury, social displacement, or underlying disease processes.

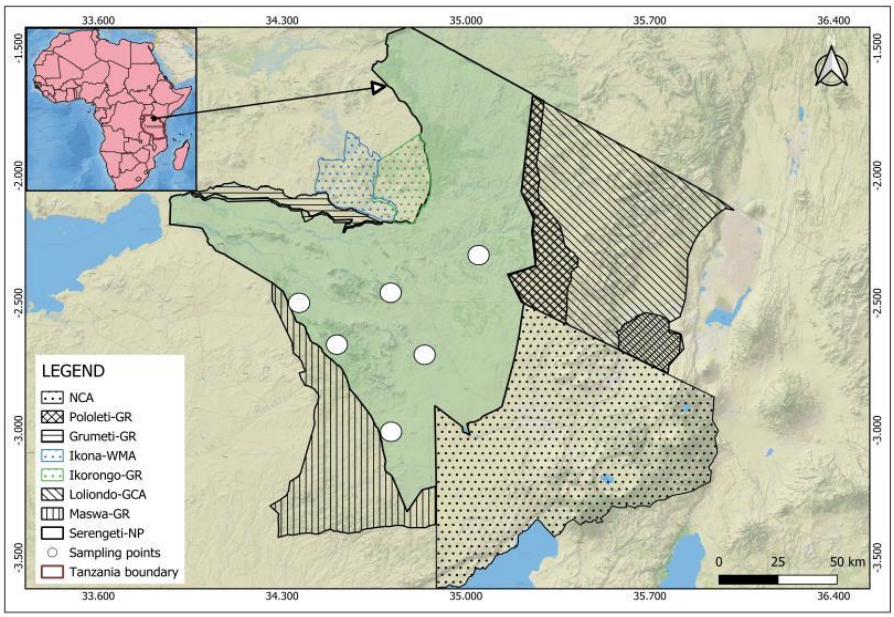


Figure 1. Location of the study sites in the Serengeti National Park within the Serengeti ecosystem, Tanzania

Necropsy and ecological observations

The isolation of nematodes was performed following standard gastrointestinal parasitological necropsy procedures with minor modifications (Anderson, 2000; Wobeser, 2007). The abdominal cavity was opened using sterile dissection tools, and the stomach and small intestine were removed sequentially to minimize cross-contamination between organs. Careful separation of gastrointestinal compartments was maintained throughout the procedure to ensure accurate parasite recovery and identification (Wobeser, 2007). The nematode worms were recovered using fine forceps, rinsed in 0.85% physiological saline solution (Oxoid Ltd., United Kingdom), counted, and preserved in 70% ethanol (Sigma-Aldrich, USA) for molecular studies and in 10% neutral buffered formalin (Sigma-Aldrich, USA) for subsequent morphological examination, following standard helminth preservation protocols (Wobeser, 2007). During necropsy, stomach content composition, parasite burden, gross pathological changes, and overall organ condition were recorded.

Coprological investigation

Fecal samples collected from the rectum were examined using the formalin ether concentration technique (Sato et al., 2014; Khanna et al., 2018) with minor modifications. Briefly, approximately 10g of fecal sample was thoroughly homogenized to ensure uniform distribution of parasite stages, after which 1g aliquots were transferred into 15 mL conical-bottom polypropylene tubes (Falcon®, USA) containing 7 mL of 10% formalin. The reduced aliquot volume was used to optimize sedimentation efficiency and microscopic clarity during concentration procedures. Subsequently, 3 mL of diethyl ether (Sigma-Aldrich, USA) was added, and the suspension was vigorously mixed for approximately one minute with intermittent venting to release internal pressure. Samples were centrifuged at 500 × g for 10 minutes, after which the supernatant layers were carefully decanted. The sediment was washed twice to improve preparation clarity and then resuspended in 200 µL of formalin-saline. The remaining pellets were resuspended in 200 µL of formalin-saline. Two slides per sample were prepared using 30 µL aliquots and examined systematically under a light microscope (Olympus, Japan) at 100× and 400× magnification. Helminth eggs were identified based on morphological characteristics using standard diagnostic keys (Sohn and Chai, 2024).

Morphological identification of *Physaloptera* species

Prior to morphological identification, recovered nematodes were gently rinsed in 0.85% physiological saline solution (Oxoid Ltd., United Kingdom) to remove adherent debris and subsequently preserved in pre-heated 10% buffered formalin (Sigma-Aldrich, USA). Specimens were cleared in lactophenol prepared from phenol crystals

(C_6H_5OH; Sigma-Aldrich, USA), glycerol (Merck KGaA, Germany), lactic acid ($CH_3CHOHCOOH$; Thermo Fisher Scientific, USA), and distilled water in proportions of 20 g, 40 mL, 20 mL, and 20 mL, respectively, to enhance visualization of diagnostic morphological structures (Rana et al., 2020). Morphological identification was performed using a light microscope (Olympus, Japan) following established taxonomic keys (Anderson et al., 2009). Diagnostic characters examined included cephalic collarette and pseudolabia, spicule length and morphology, arrangement of pedunculate and sessile caudal papillae, and vulvar position and morphology in females. Representative specimens were measured using calibrated ocular micrometry, and images were captured for documentation (Maggenti, 2020).

Molecular identification of *Physaloptera* species

PCR and DNA sequencing

Nine adult nematodes (three specimens per host) obtained from three representative lions were selected for molecular analyses to assess species identity and intra-population genetic variation. Specimens were rinsed three times in sterile phosphate-buffered saline (PBS) solution (Thermo Fisher Scientific, USA) to remove residual host material. Worms were then mechanically homogenized in 180 µL ATL tissue lysis buffer (Qiagen, Germany) supplemented with 20 µL proteinase K (20 mg/mL) and incubated overnight at 56°C until complete tissue digestion. Genomic DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, USA) following the manufacturer's protocol and eluted in 50 µL of TE buffer composed of 10 mM Tris-HCl and 1 mM EDTA at pH 8.0. DNA concentration and purity were quantified using a UV–Vis spectrophotometer (NanoDrop Technologies, Thermo Fisher Scientific, USA), and DNA integrity was confirmed by agarose gel electrophoresis. Partial fragments of the *mitochondrial cytochrome c oxidase subunit I* (*cox1*) gene and the nuclear *small subunit ribosomal RNA* (*18S rRNA*) gene were amplified using species-specific conserved primers listed in Table 1. Polymerase chain reaction assays were performed in 25 µL reaction volumes containing 2.5 µL of 10× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of each deoxynucleotide triphosphate, 0.4 µM of each primer, 1 U Taq DNA polymerase (Takara Bio Inc., Japan), and approximately 50 ng of genomic DNA template with nuclease-free water added to the final volume. Thermal cycling conditions for both *cox1* and *18S rRNA* consisted of initial denaturation at 95°C for three minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at primer-specific temperatures for 30 seconds, and extension at 72°C for 60 seconds, with a final extension step at 72°C for seven minutes. Negative controls included a no-template control containing nuclease-free water instead of DNA and an extraction blank processed alongside samples to monitor contamination. Polymerase chain reaction products were separated by electrophoresis on 1.5% agarose gels in 1× TBE buffer at 100 V for 45 minutes, stained with ethidium bromide, and visualized under UV illumination. Amplified products were purified prior to bidirectional Sanger sequencing.

Table 1. Primers of *Cytochrome oxidase 1* and *18s ribosomal RNA* were used in the study

Target gene	Primer name	Primer sequence (5′→3′)	Product length (bp)	Reference
Cox 1	Cophy F Cophy R	GGGCAGGATTAGGAGGTTCTG AAGCCCCAGCCAAAACTGGAA	525	(Kalyanasundaram et al., 2018)
18srRNA	Physa F Physa R	GCGAACGGCTCATTATAA AATTTCACCTCTCAGCA	745	(Maldonado et al., 2019)

DNA sequence analyses

DNA sequences of the mitochondrial *cox1* and *18S rRNA* genes were assembled and edited using Geneious R9.1 software (Biomatters Ltd., New Zealand) following standard sequence processing workflows (Kearse et al., 2012). Consensus sequences were aligned using MUSCLE and trimmed to uniform lengths. Comparative *Cox1* sequences were retrieved from Genbank, including *Physaloptera* spp. WTL (LC596961), *Physaloptera* spp. SNM (MH752202), *Physaloptera* spp. BRM (MW517846) and *Physaloptera* spp. haplotype D KG (OP589140), *Onchocerca lupi* (JX080028), *Dirofilaria immitis* (KT282097), *Brugia malayi* (OQ727412), and the outgroup was *Gnathostoma turgidum* (KT894798) was selected as an outgroup based on its phylogenetic distance within Spirurida. For *18S rRNA* analyses, reference sequences included *Physaloptera praeputialis* (MW410927), *Physaloptera retusa* (KT894814), *Physaloptera turgida* (MH748145), *Physaloptera rara* (MH938367), *Physaloptera sibirica* (OQ846902), *Heliconema longissimum* voucher IPCAS N-862/2 (JF803949), *Abbreviata antarctica* (KX255660), *Abbreviata hastaspicula* (KX255661), *Dirofilaria immitis* (OP811190), *Brugia malayi* (OQ727413), with *Gnathostoma turgidum* (Z96948) as the outgroup.

Phylogenetic analysis using the partial sequences of *Cox1* and *18S RNA* was evaluated by the most appropriate nucleotide substitution model using the Bayesian Information Criterion in Molecular Evolution Genetics Analysis, MEGA v11.0 (Tamura et al., 2021). Maximum-likelihood (ML) phylogenies were reconstructed in MEGA with 1,000 bootstrap replicates. Bayesian phylogenetic analyses were performed using BEAST v1.10.4 (Suchard et al., 2018), which implements a Bayesian inference framework for evolutionary reconstruction. Population genetic indices, including

number of haplotypes, haplotype diversity (Hd), nucleotide diversity (π), number of polymorphic sites, total mutations, and mean pairwise nucleotide differences, were calculated using DnaSP v6.12.03 (Librado and Rozas, 2009).

RESULTS

Parasite burden and host infection intensity

A total of 157 adult spirurid nematodes were recovered from the stomachs of three infected African lions (Figure 2). The remaining four lions examined during the study were negative for *Physaloptera* infection based on necropsy and gastrointestinal inspection. Of the total parasite collection, 105 specimens were recovered from two infected hosts, a 9-year-old female (n = 57) and a 12-year-old male (n = 48), while 52 worms were recovered from a third female lion estimated to be approximately 10 years old. Both sexes exhibited a thick cuticle forming a posteriorly extended caudal sheath (Figure 2). The anterior extremity bore a well-developed cephalic collarette with partially submerged pseudolabia armed with flattened internal tooth-like projections and a single external conical tooth, a configuration characteristic of the genus *Physaloptera* (Figure 3).

Morphometrics and key diagnostic features of male specimens

Male *Physaloptera* specimens measured 48.5-50.2 mm in length and 1.8-2.1 mm in maximum width (Table 2). The muscular-glandular esophagus of male *Physaloptera* spp. was long and robust, measuring 6.8-7.1 mm, representing approximately 14% of total body length. In male *Physaloptera* spp., the nerve ring, deirids, and excretory pore were located at 1.1-1.4 mm, 1.2-1.4 mm, and 7.9-8.3 mm from the anterior extremity, respectively (Table 2). The posterior end of male *Physaloptera* spp. bore prominent lateral alae forming a wing-like bursa structure (Figure 3). Spicules of male *Physaloptera* spp. were markedly unequal and dissimilar; the left spicule was longer and lanceolate, measuring 1.5-1.7 mm in length, while the right spicule was shorter and curved, measuring 1.1-1.3 mm, without terminal dilation. The cloaca of male *Physaloptera* spp. was situated 0.9-1.2 mm from the posterior extremity (Figure 3).

Morphometrics and reproductive features of female *Physaloptera* species

Female *Physaloptera* spp. were larger than male worms, measuring 59.5-77.5 mm in length and 2.1-2.4 mm in width (Table 3). The total esophageal length in female *Physaloptera* spp. was 8.5-9.3 mm. The vulva of female *Physaloptera* spp. opened at 25.2-27.5 mm from the anterior extremity, corresponding to 34-42% of total body length. The anus of female *Physaloptera* spp. was located 1.5-1.6 mm from the posterior extremity. Uteri of female *Physaloptera* spp. were filled with numerous embryonated eggs morphologically identical to those recovered from fecal samples (Figure 4). Coprological examination of *Physaloptera* infection in African lions revealed oval, thick-shelled larvated eggs measuring 33-36 × 40-45 µm in all infected hosts.

Molecular identification of *Physaloptera* species and phylogenetic analysis

Partial fragments of the mitochondrial *cytochrome c oxidase subunit I* (*cox1*, approximately 525 bp) and nuclear *18S rRNA* (approximately 748 bp) genes were successfully amplified from all nematode specimens selected for molecular analysis and yielded clear single bands on 1.5% agarose gels (Figure 5). Sequencing was performed on representative PCR-positive amplicons, and all obtained sequences were identical across specimens with no observed intraspecific variation. Representative sequences were deposited in GenBank under accession numbers OR821828 (*cox1*) and OR821831 (*18S rRNA*).

BLAST analysis confirmed that the *Cox1* sequences belong to the genus *Physaloptera*. Pairwise sequence similarities with available *Physaloptera* references ranged from 88% to 88.6% identity, with the highest similarity to *P. retusa (*KT894803), *P. mirandai* (KP981418), and other *Physaloptera* isolates with accession numbers MW517846, LC381943, and LC596961 (Figure 6). The alignment contained 141 polymorphic sites and 193 mutational events, yielding 10 haplotypes with high haplotype diversity (Hd: 0.982 ± 0.046). Phylogenetic reconstruction based on *Cox1* sequences grouped the Tanzanian isolate within the Physalopteridae clade; however, as a distinct mitochondrial lineage, separated from clusters corresponding to *P. retusa* and *P. mirandai*, and formed a sister lineage to reference sequences identified as *Physaloptera* spp. (Figure 6).

The partial *18S rRNA* sequence exhibited high similarity of 98.1-99.5% to multiple *Physaloptera* species, including *P. praeputialis* (MW410927), *P. rara* (MH938367), *P. sibirica* (OQ846902), *P. retusa* (KT894814), and *P. turgida* (MH748145). Pairwise divergence between the Tanzanian isolate and *P. praeputialis* from domestic cats in India was 0.5%. Across 15 aligned sequences, 51 polymorphic sites and 65 mutational events were detected, generating 14 haplotypes (Hd: 0.9905 ± 0.028; π: 0.0607). Phylogenetic inference based on *18S rRNA* placed the Tanzanian isolate within a well-supported *Physaloptera clade*, clustering closely with reference sequences of *P. praeputialis* (Figure 7).

Table 2. Comparative morphometric features of male *Physaloptera* species recovered from different carnivore hosts, including specimens obtained from African lions in the Serengeti ecosystem, Tanzania, between 2023 and 2025

Species	***P. torquata***	***P. maxillaris***	***P. rara***	***P. praeputialis***	***P. praeputialis***	***P. praeputialis***
Reference	**Morgan (1942)**	**Morgan (1943)**	**Ackert (1941)**	**Linstow (1889)**	**Linstow (1889)**	**Current study**
Host	***Taxidea taxus***	***Mephitis mephitis***	***felis domestica***	***Felis catus***	***Felids***	***Panthera leo***
Body length (mm)	20 - 23	20 - 26	25 - 29	21	13 -40	48.5 - 50.2
Maximum width (mm)	0.56 - 0.79	0.55 - 0.88	0.71 - 0.80	1.5	--	1.8 - 2.1
Esophagus length (mm)	3.4 - 4.1	3.1 - 5.3	4.7 - 5.3	--	--	6.8 - 7.1
Nerve ring (mm)	--	--	--	--	--	1.1 - 1.4
Deirids (mm)	--	--	--	--	--	1.2 - 1.4
Excretory pore (mm)	--	--	--	--	--	7.9 - 8.3
Right spicule length (mm)	0.53 - 0.80	0.55 - 0.62	0.51 - 0.60	--	0.84 - 0.9	1.1-1.3
Left spicule length (mm)	0.604 - 0.854	0.84 - 0.99	0.67 - 0.83	--	1 - 1.2	1.5 - 1.7
Tail length/ cloaca (mm)	--	--	--	--	--	0.9 - 1.2

Table 3. Comparative morphometric characteristics of female *Physaloptera* species recovered from different carnivore hosts, including specimens obtained from African lions in the Serengeti ecosystem, Tanzania, between 2023 and 2025

Species	***P. torquata***	***P. maxillaris***	***P. rara***	***P. praeputialis***	***P. praeputialis***
Reference	**Morgan (1942)**	**Morgan (1943)**	**Ackert (1941)**	**Linstow (1889)**	**Current study**
Host	***Taxidea taxus***	***Mephitis mephitis***	***Felis domestica***	***Felis catus***	***Panthera leo***
Body length (mm)	30 - 38	21 - 29	27 - 44	30	59.5 - 77.5
Maximum width (mm)	1.2 - 1.6	0.84 - 1.2	0.56 - 1.1	2	2.1 - 2.4
Esophagus length (mm)	3.4 - 4.1	4.3 - 6.1	6.6 - 7.8	--	8.5 - 9.3
Muscular portion length (mm)	--	--	--	--	6.1 - 6.5
Glandular portion length (mm)	--	--	--	--	8.8 - 9.3
Nerve ring from the anterior extremity (mm)	--	--	--	--	0.8 - 1.0
Deirids (mm)	--	--	--	--	1.8 - 2.2
Excretory pore (mm)	--	--	--	--	8.9 - 9.7
Vulva opening (mm)	5.5 - 10.1	4.6 - 8.3	3.2 - 5.8	8	25.2 - 27.5
Tail length/ cloaca (mm)	--	--	--	--	1.5 - 1.6
Egg (µm)	51 - 33	47 × 32	29-35 × 42-46	33 - 55	32.5 -35.5 × 40-45

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Figure 2. Adult *Physaloptera praeputialis* recovered from African lions (*Panthera leo*) in the Serengeti ecosystem, Tanzania, between 2023 and 2025. **A:** Freshly recovered specimens of *P. praeputialis* immediately after collection from the host are maintained in a collection container prior to processing. **B:** Additional adult specimens separated during initial handling. **C:** Posterior end of adult male showing the caudal bursa with lateral alae (wing-like structures). **D:** Female specimen showing brown cement-like material adherent to the cuticle (black arrow). **E:** Specimens were transferred to a Petri dish for cleaning and detailed morphological examination, showing improved visibility of diagnostic structures compared with the initial collection state.

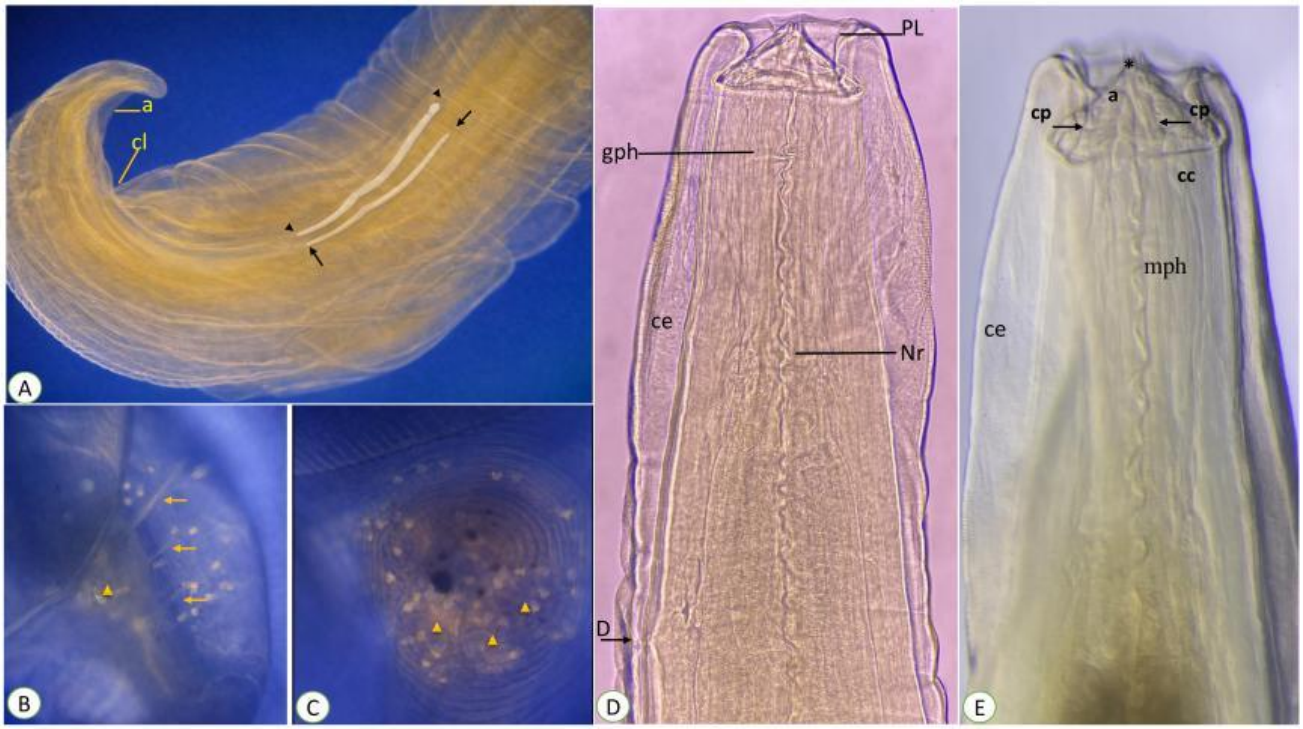


Figure 3. Morphological features of male *Physaloptera praeputialis* recovered from African lions (*Panthera leo*) in the Serengeti ecosystem, Tanzania, between 2023 and 2025. **A:** Ventral view of the posterior region showing alae (a), cloaca (cl), right spicule (arrow), and left spicule (arrowheads). **B** and **C:** Ventral view of posterior extremity showing pedunculate papillae (arrows) and sessile papillae (arrowheads). **D:** Anterior extremity showing pseudolabia (PL), glandular pharynx (gph), cuticular expansion (ce), deirid (D), and nerve ring (Nr). **E:** Cephalic region showing cephalic papillae (cp), amphids (a), cephalic collar (cc), muscular pharynx (mph), and internal tooth (asterisk). Scale bars: 200 µm **(A-D**, **F-G)**; 100 µm **(E)**.

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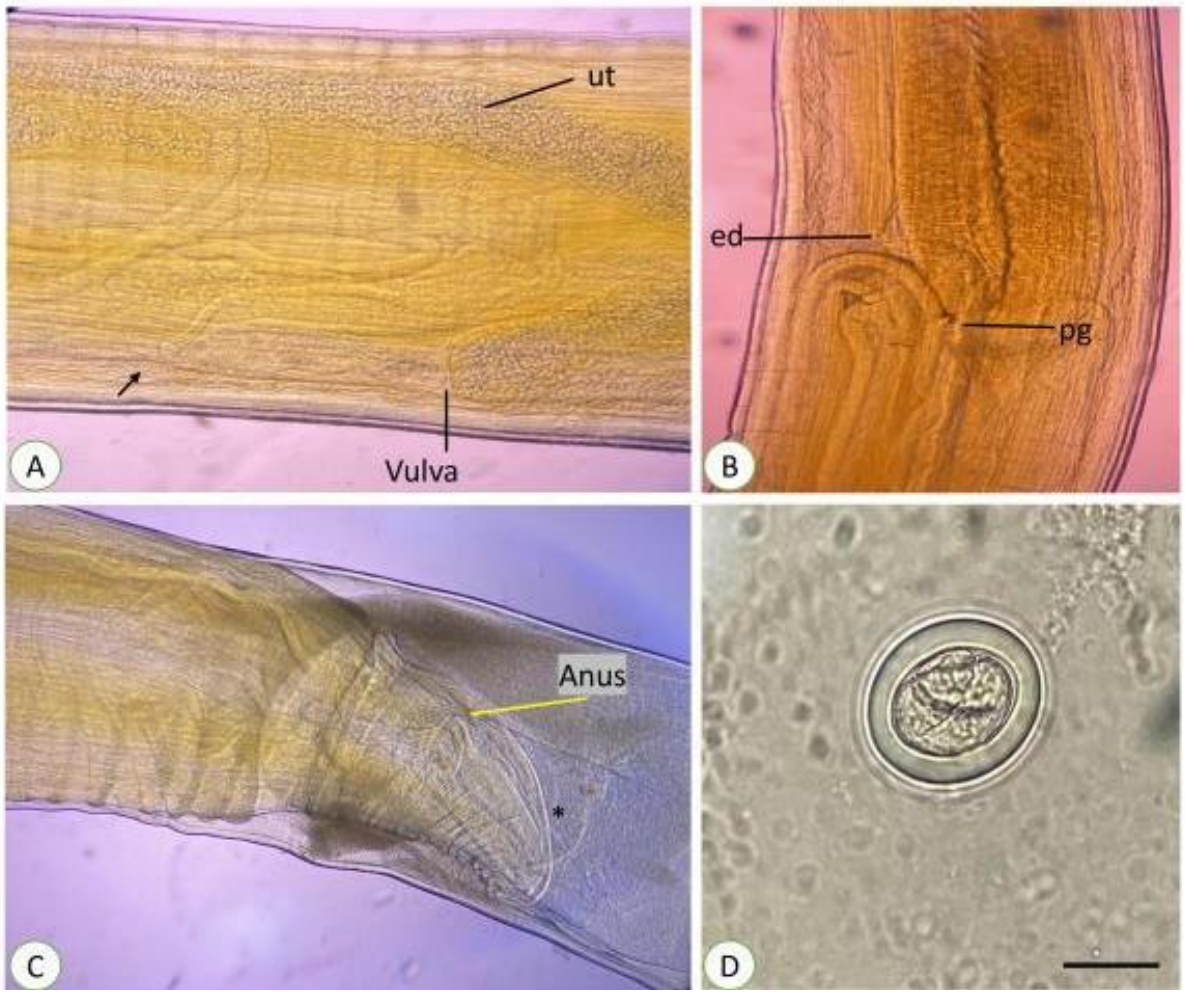


Figure 4. Female reproductive system of *Physaloptera praeputialis*. Recovered from African lions (*Panthera leo*) in the Serengeti ecosystem, Tanzania, between 2023 and 2025. **A:** Uterus (ut) and vaginal duct (arrows). **B:** Anterior region of female showing the excretory duct (ed) and pharyngeal-gastric valve (pg). **C:** Posterior end of adult female showing the anus and anal pouch (asterisk). **D:** Larvated egg recovered from fecal sample (×40 magnification).

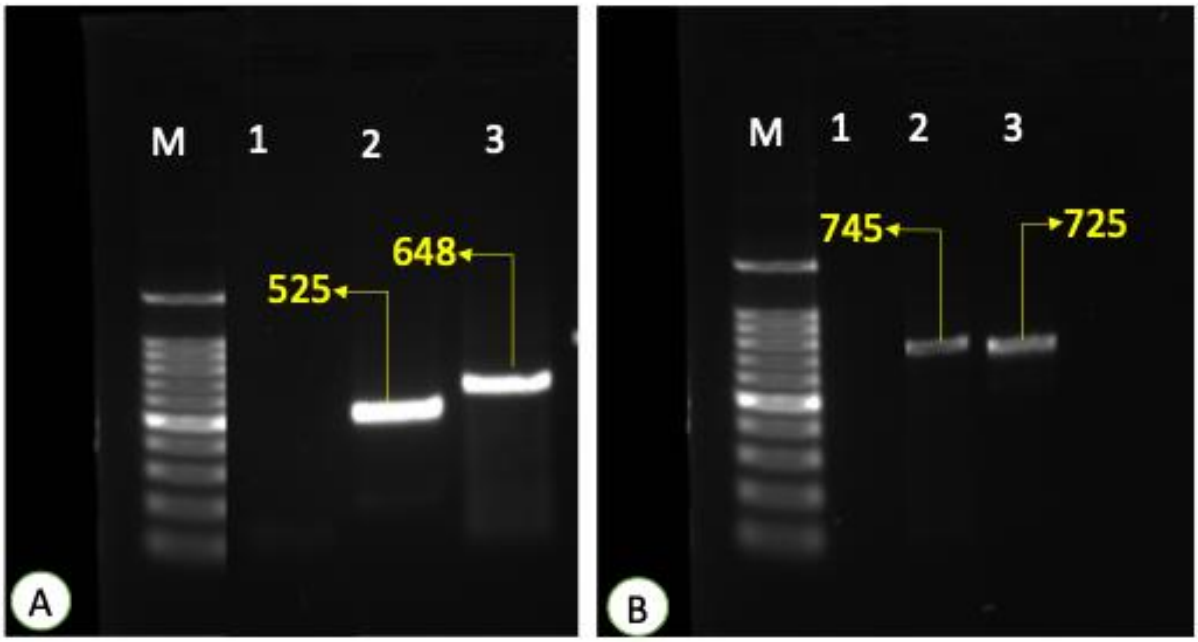


Figure 5. Agarose gel electrophoresis (1.5%) showing PCR amplification of mitochondrial *cox1* and nuclear *18S rRNA* genes of *Physaloptera praeputialis* isolated from African lions (*Panthera leo*) in the Serengeti ecosystem, Tanzania, collected between 2023 and 2025. **A:** Amplification of the *cox1* gene showing positive bands at approximately 525 bp (lane 2) and 648 bp (lane 3), negative control (lane 1; no-template control), and molecular weight marker (100 bp DNA ladder, lane M). **B:** Amplification of the *18S rRNA* gene showing positive bands at approximately 745 bp (lane 2) and 725 bp (lane 3), negative control (lane 1; no-template control), and molecular weight marker (100 bp DNA ladder, lane M).

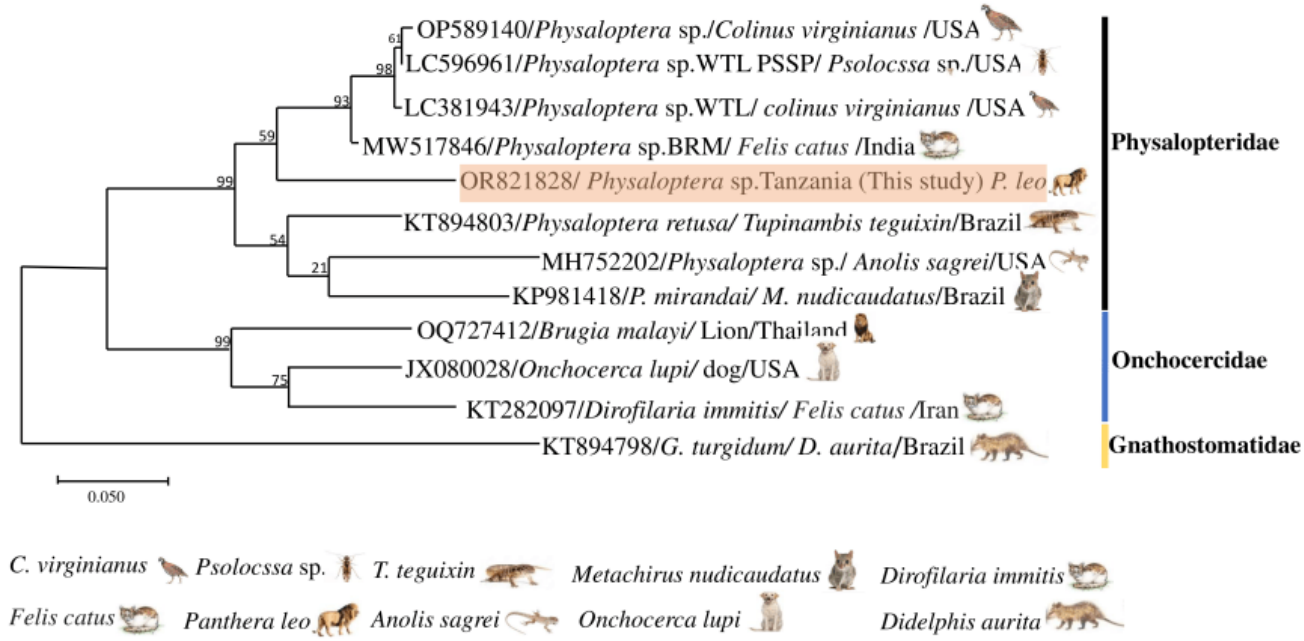


Figure 6. Phylogenetic tree based on mitochondrial *cox1* sequences showing the relationships of *Physaloptera* spp. (OR821828) isolates obtained from *Panthera leo* in the Serengeti ecosystem, Tanzania, collected between 2023 and 2025, together with reference sequences retrieved from GenBank.

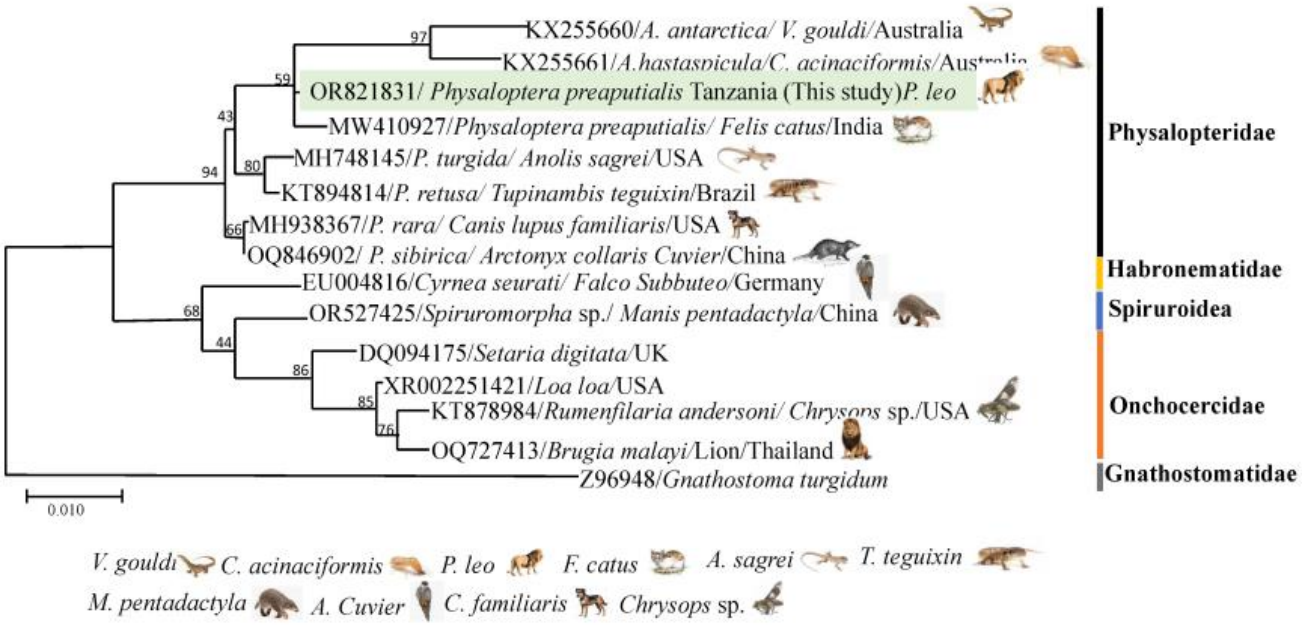


Figure 7. Phylogenetic tree based on nuclear *18S rRNA* gene sequences showing the relationships of *Physaloptera praeputialis* isolates obtained from *Panthera leo* in the Serengeti ecosystem, Tanzania, collected between 2023 and 2025, together with reference sequences retrieved from GenBank.

DISCUSSION

The combined morphological and molecular evidence supports the identification of the Tanzanian *Physaloptera* specimens as *P. praeputialis*, extending current knowledge of its host range to African lions in Tanzania. Previous

reports of physalopterid infections in Tanzania were based primarily on coprological examinations and identified parasites only to the genus level without attempting species-level identification (Müller-Graf, 1995). The present findings establish an essential taxonomic and molecular baseline for future ecological, epidemiological, and evolutionary investigations of spirurid nematodes in African wildlife.

In the current study, adult *P. praeputialis* specimens were associated with erosive and hemorrhagic gastritis in the stomach of the African lions. Similar lesions have been reported in domestic and wildlife infected with *Physaloptera* spp., where persistent mucosal attachment, mechanical abrasion, hematophagy, and secondary bacterial invasion contribute to chronic inflammation (Soulsby,1965; Naem and Asadi, 2013). Although parasite burden was not quantified, the observed mucosal damage suggests that *P. praeputialis* may contribute to subclinical morbidity in lions, particularly in aged or nutritionally compromised individuals (Anderson et al., 2009). In apex predators such as lions, chronic gastric inflammation has the potential to reduce digestive efficiency and exacerbate energy stress during periods of prey scarcity (Anderson, 2000; Wobeser, 2007). However, direct impacts on fitness, hunting success, or survival remain speculative and should be evaluated through quantitative parasitological and longitudinal health studies (Packer et al., 2005).

Morphological characterization observed in adult worms, such as cephalic structures, spicule morphology, and caudal papillae arrangement, is consistent with classical descriptions of genus *P. praeputialis*, yet precise species validation remains challenging due to morphological similarity and phenotypic plasticity (Anderson et al., 2009). In the present study, the concordance between morphology and nuclear genetic data supports placement within the *P. praeputialis* species complex, but does not clearly resolve species boundaries. Integrative taxonomy combining morphology, multiple genetic loci, host associations, and biogeography is necessary for resolving spirurid systematics (Dayrat, 2005; Ailán-Choke and Pereira, 2021). Phylogenetic analyses based on both nuclear and mitochondrial loci place the Tanzanian isolates within the *P. praeputialis* clade. The high genetic similarity observed in the *18S rRNA* gene relative to reference sequences reflects the slow evolutionary rate and confirmation of generic placement and deep phylogenetic relationships (Blouin, 2002). Mitochondrial *cox1* sequences from the present *Physaloptera* specimens exhibited substantial nucleotide divergence and high haplotype diversity compared with nuclear *18S rRNA* sequences, a pattern commonly reported in spirurid nematodes and other parasitic helminth groups, including cestodes and trematodes (Maharana et al., 2021; Chen et al., 2023). The mean sequence divergence relative to other physalopterid sequences exceeds typical intraspecific variation reported for nematodes and overlaps with divergence levels among closely related species, consistent with species-level generic differentiation (Blouin et al., 1995; Powers, 2004). Phylogenetic reconstruction places the Tanzanian isolates within the *P. praeputialis* clade, consistent with long-term population subdivision and restricted gene flow among geographically separated parasite populations (Blouin et al., 1995; Nadler and De León, 2011). The magnitude of *Cox1* divergence observed in the present *P. praeputialis* isolates falls within previously reported interspecific divergence levels for spirurid nematodes (Maharana et al., 2021). The observed pattern may reflect deep phylogeographic structuring within a broadly distributed *P. praeputialis* species complex or cryptic diversification in African populations that is not detectable using conserved nuclear markers alone (Maharana et al., 2021).

Lions occupy the apex of a highly connected trophic web linking herbivorous prey, insect communities, scavengers, and domestic carnivores at the wildlife-livestock interface (Sinclair et al., 2015). Such ecological connectivity enables *Physaloptera* spp. to utilize insects as intermediate hosts and vertebrates as paratenic hosts, expecting transmission to be strongly structured by both food-web architecture and host association (Dobson et al., 2009). The Serengeti ecosystem is characterized by high prey biomass and abundant coprophagous insects, which likely sustain stable enzootic transmission cycles (Sinclair et al., 2015). African lions may function as terminal accumulation hosts rather than key maintenance hosts, acquiring infections through repeated ingestion of infected prey (Bjork et al., 2000; Green et al., 2020; Tull et al., 2022). Such ecological connectivity can promote higher mitochondrial genetic variation while nuclear genes remain more conserved due to occasional gene flow among host species and across different habitats (Nadler et al., 2011). Although numerous parasitological investigations in East Africa have focused on domestic animals, molecular characterization of helminths infecting wildlife remains comparatively limited, highlighting the need for integrative studies to improve understanding of parasite diversity and transmission dynamics in the region (Brooks and Hoberg, 2007).

As a result, the diversity and distribution of spirurid nematodes in Tanzania remain poorly resolved. The presence of *P. praeputialis* in the Serengeti ecosystem highlights the hidden parasite diversity in protected areas and the role of apex predators as indicators of ecosystem health (Bjork et al., 2000; Han et al., 2021; Moraes et al., 2024).

Although *Physaloptera* spp. are primarily parasites of carnivorous vertebrates, human exposure may occasionally occur through accidental ingestion of infected intermediate hosts such as insects; however, confirmed human infections are extremely rare and not well documented in the literature (Anderson, 2000). While the public health risk is likely low,

the detection of *P. praeputialis* populations in human-wildlife proximity highlights the importance of incorporating spirurid nematodes into broader helminth surveillance frameworks (Ma et al., 2020). Establishing molecular reference sequences from Tanzania also strengthens regional parasite biobanking initiatives and enables future monitoring of parasite dispersal associated with land-use change, climate variability, and carnivore population dynamics.

CONCLUSION

The current study presents the first characterization of adult *P. praeputialis* recovered from the African lion in Tanzania using integrative morphological and molecular approaches. The parasite exhibits substantial mitochondrial diversity while maintaining nuclear genetic cohesion, consistent with geographically structured populations circulating within complex Serengeti food webs. The findings expand the known range of *P. praeputialis*, emphasize the ecological role of apex predators in helminth transmission networks, and provide a molecular baseline for future investigations on spirurid diversity, wildlife health, and ecosystem stability in Tanzania. Interpretation of the findings should consider the limited sample size, opportunistic recovery from naturally deceased hosts, absence of intermediate host investigations, and analysis based on only two genetic loci.

DECLARATIONS

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Authors' contributions

Barakaeli Abdieli Ndossi, Maulid Mzinga Mdaki, and Eblate Ernest Mjingo were responsible for the conceptualization. Mohammed Mebarek Bia and Barakaeli Abdieli Ndossi contributed to the formal analysis. Barakaeli Abdieli, Ndossi, and Mohammed Mebarek Biawere conducted the methodology. Barakaeli Abdieli Ndossi, Maulid Mzinga Mdaki, Heejae Yang, and Marry Wokusima Zebedayo participated in the resources. Keeseon Eom, Mohammed Mebarek Bia, and Eblate Ernest Mjingo were responsible for the validation. Barakaeli Abdieli Ndossi prepared the original draft. Mohammed Mebarek Biaand Barakaeli Abdieli Ndossi contributed to writing, reviewing, and editing. All authors have read and approved the final version of the manuscript before publication in the present journal.

Availability of data and materials

The data to support the findings of the present study are available upon reasonable request from the corresponding author.

Competing interests

We have no conflict of interest related to the present study.

Ethical considerations

The authors declared that the manuscript is original, has not been published elsewhere, and confirmed the latest version before publication. The authors declared that no AI tools were used for writing and preparing the article.

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Genotypic Characterization of *Escherichia coli* Pathotypes Based on Antibiotic Resistance Genes

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ABSTRACT

Diarrhea represents a major health challenge in young calves and is a leading cause of mortality, arising from infectious and non-infectious factors. The present study aimed to investigate antibiotic-resistant, multi-virulent diarrheagenic *Escherichia coli* (*E. coli*) strains isolated from human and animal samples. The current study included 23 *E. coli* strains obtained from calves across four different farms in Egypt, as well as nine *E. coli* isolates derived from 18 stool samples from workers. These samples were transferred to the microbiological laboratory for bacteriological and molecular analysis utilizing polymerase chain reaction (PCR). Molecular analysis of the antibiotic resistance gene was performed using PCR, followed by antibiotic sensitivity testing against ciprofloxacin, enrofloxacin, norfloxacin, ampicillin, cephalosporin, and colistin. Among 23 *E. coli* isolates obtained from calves, the resistance genes, including *qnrS* (21.7%), *gyrA* (34.7%), and *blaTEM* (13%), were identified. Conversely, *qnrA*, *blaOXA-1*, and *blaSHV* were not detected. Only one isolate exhibited colistin resistance, which was uncommon. Human *E. coli* isolates demonstrated moderate prevalence of *qnrA* (55.5%), *qnrS* (33.3%), *gyrA* (33.3%), and *blaTEM* (22.2%), while *blaOXA* and *blaSHV* were not detected, and a colistin resistance isolate (*mcr-1*) was rare. The antibiotic sensitivity test of *E. coli* isolates from calves exhibited that 43% were resistant to ciprofloxacin and 35% to enrofloxacin. Meanwhile, 22% of isolates demonstrated resistance to norfloxacin. The β-lactam group revealed 13% resistance to ampicillin and cephalosporin. Only 4% of all isolates exhibited resistance to colistin. Antibiotic sensitivity test of *E. coli* isolates from humans revealed significant resistance patterns. Among the quinolones, 78% demonstrated resistance to ciprofloxacin, 56% to enrofloxacin, and 45% to norfloxacin. In contrast, only 22% of isolates were resistant to ampicillin and cephalosporin, while resistance to colistin was detected in just 11% of isolates. The present study confirmed that the misuse of different antibiotics is a significant factor in the development of antimicrobial resistance genes, especially in *E. coli* strains isolated from both humans and infected calves.

Keywords: Antibiotic sensitivity test, *Escherichia coli*, Multidrug resistance, PCR

INTRODUCTION

Diarrhea causes substantial morbidity and mortality in developing nations. Given that several distinct phenotypic enteric bacteria have been identified as causative agents of severe gastroenteritis (Davis and Brown, 2016). *Escherichia coli* (*E. coli*) is the main cause of diarrhea and can be categorized as either commensal or pathogenic (Davis and Brown 2016). The pathogenic group of *E. coli* comprises two pathotypes, including diarrheagenic *E. coli* (DEC) and extraintestinal pathogenic *E. coli* (ExPEC), each responsible for distinct diseases in humans and animals (Kunert et al., 2015). Neonatal meningitis and uropathogenic *E. coli* are the two pathovars of ExPEC (Bae et al., 2006). Furthermore, regarding the mechanisms underlying diarrhea, six pathovars of DEC have been identified, including diffusely adherent *E. coli*, enteropathogenic *E. coli*, enteroaggregative *E. coli*, enterohaemorrhagic *E. coli*, and entero-invasive *E. coli* (Croxen and Finlay, 2010). *Escherichia coli* has been linked to foodborne outbreaks in humans, resulting in thrombotic thrombocytopenic purpura (TTP) in adults, hemolytic uremic syndrome (HUS), and bloody diarrhea in children (Bae et al., 2006). There are two major public health crises involving *E. coli* infections. Firstly, the prevalent pathogenic *E. coli* strains in environmental waters, such as those used for irrigation and drinking, cause severe infections in humans and animals (Koczura et al., 2011). Secondly, the presence of multi-virulent and multidrug-resistant (MDR*) E. coli* strains that exhibited resistance to at least three antimicrobial medicines from different classes (Hamelin et al., 2007).

The presence of virulence genes and antimicrobial resistance was demonstrated to have a strong negative correlation (Bendary et al., 2022). The high prevalence of multiple virulence genes among MDR *E. coli* isolates from human,

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animal, and environmental sources indicated a significant pathogenic potential, making these infections difficult to treat due to their multidrug resistance (James et al., 2025). Antimicrobial resistance among *E. coli* strains has become a growing concern worldwide. In 2017, Iranian hospitals documented an unexpected rise in resistance to commonly used antibiotics such as ampicillin and ceftazidime, underscoring the growing difficulty of managing nosocomial *E. coli* infections (Eshrati et al., 2025). The number of antibiotic resistance genes (ARGs) in *E. coli* has gradually increased over recent decades, influenced by factors such as global antibiotic use, the spread of mobile genetic elements, and various climatic conditions (Wang et al., 2026). Gamal et al. (2019) analyzed different human and animal samples to identify specific pathogenic genes. In this context, the present study aimed to assess antibiotic-resistant and MDR strains of highly virulent diarrheagenic *E. coli*, which pose significant health challenges to both humans and animals.

MATERIAL AND METHODS

Ethical approval

Ethical approval was not required for the present study according to institutional and journal guidelines, as it relied solely on previously isolated *E. coli* DNA samples and did not involve any animal experimentation or handling.

Strains

In the current investigation, nine *E. coli* isolates from humans and 23 *E. coli* strains from calves were utilized. The *E. coli* isolates used in the present experiment were sourced from a prior study by Gamal et al. (2019), who collected 43 fecal samples from diarrheic calves across four farms in New Valley, Egypt, and 18 stool samples from workers in 2019. These samples were transferred to the microbiological laboratory for bacteriological and molecular examination. Polymerase chain reaction (PCR) was used to genotype all 23 calves and 9 human samples as *E. coli* (Gamal et al., 2019).

Extraction of DNA

Genomic DNA was isolated from both human and animal *E. coli* cultures utilizing the EasyPURE Bacterial Genomic DNA Kit (Cat. No. EE161-01), following the manufacturer's provided protocol. To collect bacterial cells, one mL of each overnight culture was centrifuged at 12,000 × g for one minute. The cell pellet was subjected to enzymatic lysis using the provided lysis buffer and Proteinase K at 55°C for 15 minutes. The DNA binding was performed using the kit's binding buffer, then the mixture was applied to a silica column and centrifuged at 12,000 × g for 30 seconds. The column was subsequently washed according to the manufacturer's protocol, and DNA was eluted in 50 µL of elution buffer. The concentration and purity of the extracted DNA were determined using a spectrophotometer.

Polymerase chain reaction

A reaction mixture was prepared in 0.5 mL microfuge tubes containing 5 µL of genomic DNA, 12.5 µL of Dream Taq Green Master Mix (2x Easy Taq PCR Supermix [AS111]; Thermo Fisher Scientific, China), 1 µL of each primer (50 pmole/µl; Table 1), and 5.5 µL of deionized water. Then, amplification was performed under the following conditions. The thermal cycling profile for the *qnrA*, qnrS, and *gyrA* gene primers consisted of an initial denaturation step of 95°C for five minutes. This was followed by 35 cycles, each comprising 45 seconds at 94°C (denaturation), 45 seconds at 51°C (annealing), and 45 seconds at 72°C (extension). A final extension was performed at 72°C for seven minutes. For the *blaOXA-1*, *blaSHV*, and *blaTEM* genes (Colom et al., 2003), the protocol involved an initial denaturation at 94°C for five minutes, followed by 35 cycles. Each cycle consisted of 30 seconds at 94°C, 30 seconds at 54°C, and 1 minute at 72°C. A final extension was performed at 72°C for 10 minutes. For the *mcr1* gene primer (Rebelo et al., 2018), the protocol started with a 15-minute denaturation at 94°C. This was followed by 25 cycles, each comprising 30 seconds at 94°C, 90 seconds at 58°C, and one minute at 72°C. The final step was a 10-minute extension at 72°C. The PCR products were analyzed on a 1% agarose gel prepared in 1× TAE buffer and stained with ethidium bromide (0.5 µg/mL). A 100-base pair DNA ladder was included as a molecular size marker. Electrophoresis was performed at 100 V and 80 mA for 30 minutes. After separation, DNA bands were visualized and photographed under UV transillumination.

Antibiotic sensitivity test

In compliance with the guidelines supplied by the Clinical and Laboratory Standards Institute (CLSI, 2019), the *in vitro* susceptibility patterns of all tested *E. coli* isolates from both animal and human origin were determined using Mueller-Hinton agar and standard antimicrobial discs using the Kirby-Bauer disc diffusion method.

Table 1. Resistance gene primers used in the present study

Genes	Primer sequence	Amplicon size	Reference
qnrA	F 5-ATTTCTCACGCCAGGATTTG-3 R 5-GATCGGCAAAGGTTAGGTCA-3	516	Hammad et al. (2009)
qnrS	F 5-ACGACATTCGTCAACTGCAA-3 R 5-TAAATTGGCACCCTGTAGGC-3	417	Hammad et al. (2009)
gyrA	F 5-AAATCTGCCCGTGTCGTTGGT-3 R 5-GCCATACCTACGGCGATACC-3	344	Eguale et al. (2017)
blaOXA-1	F 5-TCAACTTTCAAGATCGCA-3 R5-GTGTGTTTAGAATGGTGA-3	609	Colom et al. (2003)
blaSHV	F 5-AGGATTGACTGCCTTTTTG-3 R 5-ATTTGCTGATTTCGCTCG-3	392	Colom et al. (2003)
blaTEM	F 5-ATCAGCAATAAACCAGC-3 R 5-CCCCGAAGAACGTTTTC-3	516	Colom et al. (2003)
mcr-1	F 5-AGTCCGTTTGTTCTTGTGGC-3 R 5-AGATCCTTGGTCTCGGCTTG-3	320	Rebelo et al. (2018)

RESULTS

The present results indicated that the *qnrA* resistance gene was not detected in any of the 23 *E. coli* isolates from calves (Table 2). However, the *qnrS* resistance gene was detected in 5 of the 23 isolates, producing a 417 bp PCR product (Figure 2A and 1A). The prevalence rates for the *qnrA*, *qnrS*, and *gyrA* resistance genes in the 23 *E. coli* isolates from calves were 0%, 21.7%, and 34.7%, respectively. Specifically, the *gyrA* gene was detected in eight isolates via PCR, producing a 344 bp product (Figure 3A). Analysis of the β-lactam resistance genes revealed that all 23 calf isolates were negative for *blaOXA-1* and *blaSHV* (Figure 4A). However, *blaTEM* was detected in three isolates, producing a 516 bp PCR product (Figure 5A). The prevalence rates for *blaOXA-1* and *blaSHV* were 0%, while the prevalence for *blaTEM* was 13%. Regarding colistin resistance, only 1 of 23 calf isolates tested positive for the *mcr-1* gene, yielding a prevalence of 4.3%. This positive result yielded a 320 bp PCR product (Figure 6A).

Analysis of nine *E. coli* isolates from human sources revealed the presence of resistance genes. Specifically, three isolates were positive for *qnrS*, yielding a 417 bp PCR product (Figure 1 B), and five isolates were positive for *qnrA*, yielding a 516 bp PCR product (Table 3; Figure 2B). The *gyrA* resistance gene was detected in three human isolates, yielding a 344 bp PCR product (Figure 3B). The prevalence of *qnrA*, *qnrS*, and *gyrA* resistance genes among the human isolates was 55.5%, 33.3%, and 33.3%, respectively. In the β-lactamase gene analysis, all nine human *E. coli* isolates yielded negative results for the *blaOXA* and *blaSHV* genes (Figure 4B). In contrast, the *blaTEM* gene was found in two of the nine human strains, producing a 516 bp PCR product (Figure 5B). The prevalence rates for *blaOXA*, *blaSHV*, and *blaTEM* were 0%, 0%, and 22.2%, respectively. For colistin resistance genes, the *mcr-1* gene was detected in one out of the nine human *E. coli* isolates, yielding a 320 bp PCR product. The prevalence rate for *mcr-1* among human strains was 11.1%.

The antibiotic sensitivity test results for *E. coli* isolates from calves against quinolones, β-lactams, and colistin are presented in Table 4. Susceptibility testing for quinolones in 23 *E. coli* calf isolates indicated that resistance to ciprofloxacin was observed in 10 isolates (43%). Resistance to enrofloxacin was detected in eight isolates (35%), and resistance to norfloxacin was found in five isolates (22%). The remaining isolates demonstrated different degrees of sensitivity to these antibiotics. Resistance to ampicillin and cephalosporin antibiotics (β-lactam group) was identified in 3 of 23 calf isolates (13%). Colistin resistance was found in only one calf isolate (4%), while the other 23 isolates demonstrated sensitivity to colistin.

Antibiotic sensitivity test results of human *E. coli* isolates to quinolones, β-lactams, and colistin are presented in Table 5. For quinolones, seven of nine human *E. coli* isolates (78%) were resistant to ciprofloxacin, while two isolates (22%) were sensitive (Table 5). The current findings indicate that five of the nine human *E. coli* isolates (56%) were resistant to enrofloxacin, whereas the remaining isolates showed varying degrees of sensitivity. Additional findings indicated that four of the nine human *E. coli* strains (45%) were resistant to norfloxacin, and the remaining isolates (5/9) were sensitive to norfloxacin. Resistance to ampicillin and cephalosporin antibiotics (β-lactams) was observed in two of nine human *E. coli* isolates (22%). Colistin resistance was noted in only one human *E. coli* isolate (11%).

Table 2. PCR results of *Escherichia coli* isolated from calves in New Valley, Egypt

Calves-tested isolates		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
Target resistance genes																								
Quinolones	*qnrA*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	qnrS	-	-	+	+	-	-	+	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-
	gyrA	+	+	+	-	-	-	+	-	-	-	+	-	+	-	-	-	+	+	-	-	-	-	-
β- lactam	*blaOXA-1*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	blaSHV	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	blaTEM	-	-	+	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Colistin	*Mcr-1*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-

+: Means presence of gene, -: Means absence of genes

Table 3. PCR results of *Escherichia coli* isolated from humans in New Valley, Egypt

Human-tested isolates		5E	6E	9E	15E	51E	54E	55E	65E	67E
Resistance genes										
Quinolone group	*qnRA*	-	+	-	+	-	+	+	+	-
	qnrS	-	-	+	-	-	-	+	-	+
	gyrA	-	-	+	+	-	-	-	-	+
β-lactam group	*blaOXA-1*	-	-	-	-	-	-	-	-	-
	blaSHV	-	-	-	-	-	-	-	-	-
	blaTEM	+	-	-	-	+	-	-	-	-
Colistin	*mcr-1*	-	+	-	-	-	-	-	-	-

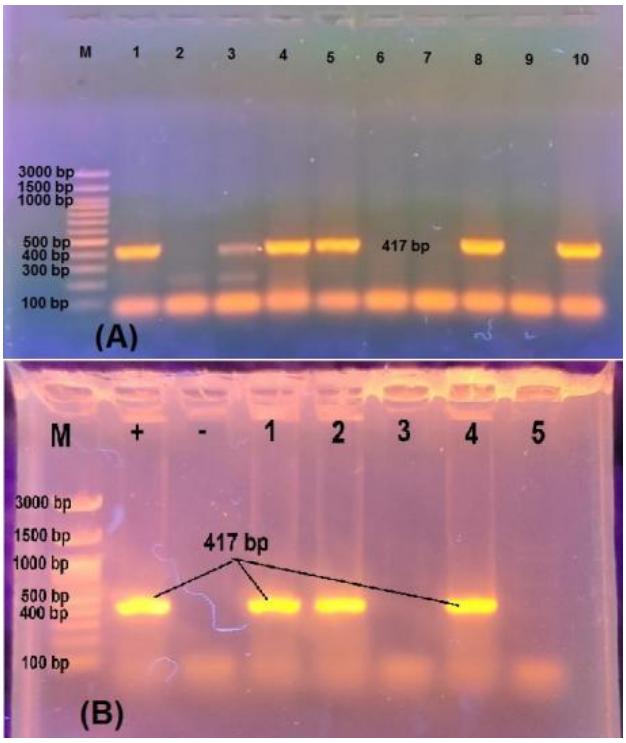


Figure 1. The *qnrS* gene of *Escherichia coli* isolated from different sources in Egypt. **A**: Calves, **B**: Humans

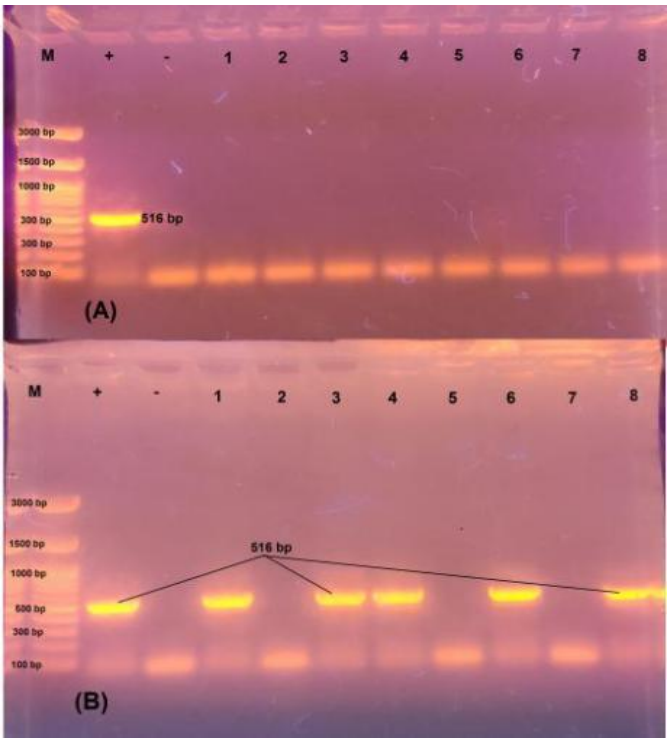


Figure 2. The *qnr A* gene of *Escherichia coli* isolated from differen sources in Egypt. **A**: Calves, **B**: Humans

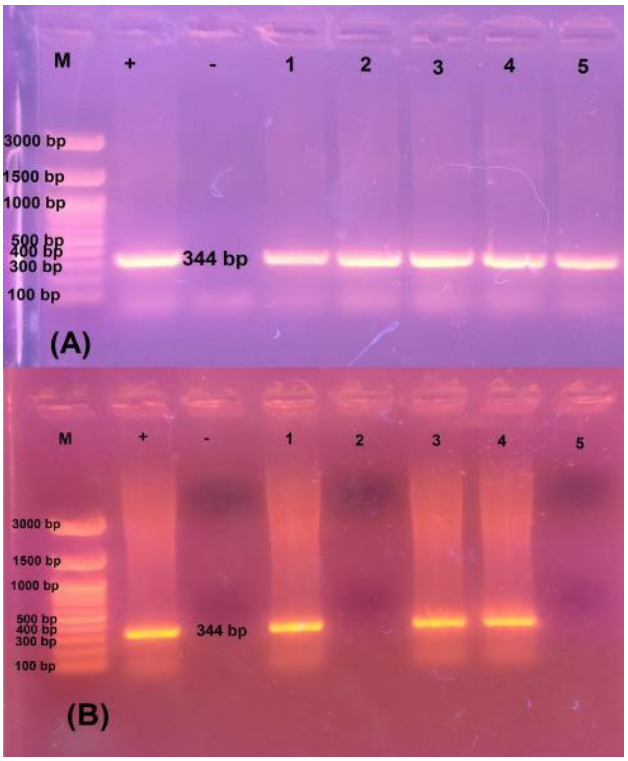


Figure 3. The *gyrA* gene of *Escherichia coli* isolated from different sources in Egypt. **A**: Calves, **B**: Humans

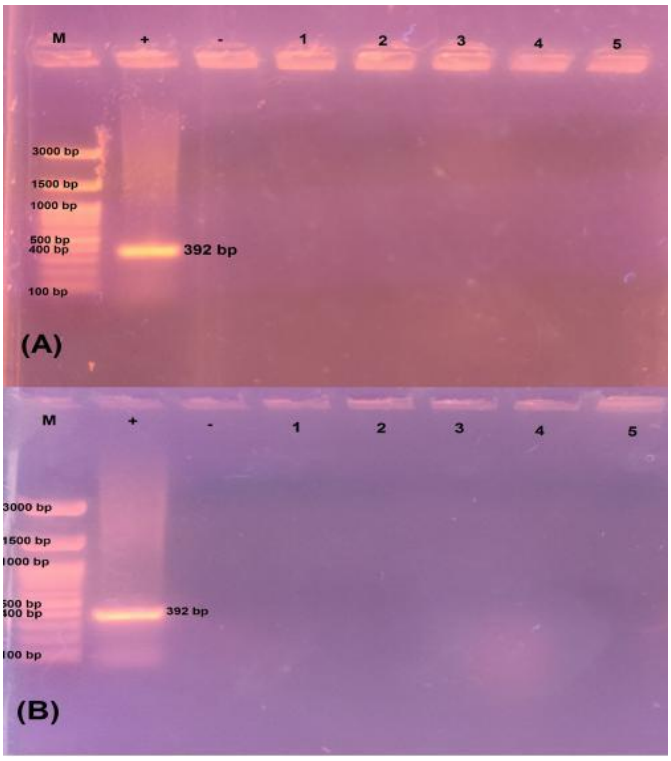


Figure 4. The *blaSHV* gene of *Escherichia coli* isolated from different sources in Egypt. **A**: Calves, **B**: Humans

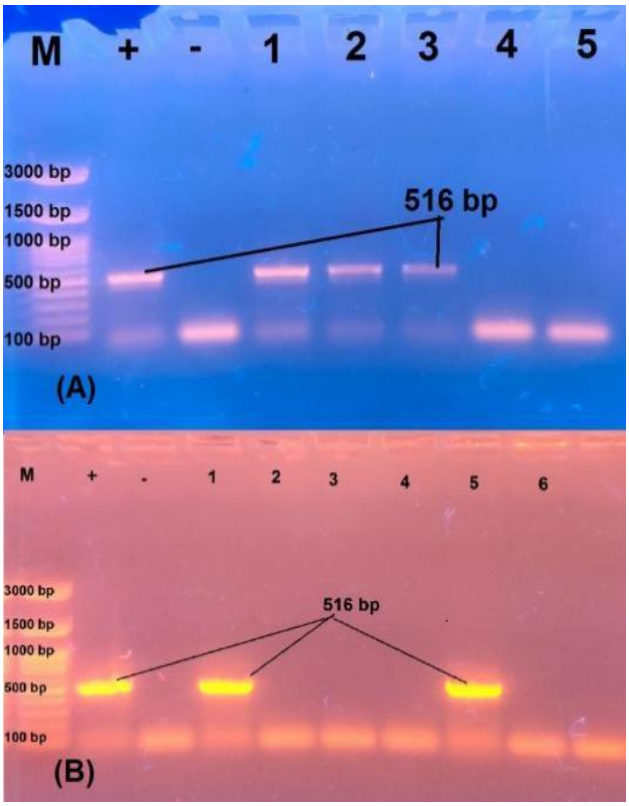


Figure 5. The *blaTEM* gene of *Escherichia coli was* isolated from different sources in Egypt. **A**: Calves, **B**: Humans

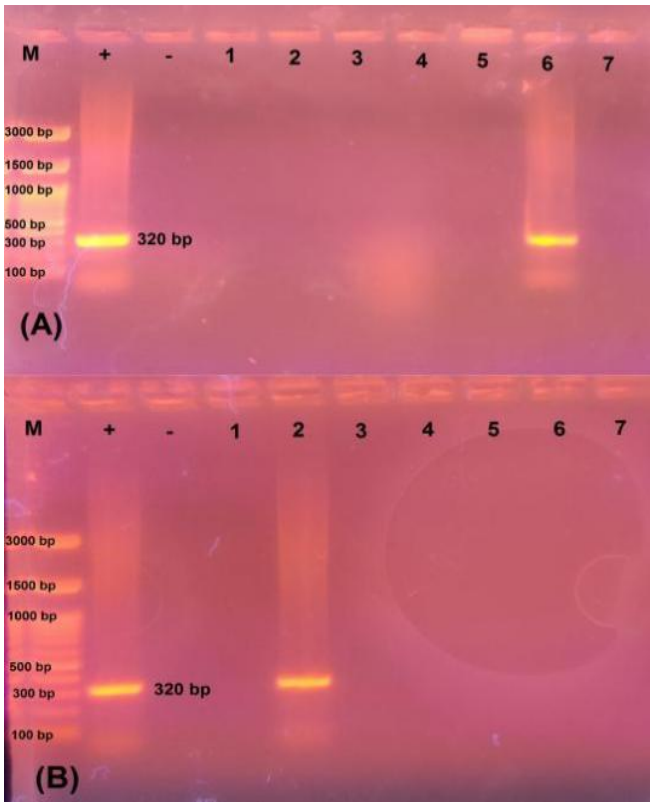


Figure 6. The *mcr-1* gene of *Escherichia coli* isolated from different sources in Egypt. **A**: Calves, **B**: Humans

Table 4. Antibiotic sensitivity test of *Escherichia coli* isolated from calves, New Valley, Egypt

Calves-tested isolates		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**
Antibiotic																								
Quino-Lons group	Ciprofloxacin	-	-	-	-	+++	+++	-	+++	+++	++	-	+++	-	++	-	++	-	-	+++	++	+++	++	++
	Enrofloxacin	-	-	-	+	++	++	-	++	+++	++	-	++	-	++	+	+++	-	-	++	+++	+++	+++	+++
	Norfloxacin	+	+	-	-	+++	+++	-	++	++	+++	+	++	-	+++	-	+++	+	+	++	+++	++	++	++
β-lactam group	Ampicillin	++	+	-	+++	-	+++	-	+	++	+	+++	+++	+	+	++	+++	+	+	+++	++	++	+	+
	Cephalosporin	+++	+	-	++	-	++	-	+++	+++	++	+	+	++	+++	+++	+	+	+++	+	+	+++	+++	++
Colistin		+	++	++	+	+	+++	+	++	+++	++	+	+	++	++	+++	++	+++	++	++	+++	-	++	++

+: Sensitive to antibiotic, -: Resistance to antibiotic

Table 5. Antibiotic sensitivity test of *Escherichia coli* isolated from humans in New Valley, Egypt

Human-tested isolates		**5E**	**6E**	**9E**	**15E**	**51E**	**54E**	**55E**	**65E**	**67E**
Antibiotic										
Quinolone group	Ciprofloxacin	++	-	-	-	++	-	-	-	-
	Enrofloxacin	++	-	-	-	+++	-	+	+	-
	Norfloxacin	+++	+	-	+	++	+	-	-	-
β-lactam group	Ampicillin	-	+++	+	+++	-	+	++	+++	++
	Cephalosporin	-	++	+	++	-	+	++	+++	+++
Colistin		+++	-	++	++	+	+++	++	+	+++

Sensitive to antibiotic, -: Resistance to antibiotic

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DISCUSSION

Escherichia coli strains in cattle can exist asymptomatically, primarily within the digestive tract. This environment facilitates the transfer of resistance traits from non-pathogenic to pathogenic strains. Increasing bacterial antibiotic resistance poses a major challenge for treating diarrheagenic *E. coli* infections, especially in low-income countries. These infections can pose a major hazard due to their widespread environmental distribution.

In the current study, resistance gene profiling of *E. coli* isolated from calves revealed prevalence rates of 21.7% and 34.7% for *qnr* and *gyrA*, respectively. In contrast to the present findings, Tabran et al. (2022) revealed that 4.76% (2/42) of the isolates tested negative for all resistance genes, and *tet(A)* and *tet(B)* were the main genes found in 64.2% (27/42) of the shiga toxin-producing *E. coli* (STEC) strains, while *sul1* and *aadA1* were present in 52.3% (22/42) of the pathogenic strains examined.

For the β-lactam group, no *blaOXA* or *blaSHV* genes were detected in the present study, whereas *blaTEM* was found in 13% of isolates. In contrast to the present results, Tabran et al. (2022) reported β-lactam resistance genes in 2.3% (1/42) of isolates for ^{bla}*SHV* and a higher prevalence of 23.8% (10/42) for *blaTEM*. For the colistin resistance group, the current results reported a prevalence rate of 4.3%. However, Zhang et al. (2019) reported notably higher prevalence rates of *E. coli* isolates in calves (57.14%), developing cows (75%), and milking cows (73.68%), which contrasted with the present findings. Additionally, Bai et al. (2016) collected 93 STEC isolates from fecal, small intestinal, and colonic contents of healthy pigs in Beijing, China. Of these 93 STEC isolates, 10 were resistant to colistin, and the *mcr-1* gene was detected in all 10 isolates.

Antibiotic sensitivity testing confirmed the PCR results for strains encoding the *qnr* and *gyr* genes, which exhibited resistance to ciprofloxacin, enrofloxacin, and norfloxacin. The current findings were inconsistent with those of Tabran et al. (2022), who reported that nalidixic acid had the highest resistance mechanism against pathogenic *E. coli*, followed by ciprofloxacin and tetracycline. In the β-lactam group, this study reported resistance rates of 13% for both ampicillin and cephalosporins, and 4% for colistin. In comparison, Tabran et al. (2022) observed a notable decrease in resistance levels within the cephalosporin class. Similar to Romania, 31% of intestinal samples from cattle exhibited resistance to cefotaxime and ceftazidime; whereas only 10% (1/3) of the isolates from France exhibited a similar resistance pattern.

The current findings on *E. coli* isolates from human samples demonstrated a high resistance to the *qnr* gene (88.8%), with 55.5% for *qnrA* and 33.3% for *qnrS*. Additionally, the *gyrA* gene was present in 33.3% of isolates. These findings were considerably higher than those reported by Tahou et al. (2017) in Côte d'Ivoire, who detected *qnr* genes in 31.2% of *E. coli* isolates, with a prevalence rate of 14.6% for *qnrB*, 9.9% for *qnrA*, and 2.7% for *qnrS*. Furthermore, in Niger, Moumouni et al. (2017) found that 44.4% of *E. coli* isolates carried *qnr* genes, with specific prevalence rates of 64.3% for *qnrS*, 26.2%for *qnrB*, and9.5%for *qnrA*. Further findings from Morocco indicated that 18.7% of *E. coli* isolates harbored *qnrB* (23%), *qnrA* (10%), and *qnrS* (3%; Bouchakour et al., 2010).

In the β-lactam group, the present findings indicated prevalence rates of 0% for *blaOXA*, 0% for ^{bla}*SHV*, and 22.2% for *blaTEM*. The present results contrast with the findings of Salah et al. (2019), who found that all *qnr*-positive *E. coli* and *Klebsiella* spp. strains were linked to the spectrum β-lactamase (ESBL) producers. Salah et al. (2019) identified CTX-M1 in 102 *qnr*-positive strains, *SHV* in 52, and *TEM* in 96. Furthermore, the characterization of *qnr*-positive isolates in Mexico revealed that the *SHV* ESBL-type (*SHV*-12, -5, -2a) was the most prevalent isolate, followed by CTX-M-15 (Silva-Sanchez et al., 2011). Notably, CTX-M-15 was the most predominant isolate among pediatric populations (Silva-Sanchez et al., 2013).

According to the present results, the prevalence of the *mcr-1* gene was 11% in the colistin group. Skov et al. (2016) reported the discovery of over 100 *Enterobacteriaceae* strains carrying *mcr* from human samples worldwide, mainly *E. coli* strains from patients who had recently visited Asian countries. Liu et al. (2016) reported the first detection of *mcr-1*, identifying 16 clinical *E. coli* strains that were *mcr-1*-positive.

Regarding the antibiotic sensitivity results of *E. coli* isolates from human samples, among strains encoding *qnr* and *gyr* genes in the present study, 78% were resistant to ciprofloxacin, 56% to enrofloxacin, and 45% to norfloxacin. In contrast to the present findings, Bouchakou et al. (2010) reported that among *E. coli* isolates carrying the *qnr* and *gyr* genes, resistance rates were 100% to ceftazidime, 71% to cefotaxime, 78% to ciprofloxacin, and 57% to nalidixic acid. In Mexico, the resistance rate for *qnr*-positive pediatric infections was 41.1% for nalidixic acid, 29.4% for ciprofloxacin, 82.3% for ceftazidim, and 100% for cefotaxim (Silva-Sanchez et al., 2013).

Based on the present results, the resistance rate for β-lactam antibiotics was 22% for ampicillin and cephalosporins. The current findings indicated a colistin resistance rate of 11%. However, Hassan et al. (2022) reported higher prevalence rates of *E. coli* isolates resistant to ampicillin (91%), cephalosporins (46-68%), nitrofurantoin (56%), gentamicin (26%), and amikacin (21%).

CONCLUSION

The present findings indicated a higher prevalence of resistance genes in humans than in calves, which was due to the smaller number of human samples included in this study. The presence of resistance genes, including *qnrS*, *gyrA*, *blaTEM*, and *mcr-1*, in humans and calves is of significant concern and requires further investigation. The current results indicated that antimicrobial resistance genes were identified through both PCR and antimicrobial susceptibility testing. Therefore, the high prevalence of resistance genes and phenotypic resistance, especially in human isolates, emphasized the urgent need for antimicrobial stewardship programs. The current findings highlighted the need to impose restrictions on the improper use of medications and antibiotics. Antibiotic use should be guided by antimicrobial susceptibility testing to reduce the spread of resistant strains and the horizontal transfer of resistance genes.

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DECLARATIONS

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Authors' contributions

Mohamed Saeed Diab, Yasser El-Naker, Nermin Awad, and Marwa Salah Eldin Diab contributed to sample collection. Fatma El Zahraa Gamal and Fatma Mohamed Gadallah have contributed to the processing and cultivation of the bacteria, as well as to the development of the PCR assay and the antibiotic sensitivity test. Selim Selim Salamawas was involved in molecular analysis, data analysis, drafting the article, and critically revising its intellectual content. Fatma Mohamed Gadallah participated in the study design and critically revised the manuscript. Selim Selim Salama and Fatma Mohamed Gadallah supervised and approved the final edition before submission. All authors read and approved the final edition of the manuscripts before publication in the present journal.

Availability of data and materials

All datasets used in the current study are available upon reasonable request from the corresponding author.

Competing interests

The authors declared no conflict of interest.

Ethical considerations

Ethical issues, including plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy, have been checked by all the authors. The authors confirmed that no AI tools were used in conducting and preparing the present study.

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Epidemiology of Ectoparasites and Gastrointestinal Parasites in Dogs Located in Tanzania: A Cross-Sectional Study

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ABSTRACT

Dogs, common companion animals, serve several cultural, social, and economic functions in society but can pose public health risks if not properly managed. The present study aimed to examine the epidemiology of canine parasites in the Mvomero district and the Morogoro municipality in Tanzania. A cross-sectional study was conducted in 2024 involving 400 randomly selected dogs, aged from 3 months to over 1 year, selected from households in the Mvomero district and the Morogoro municipality of Tanzania. The study population consisted predominantly of indigenous mongrels (n = 372), with a smaller proportion of crossbreeds (n = 28). Ectoparasites were collected through full-body examination, including the skin, ears, and interdigital spaces. Ticks, fleas, mites, and lice were manually removed using forceps and combing, with ticks carefully extracted to preserve their mouthparts. Fleas were collected using an acetone application followed by brushing onto white cloths. All samples were preserved in 70% ethanol and identified morphologically under a light microscope using standard identification keys. Fecal samples were collected from the study dogs and examined using flotation, sedimentation, direct saline/iodine wet mount, and modified Ziehl-Neelsen staining techniques. The overall prevalence of ectoparasites was 83.8%, while gastrointestinal parasites were detected in 76.8% of the dogs. Helminths included *Ancylostoma* spp. (60.5%), *Uncinaria stenocephala* (22.0%), *Toxocara canis* (11.5%), *Toxascaris leonina* (6.3%), *Ascaris* spp. (3.8%), *Taeniidae* (6.0%), and *Dipylidium caninum* (1.8%). Protozoa included *Cryptosporidium* spp. (15.5%), *Isospora* spp. (8.0%), *Cyclospora* spp. (4.3%), and *Entamoeba* spp. (3.0%). Dogs from Mvomero, Tanzania, had significantly higher parasite prevalence than those from Morogoro municipality, Tanzania. Risk factors for parasitic infection included dog age, geographic location, management practices, and lack of deworming. The current study highlighted an alarming burden of parasitic infestations among dogs in Morogoro, Tanzania, posing significant threats to animal health, public health, and animal welfare.

Keywords: Disease, Dog, Ectoparasite, Gastrointestinal parasite, Risk factor, Zoonoses

INTRODUCTION

Dogs were the first animals to be domesticated by humans and continue to play multiple roles in various cultures worldwide (Freedman et al., 2014). In many African countries, including Tanzania, dogs serve important functions such as providing security, assisting in hunting, herding livestock, and occasionally as pets (Swai et al., 2010; Whitfield and Smith, 2014). In Tanzania, the majority of dogs are local mongrels, predominantly reared in rural areas where access to veterinary services is limited (Swai et al., 2010). Pastoral and agro-pastoral communities, including the Maasai, Sukuma, Gogo, Barabaig, and Wambulu, commonly keep large numbers of dogs for security, hunting, and livestock herding purposes (Knobel et al., 2008). In urban areas, dogs are also commonly kept, but they are often mismanaged, allowed to stray, and pose significant public health risks (Swai et al., 2010). Stray dogs are frequently infested with ectoparasites and gastrointestinal parasites due to poor management and the absence of effective disease control programs (Swai et al., 2010). Globally, common ectoparasites of dogs include ticks, fleas, mites, and lice (Kumsa et al., 2011; Costa et al., 2013). Fleas, in particular, are notable vectors for *Yersinia pestis*, the causative agent of plague in humans (Gage et al., 2005). Additionally, gastrointestinal parasites such as *Ancylostomum* spp. and *Giardia* spp. are prevalent worldwide, with their prevalence and severity influenced by factors including age, geographical location, breed, and the nutritional and immune status of the host (Swai et al., 2016). Effective parasite control in dogs requires regular and appropriate use of anthelmintic and antiprotozoal medications, alongside sound husbandry practices (Swai et al., 2016).

Despite the well-established roles of dogs and the recognized health challenges affecting them worldwide, substantial gaps remain in the understanding of the epidemiology of parasitic infestations among dogs in Tanzania, particularly across rural and urban settings. Poor management practices observed in Tanzania can make dogs significant

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reservoirs and vectors for zoonotic diseases (Ernest et al., 2013). While global studies have identified risk factors for parasitic infestations in dogs, including age, sex, body condition, and husbandry practices, there is limited specific data on these aspects in Tanzania (Massei et al., 2017). The Morogoro Region, characterized by a high concentration of pastoralist and agro-pastoralist communities, supports a substantial population of stray dogs that pose a significant risk for disease transmission. Therefore, the present study aimed to determine the prevalence of parasitic infestations and associated risk factors among dogs in Mvomero District and Morogoro Municipality in Tanzania, to address existing knowledge gaps and inform effective control strategies for improved animal and public health.

MATERIALS AND METHODS

Ethics approval

A study permit was provided by the Ethics committee of Sokoine University of Agriculture (SUA/DRPSG/R/126/3/93) in Morogoro, Tanzania, and permission letters at the district level were obtained from the Executive Directors (MVDC/D.30/15/VOL.IV/54).

Study location

The study was conducted in the Mvomero District and the Morogoro Municipality to compare dog husbandry practices in rural and urban areas (Figure 1). Mvomero district, located at 06°26'S and 37°32'E, was chosen for its large population of pastoralists and agro-pastoralists who own many dogs; it has 30 wards, 115 villages, and a population of 312,109 (PHCT, 2013). Morogoro municipality, located on the lower slopes of the Uluguru Mountains between latitudes 5.7° and 10°S and longitudes 35.6° and 39.5°E, comprises 29 wards and 272 streets, with a population of 315,866 based on the 2012 census (PHCT, 2013). The area was selected to facilitate a comparative analysis of dog husbandry practices in both urban and rural settings.

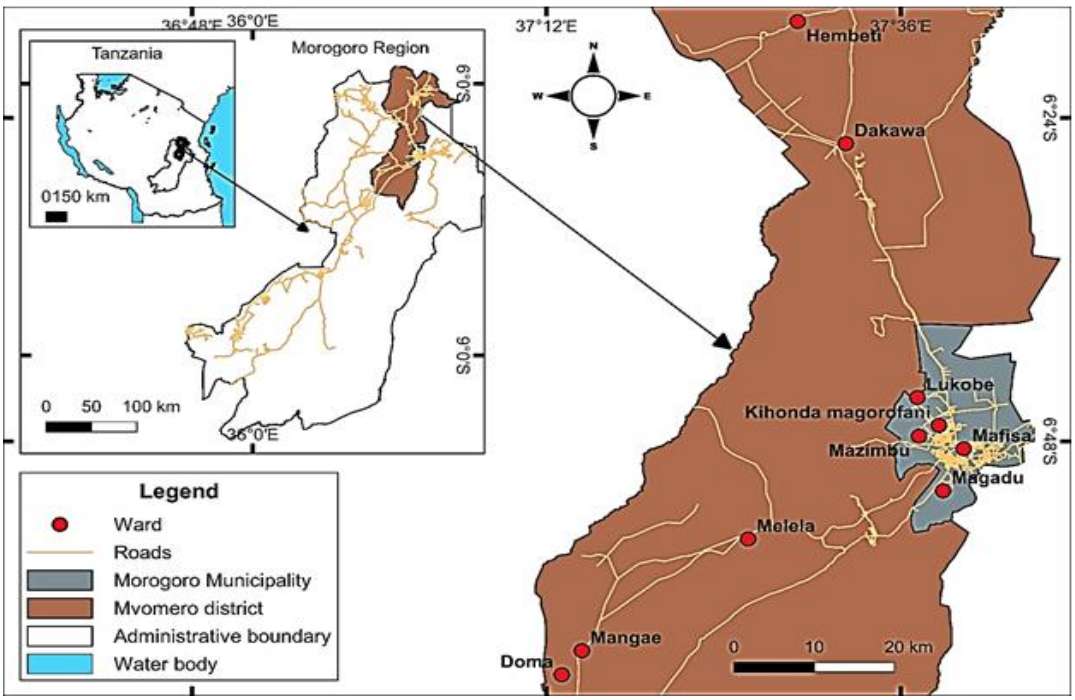


Figure 1. The Morogoro region's location, including Mvomero district and Morogoro municipality. A map was developed in QGIS version 3.29.1. Source: Data catalog for Building Disaster Resilience to Natural Hazards in Sub-Saharan Africa Regions websites (http://riskprofilesundrr.org/layers/geonode:tzwards)

Study design

A cross-sectional study was undertaken to assess the prevalence of ectoparasites, gastrointestinal parasites, and related risk factors in dogs in each district. The study included all households that kept dogs in Mvomero district and Morogoro municipality.

Sampling

Five wards from each district were purposely selected from the 15 wards in Mvomero district and the 29 wards in Morogoro municipality. The selected wards in Mvomero district were Dakawa, Mangae, Melela, Doma, and Hembeti,

whereas those in Morogoro municipality were Magadu, Lukobe, Kihonda Magorofani, Mafisa, and Mazimbu. The study covered 12 villages and 100 households in Mvomero District, as well as 27 streets and 100 households in Morogoro municipality. Only dogs aged three months and above were included in the examination. In households with one to five dogs, all eligible dogs were examined, whereas in households with more than five dogs, three to five dogs were randomly selected for inclusion.

Clinical examination

Before examination and sampling, information on age, sex, breed, management system, history of ectoparasite control, and overall body condition was collected and recorded. Dogs aged three months to one year were classified as young, and those over one year as adults, with age determined by owners' records, dentition, and body size (Lawrence et al., 2015). Dogs were categorized as having poor body condition if they were emaciated, had rough coats and showed general weakness, or as having a healthy condition if they were well-muscled, had smooth coats, and appeared alert, and healthy (Lawrence et al., 2015). Rectal temperature, heart rate, and respiratory rate were also measured by qualified veterinarians and animal health experts to assess the general health status of the dogs during examination.

Skin examination

Restrained dogs were carefully inspected for skin lesions and ectoparasites. Following examination, each dog was positioned in lateral recumbency on a clean white cloth, which facilitated the detection and collection of ectoparasites falling from the animal during brushing the fur. A thorough physical examination was conducted from head to tail, and ectoparasites found on the skin, inside the ears, and between the toes were manually collected. Ticks were removed using thumb forceps to ensure the mouthparts were retained for identification (Taylor et al., 2016; Heylen et al., 2021). To collect fleas, absolute acetone (Sigma-Aldrich Corporation, USA) was applied to the skin, and the fur was brushed to dislodge the fleas onto a white cloth (Nguyen et al., 2020). Ectoparasites were preserved in bottles containing 70% ethanol (Sigma-Aldrich Corporation, USA) labeled according to body region, and transported to Sokoine University of Agriculture for analysis. Skin scrapings from dogs suspected of mange were collected and preserved in glass tubes for laboratory examination.

Fecal sampling

Fecal samples (approximately 5-10 g per dog) were collected from the rectum of restrained dogs using a lubricated gloved finger. The samples were transported to the laboratory in a cool box with ice packs for analysis.

Laboratory identification

Ticks, fleas, and mites were identified microscopically after preparing preserved specimens in 70% ethanol using a light microscope (Carl Zeiss, Germany). Ticks were placed directly on clean slides, fleas were mounted under coverslips, and mites were cleared with lactophenol before mounting. The microscope was set to low magnification (4× to 10×) for ticks and fleas, and higher magnifications (40×) were used for mites to observe detailed structures. Key morphological features such as the capitulum and scutum in ticks, the laterally compressed body and combs in fleas, body shape, and setae in mites were examined. Observations were compared with standard identification keys to confirm genera or species (Foreyt, 2013; Krishna Murthy et al., 2017), and findings were recorded.

Qualitative analysis of gastrointestinal parasite

Each fecal sample was initially examined in a Petri dish for the presence of adult worms, larvae, and tapeworm segments. Subsequently, four qualitative techniques were used to detect gastrointestinal parasites; simple test tube flotation, sedimentation, direct saline and iodine wet mount, and modified Ziehl-Neelsen staining (Garcia et al., 2016; Henriksen et al., 2018).

Data analysis

Data were entered, stored, and analyzed using Epi-Info version 7.2.6.0 (CDC, USA). Descriptive statistics were summarized in tables. The prevalence (p) of each parasite in the dog population was calculated as $p = d/n$, where ‘d’ represents the number of dogs diagnosed with a specific parasite at the time of examination and ‘n’ is the total number of dogs examined. Associations between parasitism and categorical variables, including host characteristics and husbandry practices, were assessed using chi-square tests performed in Epi Info version 7.2.6.0 (CDC, USA). The level of statistical significance was set at 5% ($p < 0.05$).

RESULTS

Dog demographic and clinical characteristics

The results indicated that the majority of dogs were male mongrels aged between three months and one year. A significant proportion (64.3%) of the dogs were in healthy condition and had a history of external parasite control, while 30.3% exhibited skin lesions, predominantly dermatitis (Table 1).

Types of ectoparasites

The results showed that 83.8% of the examined dogs were infested with ectoparasites. Specifically, 62.5% had ticks, 64.5% had fleas, 3% had mites, and 2.5% had lice. Ticks were predominantly located on the head and neck, while fleas were most commonly found on the abdomen. A total of 250 ticks, 278 fleas, 11 lice, and 20 skin scrapings for mites were collected during the study (Supplementary Table 1).

Prevalence of ectoparasites

The overall prevalence of ectoparasites was 83.8%. Dogs from the Mvomero district showed significantly higher ectoparasite infestation rates compared to those from Morogoro municipality ($p < 0.05$), with an average of two types of ectoparasites per host observed in both areas (Table 2). Five ectoparasite species were identified, namely *Rhipicephalus sanguineus* (brown dog tick), *Ctenocephalides canis* (dog flea), *Ctenocephalides felis* (cat flea), *Sarcoptes scabiei* (mange mite), *Trichodectes canis* (biting louse), and *Pulex irritans*. Mvomero District dogs had a significantly higher tick infestation rate (74.5%) compared to Morogoro municipality dogs (50.5%; $p < 0.05$). The identified ectoparasites are shown in Figure 2.

Magnitude of ectoparasite based on risk factors

The analysis of risk factors for ectoparasite infestation in dogs revealed higher rates in Mvomero district (88.0%) than in Morogoro municipality (79.5%; $p < 0.05$). In addition, dogs with poor body condition (76.5%), those kept under free-ranging management systems (90%), and dogs with skin lesions showed significantly higher rates of ectoparasite infestation than healthier dogs (Table 3; $p < 0.05$).

Table 1. Clinical characteristics of sampled dogs from Mvomero District and Morogoro municipality, Tanzania

Parameter	Category	Number and percentage of dogs examined in the two districts		Total of dogs examined (%)
		Mvomero district (n = 200)	Morogoro municipality (n = 200)	
Sex	Male	106 (53.0)	108 (54.0)	214 (53.5)
	Female	94 (47.0)	92 (46.0)	186 (46.5)
Age (years)	Young (< 1)	139 (69.5)	120 (60.0)	259 (64.8)
	Adult (> 1)	61 (30.5)	80 (36.0)	141 (35.2)
Breed	Mongrels	198 (99.0)	174 (87.0)	372 (93.0)
	Crosses	2 (1.0)	26 (13.0)	28 (7.0)
Body condition	Good	118 (59.0)	139 (69.5)	257 (64.3)
	Poor	82 (41.0)	61 (32.0)	143 (35.7)
Management system	Free range	122 (61.0)	46 (23.0)	168 (42.0)
	Confined	58 (29.0)	154 (77.0)	212 (58.0)
History of ectoparasite control	Yes	86 (43.0)	140 (70.0)	226 (56.5)
	No	114 (57.0)	60 (30.0)	174 (43.5)
Temperature	Normal (38.5℃)	193 (96.5)	193 (95.5)	386 (96.5)
	Below normal	0 (0.0)	4 (2.0)	4 (1.0)
	Above normal	7 (3.5)	3 (1.5)	10 (2.5)
Presence of lesions on the skin	Yes	67 (33.5)	54 (27.0)	121 (30.3)
	No	133 (66.5)	146 (73.0)	279 (69.7)
Types of lesions on the skin	Alopecia	24 (12.0)	11 (5.5)	35 (8.8)
	Dermatitis	37 (18.5)	41(20.5)	78 (19.5)
	Pruritis	10 (5.0)	6 (3.0)	16 (4.0)

n: Number of dogs. Numbers in parentheses are expressed as a percentage.

Table 2. Number of ectoparasite species per host and prevalence of parasites in Mvomero District and Morogoro Municipality, Tanzania

Parameter	Category	Number and percentage of infested dogs		χ^2 test	P value
		Mvomero district (n = 200)	Morogoro municipality (n = 200)		
Ectoparasite infestation status	No parasite	24 (12.0)[a]	41 (20.5)[b]	10.906	0.012
	Single parasite species	73 (36.5)	76 (38.0)		
	Two parasites' species	95 (47.5)	82 (41.0)		
	Three parasites' species	8 (4.0)	1 (0.5)		
General ectoparasite infestation rates	Ticks	149 (74.5)[a]	101 (50.5)[b]	24.576	0.001
	Fleas	123 (61.5)	135 (67.5)	1.572	0.210
	Mites	7 (3.5)	5 (2.5)	0.344	0.558
	Lice	8 (4.0)	2 (1.0)	3.692	0.055
	Ectoparasites prevalence	176 (88.0)[a]	159 (79.5)[b]	5.309	0.021
Ectoparasite infestation according to species	*Rhipicephalus sanguineus*	149 (74.5)[a]	101 (50.5)[b]	24.576	0.001
	Ctenocephalides felis	95 (47.5)	115 (57.5)	4.010	0.045
	Ctenocephalides canis	30 (15.0)	27 (13.5)	0.184	0.668
	Pulex irritans	3 (1.5)	0 (0.0)	3.023	0.082
	Sarcoptes scabiei	7 (3.5)	5 (2.5)	0.344	0.558

n: Number of dogs. Numbers in parentheses are expressed as a percentage. [a,b] Mean different superscript letters indicate a significant difference in a row at $p < 0.05$.

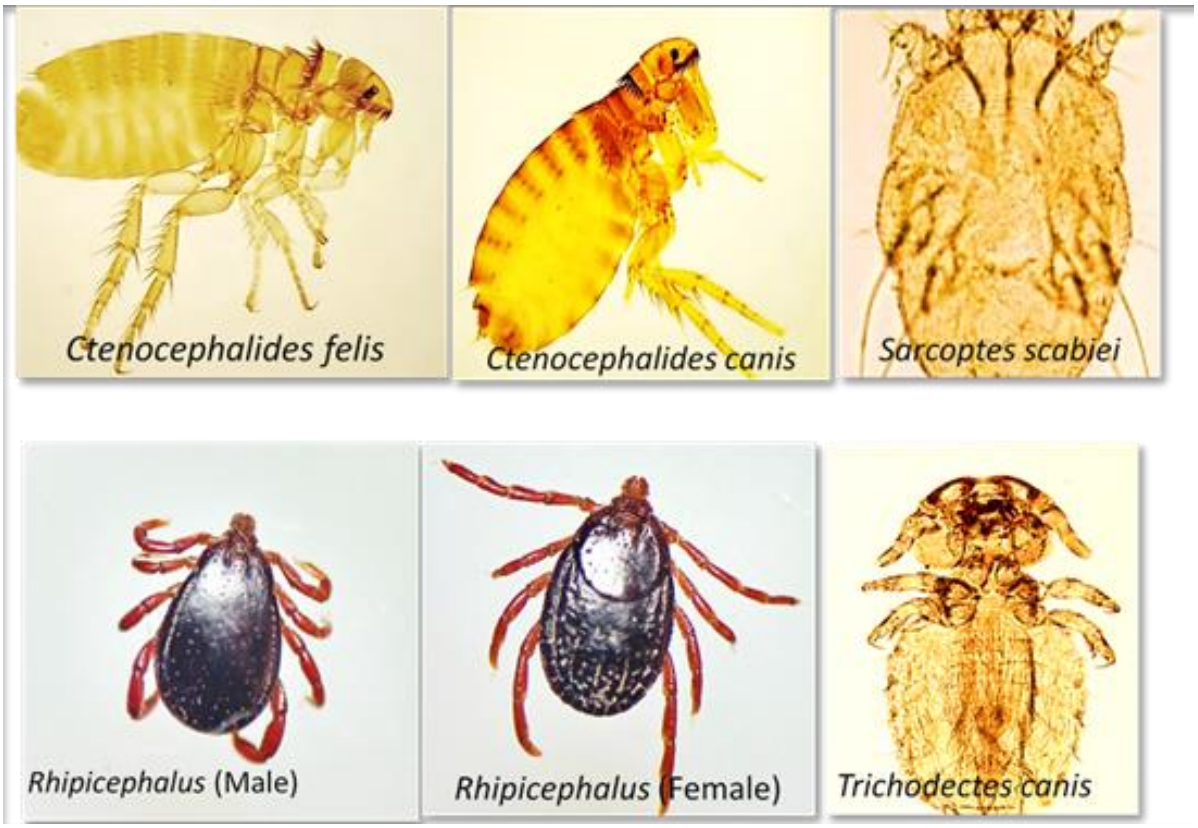


Figure 2. The ectoparasites collected from domestic dogs in Mvomero district and Morogoro Municipality, Tanzania

Ectoparasite infestations based on age, sex, and body conditions

Ectoparasite infestation in dogs varied between Mvomero district and Morogoro municipality, with Mvomero mostly demonstrating higher prevalence rates (Supplementary Table 2). Ticks (*R. sanguineus*) were most common in Mvomero, affecting up to 76.3% of young dogs and 84.0% of those in poor body condition, while fleas (*C. felis*) were more prevalent in Morogoro, especially among young dogs (59.0%) and those in poor condition (74.6%). Mites (*S. scabiae)* and lice (*T. canis*) had lower overall prevalence but were slightly more common in dogs with poor body condition, particularly in Mvomero. Risk factors influencing infestation included young age, poor body condition, and to a lesser extent, sex, with females experiencing slightly higher flea infestations (Supplementary Table 2).

Magnitude and types of gastrointestinal parasites

The study revealed that 76.8% of the 400 dogs examined were infested with gastrointestinal parasites, comprising seven genera of helminths and four genera of protozoa (Table 4; Figure 3).

Table 3. Prevalence of ectoparasite infestations based on animal and management risk factors in Mvomero District and Morogoro Municipality, Tanzania

Risk factor	Category	Number (%) of dogs with ectoparasites	Number (%) of dogs without ectoparasites	χ^2 test	P-value
Sex	Male	174 (81.3)	40 (18.7)	2.016	0.156
	Female	161 (86.7)	25 (13.4)		
Age	Young (< 1)	217 (82.8)	45 (17.2)	0.478	0.489
	Adult (> 1)	118 (85.5)	20 (14.5)		
Breed	Mongrels	312 (83.4)	62 (16.6)	0.454	0.501
	Crosses	23 (88.5)	3 (11.5)		
Location of origin	Mvomero district	176 (88.0)[a]	24 (12.0)[b]	5.309	0.021
	Morogoro municipality	159 (79.5)[a]	41(20.5)[b]		
Body condition	Good	195 (76.5)[a]	60 (23.5)[b]	27.390	0.0001
	Poor	140 (96.6)[a]	5 (3.5)[b]		
Management system	Free range	208 (90.0)[a]	23 (10.0)[b]	12.388	0.0001
	Confined	127 (75.1)[a]	42 (24.9)[b]		
History of ectoparasite control	Yes	87 (79.1)	23 (20.9)	2.420	0.120
	No	248 (85.5)	42 (14.5)		
Access to veterinary services	Yes	222 (83.8)	43 (16.2)	0.000	0.986
	No	113 (83.7)	22 (16.3)		
Skin lesions	Yes	140 (100.0)[a]	0 (0.0)[b]	41.791	0.0001
	No	195 (75.0)[a]	65 (25.0)[b]		

[a,b] Mean different superscript letters indicate a significant difference in a row at $p < 0.05$. The total number of dogs was 400. Numbers in parentheses are expressed as a percentage.

Table 4. Gastrointestinal parasites prevalence in dogs of Mvomero District and Morogoro municipality, Tanzania

Parameter	Category	Number and percentage of dogs infested		χ^2 test	P-value
		Mvomero district	Morogoro municipality		
Gastrointestinal infestation status	No parasite genera	27 (13.5)[a]	66 (33.0)[b]	33.348	0.0001
	Single parasite genus	58 (29.0)[a]	68 (34.0)[b]		
	Two parasites' genera	80 (40.0)[a]	40 (20.0)[b]		
	Three parasites' genera	30 (15.0)[a]	24 (12.0)[b]		
	Four parasites' genera	3 (1.5)[a]	2 (1.0)[b]		
	Five parasites' genera	2 (1.0)	0 (0.0)		
Types of gastrointestinal parasites	Helminthes	159 (79.5)[a]	120 (60.0)[b]	18.022	0.0001
	Protozoa	55 (27.5)[a]	35 (17.5)[b]		
Gastrointestinal spp. prevalence	*Ancylostoma caninum*	142 (71.0)[a]	100 (50.0)[b]	18.454	0.0001
	Uncinaria stenocephala	46 (23.0)	42 (21.0)	0.233	0.629
	Toxocara canis	29 (14.5)	17 (8.5)	3.537	0.060
	Toxascaris leonina	15 (7.5)	10 (5.0)	1.067	0.302
	Ascaris spp.	6 (3.0)	9 (4.5)	0.623	0.430
	Dipylidium caninum	6 (3.0)	1 (0.5)	3.635	0.057
	Taeniidae spp.	14 (7.0)	10 (5.0)	0.709	0.400
	Diphylobothrium latum	3 (1.5)	0 (0.0)	3.023	0.082
	Troglotrema salmincola	1 (0.5)	0 (0.0)	1.003	0.317
	Cryptosporidium spp.	36 (18.0)	26 (13.0)	1.909	0.167
	Cyclospora spp.	11 (5.5)	6 (3.0)	1.536	0.215
	Isospora spp.	25 (12.5)[a]	7 (3.5)[b]	11.005	0.001
	Entamoeba spp.	9(4.5)	3 (1.5)	3.093	0.079

[a,b] Mean different superscript letters indicate a significant difference in a row at $p < 0.05$. The total number of dogs was 400, 200 dogs in each district. Numbers in parentheses are expressed as a percentage.

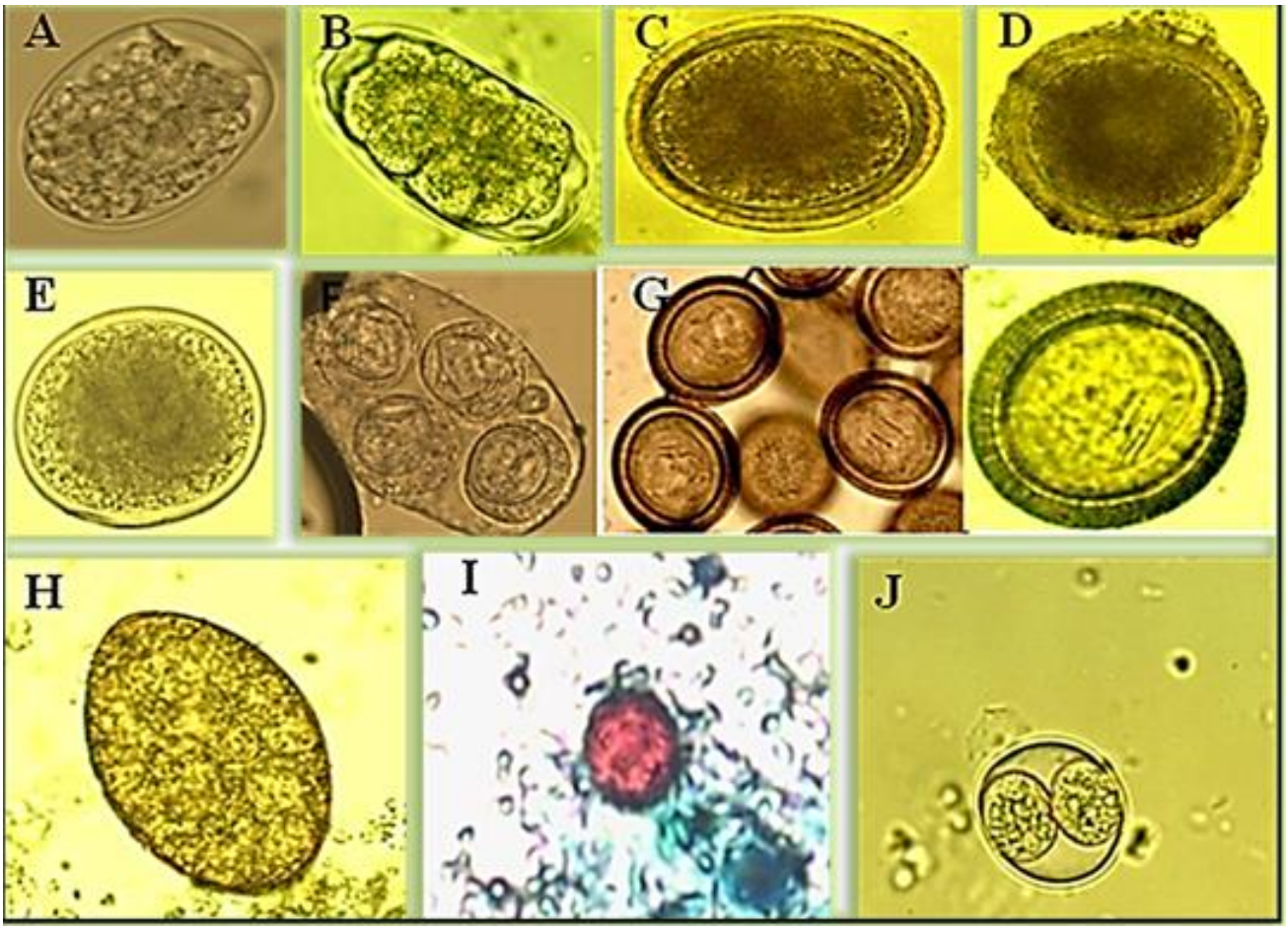


Figure 3. Eggs/oocysts of intestinal parasites derived from dog fecal samples in Mvomero District and Morogoro Municipality, Tanzania, in 2024. **A**: *Ancylostoma caninum* egg, **B**: *Uncinaria stenocephala* egg, **C**: *Toxocara canis* egg, **D**: *Ascaris* egg, **E**: *Toxascaris leonina* egg, **F**: *Dipylidium caninum* packet of eggs, **G**: *Taeniidae* spp. eggs, **H**: *Diphyllobothrium latum* egg, **I**: *Cryptosporidium* cyst, **J**: *Isospora* cyst.

Table 5. Intestinal parasites and associated risk factors for infestations in dogs in the districts of Mvomero and Morogoro, Tanzania

Risk factor	Category	Number and percentage of dogs with GIT parasites	Number and percentage of dogs without GIT parasites	χ^2 test	P-value
Sex	Male	165 (77.1)	49 (22.9)	1.641	0.200
	Female	133 (71.5)	53 (28.5)		
Age	Young (< 1)	206 (78.6)[a]	56 (21.4)[b]	6.805	0.009
	Adult (> 1)	92 (67.7)[a]	46 (33.3)[b]		
Breed	Mongrels	282 (75.4)	92 (24.6)	2.459	0.117
	Crosses	16 (61.5)	10 (38.5)		
Location of origin	Mvomero district	173 (86.5)[a]	27 (13.5)[b]	21.055	0.001
	Morogoro municipality	134 (67.0)[a]	66 (33.0)[b]		
Body condition	Good	178 (69.8)[a]	77 (30.2)[b]	8.166	0.004
	Poor	120 (82.8)[a]	25 (17.2)[b]		
Management system	Free range	184 (79.7)[a]	47 (20.3)[b]	7.644	0.006
	Confined	114 (67.5)[a]	55 (32.5)[b]		
History of deworming	Yes	44 (44.9)[a]	54 (55.1)[b]	59.872	0.001
	No	254 (84.1)[a]	48 (15.9)[b]		
Access to veterinary services	Yes	202 (76.2)	63 (23.8)	1.232	0.267
	No	39 (28.9)	96 (71.1)		
Dog feeding	Yes	288 (74.0)	101 (26.0)	1.603	0.205
	No	10 (90.0)	1 (9.0)		
Feeding system	In a container	123 (63.7)[a]	70 (36.3)[b]	22.769	0.0001
	Throw on ground	175 (84.5)[a]	32 (15.5)[b]		

GIT: Gastrointestinal parasites. [a,b] Mean different superscript letters indicate a significant difference in a row at $p < 0.05$. The total number of dogs was 400. Numbers in parentheses are expressed as a percentage.

Gastrointestinal parasites and risk factors

Age, body condition, geographic location, husbandry and housing practices, lack of routine deworming, and feeding practices were significant risk factors associated with gastrointestinal parasite infestations in dogs (Table 5; $p < 0.05$).

Helminths, protozoan species, and risk factors

Gastrointestinal (GIT) parasite infections in dogs showed significant variation depending on age, sex, and body condition in both Mvomero district and Morogoro municipality, with *Ancylostoma caninum* being the most commonly detected parasite across the two areas (Supplementary Tables 3a and b). In Mvomero, *Ancylostoma caninum* affected 58.2% of young dogs, 60.7% of adults, and more males (66.1%) than females (51.1%), with higher prevalence in dogs with poor body condition (61.7%). Similarly, in Morogoro, it affected 53.3% of young dogs, 44.9% of adults, and more males (57.4%) than females (41.3%), with 54.0% prevalence in dogs with poor body condition. *Toxocara canis* was more common in young dogs (18.7%) and females (17.6%) in Mvomero, while in Morogoro, it was slightly more prevalent in females (10.9%) and dogs with poor body condition (12.7%). *Uncinaria stenocephala* and *Toxascaris leonina* showed moderate prevalence, particularly in adults and dogs in poor condition, in both areas. Among protozoa, *Cryptosporidium* spp. was common, affecting young dogs and those with healthy body condition, with a prevalence of 21.5% in Mvomero and 20.5% in Morogoro. Less prevalent parasites, including *Cyclospora* spp. and *Isospora* spp., showed variations based on age, sex, and body condition, highlighting young dogs as a high-risk group. Overall, the findings emphasize *A. caninum* as the dominant parasite and underline the effects of age, sex, and body condition on infestation rates.

Mixed infection based on age, sex, and body conditions

The findings indicated that *Ancylostoma caninum* and *Uncinaria stenocephala* were the most common mixed parasites in dogs, with a higher prevalence observed in adult dogs compared to young ones (Supplementary Table 4).

Relationship between the prevalence of parasites and risk factors

The study identified a significant association between the prevalence of certain parasites and specific risk factors, showing that *Toxocara canis*, *Cryptosporidium*, and *Isospora* spp. affected young dogs significantly more than adult dogs (Supplementary Table 5; $p < 0.05$). Poor body condition and origin from Mvomero District were significant predictors of parasitic infestation in dogs when compared to those originating from Morogoro municipality (Table 5; $p < 0.05$).

Magnitude of zoonotic gastrointestinal parasites

The study identified 11 genera of zoonotic gastrointestinal parasites in dog fecal samples, with *Ancylostoma, Uncinaria* and *Toxocara* being the most common helminths and *Cryptosporidium* the most common protozoan parasite. Overall, a high prevalence of zoonotic gastrointestinal parasites was recorded in dogs of Mvomero District (Table 6).

Table 6. Prevalence of zoonotic gastrointestinal parasites identified in fecal samples of dogs from Mvomero and Morogoro districts, Tanzania

Type of parasites	Species of zoonotic parasites	Mvomero district (n=200)	Morogoro municipality (n=200)
		Prevalence	Prevalence
Helminthes	*Ancylostoma caninum*	142 (71.0)	100 (50.0)
	Uncinaria stenocephala	46 (23.0)	42 (21.0)
	Toxocara canis	29 (14.5)	17 (8.5)
	Ascaris lumbricodes	6 (3.0)	9 (4.5)
	Dipylidium caninum	6 (3.0)	1 (0.5)
	Taeniidae spp.	14 (7.0)	10 (5.0)
	Diphyllobothrium latum	3 (1.5)	0 (0.0)
	Troglotrema salmincola	1 (0.5)	0 (0.0)
Protozoa	*Cryptosporidium* spp.	36 (18.0)	26 (13.0)
	Cyclospora spp.	11 (5.5)	6 (3.0)
	Entamoeba spp.	9 (4.5)	3 (1.5)
	Overall prevalence of zoonotic parasites	169 (84.5)	126 (63.0)

n: Number of dogs. Numbers in parentheses are expressed as a percentage.

DISCUSSION

The present study examined the occurrence of parasitic infestations and zoonotic gastrointestinal parasites among the examined dogs in Mvomero District and Morogoro municipality. The findings revealed a substantial prevalence of ectoparasites in dogs (83.8%), including ticks, fleas, mites, and lice, with higher infestation rates observed in dogs from Mvomero District (88.0%) compared to those from Morogoro municipality (79.5%). These findings underscore challenges in dog management practices and the limited implementation of routine parasite control measures. A previous investigation conducted in Morogoro municipality by Mwangengwa and Rhanga (2023) reported an ectoparasite prevalence of 71.7% among dogs. Similarly, a study by Kumsa and Mekonnen (2011) in Hawassa, southern Ethiopia, reported an even higher prevalence of ectoparasite infestations in dogs, reaching 99.5%. Comparable findings were also reported in Nigeria, where Ugbomoiko et al. (2008) documented an ectoparasite prevalence of 60.4% among dogs, while in Maranhão State, Brazil, Costa et al. (2013) recorded prevalence rates of 73.9% in rural dogs and 51.3% in urban dogs, indicating that canine ectoparasitism is a widespread global concern. Variations in prevalence across studies may be attributed to differences in climatic conditions, environmental parasite dynamics, dog husbandry practices, and variations in sampling periods.

Rhipicephalus sanguineus, commonly referred to as the brown dog tick or kennel tick. It is the most prevalent ixodid tick species infesting dogs globally (Klimpel et al., 2010). In the present study, dogs from Mvomero District exhibited a higher prevalence of tick infestation (74.5%), predominantly *R. sanguineus*, compared to those from Morogoro municipality (50.5%), indicating suboptimal husbandry practices.

In Nigeria, *R. sanguineus* prevalence in dogs has been shown to range widely from 0.3% to 80% (Oguntomole et al., 2018). Similarly, studies from Brazil have documented diverse infestation levels depending on ecological context and host exposure. In Fortaleza and Belo Horizonte, urban dog populations showed infestation rates ranging from approximately 29.0% to 38.5%, influenced by serological grouping and exposure status rather than universal infestation (Paz et al., 2010). However, higher prevalence has also been reported in specific settings, including up to 100% infestation in urban stray dogs in Fortaleza (Klimpel et al., 2010). In Indonesia, particularly in Bogor, Jakarta, and Bandung, exceptionally high infestation levels have been observed, with reports reaching 67.9% in kennel and police dogs in Bogor and up to 100% in controlled kennel populations such as Atang Sendjaja Air Force dogs (Kesumawati et al., 2015). Beyond their veterinary importance, *Rhipicephalus* spp. have also been reported to occasionally parasitize humans (Oguntomole et al., 2018) and are recognized as potential vectors of zoonotic rickettsial diseases (Khrouf et al., 2014; Haule et al., 2023). The study found that 64.5% of examined dogs were infested with fleas, which can be attributed to inadequate husbandry practices such as irregular cleaning, disinfection, and external parasite control. Flea infestations cause symptoms such as pruritus, self-inflicted trauma, allergic dermatitis, and general discomfort, often observed in dogs with skin lesions and poor body condition (Mwangengwa and Rhanga, 2023). Flea infestations in dogs in Morogoro pose a significant public health concern due to their high population and potential for pathogen transmission. *Ctenocephalides* spp. are known vectors for *Yersinia pestis*, the causative agent of human plague (Mwangengwa and Rhanga, 2023), and serve as intermediate hosts for the zoonotic tapeworm *Dipylidium caninum* (Beugnet et al., 2014). High prevalence rates of *Ctenocephalides* spp. infestations in dogs have been reported in several African countries, including northeastern Tanzania, where Haule et al. (2023) documented a prevalence of 90.0%, and Ethiopia, where Kumsa and Mekonnen (2011) reported a prevalence of 82.8%. These infestations are influenced by management practices, seasonal variations, and the geographic distribution of parasites. Besides flea infestations, the study also identified *Sarcoptes scabiei* (3.0%) and *Trichodectes canis* (2.5%) among the ectoparasites affecting dogs. *Sarcoptes scabiei* is of particular public and veterinary health importance, as it can cause sarcoptic mange in both dogs and humans (Bandi et al., 2013). The results underscore the significance of improving dog management practices and routine parasite control measures to safeguard both canine and human health in Mvomero and Morogoro districts.

The overall high prevalence (76.8%) of gastrointestinal parasites (helminths and protozoa) in dogs of Mvomero and Morogoro districts indicates chronic health challenges in these animals. Previous studies conducted on dogs in Tanzania have reported varying prevalence rates of gastrointestinal parasites, including 50.5% and 74.0% in Ethiopia (Abere et al., 2013) and 59.3% in Arusha (Swai et al., 2010). Similar studies conducted in Nigeria (Amadi et al. 2021), Ghana (Johnson et al. 2015), and Egypt (Awadallah and Salem, 2015) have also demonstrated varying levels of gastrointestinal parasite infections among dogs. Factors such as environmental conditions, host characteristics, and husbandry practices play crucial roles in determining parasite prevalence. These findings highlight the need for comprehensive parasite control measures to protect both animal and public health in Mvomero and Morogoro districts.

The present study revealed that hookworm eggs (*Ancylostoma* spp.) are the most frequently detected parasites in both study areas, indicating their widespread occurrence. Similar findings have been reported in previous studies involving dogs from Tanzania and other African countries. In Arusha municipality, northern Tanzania, Swai et al. (2010)

reported *Ancylostoma* spp. in 59.3% of non-descript local dogs. In Morogoro, Abere et al. (2013) documented prevalence rates of 50.5% and 74.0% among domestic dogs from rural and peri-urban settings. In Lusaka, Zambia, Bwalya et al. (2011) reported hookworm infestations in 63.2% of mixed-breed owned and stray dogs, while in Egypt, Awadallah and Salem (2015) recorded *Ancylostoma* spp. infections in 44.7% of household dogs. Variations in prevalence across studies are likely associated with differences in environmental conditions, seasonal factors, dog systems, anthelmintic use, and sanitation practices. Collectively, the findings of the present study emphasize management of hookworms as the predominant intestinal helminths affecting dogs in Morogoro and comparable regions.

Other helminths identified in the present study include *stenocephala*, *Toxocara canis*, *Toxascaris leonina*, *Ascaris* spp., and *Dipylidium caninum Uncinaria*, consistent with previous reports from Tanzania and other regions. For instance, in Mvomero District, Tanzania, Swai et al. (2016) documented *Toxocara canis* in 18.7% and *Dipylidium caninum* in 9.3% of domestic dogs. In Zambia, Siwila (2016) reported *Toxocara canis* in 14.8% and *Dipylidium caninum* in 6.5% of examined dogs. Similarly, in Ghana, Amissah et al. (2016) identified multiple zoonotic gastrointestinal helminths, including *Toxocara* spp. in 22.4% and *Dipylidium caninum* in 11.6% of dogs. The study by Sowemimo and Asaolu (2009) in Ibadan, Nigeria, also reported the prevalence of various helminths in stray dogs as following, *Toxocara canis* (9.0%), *Ancylostoma* spp. (17.9%), *Toxascaris leonina* (0.6%), *Uncinaria stenocephala* (0.4%), and *Dipylidium caninum* (0.2%). The findings of the present study confirm that canine gastrointestinal helminths remain widely distributed across diverse ecological and management systems.

The current study provides the first documented evidence of the presence of two fish-associated helminths, *Diphyllobothrium latum* (1.5%) and *Troglotrema salmincola* (0.5%), in dogs in Tanzania. Their occurrence is most likely linked to the widespread practice of feeding dogs raw or undercooked fish and fish by-products, facilitating the transmission of fish-borne zoonotic helminths. Most of the sampled dogs were fed a homemade diet of maize bran and sardines, which may explain *Troglotrema salmincola* infection. These parasites (*Diphyllobothrium latum* and *Troglotrema salmincola*) were primarily found in dogs from Mvomero District, where dogs often scavenge for food in ponds and rivers, potentially consuming infested fish. Further studies are recommended to explore this phenomenon. The presence of *Taeniidae* eggs (6.0%) in dog fecal samples is of epidemiological importance and may be attributed to free-roaming dogs scavenging condemned raw meat or animal carcasses, even in cases where they are primarily fed homemade diets. Studies in Tanzania have reported high infestation rates (73.2%) of *Taeniidae* spp. in stray dogs (Swai et al., 2016), suggesting that dogs may act as reservoirs for human infections. The present study documented four protozoan parasites in dog feces, including *Cryptosporidium* spp., *Isospora* spp., *Cyclospora* spp., and *Entamoeba* spp. Similar findings have been reported in previous investigations involving domestic dogs from specific geographical locations. For instance, Perera et al. (2013) identified intestinal protozoan infections, including *Cryptosporidium* spp. and *Isospora* spp., among owned and free-roaming domestic dogs in Colombo and the surrounding urban areas of Sri Lanka. Likewise, Sarmiento-Rubiano et al. (2024) reported enteric protozoan parasites, including *Cryptosporidium* spp. and *Entamoeba* spp., in household and shelter dogs of mixed breeds in Colombia. Dogs in Mvomero District exhibited a higher prevalence (27.5%) compared to those in Morogoro municipality (17.5%), likely due to free-ranging management practices that increase exposure to a contaminated environment.

Previous studies on the epidemiology of canine parasites have identified geographic location, seasonality, demographics, and husbandry practices as key risk factors (Nijsse et al., 2015; Kidima et al., 2019). The present study observed a higher susceptibility of young dogs, likely due to immature immune systems, transplacental and trans-mammary transmission, and continuous exposure to contaminated environments (Abere et al., 2013; Swai et al., 2016). Furthermore, zoonotic intestinal parasites were highly prevalent in dogs from Mvomero District (84.5%) and Morogoro municipality (63%), posing a significant health risk. The identified zoonotic helminths in the present study included *Ancylostoma caninum*, *Uncinaria stenocephala*, *Toxocara canis*, *Dipylidium caninum*, and *Ascaris* spp., along with protozoa such as *Cryptosporidium* spp., *Cyclospora* spp., and *Entamoeba* spp. These findings are consistent with previous reports from Tanzania and other regions. Given the high levels of infection and close human-dog interactions, there is a substantial risk of zoonotic transmission. Therefore, raising public awareness is essential to prevent the spread of parasitic diseases from dogs to humans.

CONCLUSION

The study concludes that ectoparasites and gastrointestinal parasites are highly prevalent, especially in Mvomero District, with considerable zoonotic parasite occurrence in dogs across Mvomero and Morogoro, indicating a need for targeted control interventions. Importantly, fish-borne helminths (*Diphyllobothrium latum* and *Troglotrema salmincola*) were detected in Tanzanian dogs for the first time. Infection risk is influenced by age, location, husbandry practices, housing conditions, irregular deworming, and feeding behavior. Strengthening owner education on responsible dog

management, including vaccination, biosecurity, and routine deworming, is essential. Future studies should use longitudinal, seasonal, molecular, and One Health approaches to clarify transmission dynamics and zoonotic risk.

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Authors' contributions

Amina Ramadhani Issae contributed to conceptualization, data curation, formal analysis, investigation, methodology, validation, and preparation of the first draft of the manuscript, as well as reviewing and editing the manuscript. Hezron Emanuel Nonga contributed to conceptualization, funding acquisition, investigation, methodology, project administration, supervision, validation, and review and editing of the manuscript. All authors have read and approved the final edition of the manuscript before publication in the present journal.

Availability of the data and materials

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Competing interests

The authors confirmed that they have no conflicts of interest in the present study.

Ethical considerations

The present study is original and has not been submitted to or published in any other journal. The authors have verified the originality of the content using plagiarism-detection tools and confirmed that the article is based on their own original scientific research findings. Additionally, artificial intelligence tools were not used in the preparation and writing of this study.

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Supplementary Table 1. Ectoparasites collected from different body parts of dogs in Morogoro municipality and Mvomero district, Tanzania

Region of the body	Number and percentage of ectoparasites in Mvomero district				Number and percentage of ectoparasites in Morogoro municipality			
	Ticks	Fleas	Mites	Lice	Ticks	Fleas	Mites	Lice
Head & Neck	98 (67.6%)	5 (3.7%)	0 (0.0)	0 (0.0)	85 (81.0%)	0 (0.0)	0 (0.0)	0 (0.0)
Abdomen	19 (13.1%)	106 (77.4%)	2 (22.2%)	8 (100.0)	3 (2.9%)	113 (80.1%)	0 (0.0)	2 (66.7%)
Back	12 (8.3%)	26 (19.0%)	7 (77.8%)	0 (0.0)	11 (10.5%)	28 (19.9%)	11 (10.5%)	1 (33.3%)
Legs	7 (4.8%)	0 (0.0)	0 (0.0%)	0 (0.0)	5 (4.8%)	0 (0.0)	0 (0.0)	0 (0.0)
Around the genital area	9 (6.2%)	0 (0.0)	0 (0.0)	0 (0.0)	1 (1.0%)	0 (0.0)	0 (0.0)	0 (0.0)
Total	145 (100.0%)	137 (100.0%)	9 (100.0%)	8 (100.0)	105 (100.0%)	141 (100.0%)	11 (100.0%)	3 (100.0%)

Numbers in parentheses are expressed as a percentage.

Supplementary Table 2. Ectoparasite infestation in dogs based on risk factors such as age, sex, and body condition in Morogoro municipality and Mvomero district, Tanzania

Study area	Ectoparasites identified	Species identified	Dogs' factors considered					
			Age		Sex		Body condition score	
			Young (n = 122)	Adult (n = 78)	Male (n = 108)	Female (n = 92)	Good (n = 137)	Poor (n = 63)
Morogoro municipality	Ticks	*Rhipicephalus sanguineus*	57 (46.7)	44 (56.4)	56 (51.9)	45 (48.9)	60 (43.8)	41 (65.1)
	Fleas	*Ctenocephalides felis*	72 (59.0)	43 (55.1)	58 (53.7)	57 (62.0)	68 (49.6)	47 (74.6)
		Ctenocephalides canis	13 (10.7)	14 (17.9)	11 (10.2)	16 (17.4)	18 (13.1)	9 (14.3)
	Mite	*Sarcoptex scabiei*	2 (1.6)	3 (3.8)	4 (3.7)	1 (1.1)	2 (1.5)	3 (4.8)
	Lice	*Trichodetes canis*	1 (0.8)	1 (1.3)	2 (1.9	0 (0.0)	2 (1.5)	0 (0.0)
Mvomero district	Ticks	*Rhipicephalus sanguineus*	106 (76.3)	44 (72.1)	80 (75.5)	70 (74.5)	82 (68.9)	68 (84.0)
	Fleas	*Ctenocephalides felis*	65 (46.8)	21 (34.4)	49 (46.2)	37 (39.4)	54 (45.4)	32 (39.5)
		Ctenocephalides canis	20 (14.4)	12 (19.7)	16 (15.1)	16 (17.02)	17(14.3)	15 (18.5)
		Pulex irritans	0 (0.0)	3 (3.8)	2 (1.9)	1 (1.1)	0 (0.0)	3 (4.8)
	Mite	*Sarcoptes scabiae*	5 (3.6)	2 (3.3)	4 (3.8)	3 (3.2)	3 (2.5)	4 (4.9)
	Lice	*Trichodetes canis*	7 (5.04)	1 (1.6)	3 (2.8)	5 (5.3)	1 (0.8)	7 (8.6)

n: Number of dogs. The total number of dogs was 400. Numbers in parentheses are expressed as a percentage.

Supplementary Table 3a. Gastrointestinal parasite infestation in dogs based on age, sex, and body condition in Mvomero district, Tanzania

Group of GIT parasites	Species identified	Number and percentage of dogs with different species of helminths and protozoan gastrointestinal parasites					
		Age		Sex		Body condition score	
		Young (n = 139)	Adult (n = 61)	Male (n = 106)	Female (n = 94)	Good (n = 119)	Poor (n = 81)
Helminthes	*Ancylostoma caninum*	81 (58.2)	37 (60.7)	70 (66.1)	48 (51.1)	68 (57.1)	50 (61.7)
	Uncinaria stenocephala	22 (15.8)	14 (23.0)	20 (18.9)	16 (17.0)	20 (16.8)	16 (19.8)
	Toxocara canis	26 (18.7)	1 (1.6)	11 (10.4)	16 (17.6)	17 (14.3)	10 (12.4)
	Toxascaris leonina	8 (5.8)	7 (11.5)	8 (7.6)	7 (7.5)	7 (5.9)	8 (9.9)
	Ascaris spp.	5 (3.6)	0 (0.0)	4 (3.8)	1 (1.1)	5 (4.2)	0 (0.0)
	Dipylidium caninum	4 (2.9)	2 (3.3)	4 (3.8)	2 (2.2)	2 (1.7)	4 (4.9)
	Taeniidae spp.	12 (8.6)	2 (3.2)	9 (8.5)	6 (6.4)	7 (5.9)	7 (8.6)
	Diphyllobothrium latum	0 (0.0)	2 (3.2)	2 (1.9)	0 (0.0)	0 (0.0)	2 (2.5)
	Troglotrema salmincola	1 (0.7)	0 (0.0)	0 (0.0)	1(1.1)	0 (0.0)	1 (1.2)
Protozoa	*Cryptosporidium* spp.	30 (21.5)	6 (9.84)	19 (17.9)	17(18.1)	22 (18.5)	14 (17.3)
	Cyclospora spp.	11 (7.9)	1 (1.6)	5 (4.7)	7 (7.5)	8 (6.7)	4 (4.9)
	Isospora spp.	22 (15.8)	3 (4.9)	12 (11.3)	13 (13.8)	15 (12.6)	10 (12.4)
	Entamoeba spp.	8 (5.8)	1 (1.6)	4 (3.8)	5 (5.3)	5 (4.2)	4 (4.9)

n: Number of dogs. The total number of dogs was 200. Numbers in parentheses are expressed as a percentage.

Supplementary Table 3b. Gastrointestinal parasite infestation in dogs based on age, sex, and body condition in Morogoro municipality, Tanzania

Group of GIT parasites	Species identified	Number and percentage of dogs with different species of helminths and protozoan gastrointestinal parasites					
		Age		Sex		Body condition score	
		Young (n = 122)	Adult (n = 78)	Male (n = 108)	Female (n = 92)	Good (n = 137)	Poor (n = 63)
Helminthes	*Ancylostoma caninum*	65 (53.3%)	35 (44.9%)	62 (57.4%)	38 (41.3%)	66 (48.2%)	34 (54.0%)
	Uncinaria stenocephala	33 (27.0%)	9 (11.5%)	26 (24.1%)	16 (17.4%)	30 (21.9%)	12 (19.0%)
	Toxocara canis	11 (9.0%)	6 (7.7%)	7 (6.5%)	10 (10.9%)	9 (6.6%)	8 (12.7%)
	Toxascaris leonina	10 (8.2%)	7 (11.7%)	4 (3.7%)	6 (6.5%)	8 (5.8%)	2 (3.2%)
	Ascaris spp.	6 (4.9%)	3 (3.8%)	7 (6.5%)	2 (2.2%)	4 (2.9%)	5 (7.9%)
	Diphyllobothrium. caninum	0 (0.0)	1 (1.3%)	1 (0.9%)	0 (0.0)	0 (0.0)	1(1.6%)
	Taeniidae spp.	5 (4.1%)	5 (6.41%)	5 (4.63%)	5 (5.43%)	4 (2.92%)	6 (9.52%)
Protozoa	*Cryptosporidium* spp.	25 (20.5%)	1 (1.3%)	15 (13.9%)	11 (12.0%)	19 (13.9%)	7 (11.1%)
	Cyclospora spp.	2 (1.6%)	4 (5.1%)	3 (2.8%)	3 (3.3%)	4 (2.9%)	2 (3.2%)
	Isospora spp.	5 (4.1%)	2 (2.6%)	3 (2.8%)	4 (4.3%)	7 (5.1%)	0 (0.0)
	Entamoeba spp.	1 (0.8%)	2 (2.6%)	3 (2.8%)	0 (0.0)	2 (1.5%)	1 (1.6%)

n: Number of dogs. The total number of dogs was 200.

Supplementary Table 4. Prevalence of mixed infestation of gastrointestinal parasites according to age, sex, and body condition of dogs of Mvomero district and Morogoro municipality, Tanzania

Study area	Species identified	Number and percentage of dogs with mixed species of gastrointestinal parasites					
		Age		Sex		Body condition score	
		Young (n = 139)	Adult (n = 61)	Male (n = 106)	Female (n = 94)	Good (n = 119)	Poor (n = 81)
Mvomero district	*Ancylostoma caninum* and *Uncinaria stenocephala*	5 (3.6%)	21 (34.4%)	19 (17.9%)	7 (7.5%)	15 (12.6%)	10 (12.4%)
	Ancylostoma caninum and *Cryptosporidium* spp.	14 (10.1%)	3 (4.9%)	10 (9.4%)	7 (7.5%)	8 (6.7%)	9 (11.1%)
	Ancylostoma caninum and *Toxocara canis*	13 (9.4%)	4 (6.6%)	9 (8.5%)	8 (8.5%)	5 (4.2%)	11 (9.2%)
	Ancylostoma caninum, Uncinaria stenocephala, and *Cryptosporidium* spp.	3 (2.2%)	1 (1.6%)	2 (1.9%)	2 (2.1%)	3 (2.5%)	1 (1.2%)
	Ancylostoma caninum, Uncinaria stenocephala, and *Taeniidae* spp.	4 (2.9%)	2 (3.3%)	2 (1.9%)	4 (4.3%)	1 (0.8%)	5 (6.2%)
	Ancylostoma caninum and *Isospora* spp	3 (2.2%)	1 (1.6%)	2 (1.9%)	2 (2.1%)	1 (0.8%)	3 (3.7%)
	Ancylostoma caninum, Toxacaris leonina, and *Cryptosporidium* spp.	4 (2.9%)	1 (1.6%)	1 (0.9%)	4 (4.3%)	4 (3.4%)	1 (1.2%)
	Ancylostoma caninum, Toxocara canis, and *Taeniidae* spp.	2 (1.4%)	1 (1.6%)	2 (1.9%)	1 (1.1%)	0 (0.0)	3 (3.7%)
	Ancylostoma caninum and *Dipylidium caninum*	0 (0.0)	2 (3.3%)	2 (1.9%)	0 (0.0)	0 (0.0)	2 (1.7%)
	Ancylostoma caninum and *Ascaris* spp.	3 (2.2%)	1 (1.6%)	2 (1.9%)	2 (2.1%)	3 (1.5%)	1 (1.2%)
	Toxocara canis, *Ancylostoma caninum*, and *Uncinaria stenocephala*	3 (2.2%)	1 (1.6%)	1 (0.9%)	3 (3.2%)	1 (0.5%)	3 (3.7%)
Morogoro municipality	*Ancylostoma caninum* and *Uncinaria stenocephala*	12 (9.8%)	8 (10.3%)	14 (13.0%)	6 (6. 5%)	13 (9.5%)	7 (11.1%)
	Ancylostoma caninum and *Cryptosporidium* spp.	5 (4.1%)	0 (0.0)	3 (2.8%)	2 (2.2%)	4 (2.9%)	1 (1.6%)
	Ancylostoma caninum and *Toxocara canis*	6 (4.9%)	1 (1.3%)	3 (2.8%)	4 (4.4%)	3 (2.2%)	4 (6.4%)
	Ancylostoma caninum, Uncinaria stenocephala, and *Cryptosporidium* spp.	6 (4.9%)	4 (5.1%)	5 (4.6%)	5 (5.5%)	7 (5.1%)	3 (6.4%)
	Ancylostoma caninum, Uncinaria stenocephala, and *Taeniidae* spp.	5 (4.1%)	2 (2.6%)	1 (0.9%)	4 (4.4%)	2 (1.5%)	3 (6.4%)
	Ancylostoma caninum and *Entamoeba* spp.	0 (0.0)	2 (2.6%)	2 (1.9%)	0 (0.0)	0 (0.0)	2 (3.2%)
	Ancylostoma caninum and *Ascaris* spp.	3 (2.5%)	0 (0.0%)	3 (2.8%)	0 (0.0)	0 (0.0)	3 (6.4%)
	Ancylostoma caninum, Toxocara canis, and *Uncinaria stenocephala*	5 (4.1%)	2 (2.6%)	6 (5.6%)	1 (1.1%)	3 (2.2%)	4 (6.4%)
	Ancylostoma caninum and *Uncinaria stenocephala*	12 (9.8%)	8 (10.6%)	14 (13.0%)	6 (6. 5%)	13 (9.5%)	7 (11.1%)
	Ancylostoma caninum, Toxocara canis, and *Uncinaria stenocephala*	5 (4.1%)	0 (0.0)	3 (2.8%)	2 (2.2%)	4 (2.9%)	1 (1.6%)

n: Number of dogs. The total number of dogs was 400.

Supplementary Table 5. Proportional comparisons of parasites based on selected risk factors among dogs in Mvomero and Morogoro districts of Tanzania

Type of gastrointestinal parasites	Species of parasite	P values of proportional comparisons of parasite infections in dogs				
		Breeds of dogs	Body condition	Age	Sex	Location (Mvomero and Morogoro)
Helminths	*Ancylostoma caninum*	0.125	0.070	0.506	0.022	0.016
	Uncinaria stenocephala	0.584	0.443	0.157	0.761	0.450
	Toxocara canis	0.293	0.293	0.001	0.332	0.111
	Toxscaris leonina	0.601	1.000	0.464	0.114	0.302
	Ascaris spp.	0.021	0.094	0.663	0.291	0.277
	Dipylidium caninum	0.482	0.703	0.995	0.812	0.057
	Taeniidae spp.	0.632	0.148	0.901	0.946	0.400
Protozoa	*Cryptosporidium* spp.	0.543	0.846	0.0001	0.013	0.217
	Isospora spp.	0.420	0.570	0.018	0.847	0.001
	Cyclospora spp.	0.253	0.794	0.637	0.869	0.148
	Entamoeba spp.	0.794	0.102	0.917	0.981	0.079
Ectoparasites	Mites	0.724	0.026	0.053	0.914	0.558
	Lice	0.398	0.114	0.094	0.822	0.055
	Ticks	0.009	0.0001	0.284	0.525	0.0001
	Fleas	0.025	0.0001	0.231	0.544	0.125
Intestinal parasites	Protozoa	0.289	0.777	0.001	0.035	0.023
	Helminths	0.346	0.022	0.041	0.114	0.0001
Ticks	Degree of ticks' infestation	0.018	0.0001	0.463	0.693	0.0001

n: Number of dogs. The total number of dogs was 400.

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Conjunctival Cytology and Bacterial Isolation from Arabian Horses with Conjunctivitis in Saudi Arabia

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ABSTRACT

Cytological and bacterial analyses are vital for diagnosing equine conjunctival diseases. These analyses facilitate accurate pathogen detection and enable targeted treatments. The present study aimed to describe and compare the conjunctival cytology and bacterial flora of clinically healthy Arabian horses and those with conjunctivitis in the Eastern Province of Saudi Arabia. Eighteen Arabian horses, including 5 healthy and 13 with conjunctivitis, were referred to King Faisal Hospital and examined. Healthy horses were distinguished from those with conjunctivitis through case history and an ocular examination. Conjunctival swabs were collected for cytological analysis and for aerobic bacterial culture and identification, with Diff-Quik staining. Differential cell counts were compared across the groups, and the findings were correlated with bacterial isolates. Biochemical identification of bacterial isolates was performed using conventional methods, including Gram staining and catalase and oxidase tests. Healthy horses exhibited a cytological profile primarily composed of superficial epithelial cells (59 ± 12.0%), with only a small number of inflammatory cells, including neutrophils and eosinophils. In contrast, horses with conjunctivitis exhibited a significant decrease in superficial epithelial cells (28 ± 17%) and a significant increase in basal cells (5 ± 3.5%). Conjunctival samples exhibited marked neutrophilia (32 ± 12.5%) and a significant increase in eosinophils (12 ± 9.5%) compared to healthy controls, indicating an inflammatory condition in the conjunctiva. Bacterial growth was found in 40% of healthy horse samples and in 92% of infected horse samples. *Staphylococcus aureus* was the most common isolate in infected horse samples. Gram-negative bacteria, including *Moraxella* sp., *Escherichia coli*, and *Klebsiella* sp., were isolated from 7 of 13 affected horses and were associated with a severe neutrophilic response (39 ± 6.5%) and significant epithelial disruption. Conjunctivitis in Arabian horses in the Eastern Province of Saudi Arabia was characterized by neutrophilic and eosinophilic inflammation with concurrent epithelial disruption.

Keywords: Bacteria, Conjunctivitis, Cytology, Equine, Ocular, Swab

INTRODUCTION

Equine populations, especially those residing in arid regions of the Middle East, such as Saudi Arabia, are persistently exposed to environmental stressors, including hot, dry, and dusty conditions (Beech et al., 2003). Although horses lack the same specialized ocular adaptations for harsh and dusty environments, their ocular adnexa, including the eyelids and the third eyelid (nictitating membrane), are essential for protecting the eyeball from environmental particulate matter and desiccation (Williams and Miller, 2006). The third eyelid of horses, composed of T-shaped cartilage and associated lymphoid tissue, serves to distribute the tear film and provide a mechanical barrier against foreign bodies (Brooks, 2010). Ocular disorders are a significant and common problem in horses, frequently resulting in different degrees of discomfort, pain, and potential vision impairment (Stoppini et al., 2005).

In intensive veterinary management, the prevalence of ocular infections and inflammation, particularly conjunctivitis, is significantly high (Ferreira et al., 2017). This increased rate of conjunctivitis is frequently associated with several factors, including the widespread presence of dust and sand, large populations of flies serving as mechanical carriers of pathogens, and, importantly, poor management and hygiene practices in some stables that weaken the eye's natural defenses (Shawaf and Hussen, 2023). The conjunctiva is a mucous membrane that lines the inner eyelids and covers the anterior surface of the eye. Conjunctiva comprises non-keratinized stratified squamous epithelium containing goblet cells, as well as stratified columnar and stratified cuboidal epithelium (Henriksen et al., 2020; Kovalcuka et al.,

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2023). Conjunctival cytology offers a valuable, non-invasive method for rapid assessment of cellular responses within the epithelium, as well as for detecting foreign bodies, infectious agents, and inflammatory processes. This technique facilitates a greater understanding of the pathogenesis and prognosis of numerous ocular diseases (Henriksen et al., 2020; Moshtaghion et al., 2021; Kovalcuka et al., 2023).

Cytologic features of the eye have been widely documented in veterinary literature, especially in equine, bovine, canine, and feline species (Henriksen et al., 2020; Sebbag et al., 2020; Lucyshyn et al., 2021). Cytologic samples are typically studied for the presence of inflammatory cells, infectious agents, intracellular inclusion bodies, and neoplastic cells (Brooks, 2010; Kayat et al., 2017). A typical cytologic sample consists mainly of conjunctival epithelial cells with no cytoplasmic or nuclear abnormalities. In contrast, inflammatory ocular cytology demonstrates an elevated number of inflammatory cells, including neutrophils, lymphocytes, eosinophils, and mast cells (Calonge et al., 2004; Donaldson and Matas, 2011; Henriksen et al., 2020). The ocular surface naturally contains a community of microorganisms that constitute its normal microflora (Ferreira et al., 2017; Hampson et al., 2019). However, resident microorganisms, such as *Staphylococcus* spp., *Streptococcus* spp., and *Bacillus* spp., are often involved in opportunistic ocular infections, particularly when external factors such as trauma, stress, or environmental disturbances interfere with the normal commensal microbiota (Ferreira et al., 2017; Zak et al., 2018). Conjunctivitis, commonly known as pink eye, may result from different conditions affecting the eye (Stoppini et al., 2005; Saberinia et al., 2025).

While general equine ocular pathology is well documented, limited information is available on the specific cytological assessment of the equine conjunctiva under the distinct environmental and management conditions in Saudi Arabia. The present study aimed to characterize and compare the cytological analysis of conjunctival swabs from healthy horses and those with conjunctivitis in Saudi Arabia, focusing on how these findings relate to risk factors such as dust, flies, and management practices.

MATERIALS AND METHODS

Ethical approval

All experimental procedures carried out in the present study were approved by the ethics committee at King Faisal University, Saudi Arabia (Permission number KFU-REC-256002).

Animals

The present study examined 18 Arabian horses. Five horses were apparently healthy based on a general clinical ocular examination, while thirteen horses were affected by conjunctivitis. The healthy horses, comprising two males and three females, had a median age of 7 ± 5.2 years and a median weight of 320 ± 105 kg and were selected from a stable located at the Equine Research Center, King Faisal University, Al-Ahsa, Saudi Arabia. The study was conducted from October 2025 to February 2026, during which ambient temperatures fluctuated between 18°C and 38°C. To ensure the health status of the control group, a systematic and comprehensive clinical and ophthalmic examination was conducted on each horse. The assessment was performed under both ambient and darkened lighting to facilitate a comprehensive evaluation of all ocular structures and to ensure horses were free of general or ocular disorders, including signs of discharge, swelling, discomfort, or abnormalities (Brooks, 2010). The affected horses, including five males and eight females, had a median age of 5 ± 3.5 years and a median weight of 330 ± 145 kg. The affected horses, exhibiting signs such as chemosis, squinting, blinking, and ocular discharge, were brought to the Veterinary Teaching Hospital and the Equine Research Center at King Faisal University, Saudi Arabia.

Sampling

Samples were collected for bacterial analysis by swabbing the lower conjunctival sac of the affected eye without using a local anesthetic. The process employed swabs specifically designed for bacterial collection and was conducted under strict sterile conditions (Donaldson and Matas, 2011). Precautions were implemented to ensure that the swab did not contact the upper eyelid margin or adjacent facial skin, thereby reducing the risk of contamination. Subsequently, the swab was placed within the protective tube of the double-capped swab. The samples were transported to the bacterial diagnostic laboratory at the College of Veterinary Medicine, King Faisal University, Saudi Arabia, at room temperature in a modified medium and cultured within 24 hours. Conjunctival samples for cytological evaluation were obtained by carefully removing mucus and debris with sterile saline-moistened gauze. The samples were subsequently evenly and delicately smeared onto clean microscope slides, allowed to air-dry at ambient temperature, and stained using the Diff-Quik method (Hemal Stain Co., Inc., Danbury, USA).

Cytological examination

The slides were prepared for microscopic examination after staining with the Diff-Quik method and examined at different magnifications under a light microscope (Nikon Eclipse, Tokyo, Japan) to identify and perform a differential cell count. Differential cell counts of the target cells were performed using an oil-immersion lens (1000x) to highlight each cell's morphological features. Differential counts were expressed as percentages and mean values (with ranges) for neutrophils, eosinophils, lymphocytes, macrophages, and epithelial cells (including surface, mesoderm, and basophils; Henriksen et al. 2020).

Bacterial isolation and identification

To identify the bacterial species in the samples, individual smears were cultured on Columbia agar (CM331; Oxweed, Basingstoke, UK) with 5% citrate-treated sheep blood and on MacConkey agar (Oxweed, Basingstoke, UK). After inoculation, plates were incubated aerobically and anaerobically at 37°C for 18-24 hours, with an additional 24 hours when no growth was observed. Cultures with dissimilar colonies and no growth were excluded. Bacterial isolates grown on nutrient, blood, and MacConkey agar after 24-48 hours at 37°C were identified following Quinn et al. (2002). Identification of Gram-negative and Gram-positive bacteria involved biochemical tests such as catalase, coagulase, oxidase, citrate utilization, triple sugar iron, indole, methyl red, urease, H_2S production, and motility analysis.

Statistical analysis

The differences between means were analyzed employing Student's t-test with GraphPad Prism 7 statistical software to compare the healthy and affected groups. Normality was assessed using the D'Agostino and Pearson omnibus normality test. Differences with a probability level (p) of 0.05 or less were considered statistically significant, utilizing Duncan's multiple range test for mean separation.

RESULTS

The cytological evaluation of conjunctival smears revealed distinct differences between the healthy and affected groups, and microbiological culture identified a diverse range of aerobic bacteria from the conjunctival sacs. The differential cell counts derived from the conjunctival cytology of both healthy and affected horses are presented in Table 1. In healthy horses, the conjunctival epithelium mainly consisted of superficial epithelial cells, accounting for 59 ± 12.0%, while intermediate and basal cells were less common. The healthy group demonstrated a minimal inflammatory cell presence, with low mean percentages of neutrophils (8 ± 5.5%) and lymphocytes (11 ± 4.5%). Eosinophils and macrophages were rarely seen, with mean values of 1 ± 1.5% and 1 ± 0.5%, respectively. In contrast, horses with clinical signs of conjunctivitis demonstrated a significantly different cytological profile. There was a statistically significant decrease in the percentage of superficial epithelial cells (28 ± 17%) compared to the healthy group ($p < 0.05$). This was accompanied by a significant increase in basal epithelial cells (5 ± 3.5%; $p < 0.05$), indicating epithelial hyperplasia or increased cellular turnover in response to inflammation. The percentage of intermediate epithelial cells, while slightly lower in the affected group (14 ± 7.8%), did not differ significantly from the healthy group ($p > 0.05$). The most pronounced differences were observed in the inflammatory cell infiltrate. Horses with conjunctivitis exhibited a significant increase in the percentage of neutrophils (32 ± 12.5%) compared to healthy horses ($p < 0.01$). Eosinophils were significantly elevated in the affected group (12 ± 9.5%; $p < 0.001$). Conversely, the percentage of lymphoid cells was significantly lower in the conjunctivitis group (4 ± 3.1%) than in the healthy group ($p < 0.05$). The percentage of macrophages was minimal in both groups and did not exhibit a significant difference ($p > 0.05$).

A total of 18 conjunctival swabs were subjected to aerobic bacterial culture. Bacteria were isolated from 2 out of 5 (40%) healthy horses and from 12 out of 13 (92%) horses diagnosed with conjunctivitis. The isolated microorganisms are detailed in Table 2. In the healthy group, *Staphylococcus aureus* (*S. aureus*) and *Moraxella* sp. were detected. No bacterial growth was observed in three healthy horses. Among horses with conjunctivitis, a wider variety of bacterial species was isolated. Gram-positive bacteria were the most frequently identified, with *S. aureus* being the most common, detected in five horses.

Other Gram-positive isolates included *Corynebacterium* sp., *Streptococcus* sp., and *Bacillus* sp. Gram-negative bacteria were isolated from 7 out of 13 affected horses, either alongside Gram-positive bacteria in mixed cultures or as single isolates. These bacteria included *Moraxella* sp., *Escherichia coli*, and *Klebsiella* sp. The relationship between conjunctival cytology and the type of bacteria isolated is presented in Table 3. In healthy horses, ocular samples that yielded Gram-positive bacteria exhibited a cellular profile, superficial cells at 62 ± 5.5% and neutrophils at 9 ± 4.5%, similar to the average for the healthy group. Ocular samples from Gram-negative isolates demonstrated a slightly lower mean percentage of superficial cells (49 ± 8.0%) and a lower neutrophil count (5 ± 3.5%), although the limited sample

size precluded statistical analysis. In horses with conjunctivitis, ocular samples with Gram-positive bacterial isolates demonstrated marked neutrophilia (28 ± 11.5%) and eosinophilia (11 ± 7.5%), along with a reduced superficial epithelial cell count (30 ± 14%). Ocular samples from which Gram-negative bacteria were isolated exhibited an even stronger inflammatory response, characterized by the highest mean percentage of neutrophils (39 ± 6.5%) and a similarly high eosinophil count (12 ± 7.5%). These samples had the lowest mean percentage of superficial epithelial cells (25 ± 13%) and the highest percentage of basal cells (7 ± 2.5%), indicating a more severe epithelial injury associated with Gram-negative infections.

Table 1. Ocular samples from healthy horses and those affected with conjunctivitis from October 2025 to February 2026 in the Eastern province of Saudi Arabia

Cells		Healthy horses (%)	Affected horses (%)	
		Mean ± SD	Mean ± SD	P-value
Epithelial	Superficial	59 ± 12.0	28 ± 17	0.01
	Intermediate	17 ± 8.2	14 ± 7.8	0.12
	Basal cells	2 ± 0.5	5 ± 3.5	0.03
Leukocytes	Neutrophils	8 ± 5.5	32 ± 12.5	0.001
	Lymphoid cells	11 ± 4.5	4 ± 3.1	0.01
	Eosinophils cells	1 ± 1.5	12 ± 9.5	0.001
Macrophages		1 ± 0.5	2 ± 0.5	0.2

SD: Standard deviation. Data are expressed as Mean ± SD

Table 2. Microorganisms isolated from ocular horse samples from October 2025 to February 2026 in the Eastern province of Saudi Arabia

Animal numbers		Gram-positive bacteria	Gram-negative bacteria
Healthy horses	1	*Staphylococcus aureus*	-
	2	-	-
	3	-	-
	4	-	*Moraxella* sp.
	5	-	-
Horses with conjunctivitis	6	*Streptococcus sp.*	-
	7	*Staphylococcus aureus*	*Klebsiella* sp.
	8	-	*Moraxella* sp.
	9	-	-
	10	*Staphylococcus aureus*	*Moraxella* spp.
	11	*Corynebacterium sp.*	*Escherichia coli*
	12	*Staphylococcus aureus*	-
	13	*Bacillus sp.*	*Klebsiella* sp.
	14	-	*Escherichia coli*
	15	*Staphylococcus aureus*	
	16	*Corynebacterium sp.*	-
	17	*Staphylococcus aureus*	-
	18	-	*Escherichia coli*

Table 3. Relationship between conjunctival cytology and cell types and isolated bacteria from healthy horses and horses affected with conjunctivitis from October 2025 to February 2026 in the Eastern Province of Saudi Arabia

Cells		Healthy horses (Mean ± SD [%])		Affected horses (Mean ± SD [%])	
		Gram-positive bacteria	Gram-negative bacteria	Gram-positive bacteria	Gram-negative bacteria
Epithelial	Superficial	62 ± 5.5	49 ± 8.0	30 ± 14	25 ± 13
	Intermediate	14 ± 5.1	18 ± 7.5	12 ± 5.5	16 ± 6.6
	Basal cells	1 ± 0.8	2.2 ± 0.3	4 ± 3.4	7 ± 2.5
Leukocytes	Neutrophils	9 ± 4.5	5 ± 3.5	28 ± 11.5	39 ± 6.5
	Lymphoid cells	12 ± 2.5	8 ± 5.5	5 ± 2.5	4 ± 2.8
	Eosinophils cells	1.5 ± 1	0.5 ± 0.5	11 ± 7.5	12 ± 7.5
Macrophages		1.5 ± 0.5	0.5 ± 0.5	1.5 ± 0.5	2.5 ± 0.8

SD: Standard deviation. Data are expressed as Mean ± SD

DISCUSSION

The present study provided a detailed comparative analysis of conjunctival cells and aerobic bacteria in Arabian horses in the Eastern Province of Saudi Arabia, a region characterized by a dry climate that poses distinct challenges to ocular health and exposes individuals to inflammatory and allergenic agents. The present results revealed clear differences in the cellular composition of the conjunctiva between healthy and infected horses and identified several potential pathogenic bacteria associated with conjunctivitis. The cellular composition of the healthy horses in the present study was consistent with that of normal equine conjunctiva, with surface squamous epithelial cells being the predominant cell type and inflammatory cells being rare (Bonsembiante et al., 2019; Berzina et al., 2022). The low number of neutrophils and lymphocytes observed in healthy horses in the present study was consistent with previous findings on the healthy ocular surface (Braus et al., 2017; Henriksen et al., 2020). Conversely, horses with conjunctivitis exhibited a notable change in their cellular profile (Brooks, 2010; Kari and Saari, 2014). The marked decrease in surface epithelial cells, coupled with the marked increase in basal epithelial cells in the present study, indicated epithelial damage followed by regeneration. This change might be due to inflammation in the surface layers, which then triggers a proliferative response in the basal layers to rebuild the epithelial structure, as previously reported by Calonge et al. (2004) and Stoppini et al. (2005).

The principal characteristic of the inflammatory response observed in the conjunctiva of the affected horses in the present study was a marked increase in neutrophil count (Maggs, 2008; Bruschi et al., 2020). This elevation of neutrophils is a cytological indicator consistent with bacterial conjunctivitis, as these cells are the first line of defense against bacterial activity (Donaldson and Matas, 2011; Johns et al., 2011). A particular finding in the present study was the markedly elevated eosinophil count in the affected horses. While eosinophils are often associated with parasitic infections or hypersensitivity reactions, they were observed in equine conjunctival cells (Henriksen et al., 2020; Lucyshyn et al., 2021). This eosinophilic infiltration could be associated with the environmental and unsanitary conditions in which the horses resided, as well as with contact allergens such as insects, dust, and pollen (Shawaf and Hussein, 2023). The cytological findings in the present study indicated that the conjunctivitis observed in the horses might have a mixed etiology, involving infectious and hypersensitivity factors. Persistent irritation from airborne particles could be an exacerbating factor, aligning with previous findings (Williams and Miller, 2006; Zak et al., 2018). The marked decrease in lymphocytes in the affected horses compared to healthy horses in the present study was unexpected, as lymphocytes are key mediators of the adaptive immune response (Henriksen et al., 2020). This decrease could be due to the acute nature of the inflammation, in which the immediate neutrophil response surpasses the lymphocyte response, or it might result from the specific sampling method and the characteristics of the surface infiltrate (Stoppini et al., 2005). Low macrophage counts in affected and healthy horses suggested that the conjunctivitis was primarily acute and did not progress to chronic granulomatous inflammation (Lamagna et al., 2015; Moshtaghion et al., 2021). Bacterial isolation from 60% of healthy horses confirmed the presence of normal ocular microbiota, consistent with the findings of Hampson et al. (2019).

The detection of opportunistic pathogens such as *S. aureus* and *Moraxella* spp. from healthy ocular samples suggested that these commensal organisms can cause disease when ocular surface defenses are compromised (Zak et al., 2018). In horses with conjunctivitis, the bacterial isolates were more diverse. The dominance of Gram-positive bacteria, particularly *S. aureus*, was consistent with the findings of Ferreira et al. (2017) and emphasized their role as the main opportunistic pathogens. The isolation of *Escherichia coli* and *Klebsiella* sp. from affected horses was clinically

substantial. These Gram-negative bacteria are frequently associated with more severe ocular disease due to their virulence factors and capacity to cause rapid tissue damage (Brooks, 2010). The presence of these pathogens, along with *Corynebacterium* sp. and *Moraxella* sp., indicated that conjunctivitis in the affected population can be caused by a broad range of bacteria. This finding highlighted the importance of performing culture and sensitivity analyses to determine the most effective antimicrobial treatment (LaFrentz et al., 2020; Seeger et al., 2021). The correlation between cytology and bacteriology offered valuable insights into the host response to different types of infection (Shawaf and Hussen, 2023). Although the sample size in the healthy group was too small for statistical comparison, the trend in horses with conjunctivitis helped clarify the relationship between cytological changes and bacterial pathogens. Infections involving Gram-negative bacteria were associated with a stronger neutrophilic response and greater epithelial disruption (fewer superficial cells, more basal cells) than infections involving only Gram-positive isolates.

These findings supported the clinical understanding that Gram-negative bacteria frequently trigger a more severe inflammatory response, likely due to endotoxins in their cell walls (Lamagna et al., 2015), and cause more extensive tissue damage (Bourges-Abella et al., 2007). The elevated eosinophil counts observed in both bacterial types in the present study suggested that environmental allergens, such as insects and dust, might be a common underlying factor in Eastern Saudi Arabia. This could increase horses' susceptibility to developing more severe clinical signs (Shawaf and Hussen, 2023).

CONCLUSION

The current study demonstrated that conjunctivitis in Arabian horses in Eastern Saudi Arabia had a distinctive cytological pattern of neutrophilic and eosinophilic inflammation, accompanied by epithelial cell regeneration. The present findings highlighted the importance of conjunctival cytology as a rapid, readily accessible tool for characterizing the inflammatory response, particularly when combined with bacterial culture. Nevertheless, the current study encountered several limitations, notably the small sample size, particularly in the healthy control group, which constrained the statistical power and the broader applicability of the current results. The investigation was confined to a specific period and did not account for potential seasonal variations. Additionally, the study focused solely on aerobic bacteria, overlooking anaerobic bacteria, fungi, and viruses, which may contribute to conjunctivitis. Further studies are needed with larger equine samples and across different time periods, including the investigation of fungal and viral agents, to fully elucidate the multiple causes of equine conjunctivitis in arid regions.

DECLARATIONS

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Authors' contributions

Zainab Al-Mashabirah, Maryam Al-Shehab, and Turke Shawaf did the examination. Maryam Al-Shehab, Khalid Elbager, Mohammad Asvad, and Mohammad Alsalman contributed to laboratory investigations. Zainab Al-Mashabirah, Moustafa Salouci, and Turke Shawaf drafted the manuscript. The final edition of the manuscript was read and approved by all authors.

Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Competing interests

The authors declared no conflicts of interest.

Ethical considerations

Ethical issues, including plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy, have been checked by all the authors. The authors confirmed that no AI tools were used in conducting and preparing the present study.

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Prevalence and Risk Factors of *Balantioides coli* Infection in Pig-Breeding Farms in Bali, Indonesia

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ABSTRACT

Balantioides coli (*Balantidium coli* [*B. coli*]) infection poses significant health and economic burdens in pigs, causing diarrhea, intestinal dysbiosis, and increased susceptibility to secondary infections. The present study aimed to assess the occurrence of balantidiasis and pinpoint related risk factors in pigs at breeding facilities in Gianyar Regency, Bali, Indonesia. A cross-sectional study with random sampling was conducted from August 2024 to February 2025. Fecal samples were collected from 15 to 35 pigs from 16 pig-breeding farms that utilized a semi-intensive rearing system. In total, 417 fecal samples were collected from pigs in seven districts, including 202 sows and 215 piglets. The samples were preserved in 3% potassium dichromate ($K_2Cr_2O_7$) and examined using the coprological sedimentation method. Morphological identification was based on the detection of *B. coli* cysts in fecal samples. Data on potential risk factors were evaluated using logistic regression analysis, which was conducted on data obtained from structured questionnaires and direct on-farm observations. The results indicated that 43.17% of pigs were infected with *B. coli*, with a higher prevalence in sows (56.95%) than in piglets (30.23%). Logistic regression analysis indicated that sows had a 3.366 times higher risk of infection than piglets. Farms with pig populations exceeding 100 heads also exhibited a higher risk of infection compared to farms housing 100 or fewer pigs. In contrast, farmers who purchased feed from commercial suppliers and those who used ready-made feed had a significantly lower risk of *B. coli* infection, reducing the incidence by 79.3% and 60.2%, respectively, compared to farmers who produced their own feed or used mixed feed. Factors of water source, pigpen condition, and pigpen cleaning practices were not significantly associated with infection. The prevalence of *B. coli* infection in pig-breeding farms in Gianyar Regency is notably high (43.17 %). The analysis indicated that life stage, herd size, feed source, and feed type are significantly associated with the risk of balantidiasis in pigs and should be prioritized in disease prevention and control strategies by farmers in the study region.

Keywords: Balantidiasis, *Balantidium coli*, Piglet, Protozoa, Sow

INTRODUCTION

Balantioides coli Alexeieff, 1931 (*Balantidium coli* [*B. coli*]) is a ciliate protozoan that causes balantidiasis, a disease affecting a wide range of hosts that include primates, pigs, ruminants, rodents, birds, and humans (Ahmed et al., 2020). Balantidiasis is recognized as a zoonotic and neglected tropical disease (Byun et al., 2021). Although infections in pigs are often subclinical, they may also lead to severe gastrointestinal disorders such as diarrhea, ulcerative colitis, dehydration, and growth retardation (Ahmed et al., 2020; Ponce-Gordo and García-Rodríguez, 2021). While specific quantitative data on the economic impact of balantidiasis in pigs are currently lacking, the disease is believed to affect growth performance and increase mortality rates adversely (Ahmed et al., 2020; Zhang et al., 2024). Zoonotic transmission of *B. coli* can reduce productivity, increase healthcare costs, and ultimately decrease profitability, especially among smallholder farming communities (Ngoshe et al., 2022).

The economic ramifications of *B. coli* infection in swine include diminished growth rates, decreased production, and reduced reproductive efficiency, which may indirectly lead to heightened mortality (Ahmed et al., 2020; Muthu et al., 2025). *B. coli* infection compromises overall health, which in turn increases vulnerability to other diseases (Muthu et al., 2025). Nevertheless, the mortality rate in pigs remains uncertain, as *B. coli* infection frequently presents as asymptomatic or mild (Ahmed et al., 2020). In severe instances, *B. coli* infection can result in mortality among piglets (Purnama et al., 2019).

The *B. coli* has a simple life cycle with two main forms, including trophozoites and cysts (Otranto and Wall, 2024). Human and pig transmission predominantly occurs by the fecal-oral route, specifically through the ingestion of cysts that contaminate water or food sources (Ahmed et al., 2020). After passing through the stomach, cysts undergo exocytosis in the small intestine and release trophozoites, which migrate to the large intestine. In the colon, the trophozoites colonize the intestinal lumen, utilize the microflora as a source of nutrition, and reproduce through binary fission or conjugation

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(Ahmed et al., 2020). Under certain conditions, trophozoites can invade the intestinal mucosa and cause inflammation, ulceration, and fluid absorption disorders, which can clinically trigger diarrhea to dysentery (Allievi et al., 2025). The encystation process occurs in the colon and rectum, and the formed cysts are excreted with feces as a source of subsequent infection transmission (Otranto and Wall, 2024).

Currently, no standardized diagnostic method exists for the detection of *B. coli*. Identification is commonly performed through fecal examination using flotation or sedimentation techniques; however, sedimentation is considered more sensitive, as flotation procedures may damage cysts and complicate microscopic identification (Ponce-Gordo and García-Rodríguez, 2021). In addition, recent molecular studies have successfully differentiated two major genetic types of *B. coli*, as types A and B, using the internal transcribed spacer gene (Allievi et al., 2025).

B. coli infections have been widely reported in both animals and humans. In Indonesia, infections have been documented in cattle (Hastutiek et al., 2019), buffaloes (Balkis et al., 2025), long-tailed macaques (Athaillah et al., 2021), elephants (Herdaus et al., 2015), and pigs, which serve as the primary reservoir host (Apsari et al., 2023; Pinatih et al., 2024). Close exposure to pigs increases the risk of *B. coli* transmission to both humans and animals (López Arias et al., 2017; Ponce-Gordo and García-Rodríguez, 2021). Tropical and subtropical climates, coupled with poor sanitation, facilitate the persistence and spread of *B. coli* (Ahmed et al., 2020). Moreover, intensive pig farming systems, inadequate fecal waste management, and unhygienic housing conditions further elevate the risk of infection in animals within and around pig farms (Giarratana et al., 2021). Free-range farming practices without appropriate personal protective equipment also increase exposure to *B. coli* (Aninagyei et al., 2021).

In Bali, Indonesia, epidemiological studies have reported different prevalence rates of *B. coli* infection in pigs, including in the regencies of Jembrana and Buleleng (Pinatih et al., 2024), as well as Badung and Tabanan (Widisuputri et al., 2020). A study conducted in traditional pig markets in Bali reported a prevalence of 61.2% (Agustina et al., 2016), whereas a considerably lower prevalence of 3.5% was observed in pigs slaughtered at the Pesanggaran abattoir (Apsari et al., 2023). Studies on the prevalence of *B. coli* in Bali have shown substantial variation across regions, which may be influenced by geographical location, pig age, and the diagnostic methods used for fecal examination (Li et al., 2020; Ponce-Gordo and García-Rodríguez, 2021).

Pigs are a vital livestock commodity in Bali, as they contribute to the local economy through regional markets and traditional farming practices, which help preserve culture, tradition, and religion (Suarta et al., 2025). Gianyar Regency is one of the major pig-producing areas in Bali, with a pig population of 85,579 in 2022 (Pridayasa et al., 2025). Although the significance of *B. coli* infection in pigs is well recognized, data regarding its prevalence in this region remain limited, and the associated risk factors have not been thoroughly assessed. Consequently, the present study aimed to determine the prevalence of *B. coli* infection in pigs from breeding farms in Gianyar Regency and to identify the associated risk factors.

MATERIALS AND METHODS

Ethical approval

No direct interventions were conducted on the pigs in the present study. The present study was approved by the Animal Ethics Committee of the Faculty of Veterinary Medicine, Udayana University, Indonesia (No: B/146/UN14.2.9/PT.01.03/2023).

Study site and period

The study was conducted in Gianyar Regency, Bali, Indonesia (8.4667˚S, 115.2833˚E), from August 2024 to February 2025. Fecal samples were obtained from pig breeding farms located in all sub-districts of Gianyar Regency, as Payangan (139 samples), Gianyar (19 samples), Tegallalang (130 samples), Sukawati (71 samples), Blahbatuh (23 samples), Ubud (15 samples), and Tampaksiring (20 samples; Figure 1). The variation in the number of samples in the current study was due to differences in the number of pig breeding farms in each sub-district, with pig farmers being predominantly located in the Payangan and Tegallalang sub-districts.

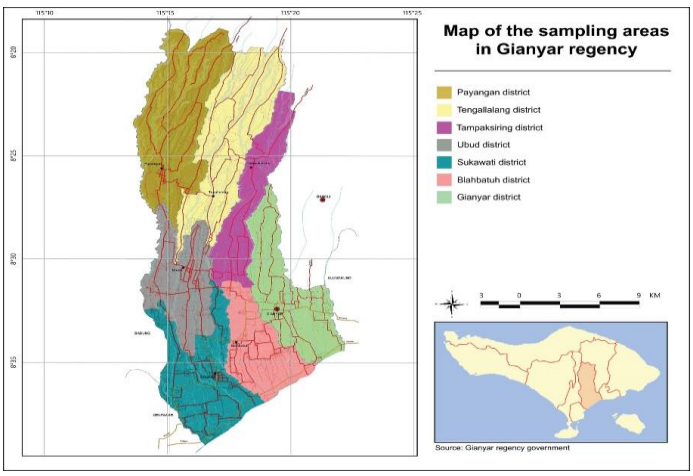


Figure 1. Geographic distribution of sampling locations in Gianyar Regency, Bali, Indonesia.

Study design and sample collection

The present study is a cross-sectional study. Pig breeding farms were selected using multistage random sampling to ensure representative coverage of each district in Gianyar Regency. In total, 16 pig farms were included in the present study, which are distributed in Payangan (4 farms), Tegallalang (4 farms), Blahbatuh (1 farm), Gianyar (1 farm), Sukawati (3 farms), Tampaksiring (1 farm), and Ubud (1 farm). The pig breeding farms in Gianyar that were used as samples implemented a semi-intensive production system. At each pig-breeding farm chosen for sampling, 15 to 35 fecal samples were collected. The variation in the number of samples and their distribution across districts was influenced by the unequal distribution of pig farms in the study area. In Gianyar Regency, pig-farming activities were primarily concentrated in Payangan and Tegallalang, where the number of pig farmers and farm populations were relatively high. In contrast, other districts had fewer pig farmers, which resulted in a limited number of farms available for sampling. Therefore, the sample distribution reflects the actual population distribution of pig farms in the study area.

Fecal samples were collected randomly from individual pigs, regardless of the presence or absence of clinical signs, because *B. coli* infection in pigs is frequently asymptomatic. Approximately 10 g of fecal material was collected from each individual pig and placed into labeled tubes containing 3% potassium dichromate ($K_2Cr_2O_7$). Each sample was labeled with the district, farm name, and sample code. The samples were immediately stored in a cooler at approximately 4˚C after collection and transported to the Veterinary Parasitology Laboratory, Faculty of Veterinary Medicine, Udayana University, Bali, Indonesia, within 24 hours.

All samples were processed within 24-72 hours after arrival at the laboratory to maintain sample integrity. The required sample size was determined using the formula $n = z^2 p (1 - p) / d^2$ (Thrusfield, 2018). In this equation, n denotes the minimum required sample size, z represents the value corresponding to the confidence level (set at 95% [1.96]), p is the expected prevalence based on prior studies (estimated at 61.2% [0.612, Agustina et al., 2016]), and d signifies the desired level of precision (set at 5% [0.05]). The calculated minimum required sample size was 365. However, the present study included 417 fecal samples in total.

Risk factor assessment

Data on potential risk factors were collected using a structured questionnaire administered by the research team and direct on-farm observations. The questionnaire underwent preliminary testing with three pig farmers who were not included in the study population to assess clarity, relevance, and face validity of the items. Based on the feedback obtained during the pilot testing, minor revisions were made to improve wording and comprehension. Minor revisions were made to the risk factor variables, specifically water sources, where the river category was excluded because of the absence of river-derived water sources in the study area. Additionally, adjustments were made to the average population categories to reflect the actual farm population better, as observed in the field. The farmers were not apprised of the specific hypotheses or objectives of the study. In instances where the responses were ambiguous, clarification was sought from the participants during the interview process; however, if the data remained unclear, they were excluded from the statistical analysis. The inclusion criteria were farmers who agreed to participate in the study, had active farms at the time of data collection, operated farms with breeding sows, and provided complete and clearly verifiable questionnaire responses during the interview process. To ensure the integrity of the questionnaire data, the completed questionnaires were cross-verified and confirmed for their completeness.

The questionnaire was administered directly by the research team to the farmers during farm visits. The questionnaire was designed by the research team based on relevant literature (Dohoo et al., 2003) as well as field conditions and practical risk factors observed in local pig farming systems. A combined literature-based and field-adapted approach was used to ensure that the variables included in the questionnaire were relevant to the actual management practices and environmental conditions in the study area. Therefore, the questionnaire was developed according to the epidemiological framework described by Dohoo et al. (2003) and adapted to the field conditions observed on pig farms in Gianyar. In total, ten risk factors were evaluated, each categorized into two or three levels. The risk factors were pig life stage, herd size, feed source, feed type, water source, pigpen cleaning frequency, pigpen cleaning method, frequency of pigpen disinfection, type of pigpen floor, and pigpen condition.

Coprological sedimentation examination

Sedimentation examination was performed using 3 g of feces per sample. The sample was placed in a beaker, mixed with 30 cc of distilled water, and thoroughly homogenized using a stirring rod (Suji Premium Handcrafted, Indonesia) before being filtered to remove debris. The filtrate was transferred into a 15 mL centrifuge tube until it reached approximately one-third of the tube volume; then, centrifugation at 1,500 rpm was performed for 5 minutes. Following centrifugation, the supernatant was gently removed, leaving the sediment undisturbed. A tiny portion of the sediment

was mounted on a glass slide, combined with a drop of distilled water, and sealed with a coverslip. The sedimentation examination was performed following a protocol for fecal sedimentation in parasitology (Zajac et al., 2021).

Microscopic (Olympus Corp, Japan) examination was conducted using a light microscope at 100× and 400× magnification to identify *B. coli* cysts, following the standard morphological criteria described by Otranto and Wall (2024) and Barbosa et al. (2018). *B. coli* cysts are typically spherical, have a diameter of approximately 40-65 µm, and possess a relatively thick cyst wall (Otranto and Wall, 2024). The cysts contain a dense nucleus with a clearly visible macronucleus, whereas the micronucleus is usually not readily observed (Barbosa et al., 2018). *B. coli* cysts can be confused with *Entamoeba* protozoa due to their similar round shape, but *Entamoeba* are smaller (4-17 µm) and have one or more nuclei depending on the species (Otranto and Wall, 2024). Molecular analysis was not performed due to limited study funding. The applied microscopic methods were sufficient for the study objectives, and the study focused on a field-based epidemiological survey.

Data analysis

Prevalence was calculated as the number of pigs positive for *B. coli* divided by the total number of examined pigs, multiplied by 100. Associations between potential risk factors and *B. coli* infection were evaluated using logistic regression analysis. Statistical significance was analyzed using the Wald test, with $p < 0.05$ being considered statistically significant. Odds ratios (ORs) were obtained from the Exp(B) values of the logistic regression model, and 95% confidence intervals (CIs) were calculated accordingly (Boateng and Abaye, 2019).

RESULTS

The results of the sedimentation examination indicated that the overall prevalence of *B. coli* infection in pigs in Gianyar Regency was 43.17%. Microscopic examination revealed spherical cysts with a thick cyst wall measuring approximately ± 45 µm and a clearly visible macronucleus, which are consistent with the morphological criteria of *B. coli* (Figure 2). The lowest prevalence was observed in Gianyar District (21.05%), whereas the highest prevalence was recorded in Blahbatuh District (56.52%; Figure 3). Risk factor analysis revealed that different variables were significantly associated with *B. coli* infection in pigs in Gianyar Regency ($p < 0.05$; Table 1).

The pig life stage had a significant effect on infection prevalence ($p < 0.05$). The prevalence of *B. coli* infection was significantly higher in sows (56.9%) compared to piglets (30.2%), with piglets used as the reference group ($p < 0.05$). Sows were 3.366 times more likely to be infected than piglets (OR = 3.366). The population size of farm pigs was also significantly associated with infection ($p < 0.050$). Farms housing more than 100 pigs had a higher prevalence of infection (48.8%) compared to farms housing 100 or fewer pigs (37.5%), and pigs from larger farms were 3.509 times more likely to be infected (OR = 3.509; $p < 0.05$).

Feed-related factors were also significantly associated with *B. coli* infection ($p < 0.05$). Pigs fed store-bought feed had a 79.3% ([1-0.207] × 100%) lower risk of infection compared with those who are fed home-produced feed (OR = 0.207; $p < 0.05$). Similarly, pigs fed ready-made feed had a 60.2% ([1-0.398] × 100%) lower risk of infection compared with those who are fed mixed feed (OR = 0.398; $p < 0.05$). Together, these data indicated that both store-bought and ready-made feeds are protective against *B. coli* infection. In contrast, the other factors of water source, pigpen cleaning frequency, pigpen cleaning method, disinfection frequency, type of pigpen floor, and pigpen condition were not significantly associated with *B. coli* infection in pigs in Gianyar Regency, Bali ($p > 0.05$).

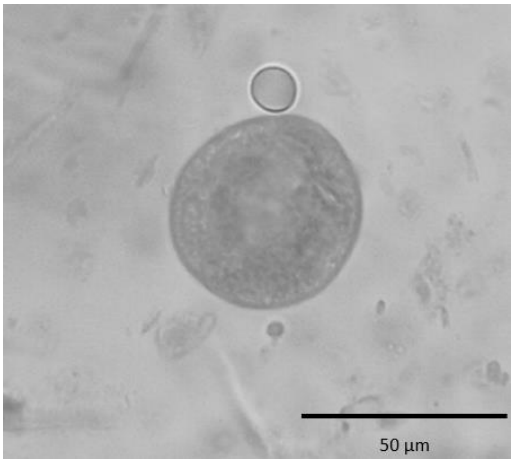


Figure 2. *Balantioides coli* cysts collected from breeding pigs across seven districts in Gianyar Regency, Bali, Indonesia

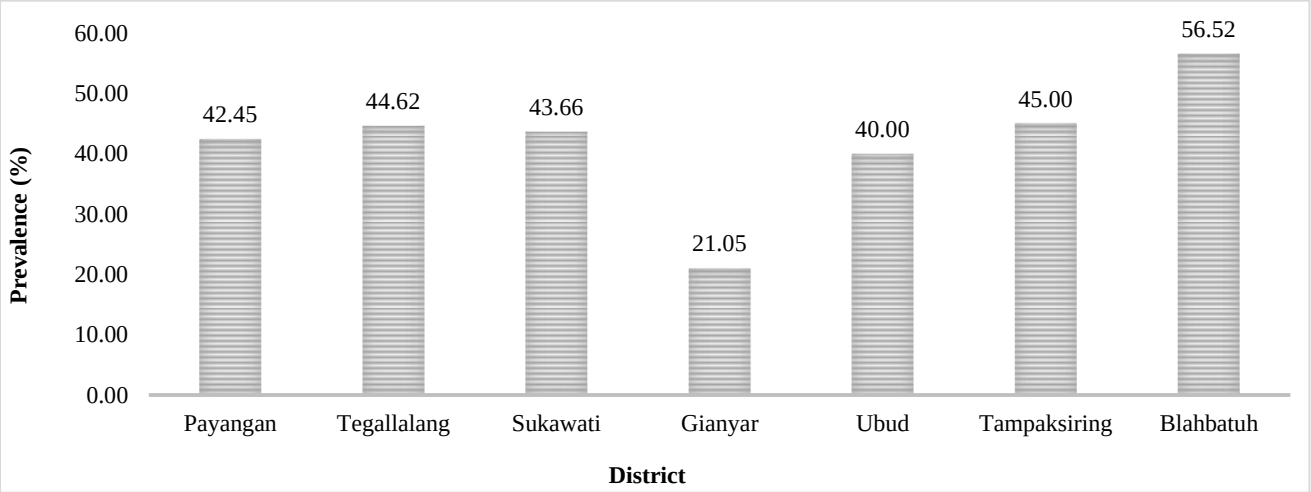


Figure 3. Prevalence of *Balantioides coli* infection in breeding pigs across seven districts in Gianyar Regency, Bali, Indonesia. Prevalence varied among districts, with the highest in Blahbatuh (56.52%) and the lowest in Gianyar (21.05%).

Table 1. Risk factors associated with *Balantioides coli* infection in pigs at breeding farms in Gianyar Regency, Bali, Indonesia, from August 2024 to February 2025

Risk factors	Category	N	Positive	Prevalence (%)	OR	CI 95%	p value
Life stage	Sow	202	115	56.931	3.366	2.194 - 5.165	0.001*
	Piglet	215	65	30.233			
Pig population on farm	≤ 100 heads	208	78	37.500	3.509	1.670 - 7.372	0.001*
	> 100 heads	209	102	48.804			
Feed source	Purchased from store	354	157	44.350	0.207	0.074 - 0.580	0.003*
	Self-produced	63	23	36.508			
Feed type	Ready-made feed	117	47	40.171	0.398	0.184 - 0.863	0.020*
	Mixed feed	300	133	44.333			
Water source	Spring	181	74	40.884	0.720	0.452 - 1.147	0.167
	Well	135	65	48.148			
	Municipal waterworks	101	41	40.594			
Pigpen cleaning frequency	Once daily	51	19	37.255	0.533	0.207 - 1.369	0.191
	Twice daily	366	161	43.989			
Pigpen cleaning method	Water spraying only	155	65	41.935	1.262	0.524 - 3.040	0.604
	Water spraying + manure removal	262	115	43.893			
Pigpen disinfection frequency	Daily	195	85	43.590	0.908	0.568 - 1.453	0.688
	Weekly	172	81	47.093			
	Irregular	50	14	28.000			
Type of pigpen floor	Cement	328	142	43.293	0.745	0.362 - 1.535	0.425
	Iron slatted floor	89	38	42.697			
Pigpen condition	Dry	265	120	45.283	0.525	0.208 - 1.324	0.172
	Occasionally wet	152	60	39.474			

N: Total sample, OR: Odds ratio, CI: Confidence interval. The Wald test was used to determine the statistical significance of each risk factor listed in the table. The results are shown in the p-value column, and significant variables are indicated with (*). Therefore, the test applies to all rows in the table rather than a specific row or column.

DISCUSSION

A prevalence of *B. coli* infection of 43.17% was observed in pigs in Gianyar Regency. The prevalence rate in the present study falls within the global prevalence range of 30-100% reported for *B. coli* infections in pigs and wild boars (Ponce-Gordo and García-Rodríguez, 2021). However, it is higher than prevalence rates previously reported in Bali, including the 3.5% in the Pesanggaran abattoir in Denpasar by Apsari et al. (2023) and the 5.1% in Buleleng Regency and Jimbaran Regency by Pinatih et al. (2024). Such prevalence rate discrepancies may be partly explained by differences in diagnostic methods. Apsari et al. (2023) employed a flotation technique, whereas the present study used the sedimentation method. Flotation techniques could damage *B. coli* cyst morphology, which would complicate microscopic identification and reduce diagnostic sensitivity (Ponce-Gordo and García-Rodríguez, 2021). The low sensitivity of the flotation method is supported by Pinilla et al. (2021), who reported that the most efficient approach for diagnosing *B. coli* infection is direct microscopic examination using Lugol's iodine solution and buffered saline. In contrast, flotation techniques, including centrifugation, flotation, and the McMaster method, were found to be ineffective for detecting *B. coli* cysts (Pinilla et al., 2021). Nevertheless, Pinatih et al. (2024), who applied the three diagnostic methods of direct examination (wet mount), sedimentation, and flotation, still reported a lower prevalence than what was observed in the present study using the sedimentation method. Compared to the higher prevalence reported by Widisuputri et al. (2020) of 79% using three methods (wet mount, sedimentation, and saturated sugar flotation) and Agustina et al. (2016) of 90% using two methods (Ritchie method and $ZnSo_4$ flotation), the prevalence observed in the present study is still lower. Such variation in prevalence rates may reflect differences in farm management practices and other risk factors that influence the transmission and persistence of infection.

The association between infection and pig life stage, herd size, feed source, and feed type highlights the importance of both host-related factors and management practices in the transmission of *B. coli.* Sows were found to be at a higher risk of infection than piglets, which may be attributable to prolonged environmental exposure and the accumulation of chronic infections over time, which potentially positions sows as a major reservoir of *B. coli* within farms (Giarratana et al., 2021). The results of the present study are consistent with previous studies reporting an age-related increase in *B. coli* prevalence (Yui et al., 2014; Symeonidou et al., 2020; Zhang et al., 2024). Yui et al. (2014) demonstrated that infection rates were significantly higher in pigs older than four months compared with those younger than one month, while suckling piglets and one-month-old piglets exhibited relatively low prevalence rates. Similar findings were reported by Symeonidou et al. (2020) in Greece, where sows had the highest risk of infection among all age groups. Both suckling piglets and weaners were indicated to have a significantly lower risk than sows, and although grower and fattening pigs had a higher risk than piglets, their risk remained lower than that of sows. Despite the general trend of increasing prevalence with age, a study had demonstrated that *B. coli* could infect pigs of all age groups, with considerable variation in prevalence. The mentioned condition may be attributed to differences in farm management practices, study locations, used diagnostic methods, sanitation conditions, and host-related factors (Paul et al., 2019). In some cases, pre-weaned piglets were indicated to have infection rates comparable to those of weaned pigs, with no statistically significant difference. Moreover, fattening pigs have been identified as the most susceptible age group in certain settings (Zhang et al., 2024). Contrasting findings have also been reported. For example in Ukraine, piglets aged 2-4 months from small-scale farms exhibited the highest prevalence at 17.2%, exceeding the rates observed in fattening pigs and sows (Bohach et al., 2023). The high prevalence observed in piglets in a study by Bohach et al. (2023) was not solely due to age, but rather a combination of the piglets' high susceptibility at that age and the conditions on small-scale farms (< 25 sows), which were characterized by poor hygiene, extensive management, and traditional farming practices. Similarly, in a study conducted in Shaanxi Province, northwestern China, a lower prevalence was reported in adult pigs (5.7%) compared to suckling piglets (7.1%), while weaners were indicated to have the highest prevalence at 18.2% (Li et al., 2020). Nevertheless, Li et al. (2020) suggested that age might not be a direct predisposing factor for *B. coli* infection. Instead, management practices, hygiene standards, stocking density, and overall farm conditions likely play a more influential role in shaping the observed prevalence patterns that are related to age.

The pig population size on a farm was also identified as a significant factor associated with *B. coli* infection in Gianyar Regency, with farms housing more than 100 pigs indicating a higher prevalence of infection. The increased infection risk in larger farms may be related to the increased stocking density and more frequent animal contact in larger farms, which can facilitate environmental contamination and the transmission of *B. coli* cysts through fecal-oral routes, including contamination of feed and water sources (Aninagyei et al., 2021). In Gianyar Regency, larger herd sizes are generally associated with more intensive husbandry systems, whereas farms with 100 or fewer pigs are typically managed at a household scale. The tendency of larger farms in Gianyar Regency to operate under more intensive husbandry systems is consistent with Giarratana et al. (2021), who reported that intensive husbandry systems are associated with an increased risk of *B. coli* infection. In contrast, Bohach et al. (2023) reported different results in the

Northern Black Sea region of Ukraine, where *B. coli* infection was not detected on large-scale farms with more than 100 sows, while moderate prevalence was observed on medium-scale farms with 20-100 sows (4.88%) and small-scale farms with fewer than 25 sows (12.7%). The observation that infection rates were lower in larger farms than in smaller farms in the study by Bohach et al. (2023) contrasts with the findings of the present study and may reflect regional differences in management practices, sanitation, and biosecurity measures.

Pig-breeding farms in Gianyar Regency predominantly implement semi-intensive production systems. Semi-intensive management practice could contribute to the occurrence of *B. coli* infection. Giarratana et al. (2021) similarly reported that the prevalence of *B. coli* infection was higher in commercial hybrid pigs (64.84%) than in local pigs (27.91%), which indicates that infections are more common in intensively-raised pigs than in those managed under extensive systems. Different mechanisms may explain why intensive husbandry systems are associated with increased *B. coli* infection. Previous studies have suggested that high animal densities may promote close contact between pigs and increase environmental contamination with cyst-containing feces, thereby facilitating fecal-oral transmission (Symeonidou et al., 2020; Giarratana et al., 2021). Maintaining optimal hygiene can also be challenging on farms with large pig populations, where fecal accumulation and contamination of feed and drinking water are likely (Giarratana et al., 2021; Mian et al., 2025). In addition, intensive systems often involve limited space and suboptimal waste management, which can result in contamination of soil and water sources that serve as reservoirs for *B. coli* cysts (Ponce-Gordo and García-Rodríguez, 2021; Patel et al., 2025). Furthermore, pigs raised under intensive conditions are frequently exposed to higher stress levels due to overcrowding and restricted movement, which may impair immune function and increase susceptibility to infection (Patel et al., 2025).

Feed management was also closely associated with *B. coli* infection in pigs at breeding farms in Gianyar Regency. Home-produced and compounded feeds were associated with a higher risk of infection compared with commercially manufactured feeds. The higher risk of infection associated with feeds that are produced and mixed on-site may be related to a greater potential for cross-contamination under unhygienic handling conditions, as suggested in a previous study (Ahmed et al., 2020), whereas commercial feeds typically undergo standardized processing and quality control. In particular, on-farm feed mixing practices in Gianyar, especially those conducted directly on the floor, may increase the likelihood of contamination with feces containing *B. coli* cysts. However, unhygienic handling conditions and on-farm feed mixing were not directly observed or quantified in the present study. Contaminated feed and water sources can serve as important vehicles for parasite transmission (Ponce-Gordo and García-Rodríguez, 2021).

In addition, compounded and home-produced feeds used in Gianyar Regency are generally rich in carbohydrates, as they are primarily composed of rice bran and corn bran. Carbohydrate-rich diets, especially those high in starch, are known to support the growth and proliferation of *B. coli* (Ponce-Gordo and García-Rodríguez, 2021). The high risk of *B. coli* infection in pigs fed high-carbohydrate diets is supported by findings from studies involving balantidiasis in symphysomal animals. The other study had demonstrated higher trophozoite counts during periods of high-starch feeding compared with low-starch feeding, which findings suggested that starch-rich diets may increase infection intensity and promote the development of clinical balantidiasis (Schovancová et al., 2013).

Although water source, pigpen cleaning frequency, pigpen cleaning method, disinfection frequency, type of pigpen floor, and pigpen condition were not statistically significantly associated with *B. coli* infection, their potential roles in parasite transmission cannot be excluded. The lack of statistical significance may be attributable to variations in husbandry practices among farms or limitations in sample size. Consequently, further studies are warranted to investigate additional and more specific risk factors, including the design and placement of drinking water systems. A practice commonly observed on small-scale family farms is the direct placement of drinking water containers on the pigpen floor. This may facilitate contamination of drinking water with feces containing *B. coli* cysts, thereby increasing the risk of reinfection (Class et al., 2025).

CONCLUSION

The present study demonstrated that the prevalence of *B. coli* infection in pigs at breeding farms in Gianyar Regency, Bali, Indonesia, is relatively high at 43.17%. This confirmed that the protozoan remains a significant concern for pig health and poses a potential zoonotic risk. The four factors of pig life stage, herd size, feed source, and feed type are significantly associated with *B. coli* infection. The finding of these four risk factors highlights the importance of prioritizing herd population management, age-related susceptibility, and improvements in feeding practices to reduce the risk of balantidiasis in pigs in Gianyar Regency. The cross-sectional design limits causal inferences between risk factors and infection. Identification based solely on morphology prevented differentiation of *B. coli* genetic types as well as co-infections with other enteric pathogens were not evaluated, which restricts the interpretation of clinical relevance. Future studies should employ molecular characterization, longitudinal designs, and comprehensive pathogen screening to

elucidate better infection dynamics and disease impact in pigs.

DECLARATIONS

Acknowledgments

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Authors' contributions

I Putu Cahyadi Putra, contributed to the sample examination, conceptualization, methodology development, statistical analysis, and writing and reviewing of the original draft. Nyoman Adi Suratma was responsible for supervision, validation, and project administration. Ni Wayan Helpina Widyasanti contributed to the investigation, data curation, and writing of the original article. Palagan Senopati Sewoyo contributed to the visualization and review of the original draft. All authors contributed to the writing and revision of the manuscript and have read and approved the final version.

Availability of data and materials

All data obtained in the present study were analyzed and are presented in tables and figures within this manuscript. Additional information or datasets are available from the corresponding author upon reasonable requests.

Competing interests

The authors confirmed that they have to competing interest to declare rated to the publication of this article.

Ethical considerations

The present study was conducted as an observational investigation without experimental intervention. Pigs were used solely as observation subjects, and fecal samples were collected for analysis. The authors have checked for ethical issues related to publication, including plagiarism, consent to publish, ethical misconduct, data fabrication, data falsification, double submission, and redundancy. The Paperpal by Editage (https://edit.paperpal.com) tool was employed to facilitate language editing and sentence structuring. The authors independently conducted the entirety of scientific content development, data interpretation, and study design.

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A Retrospective Study on Foot and Mouth Disease Outbreaks and Vaccination during 2017-2023, North Shewa, Ethiopia

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ABSTRACT

Foot-and-mouth disease (FMD) is a highly contagious viral disease that reduces livestock productivity in Ethiopia. The present study aimed to assess the spatial and temporal patterns of FMD outbreaks and associated morbidity, mortality, case-fatality rates, and vaccination coverage in the North Shewa Zone from 2017 to 2023. Data were collected from the Disease Outbreak and Vaccination Activities Reports (DOVAR) of the North Shewa Zone Livestock Office and analyzed using descriptive statistics. A total of 162 outbreaks were recorded across 24 districts and 374 peasant associations (PAs) over the study period, resulting in an overall outbreak proportion of 43.4% (162/374 PAs), with considerable variation between years. The highest number of outbreaks was recorded in 2023 (30.9%), followed by 2021 (22.2%), while the lowest was in 2020. Overall, there were 5,246 recorded FMD cases and 67 deaths among 269,704 cattle at risk, yielding morbidity, mortality, and case-fatality rates of 1.95%, 0.02%, and 1.28%, respectively. Outbreak occurrence differed by season, peaking in the dry season (October to January) at 57% and reaching its lowest in the rainy season (June to September) at 21%. Morbidity and mortality were highest in the dry season (3.05% and 0.05%, respectively). Outbreaks and cases were unevenly distributed across districts, with BassonaWorana, Midda, and Menz-Gera reporting the highest outbreak frequencies, while Merhabet and Siyadeber exhibited relatively higher morbidity rates relative to their cattle populations. Over the seven years, vaccine coverage was limited, reaching only 12.3% of the at-risk population. Years with a high incidence of FMD were generally correlated with low vaccination coverage. Endemic FMD across the North Shewa Zone, along with increasing outbreaks, dry-season peaks, district hotspots, and inadequate vaccination coverage, highlighted FMD as a significant obstacle to livestock productivity. The results indicated the critical need for targeted vaccination programs and strengthened control strategies, particularly in high-risk districts.

Keywords: Epidemiology, Foot and mouth disease, Morbidity, Mortality, Outbreak, Vaccination

INTRODUCTION

Ethiopia has the largest livestock population in Africa, with approximately 70 million cattle, 42.9 million sheep, and 52.5 million goats (Legesse et al., 2023). The Amhara Region alone accounts for about 16.4 million cattle, while the North Shewa Zone of Ethiopia has an estimated 1.7 million cattle (North Shewa Livestock Office, 2022/23). The livestock sector plays a significant role in the country's economy, contributing roughly 45% of agricultural gross domestic product (GDP), about 19% of total national GDP, and between 16% and 19% of foreign exchange earnings (Seifu et al., 2023). In addition to its economic benefits, livestock serves as a vital source of high-quality protein and provides essential industrial raw materials, including milk, meat, hides, and skins (Countryman et al., 2024; Getiso and Mijena, 2024).

Despite the substantial economic and nutritional contributions of the livestock sector, its productivity and market potential are considerably hindered by transboundary animal diseases (TADs), which restrict the movement and export of animals and animal products, thereby restricting Ethiopia's participation in profitable international markets (Jemberu et al., 2016; Shapiro et al., 2017). Among TADs, foot-and-mouth disease (FMD) is particularly important due to its highly contagious nature, widespread distribution, and substantial socioeconomic impacts. Foot-and-mouth disease is caused by the foot-and-mouth disease virus (FMDV), a member of the *Picornaviridae* family. The virus has seven immunologically distinct serotypes, including O, A, C, Asia-1, Southern African Territories (SAT)-1, SAT-2, and SAT-3, none of which confer cross-protective immunity (Sangula et al., 2010; Chestley et al., 2022). In Ethiopia, five serotypes, O, A, C, SAT-1, and SAT-2, have been identified. Among these, serotypes O, A, SAT-1, and SAT-2 continue to cause recurring outbreaks (Jemberu et al., 2016; Seyoum and Tora, 2023).

Foot-and-mouth disease is characterized by very high morbidity (up to 100% in susceptible herds), while mortality in adult animals is generally low (1-5%); however, mortality in young animals may reach or exceed 20%, often due to acute myocarditis (WOAH, 2023). In addition to mortality, FMD leads to substantial production losses, including

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reduced milk yield, abortion, and decreased fertility, thereby severely affecting household livelihoods and national productivity.

Transmission occurs through direct contact, aerosols, fomites, animal products, and mechanical vectors, with the virus shed in nearly all bodily secretions and excretions, including saliva, milk, semen, and exhaled air, facilitating rapid spread within and between herds (WOAH, 2023). Although diagnosis is often based on clinical signs, laboratory confirmation is essential for accurate serotype identification. There is no specific treatment for FMD; therefore, control relies on movement restrictions, quarantine, strategic vaccination, surveillance, and biosecurity measures rather than slaughter policies, which are economically unfeasible in Ethiopia (Jemberu et al., 2014; Zewdie et al., 2023). However, inconsistent vaccine supply, limited vaccine strain matching, and weak coordination continue to undermine effective and sustainable disease control efforts (Jemberu et al., 2016; Tesfaye et al., 2020).

The North Shewa Zone of the Amhara Region of Ethiopia is at high risk for FMD outbreaks due to different well-established epidemiological risk factors, including a large livestock population, communal grazing systems, frequent animal movements through markets, shared watering points, and low vaccination coverage (Zewdie et al., 2023). Such conditions are known to facilitate the introduction, transmission, and persistence of FMD within endemic settings by increasing contact rates among susceptible and infected animals and enabling virus circulation across herds and locations (Woldemariyam et al., 2022). The present study aimed to assess the temporal and spatial trends of FMD outbreaks in domestic ruminants in North Shewa Zone, Amhara Region, Ethiopia, from 2017 to 2023.

MATERIAL AND METHODS

Ethical approval

The present study was based on a retrospective analysis of secondary surveillance and vaccination data obtained from the North Shewa Zone Animal Health Office. The data were aggregated at the peasant association (PA) and district levels and did not include individual animal identifiers or personal information. As the study did not involve experimental investigations on animals or human participants, formal ethical approval from an institutional review board or ethics committee was not required. Permission to access and use the data for study and publication purposes was obtained from the relevant authorities.

Study location

The present study was conducted in the North Shewa Zone of the Amhara Region, Ethiopia, located 130 km northeast of Addis Ababa. The zone encompasses 24 districts and includes 374 peasant associations. The area is geographically located at approximately 9˚46′8.4″ N latitude and 39°40′4.8″ E longitude (Figure 1). North Shewa is bordered by multiple administrative regions, including the south and southeast by the North Shewa and East Shewa zones of the Oromia Region; to the west, north, and northeast by the East Gojjam Zone, South Wollo Zone, and the Oromia Special Zone of the Amhara Region, respectively. To the east, the zone is bordered by the Afar region. Topographically, the zone ranges from 1500 m in the lowland to over 3000 m in the highland. The differences in topography lead to variations in climate. In the highlands, the temperatures range between 10 and 20˚C, while in the lowlands, temperatures reach 20-30˚C. The rainfall pattern is bimodal, with a long rainy season extending from June to September, a shorter rainy season occurring from February to May, and a dry period from October to January. The average annual rainfall in the zone ranges between 1085 and 1199 mm (Kassa, 2015).

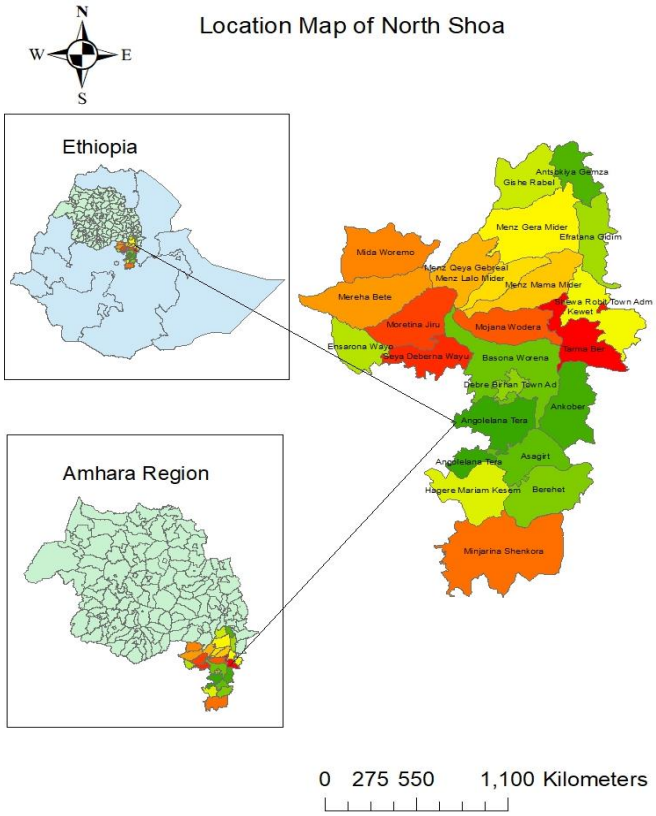


Figure 1. Geographical location of the study area, Amhara region, North Showa zone districts, Ethiopia (ArcGIS, 2021)

Study population

During the study period, no FMD cases were reported in small ruminants due to the absence of visible clinical signs; therefore, the study focused on cattle. The total cattle population at risk in affected PAs across the study districts was 269,704. All reported PA-level outbreaks were included, and all cattle within affected PAs were considered at risk, irrespective of age. The cattle were managed predominantly under different production systems, including extensive, semi-intensive, and intensive, and comprised indigenous, exotic, crossbred, and local breeds.

Study design

A retrospective observational study was conducted to assess the patterns of FMD outbreaks and associated vaccination activities in the North Shewa Zone, Amhara Region, Ethiopia, using data collected from 2017 to 2023. The PAs served as the primary spatial units, and reported FMD outbreaks constituted the units of analysis. The outcome variables included FMD occurrence, case counts, and the spatial and temporal distribution of outbreaks. The explanatory variables included the at-risk cattle population, PA-level features, and vaccination status.

Data collection

Data on FMD outbreaks and vaccination trends were obtained from the North Shewa Zone Animal Health Office through the Disease Outbreak and Vaccination Activity Report (DOVAR), which was compiled from reports submitted by government animal health services across all 24 districts of Ethiopia. The data, organized by month, year, and district, included information on FMD cases, deaths, and populations at risk, as well as morbidity, case fatality, and the number of cattle vaccinated with the A, O, and SAT2 serotypes.

Statistical analysis

The retrospective data were entered into Microsoft Excel for data management, visualization, and the calculation of key epidemiological parameters, including the frequency of outbreaks, morbidity, mortality, and case fatality rates of FMD. The morbidity, mortality, and fatality rates were calculated using the following formulas (Thrusfield, 2005).

Morbidity rate = (number of cases/total population at risk) × 100

Mortality rate = (number of deaths/total population at risk) × 100

Fatality rate = (number of deaths/number of cases) × 100

RESULTS

Temporal distribution

Across 24 districts and 374 PAs, a total of 162 outbreaks were reported between 2017 and 2023, with marked year-to-year variation. An overall outbreak proportion of 43.4% was observed. The highest outbreak burden was observed in 2023, with 50 outbreaks (30.9%) and the highest occurrence rate (13.4%), followed by 2021, which recorded 36 outbreaks (22.2%) and an occurrence rate of 9.65%. Earlier years contributed substantially fewer outbreaks, with 2017 reporting 32 outbreaks (19.8%), while 2018 and 2019 recorded relatively low proportions at 5.6% and 8.6%, respectively. The lowest outbreak activity was observed in 2020, with only 2 outbreaks (1.2%) and an occurrence rate of 0.5%. Although outbreaks declined from 36 (22.2%) in 2021 to 19 (11.7%) in 2022, the 2022 level remained higher than in previous years. Overall, despite constant geographic coverage, outbreak occurrence increased markedly in the later years, peaking in 2023 (Table 1 and Figure 2).

Table 1. Yearly distribution of outbreak occurrence and proportion across 24 districts and 374 kebeles in North Shewa Zone, Ethiopia, from 2017 to 2023

Year	Outbreaks numbers	Occurrence of outbreaks (%)	Proportion outbreaks (%)
2017	32	8.6	19.8
2018	9	2.4	5.6
2019	14	3.75	8.6
2020	2	0.5	1.2
2021	36	9.65	22.2
2022	19	5.09	11.7
2023	50	13.40	30.9
Total	162	43.4	100

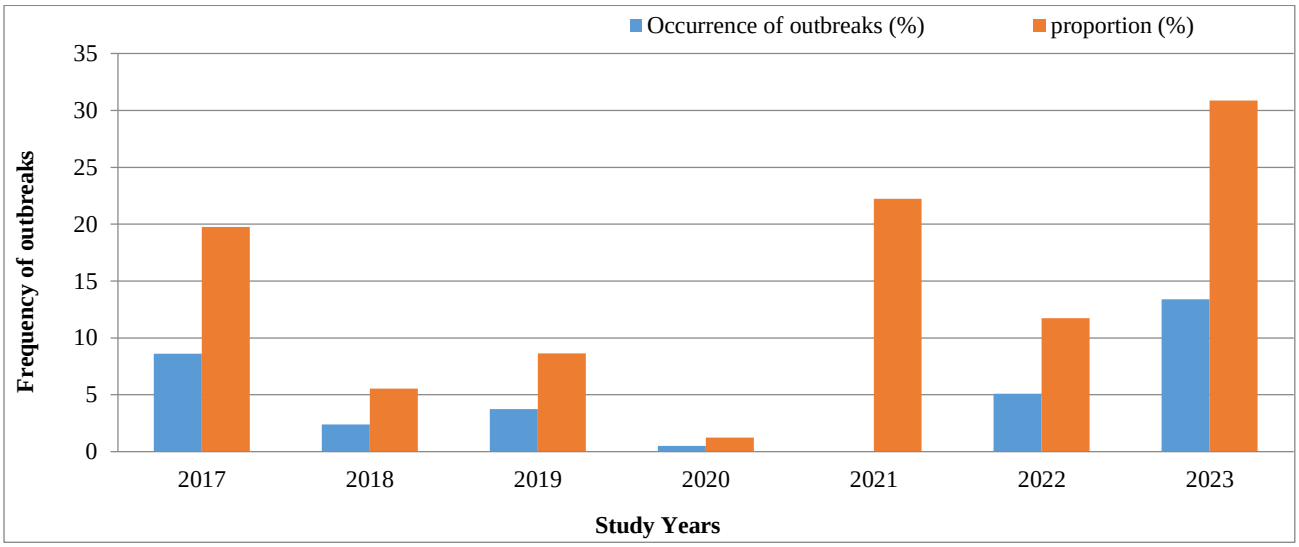


Figure 2. Number of foot-and-mouth disease outbreaks by year in North Shewa Zone, Ethiopia, from 2017 to 2023

Seasonal analysis indicated variation in FMD occurrence across the study period. When the overall data were categorized by season, the highest number of outbreaks occurred during the dry season (October-January; n = 93, 57%), followed by the short rainy season (February-May; n = 35, 22%). The fewest outbreaks were recorded during the long rainy season (June-September; n = 34, 21%; Figure 3). Of the 269,704 cattle considered at risk, 5,246 were affected, and 67 deaths were documented. The outbreak resulted in an overall morbidity rate of 1.95%, a mortality rate of 0.02%, and a case fatality rate of 1.28%. The highest numbers of cases and deaths were recorded during the dry seasons, with 2,839 reported cases and 45 deaths in the 93,104 cattle at risk. The corresponding values for the morbidity rate, mortality rate, and case fatality rate for the dry season were 3.05%, 0.05%, and 1.59%, respectively. The longer rainy season had a relatively lower impact, with 1,472 cases and 8 deaths reported among the 115,191 cattle at risk. Hence, the lower values for the morbidity rate, mortality rate, and case fatality rate for the longer rainy season were 1.28%, 0.007%, and 0.54%, respectively. During the short rainy season, there were 935 cases and 14 deaths among 61,409 cattle at risk, resulting in morbidity, mortality, and case fatality rates of 1.52%, 0.02%, and 1.49%, respectively (Table 2).

A total of 5,246 cases of FMD and 67 deaths were recorded among the 269,704 cattle at risk over the seven-year study period of 2017-2023, resulting in an overall morbidity rate of 1.95%, a mortality rate of 0.02%, and a case fatality rate of 1.28%. Annual FMD occurrence varied considerably across the years. The highest numbers of cases were reported in 2023 (1,796 cases) and 2017 (1,374 cases), corresponding to morbidity rates of 1.93% and 2.27%, respectively. Similarly, 2021 recorded 1,355 cases with a morbidity rate of 2.17%. In contrast, lower case numbers were observed in 2018 (157 cases) and 2019 (242 cases), with morbidity rates of 1.03% and 1.51%, respectively. The lowest number of cases was recorded in 2020, with 17 cases from a relatively small at-risk population of 450 cattle; however, this year revealed the highest morbidity rate (3.78%) among all study years. Deaths were reported in all years except 2020, with the highest number observed in 2023 (33 deaths), followed by 2021 (18 deaths) and 2017 (6 deaths). Annual mortality rates remained low throughout the study period, ranging from 0.00% to 0.04%. Case fatality rates differed across years, with the highest recorded in 2019 (2.07%) and 2023 (1.84%), while no fatalities were reported in 2020 (Table 3).

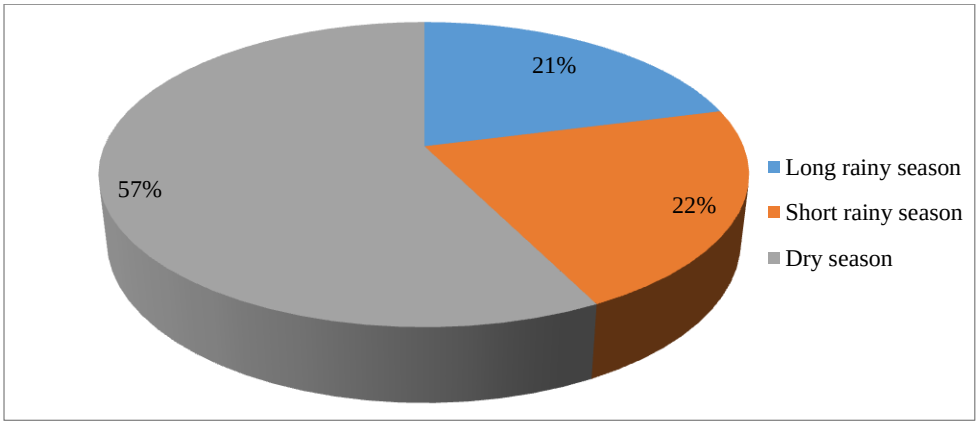


Figure 3. Foot-and-mouth disease outbreaks by season in the North Shewa Zone, Ethiopia, from 2017 to 2023

Table 2. Number of foot-and-mouth disease cases and deaths, and the rates of morbidity, mortality, and fatality across seasons in the North Shewa zone, Ethiopia, from 2017 to 2023

Seasons	Population at risk	Cases (number)	Deaths (number)	Morbidity rate (%)	Mortality rate (%)	Case fatality rate (%)
Long rainy season	115,191	1,472	8	1.28	0.007	0.54
Dry season	93,104	2,839	45	3.05	0.05	1.59
Short rainy season	61,409	935	14	1.52	0.02	1.49
Total	269,704	5,246	67	1.95	0.02	1.28

Table 3. Foot-and-mouth disease morbidity, mortality, and fatality rate across years in the North Shewa zone, Ethiopia, from 2017 to 2023

Year	Population at risk	Cases (number)	Deaths (number)	Morbidity rate (%)	Mortality rate (%)	Case fatality rate (%)
2017	60479	1374	6	2.27	0.01	0.44
2018	15192	157	1	1.03	0.01	0.64
2019	15943	242	5	1.51	0.03	2.07
2020	450	17	0	3.78	0.00	0.00
2021	62500	1355	18	2.17	0.03	1.33
2022	22288	305	4	1.37	0.02	1.31
2023	92852	1796	33	1.93	0.04	1.84
Total	269704	5246	67	1.95	0.02	1.28

Spatial distribution

In total, 162 outbreaks were reported in the 24 districts of the North Shewa zone, Ethiopia, during the study period, with 5,246 animals infected and a population at risk of 269,704 cattle. The overall morbidity, mortality, and case fatality rates were 1.95%, 0.02%, and 1.28%, respectively, indicating widespread disease but low mortality. Outbreaks were not evenly distributed across the study area. The highest number of outbreaks was reported in Bassonaworana (27 outbreaks; 16.7%), followed by Midda (17; 10.5%) and Menz-gera (15; 9.3%), whereas Shewarobit and Gishi recorded only one outbreak each (0.6%). Similarly, the number of cases ranged from the highest in Siyadeber (544), Hagermariam (531), and Minjar (542) to the lowest in Shewarobit (6) and Gishi (7).

District-level analysis indicated that FMD morbidity was generally low (<5%) across most districts in the North Shewa Zone, Ethiopia. In contrast, markedly higher morbidity was recorded in Merhabet (17.07%), Siyadeber (9.06%), and Minjar (4.99%), indicating more intense outbreaks in these districts. Mortality rates were consistently low across all districts, with the highest value (0.15%) observed in Merhabet. Although overall case fatality rates were low, comparatively higher rates were noted in Angolela (5.09%), Ankober (4.26%), and Moretena-Jiru (3.7%; Table 2). These findings indicated that FMD was widely distributed throughout the North Shewa zone of Ethiopia, but exhibited pronounced spatial heterogeneity in morbidity and outbreak severity, underscoring the need for targeted vaccination and control interventions in high-risk districts (Table 4).

Table 4. Spatial distributions of foot-and-mouth disease cases, deaths, and rates of morbidity, mortality, and fatality in districts in North Shewa Zone, Ethiopia, from 2017 to 2023

Districts name	Frequency of outbreak	Proportion (%)	Cases (number)	Deaths (number)	Population at risk	Morbidity rate (%)	Mortality rate (%)	Fatality rate (%)
Deberbirhan	6	3.70	128	0	6700	1.91	0	0
Bassonaworana	27	16.7	416	13	29164	1.43	0.44	3.13
Ankober	4	2.47	47	2	4712	0.99	0.04	4.26
Mojanawodera	8	4.94	228	4	24686	0.92	0.01	1.75
Taremaber	5	3.09	138	0	15456	0.89	0	0
Kewot	3	1.85	74	1	15429	0.48	0.01	1.35
Shewarobit	1	0.62	6	0	580	1.03	0	0
Eferatagidem	3	1.85	51	0	5525	0.92	0	0
Antsokeya	2	1.23	25	0	5656	0.44	0	0
Gishi	1	0.62	7	0	1240	0.56	0	0
Menz-qeya	11	6.79	215	7	14787	1.45	0.05	3.26
Menz-gera	15	9.26	229	0	27572	0.83	0	0
Menz-lalo	4	2.47	385	7	8006	4.80	0.09	1.82
Menz- mama	2	1.23	160	0	3550	4.50	0	0
Angolela	9	5.56	157	8	29900	0.53	0.03	5.10
Asagiret	5	3.09	280	3	6948	4.03	0.04	1.07
Hagermariam	9	5.56	531	5	11300	4.69	0.04	0.94
Minjar	8	4.94	542	6	10847	4.99	0.06	1.11
Berehet	4	2.47	216	0	9652	2.24	0	0
Siyadeber	7	4.32	544	2	6005	9.06	0.03	0.37
Moretena-jiru	2	1.23	54	2	3253	1.66	0.06	3.70
Ensaro	7	4.32	91	0	2750	3.31	0	0
Merhabet	2	1.23	350	3	2050	17.07	0.15	0.86
Midda	17	10.49	372	4	23936	1.55	0.02	1.08
Total	162	100	5246	67	269704	1.95	0.02	1.28

Vaccination utilization

From 2017 to 2023, FMD in the North Shewa Zone of Ethiopia revealed marked annual variation. Overall, 162 outbreaks were recorded, with a peak in 2023 (50 outbreaks across 15 districts) and a minimum in 2020 (two outbreaks across two districts). The population at risk differed over time, reaching its highest level in 2023, coinciding with the outbreak peak. During the study period, 33,155 cattle of all age groups were vaccinated against FMD using the A, O, and SAT2 serotypes. However, overall vaccination coverage remained low, reaching only 12.3% of the at-risk population. Vaccination coverage was relatively higher in 2017 (19.2%) and 2022 (17.9%) but notably lower in 2018 (3.6%) and 2021 (5.1%), with no vaccination conducted in 2020. Years with a high number of outbreaks often coincided with low vaccination coverage, suggesting that inadequate vaccination may have contributed to the persistence of FMD outbreaks (Table 5 and Figure 4).

Table 5. Foot-and-mouth disease outbreaks in North Shewa, Ethiopia, showing annual occurrence, number of affected districts, and number of vaccinated animals from 2017 to 2023

Year	Outbreaks (number)	Districts with FMD number	Population at risk	Vaccinated cattle (number)	Vaccine coverage (%)
2017	32	15	60479	11610	19.2
2018	9	5	15192	550	3.6
2019	14	6	15943	2750	17.2
2020	2	2	450	0	0
2021	36	15	62500	3180	5.1
2022	19	7	22288	4000	17.9
2023	50	15	92852	11065	11.9
Total	162	-	269,704	33,155	12.3

FMD: Foot and mouth disease

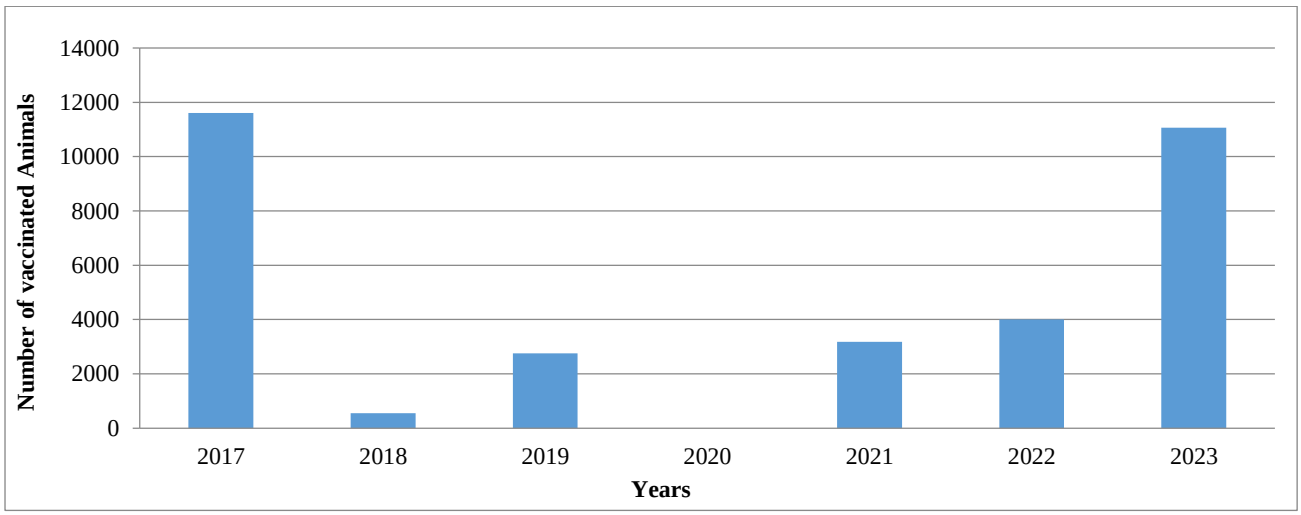


Figure 4. Number of foot-and-mouth disease-vaccinated animals in North Shewa, Ethiopia, from 2017 to 2023

DISCUSSION

The present study indicated that FMD was widespread and recurrent in the North Shewa Zone, Ethiopia, with 162 outbreaks reported across 24 districts and 374 kebeles between 2017 and 2023. Outbreak frequency differed markedly over time, peaking in 2023 (50 outbreaks; 30.9%), followed by 2021 (36 outbreaks; 22.2%) and 2017 (32 outbreaks; 19.8%). The fewest outbreaks occurred in 2020 (2 outbreaks; 1.2%), which may reflect reduced transmission, underreporting, or disruptions in animal movement and surveillance during that year. The temporal fluctuations aligned with recent Ethiopian studies documenting irregular but recurrent FMD patterns with intermittent annual peaks (Woldemariyam et al., 2022; Zewdie et al., 2023; Gizaw et al., 2025).

Seasonal analysis revealed that FMD cases and deaths were highest during the dry season, with morbidity, mortality, and case fatality rates of 3.05%, 0.05%, and 1.59%, respectively. In contrast, lower rates were observed during the long and short rainy seasons, indicating a clear influence of seasonal factors on FMD transmission dynamics. These findings are consistent with previous studies in Ethiopia, which reported increased FMD incidence during the dry season, largely attributed to intensified animal movement, livestock marketing, and congregation of animals at communal grazing and

watering points, where close contact facilitated viral transmission (Aman et al., 2020; Woldemariyam et al., 2022; Demil and Tadesse, 2025; Gizaw et al., 2025). The current findings align with previous studies, supporting the fact that dry-season environmental conditions and livestock management practices facilitated the spread of FMD. However, some studies have indicated that seasonal variation alone did not fully explain outbreak dynamics, as sporadic introductions of infected animals, heterogeneous vaccination coverage, and gaps in herd immunity may contribute to outbreaks (Aman et al., 2020). Therefore, although the dry season represented a clear high-risk period, FMD epidemiology in the study area was likely multifactorial, driven by interactions among animal movement patterns, production systems, and immunization status. The current findings highlighted the importance of implementing seasonally targeted control strategies, particularly pre-dry-season vaccination campaigns, strengthened animal movement control, and improved surveillance systems, to reduce the risk and impact of FMD outbreaks in the region.

The study found an overall morbidity of 1.95%, mortality of 0.02%, and a case-fatality rate of 1.28%, indicating widespread infection of FMD, but low fatality. District-level analysis revealed a heterogeneous distribution of outbreak severity. Bassonaworana, Midda, and Menz-Gera experienced the most outbreaks, whereas Shewarobit and Gishi recorded fewer. Elevated morbidity rates were noted in Merhabet (17.1%) and Siyadeber (9.1%), whereas most districts had rates below 5%. The current results supported the findings of Aman et al. (2020) and Gizaw et al. (2025), highlighting diverse patterns of FMD distribution across Ethiopian districts and pointing out specific high-risk zones.

Trends of vaccinations

Vaccination coverage for FMD in the North Shewa Zone, Ethiopia, was generally low and inconsistent, with only 12.3% of the at-risk cattle population vaccinated over the seven-year study period. Vaccination coverage indicated notable fluctuations, peaking in 2017 (19.2%) and 2022 (17.9%), with marked declines in 2018 (3.6%) and 2021 (5.1%). No vaccination activity was recorded in 2020, indicating a complete interruption in immunization efforts during that year.

Periods of high outbreak incidence, particularly in 2021 and 2023, coincided with low vaccination coverage, suggesting that inadequate and irregular immunization may have contributed to the persistence and recurrence of FMD outbreaks in the study area. The temporal overlap between reduced vaccination and increased outbreak occurrence highlighted the potential role of herd immunity gaps in sustaining viral transmission. The current findings were consistent with previous studies in Ethiopia, which have reported that FMD control was constrained by limited vaccine availability, irregular vaccination campaigns, and logistical challenges in delivery systems (Zewdie et al., 2023; Demil and Tadesse, 2025). Although vaccination is a key component of FMD control, outbreak dynamics are influenced by other epidemiological drivers, including animal movement patterns, production systems, and environmental conditions, as noted by Aman et al. (2020). The low and uneven vaccine coverage observed in the present study underscored the need for stronger, consistently implemented vaccination programs, particularly targeted to high-risk periods and high-incidence districts, to effectively reduce FMD transmission in the North Shewa Zone, Ethiopia.

CONCLUSION

The present seven-year retrospective study (2017-2023) demonstrated that FMD was endemic and widely distributed in North Shewa Zone, Ethiopia, with considerable spatial and seasonal variation in outbreak occurrence. A total of 162 outbreaks were reported across all districts, indicating persistent FMD circulation within the study area. The highest numbers of outbreaks and cases were recorded in 2021 and 2023, respectively, suggesting an upward trend in recent years. Major hotspot districts with the highest outbreak frequencies were Bassonaworana, Midda, and Menz-gera, while Merhabet and Siyadeber exhibited particularly high morbidity rates relative to their cattle populations, reflecting intense local disease transmission. Outbreaks occurred more frequently during the dry season, likely due to increased animal movement, livestock marketing, and congregation at communal grazing and watering points. Although mortality and case-fatality rates were generally low, the disease caused substantial morbidity and likely contributed to considerable economic losses through reduced livestock productivity and trade limitations. Low and inconsistent vaccination coverage throughout the study period appeared to play a major role in the persistence and recurrence of outbreaks. However, the present study relied on retrospective surveillance records, which may have underestimated the true burden of disease because of underreporting, incomplete records, and limited laboratory confirmation. The current findings emphasized the urgent need for strengthened surveillance and reporting systems, improved vaccine coverage and serotype matching, targeted strategic prophylactic vaccination of animals before the dry season in hotspot districts, enhanced farmer awareness, and strategic animal movement control measures. Further epidemiological studies are needed to understand transmission dynamics and reduce the impact of FMD in the North Shewa Zone, Ethiopia.

DECLARATIONS

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Authors' contributions

All aspects of the study, including study design, data collection, data analysis, interpretation of results, and manuscript preparation, were conducted solely by the author. The author independently conceptualized and designed this retrospective study on FMD in North Shewa, Ethiopia. All data were acquired and summarized solely from Disease Outbreak and Vaccination Activity Report (DOVAR) records and office-documented sources. The author conducted all data cleaning and statistical analyses, including the spatial heterogeneity in morbidity, outbreak patterns, and fatality rates, as well as the interpretation of the results. No external contributions were involved. The manuscript was drafted, critically revised, and finalized by the author, who also read and approved the final edition.

Availability of data and materials

All data are included in this article and its supplementary information files, available from the corresponding author upon reasonable request.

Conflict of interests

The author declared no competing interests.

Ethical considerations

Ethical issues, including plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy, have been checked by all the authors. In addition, an AI tool (ChatGPT) was used solely to assist in organizing the flow of ideas and improving the manuscript's clarity, while all scientific content and interpretations were developed and verified by the authors. Following the utilization of AI (Grammarly) for removing grammatical errors, the author has thoroughly reviewed and revised the manuscript and accepted full responsibility for the originality of the submitted manuscript.

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Fabrication and Evaluation of Absorbable Sutures Derived from Cellulose Fibers of Pineapple (*Ananas comosus*) Leaves

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ABSTRACT

Developing sustainable and biodegradable veterinary biomaterials is crucial for addressing limitations of synthetic sutures and avoiding secondary suture removal. The present study aimed to develop a novel, eco-friendly suture derived from pineapple leaves as an alternative to synthetic surgical threads and to assess its functional and clinical potential. The present study fabricated absorbable veterinary sutures from pineapple leaf fibers (PALF) via alkaline treatment and 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO)-mediated oxidation. The purified fibers, characterized by an average diameter of 53.61 ± 1.89 µm were successfully isolated via alkaline treatment and TEMPO-mediated oxidation, dyed naturally with *Peristrophe bivalvis*, coated with 1% chitosan, and subsequently twisted into size 3/0 sutures. *In vitro* degradation in phosphate buffer solution over four weeks confirmed their biodegradability, as demonstrated by increased fragility and strand separation. For the *in vivo* trial, 24 male mice (20-24 grams) were housed at a density of three mice per cage throughout the cultivation periods of 7, 14, 28, and 45 days. The mice were divided into two groups, including those that underwent surgery and were treated with PALF sutures (experimental group) and those that were treated with Polyglactin 910 (control group). A 2-3 cm dorsal-to-abdominal skin incision was made to expose the subcutaneous tissue, followed by a 1-2 cm connective tissue incision to establish the wound model for testing PALF sutures. Specific parameters, including the degree of edema and erythema, localized temperature, and the presence of exudate, suppuration, secondary infection, and hemorrhage, were assessed for each group. Critical complications, including wound dehiscence or marginal necrosis, were recorded. The current results demonstrated that PALF sutures were biocompatible and followed a healing pathway similar to that of Polyglactin 910. The present results revealed that PALF sutures had significantly lower secondary infection scores (0.08 ± 0.28) than Polyglactin 910 (0.58 ± 0.90), indicating superior anti-infective property. All clinical inflammatory signs completely resolved by day 45. The TEMPO-oxidized PALF sutures were a viable, biocompatible, and sustainable alternative to absorbable veterinary sutures, demonstrating effective tissue healing and a significantly lower incidence of secondary infection.

Keywords: Absorbable suture, *Ananas comosus*, Cellulose, Pineapple leaf, Mice

INTRODUCTION

Recent studies on bio-based materials have highlighted their safety and eco-friendly characteristics (Jain and Sinhan, 2022). Pineapple leaf fiber (PALF) is a natural fiber that can be processed to achieve a high cellulose content (Abu et al., 2019). The PALF cellulose has hydroxyl (-OH) groups that form stable polymer networks (Yadav et al., 2025). As a result of these polymer networks, cellulose fibers were macerated and developed into a productive technology, which was applied to composite materials across many fields, such as textiles, paper, and reinforced plastics (Mayakannan et al., 2023). A wide range of physicochemical and biotechnological approaches, such as alkaline treatment and hydrothermal pretreatment at high pressure, have been developed to accurately extract and isolate the components of fiber matrices, thereby optimizing the use of pineapple leaf waste in high-value applications (Saini et al., 2022; Esquivel et al., 2025; Liao et al., 2025). In Vietnam, the total area of pineapple cultivation encompasses approximately 52,000 hectares, of which 48,000 hectares are harvested annually (Pham et al., 2024). This large-scale production generated substantial agricultural by-products. Kien Giang, one of the Mekong Delta's major provinces in Vietnam, has about 7,850 hectares and an annual production of over 115,000 tons (Pham et al., 2024). Traditionally, post-harvest pineapple foliage has been regarded as a low-value residue, typically either left in the field or incorporated into the soil as green manure (Saini et al., 2023; Lalhruaitluangi and Mandal, 2024). Moreover, in fruit and vegetable processing facilities, most pineapple by-products are discarded in landfills, thereby imposing a significant environmental burden. A previous

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study has indicated that PALF was applied in the processing of high-value products, including textile-grade fibers, fiber-reinforced composites, and bromelain enzymes (Hazarika et al., 2018).

In surgery, absorbable sutures play a pivotal role in securing tissues, supporting the wound-healing cascade, and facilitating suture removal post-operation (Nappi, 2025). There are currently natural absorbable sutures, such as catgut and collagen (Gupta, 2025); neither is an ideal suture material for clinical use. Catgut suture and collagen sutures demonstrated unpredictable degradation during inflammation and suboptimal mechanical performance, including hydration-induced swelling and unreliable knot security (Pillai and Sharma, 2010). Besides natural absorbable sutures, polyglactin 910 is a synthetic absorbable suture composed of a 9:1 copolymer ratio of glycolide and lactide (Öksüz, 2024). Using Polyglactin 910 for suturing subcutaneous tissue after cesarean section is an effective solution to prevent complications such as fluid accumulation or wound infection. With clinical similarities to Polyglycolic acid, Polyglactin 910 has demonstrated its role as a reliable suture material, supporting patients' return to normal activities within a similar timeframe (Devi et al, 2023). Consequently, there is strong potential to develop bio-based absorbable sutures derived from natural sources and environmentally sustainable. Cellulose extracted from PALF is considered a highly promising material due to its superior mechanical strength, which optimizes biocompatibility and degradation profiles (Asim et al., 2015). Additionally, PALF benefits from the plentiful supply of raw materials derived from agricultural by-products. Therefore, the present study aimed to develop sustainable absorbable veterinary sutures from PALF via alkaline extraction and 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO)-mediated oxidation, and subsequently validate their mechanical performance, *in vitro* biodegradation kinetics, and *in vivo* tissue-healing efficiency in mice.

MATERIALS AND METHODS

Ethical approval

The study employed an incisional wound model, including wound closure, postoperative monitoring, clinical evaluation, and post-mortem analysis, in accordance with the guidelines of the Animal Welfare Council at Nong Lam University, Ho Chi Minh City, Vietnam. The authors considered ethical concerns, consent, animal welfare, and safety procedures before conducting the study.

Materials

A total of 3 kg of fresh pineapple leaves (*Ananas comosus* var. Queen) were collected after fruit harvesting from a pineapple farm in An Giang Province, Vietnam, during the harvest season (July to August, 2025). A total of 200 g of fresh leaves of *Peristrophe bivalvis*, locally known as Magenta, harvested from the same farm in An Giang Province, was used as a natural colorant for the chromogenic functionalization of cellulose filaments (Evitasari et al., 2019).

The materials used in the present study included TEMPO (Cool Chemical Science and Technology Company, China) chitosan (Biobasic Company, Canada), sodium bromide (NaBr; Xilong Scientific Company, China), sodium hypochlorite (NaClO; Xilong Scientific Company, China), hydrochloric acid (HCl; Xilong Scientific Company, China), acetic acid (CH_3COOH; Xilong Scientific Company, China), ethanol (Xilong Scientific Company, China), phosphate-buttered saline 10X solution (PBS; pH 7.2-7.6; Fisher Scientific Company, USA), and Sodium hydroxide (NaOH; Guangdong Guanghua Sci-Tech Company, China). Experimental equipment included a Cobalt-60 gamma irradiator, a Fourier transform infrared (FTIR) spectrometer (FTIR-6X1 type A, Serial No. A004962116, Jasco, Japan), and a field-emission scanning electron microscope (FE-SEM; S-4800, Hitachi, Japan). The tensile properties of PLAF single-strand suture samples were evaluated using a manual dead-weight loading configuration adapted from the ASTM D2256/D2256M protocols (ASTM, 2002).

Pineapple leaf fiber-derived suture fabrication

Decortication of pineapple leaf cellulose fibers

A total of 3 kg of pineapple leaves were mechanically decorticated by manually scraping the surface using a metal spoon to remove the outer waxy epidermis and parenchyma tissues. This process exposed the longitudinal vascular bundles of cellulose fibers. The decortication process yielded approximately 45 g of fiber bundles, which were then thoroughly rinsed with distilled water to remove any remaining impurities.

Alkaline treatment of pineapple leaf fibers

Alkaline treatment was performed by immersing the cellulose fibers in a 3% (w/v) NaOH solution at room temperature (22°C) for 60 minutes, maintaining a liquor ratio of 1:20. This process aimed to disrupt the hydrogen bonding in the cellulose fiber's network structure, adapted from Motaleb et al. (2018), and solubilize non-cellulosic components, such as lignin and hemicellulose. Consequently, alkaline treatment yielded higher-purity cellulose fiber, defibrillated clustered bundles, and increased surface roughness, thereby enhancing interfacial adhesion (Gnanasekaran

et al., 2021), potentially increasing inter-fiber friction during the braiding process. Finally, all 45 g of treated fibers were thoroughly rinsed with distilled water to remove residual NaOH, then air-dried at room temperature (22°C) for 15 minutes.

TEMPO-mediated oxidation of pineapple leaf fiber

The TEMPO-mediated oxidation of the PALF cellulose fibers was conducted in the TORC system (TEMPO/NaBr/NaClO; Araya-Chavarría et al., 2022). The TEMPO/NaBr catalyst mixture was prepared with a molar ratio of 1:10 mmol/g of fiber and dissolved in an appropriate volume of distilled water until the fibers' viscosity disappeared. After the alkaline treatment, the fibers were added to the TEMPO/NaBr solution and stirred continuously to ensure the fibers were fully wetted (Araya-Chavarría et al., 2022). Before initiating the oxidation reaction, the pH was adjusted to 10-11 with 0.5 M NaOH to optimize the selective oxidation of cellulose (Lin et al., 2018). Once the target pH was reached, NaClO at a concentration of 5 mmol/g of fiber was added to the solution by slow, dropwise addition. The mixture was stirred continuously for 45-60 minutes to control the oxidation rate and maintain pH stability. During the reaction, 0.5 M NaOH was added as required to maintain a pH of 10-11, which was continuously monitored with litmus paper. After the addition of NaClO, the mixture was continuously stirred for 10-15 minutes to ensure complete oxidation. Subsequently, the reaction was stopped by adding a 2:1 ethanol-NaClO mixture, and the mixture was stirred for 2-5 minutes to neutralize residual NaClO and stabilize the oxidized product. Finally, the mixture was neutralized to pH 7.0 by dropwise addition of 0.1 M HCl. The PALF cellulose fibers were then thoroughly rinsed with distilled water and air-dried at room temperature for 15 minutes.

Natural dyeing process of pineapple leaf fiber

The natural dyeing process of the PALF-derived cellulose fibers was conducted using an anthocyanin-rich extract from magenta leaves (Le et al., 2021). The dye was prepared by aqueous extraction, in which dried leaves were immersed in distilled water at a 1:10 (w/v) material-to-solvent ratio. The mixture was heated at 80-90°C for 10-15 minutes, then filtered through Whatman No. 1 filter paper (Merck Company, Germany) with a pore size of 11 µm to obtain a clear pigment solution. The dye bath pH was adjusted to 3-4 using acetic acid (CH3COOH) to stabilize the anthocyanins and preserve their purple hue. The cellulose fibers were then immersed in the dye solution at 60-70°C for 30-60 minutes to enhance dye adsorption. After dyeing, the fibers were gently rinsed with cold distilled water and air-dried at room temperature. This procedure was based on findings that anthocyanins were most stable in acidic media and are effective as natural colorants for cellulosic substrates (Le et al., 2021).

Suture fabrication and chitosan coating of pineapple leaf fiber

The TEMPO-oxidized cellulose monofilaments were twisted (2-3 strands) to achieve a 3/0 suture size. Subsequently, a 1% (w/v) chitosan solution, characterized by a molecular weight of 10-20 kDa and a degree of deacetylation of at least 90%, was prepared by dispersing 1g of chitosan in 100 mL of 1% acetic acid buffer. The mixture was stirred at 400 rpm for six hours to ensure homogeneity (Akter et al., 2020). The twisted filaments were immersed in the chitosan solution at a 1:20 (w/v) ratio for 60 minutes at room temperature to ensure a uniform polymeric layer. Following a brief rinse with distilled water, a secondary coating was applied for another 60 minutes. After the coating process, the fibers were rinsed three times with distilled water to remove excess surface chitosan and dried in a laboratory drying oven (UF55, Memmert, Germany) at 40°C for 60 minutes.

Gamma irradiation sterilization of pineapple leaf fiber sutures

The radiation processing of the developed PALF sutures was performed at the Biotechnology Center of Ho Chi Minh City, Inc., Vietnam, using a Co-60 gamma source at a dose rate of 5 kGy/h for four hours, yielding a total dose of 20 kGy. The baseline understanding of radiation-induced chemical effects, including macromolecular chain scission and free-radical mechanisms in polymer backbones, was derived from studies on gamma-irradiated polylactic acid (PLA) and PLA/cellulose fibers (Aouat et al., 2019), along with established biopolymer radiation standards (Al-Assaf et al., 2016).

Evaluation of breaking force

The tensile properties of the single-strand suture samples were evaluated using a manual dead-weight loading configuration, adapted from ASTM D2256/D2256M protocols (ASTM, 2002). One end of the thread was secured to a fixed point with a type O knot to establish the initial gauge length (cm), while the opposing end was progressively loaded with calibrated metal weights until complete rupture occurred. The time-to-break (seconds) was recorded to ensure consistency.

The characteristics of pineapple leaf fiber sutures

Morphology and structure of pineapple leaf fiber sutures

An FTIR equipped with an ATR PRO ONE X tool (S/N: A002962140, JASCO, Japan) was employed to assess the analysis of fiber chemical composition. This analysis aimed to examine the chemical functional composition of the fibers

before and after chemical treatment in the wavenumber range of 4,000-400 cm^{-1} (Dharanendra et al., 2025). Observation of surface morphology and fiber dimensions was carried out using the FE-SEM to observe the surface morphology of the fibers post-chemical treatment. The SEM micrographs were subsequently processed in ImageJ version 1.53 to calculate fiber diameters (Götz et al., 2021).

In vitro degradation of pineapple leaf fiber sutures

The sutures were immersed in 50 mL of PBS with a pH range of 7.2 to 7.6 during the two- and four-week periods. The degradation level of the sutures was subsequently analyzed using FTIR spectroscopy (4,000- 400 cm^{-1}) at the Ho Chi Minh City Biotechnology Center, Vietnam, following the methodology described by Antoniac et al. (2021).

***In vivo* evaluation and tissue compatibility of pineapple leaf fiber sutures**

Experimental design

A one-factor randomized complete block design was employed, which consisted of two suture types, including the bio-based PALF sutures 3/0 and the commercial Polyglactin 910 3/0 (Medico, China) as the control. The clinical performance of tissues was assessed based on five key inflammatory parameters, including edema, erythema, exudate formation, secondary infection, and hemorrhage, which were evaluated at 7, 14, 28, and 45 postoperative days, using a semi-quantitative scoring system and a clinical scoring system for evaluating local tissue reactions to sutures in mice (Karner et al., 2020). These time points were strategically selected to encompass the entire wound-healing process, from the acute and subacute inflammatory phases to complete tissue regeneration. Scores ranged from 0 to 3, corresponding to absent, mild, moderate, and severe reactions, respectively, adopted from the study of Karner et al. (2020; Table 1). Following a completely randomized design (CRD), mice were divided into two treatment groups, with three independent biological replicates per group at each time point.

Table 1. Clinical evaluation scale for *in vivo* suture response in mice

Score	Edema	Erythema	Exudate formation	Secondary infection	Hemorrhage
0	None	None	None	None	None
1	Mild swelling	Pale pink	Serous fluid	Localized infection	Mild peri-sutural redness
2	Moderate swelling	Distinct redness	Slightly cloudy discharge	Extension to adjacent tissues	Slight oozing of blood
3	Severe swelling, suture site deformation	Deep red, extensive spreading	Purulent discharge (pus)	Systemic involvement	Active bleeding

Scores: 0: None, 1: Mild, 2: Moderate, 3: Severe. Source of scoring adopted from the study of Karner et al. (2020).

Animals

A total of 24 healthy adult male albino mice, each 2 months old and weighing between 20 and 24 g, were randomly assigned to two groups (n = 12 per group, with three replicates) corresponding to two suture types (Karner et al., 2020). One group was treated exclusively with PALF sutures (experimental group), and the other group was treated with Polyglactin 910 (control group).

The experimental setup was specifically designed to eliminate immune interference by separating groups, ensuring that inflammatory or healing responses were solely due to the suture material and preventing cross-interaction or signal interference. Only male mice were used in the present experiment to minimize biological variability associated with hormonal fluctuations. Female mice undergo estrous cycles, during which estrogen and progesterone levels vary and can significantly influence inflammatory responses and tissue repair processes (Beery et al., 2011). These hormonal variations may introduce additional variability in wound healing outcomes. Therefore, using male mice ensured more stable physiological conditions and improved the reliability of the experimental results. Three mice were euthanized at each time point (at 7, 14, 28, and 45 days) to collect tissue samples. The sample size was determined in accordance with ethical considerations to minimize animal use, while ensuring an adequate number of biological replicates for histological and inflammatory evaluations, in alignment with the principles of the Replacement, reduction, and refinement (3Rs) in animal studies (Kilkenny et al., 2010; MacArthur, 2018). This design ensured independent measurements and avoided repeated sampling bias.

Housing and feeding

Prior to the surgical procedure, the mice underwent a 7-day acclimatization period under controlled environmental conditions to ensure physiological and psychological stability (Obernier et al., 2006). The mice were housed in eight iron cages (three mice per cage, grouped by treatment and designated evaluation time point) measuring 47 × 30 × 30 cm, with rice husk used as bedding material. The rice husk bedding was completely replaced every 2-3 days or spot-cleaned earlier when excessive feces or urine accumulated. Established protocols for nutrition and hygiene were strictly maintained to ensure uniformity across all mice, eliminating potential confounding variables that could interfere with the experimental outcomes. The feed, manufactured by Vienovo in Vietnam, comprised pellets containing 17% crude protein, 1.5% calcium, and 2,700 kcal of energy, in accordance with the National Research Council (NRC, 2011).

Anesthesia and surgical procedure

Pre-operative preparations were carefully performed for all surgical procedures. Before surgery, the mice's health status, including normal dietary intake, active locomotion, and the absence of aggressive behavior or fighting, was thoroughly re-evaluated to ensure the absence of any pathologies. To reduce handling-related stress, the daily welfare of animals was evaluated using four key visual clinical indicators, including activity level, respiratory health, coat condition, and body posture (University of Queensland, 2021). Following a general health assessment, general anesthesia was induced using a combination of tiletamine 25 mg/mL and zolazepam 25 mg/mL (Zoletil 50, Virbac Company, Vietnam) at 1 mg/kg (Cagle et al., 2017). When the mice achieved a deep state of anesthesia, the dorsal-lateral region was shaved and disinfected with alcohol and Povidone-iodine 10%. A 2-3 cm skin incision was made from the dorsal midline towards the abdominal area. This incision was carefully performed to an appropriate depth to expose the subcutaneous connective tissue, followed by a 1-2 cm incision through the underlying connective tissue layer to establish the wound model (Kandimalla et al., 2016). In the experimental group, the subcutaneous incisions were closed using PALF 3/0 sutures. For the control group, the same layer (the underlying connective tissue) was sutured with 3/0 Polyglactin 910. The skin layer was closed with 3/0 nylon (Medico, China). All wounds were sealed with a simple interrupted pattern; this method provided strong security for each strand but increased the volume of foreign bodies at the wound site due to multiple knots, thereby increasing closure times (Nair et al., 2023). For postoperative prophylactic care, enrofloxacin (50 mg/mL; Bio Pharmachemie Company, Vietnam) was diluted 1:50 in sterile 0.9% NaCl to a final concentration of 1 mg/mL. The diluted solution was administered subcutaneously once daily at a dose of 5 mg/kg, which was within the reported dosage range for veterinary enrofloxacin use (Plumb, 2018). Treatment was continued for five consecutive days following surgery. The five-day administration period was selected as part of the present postoperative management protocol to provide antimicrobial coverage during the early phase of wound healing and was not intended to represent a universally recommended duration. Additionally, meloxicam (Bio Pharmachemie Company, Vietnam) was administered via intraperitoneal injection at a dose of 28.4 µmol/kg every 24 hours for 5 consecutive days following the surgical procedure (Santos et al., 1998).

Statistical analysis

All statistical analyses were performed using GraphPad Prism 9.0 (GraphPad Software, San Diego, CA, USA). Data were expressed as the mean ± standard deviation (SD). A two-way analysis of variance (ANOVA) was conducted to evaluate the effects of suture type (PALF versus Polyglactin 910), observation day (7, 14, 28, and 45 days post-surgery), and their interaction on each clinical parameter (erythema, edema, exudate formation, secondary infection, and hemorrhage). Before ANOVA, the assumption of normality was assessed using the Shapiro-Wilk test, and homogeneity of variances was assessed using Levene's test. When a significant main effect was detected, post hoc comparisons were performed using Tukey's honestly significant difference (HSD) test for multiple comparisons across observation days. Independent t-tests were used to compare suture types at each individual time point. Statistical significance was defined at a p-value less than 5% ($p < 0.05$).

RESULTS

Chemical composition of untreated pineapple leaf fibers

The FTIR spectra of raw PALF exhibited typical absorption bands of lignocellulosic materials (Figure 1). The band at 1,590.5 cm^{-1} represented the C=C aromatic skeletal vibrations of lignin, which constituted a significant part of pineapple leaves. Additionally, a prominent absorption peak at 1,029.33 cm^{-1} was attributed to the C–O and C–O–C stretching vibrations in β-1,4-glycosidic bonds within cellulose and hemicellulose, reflecting the presence of polysaccharide components in the fiber structure. Meanwhile, the absorption band at 2,360.44 cm^{-1} did not represent chemical bonds in cellulose, hemicellulose, or lignin and was likely due to atmospheric CO_2 or background interference. Thus, the major absorption bands in the FTIR spectra confirmed the lignocellulosic nature (cellulose, hemicellulose, and lignin) of the PALF.

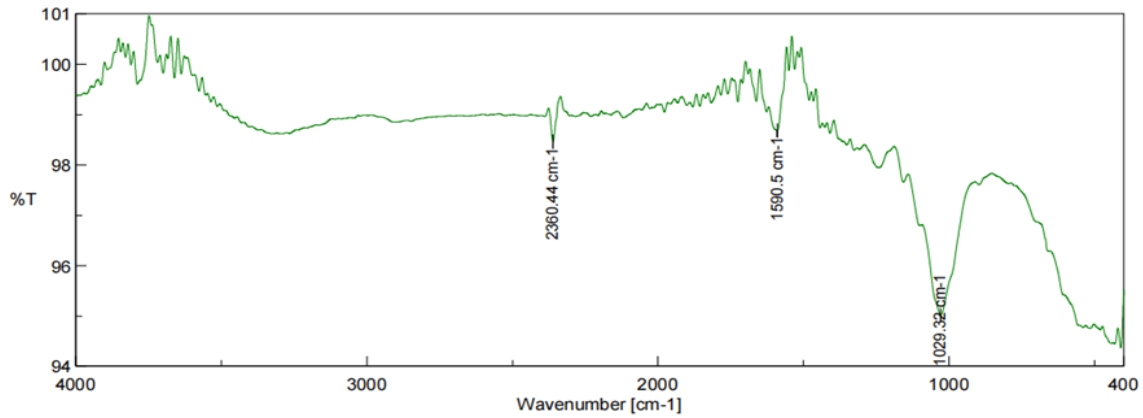


Figure 1. Fourier-transform infrared spectroscopy of untreated pineapple leaf fibers. Fourier-transform infrared spectra of untreated pineapple leaf fibers with major absorption peaks at 1590 cm-1 for C=C stretching of lignin and 1029.33 for C–O and C–O–C glycosidic linkages. The peak at 2360.44 cm-1 represents background CO_2 absorption.

Structure and morphology of treated pineapple leaf fibers

The SEM micrographs revealed that at 1,000x magnification, following 3% NaOH alkaline treatment and TORC/TEMPO-mediated oxidation, the inter-fiber binding components, such as hemicellulose and lignin, were effectively removed. This resulted in a significant amount of treated fiber with bundle defibrillation and a more porous structure, with an average fiber diameter of 53.61 ± 1.89 µm across five measured fibers ($p < 0.05$; Figure 2A). At 2,000x magnification, the fiber surface exhibited prominent longitudinal grooves and a high degree of roughness, characterized by numerous furrows and microstructures extending along the fiber axis. These characteristics enhanced frictional resistance and mechanical interlocking during the fiber spinning process (Figure 2B). The transformation from a smooth surface to a porous, rough structure in treated fibers demonstrated the efficacy of the alkaline treatment and TEMPO-mediated oxidation (chemical treatments) in increasing surface contact area. This structural modification resulted in fundamental biodegradability and tissue-adhesion properties, which were highly critical for its application as a suture material in veterinary surgery. In Figures 2A and 2B, small scattered particles were observed on the fiber surface, possibly attributable to residual debris from the chemical treatments. However, these particles did not form a continuous coating and required further FTIR spectroscopic analysis to identify their chemical nature.

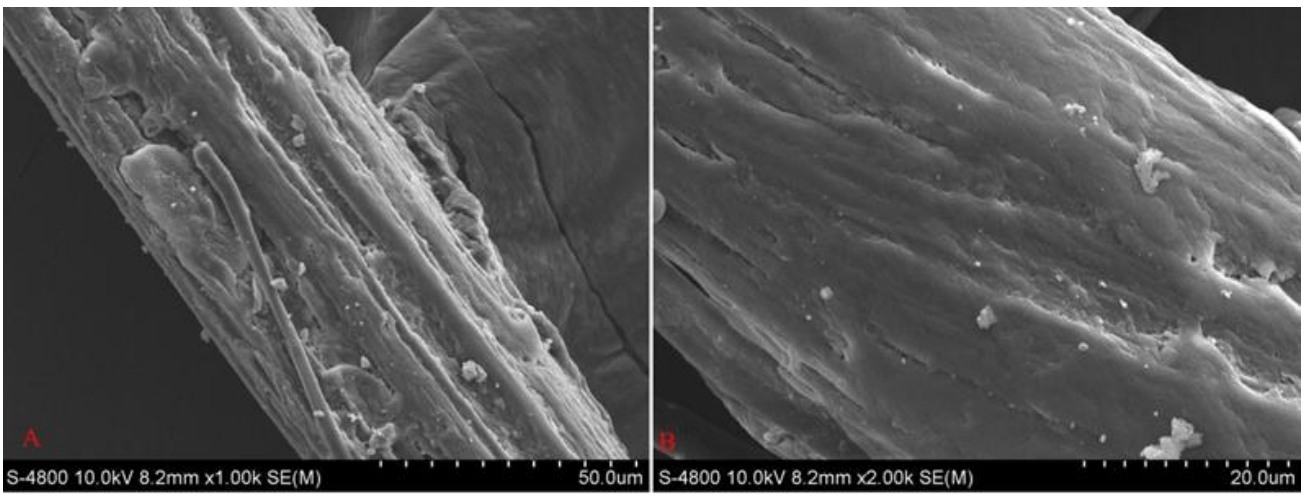


Figure 1. Scanning electron microscope micrographs of pineapple leaf fibers after alkaline treatment and TEMPO-mediated oxidation (TORC). **A**: Fiber structure and bundle defibrillation at 1,000x magnification, **B:** Rough fiber surface and longitudinal grooves at 2,000x magnification.

Figure 3 illustrates the FTIR spectra of treated fibers, revealing a broad band in the 3,500-3,200 cm^{-1} range, which corresponds to the -OH stretching of cellulose molecules. Following chemical treatment, the increased intensity of the peak near 3,300 cm^{-1} indicated greater exposure of hydroxyl groups within the polysaccharide backbone of PALF. The peak at 2,915.84 cm^{-1} corresponded to the symmetric C–H stretching vibrations of CH_2 groups, typical of cellulose and hemicellulose. In the spectra of the PALF-treated fibers, only a weak absorption peak at 1,158.53 cm^{-1} was observed, attributed to the arabinose side chains in hemicellulose. The peak at 1,590.5 cm^{-1}, which indicated C=C aromatic skeletal vibrations of lignin, disappeared after treatment. This indicated a significant decrease in intensity compared to the raw

PALF spectrum, in which the lignin-associated peak was observed at 1,590.5 cm^{-1} ($p < 0.05$; Figure 1). This finding confirmed that lignin was partially removed from the fiber structure. The peak at 2,359.96 cm^{-1} was attributed to atmospheric carbon dioxide (CO_2) interference from the environment or equipment, rather than to chemical bonds in the lignocellulosic components. The emergence of a peak at 431.012 cm^{-1} in the 500-150 cm^{-1} range, characteristic of metal-halogen bond vibrations (M-Cl, M-Br, M-F), suggested the potential presence of inorganic salt residues, such as NaCl or NaBr, remaining in the sample after treatment. Furthermore, absorption peaks at 1,630.52 cm^{-1} and 1,314.25 cm^{-1} indicated the asymmetric stretching vibrations of carboxyl groups ($–COO^–$). Consequently, regenerated cellulose produced via TEMPO-mediated oxidation (TORC) yielded a fiber material suitable for sutures, exhibiting both biodegradability and optimal mechanical properties.

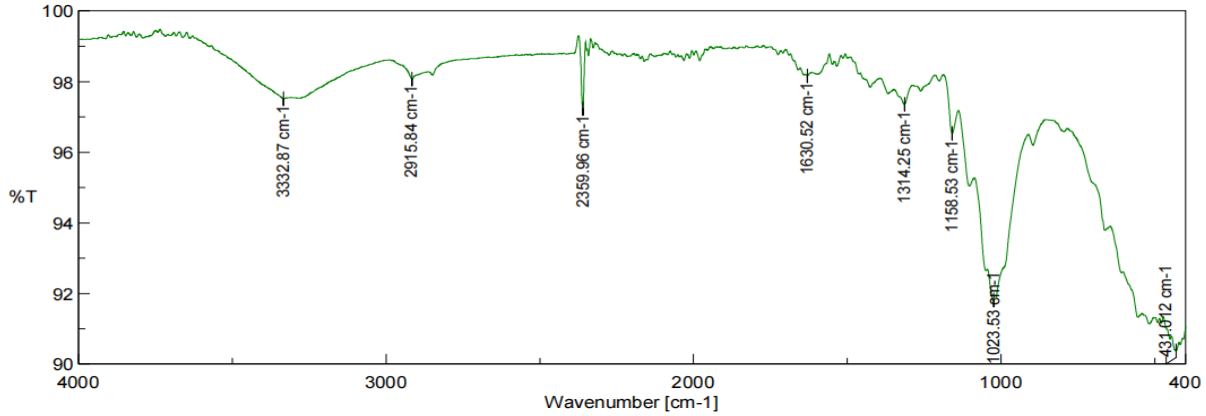


Figure 2. Fourier-transform infrared spectroscopy spectra of pineapple leaf fibers after treatment with alkaline and intermediate oxidation (TEMPO). Fourier-transform infrared spectra of treated pineapple leaf fibers with major absorption peaks at 3500-3200 cm^{-1} for O–H stretching vibrations of cellulose, 2915.84 cm^{-1} for symmetric C–H stretching of CH_2 groups, and 1630.52 cm^{-1} and 1314.25 cm^{-1} for asymmetric stretching of carboxylate groups ($–COO^–$). Additionally, the peaks at 1158.53 cm^{-1} and 431.01 cm^{-1} represent arabinose side chains of hemicellulose and metal–halogen bond vibrations (M–Cl, M–Br, M–F), respectively. The peak at 2359.96 cm^{-1} represents atmospheric CO_2 absorption.

In vitro degradation evaluation of pineapple leaf fiber suture

After two weeks of *in vitro* experiment, the PLAF suture retained its fibrous form; however, the PLAF suture exhibited a significant reduction in tensile strength compared to the original sample of PLAF ($p < 0.05$). By the fourth week, the fiber surface had lost its structural homogeneity, with fibrillation and strand separation observed at multiple sites. The current findings indicated a clear degradation in mechanical properties, with the fiber becoming more brittle, exhibiting decreased elasticity, and being prone to fracturing under minimal mechanical stress. Empirical results indicated that the single-strand suture samples of PALF had a maximum breaking force of about 2.5 ± 0.6 N (approximately 180-330 gf), effectively establishing the mechanical limits and breaking strength of these samples. A high length-to-diameter ratio facilitated the development of an effective fiber network, enabling small-diameter fibers (average diameter of 53.61 ± 1.89 µm) to be transformed into 3/0-grade medical sutures.

Animal bio-assay and *in vivo* analyses of pineapple leaf fiber sutures

Erythema and edema

The present results indicated that observation day significantly affected erythema scores ($F = 18.83$, $p < 0.05$), while suture type did not have a significant main effect ($F = 1.50$, $p > 0.05$; Table 2). There was no significant interaction between suture type and observation day ($F = 0.61$, $p > 0.05$). The current results revealed that scores on days 7 (2.00 ± 0.63) and 14 (1.50 ± 0.55) were significantly higher than those on days 28 (0.67 ± 0.51) and 45 (0; $p < 0.05$), with no significant difference between days 7 and 14 or between days 28 and 45 ($p > 0.05$). Both suture types elicited peak inflammatory responses on day seven post-surgery. Sites sutured with PALF sutures had an erythema score of 2.33 ± 0.58, compared to 1.67 ± 0.58 for those with Polyglactin 910. However, this difference was not statistically significant ($p > 0.05$). Erythema gradually decreased over time, with minimal signs by day 28 (0.67 in both groups) and complete resolution by day 45. Similarly, a significant main effect of observation day was found for edema scores ($F = 11.46$, $p < 0.05$), whereas no significant main effect of suture type ($F = 3.13$) or interaction effect ($F = 1.13$) was observed ($p > 0.05$). The current results indicated that edema scores on day seven (1.83 ± 0.75) were significantly higher than those on days 28 (0.67 ± 0.52) and 45 (0; $p < 0.05$), while day 14 score (1.33 ± 0.82) was not significantly different from any other time point ($p > 0.05$). Edema peaked on day seven, with PALF sutures scoring higher (2.33 ± 0.58) than Polyglactin 910 (1.33 ± 0.58), but the difference was not statistically significant ($p > 0.05$). By day 45, edema had completely resolved in both groups.

Exudation and clinical complications

Exudate formation demonstrated a significant effect based on the observation day ($F = 11.46$, $p < 0.05$), but there was no significant effect of suture type ($F = 1.12$, $p > 0.05$; Table 2). The current findings demonstrated that exudate scores on day seven (1.67 ± 0.81) were significantly higher than those on days 28 and 45 (0; $p < 0.05$), while the day 14 score (0.83 ± 0.75) was not significantly different from any other time point ($p > 0.05$). Exudation was primarily observed within the first 14 days post-surgery. At day seven, PALF sutures exhibited higher exudate scores (2.00 ± 1.00) than Polyglactin 910 (1.33 ± 0.58), although this difference was not statistically significant ($p > 0.05$). From day 28 onward, exudation was absent in both groups.

Secondary infection

Both suture type and observation day significantly affected secondary infection scores ($p < 0.05$), with effects having F values of 6.00 and 5.33, respectively, indicating their individual impact on infection rates. However, the interaction effect was not statistically significant ($F = 2.44$, $p > 0.05$; Table 2). The current results indicated that infection scores on day seven (1.00 ± 0.89) were significantly higher than those on days 28 and 45 (0; $p < 0.05$), while the day 14 score (0.33 ± 0.82) was not significantly different from any other time point ($p > 0.05$). Notably, PALF sutures demonstrated superior performance against secondary infection, with a significantly lower mean infection score (0.08 ± 0.28) than Polyglactin 910 (0.58 ± 0.90) throughout the observation period ($p < 0.05$). Independent t-tests conducted at each time point indicated that the most significant difference occurred on day seven, with PALF sutures having lower infection scores (0.33 ± 0.58) than Polyglactin 910 (1.67 ± 0.58).

Hemorrhage

The observation day had a significant impact on hemorrhage scores ($F = 4.50$, $p < 0.05$). The present results demonstrated that hemorrhage scores on day seven (0.50 ± 0.55) were significantly higher than those on days 14, 28, and 45 (0; $p < 0.05$). In contrast, suture type demonstrated no significant effect ($F = 0.50$), and there were no interaction effects ($F = 0.50$, $p > 0.05$). Hemorrhages were mild and limited to the first seven days after surgery, with complete recovery by day 14 in both groups.

Overall clinical outcomes

From day 28 to day 45, no signs of systemic infection or tissue necrosis were recorded in either group. By day 45 after surgery, all clinical signs of inflammation, such as erythema, edema, exudate, secondary infection, and hemorrhage, had completely resolved in both treatment groups. No wound dehiscence or marginal necrosis was observed in any of the mice.

Table 2. Assessment of clinical parameters for pineapple leaf-derived absorbable sutures and polyglactin 910 sutures at 7, 14, 28, and 45 days post-implantation

Clinical parameters	Suture type (L)	Observation day (N)				Mean (L)
		7	14	28	45	
Erythema	PALF	2.33 ± 0.58	1.67 ± 0.58	0.67 ± 0.58	0	1.67 ± 1.03
	Polyglactin	1.67 ± 0.58	1.33 ± 0.58	0.67 ± 0.58	0	0.92 ± 0.79
Mean (N)		2.00 ± 0.63^{a}	1.50 ± 0.55^{a}	0.67 ± 0.51^{b}	0^{b}	
$F_L = 1.50^{ns}$, $F_N = 18.83^{***}$, $F_{L\times N} = 0.61^{ns}$						
Edema	PALF	2.33 ± 0.58	1.67 ± 1.15	0.67 ± 0.58	0	1.17 ± 1.11
	Polyglactin	1.33 ± 0.58	1.00 ± 0.00	0.67 ± 0.58	0	0.75 ± 0.62
Mean (N)		1.83 ± 0.75^{a}	1.33 ± 0.82^{ab}	0.67 ± 0.52^{bc}	0^{c}	
$F_L = 3.13^{ns}$, $F_N = 11.46^{***}$, $F_{L\times N} = 1.13^{ns}$						
Exudate formation	PALF	2.00 ± 1.00	1.00 ± 1.00	0	0	0.75 ± 1.05
	Polyglactin	1.33 ± 0.58	0.67 ± 0.58	0	0	0.50 ± 0.67
Mean (N)		1.67 ± 0.81^{a}	0.83 ± 0.75^{ab}	0^{b}	0^{b}	
$F_L = 1.12^{ns}$, $F_N = 11.46^{***}$, $F_{L\times N} = 0.46^{ns}$						
Secondary infection	PALF	0.33 ± 0.58	0.67 ± 1.16	0	0	0.08 ± 0.28^{B}
	Polyglactin	1.67 ± 0.58	0	0	0	0.58 ± 0.90^{A}
Mean (N)		1.00 ± 0.89^{a}	0.33 ± 0.82^{ab}	0^{b}	0^{b}	
$F_L = 6.00^{*}$, $F_N = 5.33^{**}$, $F_{L\times N} = 2.44^{ns}$						
Hemorrhage	PALF	0.67 ± 0.58	0	0	0	0.17 ± 0.39
	Polyglactin	0.33 ± 0.58	0	0	0	0.08 ± 0.28
Mean (N)		0.50 ± 0.55^{a}	0^{b}	0^{b}	0^{b}	
$F_L = 0.5^{ns}$, $F_N = 4.5^{*}$, $F_{L\times N} = 0.5^{ns}$						

PALF: Pineapple leaf-derived absorbable sutures, Polyglactin: Polyglactin 910 sutures, L: Suture type factor, N: Observation day factor, F_L: F-value for the main effect of suture type, F_N: F-value for the main effect of observation day, $F_{L\times N}$: F-value for the interaction effect between suture type and observation day, ns: Not significant ($p > 0.05$), *****: $p < 0.05$, ******: $p < 0.01$, *******: $p < 0.001$. Data are expressed as mean ± standard deviation (SD). ^{a,b,c} Mean different superscript letters within the same row indicate statistically significant differences between observation days at $p < 0.05$. $^{A, B}$ Mean different uppercase superscript letters within the same column indicate statistically significant differences between suture types at $p < 0.05$.

DISCUSSION

In the present study, PALF were mechanically decorticated by scraping and subjected to an alkaline treatment (3% NaOH) to solubilize non-cellulosic components, such as lignin and hemicellulose. This process defibrillated clustered bundles and increased surface roughness, which in turn improved the inter-fiber friction during fabrication. These characteristics, referring to the length and strength of cellulose, remain unchanged under mechanical scraping and alkaline treatment (3% NaOH), consistent with the findings of Amornsakchai et al. (2023). The FTIR spectra demonstrated a strong presence of cellulose in PALF. Furthermore, the PALF/PBS material exhibited a flexural strength of 70.7 MPa, a flexural modulus of 2.0 GPa, and a heat deflection temperature of 107.3 °C, which were consistent with the findings of Amornsakchai et al. (2023).

The TEMPO-mediated oxidation system (TORC) successfully converted hydroxyl groups to carboxyl groups (–COO–), which was verified by FTIR absorption peaks at 1630.52 cm^{-1} and 1314.25 cm^{-1}. This TORC technology was similarly utilized by Park et al. (2023) to produce TEMPO-oxidized lignocellulose nanofibrils (TOLCNF). Furthermore, Park et al. (2023) reported that integrating silver nanoparticles was a promising approach to imparting antibacterial properties. Mathew et al. (2024) suggested that these antibacterial effects could be essential in reducing secondary infections observed on day seven in the PALF suture site. The biocompatibility of PALF sutures was comparable to that of the commercial control, Polyglactin 910, with no notable differences observed in erythema or edema responses. *Peristrophe bivalvis* (Magenta) extract was used solely as a natural coloring agent to enhance the visual contrast of sutures against surrounding tissues and blood during surgical observation. This purple color facilitated suture identification and handling rather than serving as an experimental factor. In the present study, there were no significant effects on the degradation behavior or inflammatory response of the PALF suture.

The degradation mechanism of PALF, determined by TEMPO-mediated oxidation, revealed hydrolytic behavior comparable to that of synthetic polymers. Antoniac et al. (2021) reported that in PBS medium (pH 7.2–7.6), fibrillation and fiber separation occurred, leading to a decrease in tensile strength by the fourth week. Antoniac et al. (2021) demonstrated that polyglycolic acid samples and poly lactic-co-glycolic acid entirely lost their load-bearing capacity within 4-6 weeks of immersion due to their highly hydrophilic properties. Because the purified PALF in the present study had a high cellulose content and an optimal crystallinity index, it ensured knot security and biosafety for modern veterinary surgery.

The FTIR spectrum of untreated PALF exhibited characteristic absorption bands indicative of its lignocellulosic nature. The broad absorption band in the 3,200- 3,600 cm^{-1} region corresponded to O–H stretching associated with hydrogen bonding in cellulose, hemicellulose, and lignin, which was consistent with the FTIR characteristics of lignocellulosic fibers reported by Mandal and Chakrabarty (2011). Similarly, the presence of lignin in the untreated PALF was confirmed by the C=C aromatic skeletal band at 1,590.5 cm^{-1}, comparable to the lignin-associated peaks previously observed in natural plant fibers. Furthermore, the dominant absorption peak at 1,029.33 cm^{-1} corresponded to the C–O–C stretch of β-1,4-glycosidic bonds, consistent with the cellulose polysaccharide backbone reported by Kacuráková et al. (2000). These functional groups confirmed that the untreated PALF possessed a typical lignocellulosic structure composed mainly of cellulose, hemicellulose, and lignin. After alkaline treatment and intermediate oxidation (TEMPO), a broad absorption band in the 3,500-3,200 cm^{-1} region appeared in both untreated and treated PALF, consistent with the hydroxyl (-OH) stretching vibrations in the cellulose structure of plant fiber reported by Mandal and Chakrabarty (2011). Although cellulose and hemicellulose both contain similar oxygen-containing functional groups (Yang et al., 2007), the effectiveness of the purification was verified by the disappearance or only a very weak hemicellulose-specific peak at 1,158.53 cm^{-1} (Zhang et al., 2021). The findings reported by Zhang et al. (2021) were consistent with the present study, demonstrating that the chemical treatment successfully removed the majority of the hemicellulose from the fiber structure. Notably, the appearance of peaks at 1,630.52 cm^{-1} and 1,314.25 cm^{-1} corresponded to the stretching vibrations of carboxyl groups ($–COO^-$). These findings aligned with the typical locations of carboxyl groups as reported by Kaya (2025). The formation of carboxyl groups on the surface of regenerated cellulose not only confirmed the effectiveness of the fiber functionalization process but also provided a critical basis for producing suture materials with biodegradability and ideal mechanical properties for biomedical applications.

The surface morphology of the PALF sutures after chemical treatment was crucial to understanding the material's biomechanical performance in surgical use. The presence of deep longitudinal grooves and distinct fibrillation indicated that amorphous binding components, mainly hemicellulose and lignin, have been effectively removed. These morphological changes were consistent with previous studies of Tanpichai and Witayakran (2017) and Gnanasekaran et al. (2021), who reported increased surface roughness and enhanced fiber bundle separation following chemical treatment of PALF. From a veterinary surgical perspective, this PALF surface restructuring offered particularly crucial clinical advantages. First, the highly roughened surface would increase the friction coefficient between the suture loops.

According to Wong and McGrouther (2023), surface roughness was identified as a key factor enhancing knot security compared with smooth synthetic monofilament sutures, thereby decreasing the risk of wound dehiscence due to strong postoperative movement in animals. Second, the presence of microgrooves and a porous structure notably increased the material's specific surface area. Ashworth et al. (2014) demonstrated that material porosity directly influenced fluid diffusion capacity and degradation rate, thereby allowing the infiltration of biological fluids and cellular proteolytic enzymes during *in vivo* degradation. In an *in vivo* environment, these microgrooves and porous structures enabled tissue fluids to permeate quickly and allowed inflammatory cells, such as macrophages and foreign-body giant cells, to infiltrate more easily. Märtson et al. (1999) indicated that the presence of these biological fluids and cellular components led to a gradual, multi-faceted degradation process. This process involved chemical and biological breakdown of the cellulose matrix, accompanied by microstructural fragmentation. Thus, the porous microstructure and improved fluid permeation supported the suture's stable, controlled degradation, meeting the key criteria for an ideal absorbable biomaterial.

Erythema and edema reached their highest levels on day seven, aligning with the acute inflammatory phase. Pathologically, the presence of suture material attracted macrophages and neutrophils to the foreign-body site to clear cellular debris, resulting in vasodilation, serous exudation, and surrounding soft-tissue edema (Fawi et al., 2023). The rapid decline in inflammatory indices from days 7 to 28 correlated with the initiation of fibroblast proliferation and granulation tissue formation as the mucosal barrier gradually closed. By day 45 post-surgery, all inflammatory scores had returned to zero, indicating the normal physiological state of a healthy subject. This baseline score confirmed that the immune system has successfully cleared the foreign material through phagocytosis. Furthermore, the connective tissue at the surgical site has fully recovered and remodeled, with no clinical complications observed. These clinical outcomes of the present study confirmed that PALF sutures and Polyglactin 910 sutures were safe, medical-grade materials with excellent biocompatibility, and followed the same healing process. The absence of major complications, evidenced by the lack of systemic infection or tissue necrosis in all mice, further demonstrated that both materials supported normal wound healing without triggering severe immune rejection. However, in terms of mean values, Polyglactin 910 material consistently demonstrated superiority, with lower scores for edema, erythema, and exudation than PALF sutures, particularly during the first 14 post-operative days. Both PALF sutures and Polyglactin 910 were multifilament materials; this discrepancy in inflammation levels was primarily due to differences in manufacturing technology. Multifilament sutures consist of numerous braided or twisted fibers. This structure created microscopic spaces that act similarly to capillaries, absorbing tissue fluids and potentially harboring bacteria (Katz et al., 1981; Nappi, 2025). The increased exudation in PALF sutures could be due to a looser braided structure, which enhanced capillarization and led to localized accumulation of inflammatory exudate (Nappi, 2025). Moreover, to reduce the surface roughness of multifilament sutures, manufacturers typically apply a surface coating. The fact that Polyglactin 910 elicited less edema and erythema demonstrated that a smoother coating can reduce tissue drag and limit micro-trauma to nearby capillaries during needle penetration (Utami et al., 2025).

Consistent with the findings of Freudenberg et al. (2004), hydrolytic degradation of absorbable sutures can alter the local chemical environment, potentially affecting tissue response and contributing to the peak inflammation observed at day seven in the present study. These findings were further elucidated by the correlation between the crystallinity index and tissue response. According to Guambo et al. (2020), the mechanical performance and biological resistance of natural fibers are highly dependent on their crystalline structure. Guambo et al. (2020) indicated that high-crystallinity fibers, such as sisal (73%), exhibited superior mechanical properties and a higher ability to prevent biofilm formation compared to more porous, low-crystallinity fibers such as coconut fiber (56%). The removal of hemicellulose and lignin from PALF increased its cellulose purity, thereby enhancing its crystallinity index and optimizing tensile strength and resistance to microbial colonization in surgical applications. Pineapple leaf fiber sutures not only reduced tissue reactions but also eliminated the need for post-healing stitch removal, indicating that these materials could serve as durable and biocompatible options.

CONCLUSION

The present study successfully fabricated absorbable veterinary sutures from PALF through alkaline extraction, TEMPO-mediated oxidation, and a 1% chitosan coating. The chemical treatments successfully eliminated non-cellulosic components, resulting in a micro-grooved surface that greatly improved mechanical quality and knot security during suturing. The sutures were sterilized using gamma irradiation at a dosage of 20 kGy to ensure aseptic conditions for surgical applications. *In vitro* degradation assays confirmed the reliable biodegradation profile of the material in a simulated physiological environment over four weeks. *In vivo* biocompatibility assessments in an albino mouse model demonstrated that the PALF sutures effectively supported tissue healing without inducing systemic inflammatory

responses. Notably, the PALF sutures exhibited superior anti-infective properties compared to the Polyglactin 910 control, reflected by significantly lower secondary infection scores (0.08 ± 0.28 versus 0.58 ± 0.90). By day 45 post-operation, all clinical inflammatory parameters, including erythema and edema, had completely resolved. No wound dehiscence, tissue necrosis, or material-related issues were observed at any point during the entire monitoring period. Bio-processed PALF offered a promising, sustainable biomaterial for veterinary surgery, serving as a basis for future improvements. Despite these clinical results, this study acknowledged limitations due to the absence of X-ray diffraction analysis to quantify changes in the fiber's crystallinity index. Moreover, the potential effects of 20 kGy gamma irradiation on the molecular structure and long-term mechanical stability of cellulose fibers remain to be fully elucidated. Extending clinical validation in canines and felines is crucial for veterinary use. Since PALF eliminated the need for suture removal, further surface-coating optimizations could enhance its biostability and performance across diverse conditions.

DECLARATIONS

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Authors' contributions

Chau Buu Tran and Thuy Thanh Nguyen conceptualized the present study. Thuong Thi Nguyen, Chau Buu Tran, Thuy Thanh Nguyen, and Khiem Gia Mai collected Herb and pineapple leaves. Thuong Thi Nguyen, Chau Buu Tran, Chau Ngoc Thuy Nguyen, and Khiem Gia Mai prepared the materials and designed the study, performed the procedures, and conducted experiments. Thuong Thi Nguyen, Chau Buu Tran, and Chau Ngoc Thuy Nguyen analyzed and interpreted the data generated. Thuong Thi Nguyen, Chau Buu Tran, Chau Ngoc Thuy Nguyen, Khiem Gia Mai, and Thuy Thanh Nguyen critically reviewed the study. Thuong Thi Nguyen supervised the study. All authors revised and approved the final edition of the manuscript.

Availability of data and materials

The authors of this article confirmed that all data supporting the study's findings are available upon reasonable request.

Competing interests

The authors declared no conflicts of interest.

Ethical considerations

This article was written originally without copying from previously published articles or books, submitted only to the present journal, and did not use any AI tools in preparing or writing the manuscript.

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Effects of Dietary Supplementation of Turmeric Powder on Haematological Profiles and Serum Biochemical Parameters in Local Sheep in Indonesia

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ABSTRACT

Productivity and livestock health are significantly affected by nutritional strategies, especially natural feed additives with immunomodulatory and antioxidant properties. Turmeric (*Curcuma domestica*) is considered a sustainable, safe alternative to synthetic additives. The present study aimed to evaluate the haematological and serum biochemical parameters in local sheep following the inclusion of turmeric powder in their diet. The experiment involved 12 male local thin-tailed sheep (*Ovis aries*), approximately 1 year old, with an average body weight of 24 kilograms. The present study was carried out at a private farm in Pringsewu, Lampung, Indonesia, using a completely randomized design with three dietary treatments, including a basal diet without turmeric supplementation (P0), a basal diet supplemented with 2.5% dry matter (DM) of turmeric powder (P1), and a basal diet supplemented with 5% DM of turmeric powder (P2). Hemoglobin, packed cell volume (PCV), total plasma protein (TPP), blood glucose, and low-density lipoprotein (LDL) levels were assessed. Dietary supplementation with turmeric powder in Group P2 modulated haematological parameters, as indicated by increased hemoglobin (14.30 ± 0.48 g/dL) and PCV levels (43.43 ± 1.48%) compared to the control group. Serum biochemical parameters were affected in Group P2. Total plasma protein remained within the physiological range in all groups (6.50-6.80 g/dL), while blood glucose levels decreased as turmeric inclusion increased, with a level of 60.00 ± 6.27 mg/dL in Group P0 compared with groups P1 and P2. The LDL concentrations demonstrated a moderate increase across the treatment groups, reaching the highest level in Group P2 at 23.25 ± 5.05 mg/dL. Supplementing local sheep diets with 5% DM of *Curcuma domestica* powder influenced hemoglobin, PCV, glucose, and LDL.

Keywords: *Curcuma domestica*, Haematological parameter, Phytogenic feed additive, Serum biochemical parameter, Sheep

INTRODUCTION

The increasing restriction on the use of antibiotic growth promoters has accelerated the exploration of phytogenic feed additives as natural alternatives in small ruminant production systems. In Asia, plant-based supplements have garnered significant interest due to their potential to enhance rumen function, metabolic efficiency, and physiological resilience under practical farm conditions (Nastoh et al., 2024; Wang et al., 2024). Among these plant-based supplements, turmeric (*Curcuma domestica syn*. *Curcuma longa* L.) is widely recognized for its bioactive compounds, particularly curcuminoids and essential oils, which exhibit antioxidant, anti-inflammatory, and hepatoprotective activities (Amalraj et al., 2016; Sureshbabu et al., 2023). These biological properties indicate that turmeric supplementation could affect overall physiological responses, not just growth performance (Jiang et al., 2019).

Haematological parameters such as hemoglobin (Hb) concentration and packed cell volume (PCV) reflect oxygen-carrying capacity and erythrocyte status, whereas serum biochemical indices, including total plasma protein (TPP), blood glucose, and lipid fractions, serve as metabolic balance indicators, nutritional adequacy, and hepatic function (Khalil et al., 2024; Korelidou et al., 2026). In local sheep production systems, particularly under smallholder management in tropical regions, these physiological traits may differ due to dietary variability, environmental stress, and management constraints. Therefore, nutritional interventions that stabilize or modulate these parameters are essential to sustain productivity and animal health (Sultan, 2022; Okoruwa et al., 2025).

Although dietary turmeric supplementation has been investigated in different livestock species, existing studies have primarily concentrated on growth performance, feed efficiency, and immune responses, with inclusion levels typically assessed up to 3% of the diet (Jaguezeski et al., 2018; Odhaib et al., 2021; Okoruwa et al., 2025). Limited evidence

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exists regarding the dose-dependent effects of turmeric on haematological and serum biochemical responses in locally adapted sheep. In particular, the common inclusion levels used in field conditions, such as 2.5% and 5% of turmeric powder in the diet, have not been extensively studied for their effects on systemic physiological indicators. Therefore, the present study aimed to evaluate the effects of turmeric (*Curcuma domestica*) powder supplemented to the diet of local sheep at inclusion levels of 2.5% and 5% on haematological and serum biochemical parameters.

MATERIALS AND METHODS

Ethical approval

Ethical approval was obtained from the Ethical Review Committee of the Faculty of Agriculture, University of Lampung, Indonesia (Approval No. 117a/UN26.14/TU/2025). All experimental procedures were rigorously designed and monitored to minimize animal stress and discomfort, ensuring adherence to animal welfare principles throughout the study.

Location and animals

The experiment was conducted at a private sheep farm in Pringsewu, Indonesia, under an intensive management system following standard husbandry and feeding practices. A total of 12 clinically healthy local sheep with relatively uniform age and body weight were used in the present study. The animals were housed individually in pens measuring 0.8 × 1.5 m to facilitate feeding and monitoring throughout the study. Feed and drinking water were provided separately. Feed was offered twice daily, in the morning and afternoon, while clean drinking water was available *ad libitum*.

Study design

The present experiment was conducted from October to December 2024, using 12 male local thin-tailed sheep (*Ovis aries*), aged approximately 1 year, with an average body weight of around 24 kg. Each sheep served as an experimental unit and was randomly assigned to one of three dietary treatments, with four replicates per treatment. The treatment groups were a basal diet without turmeric supplementation as the control group (P0), a basal diet supplemented with 2.5% DM of turmeric powder (P1), and a basal diet supplemented with 5% DM of turmeric powder (P2). The compositions of the basal and experimental diets are provided in Table 1.

Table 1. Chemical composition of sheep experimental diets containing different levels of turmeric powder

Parameters \ Treatment	P0	P1	P2
Dry matter (%)	58.0	58.2	58.3
Crude protein (% DM)	12.7	12.9	13.1
Ether extract (% DM)	3.0	3.0	3.1
Crude fiber (% DM)	20.3	20.5	20.7
Ash (% DM)	11.0	11.1	11.3
Total digestible nutrients (% DM)	61.2	60.9	60.6
Metabolizable energy (Mcal kg^{-1} DM)	2.22	2.21	2.19

DM: Dry matter, P0: Basal diet without turmeric supplementation (control), P1: Basal diet + 2.5% of turmeric powder, P2: Basal diet + 5.0% of turmeric powder. The diets were formulated to provide approximately 13% crude protein and 2.2 Mcal kg^{-1} DM of metabolizable energy, meeting the nutrient requirements for growing sheep as recommended by NRC (2007).

Parameters

The hematological and serum biochemical parameters, including Hb (g/dL), PCV (%), TPP (g/dL), blood glucose (mg/dL), and low-density lipoprotein (LDL, mg/dL), were measured. These parameters were analyzed to assess the metabolic and physiological status of the sheep.

Data collection

Approximately 5 mL of blood was collected from the jugular vein of each animal. Of the total volume, 3 mL was transferred into sterile tubes containing ethylenediaminetetraacetic acid (EDTA) for assessing haematological parameters, including Hb and PCV, following standard procedures (Shaikat et al., 2013). The remaining 2 mL was transferred into Vacutainer tubes without anticoagulant for serum preparation. Samples were centrifuged at 3000 rpm for 15 minutes using a laboratory centrifuge to separate the serum, which was subsequently transferred into Eppendorf tubes and stored at −20°C until analysis. Haematological parameters were analyzed using a Haematology Analyzer RD-7021 (China), and serum biochemical parameters, including TPP, blood glucose, and LDL, were determined using an automatic biochemical analyzer in accordance with standard laboratory protocols. All hematological and serum biochemical analyses were conducted at the Lampung Veterinary Center in Indonesia.

Statistical analysis

All data are expressed as mean ± standard deviation (SD). Data management and basic computations were performed using Microsoft Excel (Microsoft Corporation, USA). Data evaluation was limited to descriptive statistical analysis, in which treatment means for each parameter were qualitatively compared with established physiological reference ranges for sheep. This descriptive approach aimed to determine whether dietary turmeric supplementation maintained haematological and serum biochemical parameters within normal physiological limits, rather than evaluating statistically significant differences among the treatment groups

RESULTS AND DISCUSSION

An increase in Hb and PCV was observed in Group P2 compared to groups P1 and P0. The TPP level remained stable in Group P2, while blood glucose levels decreased and LDL levels increased (Table 2).

Table 2. Effects of dietary turmeric on haematological and blood biochemical parameters in sheep

Parameters \ Treatment	P0	P1	P2
Hemoglobin (g/dL)	13.13 ± 1.99	14.13 ± 0.49	14.30 ± 0.48
Packed cell volume (%)	38.95 ± 6.77	42.45 ± 0.98	43.43 ± 1.48
Total plasma protein (g/dL)	6.70 ± 0.26	6.50 ± 0.40	6.80 ± 0.48
Blood glucose (mg/dL)	60.00 ± 6.27	57.00 ± 7.61	48.25 ± 16.50
Low-density lipoprotein (mg/dL)	19.00 ± 4.54	21.75 ± 2.36	23.25 ± 5.05

P0: Basal diet without turmeric supplementation (control), P1: Basal diet + 2.5% of turmeric powder, P2: Basal diet + 5.0% of turmeric powder. Data are presented as mean ± SD.

Hemoglobin and packed cell volume

Hemoglobin concentration increased progressively with increasing levels of dietary supplementation (Table 2), rising from 13.13 ± 1.99 g/dL in the control group to 14.30 ± 0.48 g/dL in Group P2. Similarly, PCV level increased from 38.95 ± 6.77% in the control group to 43.43 ± 1.48% in Group P2. In sheep, the physiological reference range for Hb typically ranges from 9 to 15 g/dL, while the PCV generally ranges from 27% to 45%, depending on factors such as breed, age, and physiological condition (Mohamed et al., 2021; Nastoh et al., 2024). All values recorded in the present study remained within these established physiological limits, indicating that turmeric inclusion up to 5% did not induce hematological imbalances and might even suggest an improvement in hematological status (Nastoh et al., 2024). Hemoglobin and PCV are primary indicators of erythrocyte mass and oxygen-carrying capacity in small ruminants and are widely used to evaluate nutritional adequacy and systemic health (Nastoh et al., 2024). Increases in these parameters are commonly associated with enhanced erythropoiesis and improved nutrient availability, particularly protein and iron (Mohamed et al., 2021). Recent studies have indicated that phytogenic turmeric, as a feed additive, can positively influence hematological indices in small ruminants by improving nutrient digestibility and metabolic efficiency (Swelum et al., 2021; Ntsongota et al., 2025). The progressive increase observed in the 2.5% and 5% turmeric groups indicated enhanced erythrocyte production and physiological adaptation.

Curcuma longa contains curcuminoids and essential oils with strong antioxidant and anti-inflammatory properties, which may contribute to improved hematological profiles. *Curcumin* has been reported to enhance antioxidant defense mechanisms, decrease oxidative damage to erythrocyte membranes, and promote systemic physiological stability (Islam et al., 2018). In ruminants, oxidative stress can shorten erythrocyte lifespan and impair hematological parameters; therefore, dietary antioxidants derived from phytogenic sources may help preserve erythrocyte integrity and support optimal PCV levels (Piao et al., 2023; Wang et al., 2024). Recent feeding trials in sheep supplemented with herbal additives, including turmeric-based formulations, demonstrated improvements in Hb and PCV levels within physiological ranges, consistent with the findings of the present study (Valenzuela-Grijalva et al., 2017).

From a nutritional perspective, Hb synthesis in ruminants is closely linked to protein metabolism. Microbial protein produced in the rumen is the primary source of absorbable amino acids for the animals and is crucial for supporting erythropoiesis (Priyashantha et al., 2026). Phytogenic additives such as turmeric may modulate rumen fermentation patterns, enhance microbial efficiency, and improve nitrogen utilization (Mapiye et al., 2026). Improved rumen microbial protein synthesis increases the availability of essential amino acids required for globin formation, thereby facilitating Hb synthesis and erythrocyte maturation (Tian et al., 2023). The sustained increase in Hb and PCV in sheep fed 5% turmeric indicated improved protein utilization and an enhanced metabolic state. Importantly, erythrocytes in sheep are relatively small but occur in large numbers, making the PCV particularly sensitive to minor alterations in erythropoietic activity and plasma volume dynamics (Tüfekci and Sejian, 2023). The observed increases remained

within normal physiological limits (Jiang et al., 2019), indicating improved hematological homeostasis rather than pathological polycythemia. Collectively, the current findings indicated that dietary turmeric at 2.5% and 5% may enhance erythropoietic activity and systemic physiological resilience, potentially through antioxidant mechanisms, and improve rumen microbial protein synthesis.

Total plasma protein

Total plasma protein values in Table 2 ranged from 6.50 to 6.80 g/dL across dietary turmeric supplementation groups, remaining within the established physiological reference range for sheep (6.0-7.9 g/dL; Sultan, 2023). The current findings indicated that turmeric supplementation up to 5% did not adversely affect hepatic synthetic function or systemic hydration status. In small ruminants, the concentration of TPP is frequently utilized as a reliable indicator of liver function, nutritional status, immune competence, and fluid balance (Newcomer et al., 2021; Očenáš et al., 2025).

Plasma proteins in sheep consist primarily of albumin and globulins. Albumin plays a vital role in maintaining colloid osmotic pressure and transporting endogenous and exogenous compounds, whereas globulins are largely associated with immune function and inflammatory responses (Newcomer et al., 2021). Notable deviations in TPP levels are frequently associated with inflammatory disorders, parasitic infestations, dehydration, or hepatic dysfunction (Očenáš et al., 2025). The lack of notable changes observed in the current study indicated that the inclusion of dietary turmeric did not induce systemic inflammatory stress or liver dysfunction.

Phytogenic feed additives, such as turmeric, are recognized for their hepatoprotective and antioxidant properties, which are attributed to bioactive compounds, including curcuminoids and essential oils (Hashemzadeh, 2022). Curcumin has been reported to enhance hepatic antioxidant defense systems, reduce oxidative stress, and stabilize cellular membranes (Ntsongota et al., 2025). In ruminants, oxidative stress and subclinical inflammation can negatively affect protein metabolism and plasma protein synthesis (Amalraj et al., 2016). Thus, the consistent TPP levels in sheep given 2.5% and 5% turmeric likely indicated preserved liver synthetic function and stable protein metabolism. Furthermore, in ruminants, plasma protein levels are strongly linked to dietary protein intake and to rumen microbial protein production, which provides most of the animal's absorbable amino acids (Wang et al., 2024). Phytogenic additives have demonstrated the ability to modulate rumen fermentation and enhance nitrogen utilization efficiency (Mapiye et al., 2026). The maintenance of TPP within the normal physiological range in the present study suggested that turmeric supplementation did not compromise rumen protein metabolism but instead supported stable systemic protein homeostasis. Therefore, the consistent plasma protein profile across treatment groups suggested that incorporating up to 5% dietary turmeric preserved protein levels, further supporting the safety and physiological compatibility of turmeric as a phytogenic feed additive in ovine nutrition.

Blood glucose

Blood glucose concentration decreased progressively across dietary turmeric treatment groups, from 60.00 ± 6.27 mg/dL in the control group to 48.25 ± 16.50 mg/dL in Group P2 (Table 2). In ovine species, physiological blood glucose concentrations are generally lower than those observed in monogastric species, typically ranging from 40 to 70 mg/dL (Newcomer et al., 2021). All values recorded in the present study remained within the normal physiological range, indicating that the inclusion of turmeric up to 5% did not induce hypoglycemia or metabolic imbalance (Mapiye et al., 2026).

The relatively low glucose levels in ruminants reflect their metabolic adaptation, in which dietary carbohydrates are fermented by rumen microbes rather than digested directly by enzymes (Wang et al., 2024). This fermentation produces volatile fatty acids, mainly acetate, propionate, and butyrate, which function as the primary energy sources (Wang et al., 2024). Propionate serves as the primary glucogenic precursor, supporting hepatic gluconeogenesis and thereby maintaining systemic glucose homeostasis. Therefore, in sheep, blood glucose levels primarily depend on rumen fermentation and hepatic gluconeogenesis, rather than on direct glucose absorption (Chen et al., 2025). The moderate reduction in glucose concentration observed in the turmeric-supplemented groups may reflect improved metabolic efficiency or enhanced peripheral glucose utilization, rather than indicating a pathological effect. Phytogenic additives such as turmeric can modify rumen fermentation and improve nutrient utilization (Mapiye et al., 2026). In addition, curcumin (the main bioactive compound in turmeric) has been associated with improved insulin sensitivity and carbohydrate metabolism through antioxidant and anti-inflammatory mechanisms (Ntsongota et al., 2025).

The high SD observed in Group P2 indicated considerable individual differences in glucose response. In sheep, such variability may result from differences in feed intake, social hierarchy, stress responses, and rumen microbial composition, all of which can affect propionate production and hepatic gluconeogenesis (Mapiye et al., 2026). Therefore, differences in glucose levels likely reflect normal biological fluctuations rather than metabolic instability due to the treatment. Overall, the reduction in blood glucose levels with increasing turmeric inclusion (0-5%) stayed within

normal physiological ranges (Jiang et al., 2019), possibly indicating an adaptive metabolic response linked to rumen fermentation efficiency and peripheral energy utilization. The current findings suggested that dietary turmeric supplementation may influence carbohydrate metabolism without compromising metabolic homeostasis in sheep.

Low-density lipoprotein and lipid metabolism

Low-density lipoprotein concentration in Table 2 increased from 19.00 ± 4.54 mg/dL in the control group to 23.25 ± 5.05 mg/dL in Group P2. In ruminants, including sheep, lipid metabolism differs substantially from that of monogastric species due to the extensive ruminal biohydrogenation of dietary unsaturated fatty acids. Consequently, the circulating levels of total cholesterol and lipoprotein fractions in sheep are typically lower and are metabolically regulated in a distinct manner compared to humans and other monogastric species (Cheng et al., 2025; Mapiye et al., 2026). In ruminants, lipoproteins primarily function to transport endogenously synthesized lipids rather than directly absorbed dietary lipids. Low-density lipoprotein plays a key role in delivering cholesterol from the liver to peripheral tissues, where it supports membrane formation, steroid hormone production, and cellular metabolism. Studies conducted in Asia indicated that moderate increases in LDL concentration in sheep and goats can reflect adaptive lipid mobilization associated with changes in dietary composition and energy balance rather than pathological dyslipidemia (Xue et al., 2021; Cheng et al., 2025).

Phytogenic feed additives, including turmeric, contain bioactive compounds such as curcuminoids that may influence lipid metabolism by modulating hepatic lipid synthesis and antioxidant status. Studies from Asian livestock management systems have demonstrated that plant-based feed additives can modify serum lipid profiles in small ruminants without inducing adverse metabolic effects, particularly when values remain within physiological ranges (Mapiye et al., 2026; Zhao et al., 2023). Additionally, curcumin has been reported to regulate lipid metabolism by modulating hepatic enzymes involved in cholesterol synthesis and lipoprotein transport, thereby promoting metabolic adaptation rather than pathological lipid accumulation (Cheng et al., 2025). In ruminants, it is crucial to interpret LDL concentrations considering the species-specific physiological limits (Jiang et al., 2019). When there are no elevated inflammatory markers or signs of hepatic dysfunction, moderate increases in LDL are generally regarded as a normal metabolic response to dietary interventions (Očenáš et al., 2025). In the present study, LDL values remained within the normal range for sheep (Jiang et al., 2019), indicating that including turmeric up to 5% might affect lipid transport without causing metabolic disorder. Collectively, the observed increase in LDL concentration in Group P2 likely represented an adaptive response in hepatic lipid transport and peripheral cholesterol utilization associated with dietary turmeric supplementation, rather than a pathological alteration in lipid metabolism.

CONCLUSION

Dietary supplementation with turmeric (*Curcuma domestica*) at 2.5% and 5% in sheep feed modulated hematological and metabolic parameters without exceeding physiological reference ranges. The increases in Hb, PCV, and LDL concentrations, along with stable TPP and reduced blood glucose levels within normal limits, indicated adaptive improvements in erythropoiesis, protein utilization, lipid transport, and energy metabolism rather than pathological alterations. However, the present study was limited by a relatively small sample size and a short experimental duration, which may restrict the generalizability of the findings regarding long-term physiological responses to turmeric supplementation. Therefore, further studies involving larger animal populations, extended feeding periods, and additional biomarkers related to antioxidant status, immune response, and rumen fermentation are recommended to elucidate the underlying mechanisms and optimize the dietary inclusion level of turmeric in small ruminant production systems.

DECLARATIONS

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Authors' contributions

Aini Alfisyahri, Anhar Sukron Hanif, Fathma Choir Andini, and Abdurrochman Sholeh contributed to data curation, formal analysis, investigation, and writing the original draft of the study. Purnama Edy Santosa and Sri Suharyati contributed resources, supervision, and validation, and participated in the drafting, review, and editing processes. Arif Qisthon, Liman Liman, Lusia Komala Widiastuti, and Anggi Derma Tungga Dewi contributed to visualization, data

curation, and writing the original draft of the study. Kusuma Adhianto and Muhtarudin Muhtarudin were responsible for conceptualization, methodology, project administration, and writing, reviewing, and editing the manuscript. All authors have read and approved the final edition of the manuscript.

Availability of data and materials

All data generated or analyzed during the present study are included in this published article. Additional data supporting the findings of the present study are available from the corresponding author upon reasonable request.

Competing interests

The authors declared no conflict of interest regarding the publication of this study.

Ethical considerations

All authors have carefully reviewed and confirmed that the manuscript complies with ethical standards in scientific publication. Issues related to plagiarism, consent to publish, research misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy have been thoroughly examined and are not present in the current manuscript. The authors declared that the manuscript is original, has not been published previously, and is not under consideration for publication elsewhere. The authors confirmed that no AI tools were used to conduct and prepare the present study.

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Association between Dietary Mineral Profiles and Hypercalcemia in Cats with Chronic Kidney Disease: A Retrospective Study

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ABSTRACT

Hypercalcemia is an important clinical problem in feline veterinary medicine due to its association with a wide range of pathological conditions, particularly chronic kidney disease (CKD). The present study aimed to characterize the clinical and biochemical characteristics of hypercalcemic cats and to investigate the association between the dietary calcium-to-phosphorus (Ca:P) ratio and CKD, urolithiasis, and total and ionized calcium levels. A retrospective analysis was conducted of 50 hypercalcemic domestic cats presented to the Veterinary Clinic in Kyiv, Ukraine, between 2023 and 2025. For each cat, data on age, breed, total calcium, and ionized calcium were collected. Additionally, serum phosphorus levels and any concurrent diseases were documented from medical records. Dietary composition was assessed based on manufacturer-reported analytical values, including calcium, phosphorus, the Ca:P ratio, and vitamin D content. Cats were divided into two groups based on their dietary Ca:P ratio; one with a ratio of 1.4 or less (≤ 1.4) and the other with a ratio greater than 1.4. (>1.4). The majority of cats (70%) were over six years old. The median total calcium concentration was 3.05 mmol/L, while the median ionized calcium concentration was 1.60 mmol/L. Elevated ionized calcium levels, despite normal total calcium levels, was observed in 16% of cases. Chronic kidney disease was observed in 60% of cats with hypercalcemia, while 18% had urolithiasis, mainly calcium oxalate. Additionally, 18% of the cats had both conditions simultaneously. Comparative analysis demonstrated that cats fed diets with Ca:P ratios > 1.4 had a significantly higher prevalence of CKD (20/25, 80%) than cats fed diets with Ca:P ratios ≤ 1.4 (6/25, 24%). However, total and ionized calcium concentrations did not differ significantly between the groups. The current findings highlighted the diagnostic value of total calcium, the importance of measuring ionized calcium, and suggested a possible association between high dietary Ca:P ratios and concurrent CKD in hypercalcemic cats.

Keywords: Calcium-to-phosphorus ratio, Chronic kidney disease, Hypercalcemia, Ionized calcium, Vitamin D

INTRODUCTION

Disorders of calcium homeostasis are significant in feline veterinary medicine because hypercalcemia can be associated with different pathological conditions and may influence disease progression (de Brito Galvão et al., 2017; Coady et al., 2019). In cats, hypercalcemia frequently occurs concurrently with chronic kidney disease (CKD), which is one of the most common chronic conditions in felines and for which staging and treatment protocols are well established (IRIS, 2023). Tang et al. (2021) reported that changes in calcium-phosphorus balance in CKD are linked to a complex pathophysiological condition involving hormonal dysregulation and the development of extraosseous calcification. Disturbances in phosphorus and calcium regulation in CKD are associated with changes in parathyroid hormone and fibroblast growth factor 23 (FGF-23), which play key roles in maintaining mineral homeostasis. Geddes et al. (2013) and Lin et al. (2021) reported that FGF-23 levels gradually rise with advancing CKD stages in cats. Based on studies, elevations frequently precede improvement in hyperphosphatemia, suggesting that FGF-23 may play a role in early mineral imbalance.

Dietary modification plays an important role in the management of CKD in cats (Parker, 2021; Stockman, 2024). The calcium-to-phosphorus (Ca:P) ratio has been recognized as a key parameter influencing disease progression and mineral homeostasis in cats, and different studies have suggested that dietary adjustment may normalize ionized hypercalcemia in cats with idiopathic hypercalcemia or CKD (Ehrlich et al., 2024; Stockman, 2024). Recommendations for feline diets, including optimal Ca:P ratios, were provided in international nutritional guidelines, highlighting the importance of controlling dietary mineral composition in clinical practice (FEDIAF, 2024). Furthermore, dietary mineral composition has been recognized as a contributing factor in the development of feline urolithiasis, particularly calcium oxalate stones (FEDIAF, 2024). The American College of Veterinary Internal Medicine (ACVIM) guidelines identified dietary mineral composition as a contributing factor in calcium oxalate urolithiasis in cats (Lulich et al., 2016).

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Despite the availability of individual studies, the prevalence of hypercalcemia in cats and its association with feeding type, dietary Ca:P ratio, and concurrent diseases, particularly CKD, remain insufficiently studied in clinical practice. Additionally, there is limited data on isolated ionized hypercalcemia in cats with normal total calcium levels, which may lead to a missed diagnosis of a calcium ionization disturbance. The present study aimed to characterize the clinical and biochemical profiles of hypercalcemic cats and to evaluate the relationships among dietary mineral composition, particularly the Ca:P ratio and vitamin D content, concurrent diseases, and serum calcium parameters in clinical practice.

MATERIALS AND METHODS

Ethical approval

The present study was approved by the Bioethics Committee of the National University of Life and Environmental Sciences of Ukraine (Protocol No. 007/2024 dated 28 March 2024). The present study was conducted without any intervention in the treatment of animals. Data were collected from the medical records of animals at the Zoolux Veterinary Clinic (Kyiv, Ukraine), in strict accordance with confidentiality principles.

Study design

The study retrospectively analyzed medical records of cats with hypercalcemia that presented to the Veterinary Clinic in Kyiv, Ukraine, from 2023 to 2025. Cats of both sexes, different breeds, and aged between 3 and 19 years were considered for inclusion. To assess clinically relevant factors related to disturbances in calcium metabolism, data on breed, age, sex, biochemical results, dietary nutrients (calcium, phosphorus, Ca:P ratio, and vitamin D content), and the presence of concurrent diseases were extracted from clinical records. A total of 86 cats with confirmed hypercalcemia, characterized by elevated total and/or ionized serum calcium levels according to de Brito Galvão et al. (2017), presented to the clinic during the study period and were initially screened for inclusion.

Inclusion and exclusion criteria

Cats were included in the final study population for which the total and ionized serum calcium concentrations were available. Detailed dietary information, including the commercial product name and manufacturer-declared analytical composition (calcium, phosphorus, vitamin D content), was documented. Additionally, clinical history, encompassing data on concurrent diseases such as CKD, urolithiasis, polycystic kidney disease, and other systemic conditions, was recorded. Cats with a confirmed or suspected malignant neoplasm, those on calcium-containing phosphate binders, or those receiving systemic glucocorticosteroids were excluded because these factors independently influence calcium homeostasis and could confound the interpretation of dietary effects. Following the application of these criteria, 36 cats were excluded, leaving a final group of 50 cats.

Data extraction

The extracted parameters from clinical records included age (in years), breed (based on owner reports and confirmed through clinical examination), sex, body weight, total and ionized serum calcium levels, serum phosphorus levels, and the presence of any concurrent diseases. The dietary Ca:P ratio was calculated as the mass ratio of calcium to phosphorus, expressed as a percentage on a fed basis. Manufacturer-declared analytical values, as indicated on the product label, were used directly to calculate the Ca:P ratio. Serum total calcium and phosphorus levels were assessed in samples collected through jugular venipuncture and analyzed within four hours using an automated biochemistry analyzer (Mindray, China). Ionized calcium was measured separately on an electrolyte analyzer (High Technology, Inc., USA) using an ion-selective electrode method. Reference intervals for serum total calcium (2.2-2.7 mmol/L) and ionized calcium (1.10-1.38 mmol/L) were used, as described by de Brito Galvão et al. (2017). For comparative analysis, the cats were divided into two groups based on their dietary Ca:P ratio, one group with a ratio of 1.4 or less and the other with a ratio exceeding 1.4 (≤ 1.4 or > 1.4). The cutoff of 1.4 was selected based on the findings of Ehrlich et al. (2024) and Stockman (2024).

Statistical analysis

Categorical variables were compared using Fisher's exact test, while continuous variables were analyzed using the Mann-Whitney U test due to non-normal data distribution, as confirmed by the Shapiro-Wilk test ($p < 0.05$). Results were presented as absolute values, percentages, and median values. Differences were considered statistically significant at a p-value less than 5% ($p < 0.05$).

RESULTS

Study population characteristics

A total of 50 cats with hypercalcemia were included in the present study. Mixed-breed cats were the most prevalent in the study population (23/50, 46%), followed by Scottish Straight cats (11/50, 22%). British Shorthair and Exotic Shorthair cats accounted for 3/50 (6%) cases each, while other breeds, including Maine Coon, Persian, Bengal, Siamese, and Sphynx cats, were represented by isolated cases. The majority of cats (35/50, 70%) were older than six years, whereas cats younger than six years accounted for 15/50 (30%).

Renal and urinary disorders

In the total study population (2023-2025), the median concentrations were 3.05 mmol/L for total calcium (ranging from 2.27 to 3.74), 1.60 mmol/L for ionized calcium (ranging from 1.42 to 1.96), and 1.42 mmol/L for phosphorus (ranging from 0.73 to 2.71). Elevated ionized calcium levels with normal total calcium were observed in 8 out of 50 cases (16%). Chronic kidney disease was diagnosed in 30 out of 50 cats (60%), with 26 (52%) having CKD stages II-IV based on the International Renal Interest Society (IRIS) classification. Additionally, 4 cats (8%) had CKD stage I along with polycystic kidney disease. Urolithiasis, mainly calcium oxalate, was found in 9 out of 50 cats (18%). The distribution and co-occurrence of renal and urinary disorders are summarized in Table 1.

Dietary mineral composition

The dietary analysis revealed that the Ca:P ratio ranged from 1.00 to 2.33, with a median of 1.50. In 25 of the 50 cases (50%), the Ca:P ratio was greater than 1.4. Additionally, similar patterns in dietary mineral composition were identified. The most common commercial diets contained 0.6% calcium and 0.3% phosphorus (Ca:P ratio of 2.0), as well as diets with 0.6/0.33 (Ca:P ratio of 1.82) and 0.6/0.35 (Ca:P ratio of 1.71; Table 2).

To assess the effect of dietary mineral composition, animals were divided into two groups based on the Ca:P ratio, including a group with a ratio of 1.4 or less (≤ 1.4) and the other with a ratio exceeding 1.4 (> 1.4), with 25 cats in each group. The groups were evaluated based on total and ionized calcium levels, phosphorus concentration, the prevalence of CKD and urolithiasis, and dietary vitamin D intake (Table 3).

Dietary calcium-to-phosphorus ratio comparison

Comparison of biochemical and clinical parameters between the two groups was summarised in Table 3. Total calcium, ionized calcium, serum phosphorus, and dietary vitamin D levels were not significantly different between cats with a Ca:P ratio of ≤ 1.4 and those with > 1.4 ($p > 0.05$; Table 3). However, the prevalence of CKD was significantly higher in the Ca:P > 1.4 group than in the Ca:P ≤ 1.4 group ($p < 0.05$), whereas the prevalence of urolithiasis did not differ significantly between groups ($p > 0.05$). The degree of total calcium level exceeded the upper reference limit by about 13% in cases with Ca:P > 1.4 and by around 10% in cases with Ca:P ≤ 1.4. Median dietary vitamin D levels were similar between groups; however, a greater variability was observed in cats with Ca:P ≤ 1.4.

Table 1. Distribution of renal and urinary disorders in hypercalcemic cats during the study period from 2023 to 2025

Diagnostic category	Number of cats
CKD without urolithiasis	20
Urolithiasis without CKD	3
CKD with urolithiasis	6
CKD with polycystic kidney disease	4
No documented concurrent renal or urinary disease	17

CKD: Chronic kidney disease

Table 2. Most frequently observed commercial diets fed to hypercalcemic cats during the study period from 2023 to 2025

Diet (manufacturer)	Calcium	Phosphorus	Ca:P ratio	Number of cats
Renal feline (Royal Canin)	0.6	0.3	2.0	15
Renal function - advanced care (Purina)	0.6	0.33	1.82	6
Renal function early care feline (Purina)	0.6	0.35	1.71	3

Ca:P: Calcium-to-phosphorus ratio

Table 3. Comparison of serum calcium, phosphorus, vitamin D content, and prevalence of chronic kidney disease and urolithiasis according to dietary calcium-to-phosphorus ratio in hypercalcemic cats from 2023 to 2025

Parameter	Ca:P ratio (≤ 1.4)	Ca:P ratio (> 1.4)	p-value
Total calcium (mmol/L)	2.96 (2.44-3.55)	3.05 (2.27-3.74)	0.265
Ionized calcium (mmol/L)	1.64 (1.46-1.96)	1.63 (1.42-1.83)	0.741
Phosphorus (mmol/L)	1.41 (0.73-2.02)	1.55 (0.81-2.71)	0.201
Vitamin D (IU)	800 (190-1800)	800 (600-1200)	0.870
CKD	6 (24%)	20 (80%)	< 0.001
Urolithiasis	5 (20%)	4 (16%)	0.496

Ca:P: Calcium-to-phosphorus ratio, CKD: Chronic kidney disease, IU: International units. P-values were calculated using the Mann-Whitney U test for continuous variables and Fisher's exact test for categorical variables. Numbers in parentheses are presented as the median, ranging from minimum to maximum.

DISCUSSION

The high number of mixed-breed cats in this study probably indicated their greater overall presence in the general cat population, rather than a specific breed susceptibility to hypercalcemia. The relatively high number of Scottish Straight cats might be linked to the breed's popularity among owners and potential differences in management and feeding practices.

The observed age distribution, in which 70% of animals were older than six years, was consistent with previously reported data. Tang et al. (2021) reported that calcium metabolic disorders were more commonly observed in adult and senior cats, particularly in those with CKD. Other retrospective studies of feline hypercalcemia have reported a similar age distribution. Savary et al. (2000) reported a median age of 13 years among 71 cats. Meanwhile, Midkiff et al. (2000) observed idiopathic hypercalcemia mainly in adult and middle-aged animals, supporting the link between age and this condition.

Detecting isolated ionized hypercalcemia in 16% of cases suggested that measuring total calcium alone may not be sufficient to identify disturbances in calcium metabolism in cats, as some abnormalities may be limited to the ionized fraction. Similar findings were reported by Broughton et al. (2023), indicating that total calcium had limited sensitivity in detecting ionized hypercalcemia in cats. Schenck and Chew (2010), in an analysis of 434 feline serum samples, indicated that total calcium exhibited only a moderate correlation with the ionized fraction and that ionized hypercalcemia may occur despite normal total calcium levels. In a comprehensive review, de Brito Galvão et al. (2017) highlighted that ionized hypercalcemia was the primary diagnostic marker, reflecting the biologically active component rather than total calcium levels. Coady et al. (2019) established that the severity of the condition correlated with its etiology in a study with 1,373 cases of ionized hypercalcemia. Mild cases were most frequently associated with CKD and idiopathic hypercalcemia, while more severe forms were linked to paraneoplastic syndromes.

The high prevalence of hypercalcemia in CKD (52%) in the present study was consistent with current understanding of mineral metabolism disturbances in cats with CKD (Tang et al., 2021). Parker (2021) emphasized that alterations in calcium-phosphorus balance in cats with CKD were complex and predominantly influenced by dietary composition, a crucial factor in their management. Savary et al. (2000) found that the most frequent diagnoses were neoplasia, renal disease, and urolithiasis. The conditions mentioned largely aligned with the present results and highlighted the critical role of renal pathology among the associated diseases.

The detection of urolithiasis, especially calcium oxalate stones, in some cases might be linked to disruptions in calcium metabolism. According to ACVIM recommendations, calcium oxalate urolithiasis in cats is a multifactorial condition involving hypercalcemia, hypercalciuria, decreased urine volume, and dietary mineral composition (Lulich et al., 2016).

The data on dietary Ca:P ratios indicated that the majority of cases received diets with higher-than-normal Ca:P ratios. Similar findings have been reported by Pusoonthornthum et al. (2011), where increased Ca:P ratios were regarded as a factor influencing calcium-phosphorus homeostasis in felines, particularly within the context of renal disease. The identical Ca:P values observed in many cases suggested a dependence on a narrow range of diets with similar mineral compositions, likely because a few commercial manufacturers dominated the market.

The 1.4 threshold for group division was derived from Ehrlich et al.'s (2024) study, which indicated that switching hypercalcemic cats to diets with a Ca:P ratio below 1.4 generally normalized serum calcium levels within 3 to 20 weeks. Stockman (2024) further supported this threshold by examining the regulatory role of dietary Ca:P in feline mineral balance and highlighting the clinical significance of preventing excessive phosphate restrictions.

The similar ionized calcium levels observed between groups suggested a multifactorial nature of disturbances in calcium metabolism, indicating that dietary mineral composition alone, without consideration of additional factors, did not determine ionized calcium levels. However, the markedly higher prevalence of CKD in the Ca:P > 1.4 group (80%) may suggest an association between elevated dietary Ca:P ratios and concurrent renal pathology. Stockman (2024) described the Ca:P ratio as a dietary factor influencing mineral metabolism in cats, particularly in the context of kidney disease, consistent with the observed link between higher dietary Ca:P ratios and CKD in the present study. The observed tendency for higher total calcium levels in the Ca:P > 1.4 group, with 13% of animals exceeding the upper reference limit, suggested a possible association between dietary mineral ratios and calcium regulation. However, the influence of concurrent diseases and other confounding factors cannot be disregarded.

Since vitamin D plays an important role in calcium regulation, differences in dietary vitamin D intake might affect calcium levels in cats. Zafalon et al. (2020) emphasized that vitamin D is a key regulator of calcium homeostasis and may contribute to the development of mineral metabolism disorders in cats. Crossley et al. (2017) documented a series of cases of hypercalcemia accompanied by soft-tissue calcification in cats fed a commercial diet high in cholecalciferol, in which clinical signs completely resolved after dietary adjustment. The mentioned findings highlighted the importance of monitoring actual vitamin D levels in commercial diets and carefully assessing manufacturer-reported values.

It is important to note that the present study design differed mainly from previous retrospective studies that focused on identifying the causes of hypercalcemia. Savary et al. (2000) reported neoplasia (29.6%), renal failure (25.4%), and urolithiasis (15.5%) as the primary etiologies in 71 cats, whereas Coady et al. (2019) identified malignancy-associated (22.7%) and renal-associated (13.4%) forms as the most prevalent causes in 1,373 cats.

In the current study of 50 cats, the prevalence of CKD was 52%, and urolithiasis was 18%, both exceeding the referenced values. The observed distribution reflected the specific focus of the study, in which dietary mineral composition, particularly the Ca:P ratio, was the primary variable. Concurrent diseases were documented as clinically relevant conditions that could either occur before hypercalcemia or result from it, such as nephrocalcinosis and calcium oxalate urolithiasis (Lulich et al., 2016; de Brito Galvão et al., 2017).

CONCLUSION

A retrospective analysis of hypercalcemia in cats found that CKD (60%) and urolithiasis (18%) were common concurrent conditions, predominantly in older cats. A significant number of cases demonstrated isolated ionized hypercalcemia despite normal total calcium levels, highlighting the importance of measuring ionized calcium for accurate diagnosis. An elevated dietary Ca:P ratio (> 1.4) was associated with a significantly higher incidence of CKD; however, the elevated Ca:P ratio alone did not predict ionized calcium levels, highlighting the complex, multifactorial nature of calcium metabolic disorders. The significant variability in vitamin D levels among commercial diets could further affect calcium balance in cats, underscoring the need for laboratory monitoring. The retrospective single-center design, the relatively small sample size, and the reliance on manufacturer-declared dietary composition data without laboratory verification were limitations of the present study. In addition, hormonal markers of mineral metabolism, including FGF-23, parathyroid hormone, and 25-hydroxyvitamin D, were not evaluated. Further prospective multicenter studies with standardized dietary assessments and hormonal monitoring are necessary to understand the link between dietary mineral content and calcium metabolism in cats.

DECLARATIONS

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Authors' contributions

Mykhailo Umanets participated in conducting the study, collecting and analyzing clinical data, and drafting the initial manuscript. Valerii Tsvilikhovskyi contributed to the conceptualization and design of the study, the methodology, supervision, and the review and editing of the manuscript. All authors have read and approved the final edition of the manuscript.

Availability of data and materials

The data supporting the findings of the present study are available from the corresponding author upon reasonable request.

Competing interests
The authors declared no competing interests.

Ethical considerations
The authors declared that the manuscript is original, has not been published elsewhere, and that the last edition was confirmed before publication. The authors confirmed that no AI tools were used for writing and preparing the article.

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Prevalence and Molecular Characterization of Antimicrobial Resistance Genes in *Escherichia coli* Isolated from Chicken Meat in Mosul City, Iraq

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ABSTRACT

Antimicrobial resistance (AMR) in poultry is a growing concern, driven by the widespread use of antibiotics in farming, which can promote the development of resistant bacteria. Resistant pathogens in the food chain can spread AMR and impair the treatment of infectious diseases. The present study aimed to examine the incidence of *Escherichia coli* (*E. coli*) and the presence of AMR genes in chicken meat samples collected from different markets in Mosul City, Iraq. Of 120 samples, 27 isolates (22.5%) tested positive for *E. coli*, identified by chromogenic agar, the VITEK® system, and polymerase chain reaction (PCR) targeting the *uidA* gene. All isolates (27/27; 100%) were confirmed positive for *uidA* detection. The positive isolates were evaluated for five AMR genes, including *dihydrofolate reductase A* (*dfrA*), *β-lactamase* TEM (*bla*TEM), *aminoglycoside acetyltransferase (aac*), *DNA gyrase subunit A* (*gyrA*), and *DNA gyrase subunit B* (*gyrB*). The current findings indicated that 66.7% of the isolates carried at least one resistance gene, and 14.8% carried more than one. The most commonly detected gene was *dfrA* (37.0%), followed by *bla*TEM (29.6%) and *aac* (14.8%). The *gyrA* gene was found in one isolate (3.7%); however, the *gyrB* gene was not detected. Identifying resistance genes causing resistance to commonly used antibiotics, such as trimethoprim, β-lactams, and aminoglycosides, represented a significant public health concern. The current findings can help identify and reduce the spread of AMR across the food supply chain in Iraq.

Keywords: Antimicrobial resistance gene, Chicken meat, *Escherichia coli*, Polymerase chain reaction, Public health

INTRODUCTION

Antimicrobial resistance (AMR) is widely recognized as a major global public health threat. The AMR reduces treatment efficacy, leading to increased morbidity and mortality and substantially increasing healthcare and production costs (WHO, 2023). Antimicrobial medications are extensively used in agriculture, veterinary medicine, and human health care (Marshall and Levy, 2011). Resistant bacteria and their resistance genes can be transferred among humans, animals, food, and the environment (Fusaro et al., 2025). International organizations, such as the World Health Organization, underscored the importance of adopting a One Health approach to effectively address AMR (WHO, 2023). Consequently, efforts should be directed towards monitoring and regulating AMR in humans, animals, and the environment (QJSAR, 2024).

Escherichia coli (*E. coli*) is an important bacterium for studying antibiotic resistance (EFSA and ECDC, 2026). *Escherichia coli* is commonly isolated from the intestines of humans and animals and can cause gastrointestinal infections (EFSA and ECDC, 2026). To assess AMR in livestock, particularly avian species, *E. coli* is frequently examined because it readily acquires and spreads resistance genes via mobile genetic elements such as plasmids and integrons (Marshall and Levy, 2011). *uidA* is a specific gene used to identify *E. coli*; this gene encodes *β-glucuronidase* and is widely recognized for its high specificity for *E. coli* (Bej et al. 1991). The presence of AMR *E. coli* in poultry and poultry products is a significant public health concern, as these bacteria can be transmitted to humans through contaminated food, direct contact with animals, or environmental exposure (Muloi et al., 2018).

The *β-lactamase* TEM (*bla*TEM) gene, which encodes the β-lactamase enzyme, is prevalent in *E. coli* and confers resistance to numerous β-lactam antibiotics (Bush and Bradford, 2016), including penicillin and early cephalosporins such as cephalexin (Katonge and Ally, 2025). The spread of bacteria harboring the *bla*TEM gene compromises the efficacy of β-lactam antibiotics, which are extensively used in human and veterinary medicine (Bush and Bradford, 2016). Therefore, the presence of *bla*TEM-positive *E. coli* in poultry and poultry products indicates a risk of transmitting β-lactam-resistant pathogens from livestock to humans through the food supply (Carattoli, 2013).

Additionally, *dihydrofolate reductase A* (*dfrA*) genes are often identified as a key mechanism by which bacteria acquire resistance to trimethoprim, a medicine frequently combined with sulfonamides to treat bacterial infections

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(Wang et al., 2025). The *dfrA* gene encodes a modified *dihydrofolate reductase* enzyme that antibiotic agents cannot inhibit (Huovinen, 2001). Plasmids and integrons, which often carry multiple resistance genes, are the most common locations for these genes (Wang et al., 2025). The *dfrA* gene causes resistance to multiple antibiotics in *E. coli*. *Escherichia* coli strains isolated from poultry often harbor *dfrA* genes, reflecting the impact of antimicrobial selection pressure in animal production (Schwarz et al., 2010).

Furthermore, aminoglycoside-modifying enzymes, especially those encoded by *aminoglycoside acetyltransferase* (*aac*) genes, represented a significant mechanism of resistance in *E. coli* (Hemmings et al., 2025). The *aac* enzyme chemically modifies aminoglycoside antibiotics, preventing them from effectively binding to bacterial ribosomes (Hemmings et al., 2025). Consequently, aminoglycoside antibiotics become less effective at eliminating bacteria (Ramirez and Tolmasky, 2010). Additionally, *acc* resistance genes have been commonly identified in *E. coli* isolates derived from chickens, beef, and lamb (Schwarz et al., 2010), indicating that these resistance genes are prevalent in animal production sectors (Schwarz et al., 2010).

Furthermore, mutations in the genes encoding *DNA gyrase subunits A (gyrA)* and *B* (*gyrB*) frequently confer fluoroquinolone resistance in *E. coli* (Jose and Joseph, 2026). The quinolone antibiotics, including levofloxacin and norfloxacin, exert their therapeutic effects by specifically targeting DNA gyrase, a protein complex composed of the *gyrA* and *gyrB* genes. Alterations in DNA gyrase genes subsequently modify the antibiotic's binding site, thereby reducing the medicine's efficacy and the likelihood of fluoroquinolone-induced illness (Hooper and Jacoby, 2016). The proliferation of quinolone-resistant *E. coli* strains in poultry raised significant public health issues, given the importance of quinolones in human medicine (Van Boeckel et al., 2015).

Detecting antibiotic resistance genes in *E. coli* isolates from chicken posed a significant public health concern (EFSA and ECDC, 2026). Poultry products are extensively consumed worldwide, and resistant *E. coli* strains may be transmitted to people via contaminated meat, improper food handling, or environmental contamination during processing and distribution (Islam et al., 2024). Monitoring the occurrence and spread of AMR genes offers vital information. This data can inform antimicrobial stewardship, improve biosecurity and hygiene practices on farms, and strengthen the safety of the food supply chain (WHO, 2025; EFSA and ECDC, 2026). Therefore, the present study aimed to investigate the presence of AMR genes, including *bla*TEM, *dfrA*, *gyrA*, *gyrB*, and *aac*, in *E. coli* isolates collected from chicken meat samples across different markets in Mosul City, Iraq.

MATERIALS AND METHODS

Ethical approval

The present study was approved by the Scientific Committee of the Department of Veterinary Public Health at Mosul University, Iraq (Approval No. 10.9.2025-3). The study was observational and did not employ live or laboratory animals.

Sample collection

A total of 120 chicken meat samples were randomly collected from 15 markets, selected through simple random sampling from available markets across Mosul City, Iraq, between October 2025 and February 2026. Raw and fresh chicken meat samples were aseptically collected using sterile gloves from selected markets. Fresh, packaged chicken samples were purchased and transported to the laboratory under refrigerated conditions (4-6°C) within 12-24 hours. Upon arrival, each package was opened under aseptic conditions, and about 10 mL of meat exudate (drip fluid) was collected using a sterile pipette into sterile containers for microbiological analysis (Martínez-Moreno et al., 2025).

Isolation

Each sample was initially pre-enriched in buffered peptone water (BPW); 1 mL of drip fluid was combined with 9 mL of BPW and incubated aerobically at 37°C for 18 to 22 hours. The enriched broth was streaked onto MacConkey agar (Neogen, UK) using a loop and incubated at 37°C for 24 hours. Lactose-fermenting colonies (pink colonies) suggestive of *E. coli* were selected and sub-cultured for purification. For selective identification, purified isolates were streaked onto chromogenic agar specific for *E. coli* (CHROMagar™ ECC (Himedia, India) and incubated at 37°C for 18-24 hours. Colonies exhibiting typical color changes, from blue to purple, were presumed to be *E. coli*.

Identification using the VITEK system

The VITEK 2 Compact system (bioMérieux, France) was employed to automatically identify presumptive *E. coli* isolates. Pure colonies were subcultured on nutrient agar and incubated at 37°C for 18-22 hours after being isolated from a selective, chromogenic medium. A new colony was suspended in sterile saline and adjusted to a 0.5 McFarland turbidity using a densitometer. The standardized bacterial suspension was inoculated onto VITEK 2 Gram-negative

identification cards. The cards were loaded into the VITEK 2 system, where identification was performed automatically using a series of biochemical reactions and metabolic profiles. The system software interpreted the results, and isolates identified as *E. coli* with high confidence (> 95%) were confirmed.

DNA extraction

Genomic DNA was extracted from confirmed *E. coli* isolates using a commercial bacterial DNA extraction reagent (Addbio, Korea) in accordance with the manufacturer's guidelines. In brief, the lysis buffer was used to resuspend overnight cultures freshly grown on MacConkey agar. The mixture was incubated at 56°C for 20-30 minutes after the addition of Proteinase K, to ensure complete cell dissociation. Ethanol was added to promote DNA binding following lysis, and the lysate was then transferred to a spin column. The column was centrifuged at 10,000 rpm for one minute to facilitate DNA adsorption onto the silica membrane. The column was subsequently cleaned using the provided wash buffers to eliminate contaminants and impurities. Finally, genomic DNA was eluted in 50 µL of elution buffer through centrifugation at 8,000 rpm for one minute. The NanoDrop spectrophotometer (Thermo Scientific, USA) was used to assess DNA integrity and concentration by measuring absorbance at 260/280 nm. The extracted DNA samples were stored at -20°C for polymerase chain reaction (PCR) analysis.

Detection of the *uidA* gene

To confirm the *E. coli* isolates, PCR was employed to amplify the *uidA* gene. Afterward, the isolates were tested for specific AMR genes, including *bla*TEM, *dfrA*, *gyrA*, *gyrB*, and *aac*. A total of 25 µL of PCR reaction mixture was prepared, comprising 12.5 µL of 2x PCR master mix, 1 µL of each primer (forward and reverse), 2 µL of template DNA, and enough nuclease-free water (Al-Mahmood and Fraser, 2023). Table 1 presents the primer sequences, target genes, amplicon sizes, and corresponding references used for the PCR detection of AMR genes in *E. coli* isolates. The PCR was carried out in an Applied Biosystems thermal cycler (USA). The process began with an initial denaturation at 94-95°C for 3-5 minutes, followed by 30-35 cycles of denaturation at 94°C for 30 seconds, annealing of *uidA* at 62°C, *bla*TEM at 59°C, *dfrA* at 61°C, *gyrA* at 58°C, *gyrB* at 55°C, *aac* at 57°C for 30 seconds, and extension at 72°C for 45-60 seconds. A final extension at 72°C lasted 5-7 minutes. The PCR products were then analyzed on a 1.5% agarose gel and compared with a 100 bp DNA ladder.

Table 1. Primer sequences and anticipated amplicon sizes for conventional polymerase chain reaction of *Escherichia coli* isolates

Gene	Primer	Sequence (5′-3′)	Product size (bp)	Reference
uidA	Forward	CCGATCACCTGTGTCAATGT	380	Bower et al. (2005)
	Reverse	GTTACCGCCAACGCGCAATA		
*bla*TEM	Forward	ATCAGCAATAAACCAGC	516	Mabilat and Courvalin (1990)
	Reverse	CCCCGAAGAACGTTTTC		
dfrA	Forward	TGGTAGCTATATCGAAGAATGGAGT	425	Grape et al. (2007)
	Reverse	TATGTTAGAGGCGAAGTCTTGGGTA		
gyrA	Forward	AAATCTGCTCGTGTCGTTGG	349	Ardebili et al. (2015)
	Reverse	GCCATACCTACAGCAATACC		
gyrB	Forward	TACCAACAACATTCCGCAGC	238	De la Fuente et al. (2007)
	Reverse	CGCCGATTTCACCTCAGAAG		
aac	Forward	ATATCGCGATGCATACGCGG	877	Arpin et al. (2003)
	Reverse	GACGGCCTCTAACCGGAAGG		

dfrA: *Dihydrofolate reductase A*, *bla*TEM: *β-lactamase TEM*, *aac*: *Aminoglycoside acetyltransferase*, *gyrA*: *DNA gyrase subunit A*, *gyrB*: *DNA gyrase subunit B*

Statistical analysis

Data obtained from microbiological and molecular analyses were entered into Microsoft Excel and analyzed using John's Macintosh Project **(**JMP) statistical software (SAS Institute Inc., Cary, NC, USA). Descriptive statistics were used to summarize the results. The prevalence of *E. coli* isolates confirmed by the *uidA* gene and AMR genes (*bla*TEM, *dfrA*, *gyrA*, *gyrB*, and *aac*) was calculated as frequencies and percentages.

RESULTS

Escherichia coli isolation

A total of 120 chicken meat samples were examined for the presence of *E. coli*, of which 27 isolates (22.5%) were identified. Initial identification was performed using chromogenic agar, in which presumptive *E. coli* isolates produced blue-to-purple colonies, indicating β-glucuronidase activity typical of *E. coli* (Figure 1). The results of *E. coli* isolates were subsequently confirmed using the VITEK 2 automated identification system (Figure 2).

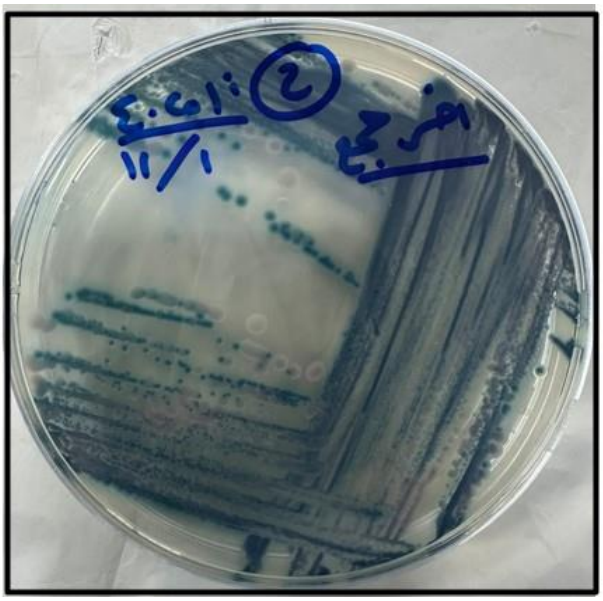


Figure 1. *Escherichia coli* isolates derived from chicken meat samples in Mosul City, Iraq, exhibited blue colonies on chromogenic agar.

bioMérieux Customer: Dr.Radhwan Al-Jammas LAB.
Microbiology Chart Report
Printed February 20, 2026:37:15 PM GMT-06:00

Patient Name: 5, .
Location:
Lab ID: 319
Isolate number:

Organism Quantity:
Selected Organism : Escherichia coli
Source: swab
Collected:

Comments:	

Identification Information	**Analysis Time:**	4.08 hours	**Status:**	Final
Selected Organism	98% Probability	**Escherichia coli**		
	Bionumber:	0405610570526600		
ID Analysis Messages				

Biochemical Details

2	APPA	-	3	ADO	-	4	PyrA	-	5	IARL	-	7	dCEL	-	9	BGAL	+
10	H2S	-	11	BNAG	-	12	AGLTp	-	13	dGLU	+	14	GGT	-	15	OFF	+
17	BGLU	-	18	dMAL	+	19	dMAN	+	20	dMNE	+	21	BXYL	-	22	BAlap	-
23	ProA	-	26	LIP	-	27	PLE	-	29	TyrA	+	31	URE	-	32	dSOR	+
33	SAC	+	34	dTAG	+	35	dTRE	+	36	CIT	-	37	MNT	-	39	5KG	-
40	ILATk	+	41	AGLU	-	42	SUCT	+	43	NAGA	-	44	AGAL	+	45	PHOS	-
46	GlyA	-	47	ODC	+	48	LDC	+	53	IHISa	-	56	CMT	+	57	BGUR	+
58	O129R	-	59	GGAA	-	61	IMLTa	-	62	ELLM	-	64	ILATa	-			

Figure 2. A VITEK 2 automated identification report confirming *Escherichia coli* isolates derived from chicken meat samples in Mosul City, Iraq

Molecular confirmation and antimicrobial resistance gene profiling

All presumptive isolates were confirmed using a molecular method (PCR) to amplify the *uidA* gene, which was unique for *E. coli.* All 27 isolates (100%) were positive for the *uidA* gene, which served as a specific genetic marker for confirming their identity as *E. coli* (Figure 3). The confirmed isolates were subsequently screened for the presence of

five AMR genes (*bla*TEM, *dfrA*, *gyrA*, *gyrB*, and *aac*) using PCR. The present results revealed that all isolates carried resistance genes, and some isolates carried more than one. Among the tested genes, *dfrA* was the most frequently detected (37.0%), followed by *bla*TEM (29.6%) and *aac* (14.8%), whereas *gyrA* was detected in only one isolate (3.7%). The *gyrB* gene was not detected in any of the isolates (Table 2; figures 4-7). The distribution of AMR genes among the 27 *E. coli* isolates revealed that 18 isolates (66.7%) carried at least one resistance gene, indicating a high prevalence of AMR within the studied population, whereas nine isolates (33.3%) did not carry any of the tested resistance genes (Table 3).

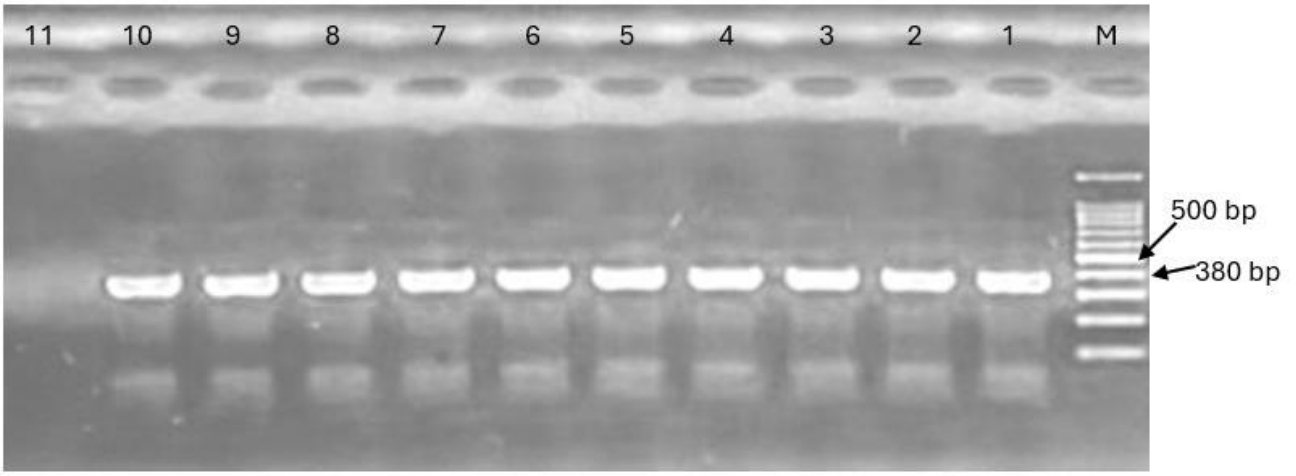


Figure 3. The PCR amplification of the *β-glucuronidase* (*uidA*) gene at a molecular size of 380 bp. Lanes 1-10: Positive samples for *Escherichia coli*, Lane 11: Negative control, Lane M: 100 bp DNA ladder

Table 2. Detection of antimicrobial resistance genes in *Escherichia coli* isolates derived from chicken meat samples in Mosul city, Iraq

Antimicrobial resistance genes	Positive isolates (number)	Percentage (%)
*bla*TEM	8	29.6
dfrA	10	37.0
gyrA	1	3.7
gyrB	0	0
aac	4	14.8

*bla*TEM: *β-lactamase* TEM, *dfrA*: *Dihydrofolate reductase A*, *gyrA*: *DNA gyrase subunit A*, *gyrB*: *DNA gyrase subunit B*, *aac*: *Aminoglycoside acetyltransferase*

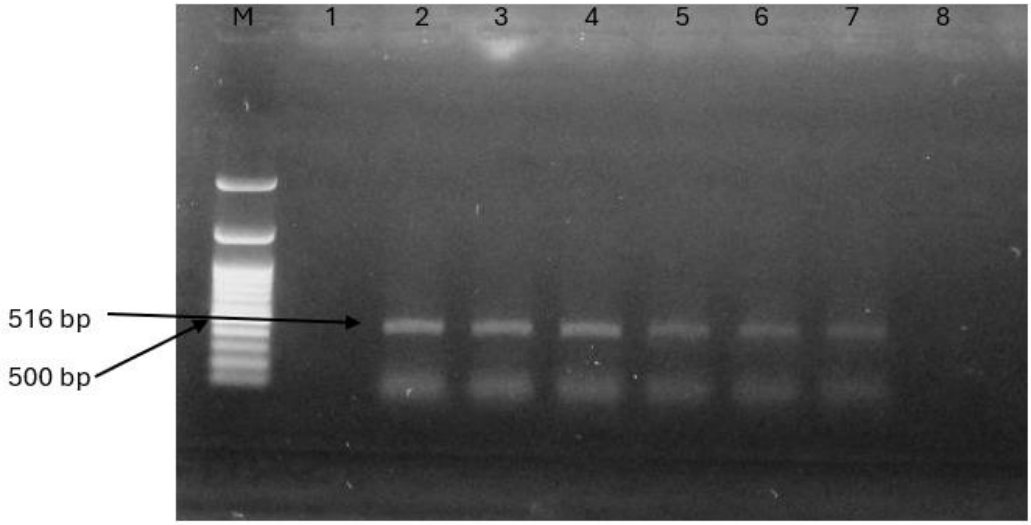


Figure 4. The PCR amplification of the *β-lactamase* TEM (*bla*TEM) gene at a molecular size of 516 bp. Lanes 2-7: Positive samples for *Escherichia coli*, Lane 1: Negative control, Lane 8: Negative sample, Lane M: 100 bp DNA ladder

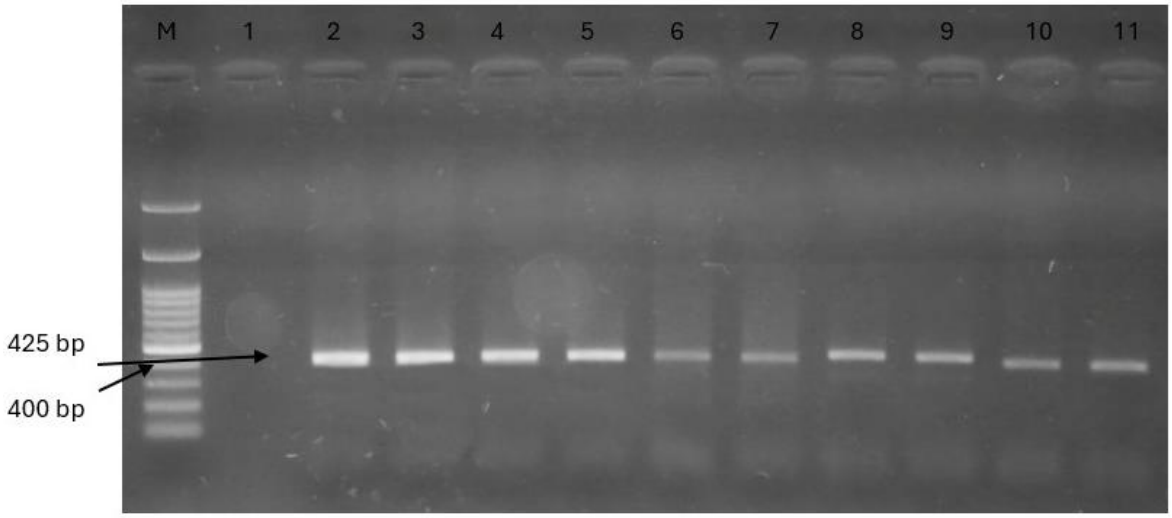


Figure 5. The PCR amplification of the *dihydrofolate reductase A* (*dfrA*) gene at a molecular size of 425 bp. Lanes 2-11: Positive samples for *Escherichia coli*, Lane 1: Negative control, Lane M: 100 bp DNA ladder

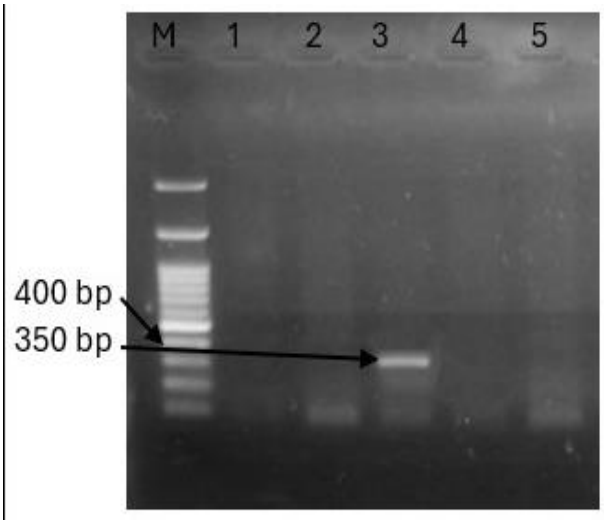


Figure 6. The PCR amplification of the *DNA gyrase subunit A* (*gyrA*) gene at a molecular size of 349 bp. Lane 3: Positive sample for *Escherichia coli*, Lanes 1, 2, 4, 5: Negative samples, Lane M: 100 bp DNA ladder

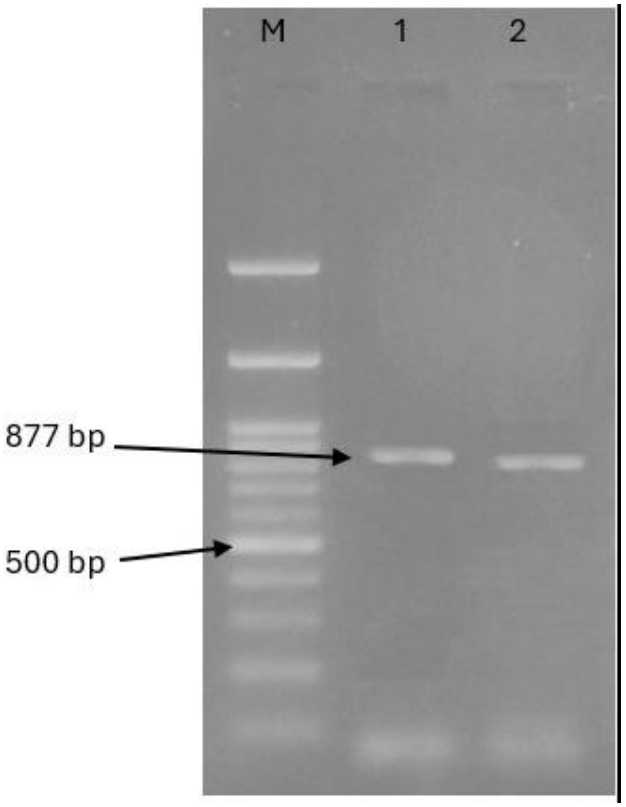


Figure 7. The PCR amplification of the *aminoglycoside acetyltransferase* (*aac*) gene at a molecular size of 877 bp. Lanes 1 and 2: Positive sample for *Escherichia coli*, Lane M: 100 bp DNA ladder

Table 3. The antimicrobial resistance gene distribution in *Escherichia coli* isolates derived from chicken meat samples in Mosul city, Iraq

Category	Number of isolates	Percentage
Isolates with no resistance genes	9	33.3%
Isolates with exactly 1 resistance gene	14	51.9%
Isolates with exactly 2 resistance genes	4	14.8%

DISCUSSION

The widespread presence of *E. coli* in chicken meat and the emergence of AMR genes in its isolates are growing public health challenges globally (Croxen et al., 2013). The use of chromogenic agar, the VITEK® automated identification system, and PCR amplification of the *uidA* gene in the identification process ensured high specificity and sensitivity, which were critical for the surveillance of foodborne pathogens (Fusaro et al., 2025). In the present study, 22.5% of chicken meat samples were contaminated with *E. coli*, consistent with previous studies reporting *E. coli* prevalence in poultry meat ranging from 10% to 48%. In these studies, approximately 100 chicken meat samples were analyzed using microbiological culture, followed by antimicrobial susceptibility testing and PCR to assess resistance patterns (Nhung et al., 2016; Brătfelan et al., 2023).

The detection of AMR genes in 66.7% of *E. coli* isolates was particularly alarming. The present findings reflected global trends of increasing AMR in foodborne pathogens, which have been attributed to the excessive use of antibiotics in poultry production (Van Boeckel et al., 2015; Tang et al., 2017). The detection of *dfrA*, *bla*TEM, and *aac* genes, particularly *dfrA*, aligns with findings from other countries. For instance, in Hong Kong, 180 fresh half-chickens from 29 wet markets were analyzed, and in China, 300 chickens were examined. These studies reported widespread resistance to trimethoprim, β-lactam, and aminoglycoside antibiotics among *E. coli* isolates collected from poultry samples (Hasib et al., 2024; Zhou et al., 2024).

Among the detected genes, *dfrA* had the highest prevalence, at 37.0%, indicating widespread trimethoprim resistance. The current finding was consistent with previous studies demonstrating that trimethoprim resistance genes were commonly found in *E. coli* isolates derived from livestock products, likely due to the widespread use of sulfonamide-trimethoprim combinations in veterinary medicine (Huovinen, 2001; Schwarz et al., 2010). The *dfrA* gene is frequently located on mobile genetic elements such as plasmids and transposons, thereby facilitating their dissemination among bacterial populations (Partridge et al., 2018).

The detection of the *bla*TEM gene in 29.6% of the isolates was substantial, as this gene provided resistance to extended-spectrum β-lactam antibiotics, which are essential in human medicine (Paterson and Bonomo, 2005; Bush and Jacoby, 2010). Faridah et al. (2023) conducted a study in Indonesia involving 115 cloacal swab samples from broiler chickens and found that 38.2% of isolates carried the *bla*TEM gene. This finding indicated that chicken meat could serve as an important reservoir of antibiotic-resistant bacteria, posing potential public health issues. The marginally elevated rate reported in Indonesia may be attributable to the isolates being obtained from live animals (cloacal samples), whereas in the present study, the isolates were derived from chicken meat.

The *aac* gene, associated with aminoglycoside resistance, was detected in 14.8% of isolates, consistent with a previous study reporting a 10% prevalence of aminoglycoside resistance genes in poultry-derived *E. coli* isolates (Brătfelan et al., 2023). The co-occurrence of multiple resistance genes in some isolates (14.8%) further highlighted the potential for multidrug resistance, likely facilitated by plasmid-mediated gene transfer (Carattoli, 2013; Partridge et al., 2018).

The absence of *gyrB* and the low prevalence of *gyrA* (3.7%) among *E. coli* isolates suggested a limited distribution of fluoroquinolone resistance, which could reflect either careful use or limited access to these antimicrobials in Middle Eastern countries (Jacoby, 2005; Chaname Pinedo et al., 2020). However, even a low frequency of fluoroquinolone resistance remains concerning, given the rapid potential for horizontal gene transfer among bacteria (Carattoli, 2013). Consumption of poultry products contaminated with multidrug-resistant *E. coli* can lead to infections that are difficult to treat, increase healthcare costs, and contribute to the global AMR crisis (WHO, 2014; Collignon et al., 2018). The detection of multiple resistance genes in some isolates emphasized the risk of co-selection and the persistence of resistant pathogens in the food chain (Marshall and Levy, 2011; Al-Mahmood, 2023). The current findings indicate that poultry products are a vital reservoir of AMR genes.

CONCLUSION

The current study revealed a high prevalence of AMR among *E. coli* isolates obtained from chicken meat, emphasizing a major concern for food safety and public health in Iraq. The presence of AMR genes in 66.7% of isolates indicated that poultry products could serve as a reservoir for resistant bacteria. The predominance of *dfrA* and *bla*TEM genes suggested widespread resistance to commonly used antimicrobial agents, particularly trimethoprim and β-lactam antibiotics. The presence of multiple resistance genes in some isolates highlighted the potential for multidrug resistance, thereby complicating treatment options and elevating the risk of transmission throughout the food supply chain. Although the prevalence of fluoroquinolone resistance genes was low, their presence still represented a potential threat due to their ability to spread rapidly via mobile genetic elements. The present study had some limitations, including a small sample

size, which may impede the generalization of the results to broader populations. Additionally, the samples were collected exclusively from a single city in Iraq, thereby limiting the ability to demonstrate regional variability. The absence of real-time PCR analysis complicated quantification of antibiotic resistance gene expression, underscoring the need for future studies that employ multi-regional sampling and next-generation sequencing to further assess AMR in *E. coli* isolates derived from poultry samples.

DECLARATIONS

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Authors' contributions

Omar Ahmad Al-Mahmood contributed to conceptualization, study design, analysis, and writing. Mohammed Jasim Mohammed Awed contributed to the investigation and analysis. Dhyaa Mohammed Jwher contributed to the investigation. All microbiological and molecular analyses were performed by Omar Al-Mahmood. All authors reviewed and approved the final edition of the manuscript.

Availability of data and materials

All datasets underlying the current study are within the manuscript and available upon reasonable request from the corresponding author.

Competing interests

The authors declared no financial or personal conflicts of interest.

Ethical considerations

All authors affirmed that the current study is original, unpublished, not under review elsewhere, and prepared and written without the use of AI tools.

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Assessment of *PPAR-α* Gene Expression in Hens' Liver After Administration of Q10 and Endosulfan

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ABSTRACT

Endosulfan is a long-lasting organochlorine pesticide, whereas coenzyme Q10 (CoQ10) is a widespread antioxidant mainly found in mitochondria. *Peroxisome proliferator-activated receptor alpha* (*PPAR-α*), a regulatory transcription factor, plays a crucial role in hepatic regulation. The current study aimed to evaluate the effect of CoQ10 and endosulfan on *PPAR-α* gene expression in broiler chicken livers. A total of 40 one-day-old broiler chickens weighing 42 ± 2 grams were randomly divided into four groups. The first group served as the control and received distilled water by gavage (T1). The second group received CoQ10 orally at 40 mg/kg body weight (T2), the third group received endosulfan orally at 30 ppm (T3), and the fourth group received the combination of CoQ10 at 40 mg/kg body weight and 30 ppm of endosulfan (T4). On days 27 and 42 of the study, 20 chickens were euthanized, and liver samples were collected for analysis of *PPAR-α* gene expression by quantitative polymerase chain reaction (qPCR). On days 27 and 42, all treatments exhibited significant downregulation of *PPAR-α* gene expression compared to the control group. The most significant suppression was observed in the endosulfan with CoQ10 (T4) group, followed by the endosulfan group (T3), whereas CoQ10 (T2) exhibited the lowest level of downregulation among the treatment groups. On day 42, all treatment groups exhibited elevated *PPAR-α* gene expression compared to the control group. The highest *PPAR-α* expression level was observed in Group T2, followed by Group T3, while Group T4 demonstrated the lowest degree of upregulation among the treatment groups. The current findings indicated an age-dependent reversal pattern in *PPAR-α* expression. On day 27, chickens demonstrated a consistent downregulation of *PPAR-α*, whereas on day 42, an upregulation was observed. The current results indicated that CoQ10 administration produced differential stimulatory effects on *PPAR-α* gene expression.

Keywords: Coenzyme Q10, Endosulfan, Liver, *PPAR-α*

INTRODUCTION

Endosulfan is a prominent chlorinated hydrocarbon insecticide, chemically similar to heptachlor, chlordane, and aldrin, used to suppress insect pests in farms and houses (Berdowska and Bandurska, 2025). Due to its high persistence and capacity to accumulate in food chains, endosulfan negatively impacts wildlife populations, leading to regulatory restrictions and bans on its use (Berdowska and Bandurska, 2025). Numerous studies have indicated that pesticide residues and their metabolites have contaminated water, ground surfaces, and soil worldwide (Moore et al., 2014; Suliman et al., 2020). Pesticide compounds exert toxic effects on cells, including immunotoxicity, and inhibit the metabolic activity of splenocytes and blood lymphocytes in poultry (Tiwari et al., 2016; Cestonaro et al., 2022). Coenzyme Q10 (Q10) is a cofactor essential for numerous enzymatic activities within the body. Ubiquinone, commonly known as Q10, is essential for the respiratory chain and oxidative phosphorylation (Gopi et al., 2015).

Structurally, CoQ10 consists of a benzoquinone ring attached to an isoprenoid side chain. The letter Q indicates the quinone structure, while the length of the isoprenoid side chain ranges from 7 to 10 units. In mammals and humans, CoQ10specifically contains 10 isoprene units (Mantle et al., 2025). The reduced form of CoQ10, ubiquinol, is a powerful lipophilic antioxidant that can regenerate both itself and other antioxidants, including ascorbate and tocopherol (Gopi et al., 2015). In recent years, Q10 has gained popularity, and its pharmacodynamics and pharmacokinetics, including dosage, safety, and drug interactions, have been examined (Bayril et al., 2020). The *peroxisome proliferator-activated receptor alpha* (*PPAR-α*) gene has been proven to be highly expressed in laying hens (König et al., 2008). The *PPAR-α* gene plays a substantial role in lipid metabolism and energy homeostasis during fasting (Fuior et al., 2023). The *PPAR-α* gene encodes a nuclear receptor transcription factor that is essential for hepatic metabolic adaptation during fasting. The *PPAR-α* gene promotes the expression of key enzymes involved in peroxisomal and mitochondrial fatty acid oxidation, including acyl CoA oxidase (ACO) and carnitine palmitoyl transferase 1 alpha (CPT1α; Pawlak et al., 2015). Fatty acid oxidation involves genes for acyl-CoA dehydrogenases with different chain lengths, including medium-chain (MCAD) and long-chain (LCAD; Fuior et al., 2023). Therefore, *PPAR-α* is essential for fatty acid oxidation in mammals

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(Strömqvist et al., 2012; Du et al., 2022). The level of liver-type fatty acid-binding protein is regulated by *PPAR-α* activity in poultry, and the *liver fatty acid-binding protein* (*L-FABP*) gene may be linked to fat deposition in chickens (Zhang et al., 2023). The present study aimed to assess the effects of endosulfan and CoQ10 on *PPAR-α* gene expression in the hepatic tissue of broiler chickens.

MATERIALS and METHODS

Ethical approval

The current study was conducted within the animal laboratory of the College of Veterinary Medicine at the University of Mosul, Mosul, Iraq. The present study adhered to the ethical approval granted by the Animal Welfare Committee on July 20, 2025, with the approval number UM.VET.2025.061.

Experimental animals

A total of 40 one-day-old Ross 308 broiler chickens, weighing 40-45 g, were purchased from the Al-Walid Hatchery in the Al-Hamdaniya area of Mosul, Iraq. The broiler chickens were raised in controlled enclosures and randomly assigned within these areas, and fed a commercially mashed diet. The temperature started at 32°C and gradually decreased by 2-3°C each week. The cage environment was well controlled, with ventilation, humidity maintained at 50%-70%, and a 24-hour lighting system.

Study design

Forty chickens were randomly allocated into four groups. The first group served as the control (T1), the second group received CoQ10 at 40 mg/kg body weight (T2), the third group received endosulfan at 30 ppm (T3), and the fourth group received a combination of CoQ10 and endosulfan (T4). All groups were treated orally from day 1 to day 42. On days 27 and 42, the broiler chickens were euthanized via jugular vein incision, and liver tissue samples were collected and promptly frozen at -20°C for subsequent analysis.

RNA extraction

Approximately 50-100 mg of frozen liver tissue was placed in a microcentrifuge tube. Subsequently, 400 µL of lysis buffer, 2 µL of β-mercaptoethanol, and 20 µL of proteinase K solution were added to a 1 mL microcentrifuge tube containing the samples. The mixture was then vortexed and sonicated to homogenize the sample. After 10 minutes of incubation at 26°C, the mixture was centrifuged for three minutes at 13,000 rpm. A 2.0 mL collection tube was used to carefully transfer the supernatant to the upper reservoir (spin column 1). The mixture was then subjected to pulse-vortexing for 30 seconds and subsequently centrifuged at 13,000 rpm. The flux-through was kept constant. Subsequently, 400 µL of binding buffer was added to a collection tube and mixed thoroughly for 10 seconds using pulse-vortexing. The mixture was then centrifuged at 13,000 rpm for one minute. Afterward, 500-600 µL of the supernatant was transferred to a new micro-centrifuge tube (1.5 µL). Then, 200 µl of absolute ethanol (99.99%) was added and mixed thoroughly, followed by the addition of 600 µl of lysate buffer to the upper reservoir of spin column 2 (green ring) using a 2.0 ml collection tube. The mixture was centrifuged at 13,000 rpm for 10 seconds. The spin column was assembled and subsequently transferred into the 2 mL tube. The spin column was then filled with 500 µl of the washing solution and centrifuged for 10 seconds at 13,000 rpm. The spin column was assembled with the 2.0 mL collecting tube after the flow-through was poured out, and incubated for 15 minutes at room temperature (25°C). To eliminate any residual ethanol, the spin column was further centrifuged and dried for one minute at 13,000 rpm after the addition of 700 µl of washing solution. The spin column was moved to a 1.5 mL tube, then the total RNA was centrifuged at 13,000 rpm for one minute, and at -20°C, the extracted RNA was stored (Sharideh et al., 2020).

Quantitative polymerase chain reaction

The master kit was supplied by Addbio (Korea) for the real-time PCR. The kit was used for all procedures involving reverse transcription of RNA into cDNA, followed by real-time PCR amplification. Only primers and RNA samples were introduced into the single RT-PCR SYBR Master mix. The reference sequence used was NM_001001464.1, with the primer pair originally designed and validated by Sharideh et al. (2020). The forward and reverse primer sequences for *PPAR-α* were 5′-GGATGCTGGTAGCCTATGGA-3′ and 5′-GGACGATCTCCACAGCAAAT-3′, respectively (Wang et al., 2020). To design chicken *GAPDH* primers, the reference sequence K01458.1 was employed. The forward and reverse primer sequences were 5′-TGGTGACAGCCATTCTTCCA-3′ and 5′-TCCAACAAAGGGTCCTGCTT-3′, respectively (Wang et al., 2020). The total volume was 20 µl, which included 10 µl of One RT-PCR SYBR Master Mix, 0.5 µl of each primer, 3 µl of RNA sample, and 6 µl of nuclease-free water. According to the manufacturer's instructions,

optimal reaction conditions included reverse transcription for 20 minutes at 50°C, RNA hybrid denaturation and reverse transcriptase inactivation at 95°C for 10 minutes, followed by 40 cycles of qPCR. Each cycle consisted of denaturation for 15 seconds at 95°C and annealing for 60 seconds at 60°C. For real-time PCR, the Step One Plus Real Time PCR kit (USA) was employed.

Statistical analysis

The *PPAR-α* gene expression was quantified by qRT-PCR and expressed as fold change relative to the control group. Differences among groups were assessed by one-way ANOVA, followed by Tukey's post hoc test for comparison between groups. A p-value less than 5% ($p < 0.05$) was considered statistically significant.

RESULTS

On day 27, the control group exhibited ΔCt values ranging from 0.5 to 1.1, with a mean ΔCt of 0.9. All treatment groups demonstrated a significant downregulation of *PPAR-α* expression compared to the control group ($p \leq 0.05$; Figure 1). Group T3 exhibited ΔCt values ranging from 5.0 to 6.4, yielding ΔΔCt values between 4.1 and 5.5, which related to the extremely low fold changes (0.02-0.06). The present results indicated a strong suppressive effect of endosulfan on *PPAR-α* transcription. Group T3 resulted in a mean fold change of 0.04, indicating an approximate 96% reduction in *PPAR-α* expression compared to the control group. CoQ10 treatment (T2) decreased *PPAR-α* levels, but the effect was less pronounced compared to endosulfan treatment (T3). The ΔCt values ranged from 4.0 to 5.2, resulting in ΔΔCt values of 3.1-4.3 and fold changes of 0.05-0.12 in Group T2. Group T2 demonstrated a significant suppression of *PPAR-α* expression, with a mean fold change of 0.083, although this effect was milder than that observed in Group T3 ($p \leq 0.05$). Group T4 demonstrated the most significant downregulation of *PPAR-α*, with ΔCt values of 5.6-7.4, ΔΔCt values of 4.7-6.5, and fold changes ranging from 0.01 to 0.04. The mean fold change of 0.027 in Group T4 confirmed that the combination of endosulfan and CoQ10 exerted a synergistic downregulatory effect. The co-administration of CoQ10 with endosulfan did not mitigate the toxicity on day 27. The present result demonstrated that all groups significantly downregulated *PPARα* gene expression, with the greatest suppression observed in Group T4, followed by T3 and T2 on day 27 of age ($p \leq 0.05$).

On day 42, the ΔCt values of control samples ranged from 3.20 to 4.40, resulting in a mean ΔCt of 3.93. In contrast to the observations at day 27, all treatment groups exhibited negative ΔΔCt values on day 42, indicating the upregulation of *PPAR-α* in older broiler chickens (Figure 2). Endosulfan induced a moderate upregulation, with ΔΔCt values ranging from –3.43 to –0.93 and fold changes ranging from 1.9 to 10.8 (mean 5.3). The highest *PPAR-α* upregulation was observed in Group T2, which recorded ΔΔCt values of –4.53 to –2.53, corresponding to fold changes of 5.7–23.1 and a mean of 14. Group T4 exhibited mild to moderate upregulation, with a fold change ranging from 1.3 to 6.6 (mean 3.2), indicating that the antagonistic effect of endosulfan partially mitigated the stimulatory action of CoQ10 at 42 days. Notably, age differences resulted in an inverted expression pattern, in which 27-day-old chickens demonstrated downregulation of *PPAR-α* expression, whereas 42-day-old chickens exhibited significant upregulation of *PPAR-α* expression ($p < 0.05$).

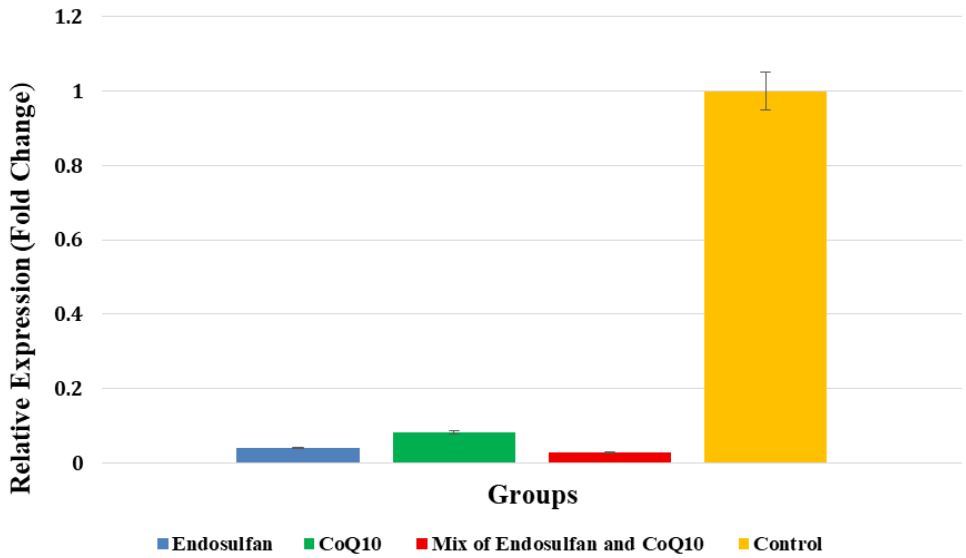


Figure 1. The *PPAR-α* gene expression across the groups at day 27

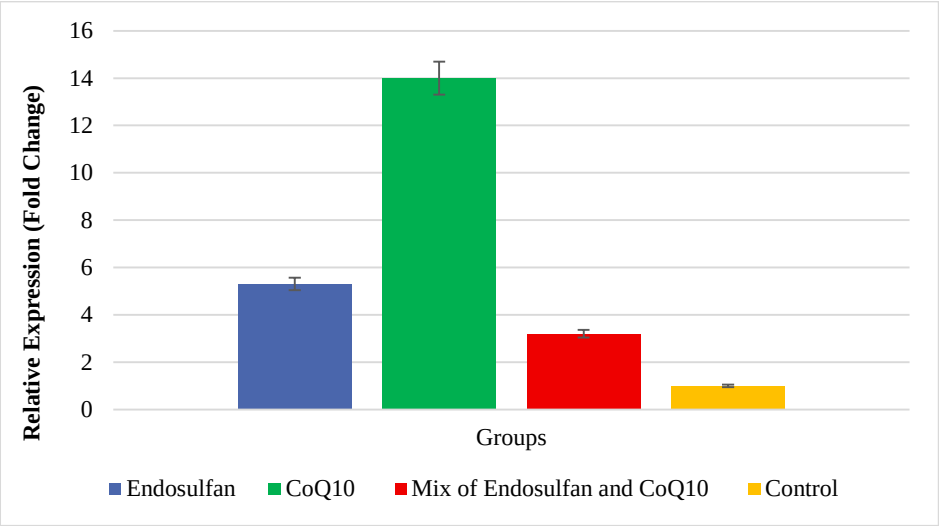


Figure 2. *PPAR-α* gene expression across the groups at day 42

DISCUSSION

The *PPAR* genes are nuclear transcription-modulating receptors that play vital roles in hepatic and overall body energy balance (Wang et al., 2020). In addition to their functions in glucose and lipid metabolism, multiple studies indicated that *PPAR* genes modulate inflammatory responses (Tan et al., 2017; Vázquez-Carrera and Wahli, 2022). New technologies, such as single-cell RNA sequencing, have helped reveal the complexity of *PPAR* expression, regulation, and signaling (Berthier et al., 2021). CoQ10 consistently enhanced *PPAR-α* expression and elicited the strongest induction in hepatic tissue, thereby indicating dose-dependent efficacy and age-related metabolic response in broiler chicken liver. The Q10 has been shown to reduce reactive oxygen species (ROS) production, thereby enhancing mitochondrial function and influencing the sensitivity of specific genes to oxidative stress, including adiponectin and *PPAR-α* (Vázquez-Carrera and Wahli, 2022). Adiponectin and *PPAR-α* are known as crucial factors in cholesterol and lipid metabolism (Zhang et al., 2023). As with other nuclear receptor ligands such as metabolic sensors, Q10 may be transported by proteins that facilitate its entry into the nucleus (Mantle, 2024). Two proteins known to bind Q10 are Q10p and Saposin B (SapB; Jin et al., 2008). The Saposin B protein facilitates the control and transport of Q10 within human cells and acts as a crucial Q10-binding protein (Jin et al., 2008). Additionally, previous studies have shown that *PPAR coactivator-1α* (*PGC-1α*) and *PPARα* bind to the D-loop promoter within mitochondria and are linked to enhanced *PPARα* gene expression (Puigserver 2005; Dominy and Puigserver 2013). The distinct responses observed between day 27 and day 42 suggested a developmental shift in hepatic sensitivity. Broiler chickens exhibited strong susceptibility to endosulfan, affecting nervous system receptors and suppressing metabolic genes, such as *PPARs*. In contrast, older broiler chickens exhibited adaptive metabolic activation, likely facilitated by an enhanced hepatic detoxification capacity and *PPAR-α*–mediated lipid mobilization. It has been indicated that *PPAR-α* regulates multiple genes involved in lipid metabolism (Pawlak et al., 2015).

Although gene regulation of liver lipid metabolism has been extensively investigated, the regulation of *PPAR* genes in the gastrointestinal tract has been poorly explored (Grabacka et al., 2024). The *PPAR-α* is one of the key receptors involved in regulating the metabolism of carbohydrates and lipids (Hernández-Valdez et al., 2023). Besides the similarity in expression patterns, RT-PCR analysis revealed that genes associated with transport and metabolism were downregulated in the layer exposed to quinalpos (Arjun et al., 2026). While endosulfan suppressed lipid metabolism genes in younger chickens by day 27, older chickens exhibited partial compensatory upregulation of the *PPAR-α* gene by day 42. The current findings might be attributed to the pesticides' mechanism of action and their effects on carbohydrate and lipid metabolism. The co-administration of CoQ10 and endosulfan demonstrated mild to moderate upregulation, suggesting that antagonistic effects of endosulfan partially mitigate the stimulatory action of CoQ10 at 42 days of age. Mild to moderate upregulation of gene expression might be attributed to oxidative stress induced by endosulfan and the protective and metabolic-enhancing effects of CoQ10.

CONCLUSION

The different responses of the *PPAR-α* gene expression at days 27 and 42 indicated an age-related transition in hepatic sensitivity, marked by suppression at day 27 and upregulation at day 42. Co-administration of CoQ10 and endosulfan in

broiler chickens affected *PPAR-α* gene expression, potentially due to mitochondrial metabolism and energy production modifications observed on day 42. The *PPAR-α* gene expression in broiler chickens was influenced by environmental variability, tissue-specific regulation, metabolic differences, and the inability of mRNA expression to fully reflect functional receptor activity. Further studies are necessary to examine the effects of CoQ10 and endosulfan on laying and breeder hens throughout the production phase.

DECLARATIONS

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Authors' contributions

Hamid and Al-Sabaawy contributed to the study design and conception, data analysis, and interpretation of results, prepared the initial draft of the manuscript, and critically reviewed it to ensure its scientific integrity. All authors have read and approved the final edition of the manuscript prior to publication in the current journal.

Availability of data and materials

The data are available upon reasonable request from the corresponding author.

Conflict of interests

The authors have declared that there is no conflict of interest related to this study.

Ethical considerations

All ethical aspects of the present study, including plagiarism, data integrity, consent to publish, and avoidance of misconduct or duplication, were carefully considered and approved by the authors. No AI tools were used in the preparation and writing the present study.

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Identification of Multidrug-Resistant *Aeromonas salmonicida* from Imported Seafood in Mosul City Shops via Microarray Technology

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ABSTRACT

The widespread emergence of multidrug-resistant (MDR) pathogens in imported seafood is an increasing public health concern. The present study aimed to detect *Aeromonas salmonicida* (*A. salmonicida*) in seafood samples and to assess the antibiotic resistance gene profiles of the isolated bacteria using conventional polymerase chain reaction (PCR) and DNA microarray analysis. Twenty samples consisting of shrimp, mussels, oysters, and salmon (five of each) were collected from shops in Mosul city, Iraq. The biochemical characteristics of the strains were determined using the VITEK 2 compact system, and their identities were confirmed by *16S rRNA* gene sequencing, which showed ≥99% identity to database sequences. Nine samples tested positive for *A. salmonicida*, with a prevalence of 45 percentage, with shrimp and salmon each accounting for three isolates (60%), mussels for two (40%), and oysters for one (20%). Microarray testing identified seven isolates as MDR, whereas two strains had no resistance genes and were antibiotic-sensitive. The current results revealed the absence of genes encoding extended-spectrum β-lactamases (ESBLs), including *bla*SHV, *bla*CTX-M, *bla*FOX, *bla*PER, *bla*VEB, and *bla*CMY, in all MDR strains. Tetracycline resistance was observed in 28.6% (2/7) of MDR isolates, with the *tetA* gene present, whereas the *tetB* gene was completely absent from all MDR isolates. Concerning sulfonamide resistance, all MDR isolates were found to carry sul1, whereas none carried sul2. The *aphA* and *aadA1* genes were detected in all seven MDR strains, while the *aac(6')-Ib* gene was found in only three of the seven MDR isolates (42.9%). Additionally, each of these strains carried *intI1* and *qacEΔ1* genes. The widespread occurrence of MDR *A. salmonicida* carrying diverse resistance genes in imported seafood presented a significant public health risk.

Keywords: *Aeromonas salmonicida*, Multidrug-resistant, Microarray, Public health, Seafood

INTRODUCTION

Seafood is a crucial component of human nutrition because it provides high-quality protein, minerals, and essential fatty acids that support healthy metabolism and cardiovascular health (Jayasekara et al., 2020). Global seafood consumption is rising steadily as more people recognize its health benefits. Consequently, several countries, including Iraq, are importing large quantities of frozen and chilled fish products (Al-Busaidi et al., 2016). However, globalization of the seafood supply chain has enabled the cross-border spread of microbial contaminants, including antibiotic-resistant bacteria, which are important for clinical and veterinary health. In recent decades, different non-cholera pathogenic *Vibrio* species have attracted increasing attention from public health authorities (Muzembo et al., 2024). *Aeromonas* species, particularly *Aeromonas salmonicida* (*A. salmonicida*), are significant because they thrive in aquatic environments, cause fish infections, and may harbor multidrug-resistant (MDR) genes that might be transmitted to humans through seafood consumption (Tao et al., 2026).

Aeromonas salmonicida was previously identified as the causative agent of furunculosis (Elbaiomy et al., 2025). *Aeromonas salmonicida* is a Gram-negative, facultatively anaerobic, psychrophilic bacterium that lives in freshwater and seawater. Several species of *Aeromonas* are recognized as causative agents of human infections. Although primarily identified as pathogens in fish, numerous species have been associated with gastroenteritis, wound infections, septicemia, and illnesses affecting the skin and soft tissues (Ali Eltabey, 2023).

The emergence of MDR in *A. salmonicida* is potentially linked to the widespread use of antimicrobial agents in aquaculture, including tetracycline, fluoroquinolones, sulfonamides, β-lactams, and aminoglycosides, as well as to environmental contamination from wastewater and other aquatic pollutants (Santos and Ramos, 2018).

Aeromonas salmonicida can acquire resistance genes via mobile genetic elements such as plasmids, integrons, and transposons under the selective pressure of antimicrobial agents. These mechanisms can promote MDR and the spread of resistant strains through imported fish products (Mutuku et al., 2022).

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The increasing consumption of seafood in Iraq, particularly in Mosul, has been observed over the past decade, linked to evolving consumption trends and the availability of imported frozen fish from regional and international markets. There is significant demand for monitoring; however, microbiological screening of imported seafood is rarely conducted. The current gaps highlighted the need for advanced, rapid molecular tools capable of detecting antibiotic resistance markers and distinguishing resistant strains from susceptible strains with high accuracy (Butz et al., 2019). Bacteriological techniques such as culture, biochemical identification, and the disk diffusion assay (Barker and Kehoe 1995) provide valuable information, but they are time-consuming, often taking several days to complete. Additionally, bacteriological techniques generally lack the sensitivity necessary to characterize complex resistance profiles (Vasala et al., 2022). Molecular techniques such as polymerase chain reaction (PCR) and real-time PCR (qPCR) have improved detection capabilities. However, these methods are limited to assessing only a select number of genetic markers per reaction (Butz et al., 2019). In contrast, the DNA microarray technique offers a rapid, high-volume screening system that enables concurrent screening of hundreds of virulence factors, species-specific sequences, and antibiotic resistance genes in a single assay (Peterson et al., 2010). The DNA microarray technique is considered more efficient and faster at identifying MDR pathogens, supporting regulatory compliance and food safety decisions (Ali Eltabey, 2023). The use of microarray technology to identify *A. salmonicida* in imported seafood sold in Mosul City, Iraq, is a significant concern, given the lack of local data on the prevalence of resistant aquatic pathogens in the retail environment. Fish products imported from countries with high antibiotic use in aquaculture may carry resistance genes, contributing to the spread of MDR organisms worldwide. Studying *A. salmonicida* in seafood products is essential for assessing potential transmission pathways (Cabello et al., 2016). Therefore, the present study aimed to identify *A. salmonicida* in seafood samples and to evaluate antibiotic resistance genes in the isolated bacteria using conventional PCR and DNA microarray analysis.

MATERIAL AND METHODS

Ethical approval

The Scientific Committee of the College of Veterinary Medicine at the University of Mosul, Iraq, approved this study under reference number VET.MED/2026/0173 on January 5, 2026. All procedures were conducted in strict accordance with the institution's animal ethics guidelines.

Sampling

Twenty samples of imported seafood were collected randomly from retail shops and batches in Mosul City, Iraq, for the present investigation. The sampling consisted of four species, including shrimp (n = 5), mussels (n = 5), oysters (n = 5), and salmon (n = 5). The selected samples were representative of the different seafood commonly consumed by the local population. Furthermore, the sampling was practically feasible, as supported by preliminary studies on microbial contamination, dosage, and antimicrobial resistance in seafood products. Although a formal power calculation was not conducted, the sample size was adequate to acquire multiple bacterial isolates for subsequent molecular characterization. Each sample was transported in a sterile, insulated container at 4°C to the laboratory. All processing steps were initiated within four hours of collection to prevent any fluctuations in bacterial proliferation (Odour-Odote, 2020).

Preparation and isolation of *Aeromonas* spp.

About 10 g of each seafood sample was collected using an aseptic technique and placed in a sterile stomacher bag containing 90 mL of phosphate-buffered saline (PBS) for two minutes of homogenization (Bolivar et al., 2016). Subsequently, the homogenates were kept at 37°C for 18 to 24 hours to facilitate pre-enrichment. *Aeromonas salmonicida* is psychrophilic; therefore, the incubation temperature of 37°C might have favored the proliferation of mesophilic contaminants or induced stress on the target organism (Boziaris et al., 20214). Then, a loopful of the enriched suspension was streaked onto the selective medium (Chromogenic agar). Colonies exhibiting characteristics typical of *Aeromonas*, such as smoothness, convexity, and a greyish to translucent appearance, were subcultured onto tryptic soy agar (TSA) to obtain pure cultures for subsequent analysis (Sarder, 2017).

VITEK 2 compact system

The automated VITEK 2 compact system (bioMérieux, France) was used to identify *A. salmonicida* isolates in accordance with the manufacturer's instructions. Initially, presumptive *Aeromonas* isolates were subcultured onto TSA and incubated at 37°C for 24 hours to obtain pure, fresh culture colonies (Alghabshi et al., 2018). A standardized bacterial suspension was made by transferring a well-isolated colony into sterile 0.85% saline solution. The turbidity of the solution was then adjusted to a range of 0.50 to 0.63 McFarland, using the DensiCHEK™ turbidity meter. The prepared inoculum was used to inoculate the VITEK 2 Compact system, specifically the Gram-negative identification card, which incorporates multiple biochemical substrate panels for identifying Gram-negative bacteria. The Gram-negative cards evaluated several biochemical reactions, such as carbohydrate utilization, enzyme activities, decarboxylation, and fermentation. The biochemical reaction group demonstrated predominantly positive responses, whereas the other groups exhibited negative responses (Table 1). Enzymatic and assimilation tests, including ornithine

decarboxylase (ODC), citrate utilization (CIT), gamma-glutamyl transferase (GGT), and β-galactosidase, further reinforced species-level identification (Table 1). The identification of isolates was performed by comparing their biochemical profiles with the system's database. Isolates having a confidence level of ≥ 95% was confirmed as *A. salmonicida* (Nakasone et al., 2007).

Table 1. Biochemical reaction profile of *Aeromonas salmonicida* using the VITEK 2 compact Gram-negative identification system

No.	Biochemical test	Reaction	No.	Biochemical test	Reaction	No.	Biochemical test	Reaction	No.	Biochemical test	Reaction	No.	Biochemical test	Reaction
1	APPA	Negative (-)	**11**	OGT	Positive (+)	**21**	PLE	Negative (-)	**31**	ILATk	Positive (+)	**41**	CIT	Positive (+)
2	ADO	Positive (+)	**12**	dXYL	Positive (+)	**22**	TYR	Positive (+)	**32**	AGLU	Negative (-)	**42**	BGLU	Positive (+)
3	PyrA	Positive (+)	**13**	IGLU	Positive (+)	**23**	URE	Positive (+)	**33**	SUCT	Positive (+)	**43**	O129R (vibriostatic)	Resistant (+)
4	LARL	Positive (+)	**14**	dMAL	Positive (+)	**24**	dSOR	Positive (+)	**34**	NAGA	Positive (+)	**44**	GGAA	Positive (+)
5	dCEL	Negative (-)	**15**	dMAN	Positive (+)	**25**	SAC	Positive (+)	**35**	AGAL	Positive (+)	**45**	MLTa	Negative (-)
6	BGAL	Positive (+)	**16**	dMNE	Positive (+)	**26**	dTAG	Negative (-)	**36**	PHOS	Positive (+)	**46**	ELLM	Positive (+)
7	H2S	Negative (-)	**17**	IXYL	Negative (-)	**27**	CIT	Positive (+)	**37**	GlyA	Positive (+)	**47**	ILATa	Negative (-)
8	BNAG	Positive (+)	**18**	BAlap	Negative (-)	**28**	CMT	Positive (+)	**38**	ODC	Positive (+)			
9	AGLTp	Negative (-)	**19**	ProA	Positive (+)	**29**	MNT	Negative (-)	**39**	LDC	Positive (+)			
10	dGLU	Positive (+)	**20**	LIP	Negative (-)	**30**	5KG	Negative (-)	**40**	IHISa	Negative (-)			

Positive (+) and negative (−) reactions represented substrate utilization and enzymatic activities used for species-level identification.

Molecular identification

The molecular identification of *A. salmonicida* isolates was performed by amplifying and sequencing the *16S rRNA* gene (Zhu et al., 2025). Genomic DNA from each isolate, obtained from a pure overnight culture, was extracted employing a commercial bacterial DNA extraction kit (Bangalore Genei, India) in accordance with the manufacturer's protocols. Universal bacterial forward and reverse primers were designed using conserved *16S rRNA* sequences, targeting the conserved regions on both sides of the gene. The *16S rRNA* (~600 base pairs) was amplified using PCR (Table 2). A 25 µL PCR reaction mixture was prepared, comprising 5 µL of template DNA, 5 µL of each forward and reverse primer at 10 pM, 1 µL of PCR master mix, and 12.5 µL of nuclease-free water. The PCR amplification was conducted for 35 cycles, starting with an initial denaturation step at 95°C, followed by 30 cycles consisting of denaturation at 95°C, annealing at 65°C, and extension at 72°C. The PCR process concluded with a final extension step (Kumar 2022). The PCR products were confirmed by gel electrophoresis (1%) at 120 volts on a 400 mm gel, and then purified for Sanger sequencing. The sequences were further analyzed using the BLAST tool of the National Center for Biotechnology Information (NCBI) GenBank (NCBI, USA). The species-level identification of the isolates was confirmed through molecular methods, exhibiting over 99% sequence similarity (Wojnarowski et al., 2024).

Table 2. Primer sequences used for PCR amplification of the *16S rRNA* gene in *Aeromonas salmonicida*

Primers	Oligonucleotide sequence (5′–3′)	Reference
Forward	AGAGTTTGATCMTG CTCAG	Khalaf et al. (2025)
Reverse	TACGGYTACCTTGTTACGACTT	Khalaf et al. (2025)

Microarray technique

Aeromonas salmonicida has been examined for antibiotic resistance genes utilizing a DNA microarray platform. In the present study, 41 gene-specific oligonucleotide probes were designed using reference sequences from the NCBI GenBank database. These probes were resistant to β-lactams, tetracyclines, fluoroquinolones, aminoglycosides, sulphonamides, phenicols, and polymyxins, all of which were classified as MDR factors according to the British Pharmacopeia (Lough, 2025). The probes target the *intI1*, *qacEΔ1*, *acrB*, *tolC*, *marA*, and *soxS* genes, which were involved in the resistance strain. To confirm probe specificity, an in silico BLASTn search was conducted, and no significant matches to non-target sequences were observed. Positive-control strains containing each target gene and negative-control strains lacking these genes were used to experimentally validate probe performance, along with no-template controls. Genomic DNA from different bacterial isolates, labeled with Cy5 and Cy3 fluorescent dyes, was hybridized to the array under optimized conditions, then washed post-hybridization to remove nonspecific binding. Microbial Analysis Software was utilized to scan slides and analyze fluorescence. Three housekeeping genes (*rpoB*, *gyrB*, and *recA*) were assessed for controls. The detected resistance profiles are shown in Table 3.

Table 3. Oligonucleotide probes used in the DNA microarray for the detection of antibiotic and multidrug resistance genes in *Aeromonas salmonicida*

No.	Gene symbol	Antibiotic	Antibiotic group	Resistance mechanism	Oligonucleotide probe sequence (5′–3′)
1	*bla*TEM	Ampicillin	β-Lactams	β-lactamase production	ATGAGTATTCAACATTTCCGTGTCGCCCTTATTCCCTTTTTGATGCGTCT
2	*bla*SHV	Amoxicillin	β-Lactams	β-lactamase production	TTGACCGCTGTTGTTAGCGTTGCTGTTATCGCTCATGGTGTTGTTAT
3	*bla*CTX-M	Cefotaxime	ESBL	Extended-spectrum β-lactamase	GCTGATGAAGGTCGCTGATACCGCTGATGATGCGTCTGCTGTTAT
4	*cphA*	Imipenem	Carbapenems	Metallo-β-lactamase	CGCTTATCGGCTGTTACGATGCTGATGACGTTGTTACCGATGCT
5	*bla*FOX	Cefoxitin	AmpC β-lactams	Cephamycinase	GCTGTTGATCGCTGATGATGACGTTGCTGATGTTGTTACGCT
6	*bla*OXA	Oxacillin	β-Lactams	Oxacillinase	ATGTTGATCGCTGATGTTGCTGACGTTGATGATGCGTTGCT
7	*tetA*	Tetracycline	Tetracyclines	Efflux pump	CGCTGATGTTGATGATGCTGTTGCTGACGTTGCTGATGTTGTT
8	*tetB*	Tetracycline	Tetracyclines	Efflux pump	TTGATGCTGTTGACGTTGATGATGCTGTTGCTGATGTTGAT
9	*tetE*	Tetracycline	Tetracyclines	Efflux pump	GATGCTGTTGACGTTGCTGATGTTGATGATGCTGTTGCT
10	*tetD*	Doxycycline	Tetracyclines	Efflux pump	GCTGATGTTGTTGACGTTGCTGATGATGCTGTTGATGCT
11	*gyrA*	Ciprofloxacin	Fluoroquinolone	Target site mutation	GCTGATGACGTTGCTGTTGATGCTGATGTTGATGCTGTT
12	*parC*	Nalidixic acid	Quinolones	Target site mutation	GTTGATGCTGATGTTGCTGTTGATGACGTTGCTGAT
13	*qnrA*	Ciprofloxacin	Fluoroquinolone	Target protection	GCTGTTGATGCTGATGTTGACGTTGCTGATGTTGTT
14	*qnrB*	Ciprofloxacin	Fluoroquinolone	Target protection	TTGCTGATGTTGACGTTGATGCTGTTGATGCTGAT
15	*qnrS*	Ciprofloxacin	Fluoroquinolone	Target protection	GATGTTGCTGATGACGTTGATGCTGTTGATGCT
16	*aac(6')-Ib*	Gentamicin	Aminoglycoside	Acetyltransferase	GCTGATGTTGCTGACGTTGATGCTGTTGATGCTGTT
17	*aphA*	Kanamycin	Aminoglycoside	Phosphotransferase	GTTGATGCTGATGTTGCTGATGACGTTGCTGTT
18	*aadA1*	Streptomycin	Aminoglycoside	Adenyltransferase	GCTGATGTTGACGTTGCTGATGTTGATGCTGTT
19	*armA*	Amikacin	Aminoglycoside	*16S rRNA* methylation	TTGCTGATGTTGATGCTGACGTTGCTGATGTT
20	*sul1*	Sulfamethoxazole	Sulfonamides	Folate pathway inhibition	GATGCTGTTGATGACGTTGCTGATGTTGCTGTT
21	*sul2*	Sulfadiazine	Sulfonamides	Folate pathway inhibition	TTGCTGATGTTGACGTTGCTGATGTTGATGCT
22	*dfrA*	Trimethoprim	Diaminopyrimidines	DHFR modification	GCTGATGTTGCTGACGTTGATGCTGTTGAT
23	*floR*	Florfenicol	Phenicols	Efflux pump	GTTGATGCTGATGTTGCTGACGTTGATGCT
24	*cat*	Chloramphenicol	Phenicols	Acetyltransferase	GCTGATGTTGACGTTGCTGATGCTGTT
25	*mcr-1*	Colistin	Polymyxins	Lipid A modification	GTTGCTGATGTTGACGTTGATGCTGATGTT
26	*arnT*	Polymyxin B	Polymyxins	LPS modification	GCTGATGTTGCTGACGTTGATGCTGTT
27	*intI1*	—	Integrons	Gene cassette integration	GATGCTGTTGATGACGTTGCTGATGTT
28	*qacEΔ1*	Disinfectants	QAC resistance	Efflux pump	TTGCTGATGTTGACGTTGATGCT
29	*acrB*	Multiple	MDR	RND efflux pump	GCTGATGTTGACGTTGCTGATGTT
30	*tolC*	Multiple	MDR	Efflux channel	GTTGCTGATGTTGACGTTGAT
31	*marA*	Multiple	MDR	Transcriptional regulator	GCTGATGTTGCTGACGTTGAT
32	*soxS*	Multiple	MDR	Stress response regulator	TTGCTGATGTTGACGTTGCT
33	*strA*	Streptomycin	Aminoglycoside	Phosphotransferase	GCTGATGTTGACGTTGCT
34	*strB*	Streptomycin	Aminoglycoside	Phosphotransferase	GTTGCTGATGTTGAC
35	*ant(3")-Ia*	Gentamicin	Aminoglycoside	Nucleotidyltransferase	GCTGATGTTGCTGAC
36	*bla*CMY	Cephalosporins	AmpC β-lactams	β-lactamase	GTTGCTGATGTTGAC
37	*bla*PER	Ceftazidime	ESBL	β-lactamase	GCTGATGTTGAC
38	*bla*VEB	Cephalosporins	ESBL	β-lactamase	GTTGCTGATGTT
39	*rpoB*	—	Control	Housekeeping gene	GCTGATGTTGACGTTGCT
40	*gyrB*	—	Control	Housekeeping gene	GTTGCTGATGTTGAC
41	*recA*	—	Control	DNA repair protein	GCTGATGTTGAC

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RESULTS

A total of nine different strains were successfully isolated. Three strains were isolated from shrimp, and three strains were isolated from salmon fish, two strains from mussels, and one strain from oysters. All isolates grew successfully on the selective agar. *Aeromonas salmonicida* isolates were distinguished phenotypically by colony morphology, such as shape, size, and growth features, rather than by the green coloration of the colonies (Figure 1). All observations confirmed the presence of *A. salmonicida* isolates in different types of imported seafood, with colony morphology on selective agar used as a preliminary identification step, followed by confirmatory biochemical and molecular characterization (Table 4).

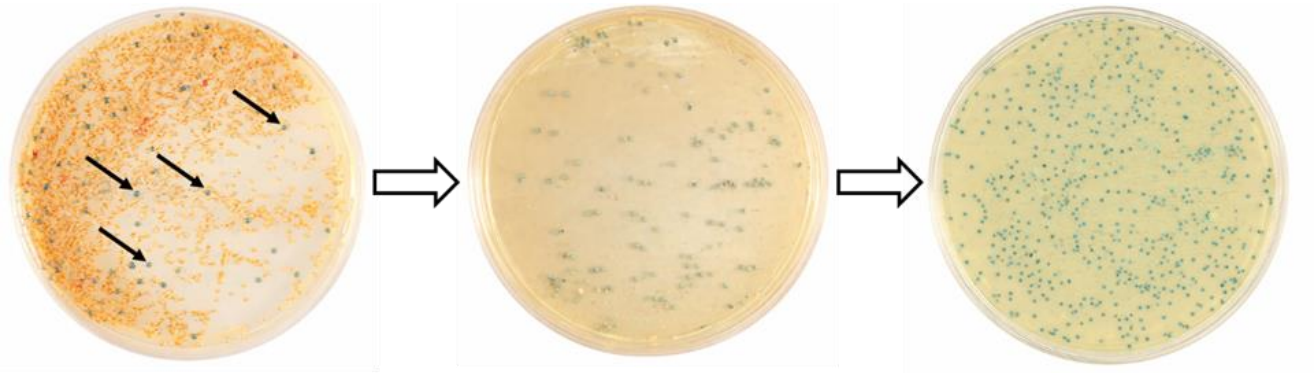


Figure 1. Isolation and identification of *Aeromonas salmonicida* from imported seafood in Mosul city, Iraq. Selective plating was used to isolate presumptive colonies (marked by arrows), which were then sub-cultured to reduce background flora. The last re-streak has produced well-isolated, shiny green colonies characteristic of *Aeromonas salmonicida*, aiding identification.

Table 4. Distribution of *Aeromonas salmonicida* isolates obtained from imported seafood samples in Mosul city, Iraq

Seafood Type	Samples (number)	Isolates (number)	Isolation rate (%)
Shrimp	5	3	60%
Mussels	5	2	40%
Oysters	5	1	20%
Salmon fish	5	3	60%
Total	20	9	45%

VITEK 2 compact system identification

The VITEK 2 compact system was used to further identify nine presumptive *A. salmonicida* isolates recovered on selective agar. Biochemical analysis revealed a similar reaction pattern across all isolates. The isolates exhibited positive reactions in different key enzymatic and metabolic assays, including pyrrolidonyl arylamidase (PyrA), glycine arylamidase (GlyA), ODC, lysine decarboxylase (LysA), D-mannose utilization/fermentation (dMNE), tyrosine arylamidase (TyrA), sucrose fermentation/utilization (SAC), D-glucose utilization/acid production (dGLU), oxidative–fermentative test for glucose metabolism (OFF), and alanine arylamidase (AlaA). Isolates revealed no significant differences; thus, nine strains were phenotypically homogeneous. According to the identification results obtained with the VITEK 2 compact system, all green colonies isolated from selective agar were confirmed as *A. salmonicida*. Selective culturing and automated biochemical identification were accurate methods for detecting *A. salmonicida*, as indicated by biochemical cassette results from imported seafood (Figure 2).



Figure 2. Identification card of the VITEK 2 compact system used for biochemical profiling of the bacterial isolates. The cassette contains a range of micro-wells containing dehydrated substrates that generate colorimetric signals upon inoculation and incubation, enabling automated identification of *Aeromonas salmonicida* in the VITEK 2 compact system.

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Molecular identification

All nine *A. salmonicida* isolates successfully underwent PCR amplification of the *16S rRNA* gene. The distinct bands of DNA were clearly visible in all nine lanes of the agarose gel electrophoresis (Figure 3). The size of each DNA band was approximately 600 base pairs in comparison with the 1 kb DNA ladder (Lane M). No nonspecific bands or primer-dimer formation was observed, indicating high primer specificity and optimal PCR conditions. The current results demonstrated that genomic DNA was successfully isolated and that the PCR assay employed was dependable. Amplification of the *16S rRNA* gene confirmed the molecular identification of the bacterial isolates as *A. salmonicida*. These findings suggested that *16S rRNA*-based PCR was an effective method for identifying *Aeromonas* species in bacterial isolates.

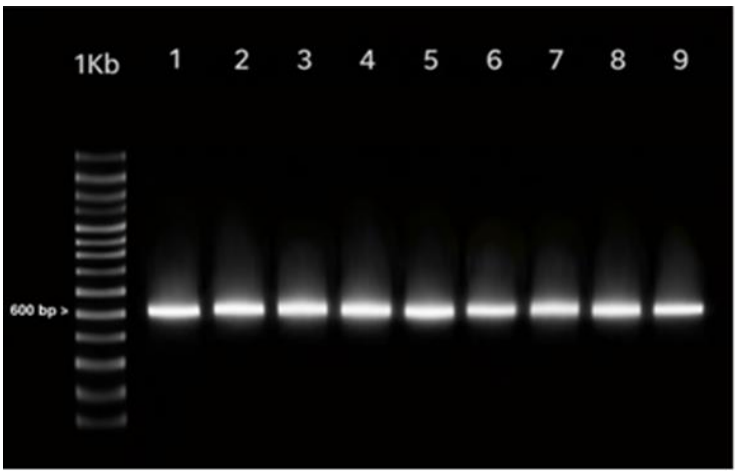


Figure 3. Gel electrophoresis results of nine *Aeromonas salmonicida* isolates obtained from imported seafood samples in Mosul city, Iraq. Lane M: 1 kb DNA ladder. Lanes 1-9: DNA fragments with a size of approximately 600 bp, indicative of *Aeromonas salmonicida*

Phylogenetic tree relationships of *Aeromonas salmonicida* strains

The evolutionary relationships among *A. salmonicida* strains based on *16S rRNA* gene sequences are illustrated in Figure 4. A phylogenetic tree was determined to be closely related to the *A. salmonicida* ATCC 33658 strain and the *A. salmonicida* strain NCIMB 1102. The CECT 894 strain demonstrated an earlier divergence, establishing a separate lineage distinct from the ATCC and the NCIMB strains. These strains formed a cluster with strong bootstrap support (85%), indicating close relationships and suggesting a common ancestry with high confidence. Based on isolate analysis, the wild-type *A. salmonicida* genome from NCBI was identified as the most distantly related among the four strains. The scale bar represented 0.00200 substitutions per nucleotide site, with shorter branch lengths indicating greater sequence similarity and closer phylogenetic relatedness.

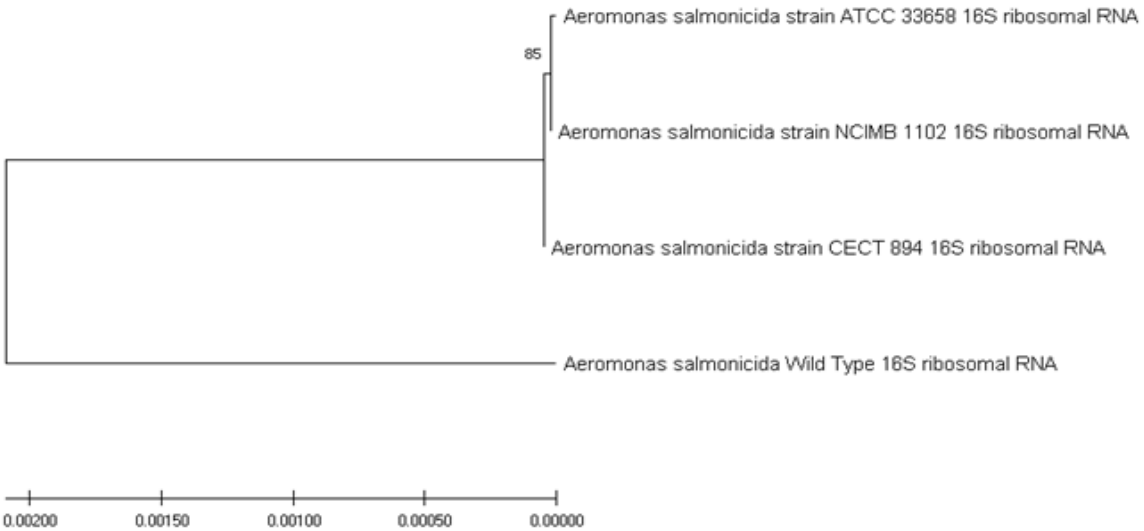


Figure 4. Phylogenetic tree of the *Aeromonas salmonicida* strains based on sequences of the *16S rRNA* gene. The tree was constructed by the Neighbor-Joining method, and bootstrap values (based on 1000 replications) are shown beside the branches.

Microarray detection of antibiotic resistance genes

Microarray analysis revealed significant variation in the distribution of antibiotic resistance genes among the nine strains of *A. salmonicida* isolated from imported seafood. All seven strains (samples 1 to 7) were identified as MDR, exhibiting resistance against common antibiotics. In contrast, samples 8 and 9 exhibited no antibiotic resistance genes (0 out of 38 screened) and were classified as antibiotic-sensitive isolates.

All seven MDR isolates were positive for *bla*TEM, *cphA*, and *bla*OXA(I), demonstrating broad-spectrum β-lactamase activity across the entire group of MDR. None of the MDR isolates carried genes coding for extended-spectrum β-lactamases (ESBLs) such as *bla*SHV, *bla*CTX-M, *bla*FOX, *bla*PER, *bla*VEB, or *bla*CMY. Sensitive isolates had no resistance genes detected (samples 8 and 9). Importantly, all the isolates, including the MDR strains, were devoid of any ESBL-associated genes, suggesting that the resistance patterns resulting from these mechanisms are unlikely in the present case. The consistent presence of *bla*OXA(I) in all MDR isolates indicated that this oxacillinase plays a specific role in β-lactam resistance. In contrast, non-MDR strains exhibited multiple *bla*OXA gene sequences (Figure 5).

All the MDR strains (1-70) carried the *tetE* genes, detected in all samples. In contrast, *tetA* was detected in only two, specifically in samples 1 and 4 (28.6%), which had the highest number of resistance genes. The current finding indicated that all MDR isolates lacked the *tetB* and *tetD* genes. The present investigations identified the sulfonamide resistance gene *sul1* in all seven MDR isolates. However, *sul2* was absent in all of the MDR strains. Even though sul2 was part of the screened targets onto the micro-array panel, it was not detected in any isolate. Significantly, the sensitive strains (samples 8 and 9) showed the absence of resistance genes since sul2 and other resistance genes were not found in strains. All MDR strains exhibited resistance to trimethoprim, which was attributed to the presence of the *dfrA* gene (Figure 6).

The MDR isolates exhibited a broad distribution of aminoglycoside resistance genes. Comprehensive gene profile analysis demonstrated that all seven MDR strains contained the *aphA* and *aadA1* genes. Additionally, three of the seven strains (42.9%), specifically samples 1, 4, and 5, possessed the *aac(6')-Ib* gene. Moreover, the *armA* gene, associated with high-level aminoglycoside resistance, was absent from all MDR isolates. The *floR* gene, conferring resistance to phenicol, was identified in all seven MDR isolates, whereas the cat gene was not detected in any MDR isolate (Table 5).

The present results confirmed that *gyrA* plays a crucial role in fluoroquinolone resistance within the MDR group. In comparison, *parC* was identified in three of seven strains (42.9%), specifically in samples 1, 4, and 5. Additionally, *qnrA*, *qnrB*, and *qnrS* genes were co-detected in these samples 1, 4, and 5, indicating a combined resistance mechanism. The presence of *intI1* and *qacEΔ1* genes, associated with integrons, was noted in all seven strains. *AcrB* and *TolC* components of the efflux pump were found in all MDR strains, suggesting that efflux-mediated resistance was a widespread mechanism across MDR strains.

The MDR isolates harbored more resistance genes than the sensitive isolates. The average number of resistance genes in MDR isolates (16.43 ± 3.05) was higher than in sensitive isolates (0.00 ± 0.00). According to the present results, microarray-based profiling can distinguish between resistant and sensitive isolates and characterize the distribution of antibiotic resistance genes (Figure 7).

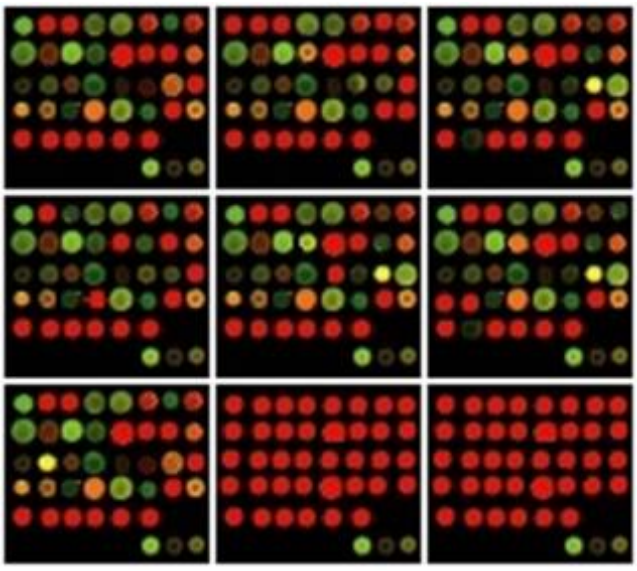


Figure 5. Antibiotic resistance genes' hybridization patterns of *Aeromonas salmonicida* strains on a DNA microarray chip. Green spots indicated the gene is present, red spots indicated the gene was absent. Samples 1 to 7 were multidrug-resistant, and samples 8 and 9 contained no resistance genes. Internal controls were housekeeping genes.

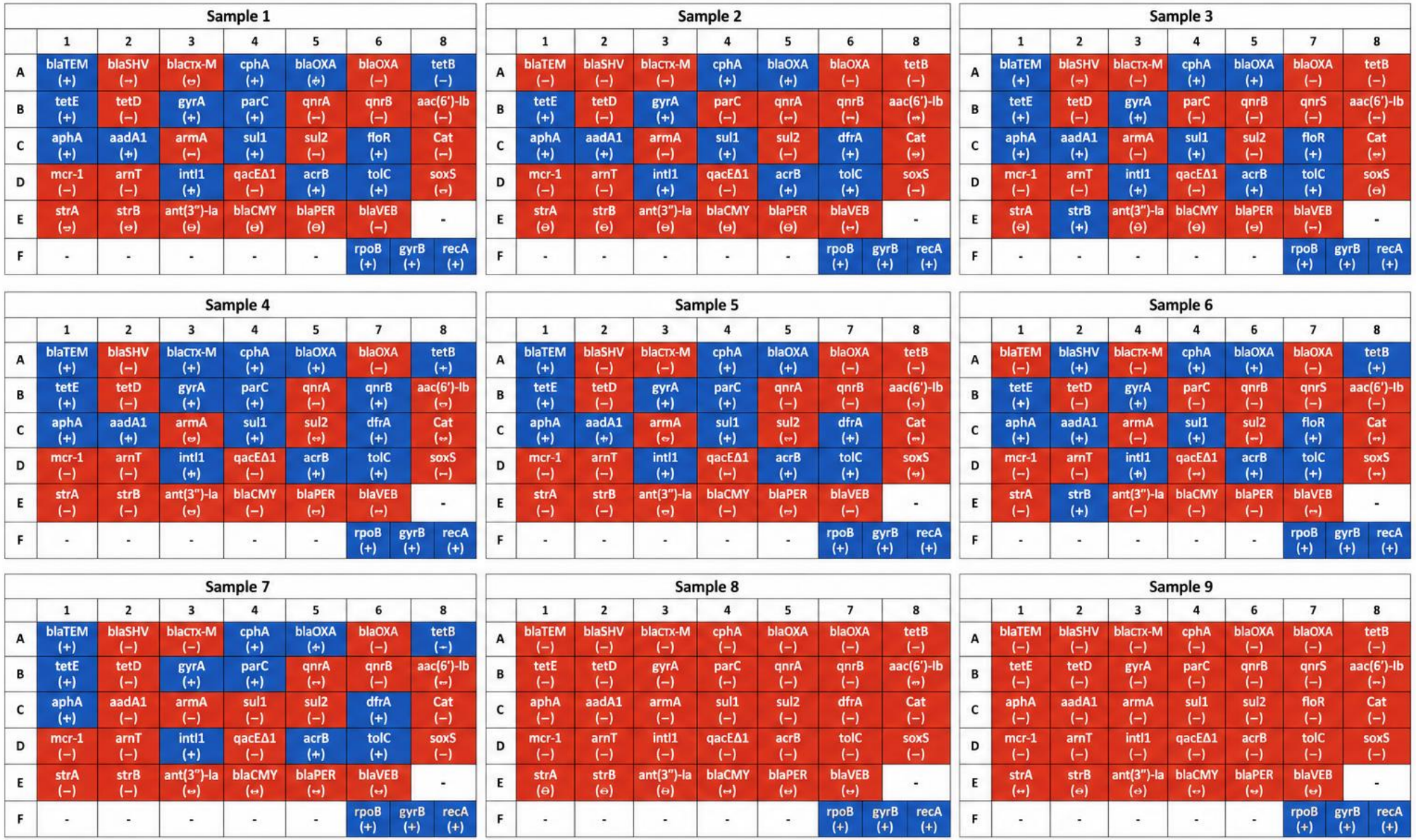


Figure 6. Microarray detection of antibiotic resistance and multidrug resistance genes in nine *Aeromonas salmonicida* strains isolated from imported seafood in Mosul city, Iraq. The layout shows the distribution of gene probes in a 6 × 8 array. Rows A-F and columns 1-8 represent positional coordinates used to identify individual probe locations

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Table 5. The microarray analysis results based on antibiotic resistance probes

Sample ID	Status	Resistance genes (n)	Susceptible loci (n)	Resistance genes detected
1	MDR	20	18	*bla*TEM, *cphA*, *bla*OXA(I), *tetA, tetE, gyrA, parC, qnrA, qnrB, qnrS, aac(6')-Ib, aphA, aadA1, sul1, dfrA, floR, intI1, qacEΔ1, acrB, tolC*
2	MDR	14	24	*bla*TEM, *cphA*, *bla*OXA(I), *tetE*, *gyrA*, *aphA, aadA1, sul1, dfrA, floR, intI1, qacEΔ1, acrB, tolC*
3	MDR	14	24	*bla*TEM, *cphA*, *bla*OXA(I), *tetE, gyrA, aphA, aadA1, sul1, dfrA, floR, intI1, qacEΔ1, acrB, tolC*
4	MDR	20	18	*bla*TEM, *cphA*, *bla*OXA(I), *tetA, tetE, gyrA, parC, qnrA, qnrB, qnrS, aac(6')-Ib*, *aphA, aadA1, sul1, dfrA, floR, intI1, qacEΔ1, acrB, tolC*
5	MDR	19	19	*bla*TEM, *cphA*, *bla*OXA(I), *tetE, gyrA, parC, qnrA, qnrB, qnrS, aac(6')-Ib, aphA, aadA1, sul1, dfrA, floR, intI1, qacEΔ1, acrB, tolC*
6	MDR	14	24	*blaTEM*, *cphA*, *bla*OXA(I), *tetE*, *gyrA*, *aphA, aadA1, sul1, dfrA, floR, intI1, qacEΔ1, acrB, tolC*
7	MDR	14	24	*bla*TEM, *cphA*, *bla*OXA(I), *tetE, gyrA, aphA, aadA1, sul1, dfrA, floR, intI1, qacEΔ1, acrB, tolC*
8	Sensitive	0	38	None detected
9	Sensitive	0	38	None detected

MDR: Multidrug-resistant. Resistance genes (n): The number of resistance determinants detected per isolate. Susceptible loci (n): The number of screened panel targets returning no detectable resistance gene (total panel = 38 targets). The two susceptible isolates (Samples 8 and 9) had no detectable resistance genes (n = 0).

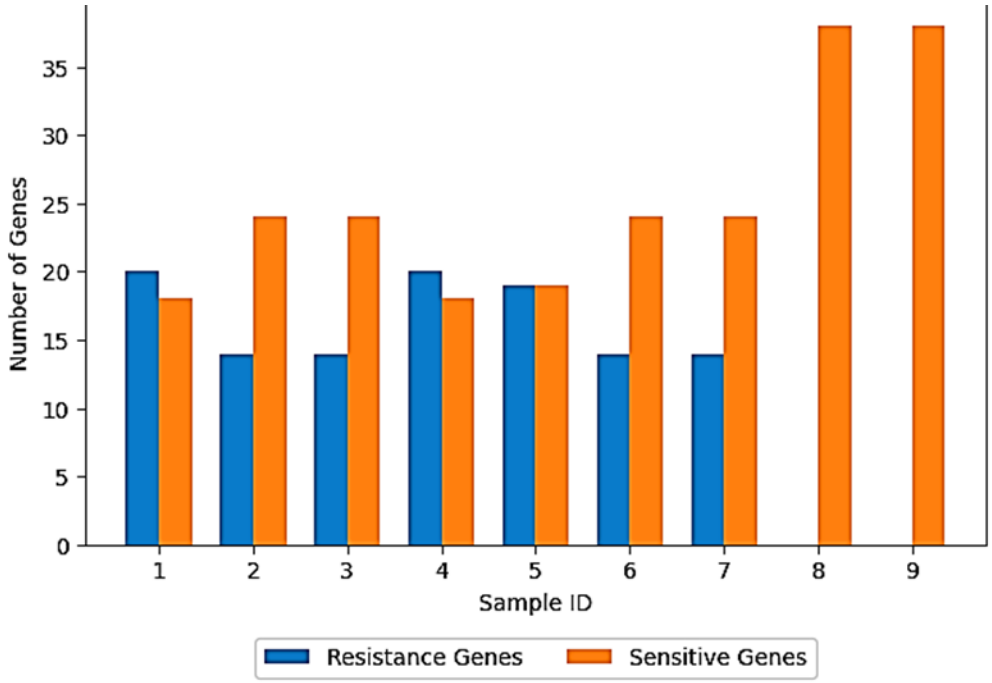


Figure 7. Distribution of resistance and sensitive gene across nine *Aeromonas salmonicida* strains isolated from imported seafood in Mosul city, Iraq, via the microarray technique

DISCUSSION

Antibiotic-resistant aquatic pathogens pose a major public health threat to humans due to the increasing international seafood trade (Odeyemi et al., 2023). Similar to the present study, Tate et al. (2022) identified *A. salmonicida* in retail seafood at a relatively high prevalence of 45%, and assessed its potential impact on food safety and public health. The detection rate for *A. salmonicida* in imported seafood, ranging from 30% to 50%, was similar across Asia and Europe, likely due to their shared origins and characteristics (Pradhan et al., 2023). The high detection levels might be attributed to extensive use of antibiotics in aquaculture, insufficient monitoring of imported products, and virulence factors (Bondad-Reantaso et al., 2023). Although the current study did not provide experimental confirmation, Lerfall et al. (2024) indicated that MDR isolates can survive refrigerated and frozen storage, potentially enabling their persistence in the seafood supply chain and increasing consumer exposure. Notably, shrimp and salmon had the highest detection rates for *A. salmonicida*, at 60% each, with mussels at 40% and oysters at 20%. These differences might be attributed to differences in farming practices, environmental exposure, and post-harvest handling (Blosser, 2022). The isolation rates in oysters and mussels might be due to their harvest from natural or semi-natural environments (Blosser, 2022). Intensive aquaculture systems are employed to cultivate shrimp and salmon, which frequently use antibiotics for prophylaxis and growth promotion (Getahun et al., 2025). Selective culture media facilitated preliminary identification, and the VITEK 2 compact system biochemically verified the isolates as *A. salmonicida*. Homogeneous biochemical profiles with a confidence level of ≥ 95% across all isolates supported the validity of this method (Feregrino et al., 2025). The VITEK 2 compact system is well-documented for its rapid and reliable identification of *Aeromonas* species from food and environmental samples, corroborating the previous observations of Kitagawa et al. (2022).

During the present study, molecular confirmation using the *16S rRNA* gene identified nine isolates as *Aeromonas* species. However, phenotypic identification with the VITEK 2 compact system consistently classified all strains as *A. salmonicida*. Phylogenetic analysis revealed that the isolates clustered closely with reference strains from different geographical origins (Long et al., 2023). According to the DNA microarray analysis in the present study, 77.8% of isolates carried multiple antibiotic resistance genes, whereas only 22.2% were entirely susceptible (Connor et al., 2023). The presence of MDR bacteria in retail seafood poses a major health threat to consumers, particularly immunocompromised individuals, and indicates a growing public health concern due to antimicrobial resistance (Sharma et al., 2023).

Although β-lactam resistance was the most frequently detected phenotype among the isolates, no ESBL genes were identified in any of the MDR strains. However, the presence of *bla*TEM, *cphA*, and *bla*OXA was concerning because these enzymes confered resistance to critically important antibiotics used in both human and veterinary medicine. Consistent with the present findings, Long et al. (2023) reported detecting *Aeromonas* isolates from aquaculture

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environments, emphasizing the widespread presence of β-lactam resistance, likely due to the extensive use of these antibiotics in fish farming.

Among the tetracycline resistance genes, *tetE* was identified in all isolates, *tetA* in 28.6% of isolates, and *tetB* was not observed. The widespread presence of the *tetE* gene indicated extensive exposure to tetracycline and the spread of tetracycline resistance genes in the aquaculture environment. The most commonly used antibiotic on fish farms is tetracycline due to its low cost and broad-spectrum activity (Amangelsin et al., 2023). The frequent administration of these antibiotics has driven the development of resistant strains, reflected in the high detection rates of efflux pump resistance genes (Vilela et al., 2022).

The sulfonamide resistance gene *sul1* was detected in 100% of MDR isolates, whereas *sul2* was absent in all strains, consistent with the established association between sulfonamide resistance and integrons (Bhat et al., 2023). The detection of sulfonamide- and β-lactam-resistance genes in the same strains indicated the concurrent accumulation of diverse resistance determinants, raising notable epidemiological concerns (Elbaiomy et al., 2025).

Aminoglycoside resistance genes were identified in 100% of MDR isolates; the *aphA* and *aadA1* genes were universally present, while *aac(6')-Ib* was detected in 42.9% (3/7) of strains. These genes encode enzymes that modify and deactivate aminoglycosides. These genes are predominantly carried on integrons and plasmids (Ali et al., 2024). These resistance genes indicated that *A. salmonicida* was developing MDR, potentially reducing the effectiveness of treatments in human infections.

Integrons are essential for the acquisition, expression, and dissemination of resistance gene cassettes. In the present study, all seven MDR isolates (77.8% of the nine total isolates) contained integron-associated genes (*intI1* and *qacEΔ1*). The detection of *A. salmonicida* underscored the potential for horizontal gene transfer within the *Aeromonas* genus and to other enteric pathogens through consumption of contaminated seafood (Pessoa et al., 2022), thereby exacerbating the public health concern associated with MDR bacteria in food. The overall prevalence of MDR strains distinctly indicated deficiencies in the surveillance and regulation of imported seafood.

The present findings indicated that marine seafood was a major reservoir of MDR *A. salmonicida*. The current study employed DNA microarray technology, which was advantageous for comprehensive resistance profiling and enabled the detection of multiple resistance factors within a single assay (Yamin et al., 2023). The high prevalence of MDR *A. salmonicida* in imported fish sold in Mosul city, Iraq, posed a notable public health risk. The current findings highlight the urgent necessity to enhance regulatory policies on antibiotic use in aquaculture, as indicated by Ljubojević Pelić et al. (2024).

CONCLUSION

The current study indicated that imported seafood in Mosul City, Iraq, was a potential reservoir for MDR *A. salmonicida*. *Aeromonas salmonicida* was detected in 45% of seafood samples, predominantly in shrimp and salmon, with the highest contamination levels. Molecular and phenotypic investigations indicated that the majority of isolates (77.8%) harbored genes conferring multiple antibiotic resistance, mainly for beta-lactams, tetracyclines, sulphonamides, and aminoglycosides. Genes associated with integrons have been identified, indicating the potential of horizontal transfer of *A. salmonicida* within the food chain. The current results indicated a public health threat associated with imported seafood sold in Mosul, Iraq. The primary constraint of the present study was its limited sample size, which might not adequately represent the prevalence of MDR *A. salmonicida* in imported seafood. Future studies should focus on improved regulation and consistent monitoring of imported seafood in markets. Furthermore, it is essential to ensure enhanced stewardship of antibiotic use in aquaculture to mitigate the dissemination of antimicrobial resistance.

DECLARATIONS

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Authors' contributions

Ayman Albanna, Aseel A. H. Al-Layla, and Sahar Lukman Al-Saleem equally contributed to the study's design, data collection, analysis, and manuscript preparation. All authors collaborated in a distinct revision of the manuscript's final edition. All authors have read and approved the final edition of the manuscript.

Availability of data and materials

The data that support the present study are available within the article. The corresponding author can provide additional datasets upon reasonable request.

Competing interests

The authors declared no conflict of interest.

Ethical considerations

All procedures were carried out following institutional standards and data confidentiality. The authors confirmed that the study is original, not under consideration elsewhere, complies with data integrity and anti-plagiarism standards, and that no artificial intelligence tools were used in the preparation of the manuscript.

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Hematological and Biochemical Indices and Performance of Sasso T_{44} Chicken Fed on Sweet Lupine-Based Formulated Feed

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ABSTRACT

Protein sources are essential components of poultry diets, with Soybean meal (SBM) being the most widely used due to its high protein content and balanced amino acid profile. However, its increasing cost and limited availability have encouraged the search for alternative plant protein sources such as sweet lupine (*lupinus angustifolius*). Dietary protein plays a crucial role in growth, performance, and blood profile parameters in determining the overall effectiveness of dietary treatments. Hematological and biochemical indices are valuable indicators of the physiological condition, metabolic activity, and health status of poultry. The current study aimed to evaluate the effect of replacing SBM with sweet vitabor lupine (SVL) in Sasso T_{44} dual-purpose chicken diets on growth performance and blood hematological and biochemical indices. A total of 180 chickens with five dietary treatments (Ts) were used. SBM was replaced by SVL at 0% (T1, control), 15% (T2), 25% (T3), 30% (T4), and 50% (T5). Each treatment had three replicates of 12 chickens and was fed for 90 days. Blood hematological parameters and biochemical indices of experimental chickens were measured on day 90. Results revealed that increasing SVL inclusion significantly affected the hematological, biochemical, and growth performance of experimental chickens. Live weight, carcass weight, and dressing percentage were significantly improved in T5 chickens compared to T1, T2, T3, and T4, indicating enhanced production performance with SVL inclusion. However, feed conversion efficiency remained statistically unchanged across treatments. Compared with the control diet (T1), chickens fed the highest SVL level (T5) had higher red blood cell counts and hemoglobin concentrations; however, despite the hematocrit value (36.18%) remaining within the normal standard reference range (25-45%), it was significantly lower. Diets T4 and T5 also resulted in higher total protein and lower uric acid and creatinine levels relative to the control, while glucose and triglycerides were unaffected; however, cholesterol was higher in T5. Overall, the results of the present study suggest that SVL may serve as a potential alternative plant protein source to partially replace SBM in diets of Sasso T_{44} dual-purpose chickens, with no observable negative effect on growth performance and almost all hematological and biochemical parameters.

Keywords: Biochemical indices, Hematological parameter, Growth performance, Sasso T_{44} chicken, Soybean meal, Sweet lupine

INTRODUCTION

Genetically improved chicken breeds have been selectively developed for either meat (broiler) or egg (layer) production, requiring intensive nutritional and health management to fully realize their genetic potential (FAO, 2014). On the other hand, dual-purpose breeds are designed to serve both functions; egg and meat production, although achieving this balance often involves nutritional and performance trade-offs (Wilson et al., 2023). Compared to specialized breeds, dual-purpose chickens generally exhibit moderate growth rates and egg production, making it challenging to formulate diets that adequately support both traits without compromising one. Therefore, appropriate nutritional strategies are essential to optimize productivity in such breeds (Wilson et al., 2023). Feed remains a critical factor in poultry production, accounting for approximately 65 to 75% of total production costs (Kim et al., 2022), while growth performance and hematological characteristics are influenced by both dietary composition (Das et al., 2025) and genetic factors (Tiemann et al., 2020).

The poultry industry relies heavily on conventional feed ingredients such as maize and soybean meal (SBM) as primary sources of energy and protein. However, constraints such as increasing cost, fluctuating supply, and limited availability of SBM driven by climatic variability, rising global demand, and disruptions in international trade pose significant challenges to sustainable poultry production (David et al., 2024; Jiang, 2025). Such constraints have intensified the search for cost-effective, locally available alternative protein sources.

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Non-oilseed grain legumes, including sweet lupine, are recognized as promising partial substitutes for soybean meal due to their substantial protein content, essential amino acids, and energy value. Advances in the development of low-alkaloid lupine cultivars have further enhanced their suitability as vegetable protein sources in animal feeding systems (Andrianova et al., 2019). In this context, sweet lupine (*Lupinus angustifolius*) has gained considerable attention due to its high protein content, balanced amino acid profile, and adaptability as a plant-based protein source in poultry diets (Ayeni et al., 2024; Tiruneh et al., 2025). Sweet lupine is particularly relevant in countries like Ethiopia, where the scarcity and high cost of conventional protein feed ingredients necessitate the evaluation of alternative locally available feed resources (Bachano, 2021).

Dietary composition plays a crucial role in maintaining the physiological and metabolic status of poultry. Hematological and biochemical parameters are widely used as reliable indicators of health, nutritional adequacy, and metabolic function of chicken, especially when evaluating novel feed ingredients (Toghyani et al., 2010); however, these values may vary depending on breed, age, sex, nutrition, environmental conditions, and laboratory methods (Thrall et al., 2012). Balanced diets support immune and antioxidant defenses, whereas nutrient imbalances or anti-nutritional factors may alter blood profiles, reflecting physiological stress or organ dysfunction (Etim et al., 2014). Sweet lupine, in particular, has been associated with improved immune response, antibody production, and disease resistance in poultry (Sufiriyanto et al., 2019). Therefore, the present study aimed to evaluate the potential of sweet lupine as an alternative protein source in the diets of Sasso T_{44} dual-purpose chickens by assessing its effects on feed intake, growth performance, hematological parameters, and serum biochemical indices as indicators of nutritional adequacy and overall physiological health.

MATERIALS AND METHODS

Ethical approval

In the current study, chicken handling and experimental procedures were conducted in accordance with the ethical standards for the care and use of experimental animals approved by Bahir Dar University Institutional Review Board of Research Ethics Committee (BDUIRBREC /1207335), dated April 2, 2024, Ethiopia. Adequate measures were taken to minimize pain, stress, and discomfort to the experimental chickens throughout the study period.

Experimental design, ingredient inclusion rate, and treatment layout

A completely randomized design (CRD) was used in the present study. A total of 180 Sasso T_{44} dual-purpose chickens were randomly assigned to five dietary treatments, each with three replications. Each replication consisted of 12 chickens, resulting in 36 chickens per treatment group. Chickens were randomly allocated to the treatment groups using a simple randomization procedure to ensure equal probability of assignment to each treatment. Regarding the experimental treatments, rations of adequate quantity and appropriate quality were formulated and prepared, and the treatment groups were established according to the predetermined experimental layout.

Feed ingredients preparation

Both macro and micro feed ingredients were collected from different sources, where they easily available and these include maize grain, wheat short, noug seed cake, soybean meal and finger millet, and the cultivar sweet vitabor lupine as macro ingredients, and limestone, meat and bone meal, vitamin premixes, and salt as micro ingredients, which bought from Mulie animal feed processing and manufacturing industry, Ethiopia. Maize grain, finger millet, and sweet lupine were slightly roasted in a flat griddle heated from 140 to 150 ($^{\circ}$C) by a burned wood flame for five to seven minutes until a part-brownish color was observed for maize grain and sweet lupine. However, the other ingredients, such as soybean meal (SBM), noug seed cake (NSC), and wheat shorts, were not roasted since they were already processed from feed processing industries. The macro ingredients were milled by a grinder (miller) to reduce the particle size, which in turn is thought to increase the palatability and digestibility of feed.

Feed ingredients were processed at Zenzelma Dairy and chicken feed processing plant, Bahir Dar University, Ethiopia. The macro-ingredients were first ground and subsequently mixed using a mechanical mixer with adjustable settings to ensure that the experimental diets were isocaloric and isonitrogenous. In contrast, micro-ingredients were incorporated manually in accordance with the recommended inclusion levels to achieve uniform distribution.

Chemical analysis of feed ingredients and experimental treatments

Representative samples of each ingredient and experimental treatment were analyzed in triplicate for dry matter, ether extract, crude fiber, and crude ash following the procedures described by AOAC (1990). The nitrogen content of

the samples was measured using the Kjeldahl method, and crude protein (CP) was estimated by multiplying the nitrogen value by a factor of 6.25. The Metabolizable energy (ME) of the experimental diets was subsequently estimated using the following formula prediction equation proposed by Wiseman (1987).

$$ME\left(\frac{kcal}{kg}\text{Dry matter}\right) = 3951 + 54.4EE - 88.7CF - 40.8\ Ash \quad \text{(Formula 1)}$$

Experimental diet formulation

The diets have been formulated using linear programming through WinFeed (2.8 Demo version) feed formulation software to ensure that the experimental diets meet the intended nutrient requirements of experimental chickens and provided a more rigorous basis for comparison among treatments. Regarding the preparation of the experimental diets, both major and minor feed ingredients were procured from various readily accessible sources. The major ingredients included maize grain, wheat shorts, noug seed cake, soybean meal, finger millet, and the cultivar sweet vitabor lupine (SVL), while the minor ingredients consisted of limestone, meat and bone meal, vitamin premixes, and salt, which were purchased from a private company in Ethiopia called Mulie Animal Feed Processing and Manufacturing Industry.

Experimental chickens and management

A total of 180 day-old Sasso T_{44} dual-purpose chickens were used in this experiment. The chickens were randomly assigned to five dietary treatment groups, each comprising three replicates (12 chickens per replicate). After the chickens arrived at the experimental site, different vaccinations supplied by Kemin Industries, Inc., Des Moines, Iowa, USA, were administered according to the standard poultry health schedule. Newcastle disease (HBI strain) at 7 days of age, Newcastle disease (Lasota strain) at 21 days, and fowl pox at 28 days. Furthermore, the chickens were routinely monitored for any signs of internal or external pathological conditions or other abnormalities, and appropriate prophylactic or therapeutic measures were undertaken in accordance with prevailing veterinary recommendations. Apart from the ingredients listed in the diet formulation, no additional medications, antibiotics, growth promoters, or other feed additives were included in the experimental diets. The chickens were fed *ad libitum* from day 1 to day 90. Cumulative body weight and weight gain were recorded to monitor growth performance, whereas blood hematology and serum chemical indices were recorded at the end of the feeding trial period (day 90).

The experimental chickens were reared under standard management conditions in a well-ventilated house. Each treatment group was allocated to pens measuring l.5 × $2m^2$, equipped with a separate feeder and drinker per pen. The ambient temperature of each pen was adjusted and maintained by hanging a 60-watt bulb during the first 3 weeks. Chickens were brooded under supplemental heat immediately after placement. The brooding temperature was maintained at approximately 30˚C during the first week of age and then gradually reduced by about 2 to 3˚C per week as the chickens grew and feather development progressed. Accordingly, the temperature was maintained at 28˚C in the second week, 26˚C in the third week, and 23˚C in the fourth week. During the experimental period, the chickens were managed under a controlled lighting program. Both natural daylight and supplemental artificial lighting were used to maintain the required photoperiod in the experimental house.

A photoperiod of 23 hours of light and 1 hour of darkness (23L: 1D) was provided during the first week of age to stimulate early feed and water intake and help chickens adapt to the rearing environment. After the brooding period, the lighting schedule was gradually adjusted to 20 hours of light and 4 hours of darkness (20L: 4D; Aviagen, 2018). Clean water and feed were provided *ad libitum*, and proper hygiene and biosecurity measures were strictly followed throughout the experimental period.

Measurements of experimental variables

The present study evaluated key response variables, including growth performance (feed intake, body weight gain, and feed conversion ratio), hematological and biochemical indices to assess physiological and metabolic status, and carcass characteristics to determine dressing percentage and relative organ weight. Dressing percentage (DP), calculated as the carcass weight divided by the live weight.

Growth performance

The growth performance of experimental chickens was evaluated through measurements of feed intake, body weight gain, and feed conversion ratio (FCR) throughout the experimental period. Feed intake was recorded on a pen basis. A known quantity of feed was offered, and refusals were measured on an as-fed basis to each pen daily, while feed intake was subsequently calculated and expressed on a dry matter basis to eliminate variation associated with moisture content among experimental diets. Mean daily dry matter intake per chicken was then computed by Formula 2.

$$\text{Mean dry matter}\frac{\text{Intake}}{\text{Chicken}} = \frac{\text{Feed offered-residue}}{\text{Number of chickens}} \quad \text{(Formula 2)}$$

Feed intake was recorded on a pen basis throughout the experimental period. The amount of feed offered to each pen was weighed daily, and the refusals were collected and weighed the following day. Daily feed intake for each pen was calculated as the difference between the amount of feed offered and the refusals. Mean daily dry matter intake per chicken was then determined by dividing the total pen feed intake by the number of chickens present in the pen during the respective period.

In the present study, body weight measurements were taken on a pen (group) basis rather than individually. All chickens within each pen were weighed collectively using a digital weighing scale, and the average body weight per chicken was then calculated by dividing the total pen weight by the number of chickens in the pen. Body weight measurements were taken using a digital weighing balance with appropriate precision. All chickens were weighed at the beginning of the experiment and subsequently at weekly intervals until the end of the experimental period. Chickens were weighed in the morning before feeding to minimize variation due to gut fill. The average body weight gain was calculated by the following formula.

$$\text{Average body weight gain (ABWG)} = \frac{\text{Final body weight-Initial body weight}}{\text{Number of experimental days}} \quad \text{(Formula 3)}$$

Feed conversion ratio (FCR) was used as an indicator of feed utilization efficiency during the experimental period. It was calculated on a pen basis by dividing the total dry matter feed intake by the corresponding body weight gain of the chickens over the same period. Thus, FCR was computed using formula 4.

$$\text{Feed conversion ratio (FCR)} = \frac{\text{Total dry matter feed intake (g)}}{\text{Total body weight gain (g)}} \quad \text{(Formula 4)}$$

Sample collection for hematological and biochemical analysis

After the feeding experiment (day 90), six chickens per treatment were utilized for hematological and biochemical analysis. Blood samples were collected through the wing vein using sterile disposable 5 mL syringes fitted with 23-gauge needles, following standard blood collection procedures as described by Parasuraman et al. (2010). Approximately 3 mL of blood was collected from each chicken. Of this, 1.5 mL was transferred into tubes containing ethylenediaminetetraacetic acid (EDTA) as an anticoagulant for hematological analysis, while the remaining 1.5 mL was placed into plain tubes without anticoagulant to allow serum separation for biochemical analysis. Samples were kept on ice and transported immediately to the laboratory. Serum was separated after centrifugation at 3,000 rpm for 10 minutes (Hettich, Tuttlingen, Germany) and stored at -20˚C until analysis.

Hematological measurements, including hemoglobin concentration, packed cell volume (PCV), and counts of red and white blood cells (RBC and WBC), were performed using a BC-2800Vet Automated Hematology Analyzer (Mindray, Shenzhen, China). Serum biochemical parameters were analyzed using commercial diagnostic kits (Randox Laboratories, Crumlin, UK), and absorbance was read with a UV-Visible Spectrophotometer (Shimadzu, Kyoto, Japan). Blood smears were prepared immediately after collection from the EDTA-anticoagulated samples. A small drop of blood was placed on a clean glass slide and spread thinly using the edge of another slide at an angle of approximately 30-45˚. The smears were then air-dried at room temperature, fixed in methanol for 2-3 minutes, and subsequently stained using Wright's stain following standard hematological procedures. The stained smears were examined under a light microscope at 1000 times magnification with oil immersion to evaluate erythrocyte morphology, leukocyte differential counts, and other hematological characteristics.

Carcass evaluation

At day 90, the completion of the feeding trial, six chickens per treatment (n = 6) with a total of 30 chickens used for hematological and biochemical analysis were also used for carcass evaluation. Each chicken was weighed, then fasted for 12 hours before being humanely slaughtered by severing the jugular vein. Prior to exsanguination, the chickens were humanely handled and manually restrained, but no electrical or mechanical stunning was applied. The method of slaughter was performed in accordance with standard poultry handling procedures, ensuring minimal stress to the chickens during the terminal procedure. The carcasses were briefly immersed in hot water boiled at 100˚C for approximately one minute to facilitate feather removal. Following slaughter, the carcasses were allowed to cool at room temperature for a short period (approximately 30 minutes) before dissection. The cooling period was intended to allow the muscles to firm up prior to weighing and cutting, thereby minimizing immediate postmortem changes that could affect carcass and cut weights. Subsequently, slaughter weight, carcass weight, dressing percentage, and the weights of major cuts, including thigh, drumstick, breast, back, wings, neck, and skin, were recorded.

Statistical analysis

Data for the key experimental parameters were analyzed using a one-way analysis of variance (ANOVA) with SAS software (version 9.4). Differences between treatment means were separated using the Tukey range test at a 5% significance level. Prior to statistical analysis, the assumptions of normality and homogeneity of variance were verified. The normality of residuals was assessed using the Shapiro-Wilk test, and homogeneity of variances among treatment groups was evaluated using Levene's test. As these assumptions were met ($p > 0.05$), the data were subsequently analyzed using the following model. The model used for the experiment was as follows.

$$Yi = u + Ti + ei$$

Where Yi is the observed response variable of chickens in the ith treatment, μ is the overall mean, T_i represents the fixed effect of the ith treatment, and e_{ie} is the random error associated with the observation.

RESULTS AND DISCUSSION

Chemical compositions of feed ingredients and experimental treatments

The proximate compositions of the feed ingredients used for diet formulation (Table 1) were analyzed for DM (Dry matter), CP, CF (Crude fiber), EE (Ether extract), and crude ash. The CP, which was highest in SBM (42.6%) followed by SVL (35.9%) and NSC (31.9%), while the lowest CP was observed in Finger millet (9.5%) and Maize (12.2%). The EE was notably higher in SVL (10.2%) compared to other ingredients, whereas ME was highest in Maize (3.760 kcal/kg) and lowest in NSC (2,258 kcal/kg). The CF was highest in NSC (17.2%) and lowest in Maize and Finger millet (4.2%), and ash content was greatest in SBM (9.9%) and lowest in maize (2.6%).

Table 1. Chemical compositions of feed ingredients used for diet formulation

Feed ingredients / Chemical composition (%)	Maize grain	Wheat short	Finger millet	SBM	NSC	SVL
DM	90.73	91.2	92.2	91.3	92.9	91.2
CP	12.2	15.3	9.5	42.6	31.9	35.9
CF	4.2	8.7	4.2	9.8	17.2	9.6
EE	5.35	3.2	4.4	2.7	2.9	10.2
Ash	2.6	4.2	3.5	9.9	7.9	6.3
OM	97.4	95.8	96.5	90.1	92.1	93.7
NFE	66.3	59.8	70.5	26.2	32.9	29.2
ME (kcal/kg)	3760.1	3179.5	3674.8	2822.3	2257.7	3399.1

DM: Dry matter, CP: Crude protein, EE: Ether extract, CF: Crude fiber, OM: Organic matter, NFE: Nitrogen-free extract, ME: Metabolizable energy, NSC: Noug seed cake, SBM: Soya bean meal, SVL: Sweet vitabor lupine

The differences observed in the proximate composition of the evaluated feed ingredients, particularly in DM, CP, CF, EE, ash, and nitrogen-free extract, highlight the nutritional variability among conventional and alternative feed resources. Such variability has important implications for diet formulation, as the substitution of conventional protein sources like SBM with alternative ingredients such as sweet lupine or NSC can substantially influence the overall dietary balance of protein, energy, and fiber. The current proximate analyses are consistent with previous reports indicating that alternative plant protein sources differ markedly in their nutrient profiles and digestibility, thereby affecting the nutritional adequacy of compounded diets (El-Deek et al., 2020). Consequently, careful consideration of these compositional differences is essential to ensure that formulated diets remain balanced and isonitrogenous, thereby supporting optimal animal performance and feed efficiency. Overall, the observed variation underscores the nutritional diversity of the ingredients and provides the basis for designing diets that meet specific protein and energy targets in chicken feeding trials.

Table 2 presents the ingredient composition and nutrient profile of the finisher diet formulated using WinFeed (2.8 Demo version) feed formulation software for a 500 kg batch. The diet consisted primarily of maize grain, finger millet, and wheat short as energy sources, while soybean meal (SBM), sweet Vitabor lupine (SVL), noug seed cake (NSC), and bone and meat meal (BMM) served as the major protein sources.

Table 2. Ingredient's inclusion rate to make 500 kg chicken finisher diet

Ingredients	Proportion/Rounded	Ingredients (%)
Maize grain	107.14	21.43
Wheat short	91.03	18.21
Finger millet	109.31	21.86
SBM	43.87	8.77
NSC	48.51	9.7
SVL	65.14	13.03
BMM	25	5
Vitamin premix	2.5	0.5
Limestone	5	1
Salt	2.5	0.5
Total	500 kg	100%

Nutrients	Nutrient requirements		Analysed result
	Minimum	Maximum	
CP	18	19	19
ME	3100	3200	3100

CP: Crude protein, ME: Metabolizable energy, NSC: Noug seed cake, SBM: Soya bean meal, SVL: Sweet vitabor lupine, BMM: bone and meat meal. The diet is formulated by WinFeed (2.8 Demo version) feed formulation Software.

Table 3. Treatment replacement levels and layout for the present study by replacing soybean meal with sweet vitabor lupine

Experimental treatments	Level of replacement
T_1	0% SVL + 100% SBM based
T_2	15% SVL + 85% SBM based
T_3	25% SVL + 75% SBM based
T_4	30% SVL + 70% SBM based
T_5	50% SVL + 50% SBM based

SVL: Sweet vitabor lupine, SBM: Soybean meal. Other ingredients (Table 2) were kept constant in type for all experimental treatments, and these included: Maize grain, wheat shorts, finger millet, and noug seed cake.

Table 3 presents the experimental treatment structure used to evaluate the effects of replacing soybean meal (SBM) with Sweet Vitabor lupine (SVL) in Sasso T_{44} chicken diets. Five dietary treatments were formulated with increasing levels of SVL substitution, ranging from 0% (control) to 50% replacement of SBM. To ensure that any observed differences in performance, carcass characteristics, hematological, and biochemical parameters were attributable primarily to the varying levels of SVL inclusion, all other feed ingredients were maintained constant across treatments.

On the other hand, the proximate composition of the experimental diets revealed numerical variations across treatments T1 to T5 (Table 4). The DM remained relatively stable, ranging from 91.90% in T2 to 92.43% in T5, whereas the CP decreased progressively from 21.67% in T1 to 20.40% in T5, corresponding to the stepwise replacement of SBM with SVL, which contains lower protein. Concurrently, the EE increased from 4.63% in T1 to 6.70% in T5, while CF decreased from 8.03% to 6.40%, reflecting the higher lipid and lower fiber contribution of SVL compared to SBM.

Table 4. The average proximate composition values of the treatments fed to the experimental chicken

Treatment	DM (%)	CP (%)	CF (%)	EE (%)	Ash (%)	OM (%)	NFE (%)	ME (kcal/kg)
T1	92.30	21.67	8.03	4.63	4.50	95.50	53.43	3128.53
T2	91.90	21.43	7.67	4.77	4.37	95.73	53.67	3148.74
T3	92.07	21.20	7.17	5.40	4.10	95.77	54.17	3162.20
T4	92.30	20.83	6.67	5.73	3.27	96.73	55.73	3168.94
T5	92.43	20.40	6.40	6.70	2.97	97.07	55.97	3195.88

T1: 0% SVL + 100% SBM based, T2: 15% SVL + 85% SBM based, T3: 25% SVL + 75% SBM based, T4: 30% SVL + 70% SBM based, T5: 50% SVL + 50% SBM based, DM: Dry matter, CP: Crude protein, EE: Ether extract, CF: Crude fiber, OM: Organic matter, NFE: Nitrogen free extract, ME: Metabolizable energy.

Ash content declined, resulting in an increase in organic matter from 95.50% to 97.07%, and nitrogen-free extract increased from 53.43% to 55.97% in T1 and T5, respectively. The ME of diets also increased progressively from 3,128.53 kcal/kg in T1 to 3,195.88 kcal/kg in T5. The chemical compositional changes of experimental diets demonstrate that incremental replacement of SBM with SVL altered the dietary protein, energy, and fiber profiles, which is critical for interpreting subsequent growth performance, feed utilization, and nutrient metabolism in the experimental chickens.

The inverse relationship between dietary energy and CP content is consistent with findings by Musigwa et al. (2024), who reported that reducing dietary protein while maintaining appropriate energy and amino acid balance can optimize broiler performance without compromising growth. The CF content decreased from 8.03% in T1 to 6.40% in T5, in which T5 revealed better performance of chickens in the current study, and this was aligned with report of Zhang et al. (2023) who reported that high fiber diets can negatively impact nutrient digestibility by diluting nutrient concentration and increasing the bulk of the diet, which can lead to reduced feed intake and growth performance in broilers.

Ash content declined progressively from 4.50% in T1 to 2.97% in T5, while the organic matter content increased correspondingly from 95.50% to 97.07%. The reduction in ash percentage may reflect a lower inclusion of mineral-rich feed components, which may enhance the digestibility of the feed (Salo et al., 2025), and a diet rich in organic matter can lead to better nutrient absorption and overall health, potentially resulting in improved growth rates and productivity (Maruthamuthu et al., 2024). Likewise, the nitrogen-free extract increased from 53.43% in T1 to 55.97% in T5, and the ME values increased from 3,128.53 kcal/kg to 3,195.88 kcal/kg, indicating progressive enrichment of available carbohydrates and fats in the diets. The progressive increase in ME recorded in the present study agreed with the observations of Infante-Rodríguez et al. (2016), who also reported enhanced dietary energy levels when higher-energy feed ingredients were incorporated into poultry diets. Overall, the observed nutrient variations among treatments suggest that the formulated diets were progressively adjusted to achieve an optimal balance between protein and energy sources. The nutrient adjustment aligns with the modern poultry feeding strategy that emphasizes the optimization of net energy and digestible amino acid profiles rather than increasing CP levels alone (Musigwa et al., 2024).

Formulated feeds

For the experimental feed formulation (Table 2), the formulated diets were adjusted to contain a minimum of 3,100 to 3,200 kcal/kg ME and to contain CP from 19 to 20 percent to satisfy the experimental chicken's requirement for finishers. According to National Research Council (NRC, 1994), with the standard specifications for broiler and dual purpose grower and finisher feed requirements, chicken diets were formulated to contain a 3,050 to 3,100 Kcal/kg ME and 210 to 220g CP/kg for the growers whereas, 3,100 to 3,200 Kcal ME and 180g CP/kg to 190 g/kg for the finisher to satisfy their requirements. Both the ME and CP were approximately obtained to meet the requirement.

Feed intake

The influence of the experimental diets on average dry matter intake, body weight, body weight gain, and feed conversion of Sasso T_{44} dual-purpose chickens throughout the feeding trial is observed in Table 5. Statistical analysis indicated significant ($p < 0.05$) differences among dietary treatments for all measured parameters, with the exception of feed conversion ratio. Both daily and cumulative dry matter intake revealed a significant ($p < 0.05$) increase as the replacement level of sweet lupine in the diets increased. Chickens fed the diet T5 recorded the highest mean daily (62.4 g/chicken) and total dry matter intake (5,611.8 g/chicken), whereas those fed the control diet (T1) had the lowest values (54.8 g/chicken and 4,933.1 g/chicken, respectively).

Table 5. The average dry matter intake, body weight, body weight gain, and feed conversion ratio of the chicken during the 90-day feeding trial

Parameters \ Treatments	T1	T2	T3	T4	T5	SEM	P -value
Mean daily DMI (g/chicken)	54.8^{c}	56.6^{bc}	57.2^{bc}	59.3^{b}	62.4^{a}	1.25	0.0001
Mean total DMI (g/chicken)	$4,933.1^{e}$	$5,091.5^{d}$	$5,148.3^{c}$	$5,335.9^{b}$	5611.8^{a}	3.35	0.0001
Mean final body weight (g/chicken)	$1,559.0^{e}$	$1,631.5^{d}$	$1,654.0^{c}$	$1,849.5^{b}$	$1,921.5^{a}$	2.92	0.0001
Mean daily BWG (g/chicken)	17.0^{c}	18.5^{bc}	18.4^{bc}	20.1^{ab}	21.5^{a}	0.57	0.0002
Mean weekly BWG (g/chicken)	127.7^{d}	138.8^{c}	139.8^{c}	150.4^{b}	161.2^{a}	1.88	0.0001
Mean total BWG (g/chicken)	$1,532.3^{e}$	$1,665.6^{d}$	$1,678.1^{c}$	$1,807.0^{b}$	$1,935.1^{a}$	5.31	0.0001
FCR (g DMI/g gain)	3.2^{a}	3.1^{a}	3.1^{a}	2.9^{a}	2.9^{a}	0.06	0.5962

[a,b,c] Different superscript letters in a row indicate significantly different ($p < 0.05$). g: Gram, MDMI: Mean dry matter intake, BW: Body weight, IBW: Initial body weight, BWG: Body weight gain, FCR: Feed conversion ratio, SEM: Standard error of the mean, T1: 0% SVL + 100% SBM based, T2: 15% SVL + 85% SBM based, T3: 25% SVL + 75% SBM based, T4: 30% SVL + 70% SBM based, T5: 50% SVL + 50% SBM based.

The observed increase in feed intake with higher levels of SVL could be attributed to improved palatability and the balanced nutrient composition of sweet lupine-based diets. Comparable results have been documented by Al-Sagan et al. (2020), who reported a linear rise in voluntary feed intake in broiler chickens offered diets containing blue lupine (*Lupinus angustifolius*) seed meal. In a similar study, Dida and Melesse, (2024) found that partially replacing soybean meal with heat-treated sweet lupine seed meal improved feed consumption and palatability in broilers without negatively affecting growth performance. Moreover, David et al. (2024) emphasized that modern lupine varieties possess lower levels of alkaloids and improved amino acid profiles, which enhance diet palatability and digestibility in poultry.

Body weight and feed conversion ratio

Body weight and feed conversion ratio are presented in Table 5. The average final body weight and total body weight gain exhibited trends comparable to those observed for feed intake. Chickens in treatment T5 recorded the greatest final body weight (1,921.5 g/chicken) and total body weight gain (1,935.1 g/chicken), whereas chickens in T1 indicated the lowest values (1,559.0 g/chicken and 1,532.3 g/chicken, respectively). Mean daily and weekly weight gains also increased progressively with sweet lupine inclusion. Brand et al. (2019) reported that a 30% inclusion of lupine in the diet resulted in improved average daily gains and final body weights. The current study suggested that, the increased performance in the mean daily and weekly weight gains could be attributed to improved nutrient utilization efficiency and the favorable amino acid balance of sweet lupine, which may have enhanced protein synthesis and growth potential and this is consistent with Gresta et al. (2023) study**,** who reported that white lupine (*Lupinus albus*) provided a high-quality protein source capable of replacing soybean meal in poultry diets when appropriately processed. Furthermore, Straková et al. (2024) found that dehulled lupine seed inclusion improved muscle protein quality and growth parameters in broilers, reinforcing its potential as an alternative plant protein. The positive growth response in the present study, therefore aligned with earlier reports suggesting that lupine inclusion up to moderate levels supports optimal growth without compromising nutrient digestibility (Al-Sagan et al., 2020; Dida and Melese, 2024; David et al., 2024).

Although there was a numerical improvement in feed conversion ratio values with increasing dietary inclusion of sweet lupine, the differences among treatments were not statistically significant. Chickens fed T4 and T5 diets exhibited the lowest feed conversion ratio values (2.9 g DM intake/g gain), indicating more efficient feed utilization compared to T1 (3.2 g DM intake/g gain). The lack of statistical significance suggested that while sweet lupine inclusion improved intake and growth, the feed-to-gain efficiency remained relatively stable across treatments. The lack of statistical significance in feed conversion ratio implies that the diets were adequately balanced in energy and protein to support efficient utilization, consistent with the findings of Al-Sagan et al. (2020) and Dida and Melese (2022), who reported similar non-significant effects of blue lupine inclusion on feed conversion ratio despite improved growth rates.

Carcass traits and organ weights

The carcass performance of the experimental chickens, including carcass weight and the weights of major cut portions obtained from chickens sampled for blood hematological and serum chemistry evaluation, is presented in Table 6 for treatments T1 through T5. Significant ($p < 0.05$) treatment effects were observed for all evaluated traits.

Table 6. Carcass traits and organ weights of chickens during the 90-day feeding trial

Parameters \ Treatments	T1	T2	T3	T4	T5	SEM	P -value
Live weight (g)	1,401.8^{e}	1,478.3^{d}	1,512.2^{c}	1,858.0^{b}	1,946.2^{a}	2.026	0.001
Slaughter weight (g)	1,347.4^{e}	1,418.0^{d}	1,471.5^{c}	1,812.6^{b}	1,912.5^{a}	0.86	0.001
Carcass weight (g)	935.5^{e}	1,048.2^{d}	1,166.5^{c}	1,458.0^{b}	1,580.2^{a}	2.035	0.001
DP (%)	66.7^{d}	72.8^{c}	76.1^{b}	77.5^{b}	80.8^{a}	1.50	0.001
Breast weight (g)	236.5^{e}	280.6^{c}	285.5^{d}	353.3^{b}	381.2^{a}	1.84	0.001
Thigh weight (g)	145.3^{e}	170.9^{d}	174.9^{c}	211.5^{b}	230.2^{a}	1.5	0.001
Drumstick weight (g)	192.7^{b}	140.8^{e}	150.9^{d}	189.3^{c}	219.6^{a}	0.34	0.001
Wings weight (g)	121.8^{e}	151.6^{c}	152.0^{d}	174.9^{ba}	185.5^{a}	0.17	0.001
Neck weight (g)	57.6^{e}	62.8^{c}	63.6^{d}	93.5^{a}	89.6^{b}	0.12	0.001
Rib back weight (g)	33.8^{e}	44.2^{d}	44.5^{c}	52.8^{b}	75.7^{a}	0.47	0.001
Tail back weight (g)	68.5^{e}	93.7^{c}	85.2^{d}	110.5^{a}	108.2^{b}	0.23	0.001
Skin weight (g)	78.3^{d}	84.3^{d}	98.0^{c}	123.6^{b}	130.5^{a}	0.22	0.001

^{a,b,c,d,e} Different superscript letters in a row indicate significantly different ($p < 0.05$). g: Gram, DP: Dressing percentage, SEM: Standard error of the mean, T1: 0% SVL + 100% SBM based, T2: 15% SVL + 85% SBM based, T3: 25% SVL + 75% SBM based, T4: 30% SVL + 70% SBM based, T5: 50% SVL + 50% SBM based.

Live body weight increased progressively across the treatments, with the lowest value recorded in T1 (1,401.8 g) and the highest in T5 (1,946.2 g). Likewise, the slaughter weight also indicated an upward trend, with T5 exhibiting the highest weight (1,912.5 g). In terms of carcass weight, the results reflected a similar pattern, with T1 having the lowest weight (935.5 g) and T5 the highest (1,580.2 g), whereas the carcass weight of chickens fed T2, T3, and T4 was between the interval values of T1 and T5. T1 had the lowest DP at 66.7%, while T5 demonstrated the highest DP at 80.8%. The present result for the DP is aligned with the studies, which reported that the carcass weight of chickens fed diets with sweet lupine was generally similar to that on traditional soybean diets (Suchý et al., 2010; Nuriev et al., 2021).

When examining individual cut parts, the breast weight followed a significant ($p < 0.05$) increasing trend, with T1 indicating the lowest weight (236.5 g) and T5 the highest (381.2 g). Similarly, the thigh weight increased from 145.3 g in T1 to 230.2 g in T5. The drumstick weight also varied significantly ($p < 0.05$) between treatments, with T5 having the heaviest drumsticks (219.6 g), while T1 had the lightest (140.8 g). The wing weight was significantly ($p < 0.05$) higher from 121.8 g (T1) to 185.5 g (T5). The neck weight followed a significantly ($p < 0.05$) higher trend, with T1 having the lowest at 57.6 g and T5 the highest at 93.5 g. A consistent upward trend was observed in the weights of rib back, tail back, and skin across the dietary treatments, increasing from 33.8 g to 75.7 g, 68.5 g to 108.2 g, and 78.3 g to 130.5 g, respectively, from T1 to T5. Overall, the values for all parameters indicated that as the levels of sweet lupine replaced soybean meal from T1 (0%) to T5 (50%), there was a consistent increase in live weight, slaughter weight, carcass weight, dressing percentage, and individual cut-up part weights, with significant ($p < 0.05$) differences observed across all parameters, suggesting a positive correlation between treatment groups and overall body mass and distribution of parts chickens.

In the findings of the present study, improved performance was observed at higher levels of sweet lupine substitution. The improved crude protein content and lower anti-nutritional factors of sweet lupine could be attributed to chickens' improved performance, and this aligns with the study by Sedláková et al. (2016), who stated that 33-40% crude protein content and lower anti-nutritional factors make it a valuable protein source and promote its use in animal feed.

Another study conducted by Suchý et al. (2010) demonstrated that substituting one-third to two-thirds of soybean meal with sweet lupine in broiler diets significantly influenced live weight during the fattening period. Evaluation of the dressed carcasses of slaughtered chickens also revealed significant variations in the weights of different cut-up parts. Chickens in treatment five exhibited the highest carcass weight and dressing percentage, whereas those in treatment one revealed the lowest values. The findings for carcass weight and dressing percentage are consistent with the report of Pietras et al. (2021), who indicated that replacing 60% of soybean meal with low-alkaloid lupine did not adversely affect the growth performance or dressing percentage of broiler chickens. However, the average weights of individual carcass cuts indicated irregular variation among treatments. The present results for carcass, thigh, and breast weights are also in close agreement with the observations of Dida and Melesse (2024), who evaluated Cobb 500 broilers fed diets containing sweet lupine. Additionally, Olver (2009) reported that incorporating sweet lupine at levels of up to 40% per kilogram of diet positively affected growth performance, feed efficiency, and carcass composition of broilers from hatch to 8 weeks of age.

Hematological parameters

Table 7 indicates that the hematological parameters measured in the present study, including white blood cell count (WBC), lymphocytes, mid-sized cells (MID), granulocytes, red blood cells (RBC), hemoglobin, and hematocrit, differed significantly ($p < 0.05$) among the treatments. The highest WBC value (30.42×10^9/L) was recorded in chickens under T1, whereas the lowest value (24.13×10^9/L) was observed in T5. Similarly, variation was also observed in granulocyte percentages, with the highest value in T5 (65.38%) and the lowest in T2 (30.38%). Changes in WBC and lymphocyte counts may be associated with differences in the immune status of chickens, as these parameters are often used as indicators of immune function (Suman and Prasad, 2020). Likewise, granulocytes are commonly linked with physiological responses to stress or inflammation (Ribeiro et al., 2024). Therefore, the differences observed among treatments in the present study may suggest possible variations in the physiological and immune responses of the chickens under the different dietary treatments.

Higher inclusion levels of sweet lupine in poultry diets have been reported to affect granulocyte profiles, which may be associated with changes in immune or inflammatory responses. It is supported by the Scientific Committee on Animal Nutrition (SCAN, 2024) as replacing soybean meal with lupine meal has been associated with improved health outcomes in poultry, including enhanced fat quality in meat and eggs, which may indirectly support immune function. In the present study, the proportion of MID varied significantly ($p < 0.05$) among treatments. The highest MID value was recorded in T1 (18.38%), followed by T2 (15.56%), whereas the lowest value occurred in T4 (10.60%). Despite such

variations, all hematological values remained within the normal physiological ranges reported by Ritchie et al. (1998), and these findings are consistent with the report of Olver (2009), who indicated that sweet lupine can be incorporated into broiler diets at levels of up to 40% without detrimental effects on hematological parameters. Similarly, Al-Sagan et al. (2020) found that replacing soybean meal with up to 30% blue lupine seed in broiler diets can serve as a cost-effective protein source without compromising growth performance, while also contributing to improvements in immune organ development.

Table 7. Hematological parameters of the chickens during the 90-day feeding trial

Hematological parameters \ Treatments	T1	T2	T3	T4	T5	SEM	P -value
WBC (×10^9/L)	30.42^{a}	28.95^{ab}	27.76^{b}	25.19^{c}	24.13^{c}	2.775	0.01
Lymph (%)	42.78^{b}	38.47^{bc}	36.28^{c}	58.38^{a}	45.56^{b}	3.465	0.01
MID (%)	18.38^{a}	15.56^{ab}	11.71^{bc}	10.60^{c}	12.24^{bc}	0.763	0.02
GRA (%)	40.28^{b}	30.38^{c}	42.28^{b}	46.46^{b}	65.38^{a}	2.741	0.01
RBC (×10^{12}/L)	2.54^{b}	2.83^{b}	2.90^{b}	3.14^{ab}	3.56^{a}	0.095	0.016
Hemoglobin (g/dL)	10.92^{b}	10.59^{b}	10.92^{b}	11.19^{b}	13.38^{a}	0.263	0.01
MCHC (g/dL)	28.74^{a}	29.59^{a}	29.67^{a}	28.26^{a}	29.93^{a}	0.223	0.07
MCH (pg)	37.35^{a}	38.27^{a}	37.78^{a}	35.0^{a}	38.72^{a}	0.488	0.11
MCV (fL)	129.59^{a}	129.85^{a}	129.83^{a}	124.37^{a}	129.08^{a}	0.773	0.09
Hematocrit (%)	45.45^{a}	37.94^{b}	36.88^{b}	38.18^{b}	36.18^{b}	0.948	0.02
Platelet (×10^9/L)	317.83^{a}	263.98^{a}	274.59^{a}	228.17^{a}	275.53^{a}	5.476	0.17
Plateletcrit (%)	0.27^{a}	0.24^{a}	0.26^{a}	0.24^{a}	0.25^{a}	0.005	0.21

^{a,b,c} Different superscript letters in a row indicate significantly different ($p < 0.05$). WBC: White blood cells, MID: Middle cells, GRA: Granulocytes, RBC: Red blood cells, MCHC: Mean corpuscular hemoglobin concentration, MCH: Mean corpuscular hemoglobin, MCV: Mean corpuscular volume, pg: Pictograms, T1: Treatment one, T2: Treatment two, T3: Treatment three, T4: Treatment four, T5: Treatment five.

The RBC counts and hemoglobin concentrations increased with higher SVL inclusion, with the highest values observed in T5 (RBC: 3.56 × 10^{12}/L; Hb: 13.38 g/dL), indicating enhanced erythropoiesis and oxygen-carrying capacity. The RBC counts and hemoglobin concentrations are consistent with reports by Suchý et al. (2010) and Lim and Choi (2023), who demonstrated that sweet lupine inclusion in broiler diets supports hematological stability and overall health without compromising erythrocyte integrity. Similarly, Sedláková et al. (2016) reported that higher dietary inclusion of sweet lupine may promote erythropoiesis, likely due to improved nutrient availability.

In contrast to T5, the lower RBC count observed in T1 suggests that exclusive reliance on soybean meal may not optimally support red blood cell production, which aligns with the report by David et al. (2034) that replacing soybean meal with the Zulika variety of lupine seed meal had no adverse effects on performance and was associated with improved health in fattening chickens. Conversely, hematocrit values decreased in T5 (36.18%), possibly reflecting a dilution effect associated with increased plasma volume. Similar erythropoietic-hematocrit trade-offs in lupine-based diets have been reported by Lim and Choi (2023). However, the hematocrit results observed in the present study differ from the findings of Lim and Choi (2023), who reported that partially replacing soybean meal with sweet lupine did not adversely affect hematocrit values or overall performance.

Regarding hemoglobin concentration, as a measure of the blood's oxygen-carrying capacity, T5 has the highest hemoglobin level (13.38 g/dL), significantly ($p < 0.05$) higher than all other treatments, while T1 has the lowest (10.92 g/dL). The higher hemoglobin in T5 suggests improved blood oxygenation, potentially due to better red blood cell production and overall nutritional support from the SVL-containing diet, and the current result agrees with (Jones, 2015). Mean corpuscular hemoglobin concentration (MCHC), which reflects the amount of hemoglobin present within red blood cells, revealed a slight upward trend across treatments, increasing from 28.26 g/dL in T4 to 29.93 g/dL in T5; however, the differences among treatments were not statistically significant. Likewise, no significant variations were detected among treatments for mean corpuscular hemoglobin (MCH) and mean corpuscular volume (MCV). The MCH values ranged from 35.0 pg in T4 to 38.72 pg in T5, while MCV values varied from 124.37 fL in T4 to 129.85 fL in T2, indicating only minor numerical differences among the treatment groups. A likely result was indicated by Straková et al. (2008), who reported that the hematological values, including MCH, MCHC, and MCV, remained within physiological ranges when broilers were fed with lupine meal, whether thermally treated or untreated.

Serum chemistry parameters

Table 8 of the present study provided important information regarding the effects of the different dietary treatments on the serum biochemical parameters of the experimental chickens. The mean serum chemistry values across different

treatment groups offer insights into the effects of different dietary or experimental treatments on key serum parameters of chickens. The key serum parameters include creatinine, total protein, total cholesterol, triglycerides, uric acid, and glucose. In the current study, significant differences were observed among experimental treatments for all parameters except triglyceride and glucose ($p < 0.05$). According to Campbell and Ellis (2013), the values of total protein, total cholesterol, triglycerides, glucose, uric acid, and creatinine are generally within normal physiological ranges, although they may vary depending on broiler strain, age, health status, and environmental conditions.

Certain treatments had a more profound impact on physiological processes than others. The T4 indicated the most notable effects, significantly ($p < 0.05$) increasing total protein levels (11.27 g/L), and a tendency to decrease in uric acid concentrations compared to the other treatments in the present study. Total protein levels are a marker of the overall protein metabolism in the body, which can reflect liver function and nutritional status, and this is in line with Richard et al. (2022), who reported that serum protein levels were positively influenced in some studies, suggesting that sweet lupine can enhance protein metabolism in broilers.

Table 8. Serum biochemical parameters of chickens during the 90-day feeding trial

Serum parameters \ Treatments	T1	T2	T3	T4	T5	SEM	P -value
Total protein (g/L)	8.00^{b}	8.80^{b}	9.13^{ab}	11.27^{a}	9.98^{ab}	0.328	0.005
Total cholesterol (mg/dL)	102.28^{b}	129.88^{ab}	110.58^{b}	111.30^{b}	162.98^{a}	5.875	0.004
Triglyceride (mg/dL)	188.68^{a}	165.65^{a}	205.28^{a}	221.58^{a}	221.45^{a}	8.563	0.181
Uric acid (mg/dL)	5.96^{a}	5.59^{a}	5.33^{ab}	4.07^{b}	5.17^{ab}	0.192	0.009
Creatinine (mg/dL)	1.02^{a}	0.56^{ab}	0.36^{b}	0.50^{b}	0.52^{ab}	0.069	0.012
Glucose (mg/dL)	161.85^{a}	145.90^{a}	133.08^{a}	169.43^{a}	171.65^{a}	6.508	0.286

$^{a, b}$ Different superscript letters in a row indicate significantly different ($p < 0.05$). mg: Milligram, dL: Deciliter, SEM: Standard error of the mean, T1: 0% SVL + 100% SBM based, T2: 15% SVL + 85% SBM based, T3: 25% SVL + 75% SBM based, T4: 30% SVL + 70% SBM based, T5: 50% SVL + 50% SBM based.

Creatinine concentration, an indicator of renal function and muscle metabolism Ávila (2025), varied significantly ($p < 0.05$) among treatments. The highest creatinine level was observed in T1 (1.02 mg/dL), which was significantly higher ($p < 0.05$) than those in T3, T4, and T5, while T2 exhibited an intermediate value (0.56 mg/dL). The lower creatinine levels in SVL-containing diets (T3-T5) may indicate reduced renal load or improved filtration efficiency, whereas the elevated level in T1 suggests potential renal or metabolic stress associated with exclusive soybean meal (SBM) inclusion. This contrasts with previous reports indicating that sweet lupine can replace SBM without adverse effects on creatinine levels (Olver, 2009; Lim and Choi, 2023). Ávila (2025) noted that creatinine concentration may also be influenced by dietary protein level, age, and other physiological factors, which may explain the present variation.

Significant differences were also observed in total cholesterol concentrations, which ranged from 102.28 to 162.98 mg/dL ($p < 0.05$). The T5 exhibited the highest cholesterol level significantly, whereas T1 recorded the lowest value ($p < 0.05$). No significant differences were detected among T2, T3, and T4 ($p > 0.05$). Triglyceride concentrations did not differ significantly among treatments, ranging from 165.65 to 221.58 mg/dL ($p > 0.05$). Although elevated cholesterol may indicate altered lipid metabolism, the increase observed in T5 remained within normal physiological limits, suggesting enhanced lipid intake or synthesis associated with higher SVL inclusion, and this was consistent with earlier studies reporting that diets containing lupine can positively influence lipid profiles by elevating high-density lipoprotein (HDL) levels and lowering low-density lipoprotein (LDL) cholesterol, providing potential metabolic benefits (Kim et al., 2023). Similarly, Lim and Choi (2023) reported increased polyunsaturated fatty acid (PUFA) concentrations in blood and muscle tissues of poultry fed lupine-containing diets.

The increased polyunsaturated fatty acid (PUFA) results suggest that increasing SVL inclusion may promote lipid accumulation and energy reserves, which could be advantageous depending on production objectives. However, careful formulation is required to avoid potential imbalances in mineral metabolism at higher inclusion levels (Hassan et al., 2018). In contrast, Dousa et al. (2011) reported no significant effects of legume-based diets on cholesterol levels, indicating variability among studies.

Uric acid levels differed significantly ($p < 0.05$) among treatments. Higher concentrations were observed in T1 (5.96 mg/dL) and T2 (5.59 mg/dL) than in T3, T4, and T5, whereas the lowest level occurred in T4 (4.07 mg/dL), with T5 indicating an intermediate value (5.17 mg/dL). Elevated uric acid levels in T1 may reflect increased protein catabolism or renal stress, while reduced levels in T4 suggest improved nitrogen utilization and metabolic efficiency, potentially associated with the combined inclusion of SVL and SBM. The elevated uric acid levels observed in T1 are consistent with those reported by Samofalova et al. (2023), who improved blood protein profiles and metabolic health following

SBM replacement with lupine. Although the uric acid concentration was comparatively higher in T1 and lower in T4, the values recorded in the present study remained within the normal physiological reference range and agree with Lumeij (2008), who reported that the normal serum uric acid concentration in chickens generally ranges from 2.0 to 7.0 mg/dL. Furthermore, Islam et al. (2020) reported that the physiological reference interval in avian species, including chickens, may extend up to 10 mg/dL depending on factors such as age, breed, diet, and management conditions.

Blood glucose concentrations did not differ significantly ($p > 0.05$) among treatments, although a numerical increase was observed from T3 (133.08 mg/dL) to T5 (171.65 mg/dL). The absence of significant differences indicated that varying proportions of SBM and SVL did not markedly affect glucose metabolism, suggesting stable glucose homeostasis across treatments ($p > 0.05$). However, the absence of significant differences in glucose metabolism in the current study contrasts with Zapletal et al. (2020), who reported reduced glucose and lipid metabolites following partial or complete replacement of SBM with whole lupine seeds.

CONCLUSION

The present study demonstrated that SVL can effectively replace up to 50% of SBM in the diets of Sasso T_{44} dual-purpose chickens without compromising growth performance, carcass characteristics, or hematological and biochemical health indicators. Dietary inclusion of SVL improved feed intake, body weight, carcass weight, and dressing percentage with most hematological and biochemical indices. However, significant reductions in hematocrit and increases in total cholesterol were observed at the highest SVL inclusion level, indicating that not all measured parameters responded uniformly. Therefore, while SVL indicated strong potential as an alternative protein source for dual-purpose chicken diets, careful consideration of inclusion levels is necessary. A limitation of the present study is that the effects of sweet lupine (SVL) inclusion were evaluated over the overall experimental period without separately assessing its impact during the starter, grower, and finisher phases, despite the varying nutrient requirements across growth stages. Consequently, the optimal SVL inclusion level for each production phase could not be established. Furthermore, although hematological and biochemical parameters were examined, other important indicators, such as gut health and microbiota composition, were not investigated, limiting a more comprehensive understanding of the physiological responses to SVL-based diets. Future studies should evaluate the effects of SVL inclusion during the starter and grower phases separately to determine phase-specific optimal inclusion levels that maximize growth performance and health outcomes. In addition, comprehensive investigations into gut health, intestinal morphology, and microbiota composition are warranted to better elucidate the physiological mechanisms underlying the responses of chickens to SVL-based diets.

DECLARATIONS

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Authors' contributions

Belayneh Bazie designed and performed the experiment, analyzed and interpreted the data, wrote the original draft, reviewed, edited, and validated the paper; Yeshambel Mekuriaw designed and performed the experiment, analyzed and interpreted the data, reviewed, edited, and validated the paper; Bimrew Asmare designed and performed the experiment, analyzed and interpreted the data, reviewed, edited, and validated the paper; Semahegn Yilkal designed the experiment, reviewed, and resourced. All authors read and approved the last edition of the manuscript before publication.

Availability of data and materials

All the raw data involved for analysis during the current study are available from the corresponding author upon reasonable request.

Competing interests

The authors have declared that there are no competing interests.

Ethical considerations

The author has checked the ethical issues, including plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy. The authors confirmed that no artificial intelligence (AI) tools were used to generate or assist in the writing of this manuscript. The original article, data interpretation, and analysis were conducted solely by the authors.

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Molecular Detection of Virulence Genes of *Salmonella* Agona Isolated from Diarrheic Cats in Al-Anbar Province, Iraq

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ABSTRACT

Salmonella infections are a major cause of foodborne infection in both humans and animals. Some serotypes are capable of inducing severe clinical manifestations, including gastroenteritis, septicemia, and abortion. The present study aimed to identify, isolate, and detect *Salmonella* Agona in cats with diarrhea in Al-Anbar Governorate, Iraq, and to evaluate the antimicrobial resistance profiles of the recovered isolates. Between November 2024 and May 2025, 100 fecal samples were collected from cats of different breeds presenting with diarrhea at veterinary clinics in Iraq. These samples were processed using established culture methods, including pre-enrichment in buffered peptone water followed by selective enrichment in tetrathionate broth. The enriched samples were then cultured on selective and differential media, including xylose lysine deoxycholate (XLD) agar and *Salmonella*-Shigella (SS) agar. For isolates identification, biochemical analyses, including catalase, indole, methyl red, Simmons citrate, oxidase, urease, lactose fermentation, triple sugar iron test, and phenylalanine, were performed, and the automated VITEK®2 test was used to confirm the results. The polymerase chain reaction targeted the *invA* gene to identify *Salmonella* species, followed by sequence analysis. Eventually, additional virulence genes were identified, including *Salmonella* enterotoxin (*stn*) and *Salmonella* outer protein E (*sopE*). The current results demonstrated that 4% of the samples (4 out of 100) were positive for *Salmonella* Agona. Antibiotic susceptibility testing against 16 antimicrobial agents demonstrated that the isolates were resistant to eight antibiotics (50%), namely amoxicillin, streptomycin, cefotaxime, oxytetracycline, doxycycline, tetracycline, trimethoprim-sulfamethoxazole, and gentamicin. The virulence gene results indicated that 2 out of 4 isolates (50%) possessed the *sopE* gene, whereas only 1 isolate (25%) carried the *stn* gene. The current findings indicate that *Salmonella* Agona was not prevalent among cats presenting with diarrhea; however, the existence of highly virulent, multidrug-resistant strains represented a vital public health concern for both humans and animals.

Keywords: Feline, *invA* gene, Polymerase chain reaction, *Salmonella* Agona, Virulence gene

INTRODUCTION

The White-Kauffmann classification categorized *Salmonella* (*S.*) species into two groups, including *S. bongori* and *S. enterica*, which are Gram-negative bacilli belonging to the *Enterobacteriaceae* family (Dougan and Baker, 2014). *Salmonella* classification is based on three surface antigens, including the lipopolysaccharide (O) antigen, the flagellar (H) antigen, and the capsular polysaccharide (Vi) antigen (Dougan and Baker, 2014). The genus *Salmonella* includes over 2,659 serotypes, of which approximately 1,547 were classified within the *Enterobacteriaceae* family, including species such as *S. salami*, *S. arizonae*, *S. diazoni*, *S. hotinii*, and *S. indica* (Dougan and Baker, 2014; Yada, 2023).

Salmonella Agona (*S.* Agona) is a non-typhoidal *Salmonella* species known for forming biofilms and surviving in environmental conditions and in dried food products (Song et al., 2025). Chickens, pigs, cattle, and ducks shed *S.* Agona, a pathogen linked to several outbreaks (Song et al., 2025). However, its ability to spread to humans and the role of large-scale genome rearrangements in ongoing infections remain poorly understood (Song et al., 2025). *Salmonella* Agona is a serotype linked to numerous outbreaks of foodborne and waterborne disease worldwide, commonly associated with contaminated animal products and feed (Dhakal et al., 2024). Pet food is considered unsafe for human consumption because it does not meet the same microbial safety standards as human food. This disparity raised concerns about the potential transmission of pathogens, such as *S.* Agona, to humans through direct contact or accidental ingestion. Additionally, due to the close proximity between humans and their pets, individuals often handle pet food with their bare hands and interact with their pets. In some instances, children inadvertently ingest pieces of pet food (Balachandran et al., 2012). Domestic cats can shed pathogens into the environment and contribute to the spread of zoonotic diseases, whether they exhibit clinical signs such as diarrhea or remain asymptomatic carriers (Udainiya et al., 2024).

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Salmonella was regarded as an easily treatable infection; however, it has become increasingly difficult to manage due to the global spread of antibiotic resistance (Mohamedtahir Fadlalla, 2025). Several mechanisms contribute to the emergence of antibiotic resistance, including beta-lactamase production, efflux pump overexpression, target-site modifications, and decreased membrane permeability (Mohamedtahir Fadlalla, 2025). Rapid proliferation of antibiotic resistance is occurring due to the excessive use of beta-lactamase production in human and veterinary healthcare settings (Caneschi et al., 2023). Recent surveillance data indicated that multidrug resistance is becoming more prevalent, particularly among isolates obtained from pets (Cardoso et al., 2023). Antibiotic resistance presented significant challenges due to the limited availability of treatment options and the increasing ease of access to antibiotics (Caneschi et al., 2023). Given the limited number of studies on the isolation of *S.* Agona from felines, the present study aimed to isolate *S.* Agona from cats with diarrhea to assess the antimicrobial resistance profiles of these isolates.

MATERIALS AND METHODS

Ethical approval

The present study was approved by the College of Veterinary Medicine at the University of Baghdad, Iraq, in accordance with the research ethics committee (ID: 2968 on 3/12/2025). The present study was conducted at Al-Ramadi Teaching Hospital after obtaining the necessary official documentation (P.G./ 2174 dated 11/2024 and P.G./ 2230 dated 24/11/2024). Pet owners provided their informed consent for the use of their pets' samples in the present study, and the identities of the animals and their owners were maintained confidentially.

Sampling and location

Between November 2024 and May 2025, 100 fecal samples were collected from cats with diarrhea that visited veterinary clinics in Al-Anbar province, primarily in Ramadi, Hit, and Haditha. No samples were collected from cats that had received antibiotics within the previous 10 days. Age, sex, living situation, and breed of cats were recorded. The samples were placed in buffered peptone water (Oxoid, England) and maintained in a cooled transport box at approximately 4°C until their arrival at the Microbiology Laboratory, Ramadi Teaching Hospital, Al-Anbar, Iraq. Subsequently, the samples were stored at –20°C for further analysis.

Isolation and identification of *Salmonella* Agona

Each sample in buffered peptone water was incubated at 37°C for 24 hours, then transferred to tetrathionate broth (TTB; Oxoid, England) at 42°C for 48 hours to promote bacterial growth. Samples were cultured on *Salmonella-Shigella* (SS) agar (Condalab, Spain) and xylose-lysine deoxycholate (XLD) agar (Oxoid, England) and incubated at 37°C for 24 hours. Suspected colonies were subjected to Gram staining and identified using standard biochemical tests, including urease, citrate, phenylalanine deaminase, indole, triple sugar iron (TSI) test, and catalase. The VITEK®2 Compact (GN ID card) was used to confirm the isolates. Pure colonies were obtained from selective media such as XLD agar after incubation at 37°C for 18-24 hours. A single, well-isolated colony was aseptically transferred into a sterile tube containing 3 mL of sterile saline solution (0.45-0.5% NaCl). The bacterial suspension was mixed thoroughly using a vortex mixer, and the turbidity was adjusted to 0.5 McFarland standard using a densitometer. The standardized suspension was then used to inoculate the GN identification card, which was designed for Gram-negative bacteria. The inoculated cards were loaded into the VITEK 2 system according to the manufacturer's instructions. The system automatically performed incubation and biochemical analysis of the isolates. Identification results were produced within 6-8 hours and analyzed using the system's probability percentages (Neyaz et al., 2024).

Antimicrobial susceptibility

The disk diffusion method was employed to assess antibiotic sensitivity, following the protocols established by CLSI VET01 and CLSI VET01S (2024). A total of 16 antibiotics were tested, manufactured by Bioanalyse, Turkey, including amoxicillin (25 µg), ampicillin (10 µg), streptomycin (10 µg), cefotaxime (30 µg), ceftriaxone (30 µg), piperacillin (100 µg), imipenem (10 µg), oxytetracycline (30 µg), doxycycline (30 µg), tetracycline (30 µg), norfloxacin (10 µg), enrofloxacin (5 µg), levofloxacin (5 µg), ciprofloxacin (5 µg), trimethoprim–sulfamethoxazole (SXT [1.25/23.75] 25 µg), and gentamicin (10 µg; Palladini et al., 2023).

Polymerase chain reaction

The Presto™ DNA extraction kit (Geneaid Biotech Ltd, Taiwan) was used for DNA extraction. Bacterial DNA was extracted using the Genomic DNA Mini Kit (Geneaid). One ml of an overnight culture in brain heart infusion broth was added to an Eppendorf tube (1.5 mL), centrifuged at 13000 rpm for 60 seconds to pellet the cells, and the supernatant

was discarded. A total of 180 µl of GT buffer (lysis solution) was added to the tube, the cells were gently pipetted to resuspend, and the mixture was incubated at room temperature for five minutes. In this step, 20 µl of Proteinase K was added to the sample, and the tube was incubated at 60˚C for 10 minutes, with the tube inverted every three minutes. Subsequently, 200 µL of lysis buffer (GB buffer) was added to the sample, and the mixture was shaken for five seconds. The mixture was then incubated at 70˚C for ten minutes, with the tube inverted every three minutes. Meanwhile, the TE buffer (Elution buffer) was incubated at 70˚C in a water bath (Hearn and Arblaster, 2010). A total of 200 µL of absolute ethanol was added to the sample lysate, and the mixture was vortexed immediately until a precipitate formed. At this time, a genomic DNA (GD) binding column in a collection tube (2 mL) was placed, and the whole mixture was transferred to the GD column, centrifuged at 13000 rpm for two minutes. The GD column was transferred to a new collection tube, and the first collection tube containing the flow-through was removed (Hearn and Arblaster, 2010). A washing solution (400 µL) was added to the GD column, centrifuged at 13000 rpm for 30 seconds, and the column was returned to the collection tube after the flow-through was removed. Then, wash buffer (600 µL) was added to the GD column, centrifuged at 13000 rpm for 30 seconds, and the flow-through was removed. The GD column was placed back into the collection tube (2 mL) and centrifuged for three minutes at 13,000 rpm to dry the column matrix (Menchhoff et al., 2020). Subsequently, 100 µL of preheated elution solution (TE buffer) was added to the center of the matrix in the GD column after placing it in a clean Eppendorf tube. After three minutes, the matrix absorbed the added solution, and the purified DNA was finally eluted by centrifuging at 13000 rpm for 30 seconds and stored at -20˚C until use (Menchhoff et al., 2020).

According to the method of Rahn et al. (1992), primers were designed to detect the *invA* gene, alongside the virulence genes *sopE* and *stn* (Table 1). The PCR reactions were performed in a final volume of 25 µL, consisting of 2.5 µL of 10x PCR buffer, 2.0 µL of magnesium chloride (25 mM), 0.5 µL of dNTP mix (10 mM each), 1.0 µL of each primer (10 µM), 0.2 µL of Taq polymerase (5 units/µL), 2.0 µL of DNA template, and 15.8 µL of sterile distilled water. The PCR was performed using the following thermal cycling settings. An initial denaturation was conducted at 94˚C for five minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, primer annealing at 55°C for 30-60 seconds (depending on the primers' melting temperatures), and extension at 72°C for one minute. The final extension was at 72°C for 10 minutes. Agarose gel (1.5%) was prepared by mixing agarose with 1x Tris-borate-EDTA (TBE) buffer and heating until completely dissolved. Ethidium bromide (2 µL of 10 mg/mL) was added, and the solution was cooled to about 50˚C before being poured into the gel tray. Following this procedure, 5 µL of the DNA samples and 2 µL of loading dye were added to the wells. Electrophoresis was performed for 45 minutes at 5 V/cm², and the DNA bands were visualized under ultraviolet light; then, the results were documented using a gel documentation system.

Table 1. Primers for the *invA*, *sopE*, and *stn* genes used in the present study

Gene	Primer sequence (5'-3')	Product size (bp)	Reference
invA	F: GTGAAATTATCGCCACGTTCGGGC AA R: TCATCGCACCGTCAAAGGAACC	284	Rahn et al. (1992)
sopE	F: GCAACACACTTTCACCGAGG R: TTGCTGTGGAGTCGGCATAG	485	*Primers selected from GenBank
stn	F: ATTCGTAACCCGCTCTCGTC R: GGTCGGTCCCACTGTCTTTT	420	*Primers selected from GenBank

F: Forward primer, R: Reverse primer, *Primers for *sopE* and *stn* were designed based on conserved sequences available in GenBank.

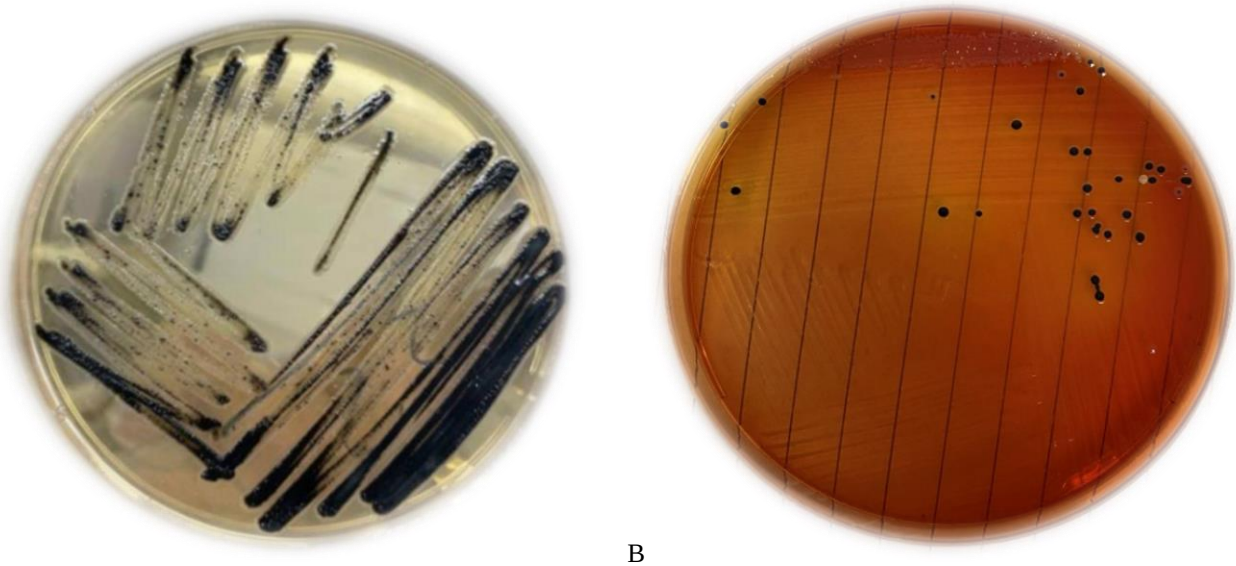
DNA sequencing and data analysis

The *invA* gene products obtained from PCR were sequenced to verify the uniformity of the *Salmonella* isolates. Four strains resembling the PCR products were purified and sent to Macrogen in Korea for sequencing. The same primers used for PCR amplification were employed for DNA sequencing. Sequencing was conducted in forward and reverse directions utilizing the Sanger method. The BioEdit, Chromas, and MEGA software applications were used to edit, assemble, and analyze the nucleotide sequences obtained, thereby generating consensus sequences. The BLASTn tool from the National Center for Biotechnology Information (NCBI, USA) was utilized to analyze the new sequences and evaluate their similarity to reference sequences available in the GenBank database. The NCBI GenBank database subsequently acquired the validated *invA* gene sequences from two isolates for inclusion.

RESULTS

Bacteriological examination

Out of 100 fecal samples, four isolates exhibited *Salmonella* colony morphology, appearing as transparent colonies with a dark center attributable to hydrogen sulfide (H_2S) production on SS agar. The colonies appeared red with a black core or, alternatively, yellow with a black center on XLD (Figure 1). The isolates tested positive for citrate and catalase activity, demonstrated an alkaline/acid TSI reaction with H_2S, and produced gas. However, the samples tested negative for urease, phenylalanine, and indole assays.



A B

Figure 1. *Salmonella* colonies isolated from cats with diarrhea in Al-Anbar province, Iraq, on selective culture media. **A**: Colonies with a black center on *Salmonella-Shigella* (SS) agar. **B**: Colonies with black center on xylose-lysine-deoxycholate (XLD) agar

Confirmation by the VITEK®2 system

All four isolates were classified as *Salmonella* group by the VITEK®2 compact system, exhibiting a high recognition probability of 98% (Table 2).

Table 2. The VITEK®2 compact system results for *Salmonella* species isolated from cats with diarrhea, in Al-Anbar province, Iraq

Number of samples	Source	Identified organism	Probability (%)
1	Cat feces	*Salmonella* spp.	98
2	Cat feces	*Salmonella* spp.	98
3	Cat feces	*Salmonella* spp.	99
4	Cat feces	*Salmonella* spp.	100

Antimicrobial resistance profile

Antibiotic susceptibility testing against 16 antibiotics revealed that all four isolates (100%) demonstrated identical resistance patterns, with resistance to eight agents (50%), including amoxicillin, streptomycin, cefotaxime, oxytetracycline, doxycycline, tetracycline, trimethoprim-sulfamethoxazole, and gentamicin. Additionally, three of the 16 antibiotics (18.75%) exhibited moderate susceptibility, including ampicillin, ceftriaxone, and levofloxacin. The remaining five antibiotics, including piperacillin, imipenem, norfloxacin, enrofloxacin, and ciprofloxacin (31.25%), were fully susceptible (Table 3).

Molecular detection of *Salmonella* species

The PCR amplification of the *invA* gene confirmed *S. enterica* subsp. *enterica* in 4 of the 30 suspected isolates, as evidenced by four bands of approximately 284 bp visualized on the agarose gel (Figure 2).

Table 3. Antibiotic susceptibility testing of *Salmonella* Agona isolated from cats in Al-Anbar province, Iraq

Antibiotic	ug/ disk	Sensitive (%)	Intermediate (%)	Resistant (%)
Amoxicillin	(AMX 25 ug)	0	0	100
Ampicillin	(AMP 10 ug)	0	100	0
Streptomycin	(ST 10 ug)	0	0	100
Cefotaxime	(CTX 30 ug)	0	0	100
Ceftriaxone	(CRO 30 ug)	0	100	0
Piperacillin	(PRL 100 ug)	100	0	0
Imipenem	(IPM 10 ug)	100	0	0
Oxytetracycline	(OXT 30 ug)	0	0	100
Doxycycline	(DO 30 ug)	0	0	100
Tetracycline	(TE 30 ug)	0	0	100
Norfloxacin	(NOR 10 ug)	100	0	0
Enrofloxacin	(ENR 5 ug)	100	0	0
Levofloxacin	(LEV 5 ug)	0	100	0
Ciprofloxacin	(CIP 5 ug)	100	0	0
Trimethoprim-sulfamethoxazole	(SXT [1.25/23.75] 25 ug)	0	0	100
Gentamicin	(CN 10 ug)	0	0	100
Total sensitivity (%)		31.25	18.75	50

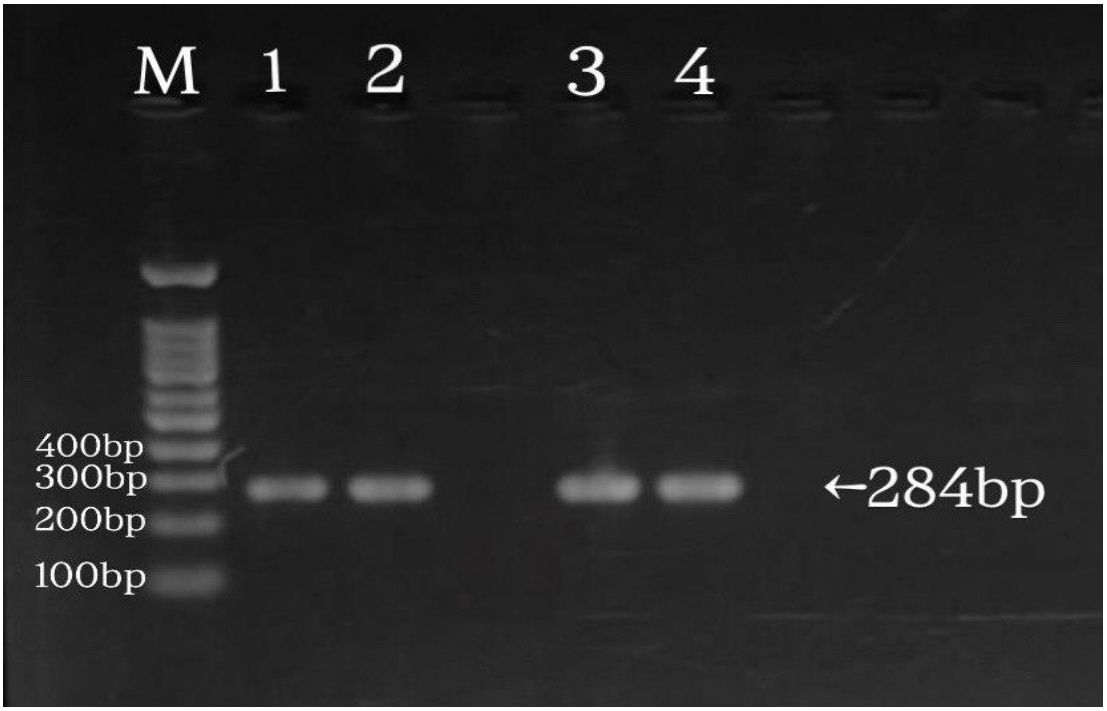


Figure 2. Agarose gel electrophoresis of PCR-amplified *invA* gene fragments from *Salmonella* spp. isolated from cats with diarrhea, in Al-Anbar province, Iraq. The PCR amplification of the *invA* gene yielded partial products of 284 bp, which were resolved by electrophoresis on a 1.5% agarose gel at 5 V/cm² for 45 minutes and visualized under ultraviolet (UV) light. Lane M: DNA ladder (1200 bp), Lanes 1-4: Positive *invA* gene amplicons from *Salmonella* spp.

DNA sequence analysis

BLAST analysis of the partial *invA* gene sequences demonstrated high genetic similarity between the *Salmonella* isolates recovered from cat fecal samples and several reference strains deposited in GenBank; however, only two sequences were formally registered. The current results demonstrated that the isolates were highly similar to each other and to reference strains of *Enterococcus enterica* serotype Agona. Isolate 1 (sequence ID: LC901557.1) had a perfect sequence match with *S.* Agona, and isolate 2 (sequence ID: LC901560.1) exhibited 100% similarity to *S.* Agona.

Detection of virulence genes

The *sopE* gene, which encodes a secreted effector protein essential for epithelial cell invasion, was identified in two of the four isolates, indicating a high prevalence of this virulence factor (Figure 3). The *stn* gene, which is associated with enterotoxin synthesis, was identified in only one isolate (Figure 4).

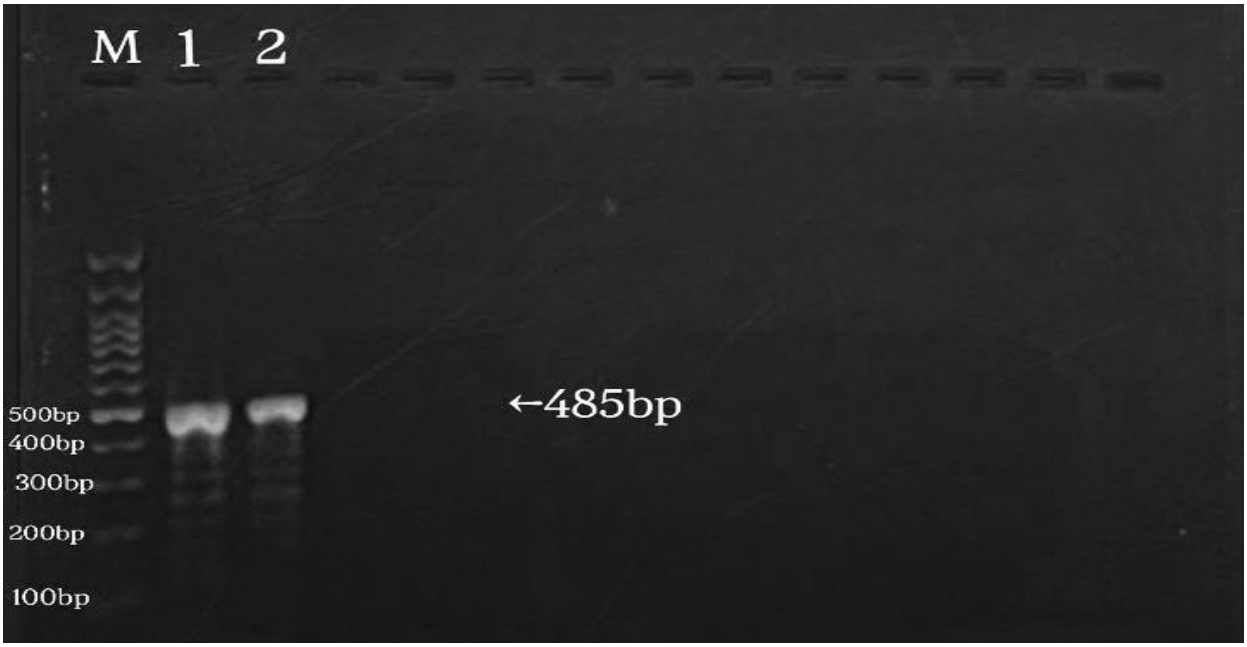


Figure 3. Agarose gel electrophoresis of *sopE* gene fragments from *Salmonella* Agona isolated from cats with diarrhea in Al-Anbar province, Iraq. The *sopE* gene was partially amplified by PCR, yielding a 485 bp product. Amplicons were resolved by electrophoresis on a 1.5% agarose gel at 5 V/cm² for 45 minutes and visualized under ultraviolet (UV) light. Lane M: DNA ladder (1200 bp), Lanes 1 and 2: *Salmonella* Agona isolates testing positive for the *sopE* gene.

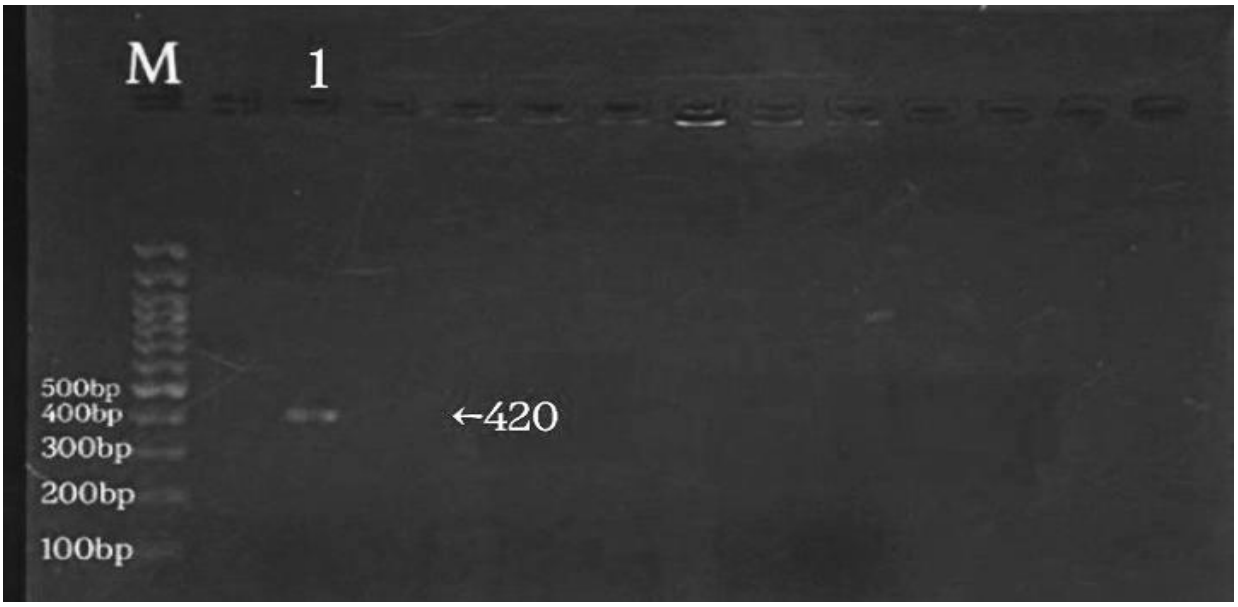


Figure 4. Agarose gel electrophoresis of the *stn* gene fragment from *Salmonella* Agona isolated from cats with diarrhea, in Al-Anbar province, Iraq. The *stn* gene was partially amplified by PCR, yielding a 420 bp product. The amplicon was resolved by electrophoresis on a 1.5% agarose gel in 1× TBE buffer at 5 V/cm² for 45 minutes and visualized under ultraviolet (UV) light. Lane M: DNA ladder (1200 bp), Lane 1: *Salmonella* Agona isolates testing positive for the *stn* gene.

DISCUSSION

Epidemiological importance and prevalence

The present study found that 4% of diarrheic cats in Al-Anbar Province, Iraq, tested positive for *S.* Agona. The findings of the current study were consistent with previous reports, as Barber et al. (2002) in Illinois, USA, identified a single *S.* Agona isolate among 26 feline-derived *Salmonella* isolates (3.8%), and Zhao et al. (2024) in Chongqing, China, detected *S.* Agona in 2 of 90 feline isolates (2.2%), representing similar incidence rates. Although the time and location of these findings differed, all support the fact that *S.* Agona is a rare serovar within the feline population. However, from an epidemiological perspective, the low incidence should not be regarded as insignificant. Cats live in close proximity to humans and may serve as silent carriers of zoonotic *Salmonella*, particularly multidrug-resistant strains. Therefore, the detection of *S.* Agona in cats, although infrequent, warrants investigation due to its potential public health implications.

Worldwide trends in antimicrobial resistance

The antimicrobial susceptibility profile observed in the present study indicated that *S.* Agona isolates exhibited resistance to 50% of the antibiotics tested (8 out of 16), including β-lactams, aminoglycosides, tetracyclines, and sulphonamides. Recent studies collecting samples from humans and food-producing animals have revealed a global increase in the prevalence of multidrug-resistant non-typhoidal *Salmonella* species (Zahra et al., 2023; Lamichhane et al., 2024). Few studies have directly examined whether *S.* Agona isolates from cats exhibit antibiotic resistance. Consistent with the present study, Song et al. (2025) reported that *S.* Agona isolates from avian and human infections exhibited resistance to multiple antibiotic classes, including β-lactams, aminoglycosides, tetracyclines, and sulphonamides. These findings may indicate the possible dissemination of resistance genes among different host species through horizontal gene transfer and environmental exposure, highlighting the epidemiological importance of antimicrobial resistance spread. The widespread use of antibiotics eradicates susceptible bacteria, but resistant bacteria survive and proliferate, thereby increasing resistance over time. The widespread use of antibiotics in animals and humans, combined with horizontal gene transfer, probably promotes the spread of resistance genes (Caneschi et al., 2023).

Zhao et al. (2024) in Chongqing, China, found that *Salmonella* strains isolated from pets exhibited greater resistance to antibiotics, particularly ampicillin, tetracyclines, and sulphonamides. Similarly, the current study found that all *Salmonella* isolates obtained from cats demonstrated complete resistance to tetracycline, doxycycline, and trimethoprim-sulfamethoxazole, indicating a consistent pattern of resistance, particularly to tetracyclines and sulfonamides. The multidrug resistance observed among the isolates in the present study may be attributed to the widespread, often uncontrolled use of antibiotics in both veterinary and human settings, which promoted the selection and persistence of resistant bacterial strains. Furthermore, horizontal gene transfer may facilitate the spread of resistance genes across diverse bacterial populations. The high resistance to tetracyclines and sulphonamides observed in the present study aligned with the findings of Zhao et al. (2024), who documented comparable resistance patterns in *Salmonella* isolates derived from companion animals such as cats and dogs. The susceptibility of the isolates to fluoroquinolones and carbapenems indicated that these antibiotics are still effective treatment options. The susceptibility of the isolates to fluoroquinolones and carbapenems indicated that these antibiotics are still effective treatment options, in line with previous reports of Zhao et al. (2024). The findings of the present study suggest that the bacterial isolates have not yet developed high levels of resistance against these antimicrobial agents. Fluoroquinolones act by inhibiting bacterial DNA replication, whereas carbapenems interfere with bacterial cell wall synthesis, making them highly effective against a broad range of Gram-negative bacteria (Aurilio et al., 2022). The observed sensitivity may be attributed to the limited spread of resistance genes associated with these antibiotic classes among the tested isolates. Similar findings were reported by Zhao et al. (2024), who demonstrated that fluoroquinolones and carbapenems remained among the most active antimicrobial agents against multidrug-resistant bacterial strains. Therefore, the fluoroquinolones and carbapenems may continue to serve as reliable therapeutic options for the treatment of infections caused by the studied isolates.

Distribution of pathogenic potential and virulence genes

Identifying virulence genes is a vital step in understanding the potential hazards posed by *S.* Agona from feline sources. All isolates in the present study possessed the *invA* gene, thereby confirming their classification as *Salmonella* spp. and indicating their capacity to invade intestinal epithelial cells, a marker of *Salmonella* pathogenicity (Rahman et al., 2004; Al-Zubaidy et al., 2015). In the present study, 50% of the isolates carried *sopE*, a gene encoding an effector protein of the type III secretion system, believed to contribute to host cell invasion and the induction of inflammation. Although the *sopE* gene was found in multiple isolates, suggesting increased virulence, the *stn* gene, which encodes an

enterotoxin, was present in only one isolate, indicating limited distribution. Differences in virulence gene profiles indicated that the *S*. Agona strains infecting cats are genetically distinct.

CONCLUSION

Salmonella Agona was identified in 4% of cats presenting diarrhea in Al-Anbar, Iraq. All isolates were subsequently confirmed by molecular analysis using *invA* gene sequencing, which demonstrated a high degree of similarity to reference strains. Whole-genome sequencing was used to examine genetic diversity and resistance. The isolates exhibited multidrug resistance to several commonly used antibiotics while carrying important virulence genes, including *sopE* and *stn*, indicating a potential public health risk despite their low prevalence. The prevalence of *Salmonella* species in Iraqi cats requires further investigation. Monitoring the development of antimicrobial resistance is recommended for future studies.

DECLARATIONS

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Authors' contributions

Ola Adnan Ismail conducted sample collection and laboratory investigations, including bacterial isolation and identification, Vitek analysis, and PCR assays, as well as data analysis and manuscript preparation. Asmaa Hamoody Abdullah supervised the study and provided scientific guidance. All authors read and approved the final edition of the manuscript prior to publication in the present journal.

Availability of the data and materials

All data generated during the study are relevant and included in this manuscript. The data supporting the findings of this study are available upon reasonable request from the corresponding author.

Competing interests

The authors declared no conflict of interest related to the present study.

Ethical considerations

All ethical aspects of the present study, including plagiarism, data integrity, consent to publish, and avoidance of misconduct or duplication, were carefully considered and approved by the authors. Artificial intelligence tools, such as Grammarly, were used only to support language improvement and understanding, without influencing the scientific content of the study. The authors have revised the manuscript using Grammarly and have taken full responsibility for its use. All scientific content, data interpretation, and conclusions were developed entirely by the authors.

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The Role of *Culicoides* Midges as Vectors of Bluetongue Virus: A Mini Review

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ABSTRACT

Culicoides biting midges (Ceratopogonidae) are the primary biological vectors of Bluetongue virus (BTV). The BTV is an orbivirus that causes substantial economic losses in livestock worldwide. Although over 50 arboviruses have been isolated from *Culicoides* species, BTV remains the most economically impactful disease. The present study aimed to evaluate the vector-virus association, including vector competence, transmission dynamics, and environmental factors influencing the spread of BTV. Vector competence in *Culicoides* is determined by midgut infection and dissemination barriers, which vary significantly among species. Transmission efficiency is influenced by temperature, extrinsic incubation period, and host factors, with cattle serving as long-term reservoirs and sheep exhibiting greater disease severity. Understanding vector-virus associations is essential for predicting outbreaks and developing effective control methods, especially as climate change promotes vector spread.

Keywords: Arbovirus, Bluetongue virus, *Culicoides*, Transmission, Vector competence

INTRODUCTION

Culicoides (*C.*) biting midges serve as the primary biological vectors for the Bluetongue virus (BTV), a pathogen classified within the genus *Orbivirus* of the family *Sedoreoviridae* (formerly Reoviridae, Payan-Carreira and Simões, 2025). Bluetongue virus causes morbidity and mortality in domestic and wild ruminants, leading to significant economic losses in the livestock industry (Maclachlan et al., 2009; Rushton, 2015). Bluetongue virus disease is notifiable to the World Organization for Animal Health (WOAH; Purse et al., 2005).

Over the past two decades, climate change has facilitated the spread of *Culicoides* vector species into geographical regions above 50° latitude (northern areas), thereby contributing to the global increase in BTV prevalence (Maclachlan, 2011). The disease has now been reported in all continents except Antarctica, exhibiting a noticeable trend of spreading towards more northern regions (Zhugunissov et al., 2025). The present study aimed to examine the association between *Culicoides* midges and BTV, focusing on vector competence, transmission mechanisms, and factors influencing viral spread.

BLUETONGUE VIRUS: KEY FEATURES RELEVANT TO VECTOR TRANSMISSION

Bluetongue virus is a small icosahedral virus measuring approximately 70 nanometers in diameter, with a genome comprising 10 segments of double-stranded RNA (Patel and Roy, 2014; Payan-Carreira and Simões, 2025). The genome segments encode seven structural proteins (VP1-7) along with four nonstructural proteins (NS1-3 and 3A; Roy, 2017). The outer capsid protein VP2 is the principal determinant of serotype specificity and the primary target of neutralizing antibodies (Bissett and Roy, 2024).

So far, 28 distinct BTV serotypes have been identified globally (Bumbarov et al., 2020). The virus demonstrated considerable genetic diversity, resulting from mechanisms such as genetic drift, reassortment, and intragenic recombination (Sanders et al., 2022). Reassortment, which involves the exchange of genome segments between different viral serotypes, can influence viral replication kinetics, cytopathogenicity, and virulence (Coetzee et al., 2014). This has been documented not only in wild-type field strains but also in live weak vaccine serotypes, prompting concerns regarding disease management (Nomikou et al., 2015). Bluetongue virus serotypes were further divided into eastern and western topotypes based on their geographic origin, indicating a long-term evolutionary divergence with limited genetic

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exchange between the regions (Maan et al., 2010). The viral RNA-dependent RNA polymerase, which lacks proofreading ability, is prone to errors, leading to increased genetic variability (Payan-Carreira and Simões, 2025).

CULICOIDES VECTORS OF BLUETONGUE VIRUS

Out of approximately 1,400 described *Culicoides* species worldwide (Harrup et al., 2015), only about 30 have been recognized as vectors of BTV (Gomulski et al., 2006). The most widely accepted major vector species of the BTV include *C. imicola*, which is distributed across Africa, southern Europe, the Middle East, and Asia, *C. brevitarsis* in Australia, *C. bolitinos* in southern Africa, *C. obsoletus* across Europe and Asia, *C. scoticus* and *C. pulicaris* in Europe, *C. sonorensis* in North America, and *C. insignis* in Central and South America. The success and extent of BTV transmission depend on the presence and density of these vector populations in regions (Mellor et al., 2000). *Culicoides* distribution is associated with warm, humid, and muddy areas rich in organic matter, and adult activity is most pronounced from approximately one hour before sunset to one hour after sunrise.

VECTOR COMPETENCE FOR BLUETONGUE VIRUS

Vector competence refers to an arthropod vector's ability to acquire, become infected with, and biologically transmit an arbovirus to a susceptible host (Carpenter et al., 2015). Competence varies significantly between different *Culicoides* species and even among individuals within a single species. For instance, only a specific proportion of *C. sonorensis* individuals can be orally infected with BTV (Jennings and Mellor, 1987).

Barriers to Bluetongue virus infection in *Culicoides*

The underlying variations in vector competence are attributed to intrinsic barriers, which include genetically determined immunological and physiological factors (Tabachnick, 1991). These barriers function at multiple stages of viral infection (Muntzer, 2017). The midgut infection barrier prevents the virus from entering or replicating within midgut epithelial cells, leaving individuals unaffected. The dissemination barrier (DB), also known as the midgut escape barrier, functions by preventing the virus from replicating in the midgut, spreading into the hemocoel, or causing infection in secondary tissues. The salivary gland barrier prevents virus transmission via saliva during subsequent blood meals, even when the virus has reached the salivary glands. Finally, the transovarial transmission barrier signified that infected females are unable to vertically transmit the virus to their offspring. The midgut is widely considered the primary determinant of vector competence and might even be the only tissue that affects the BTV infection outcomes in *Culicoides* (Fu et al., 1999).

Extrinsic incubation period

The extrinsic incubation period is the earliest time at which the virus is excreted into saliva and becomes infectious. The length of the extrinsic incubation period is influenced by viral strain, ambient temperature, viral dose ingested, vector type, body size, and nutritional status (Wittmann et al., 2002). The shortest documented incubation period for BTV is 4 days, observed with BTV-9 in *Culicoides*, whereas BTV-10 may require up to 15 days in *C. sonorensis* maintained at 25°C (Wittmann et al., 2002; Carpenter et al., 2011).

Bluetongue virus infection and dissemination in *Culicoides*

The transmission cycle initiates when female *Culicoides* ingest BTV while feeding on an infected host's blood. Then the virus replicates in both invertebrate vectors and vertebrate host cells, facilitating reproduction and transmission. Previous findings on *C. sonorensis* have reported the transmission pathway of BTV through the vector (Weaver et al., 1991; Fu et al., 1999). Following blood ingestion, virions enter the midgut, where the outer capsid protein VP2 is cleaved by intestinal proteases. Within approximately one hour, midgut epithelial cells become infected via either clathrin-mediated endocytosis or macropinocytosis (Mellor et al., 2000). Over approximately three days, viral particles replicate and spread from cell to cell within the midgut epithelium until reaching a limiting titer. Subsequently, viral particles disseminate into the hemocoel and then infect and replicate in secondary tissues, including neural tissues, the foregut, Malpighian tubules, ommatidia of the compound eyes, the fat body, the antennae, oocytes, and epithelial cells. Viral particles then enter the distal lateral lobes of the salivary glands, where they replicate and are secreted from acinar cells into saliva, a stage that lasts at least two days. Once salivary gland cells are infected, BTV is transmitted to the next host during subsequent blood feeding (Mellor et al., 2000; Muntzer, 2017).

Host factors affecting Bluetongue virus transmission

Successful vector transmission depends not only on vector competence but also on host susceptibility to BTV infection (Figure 1). Due to differences in the innate immune response, sheep exhibit significantly greater disease severity than cattle (Maclachlan, 2011; 2014). Higher and earlier levels of interferon type I (IFN-1) are associated with lower BTV titers, suggesting that early viral replication is more effective in sheep cells than in cattle cells (Hardy et al., 2023). Extended viremia in cattle makes them significant epidemiological reservoirs for BTV transmission, as indicated in Table 1 (Saminathan et al., 2020; Rodríguez-Martín et al., 2021).

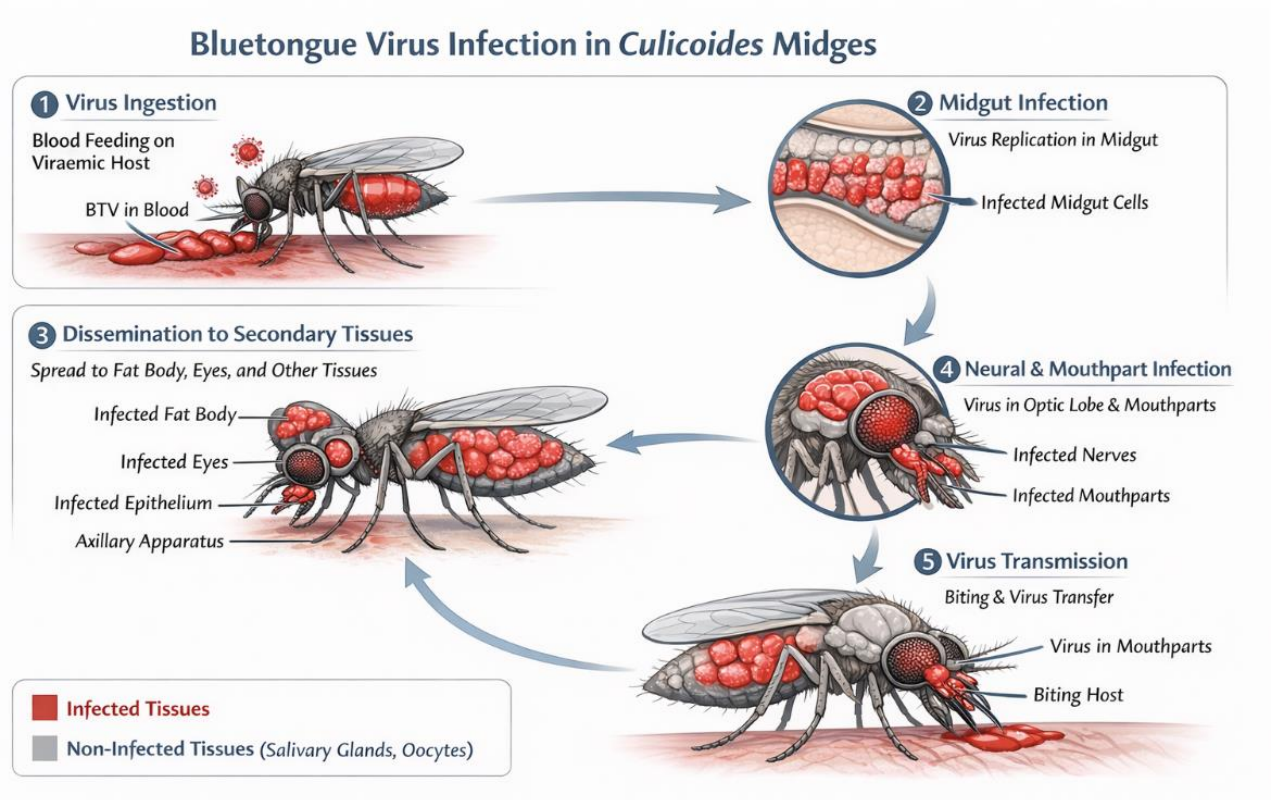


Figure 1. Bluetongue virus infection and dissemination in *Culicoides* midges. Bluetongue virus enters the midgut after blood feeding, replicates in epithelial cells, disseminates to secondary and neural tissues, and is transmitted via infected mouthparts during subsequent blood meals (Designed by the author).

Table 1. Duration of detectable viremia varies according to host species and Bluetongue virus serotype

Host	Viremia duration
Sheep	14-54 days
Goats	19-54 days
Cattle	Up to 100 days

ENVIRONMENTAL FACTORS INFLUENCING BLUETOUNGE VIRUS–*CULICOIDES* ASSOCIATION

The distribution and persistence of BTV are dependent on the presence or absence of adult *Culicoides* (Nayduch et al., 2014). Essential environmental factors include temperature, seasonality, and climate change.

Temperature

Under laboratory conditions, *C. imicola* larvae develop in 11-21 days at 20°C, 9-12 days at 25°C, and 7-9 days at 28°C. Egg and pupa development require 1-5 days, depending on temperature (Veronesi et al., 2009). In natural environments, larval development spans 10-60 days during summer and can extend through winter.

Seasonality

In Europe, active adult *Culicoides* were observed from February in oceanic climates to May in continental climates. The population peaks typically occur from May through September, with a secondary peak in the autumn following rainfall in Mediterranean climates. Populations decline in October or November, corresponding with the onset of winter temperatures (Veronesi et al., 2009). The seasonally vector-free period is defined as the duration during which the risk of BTV transmission is exceedingly low (EC, 2007).

Climate change

Mild, warm climates facilitate BTV survival during winter by expanding *Culicoides* habitats and sustaining vector activity. The emergence of multiple BTV serotypes (1, 2, 4, 9, and 16) into new regions, including Mediterranean Europe, since 1998 has led to major economic losses (Takken et al., 2007). Notably, the disease was detected in Norway in 2009, representing its farthest northern spread (Sperlova and Zendulkova, 2011).

GEOGRAPHIC SPREAD OF BLUETOUNGE VIRUS IN RELATION TO *CULICOIDES* DISTRIBUTION

Initially, BTV outbreaks were linked to tropical, subtropical, and temperate regions between 35°S and 40°N, aligning with climatic conditions conducive to *Culicoides* breeding (Sperlova and Zendulkova, 2011). Climate change has led to the expansion of *Culicoides* habitats into higher latitudes, facilitating the spread of BTV (Maclachlan, 2011). Since its initial identification in Africa in the late eighteenth century, BTV has been documented across all continents except Antarctica (Saminathan et al., 2020). Dissemination to new regions began in 1998 across the Mediterranean region of Europe (Takken et al., 2007). Previous isolated cases in Cyprus, the Greek islands, and the Iberian Peninsula likely resulted from wind-driven spread of infected *Culicoides* originating from Israel, Turkey, or North Africa (Purse et al., 2005).

CONCLUSION

The association between *Culicoides* midges and BTV represented a significant economic burden on global livestock production. Although the *Culicoides* genus comprises approximately 1,400 recognized species, only about 30 have been implicated in BTV transmission, with *C. imicola*, *C. obsoletus*, and *C. sonorensis* among the most notable vectors. Vector competence was influenced by physiological barriers, primarily the midgut infection barrier and viral dissemination barriers, which differed significantly across different species and among individuals within a population. Furthermore, species-specific differences in innate immune responses, with sheep exhibiting greater disease severity than cattle, underscore the importance of host factors in BTV transmission dynamics. The extrinsic incubation period, typically ranging from 4 to 15 days, was significantly influenced by ambient temperature and viral genotype. In vertebrates, cattle play a significant role in sustaining viral circulation, as they can remain viremic for up to 100 days and serve as strong reservoir hosts. Climate factors have enabled these vectors to move into higher latitudes, leading to the emergence of BTV in previously unaffected areas. Elucidating the specific associations between *Culicoides* species and circulating BTV serotypes is therefore important for refining predictive outbreak models and designing regionally adapted intervention strategies. Future studies should focus on regional vector competence investigations and surveillance of emerging vector-virus interactions.

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Authors' contributions

Ivana Zuber Bogdanović was responsible for conceptualization, literature collection, data interpretation, manuscript writing, review and editing, visualization, and final approval of the manuscript.The author read and approved the final edition of the manuscript before publication.

Availability of the data and materials

No new data were created or analyzed in this study. This study is a review of existing literature, and all referenced materials are cited in the reference list.

Competing interests

The author declared no conflict of interest.

Ethical considerations

The author reviewed potential ethical issues, including data falsification, multiple publications and submissions, redundancy, plagiarism, consent for publication, and misconduct, prior to publication. No AI tools were used in preparing and writing the manuscript.

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Single Nucleotide Polymorphisms of the Growth Hormone Gene and Their Association with Growth Traits in Sheep: A Systematic Review

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ABSTRACT

Growth hormone (GH) is synthesized and secreted by the pituitary gland. It is regulated by the *GH* gene, located on chromosome 11 in sheep, and is crucial for regulating postnatal growth and development in sheep. Single-nucleotide polymorphisms (SNPs) in the *GH* gene are associated with substantial differences in weight and body size in sheep. This study aimed to systematically evaluate SNPs of the *GH* gene and their association with growth parameters such as weaning weight (WW), yearling weight (YW), average daily gain (ADG), and linear body conformation in sheep. A total of 71 studies were extracted from January 2011 to March 2026. The results identified 25 distinct SNPs (with 15 SNPs reported on exon 5), of which five SNPs, A781G, A1544G, C1765A, T1772A, and G1769C, were detected in more than one breed. Eighteen SNPs were found to be significantly associated with growth traits; these associations were not uniform, as individual SNPs were linked to single or multiple traits, including body weight, birth weight, pre-weaning gain, weaning weight, 6-month weight, daily live weight gain, yearling weight, and morphometric traits. In conclusion, polymorphism at the *GH* gene influences growth traits in sheep, and several SNPs such as C1776G, A1544G, A1678G, A1558G, C1765A, G1550A, T1772A, G1769C, G1756C, 476G>A, 480 G>A, 55G>A, G871A, G1383A, A1509G, A781G, C408G and T735A associated with growth traits are promising candidate markers after validation in larger breed-specific and trait-specific validation.

Keywords: Breeding program, Growth hormone gene, Marker-assisted selection, Sheep growth, Single nucleotide polymorphism

INTRODUCTION

Globally, demand for small ruminant meat is increasing in line with population growth and changing dietary preferences toward a high-protein diet and increased red meat consumption, underscoring the urgency of improving productivity traits in sheep populations (OECD-FAO, 2025). Economically important growth traits in sheep include body weight (BWT), weaning weight (WW), and average daily gain (ADG), as they directly influence productivity and profitability (Cinar et al., 2023). Growth traits, such as BWT, ADG, WW, 6-month weight (LW6), 9-month weight (LW9), Yearling weight (YW), birth weight (BW), and morphometric traits, are quantitative traits regulated by multiple genes and environmental factors, and therefore, genetic enhancement is a complex but necessary process (Begenova et al., 2025).

Growth hormone (GH), a 191-amino acid polypeptide, is produced and secreted by the somatotrophic cells of the anterior pituitary gland and plays a crucial role in somatic growth, metabolism, protein synthesis, and tissue development (Jia et al., 2014; Bayraktar and Shoshin, 2022). In addition, GH influences major physiological processes, including reproduction, fat metabolism, and body composition (Jia et al., 2014; Bergan-Roller and Sheridan, 2018). Growth hormone is regulated by the growth hormone (*GH*) gene, which is a key candidate gene involved in regulating growth and development in livestock species (Bayraktar and Shoshin, 2022). It has five exons and four introns and is located on chromosome 11 in sheep (Bai et al., 2022). The *GH* gene regulates the synthesis and secretion of GH at the pituitary gland (Bai et al., 2022). The *GH* gene is a central regulator of growth that has been extensively studied as a possible marker to enhance economically significant phenotypes such as WW, ADG, and BWT through marker-assisted selection (Cauveri et al., 2016; Tanış and Keskin, 2025).

Recent advances in molecular genetics have enabled the identification of various single nucleotide polymorphisms (SNPs) associated with growth traits in candidate genes. Such polymorphisms can either alter gene expression or protein activity, thereby affecting phenotypic traits (Xu and Li, 2017). A systematic review of the *GH* gene is important because

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many studies have examined *GH* gene polymorphisms in various sheep breeds and their relationships with growth traits. However, the results are scattered across many studies and inconsistent, with some showing an association of specific SNPs of the *GH* gene with studied growth traits, while others do not (Cauveri et al., 2016; Esen and Elmacı, 2022; Kumar et al., 2024). These inconsistencies can be explained by the breed variations, sample size, environmental conditions, and genotyping techniques. Without a structured synthesis, it is difficult to determine which polymorphisms have a consistent effect on growth traits in sheep or have practical relevance.

There has been an increasing number of publications on *GH* gene polymorphisms in sheep, but few studies have systematically summarized the evidence for the SNPs identified and their association with sheep growth traits. To fill this gap, the aim of this study was to conduct a systematic review of the literature on the SNPs of the *GH* gene and their association with growth traits in sheep. The present review study aimed to combines and evaluates findings from many studies to identify the SNPs of the *GH* gene that are consistently associated with economically important growth traits such as weaning weight and average daily gain. As the *GH* gene plays an important role in growth traits, combining all studies to identify consistent reporting of SNPs will help in marker-assisted selection (MAS) in breeding programs.

MATERIALS AND METHODS

The population, exposure, outcome (PEO) framework is a structured way of designing a research question in a systematic review, particularly in observational studies, to enhance clarity and focus of the review (Moola et al., 2015). In order to design a study question, the PEO components of the study question were identified as explained by Moola et al. (2015), which are called Population, Exposure, and Outcome. The population was "Sheep" with exposure to "Polymorphism", and the outcome was "growth rate/body conformation/morphological traits".

Literature search

A comprehensive literature search was conducted across four databases, including Scopus, PubMed, Web of Science (WOS), and Google Scholar, on the same date from January 2011 to March 31, 2026. The chosen time frame was selected to capture studies during a significant advancement in molecular genetics and the use of more standardized and advanced genotyping methods to increase the reliability of detection of SNPs and association analysis. For each database, the same keywords were used; keywords used for searching include "growth hormone gene/*GH* gene/somatotropin gene", "single nucleotide polymorphism/SNP/polymorphisms /genetic variation", "growth traits/body weight/average daily gain/weaning weight/morphometric/body conformation/ morphological traits/biometric traits", "sheep/ovine". Boolean operators' AND ' and ' OR' were used to refine search results (Rau, 2004). The complete study selection process is shown in Figure 1.

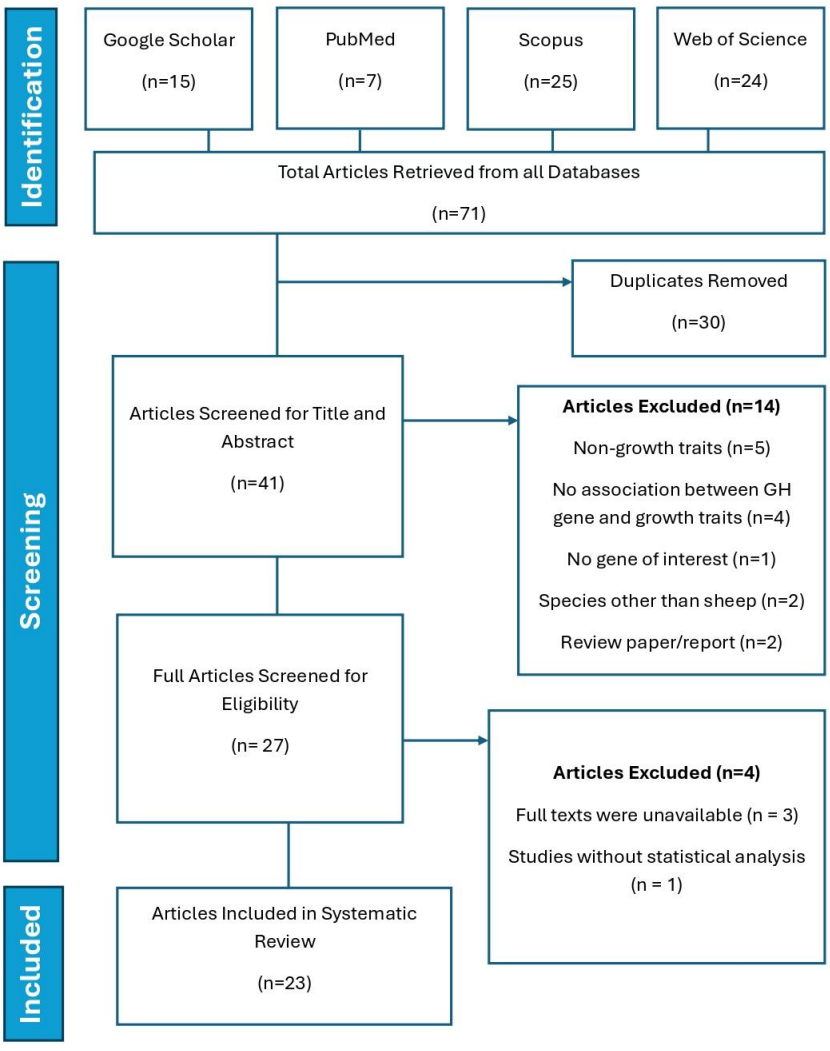


Figure 1. Study selection process for single nucleotide polymorphisms in sheep

Inclusion criteria

Two-step screening was performed, first, title and abstract screening, followed by full text screening. Studies were included based on the following criteria, including *GH* gene under investigation, reported SNPs or genetic variations, and the association between the *GH* gene and growth traits in sheep.

Exclusion criteria

Articles were excluded if they were on non-growth traits, lacked an association between *GH* gene polymorphisms and growth traits, had no gene of interest, were on species other than sheep, were review papers or reports, full texts were unavailable, or studies without statistical analysis.

Data extraction

All retrieved studies from four databases were combined, and duplicate records were removed manually. After that, the remaining articles were screened by one reviewer based on title and abstract, using predefined exclusion and inclusion criteria. After that, full articles were checked for eligibility and excluded if the full text was unavailable or if the study lacked statistical analysis. Data extracted from articles include publication characteristics (first author, journal), publication year, country, population size, sheep breed, SNPs, genomic regions, genotyping methods, genotype and genotype frequencies, allele frequencies, growth traits investigated, and the association between SNPs and growth traits, either significant or non-significant based on statistical results reported in original studies.

Methodological quality assessment

The methodological quality of included studies was assessed using the predefined criteria. Each study was evaluated based on the following criteria of adequate sample size (sample size classification was adapted based on the distribution of sample sizes across included studies, studies with fewer than 100 animals were classified as small, 100-300 as medium, and more than 300 as large), genotypic method used, reporting of genotypic frequencies, reporting of allele frequencies, and statistical analysis for association, which can affect the reliability, transparency, and interpretation of the results of association studies. Studies were divided into low, moderate, and high methodological quality based on these parameters. An adapted methodological quality scoring was used, as indicated by Hooijmans et al. (2014). Previous studies have also used this adapted methodological quality and point based scoring system (Macleod et al., 2004; Van Der Worp et al., 2005). One point was assigned for each criterion met, so the total score ranges from 0 to 5. Studies scoring 4-5 are considered to have a low methodological quality, 2-3 a moderate methodological quality, and 0-1 a high methodological quality (Macleod et al., 2004; Van Der Worp et al., 2005). Studies with a small sample size are not considered low methodological quality, even if other criteria are met, due to reduced statistical power (Zepeda Batista et al., 2018).

Study selection methodology

After searching the literature, a total of 71 articles were retrieved for this systematic review from the following electronic databases, including Google Scholar (n = 15), WOS (n = 24), Scopus (n = 25), and PubMed (n = 7). Records were combined, duplicate records (n = 30) were manually removed, and the remaining records (n = 41) were screened according to the inclusion and exclusion criteria. During initial screening of the title and abstract, fourteen (n=14) studies were removed, resulting in twenty-seven (n = 27) papers. Four articles were excluded during the full PDF screening, resulting in twenty-three articles.

Characteristics of included studies

Characteristics of the selected 23 studies are presented in Table 1. Studies included were published between 2013 and 2026. All authors published one article, except one author who published two articles, from each of the 23 studies. The studies were conducted across ten countries. A total of 40 distinct breeds and crossbreeds were investigated across the included studies in these countries. Among these, Awassi was studied in 13% (n = 3) of studies, followed by small-tailed Han sheep in 8.69% (n = 2) of studies. Sample size ranged from 50 to 632 animals, with 73.9% studies (n = 16) ranging from 50 to 192 animals. All studies investigated the *GH* gene polymorphism in relation to growth traits. The most investigated traits were birth weight (60.8%), weaning weight (56.5%), morphometric traits (52.1%), followed by body weight (43.4%), and 6-month weight (39.1%). The most frequently used genotyping method was Polymerase Chain Reaction-Restricted Fragment Length Polymorphism (PCR-RFLP), which accounted for 47.8% (n = 11) of the studies, followed by PCR combined with sequencing in 26% (n = 6) of studies, Polymerase chain reaction single strand conformation polymorphism (PCR-SSCP) in three studies (13%), PCR SSCP combined with sequencing in two studies (8.7%); however, only one study used tetra-primer amplification refractory mutation system PCR (ARMS-PCR).

Distribution of studies by country

The studies were geographically distributed across ten countries, with the highest contribution from Asia. Most of the studies were from Turkey and India (17.3% each), followed by China, Iraq, and Indonesia, each with three studies (13% each). While 8.6 % of the studies were from Russia, the fewest (4.3%) were from Brazil, Egypt, Iran, and Saudi Arabia. Figure 2 shows the distribution of articles by countries.

Distribution of studies by year

The timeline of the publications is presented in Figure 3, which indicates that the researchers' interest in this field has been growing steadily over the years; the latest studies (from 2022 to 2025) have led to the highest number of publications. Most of the articles were published in 2022 (17.4%). While the number of studies was consistent (13 % each year) in 2023 (n = 3), 2024 (n = 3), and 2025 (n = 3), followed by 2014, 2017, and 2019 with two studies each year (8.7 % each year). While one study was published in each of 2013, 2016, 2021, and 2026 (4.3% per year).

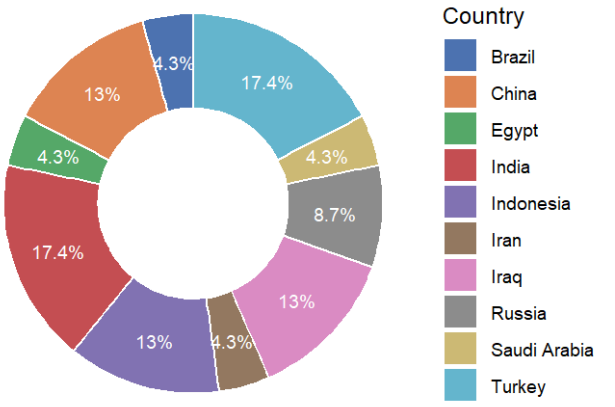


Figure 2. Geographic distribution of studies on the *GH* gene polymorphisms and growth traits in sheep.

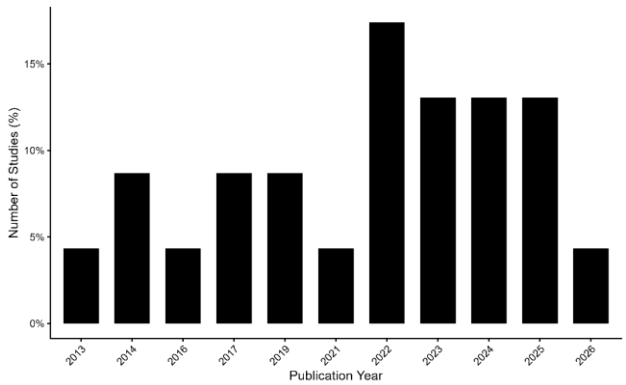


Figure 3. Distribution of studies on the *GH* gene polymorphisms and growth traits in sheep by year (2013-2026).

Distribution of studies by journal

From the findings, 19 studies were each published in one separate journal, while only four studies were published across two journals (8.7% each), as shown in Figure 4.

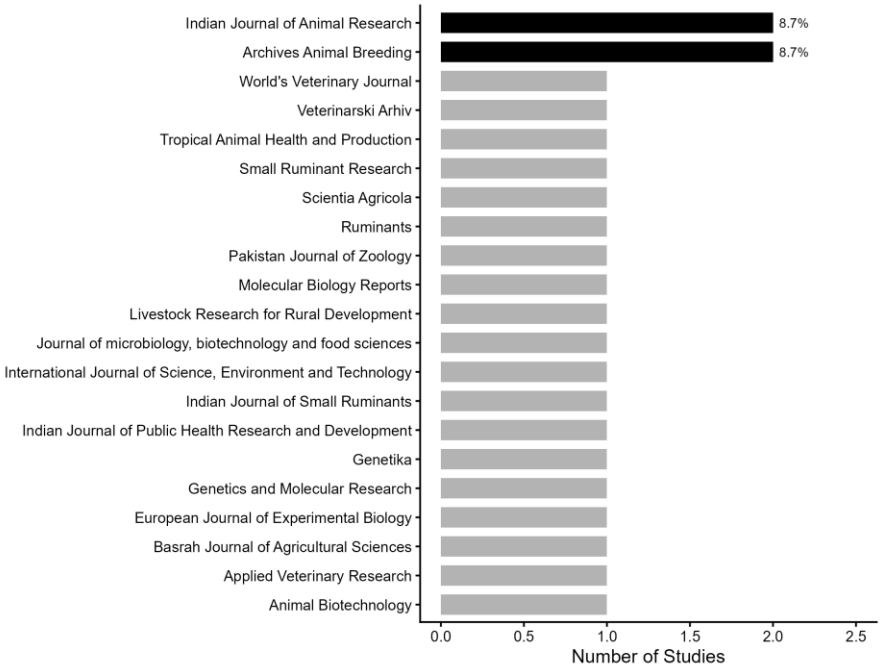


Figure 4. Journal-wise distribution of studies on the *GH* gene polymorphisms and growth traits in sheep published between 2013 and 2026.

Distribution of studies by breed

There was a high level of diversity in sheep populations in the 23 included studies. Findings revealed a total of 40 breeds across the included studies. These breeds include indigenous, commercial, and crossbreed sheep from ten countries. Some articles studied only one breed, whereas others studied more than one breed. From China, one study compared four breeds (Tibetan, Small Tail Han, German Merino, and Polled Dorset), whereas another used two breeds, Charolais and Australian White. Crossbred populations, such as the Rahmani-Barki cross, the Awassi-Suffolk cross, and the Merino cross, were also reported, suggesting that *GH* polymorphism studies were conducted in both purebred and crossbreeding systems. Moreover, certain breeds were reported in multiple studies, such as Awassi in three studies, Merino-related populations (Anatolian Merino, Karacabey Merino, Merino Cross Rams, Soviet Merino, Hampshire Down × Merino crossbreed) in five studies, and the Chinese indigenous breed in two studies. Breeds included in this review are presented in Table 1.

Table 1. Characteristics of 23 included studies on *GH* gene polymorphisms associated with growth traits in sheep

Study references (n = 23)	Breed	Sample size (N)	Year	Country	Genotyping method
Tanış and Keskin (2025)	Akkaraman, Anatolian Merino	73	2025	Turkey	PCR-RFLP
Al Qasimi et al. (2019)	Awassi	68	2019	Iraq	PCR+ sequencing
Bayraktar and Shoshin (2022)	Awassi	150	2022	Iraq	PCR-RFLP
Li et al. (2025)	Charolais, Australian White	632	2025	China	PCR + sequencing
Madikadike et al. (2024)	Dorper	50	2024	Indonesia	PCR-RFLP
Kumar et al. (2024)	Harnali	138	2024	India	PCR-RFLP
Abdelmoneim et al. (2017)	Harri	100	2017	Saudi Arabia	PCR + sequencing
Malewa (2014)	Indonesia fat-tailed	100 (genotyped =70)	2014	Indonesia	PCR-RFLP
Muniasamy et al. (2023)	Kilakarsal	99	2023	India	PCR-RFLP
Esen and Elmacı (2022)	Kıvırcık, Karacabey Merino, Ramlıç, German Black-Head Mutton × Kıvırcık, Hampshire Down × Merino crossbreed	202	2022	Turkey	PCR-SSCP + sequencing
Putra et al. (2024)	Merino Cross Rams	145	2024	Indonesia	PCR-RFLP
Bai et al. (2022)	Mongolia, Small-tailed Han, Tong, Lanzhou large-tailed, Henan large-tailed Han, Yuxi fatty-tailed	300 (50 each)	2022	China	PCR-SSCP
Dagdelen and Esenbuga (2025)	Morkaraman	123	2025	Turkey	PCR-RFLP
Rajith Reddy et al. (2023)	Nellore	50	2023	India	PCR-SSCP
Cauveri et al. (2016)	Nilagiri	60	2016	India	Tetra-primer ARMS-PCR
Skorykh et al. (2023)	North Caucasian Meat Wool, Soviet Merino	60	2023	Russia	PCR + sequencing
Al-Muhsen et al. (2019)	Nuimi, Awassi	63	2019	Iraq	PCR-RFLP
Saleh et al. (2022)	Egyptian sheep: Rahmani, Barki, Rahmani-Barki cross, Awassi-Suffolk cross Ossimi	286	2022	Egypt	PCR + sequencing
Gorlov et al. (2017)	Salsk	50	2017	Russia	PCR-RFLP
Machado et al. (2020)	Santa Ines	192	2021	Brazil	PCR + sequencing
Jia et al. (2014)	Tibetan, Small-tailed Han, German Merino, Polled Dorset	510	2014	China	PCR-SSCP + sequencing
Dagdelen (2026)	Tuj	312	2026	Turkey	PCR-RFLP
Moradian et al. (2013)	Makooei	100	2013	Iran	PCR-SSCP

PCR: Polymerase chain reaction, PCR-RFLP: Polymerase chain reaction restriction fragment length polymorphism, PCR-SSCP: Polymerase chain reaction single strand conformation polymorphism, ARMS-PCR: Amplification refractory mutation system polymerase chain reaction.

Identified single nucleotide polymorphisms and genomic regions

The SNPs and genomic regions identified are listed in Table 2. Results showed that 19 of the 23 articles reported SNPs and genomic regions, with SNPs reported in 10 studies (43.4%) and genomic regions in 20 studies (86.9%). Twenty-five distinct SNPs were identified across 23 studies, of which 18 were associated with growth traits such as BWT, BW, WW, LW6, YW, chest circumference (CC), chest depth (CD), chest width (CW), leg length (LL), hip width (HW), daily live weight gain (DLWG), body height (BH), withers height (WH), body length (BL), rump height (RH), back height, waist height and pre-weaning gain, with some influencing single traits and others influencing multiple traits. Nineteen SNPs were reported in the exonic variant region, of which 15 were in Exon 5. Of the 25 identified SNPs, 4 were in non-coding regions, and the regions for 2 SNPs were not specifically mentioned. Five SNPs, including A781G (Kilakarsal, Harnali), A1544G (Barki, Rahmani-Barki cross), C1765A (Rahmani, Awassi-Suffolk cross), T1772A (Rahmani, Ossimi), and G1769C (Rahmani, Ossimi), were repeated in two different breeds. While all other SNPs occurred only once, this indicates substantial variation in *GH* polymorphisms. As different breeds were studied, this may have contributed to variations in reported SNPs.

Table 2. Identified single nucleotide polymorphisms and corresponding genomic regions in the *GH* gene in sheep

Study References (n=23)	Breed	Sample Size (N)	SNPs	Genomic region
Tanış and Keskin (2025)	Akkaraman, Anatolian Merino	73	-	Intron 2 - 4
Al Qasimi et al. (2019)	Awassi	68	-	-
Bayraktar and Shoshin (2022)	Awassi	150	-	-
Li et al. (2025)	Charolais, Australian White	632	C408G, T364C	-
Madikadike et al. (2024)	Dorper	50	T735A	Exon 4
Kumar et al. (2024)	Harnali	138	A781G	Partial exon 2 Intron 3
Abdelmoneim et al. (2017)	Harri	100	G871A	Intron 2
			G1383A	Exon 4
			A1509G	Intron 4
Malewa et al. (2014)	Indonesia fat-tailed	100 (genotyped = 70)	-	Exon 3, Exon 4, Intron 2 - Intron 4
Muniasamy et al. (2023)	Kilakarsal	99	A781G	Exon 2
Esen and Elmacı (2022)	Kıvırcık, Karacabey Merino, Ramlıç, German Black-Head Mutton × Kıvırcık, Hampshire Down × Merino crossbreed	202	1588C > T 1603A > C 1604G > C 1606A > T 1664C > T	Exon 5
Putra et al. (2024)	Merino Cross Rams	145	55G > A	Exon 2
Bai et al. (2022)	Mongolia, Small-tailed Han, Tong, Lanzhou large-tailed, Henan large-tailed Han, Yuxi fatty-tailed	300 (50 each)	-	Exon 2
Dagdelen and Esenbuga (2025)	Morkaraman	123	-	-
Rajith Reddy et al. (2023)	Nellore	50	-	5′ regulatory region, Exon 4
Cauveri et al. (2016)	Nilagiri	60	480 G > A	Intron 1
			871 G > A	Intron 2
Skorykh et al. (2023)	North Caucasian Meat Wool, Soviet Merino	60	476G > A	Exon 5
Al-Muhsen et al. (2019)	Nuimi, Awassi	63		Exon 2
Saleh et al. (2022)	Rahmani	45	C1776G T1772A G1769C C1765A	Exon 5
	Barki	45	A1544G	
	Rahmani-Barki cross	123	A1678G A1558G A1544G	
	Awassi-Suffolk cross	38	C1765A G1550A	
	Ossimi	35	T1772A G1769C G1756C	
Gorlov et al. (2017)	Salsk	50	-	Exon 3
Machado et al. (2020)	Santa Ines	192	-	Introns 2-4, Exons 3-5
Jia et al. (2014)	Tibetan, Small-tailed Han, German Merino, Polled Dorset	510	-	5′ regulatory region, Exon 4, 3′ untranslated region
Dagdelen (2026)	Tuj	312	-	Exon 5
Moradian et al. (2013)	Makooei	100	-	Exon 4

SNPs: Single nucleotide polymorphisms, -: Not mentioned

Genotype and allele frequencies

Genotype frequencies were reported in 18/23 studies (78.2 %), whereas five studies did not report them. Of the included studies, five studies reported three genotypes per SNP, three studies reported two genotypes per SNP, and one reported one genotype per SNP. The most common genotypic pattern observed was AA, AB, and BB. Moreover, in 18 out of 23 studies (78.2%), allele frequencies were reported, and in 5 studies (21.7%), no allele frequency data were reported. In the included studies, reported allele frequencies range from 0.015 to 1.000. Genotype and allele frequencies of all the reviewed articles are presented in Table 3.

Table 3. Reported genotype and allele frequencies of *GH* gene polymorphisms in sheep

Study references (n = 18)	Breed	SNPs	Genotype frequency[a]	Allele frequency[a]
Tanış and Keskin (2025)	Akkaraman, Anatolian Merino	-	AA = 0.542, AB = 0.429, BB = 0.048; AA = 0.192, AB = 0.426, BB = 0.346	A = 0.738, B = 0.262; A = 0.423, B = 0.577
Bayraktar and Shoshin (2022)	Awassi	-	AA = 0.60, AB = 0.20, BB = 0.20	A = 0.70, B = 0.30
Li et al. (2025)	Charolais, Australian White	C408G	CC=0.01, CG=0.95, GG=0.04 CC=0.00, CG=0.93, GG=0.07	C=0.49, G=0.51 C=0.47, G=0.53
		T364C	TT=0.01, CT=0.95, CC=0.04 TT=0.00, CT=0.90, CC=0.10	T=0.49, C=0.51 T=0.45, C=0.55
Madikadike et al. (2024)	Dorper	T735A	AA = 0.70, AB = 0.30	A = 0.85, B = 0.15
Kumar et al. (2024)	Harnali	A781G	AA = 0.62, AB = 0.38	A = 0.81, B = 0.19
Abdelmoneim et al. (2017)	Harri	G871A	GG = 0.30, GA = 0.22, AA = 0.48	G = 0.41, A = 0.59
		G1383A	GG = 0.18, GA = 0.26, AA = 0.56	G = 0.31, A = 0.69
		A1509G	AA = 0.20, AG = 0.30, GG = 0.50	A = 0.35, G = 0.65
Malewa et al. (2014)	Indonesian fat-tailed: Donggala	-	AA=0.357, AB=0.357, BB=0.286	A=0.536, B=0.464
	Indonesian fat-tailed: East Java		AA=0.464, AB=0.250, BB=0.286	A=0.589, B=0.411
Muniasamy et al. (2023)	Kilakarsal	A781G	AA = 0.29; AB = 0.71	A = 0.65; B = 0.35
Putra et al. (2024)	Merino Cross Rams	55G > A	GG = 0.70; GA = 0.30	G = 0.85, A = 0.15
Bai et al. (2022)	Mongolia	-	AA=0.313, AB=0.375, BB=0.312	A= 0.500; B =0.500
	Small-tailed Han		AA=0.281, AB=0.531, BB=0.188	A= 0.547; B =0.453
	Tong		AA=0.364, AB=0.545, BB=0.091	A= 0.636; B =0.364
	Lanzhou large-tailed		AA=0.311, AB=0.311, BB=0.378	A= 0.525; B =0.475
	Henan large-tailed Han		AA=0.350, AB=0.350, BB=0.300	A= 0.511; B =0.489
	Yuxi fatty-tailed		AA=0.213, AB=0.596, BB=0.191	A= 0.467; B =0.533
Dagdelen and Esenbuga (2025)	Morkaraman	-	AA = 0.504; AB = 0.496	A = 0.752; B = 0.248
Rajith Reddy et al. (2023)	Nellore	-	AA = 0.24, AB = 0.42, BB = 0.34 AA = 0.60, AB = 0.40	A = 0.45, B = 0.55 A = 0.80, B = 0.20
Cauveri et al. (2016)	Nilagiri	480 G > A	GG=0.48, GA=0.43, AA=0.09	G=0.70, A=0.30
		871 G > A	AA=1.00	A=1.00
Skorykh et al. (2023)	North Caucasian Meat Wool	476G > A	CC=53.3%, CT=30.0%, TT=16.7%	C=0.68, T=0.32
	Soviet Merino		CC=53.3%, CT=33.3%, TT=13.4%	C=0.70, T=0.30
Gorlov et al. (2017)	Salsk	-	AA = 57%, AB = 36%, BB = 7%	A ≈ 0.75, B ≈ 0.25
Jia et al. (2014)	Tibetan	-	AA = 0.8175, AB = 0.1111, BB = 0.0714; AA = 0.0872, AB = 0.2461, BB = 0.6667; AA = 0.4286, AB = 0.3095, BB = 0.1032, and AC = 0.1578	A = 0.8532, B = 0.1468; A = 0.4206, B = 0.5794; A = 0.6623, B = 0.2589, C = 0.0788
	Small Tail Han		AA = 0.6071, AB = 0.2858, BB = 0.1071; AA = 0.1429, AB = 0.2500, BB = 0.607; AA = 0.5357, AB = 0.1429, BB = 0.1786, AC = 0.1428	A = 0.6607, B = 0.3393; A = 0.4464, B = 0.5536; A = 0.6786, B = 0.2501, C = 0.0713
	German Merino		AA= 1.000; AA = 0.6111, AB = 0.1667, BB = 0.2222; AA = 0.2222, AB = 0.6111, BB = 0.0000, AC = 0.1667	A = 1.0000; A = 0.7222, B = 0.2778; A = 0.6111, B = 0.3056, C = 0.0833
	Polled Dorset		AA= 1.000; AA = 0.5263, AB = 0.2105, BB = 0.2632; AA = 0.5000, AB = 0.2895, BB = 0.0000, AC = 0.2105	A = 1.0000; A = 0.6579, B = 0.3421; A = 0.6053, B = 0.1447, C = 0.2500
Dagdelen (2026)	Tuj	-	LL = 49.0%, LV = 39.0%, VV = 11.0%	L=0.685, V=0.315
Moradian et al. (2013)	Makooei	-	AA=0.313, AB=0.646, BB=0.01, CC=0.01, CD=0.021	A=0.63, B=0.327, C=0.028, D=0.015

[a] Genotype and allele frequencies are presented as proportions, SNPs: Single nucleotide polymorphisms, -: Not mentioned

Association of *GH* gene polymorphisms with growth traits

Of the included studies, 20 (86.9%) indicated significant associations in one or more traits of interest, while three indicated non-significant associations. Significant and non-significant associations are based on statistical analysis and p-values reported in the original studies. Traits are divided into three standard livestock trait categories, including growth traits (birth weight, weaning weight, 6-month weight, 9-month weight, 12-month / Yearling weight, Body weight at 100,120 and 240 days), growth performance traits (average daily gain, daily live weight gain, Pre and Post-weaning average daily gain, growth rate, Kleiber ratio-feed utilization efficiency of animal compared to its body size), and morphometric traits (withers height, rump height, croup height, body height, back height, sacral height, body length, limb length, leg length, neck length, rump length, chest circumference / thoracic girth, heart girth, paunch girth, cannon bone circumference, abdomen circumference, chest width, thoracic width, croup width, hip width, chest depth, body depth, head width, leg girth, foreleg height). Statistically significant and non-significant findings reported across all studies based on statistical analysis and p-value used in that study are presented in Table 4.

Methodological quality assessment

Table 5 presents the methodological quality assessment for the included studies. Of 23 studies, four were considered of low methodological quality, 17 moderate, and two of high. The predominance of moderate quality studies was primarily due to small or medium sample sizes, despite clear reporting of the genotyping method and statistical analysis. Only a limited number of studies were of low methodological quality because of large sample sizes and comprehensive reporting of genotype methods, genotype frequencies, allele frequencies, and reported associations of *GH* gene polymorphisms with studied growth traits.

Table 4. Association of identified *GH* gene polymorphisms with growth related traits in sheep

Study references (n=23)	Breed	SNPs	Genomic region	Genotypes	Traits	Association
Tanış and Keskin (2025)	Akkaraman, Anatolian Merino	-	Intron 2 - 4	AA, AB, BB	WW, WH, RH, BL, CW, CC	S
					BW, LW6, ADG, KR	NS
Al Qasimi et al. (2019)	Awassi	-	-	CT, TC	BW	S
					WW, LW6, body measurements, growth rate	NS
Bayraktar and Shoshin (2022)	Awassi	-	-	AA, AB, BB	BWT	S
					BL, CD	NS
Li et al. (2025)	Charolais, Australian White	C408G T364C	-	CC, CG, GG TT, CT, CC	BWT, BH, back height, waist height, CC, CD, CW, LML, HW	S
					SH, BL, LL, FW	NS
Madikadike et al. (2024)	Dorper	T735A	Exon 4	AA, AB	WH	S
					BWT, BL, HG, STH, RH	NS
Kumar et al. (2024)	Harnali	A781G	Partial exon 2, Intron 3	AA, AB	BW, WW, LW6, YW, BL, BH, HG, PG	NS
Abdelmoneim et al. (2017)	Harri	G871A	Intron 2	GG, GA, AA	BW, BWT120d, DLWG	S
		G1383A	Exon 4			
		A1509G	Intron 4			
Malewa et al. (2014)	Indonesia fat-tailed	-	Exon 3, Exon 4, Intron 2 - Intron 4	AA, AB, BB	Growth rate, WW	S
					BW	NS
Muniasamy et al. (2023)	Kilakarsal	A781G	Exon 2	AA, AB	YW	S
					BW, WW, LW6, LW9	NS
Esen and Elmacı (2022)	Kıvırcık, Karacabey Merino, Ramlıç, German Black-Head Mutton × Kıvırcık, Hampshire Down × Merino crossbreed	1588C>T 1603A>C 1604G>C 1606A>T 1664C>T	Exon 5	-	BL, RH, CD	S
					BWT, WH, CW, LC	NS

Putra et al. (2024)	Merino Cross Rams	55G > A	Exon 2	GG, GA	BW, CW	S
					BWT120d, BWT365d, Pre-ADG, Post-ADG, HL, HW, WH, BL, CG, RL, RW, CW, leg traits	NS
Bai et al. (2022)	Mongolia, Small-tailed Han, Tong, Lanzhou large-tailed, Henan large-tailed Han, Yuxi fatty-tailed	-	Exon 2	AA, AB, BB	BWT, CW, HH	S
					BH, BL, RH, FH, HL, CC, CBC, RL, NL, HW, CD, HD, HC, AC, waist width, back height	NS
Dagdelen and Esenbuga (2025)	Morkaraman	-	-	AA, AB	WW, ADG	S
					BW	NS
Rajith Reddy et al. (2023)	Nellore	-	5′ regulatory region	AA, AB, BB	BWT, BL, WH, HG	NS
			Exon 4	AA, AB		
Cauveri et al. (2016)	Nilagiri	480 G>A 871 G>A	Intron 1 Intron 2	GG, GA, AA	WW, pre-ADG	S
					BW, LW6, LW9, YW, post-ADG	NS
Skorykh et al. (2023)	North Caucasian meat-wool Soviet Merino	476G>A	Exon 5	CC, CT, TT	WW, growth rate (birth-weaning)	S
					BW: Soviet Merino	S
					BW: North Caucasian	NS
Al-Muhsen et al. (2019)	Nuimi and Awassi	-	Exon 2	AA, Aa, aa	BW, WW	S
Saleh et al. (2022)	Rahmani	C1776G T1772A G1769C C1765A	Exon 5	-	BW, WW, LW6	S
	Barki	A1544G				
	Rahmani-Barki cross	A1678G A1558G A1544G				
	Awassi-Suffolk cross	C1765A G1550A				
	Ossimi	T1772A G1769C G1756C				
Gorlov et al. (2017)	Salsk	-	Exon 3	AA, AB, BB	WW, LW9, ADG	S
					BW	NS
Machado et al. (2020)	Santa Ines	-	Introns 2-4, exons 3-5	-	BWT100d, BWT240d, ADG, WH, CH, BL, TG, LG, TW, CW, BD	NS
Jia et al. (2014)	Tibetan, Small Tail Han, German Merino, Polled Dorset	-	5′ regulatory region, Exon 4	AA, AB, BB	BWT, BL, HG, WH	S
			3′ UTR	AA, AB, BB, AC		
Dagdelen (2026)	Tuj	-	Exon 5	LL, LV, VV	WW, ADG	S
					BW	NS
Moradian et al. (2013)	Makooei	-	Exon 4	AA, AB, BB, CC, CD	WW, LW6, LW9	S
					BW, YW	NS

3′ UTR: 3′ untranslated region, ADG: Average daily gain, ADWG: Average daily weight gain, BWT: Body weight, BWT100: Body weight at 100 days, BWT120: Body weight at 120 days, BWT240: Body weight at 240 days, BWT365: Body weight at 365 days, BW: Birth weight, DLWG: Daily live weight gain, KR: Kleiber ratio, LW6: 6-month weight, LW9: 9-month weight, Pre-ADG: Pre-weaning average daily gain, Post-ADG: Post-weaning average daily gain, WW: Weaning weight, YW: Yearling weight, AC: Abdomen circumference, BD: Body depth, BH: Body height, BL: Body length, CBC: Cannon bone circumference, CC: Chest circumference, CD: Chest depth, CH: Croup height, CG: Chest girth, CW: Chest width, FH: Foreleg height, FW: Frontal width, HC: Hip circumference, HG: Heart girth, HH: Hip height, HL: Head length, HD: Head depth, HW: Hip width, LC: Leg circumference, LG: Leg girth, LL: Leg length, LML: Limb length, NL: Neck length, PG: Paunch girth, RH: Rump height, RL: Rump length, STH: Sternum height, SH: Sacral height, TG: Thoracic girth, TW: Thoracic width, WH: Withers height, S: Significant, NS: Non-significant.

Table 5. Methodological quality assessment of included studies (n = 23) evaluating *GH* gene polymorphisms and growth traits in sheep

Study references (n=23)	Sample size (N)	Genotyping method	Genotyping frequencies	Allele frequencies	Statistics	Methodological quality level
Tanış and Keskin (2025)	Small	PCR-RFLP	Yes	Yes	Yes	Moderate
Al Qasimi et al. (2019)	Small	PCR+ sequencing	No	No	Yes	High
Bayraktar and Shoshin (2022)	Medium	PCR-RFLP	Yes	Yes	Yes	Moderate
Li et al. (2025)	Large	PCR + sequencing	Yes	Yes	Yes	Low
Madikadike et al. (2024)	Small	PCR-RFLP	Yes	Yes	Yes	Moderate
Kumar et al. (2024)	Medium	PCR-RFLP	Yes	Yes	Yes	Moderate
Abdelmoneim et al. (2017)	Small	PCR + sequencing	Yes	Yes	Yes	Low
Malewa (2014)	Small	PCR-RFLP	Yes	Yes	Yes	Moderate
Muniasamy et al. (2023)	Small	PCR-RFLP	Yes	Yes	Yes	Moderate
Esen and Elmacı (2022)	Medium	PCR-SSCP + sequencing	No	No	Yes	Moderate
Putra et al. (2024)	Medium	PCR-RFLP	Yes	Yes	Yes	Moderate
Bai et al. (2022)	Low	PCR-SSCP	Yes	Yes	Yes	Moderate
Dagdelen and Esenbuga (2025)	Medium	PCR-RFLP	Yes	Yes	Yes	Moderate
Rajith Reddy et al. (2023)	Small	PCR-SSCP	Yes	Yes	Yes	Moderate
Cauveri et al. (2016)	Small	Tetra-primer ARMS-PCR	Yes	Yes	Yes	Moderate
Skorykh et al. (2023)	Small	PCR + sequencing	Yes	Yes	Yes	Moderate
Al-Muhsen et al. (2019)	Small	PCR-RFLP	No	No	Yes	High
Saleh et al. (2022)	Medium	PCR+ sequencing	No	No	Yes	Moderate
Gorlov et al. (2017)	Small	PCR-RFLP	Yes	Yes	Yes	Moderate
Machado et al. (2020)	Medium	PCR + sequencing	No	No	Yes	Moderate
Jia et al. (2014)	Large	PCR-SSCP + sequencing	Yes	Yes	Yes	Low
Dagdelen (2026)	Large	PCR-RFLP	Yes	Yes	Yes	Low
Moradian et al. (2013) Moradian et al. (2013)	Medium	PCR-SSCP	Yes	Yes	Yes	Moderate

PCR-RFLP: Polymerase chain reaction restriction fragment length polymorphism, PCR-SSCP: Polymerase chain reaction single strand conformation polymorphism.

DISCUSSION

This systematic review synthesized evidence from 23 studies, identified 25 SNPs, of which 18 were associated with different growth rates, investigating the polymorphism of the *GH* gene in sheep and their association with growth traits.

The compiled Table 4 indicated that *GH* variation was not confined to one location; it was distributed across exonic regions (exons 2, 3, 4, 5), intronic regions (introns 1, 2, 3), the 5′ regulatory region, and the 3′ untranslated region (3′ UTR). One–notable result of this review is the abundance of SNPs in exon 5, which explained most of the observed polymorphisms. This concentration indicates that exon 5 is a highly variable segment of the *GH* gene across sheep populations. Exon 5 is a coding region essential to the GH protein and, therefore, mutations in this region can cause amino acid replacement, which can have effects on protein structure and function. The presence of numerous SNPs in exon 5 might be the reason why several studies have found a relationship between *GH* polymorphisms and growth traits (Esen and Elmacı, 2022; Saleh et al., 2022; Skorykh et al., 2023; Dagdelen, 2026). Since variations in coding regions have more functional implications than those in non-coding regions, they influence phenotypic expression. For example, the study on Turkish meat-type lambs identified several variants in exon 5. It also identified stage-specific variations in body length (BL), rump height (RH), and chest depth (CD). Esen and Elmacı (2022) found a combined SNP effect (1588C > T, 1603A > C, 1604G > C, 1606A > T, and 1664C > T) in exon 5 and discovered breed, growth, and stage-specific correlation with BL, RH, and CD.

The association findings were evidently diverse among breeds, traits, and genomic regions. Some of the studies have reported GH-growth variations in the exon 2 region, such as the study on Nuimi and Awassi sheep by Al-Muhsen et al. (2019), which showed a statistically significant association of GH genotype with BW and WW, in both breeds. Another study on six sheep breeds from China reported an association with BWT, chest width (CW), and hip height (HH; Bai et al., 2022). Similarly, a missense SNP, 55G > A, was reported in merino cross rams by Putra et al. (2024), and it was

associated with BW and CW, with no effect on other body traits, indicating a trait-specific effect. Moreover, polymorphism at Exon 3 was also associated with growth rate in sheep. For instance, Gorlov et al. (2017) identified GH variation in Exon 3, which showed association with WW, ADG, and LW9 in Salsk sheep. Similarly, Malewa et al. (2014) identified a polymorphism in exon 3 associated with growth rate and weaning weight in Indonesian fat-tailed sheep. While variation at the exon 4 region in the *GH* gene was associated with important traits, WW, LW9, and LW6 in Makooei sheep (Moradian et al., 2013). Association of genomic regions with various growth traits in these studies showed the importance of a specific functional region with economically important traits, but these effects are breed and trait specific.

In Nilagiri sheep, of the two identified SNPs, only 480 G > A was reported to be associated with WW and pre-weaning average daily gain (pre-ADG), suggesting an effect on early sheep growth (Cauveri et al., 2016). Similarly, a study on Awassi lambs by Al Qasimi et al. (2019) revealed an effect on BW but no effect on WW, LW6, and other body measurements was observed. Also, in Dorper sheep, SNP T735A was associated with wither height (WH) only, suggesting a limited, trait-specific influence (Madikadike et al., 2024). Polymorphism in a study on Tuj sheep by Dagdelen (2026) showed a statistically significant association of GH genotype with WW and DLWG, but no association with BW or placental traits. Similarly, in Morkaraman sheep, *GH* gene polymorphism has a trait-specific effect, with no association reported with BW, whereas WW and DLWG were influenced by genotype (Dagdelen and Esenbuga, 2025). These variations in the association of SNPs with growth traits showed that association can be trait-specific if one trait is influenced by a specific SNP; other traits may show no association in the same breed.

Moreover, breed specificity is a consistent feature in the literature. Allele frequencies across breeds and genotype frequencies in the 5′ regulatory region, exon 4, and 3′ UTR in the multi-breed study of Tibetan, Small Tail Han, German Merino, and Polled Dorset sheep also supported the notion that *GH* variation is not homogeneous across populations (Jia et al., 2014). Similarly, a SNP 476G > A was reported by Skorykh et al. (2023) to be associated with WW in North Caucasian meat-wool and Soviet Merino. However, BW was significant only in Soviet Merino, indicating a breed-specific effect. According to Li et al. (2025), two SNPs, C408G and T364C, were associated with BWT, CW, BH, CC, CD, LL, HW, back height, and waist height, in Charolais and Australian White sheep breeds; however, the effects were population- and genotype-specific.

Multiple SNPs reported in Egyptian sheep, while three SNPs, A1678G, A1558G, and A1544G, were associated with BW, WW, and LW6 in the crossbred population; however, A1544G was also reported in Barki sheep, but was not associated with growth rate, which showed the effect might be breed-specific or due to the combination of the effects of SNPs (Saleh et al., 2022). One SNP, A781G, reported in two studies, was not associated with any trait in the Harnali breed (Kumar et al., 2024) but was significantly associated with yearling weight in the Kilakarsal breed (Muniasamy et al., 2023), demonstrating breed-specific SNP effects. According to Abdelmoneim et al. (2017), three SNPs, G871A, G1383A, and A1509G, were associated with BW, BWT120d, and DLWG in Harri sheep, indicating that specific SNPs on the *GH* gene have consistent effects in some populations.

Some studies did not report a significant association with growth traits in sheep, such as Machado et al. (2020), who did not find any significant associations following Bonferroni correction in the Santa Inês sheep. Similarly, Rajith Reddy et al. (2023) did not report any statistically significant association of *GH* gene polymorphism with BWT, BL, Withers height (WH), and heart girth (HG) in the Nellore breed, which may show that it does not have an effect in this breed, or maybe linked to the small sample size, which reduces statistical power to detect true effects. Also, in Harnail sheep, no association was observed with growth traits, but the effect on other traits (annual greasy fleece weight) indicates that the effect may be trait-specific (Kumar et al., 2024).

The contribution of the systematic review is to demonstrate an association between the *GH* gene and growth traits in sheep. Based on knowledge, there was a lack of systematic synthesis of the association between the *GH* gene and growth traits in sheep, so this systematic review was conducted. This review suggests that *GH* gene polymorphisms can be useful in MAS programs to enhance sheep productivity. Nevertheless, the inconsistency of SNPs between breeds and the inconsistency in association outcomes imply that *GH* markers must be used with caution. SNPs found in one population are unlikely to affect another population in the same way, due to the interaction between genetics and environment.

CONCLUSION

GH gene polymorphism affects sheep growth traits. However, there are some of the limitations found in the studies in which some studies did not specifically mentioned SNPs, incomplete reporting of allele and genotype frequencies in some studies, identified SNPs were not similar which make it impossible to do meta-analysis, smaller sample sizes in a fraction of the studies (less than 200 animals in 73.9% of studies), heterogeneity in breeds, environments and methodologies. These limitations may have contributed to inconsistent results and hindered a direct comparison across

studies. In 20 of 23 studies, several identified, C1776G, A1544G, A1678G, A1558G, C1765A, G1550A, T1772A, G1769C, G1756C, 476G > A, 480 G > A, 55G > A, G871A, G1383A, A1509G, A781G, C408G and T735A, and unidentified SNPs were reported to be associated with growth traits in sheep. A small number of SNPs were repeated across breeds, but no single SNP was shared across all studies. Findings suggest that the *GH* polymorphism is often significantly associated with growth traits, and effects are highly dependent on breed, trait, and genomic region. Although findings from 23 studies included in this review suggest an association of SNPs of the *GH* gene with growth traits in sheep, practical application in breeding programs requires validation in larger populations, across breeds, and for specific traits. Future studies are needed, with a greater focus on other traits such as reproductive performance.

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Authors' contributions

Khadija Salka wrote the original manuscript. Suyadi Suyadi performed the study conceptualization, methodology development, supervision, and assessment of the manuscript. Sohaib Akram conducted data curation and investigation. Veronica Margareta Ani Nurgiartiningsih made a conceptual contribution that enriched the study. Asrullah, Irida Novianti and Marjuki evaluated and reviewed the manuscript. All authors read and approved the final edition of the manuscript.

Availability of data and materials

The data collected or analyzed during this study are all included in this article.

Competing interests

The authors have not declared any conflict of interest.

Ethical considerations

The authors declare that this manuscript is original and has not been submitted to any other journal for consideration or publication, and have checked the ethical issues, including consent to publish, misconduct, data fabrication, and redundancy. The authors confirmed that no AI tools were used in preparing this study.

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Use of Antibiotic Alternatives in Nepal: A Strategy for Sustainable Animal Nutrition

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ABSTRACT

The routine use of antibiotics in animal nutrition has historically improved growth and productivity. However, the continued and often indiscriminate use of these compounds has accelerated the emergence of antimicrobial resistance (AMR), posing serious risks to animal health, food safety, and public health. Nearly three-quarters of antimicrobials are consumed by livestock, allowing resistant pathogens to cross animal, human, and environmental boundaries and undermining the concept of One Health. The present study aimed to assess sustainable feed-based alternatives to antibiotics and their effectiveness in enhancing animal health in the Nepalese context. Based on global evidence and national experiences, the review synthesizes the roles of probiotics, prebiotics, phytobiotics, organic acids, enzymes, precision feeding, and complementary management practices in maintaining animal performance while reducing reliance on antibiotics. Nepal presents both urgency and opportunity for this transition, as livestock production remains largely smallholder-based, regulatory enforcement is uneven, and antimicrobial residues continue to be detected in animal-derived foods. At the same time, increasing awareness, ethno-veterinary knowledge, emerging feed innovations, and recent policy commitments under the national AMR action framework signal positive momentum. The review suggests that replacing antibiotics with alternatives should not be viewed as a simple substitution but rather as a system-level transformation integrating nutrition, biosecurity, vaccination, farmer capacity building, and governance within a One Health framework. Strengthening locally adaptable, cost-effective solutions through field-based research and extension will be critical for sustaining productivity while contributing to national and global efforts to mitigate AMR.

Keywords: Antimicrobial resistance, Feed additive, Sustainable livestock production, One Health

INTRODUCTION

The use of antibiotics in animal agriculture, particularly as growth promoters, has played a central role in enhancing livestock health and productivity. Antibiotic growth promoters enhance feed efficiency and weight gain by inhibiting pathogenic bacteria and promoting intestinal health (Dibner and Richards, 2005; Adegbeye et al., 2020). Globally, almost three-quarters of antimicrobials are used in farm animals, often in non-therapeutic contexts such as prophylaxis and growth promotion (Mulchandani et al., 2023). While these practices transformed modern production systems, they have also accelerated the development of antimicrobial resistance (AMR; Enshaie et al., 2025). Excessive and inappropriate antimicrobial use in animal production systems has accelerated the emergence of antimicrobial-resistant bacteria, increasing the risk of transmission of resistant pathogens and resistance genes across animal, human, and environmental interfaces (Zhang et al., 2024). Projections indicate that, without effective intervention, the annual mortality could rise to nearly 10 million by 2050 (WHO, 2021). Resistance to One Health is further threatened by the spread of pathogens from animals to humans through food, direct contact, and environmental contamination, as exemplified by plasmid-mediated colistin resistance. The *mobilized colistin resistance* gene (*MCR-1*) was first detected in livestock and subsequently reported in humans (Liu et al., 2016). In response to the growing risks of antimicrobial resistance, the European Union implemented a major policy reform in 2006 that prohibited the use of antibiotics as growth promoters in animal production, which marked a significant transition in modern livestock management practices (Attia et al., 2015). Sweden's antibiotic-free system, established after 1985, demonstrated that high productivity can be achieved without routine antibiotic use (Grundin et al., 2020).

Globally, consumer demand for antibiotic-free products, including meat, milk, and eggs, has been increasing rapidly, prompting producers to adopt safer production systems (Hussain et al., 2025). Similarly, in Nepal, increasing concerns regarding antimicrobial resistance, rising consumer awareness about food safety, and the growing emphasis on

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sustainable livestock production have intensified the need for strategies that reduce antimicrobial dependence in animal production. Recent reports indicate a gradual decline in antimicrobial use in food-producing animals, reflecting increasing awareness and stewardship efforts toward prudent antimicrobial use and sustainable livestock management (Upadhyaya et al., 2023). Agriculture contributes approximately 25% to Nepal's national Gross Domestic Product (GDP) and remains a major source of employment and livelihood. Within the agricultural sector, livestock contributes about 25% to the Agricultural GDP (AGDP) and nearly 6% to the national GDP, while the poultry sector alone contributes around 4% to the national GDP and approximately 8% to the AGDP, highlighting its growing economic importance in Nepal (MOF, 2023; MOALD, 2024). The livestock sector contributes significantly to household income, food security, and rural livelihoods, with dairy production, largely dominated by cattle and buffalo, playing a major role in the subsector (FAO, 2023a; MOALD, 2024). Ensuring sustainable and antibiotic-free livestock production systems is not only a public health necessity but also an important economic priority for Nepal (Upadhyaya et al., 2023; WOAH, 2023).

Several studies have reported that probiotics, phytogenic feed additives, organic acids, emulsifiers, neutral feed additives, and exogenous enzymes are promising alternatives to antibiotic growth promoters for improving gut health and production performance in poultry (Vahdatpour and Babazadeh, 2016; Siyal et al., 2017). Such alternatives support gut health, immunity, and performance without contributing to AMR (Attia, 2018; Adegbeye et al., 2020; Zhu et al., 2022). Improvements in intestinal morphology and growth have been well documented in piglets supplemented with *Lactobacillus* spp., highlighting their potential in the antibiotic-free era (Yi et al., 2018; Wang et al., 2019). Alternative nutritional and management strategies should be viewed as part of a broader approach to sustainable and resilient livestock production rather than simply as replacements for antibiotics. Combined with improved biosecurity, vaccination, and precision nutrition, these approaches can help protect animal health, ensure food safety, and reduce antimicrobial resistance (Van Boeckel et al., 2019). For Nepal, adopting these strategies offers a unique opportunity to align livestock development with One Health principles while meeting domestic food security needs and international standards for safe animal products. Therefore, the current review aimed to synthesize current knowledge on antibiotic alternatives in livestock production, with emphasis on their mechanisms, efficacy, and practical applicability within Nepal as a low- and middle-income context.

METHODOLOGY

A narrative synthesis approach was employed to collate and critically interpret published evidence on antibiotic alternatives in livestock production. Relevant literature was retrieved through systematic searches of Scopus, Web of Science, PubMed, ScienceDirect and Google Scholar, including peer-reviewed research articles, review papers, meta-analyses and policy documents published primarily between 2005 and 2025. Search strategies combined terms related to antibiotic alternatives, antimicrobial resistance, livestock nutrition, probiotics, phytogenic, precision nutrition, biosecurity, and One Health, alongside Nepal-focused keywords. Studies were included if non-antibiotic interventions in food-producing animals and reported outcomes are associated with animal health, growth performance, immune function, or reductions in antimicrobial usage. In addition, policy and surveillance documents from WHO, FAO, WOAH, Codex Alimentarius, and relevant national authorities were consulted to provide regulatory and contextual perspectives. The selected literature was examined critically and synthesized thematically, with emphasis on biological plausibility, field-level feasibility, and consistency with One Health principles.

Alternatives to antibiotics

Farmers are now shifting from irrational antibiotic use to safer alternatives in livestock and poultry production due to rising concerns about AMR, regulatory restrictions, and consumer demand for safe products (Tang et al., 2017; Lambo et al., 2021; Leistikow et al., 2022; Hussain et al., 2025). At the same time, increasing consumer preference for antibiotic-free animal products has created market incentives and premium value for safer production systems (Hussain et al., 2025). Antibiotic alternatives have shown potential to improve growth, health, and productivity, as well as reduce AMR risks (Wang et al., 2024). Natural feed additives, such as phytobiotics, essential oils, and medicinal plants, are widely recognized for their antimicrobial, antioxidant, and digestive benefits (Lillehoj et al., 2018). Compounds such as eucalyptol and eugenol (Brenes and Roura, 2010), ginger extracts, tannins, and turmeric (Abd El-Hack et al., 2022), and moringa-derived bioactive compounds, including moringin (Khan et al., 2021), have demonstrated efficacy in enhancing gut health, nutrient absorption, immunity, and resilience, particularly in antibiotic-free production systems. Probiotics and prebiotics represent another robust category; probiotics improve intestinal morphology and secrete antimicrobial metabolites, such as bacteriocins, while prebiotics selectively stimulate beneficial microbes and enhance nutrient utilization (Pourabedin and Zhao, 2015; Solís-Cruz et al., 2020). Spore-forming

probiotics, including specific *Bacillus* strains, are remarkably resilient, helping to stabilize the gut microbiota and improve performance (Grant et al., 2018).

In addition to botanical and microbial alternatives, short- and medium-chain fatty acids are known to suppress harmful bacteria, such as *Escherichia coli* and Salmonella (Van Immerseel et al., 2006). Organic acids also improve carcass characteristics and overall meat quality, making them valuable components of antibiotic-free feeding strategies (Szabó et al., 2023). Insect meal has emerged as an innovative protein source with immunostimulatory benefits, reducing dependency on antibiotics. Similarly, bacteriophages that selectively target bacteria offer precise control of infections without disrupting beneficial microbiota, while antimicrobial peptides (AMPs), as components of innate immunity, provide broad-spectrum bactericidal activity with a low risk of resistance development (Kumar et al., 2020; Łusiak-Szelachowska et al., 2022). Emerging technologies, including nanotechnology and immunomodulators, further expand the toolbox of sustainable alternatives by enhancing nutrient delivery and strengthening host defenses (Adegbeye et al., 2020). In addition, natural plant-derived phytogenic feed additives have been extensively investigated and shown to support health, performance, and immunity while reducing antibiotic dependence in livestock production (Wang et al., 2024).

Table 1. Commonly used alternatives to antibiotics in livestock and poultry production systems in Nepal

Alternatives to antibiotics	Examples	References
Ethnoveterinary remedies (traditional plant-based)	Turmeric (*Curcuma longa*), garlic (*Allium sativum*), ginger (*Zingiber officinale*), neem (*Azadirachta indica*), tulsi (*Ocimum* spp.)	Uprety et al. (2022)
Herbal growth promoters / phytogenic herbs (on-farm and premixes)	Tulsi, amla (*Phyllanthus emblica*), ashwagandha (*Withania somnifera*)	Sanjyal and Sapkota (2011)
Probiotics (bacterial/yeast preparations)	*Lactobacillus* spp., *Bacillus* spp., yeast products (commercial probiotic premixes/water supplements)	Gupta et al. (2023)
Synbiotics (probiotic plus prebiotic blends)	Synbiotic premixes in feed/water (routine or targeted phases)	Acharya et al. (2024)
Organic acids/acidifiers	Citric acid, sorbic acid, formic/propionic blends; feed/water acidifiers (commercial)	Jha (2019)
Biosecurity plus hygiene (management alternative replacing "routine prophylaxis")	Footbaths, controlled entry, litter management, water sanitation, rodent control, downtime/all-in-all-out (where feasible)	Poudel et al. (2024)
Vaccination as an AMU-reduction strategy (prevention-first)	ND vaccination (poultry); routine livestock vaccines (FMD/PPR programs in ruminants)	Shahi et al. (2023)
Training/stewardship interventions (behavioral alternative)	Farmer awareness, prudent-use guidance, and reduced preventive antibiotic use	Shahi et al. (2023)

AMU: Antimicrobial use, ND: Newcastle disease, FMD: Foot and mouth disease, PPR: Peste des petits ruminants.

Alternatives to antibiotics in Nepal's livestock farming

Livestock and poultry are central to Nepal's agriculture, engaging nearly two-thirds of the population and ensuring food security (Bagale et al., 2022; Upadhyaya et al., 2023). However, antibiotic use is widespread and poorly regulated, with about 90% of poultry farms in the Kathmandu Valley administering critically important drugs, often without veterinary oversight (Koirala et al., 2021; Subedi et al., 2023). Awareness of antibiotic resistance is limited, as only one-third of farmers report understanding AMR (Lambrou et al., 2021). Notably, national antimicrobial consumption decreased from 91,088 kg in 2018 to 45,671 kg in 2020, accompanied by a decline in the use of WHO Class I drugs (Upadhyaya et al., 2023). A range of antibiotic alternatives is already in use in Nepal's livestock sector, particularly in poultry and smallholder dairy systems (Upadhyaya et al., 2023). Ethnoveterinary remedies such as turmeric (*Curcuma longa*), ginger, and garlic are commonly used in backyard production for infection control and immune support (Uprety et al., 2022), while herbal feed additives, including tulsi (*Ocimum sanctum*), moringa, and neem, are increasingly adopted to improve gut health, growth performance, and disease resistance (Sanjyal and Sapkota, 2012; Yadav et al., 2022). Probiotics and yeast-based supplements are used across poultry and dairy systems to stabilize the gut microbiota

and reduce reliance on antibiotics (Gupta et al., 2023). Preventive vaccination against major diseases and the use of phytogenic compounds from essential oils further limit the need for therapeutic antibiotics (Acharya and Wilson, 2019; Koirala et al., 2021; Poudel and Bhatt, 2024). Complementary biosecurity measures, including footbaths, litter management, and controlled farm entry, also play a key role in lowering infection pressure and antimicrobial use (Dhakal and Gompo, 2022; Dhakal et al., 2025). Despite progress in antimicrobial resistance awareness, antibiotic use regulations, and national policy development, challenges persist in residue monitoring, farmer education, and policy enforcement. Although Nepal has endorsed a One Health approach, implementation at the grassroots level remains weak, necessitating stronger awareness programs, effective regulatory mechanisms, and greater investment in sustainable alternatives (Acharya et al., 2019; Bagale et al., 2023). Common alternatives to antibiotics used in Nepal are shown in Table 1.

Biosecurity, hygiene, and antibiotic alternatives

Reducing infection pressure through strict biosecurity, as well as water, sanitation, and hygiene, remains the foundation of antimicrobial stewardship (FAO, 2021). Strict biosecurity protocols, including controlled farm entry, flock/herd compartmentalization, and pathogen exclusion at housing and equipment levels, significantly reduce pathogen pressure and unnecessary antimicrobial exposure (Postma et al., 2017). Environmental regulations play a significant role in minimizing AMR risks (UNEP, 2023). Likewise, hygiene, clean water systems, litter quality control, and routine sanitation lower the environmental pathogen load, thereby enhancing the baseline performance of alternative strategies such as probiotics or phytobiotics (Lambrou et al., 2021). Vaccination programs further reinforce the framework by establishing herd immunity against high-impact diseases (Newcastle disease virus [NDV], foot-and-mouth disease [FMD], and peste des petits ruminants [PPR]), reducing the frequency and severity of clinical outbreaks, and lowering reliance on therapeutic antibiotics (OIE, 2021). Equally important is manure and waste management, where regulated composting and controlled disposal interrupt the environmental spread of antibiotic residues, resistant bacteria, and resistance genes (He et al., 2020). Aerobic composting has been shown to reduce antibiotic resistance genes (ARGs) and mobile genetic elements by 70-90%, preventing their dissemination into soil–water ecosystems (Keenum et al., 2021; Wang et al., 2024). Once the preventive foundation is established, nutrition-based alternatives such as probiotics, phytobiotics, organic acids, yeast derivatives, and competitive exclusion cultures become more effective in supporting gut health, immunity, and production performance (Gadde et al., 2017; Diaz Carrasco et al., 2019). Probiotics, phytogenic compounds, organic acids, enzymes, and related feed additives should not be considered replacements for effective management practices; rather, such interventions function as complementary strategies integrated with strong production system controls to improve gut health, immunity, and animal resilience while reducing reliance on antimicrobial agents (Gadde et al., 2017; Yaqoob et al., 2022). The One Health approach integrates farm-level biosecurity, environmental hygiene, public health protection, and antimicrobial resistance (FAO, UNEP, WHO, WOAH, 2022).

Precision nutrition and smart farming

Precision nutrition aims to optimize nutrient supplementation based on the specific requirements of individual animals (González et al., 2018). The optimization is made possible by data-based rationing and feed supplementation, which are implemented only after assessing feed quality and reducing excretion (Zuidhof, 2020). In the case of poultry, the integration of metabolites into precision feeding has shown better performance effects, and sustainability is promising (Abd El-Hack et al., 2025). The integration of precision livestock farming technologies, sensors, analytics, and decision dashboards can detect deviations in animal health status through behavioral, acoustic, and thermal cues, thereby enabling timely interventions and reducing the need for antibiotics (Rowe et al., 2019; Schillings et al., 2021). Furthermore, advancements in biosensors have enabled the rapid detection of pathogens and increased the effectiveness of preventive systems over curative ones (Boodhoo et al., 2024).

Policies, awareness, and capacity building

A coordinated global effort to improve antimicrobial stewardship is advancing through joint leadership from the World Health Organization (WHO), the Food and Agriculture Organization of the United Nations (FAO), the World Organization for Animal Health (WOAH, formerly OIE), and the United Nations Environment Program (FAO, UNEP, WHO, WOAH, 2022). The organizations are aligning strategies to reinforce veterinary governance, laboratory systems, and AMR monitoring. Key instruments supporting this agenda include the FAO Action Plan on AMR (2021-2025), the Progressive Management Pathway for AMR (PMP-AMR), and WOAH's Performance of Veterinary Services (PVS) framework, all of which are designed to strengthen national capacities and coordinated surveillance within animal health systems (FAO, UNEP, WHO, WOAH, 2022). At the same time, the Codex Alimentarius has raised concern regarding

guidelines for monitoring and managing food-borne antimicrobials, providing a global benchmark for countries to integrate responsible antibiotic use into their food safety systems (Codex Alimentarius, 2021; FAO, 2022; WHO, 2024). Nepal has taken steps to align with these global directives. The National Action Plan on AMR (2024–2028), which adopts a One Health approach, is currently being implemented by the Ministry of Health and Population (MoHP; National Action Plan on Antimicrobial Resistance 2024-2028 endorsed by WHO and led by the MoHP), supported through a One Health approach. Similarly, a national AMR steering committee and technical working groups function as governing bodies at the national level. The regulation of veterinary antimicrobial use in Nepal is primarily governed by the Drugs Act of 1978, which provides the legal framework for the registration, production, importation, distribution, inspection, and quality control of veterinary medicines (Government of Nepal, 1978). Under this framework, relevant government agencies oversee the authorization, monitoring, and regulation of veterinary drugs in the country. Furthermore, the FAO and Codex have begun integrating AMR surveillance into the poultry value chain, thereby helping to ensure both animal and food safety (FAO, 2023b). Despite these advancements, gaps remain in residue monitoring, farmer awareness, and community-level enforcement. To achieve meaningful outcomes, provincial action plans must be strengthened, awareness campaigns expanded, and reliable alternatives promoted.

National antimicrobial resistance response and ongoing interventions

Nepal has undertaken a range of coordinated actions to address AMR through policy development, surveillance, education, and stewardship within a One Health framework. The national action plan on AMR provides the central policy platform for integrating human and animal health interventions and aligning national efforts with global AMR priorities (Khanal et al., 2023; Yadav et al., 2023). Antimicrobial resistance surveillance, initiated in 1999, focuses on priority pathogens but remains constrained by limited laboratory capacity and insufficient sentinel sites, underscoring the need for system expansion and strengthening (Frumence et al., 2021; Maharjan et al., 2023). Complementary initiatives include awareness and training programs targeting healthcare providers, veterinarians, and the public to promote rational antimicrobial use (Ayukekbong et al., 2017; Acharya and Wilson, 2019; Gahimbare et al., 2023), alongside the gradual implementation of antimicrobial stewardship programs in human and veterinary sectors to curb inappropriate prescribing and non-therapeutic antibiotic use (Saha et al., 2019; Mutua et al., 2020; Leung et al., 2024). Sustained investment, regulatory enforcement, and multisectoral collaboration supported by international frameworks and partners remain essential for effective AMR mitigation in Nepal.

Challenges and prospects

Despite growing evidence supporting antibiotic alternatives, their adoption in Nepal remains limited due to economic constraints, weak extension systems, and insufficient field-based evidence. Smallholder-dominated production systems rely heavily on low-cost antibiotics for growth promotion and disease prevention, as many alternatives, such as probiotics, phytobiotics, essential oils, and precision feeding tools, remain financially inaccessible and largely imported, limiting farmer uptake (Acharya and Wilson, 2019; Lambrou et al., 2021). Cost constraints are further exacerbated by weak rural infrastructure, fragmented supply chains, and limited local production of feed additives, collectively hindering reliable access to alternatives and reinforcing dependence on conventional antimicrobials (Saud et al., 2019; Sharma et al., 2025). In addition, gaps in farmer knowledge and technical capacity reduce confidence in non-antibiotic interventions, as limited extension services and training opportunities restrict understanding of appropriate application, expected outcomes, and long-term benefits (Subedi et al., 2023). Addressing the challenges requires specific, integrated strategies, including targeted financial incentives for antibiotic-free inputs, promotion of local production and entrepreneurship, strengthened extension and capacity-building programs of para-veterinary professionals, and further study in multi-season, on-farm field trials that evaluate the productivity and economic viability of alternative approaches under local conditions (Charoenratana and Kharel, 2024). Such measures are essential to shift antibiotic alternatives from experimental options to scalable, mainstream components of Nepal's antimicrobial stewardship and sustainable livestock development pathways.

Global collaboration for safer animal products

The transition to safer animal production systems and the effective containment of AMR critically depend on strong global collaboration, particularly to bridge implementation gaps observed across countries such as Nepal. Although many countries have developed national AMR action plans, uneven enforcement, weak surveillance, and limited institutional capacity continue to undermine their effectiveness, underscoring the need for harmonized standards, interoperable monitoring systems, and coordinated cross-border actions (Woolhouse et al., 2015; Boeckel et al., 2019). International frameworks such as the Codex Alimentarius, the FAO Progressive Management Pathway for AMR, and the WOAH Performance of Veterinary Services Pathway provide guidance for aligning national policies with global

antimicrobial resistance mitigation efforts (WOAH, 2019; FAO, 2022). In Nepal, irrational and routine antibiotic use in animal husbandry, driven by limited regulatory enforcement, inadequate laboratory infrastructure, and poor surveillance, has been identified as a major contributor to AMR, including the emergence of multidrug-resistant pathogens in animal-derived foods with direct public health implications (Acharya and Wilson, 2019; Subedi et al., 2023; Mim et al., 2024). Strengthening laboratory networks, workforce development, and traceability systems through targeted international support is therefore essential to translate farm-level interventions into population-wide reductions in AMR risk (Al Bakri et al., 2025). Moreover, facilitating international trade in animal products produced under responsible antibiotic-use standards can provide economic incentives for producers to adopt safer practices, aligning market access with global food safety expectations (Maffioli et al., 2025). The effective global collaboration against AMR must combine adherence to international frameworks with sustained investments in local capacity, stakeholder engagement, and innovation, thereby safeguarding public health while promoting resilient and sustainable animal production systems (Elbehiry et al., 2025; Obolensky et al., 2025).

CONCLUSION

Nutrition-based alternatives, including probiotics, phytobiotics, organic acids, enzymes, and precision feeding approaches, offer practical, evidence-based ways to sustain animal health and productivity while progressively reducing reliance on antibiotics. A coordinated, system-level strategy that integrates nutritional innovations with strengthened biosecurity, preventive vaccination, farmer-oriented training, and effective policy enforcement will achieve better results in AMR. Further studies on cost-effective, locally adaptable alternatives are needed to support broader adoption and achieve long-term reductions in antimicrobial resistance in Nepal.

DECLARATIONS

Authors' contributions

Arjun Acharya and Utshav Pandey wrote the article. Arjun Acharya collected the information and prepared the original manuscript draft. Both authors contributed to the review and editing of the manuscript. Both authors read and approved the final version of the manuscript before publication in the present journal.

Availability of data and materials

All information used in the current study was derived from publicly available articles, which have been appropriately cited in the manuscript.

Competing interests

The authors declare no competing interests.

Ethical considerations

Ethical issues, including plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy, have been carefully checked and confirmed by the authors. The authors confirmed that no AI website or software programs were used to prepare, write, or revise this article.

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The Impact of Climate Change on Goat and Sheep Farming Systems in Southern Africa: A Narrative Review

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ABSTRACT

Smallholder goat and sheep farming is a critical component of rural livelihoods in Southern Africa. Climate change has, however, aggravated the situations of drought, heat stress, and disease burdens, which threaten the productivity of small ruminants. The present review aimed to determine the multidimensional effects of climate change on goat and sheep farming in Southern Africa, to analyze existing adaptation measures, and identify policy measures to build resilience. The current review used Google Scholar, Scopus, and PubMed to cover peer-reviewed articles and grey literature published between 2014 and 2025. Based on the inclusion criteria, 102 articles were initially screened, and 51 articles were considered for the narrative review. The review focused on studies conducted in South Africa, Namibia, Botswana, Zimbabwe, and Lesotho. The results indicated a strong relationship between climate variability and decreased ruminant productivity, driven by pasture degradation, water shortages, heat stress, and limited access to veterinary services in Southern African countries. Additionally, the results showed that adaptive strategies include the use of indigenous breeds, drought-resistant feeding, and ethnoveterinary practices to cope with climate variations. The present narrative review highlights the importance of adaptive strategies and integrated policies in Southern African countries for sustaining the development of small ruminant farming amid climate variability.

Keywords: Climate change, Heat stress, Rangeland management, Small ruminant

INTRODUCTION

Goat and sheep farming is a significant part of rural livelihoods and food systems in Southern Africa, supplying meat, milk, income, and cultural value to millions of smallholder farmers (Mapiye et al., 2020). Furthermore, studies revealed that goats and sheep are among the most climate-resilient livestock species, which are widely reared in arid and semi-arid regions where crop agriculture is often unsustainable due to climate variability (Joy et al., 2020; Nair et al., 2021). Hence, climate changes associated with rising temperatures, droughts, erratic rainfall patterns, and variations in the frequency of extreme weather events are regarded as a serious threat to the sustainability of goat and sheep production systems across the region (Ben Moula et al., 2024).

Climate-induced challenges negatively affect pasture availability, including access to water sources, thereby reducing reproductive performance and animal health, leading to decreased productivity and increased vulnerability, especially among small-scale farmers (Bahta et al., 2019; Zhou et al., 2022). Furthermore, climate change exacerbates socio-economic challenges by limiting access to veterinary care, undermining markets and infrastructure, and disrupting agricultural support services, which disproportionately impact marginalized rural communities (Stephen and Soos, 2021; Mthembu et al., 2025). In this context, the resilience of goat and sheep farming systems depends not only on the animals' inherent adaptability but also on the implementation of specific strategies that improve management practices and supportive policy frameworks. The current study aimed to investigate the effects of climate change on goat and sheep production in Southern Africa, including challenges, adaptation options, and policy implications for sustainable, climate-resilient livestock production systems.

STUDY AREA

The present study focused on specific countries in the Southern African region, such as South Africa, Namibia, Botswana, Zimbabwe, and Lesotho, where goat and sheep production is vulnerable to climate change. Countries in this region experienced diverse agroecological conditions and shared challenges, including rising temperatures, rangeland degradation, irregular rainfall patterns, and prolonged drought. Several studies indicated that provinces such as Kgalagadi in Botswana (Mphale et al., 2014), the Eastern Cape in South Africa (Mahlalela et al., 2020; Mahlalela, 2023),

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and Kunene in Namibia (Mahlalela et al., 2020; Mahlalela, 2023) are experiencing reduced annual rainfall and increased dry spells; this trend has impaired access to water and forage (Zhou et al., 2022; Mutengwa et al., 2023). Additionally, studies by Turpie et al. (2010) and Mutengwa et al. (2023) highlighted the impacts of climate change intensification, including heat stress, decreased pasture quality, water shortages, and increased disease prevalence in goat and sheep farming systems.

According to studies of Baleseng et al. (2016) and Bolowe et al. (2022) in Southern Africa, goat and sheep farming production differ from semi-intensive mixed production to extensive communal grazing, mostly influenced by local practices and resource availability. Notwithstanding these variations, climate-related hazards are a common occurrence in all the chosen regions, necessitating the development of regional adaptation plans. To improve the resilience and sustainability of goat and sheep farming in Southern Africa, policies and interventions must be guided by an understanding of the climatic context and regional vulnerabilities.

STUDY DESIGN

A narrative review approach has been employed to synthesize the effects of climate change on goat and sheep production systems in Southern Africa. A comprehensive literature search was conducted between January and May 2025 using Google Scholar, Scopus, and PubMed databases. The search strategy incorporated Boolean operators (AND/OR) with keywords including “climate change”, “goat farming”, “sheep production”, “Southern Africa”, “drought resilience”, “adaptation strategies”, and “policy response”. Articles published between 2014 and 2025 were included in the studies, such as peer-reviewed articles and the grey literature. Geographical relevance (Southern Africa), thematic focus (climate impacts, adaptation, and policy), and publication language (English) were used as inclusion criteria.

One hundred and twenty-two records were found. Titles and abstracts were filtered after eliminating duplicates, and the full texts were finally filtered. Finally, 51 studies were included in the review, as they met the inclusion criteria. The thematic analysis was used to extract and synthesize data to determine common patterns in climate impacts, adaptation strategies, and policy responses in the region.

The narrative review considered publication titles and abstracts, obtained through a primary electronic search and was thoroughly examined for the possibility of inclusion. Duplicate articles were removed, and article titles and abstracts were thereafter screened for relevance, resulting in the exclusion of unrelated studies. Additionally, full texts were evaluated, and unrelated studies were excluded.

Table 1. The inclusion and exclusion criteria for the present study

Criteria type	Inclusion criteria	Exclusion criteria
Geographical scope	Studies conducted in South Africa, Namibia, Botswana, Lesotho, or Zimbabwe	Studies outside the Southern African region
Population	Studies focusing on goat and/or sheep farming systems in Southern African countries	Studies focused solely on cattle or poultry without mention of goats/sheep
Climate focus	Studies addressing climate change, variability, drought, temperature, or rainfall effects on farming	Articles with no reference to climate change impacts
Language	English	Non-English publication
Publication type	Peer-reviewed articles, governmental and non-governmental organization (NGO) reports, and credible grey literature	Opinion pieces, blogs, and non-scientific commentaries
Time range	2014-2025	Publications before 2010 (unless highly cited and foundational)
Themes covered	Climate impact, adaptation strategies, resilience, and policy implications	Studies not addressing at least one of the core themes of the review

CONCEPTUAL FRAMEWORK

The narrative review was guided by the Intergovernmental Panel on Climate Change (IPCC) vulnerability framework, which describes climate risk as dependent on three fundamental components: exposure, sensitivity, and adaptive capacity. Farming systems for goats and sheep in Southern Africa are increasingly at risk due to rising temperatures, uneven rainfall distribution, and frequent droughts. Such climatic stresses are exacerbated by systemic issues in ecosystems and dependence on large-scale production systems. Adaptive capacity in farming communities is uneven and is attributed to social, economic, institutional, and knowledge-based factors. The present study assessed the interactions among climate stressors, vulnerability, and systemic weaknesses, highlighting the diversity of local responses that influence resilience and long-term sustainability.

CLIMATE IMPACTS ON GOAT AND SHEEP FARMING

Climate change in Southern Africa has profound, multidimensional effects on livestock production, with goat and sheep farming systems appearing to be the most adversely affected (Zhou et al., 2022). Substantial stressors include the increasing frequency of extreme weather events, recurrent droughts, rainfall variability, and rising ambient temperatures (Nhara et al., 2020; Zhou et al., 2022). According to Mudau et al. (2022) and Zhou et al. (2022), climate fluctuations, such as droughts, rainfall variability, and the increase in extreme weather phenomena, decrease pasture availability, contribute to rangeland degradation, and reduce water sources, all of which are critical for the survival and productivity of small ruminants. Previous studies indicated that nutritional stress and elevated temperatures adversely influence reproductive performance, milk yield, and disease resistance in goats and sheep, particularly in low-input systems such as smallholder farms relying on minimal external resources (Mandleni and Anim, 2011; Kraai et al., 2022; Cooke et al., 2023). Furthermore, increased parasite infestation and reduced body weight gains have been linked to the intensity of climate-induced heat stress, thereby contributing to higher mortality rates among goats and sheep (Ben Moula et al., 2024). Studies by Ngaka (2012) and Inman et al. (2020) have demonstrated that regions such as the Eastern Cape and Free State in South Africa, as well as Kunene in Namibia, experienced substantial herd losses during periods of extreme drought.

Climate variations in Southern Africa have led to significant mortality among goats and sheep across several countries. For instance, in the KwaZulu-Natal Province of South Africa, the drought caused by El Niño during 2015-2016 led to a 29% mortality rate among goats due to insufficient food and water supply (Vetter et al., 2020). National herd assessments in Botswana documented 18.3% goat and 15% sheep mortality, which is significantly above the 2-5% (Ramabu et al., 2024). In Zimbabwe, the official report from the Ministry of Lands, Agriculture, Water, and Rural Resettlement (2020) indicated that the mortality rates of goats (23%) and sheep (17%) were directly linked to the drought-affected season (Risk, 2020). Meanwhile, in Namibia, the study by Marius et al. (2021) indicated that numerous reports of losses in goats and sheep have been documented due to drought. Furthermore, smallholder farmers in Lesotho regularly report livestock deaths due to drought, but quantifiable mortality statistics have not been recorded (Mojaki et al., 2025).

Studies conducted in Southern Africa showed that climate change exerts diverse and interconnected stressors on goat and sheep production systems (Zhou et al., 2022). The impact of climate change in Southern Africa has been observed in countries such as South Africa (Mahlalela et al., 2020; Kraai et al., 2022), Namibia (Inman et al., 2020), Botswana (Seleka, 2001; Kgosikoma and Batisani, 2014), Zimbabwe (Mhlanga et al., 2018; Bvirwa et al., 2023) and Lesotho (Gwimbi, 2015; Tsese, 2023), whereby stressors such as erratic rainfall patterns, prolonged droughts, increased temperatures as well as the intensification of extreme weather events within the region has impact negatively the goat and sheep production systems. Several studies of Halimani et al. (2021), Slayi et al. (2022), and Letsoalo et al. (2023) on the Eastern Cape in South Africa revealed that climate-induced droughts in this province have led to widespread rangeland degradation and negatively impacted herd sizes of small ruminants due to poor pasture availability and water access scarcity. In addition, the impact of heat and water stress in the Eastern Cape province of South Africa has been reported to reduce fertility and increase fertility problems in goats and sheep, resulting in decreased productivity (Halimani et al., 2021). Meanwhile, in the Kunene region of Namibia, a study by Kwembeya and Shikangalah (2023) reported a declining rainfall trend over the past decade, contributing to increased drought intensity. The effects of climate change have led to a decline in grazing lands, nutritional deficiencies, and rising mortality in goat and sheep herds, particularly among resource-poor communal farmers (Kwembeya and Shikangalah, 2023).

In Botswana, particularly in the Kgalagadi region, small farmers experiencing a prolonged dry season should consider seasonal migration as an essential strategy for securing water and forage (Kgosikoma and Batisani, 2014). Furthermore, studies by Mosalagae and Mogotsi (2013) and Kgosikoma and Batisani (2014) indicated that these conditions disrupt traditional grazing patterns and intensify competition for limited resources among cattle, goats, and sheep. In Zimbabwe, farmers in the southern provinces of Masvingo and Matabeleland South face persistent climate threats, including rising temperatures, a higher incidence of tick-borne diseases, and a significant reduction in birth rates (20-35%) during periods of climatic stress such as drought and extreme heat.

On the other hand, the high levels of climate-induced food insecurity have been reported among goat-reliant households in these two provinces, Masvingo and Matabeleland South (Homann et al., 2007; Ndlovu et al., 2020). Previous studies conducted indicated that people in the Lesotho highland primarily depend on small ruminants for food and income for their communities (Gwimbi, 2015; Grab et al., 2017; Matsepe et al., 2021). However, extreme cold events followed by warmer winter temperatures have affected the incidence of animal diseases. Furthermore, erratic snowfall and changing grazing cycles impair herd movement and health, thereby contributing to increasing helminth and ectoparasite burdens (Zhou et al., 2022). Additionally, the study of Zhou et al. (2022) demonstrated that in Southern African states, effects of climate change, such as decreased pasture quality, heat stress, water shortage, and the increased

risks of diseases, have all led to the decline in the production of goats and sheep and threaten the sustainability of such systems in the long run.

SOCIO-ECONOMIC AND INSTITUTIONAL CONSTRAINTS

Socio-economic and institutional constraints influence the degree of adaptation of goat and sheep farmers to climatic challenges in Southern African countries. These barriers differ across countries but share a number of features across the region (Mutengwa et al., 2023). In South Africa, particularly in the Eastern Cape and Limpopo provinces, the lack of access to veterinary services and extension support is sparse in remote rural areas (Zhou et al., 2022). The situation is thought to be associated with poor road infrastructure, limiting market access and disease surveillance coverage. Different studies conducted in Namibia have demonstrated that the government has been interfering with drought relief programs but has taken inadequate action (Inman et al., 2020; Treichel et al., 2021; Menestrey Schwieger, 2023). For instance, Smallholders in Kunene and Otjozondjupa regions have indicated that access to supplementary feeds and vaccines is difficult due to costly transportation and disruption in the supply chains (Treichel et al., 2021). Meanwhile, in Botswana, despite the government's efforts to provide veterinary medications, bureaucratic delays, insufficient policies, and poor planning regarding climate sensitivity undermined the overall quality of the programs in the country (Kgosikoma and Malope, 2016; Motsumi et al., 2023). Furthermore, the studies of Kgosikoma and Malope (2016) and Motsumi et al. (2023) concluded that marginalisation of small producers involved in goat and sheep production, who are majority women and youth, is regarded as a factor limiting the distribution of resources and participation in decision-making processes. While in Zimbabwe, the recurrent economic instability and hyperinflation have contributed drastically to the affordability of livestock inputs across the country, making it difficult for farmers to maintain feeding regimes for their livestock properly (Ndlovu et al., 2020). On the other hand, the majority of farmers were found to be not able to afford the implementation of climate-smart technologies. Moreover, market volatility discourages investment in small stock farming across the country due to poverty (Ndlovu et al., 2020). Land tenure insecurity, more particularly in communal grazing systems, is pointed out as a factor restricting sustainable pasture management and long-term investment in climate adaptation practices in countries such as Lesotho (Gwimbi, 2015). In addition, in mountainous regions, veterinary services were underdeveloped, which increased disease incidence, and interventions were minimal (Stephen and Soos, 2021). These issues explain why goat and sheep production systems in the Southern African region are adverse due to weak institutional support, socio-economic marginalization, and policy coordination failure.

FARMER ADAPTATION STRATEGIES

Farmers in Southern Africa have implemented localized adaptation policies, depending on the environment and the capacity of available resources. For instance, the study by Mapiye et al. (2020) revealed that in Southern Africa, communal farmers have adopted supplementary feeding systems or practices relying on crop residues and drought-tolerant forage such as lucerne and Moringa. Small-scale farmers are increasingly focusing on breeding indigenous livestock, such as Nguni and Boer goats, which are valued for their resilience to heat and disease (Mapiye et al., 2020). Concurrently, in water-scarce regions such as Kunene in Namibia, communities depend on boreholes and community-managed water systems. Additionally, as noted by Schnegg (2016) and Schwieger et al. (2022), many farmers practice seasonal transhumance, moving their herds to higher altitudes during periods of extreme heat.

Indigenous knowledge-based seasonal calendars and rotational grazing practices have been adopted in Botswana to manage pasture usage (Kgosikoma and Batisani, 2014). Furthermore, in Botswana, an agroforestry-based intervention, including the planting of trees that provide both fodder and shade, has been implemented in this area as part of an experimental study with promising results (Cooke et al., 2023). In the study by Maroyi (2012), it was shown that some smallholders have turned to zero-grazing systems for sheep and goats, especially in peri-urban zones of Zimbabwe, while others rely on alternative feeding practices such as herbal remedies and ethnoveterinary knowledge as affordable alternatives to scarce formal veterinary services or care. In Lesotho, roofed kraals, windbreak structures, and feed blocks produced by using crop residues are small-scale interventions that are being experimented with the aim of helping in improving animal welfare in situations of extreme cold and feed deficit (Mahlehla et al., 2021). The knowledge-based practices have the potential; however, they are frequently adopted without institutional resources or financial constraints, making it difficult to expand them and achieve long-term results.

POLICY AND GOVERNANCE RESPONSES

There is still insufficient and unequal policy support for the adaptation of small ruminants among the countries in Southern Africa. In South Africa, livestock is not explicitly mentioned in national climate plans. Budgetary allocation is limited, and there is no specific plan for small ruminants. Policies such as the Agriculture and Agro-processing Master Plan (AAMP) hold promise, but the execution is lagging (Zhou et al., 2022). Namibia's national drought policy and

strategy provide a framework for livestock relief, although it does not make special considerations for goats and sheep (Turpie et al., 2010). It is imperative to align financial assistance, veterinary accessibility, and training to the requirements of communal farmers (Mutengwa et al., 2023). Meanwhile, in Botswana, the Integrated Support Program for Arable Agriculture Development (ISPAAD) has shown some success, but it mainly supports crop production rather than livestock (Mosalagae and Mogotsi 2013; Kgosikoma and Malope 2016). Moreover, dryland areas still lack a regionally coordinated framework to guide climate adaptation in the livestock sector (Motsumi et al., 2023).

The National Climate Policy in Zimbabwe highlighted the vulnerability of the agricultural sector; however, persistent institutional instability hinders interventions aimed at livestock. The majority of farmers rely on non-governmental organizations for assistance in climate-resilient practices. In Lesotho, the national adaptation program of action includes livestock-related activities; however, their implementation has been considerably constrained due to insufficient funding and a shortage of extension personnel.

CONCLUSION

The production systems of goats and sheep in Southern Africa are particularly sensitive to the effects of climate change, with losses primarily caused by degraded pasture, water shortages, and shifts in disease patterns. Even though farmers have implemented various localized and indigenous-level adaptations, these measures are poorly organized and lack financial support for their long-term sustainability. Hence, integrating small ruminants into climate frameworks and nationally determined contributions (NDCs) will strengthen regional collaboration. Additionally, funding gender-sensitive climate-smart livestock for communal farmers, incorporating Indigenous knowledge into planning, and investing in climate-veterinary data systems to improve risk management will contribute to sustainable small ruminant production.

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Authors' contributions

This review was conceptualized and designed by Baitsholetsi Gloria Mokolopi and Mpinda Edoaurd Tshipamba, who conducted the search and wrote the review. All authors reviewed and approved the final edition of the manuscript.

Availability of data and materials

All data related to this study are prepared and presented in this manuscript.

Competing interests

The authors declared no conflict of interest.

Ethical considerations

The authors have verified potential ethical concerns, including plagiarism, agreement to publish, misconduct, and/or duplicate publication and/or submission, and redundancy. The authors confirm that no AI tools were used to prepare this study.

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Traditional Medicinal Plants as Sources of Broad-Spectrum Antiviral Agents Against Poultry Viral Infections: A Review

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ABSTRACT

Across Asia, poultry farming plays a critical role in ensuring food security and supporting rural communities, delivering affordable protein to billions of people. Sustainable poultry production in Asia, especially in India, has been consistently affected by viral diseases. Conventional interventions, including vaccination and biosecurity protocols, often remain inadequate against viral infections, require specific storage conditions, elicit suboptimal immune responses, and pose economic challenges and limitations. In poultry, synthetic antiviral medications are often unsuitable due to their high cost, adverse effects, and concerns about residual compounds. Plant-based remedies emerge not as direct replacements but as valuable alternatives to consider for developing broad-spectrum antiviral solutions. The present study aimed to evaluate the antiviral potential of South Asian ethnomedicinal plants against Newcastle disease virus, infectious bursal disease virus, infectious bronchitis virus, and fowl adenovirus. Compounds such as flavonoids, terpenoids, alkaloids, saponins, and lignans were targeted for their multitarget activities, including inhibition of viral entry and polymerases, immunomodulation, and antioxidant properties. Different studies on *Glycyrrhiza glabra*, *Tephrosia purpurea*, *Cyperus rotundus*, *Tribulus terrestris*, *Boerhavia diffusa*, *Azadirachta indica*, *Andrographis paniculata*, and *Ocimum sanctum* reported promising therapeutic potential against poultry viral diseases. Available *in vitro* studies demonstrated strong antiviral activity across multiple phytochemical classes. Nevertheless, *in vivo* validation and implementation at the field level remain substantially limited. The current findings confirmed the strong potential of ethnomedicinal plants as sustainable, multi-target antivirals for poultry health.

Keywords: Flavonoid, Fowl adenovirus, Infectious bronchitis virus, Infectious bursal disease virus, Saponin, Terpenoid

INTRODUCTION

Poultry farming persistently encounters challenges worldwide, particularly due to the viral effects that disrupt production systems (Muñoz-Gómez et al., 2025). Throughout Asia, poultry farming is a crucial source of income for local communities while also providing food for large populations. Millions of people rely on smallholder farmers in countries such as India and Bangladesh, even as industrial-scale farms expand rapidly (Kabir et al., 2021; Kalaria et al., 2021). However, this rapid growth has caused increased losses related to disease, especially from infectious bursal disease virus (IBDV) and infectious bronchitis virus (IBV), which contribute to chick mortality, lower egg production, hinder feed efficiency, and raise veterinary costs (Kalaria et al., 2021; Islam et al., 2022). Frequently overlooked, potential solutions reside within indigenous plant species that have long been utilized in traditional folk remedies. The antiviral potential of traditional medicinal plants is garnering attention, as recent results confirmed measurable activity against pathogens detrimental to poultry health (Muñoz-Gómez et al., 2025). Rather than relying solely on synthetic pharmaceuticals, some poultry farmers consider utilizing natural sources already available in their environment. Progress in the use of natural sources as antiviral agents remains limited, but there is increasing effort to integrate these methods into broader health strategies for sustainable poultry care management (Kabir et al., 2021; Muñoz-Gómez et al., 2025).

Currently, there are no effective curative treatments for viral diseases in poultry (Szotowska and Ledwoń, 2024). Vaccines, although effective, remain a control strategy constrained by multiple limitations (Ravikumar et al., 2022; Toka and Geinoro, 2023). Mutations in poultry respiratory and immunosuppressive viruses, especially IBV and IBDV, lead to vaccine failure against emerging strains (Toka and Geinoro, 2023; Rafique et al., 2024). Mass vaccination campaigns face challenges, including cold-chain storage, interference from maternal antibodies, high production costs, and

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inadequate coverage in rural areas (Ravikumar et al., 2022; Toka and Geinoro, 2023). Synthetic antiviral medications, including those used in human medicine, were not practical for poultry due to costs, toxicity, the risk of residues in meat and eggs, and the potential development of resistance (Szotowska and Ledwoń, 2024). The combination of incomplete vaccination, impractical antiviral medications, and growing resistance created an urgent need for safer, more cost-effective antiviral alternatives in poultry farming.

Long-standing medicinal plants can serve as broad-spectrum antiviral agents (Adeosun and Loots, 2024). Traditional poultry and livestock healthcare relies on a well-documented ethnopharmacological foundation that includes hundreds of unique herbs (Jamil et al., 2022; Pebam et al., 2022). *Azadirachta indica* (neem), *Allium sativum* (garlic), *Zingiber officinale* (ginger), *Ocimum sanctum* (holy basil), and *Andrographis paniculata* (nilavembu) are deeply embedded in South Asian poultry farming practices (Abd El-Hack et al., 2022; Pebam et al., 2022; Eftekhari Hasan Abad and Ghaniei, 2023; Djamen et al., 2024). In poultry health management and nutrition, plants are frequently utilized as feed additives, water supplements, or prophylactic agents to control viral diseases (Abd El-Hack et al., 2022; Jamil et al., 2022). Plants such as neem, ginger, and garlic are rich in flavonoids, alkaloids, terpenoids, saponins, and sulfur compounds, which are known to inhibit viral entry, interfere with replication enzymes, and stimulate host immunity (Adeosun and Loots, 2024; Tahir and Alsayeqh, 2024).

The worldwide transition to sustainable and residue-free production makes plant-based antivirals a critically important strategy for poultry healthcare (Abd El-Hack et al., 2022; Adeosun and Loots, 2024). In many developing countries, small-scale poultry farmers often lack access to resources, where phytotherapeutics can serve as a transformative solution (Jamil et al., 2022; Pebam et al., 2022). Integration of these phytotherapeutics into modern veterinary virology could lead to new opportunities to develop novel medicines and feed-based prophylactic interventions (Adeosun and Loots, 2024; Tahir and Alsayeqh, 2024). The present study aimed to review the broad-spectrum antiviral potential of traditional medicinal plants against major poultry viral diseases, including Newcastle disease virus (NDV), IBDV, IBV, and Fowl Adenovirus (FAdV), and to synthesize evidence on phytochemical mechanisms and knowledge gaps to support sustainable poultry health management.

METHODOLOGY

The present study was conducted through a comprehensive literature review to identify studies related to the antiviral potential of traditional medicinal plants in the context of poultry viral diseases. The investigation was conducted using the electronic databases, including PubMed, Scopus, Web of Science, and Google Scholar. The search terms, used individually and in combination, included traditional medicinal plants, antiviral, phytochemicals, poultry, NDV, IBDV, IBV, FAdV, flavonoids, antiviral mechanisms, terpenoids, immunomodulation, saponins, poultry health, phytogenic feed additives, and ethnomedicinal plants in South Asia.

The inclusion criteria for selecting articles were peer-reviewed original articles, review papers, and relevant case studies, published in English between 2009 and 2025, that demonstrated direct relevance to the phytochemical characterization, antiviral mechanisms, or immunomodulatory effects of traditional medicinal plants against poultry-relevant viral pathogens.

In vitro, *in silico*, and *in vivo* studies reporting antiviral activity of plant-derived compounds were all considered eligible. Studies involving closely related mammalian model viruses were also incorporated, where their findings provided translational relevance to poultry virology. Exclusion criteria included non-peer-reviewed grey literature, conference abstracts without full data, studies on non-relevant species lacking translational value, and publications lacking original phytochemical or antiviral findings. Finally, 72 articles were included for final assessment and synthesis. The collected literature was critically synthesized to evaluate the broad-spectrum antiviral potential of key phytochemical classes, including flavonoids, terpenoids, alkaloids, saponins, and lignans, and to support the multitarget mechanistic framework presented in the present study. Five plants, *Glycyrrhiza glabra* (*G. glabra*), *Tephrosia purpurea (T. purpurea)*, *Cyperus rotundus* (*C. rotundus*), *Tribulus terrestris* (*T. terrestris*), and *Boerhavia diffusa* (*B. diffusa*), were selected for a detailed case study assessment based on the following criteria.

Initially, each plant was documented for its availability and traditional application in South Asian poultry and livestock healthcare. Secondly, phytochemical evidence from scientific studies confirmed the presence of at least one class of bioactive compounds with known antiviral, immunomodulatory, or hepatoprotective properties. Thirdly, preliminary data, whether derived from *in vitro*, *in silico*, or *in vivo* investigations, were available for each plant in the context of poultry or closely related animal models. These plants were subsequently assessed for their therapeutic relevance, practicality as phytogenic feed additives, and potential integration. Into sustainable poultry health management systems in South Asia.

PHYTOCHEMICAL RESERVOIRS OF ANTIVIRAL COMPOUNDS

Based on the existing literature, many traditional medicinal plants contain bioactive secondary metabolites with broad-spectrum antiviral potential (Phillips et al., 2023; da Fonseca et al., 2025). These phytochemical compounds, unlike synthetic antiviral agents, are designed to target specific viral enzymes or pathways and exert multiple effects by modulating viral entry, replication, host immunity, and oxidative stress responses (Phillips et al., 2023; da Fonseca et al., 2025). This multitarget characteristic of phytochemical compounds has demonstrated potential against poultry viruses (Sarwar et al., 2022).

Flavonoids

The structural diversity and extensive bioactivity of flavonoids have established them as one of the most comprehensively studied categories of plant-derived antivirals agents (da Fonseca et al., 2025). Flavonoids are structurally diverse, encompassing flavonols (quercetin, kaempferol), flavones (apigenin, luteolin), flavanones (naringenin), and catechins (epigallocatechin gallate [EGCG]; da Fonseca et al., 2025). These compounds exhibited both direct and indirect antiviral effects (Wang et al., 2021a; da Fonseca et al., 2025). Quercetin has been reported to inhibit viral polymerases and proteases, interfere with viral entry by blocking hemagglutinin-like interactions, and enhance host interferon responses (Wang et al., 2021a; da Fonseca et al., 2025).

In Ross 308 broiler chickens, supplementing the diet with 200-800 ppm of quercetin, primarily sourced from flavonol-rich plants such as *Allium cepa* and *Camellia sinensis*, enhanced growth performance, improved gut microbiota, and increased antioxidant enzyme gene expression. However, no significant increase in humoral antibody titers against the avian influenza H5N1 vaccine was observed by day 35 post-supplementation. These findings suggested that the immunoadjuvant properties of quercetin warrant further focused investigation (Abdel-Latif et al., 2021). Epigallocatechin-3-gallate, the principal catechin derived from *Camellia sinensis* (green tea), has demonstrated significant activity against coronaviruses and paramyxoviruses *in vitro*, mainly through binding to viral surface proteins and inhibiting attachment (Pham, 2021; Wang et al., 2021a). Although poultry-specific data remain limited, similar mechanisms may apply to IBV and other enveloped RNA viruses, in which surface glycoproteins are vulnerable to flavonoid interference, which is significant (Wang et al., 2021a; Saadh, 2022).

Terpenoids and steroids

Terpenoids, derived from isoprene units, encompass monoterpenes, diterpenes, triterpenes, and steroids (Obianwuna et al., 2024). Terpenoids are abundant in *Andrographis paniculata* (*A. paniculate*; andrographolide) and *Ocimum sanctum* (ursolic acid; Yasmin et al., 2020; Obianwuna et al., 2024). Terpenoids exert antiviral effects by disrupting viral membranes, inhibiting viral fusion proteins, and suppressing viral transcription (Yasmin et al., 2020; Obianwuna et al., 2024). Andrographolide from *A. paniculata* interferes with NDV replication and reduces the release of inflammatory cytokines, providing dual antiviral and host-protective effects (Nagajothi et al., 2020; 2023). Neem-derived limonoids, such as nimbolide, have generally exhibited virucidal effects against paramyxoviruses by directly destabilizing the viral envelope (Wylie and Merrell, 2022; Rajendran et al., 2024). Plant steroids, particularly phytosterols, can modulate host immune responses, thereby enhancing poultry resistance to viral infections (Yasmin et al., 2020; Obianwuna et al., 2024). Beta-sitosterol, found in many medicinal plants, has been associated with improved lymphocyte proliferation and macrophage activity (Obianwuna et al., 2024; Rajendran et al., 2024).

Alkaloids

Nitrogen-rich compounds known as alkaloids exhibit diverse antiviral effects (Kang et al., 2025). These compounds include piperine, which is found in black pepper, berberine, derived from *Tinospora cordifolia*, and solasodine, present in specific Solanum species (Kang et al., 2025). Alkaloids primarily exert antiviral effects by disrupting viral genome replication, inhibiting viral protein synthesis, and preventing viral attachment to host cell receptors (Kang et al., 2025). Piperine has been associated with enhanced immune responses post-vaccination and reduced disease incidence in poultry raised for commercial purposes (Mohite et al., 2024). Berberine acts against multiple RNA-based pathogens, including influenza, hepatitis C, respiratory syncytial virus (RSV), chikungunya, and NDV, by interfering with viral enzymes and cellular signaling pathways, such as the MAPK pathway. However, the role of berberine in controlling poultry viruses remains uncertain without further laboratory investigation (Kang et al., 2025).

Lignans and coumarins

Lignans and coumarins have yet to attract significant attention; however, interest in their potential antiviral properties is increasing (Lawal et al., 2021; Wang et al., 2023). Podophyllotoxin-based lignans inhibit DNA polymerase

activity. In contrast, certain coumarins, such as umbelliferone and scopoletin, tend to disrupt viral replication and affect oxidative processes (Lawal et al., 2021; Wang et al., 2023). The conserved nature of RNA-dependent RNA polymerases (RdRp) across RNA virus families suggested that further investigations into the effectiveness of lignans and coumarins against avian viruses could yield important clinical insights (Lawal et al., 2021; Wang et al., 2023).

Saponins

Saponins, mainly found in plants such as *T. terrestris* and *Quillaja saponaria*, are glycosides with mixed solubility. Despite their structural complexity, these compounds exhibit measurable antiviral activity and enhance immune function (Wang, 2021b; Saeed et al., 2024). While often derived from legumes, saponins are notable for their surfactant properties, which disrupt viral membranes. Saponin-mediated membrane disruption occurs alongside broader immunological changes, including increased antibody levels. Saponins derived from *Quillaja saponaria* are frequently incorporated into poultry vaccines to enhance protective immune responses against IBV (Wang, 2021b; Saddoris-Clemons et al., 2024; Morais et al., 2025).

Protection increases as circulating antibodies and cellular defences respond more effectively (Wang, 2021b; Saddoris-Clemons et al., 2024; Morais et al., 2025). Supplementation with *T. terrestris* extract has enhanced macrophage activation and elevated serum antibody concentrations in experimental models (Al-Garadi et al., 2022; Saeed et al., 2024). Such findings suggested dual roles, including preventive health support and enhanced vaccine effectiveness (Al-Garadi et al., 2022; Saeed et al., 2024).

Synergistic and multifactorial mechanisms

The antiviral effectiveness of phytochemical compounds rarely results from a single compound alone (Jasim et al., 2024; Fouad et al., 2025). Most medicinal plants contain complex mixtures of flavonoids, terpenoids, alkaloids, and saponins that act synergistically (Jasim et al., 2024; Fouad et al., 2025). For instance, garlic (*Allium sativum*) contains allicin, ajoene, and multiple organosulfur compounds that combine virucidal, antioxidant, and immunostimulatory properties (Doostmohammadian et al., 2020; Adjei-Mensah et al., 2023). Similarly, turmeric (*Curcuma longa*) yields curcuminoids along with volatile oils, demonstrating both anti-inflammatory and direct antiviral effects (Aderemi and Alabi, 2023; Fouad et al., 2025). This multifactorial activity is especially important in poultry virology, where viral diversity and coinfections often occur on commercial poultry farms, creating persistent challenges for disease management and vaccine effectiveness (Jasim et al., 2024; Fouad et al., 2025).

MECHANISMS OF ANTIVIRAL ACTION

The antiviral effectiveness of traditional medicinal plants stems from diverse bioactive compounds that interfere with multiple stages of the viral life cycle while also boosting host defenses (Adeosun and Loots, 2024; da Fonseca et al., 2025). Unlike synthetic medications designed for narrow-spectrum targets, phytochemicals typically act synergistically via multiple pathways (Adeosun and Loots, 2024; da Fonseca et al., 2025). In poultry virology, this multifaceted action is especially important due to the antigenic variability of viruses such as NDV and IBV, as well as the significant immunosuppression caused by IBDV (Ravikumar et al., 2022; Tahir and Alsayeqh, 2024).

Inhibition of viral entry and attachment

Viral entry initiates with viral surface glycoproteins attaching to host cell receptors, which then leads to membrane fusion or endocytosis (Adeosun and Loots, 2024; da Fonseca et al., 2025). Inhibiting these steps is an effective strategy for infection prevention (da Fonseca et al., 2025). Flavonoids, including quercetin and EGCG, interact with viral hemagglutinin-like proteins and receptor-binding domains, thereby inhibiting adsorption (Figure 1; Pham, 2021; Wang et al., 2021a; da Fonseca et al., 2025). In poultry studies, quercetin analogues typically inhibited syncytium formation in NDV-infected cell cultures (Hasan and Ahad, 2024; Mochamad et al., 2024).

Terpenoids, such as andrographolide derived from *Andrographis paniculata*, destabilize viral envelopes and actively interfere with fusion proteins, thereby diminishing viral entry efficiency (Figure 2; Nagajothi et al., 2020; 2023). Aqueous extracts of *Azadirachta indica* (neem) bark and leaf at concentrations ranging from 50% to 100% were confirmed to reduce NDV infectivity following pre-incubation with virions (8 HA units) for 30 minutes at 27.3°C. This reduction was assessed using a haemagglutination assay in nine-day-old embryonated chicken eggs. Notably, neem bark extract achieved complete viral inactivation at the undiluted concentration (Hasan and Ahad, 2024).

Saponins disrupt lipid bilayers due to their detergent-like amphipathic structure, rendering enveloped viruses, such as IBV, non-infectious before cell entry (Sarwar et al., 2022; Hasan and Ahad, 2024; Mochamad et al., 2024).

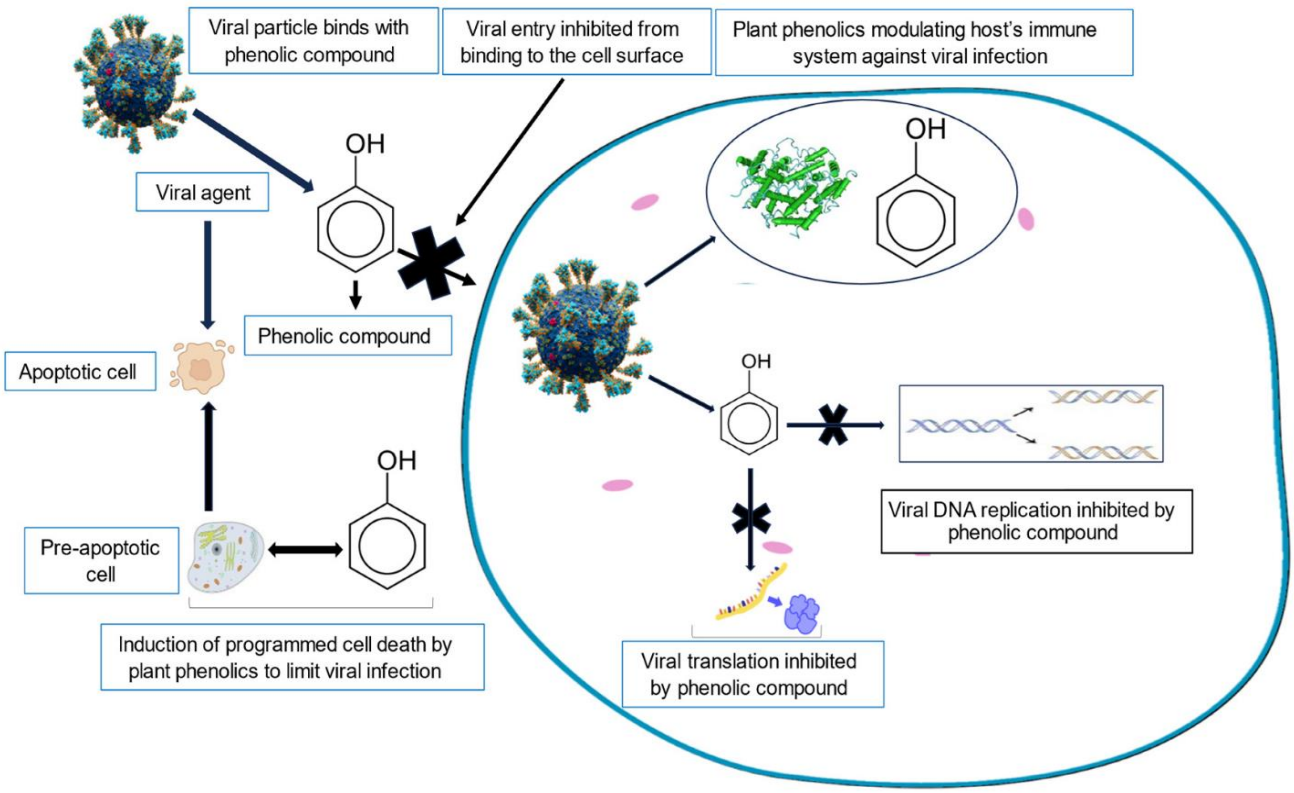


Figure 1. The mechanisms of action of the antiviral properties of phenolic compounds. Source of the image: Adeosun and Loots (2024)

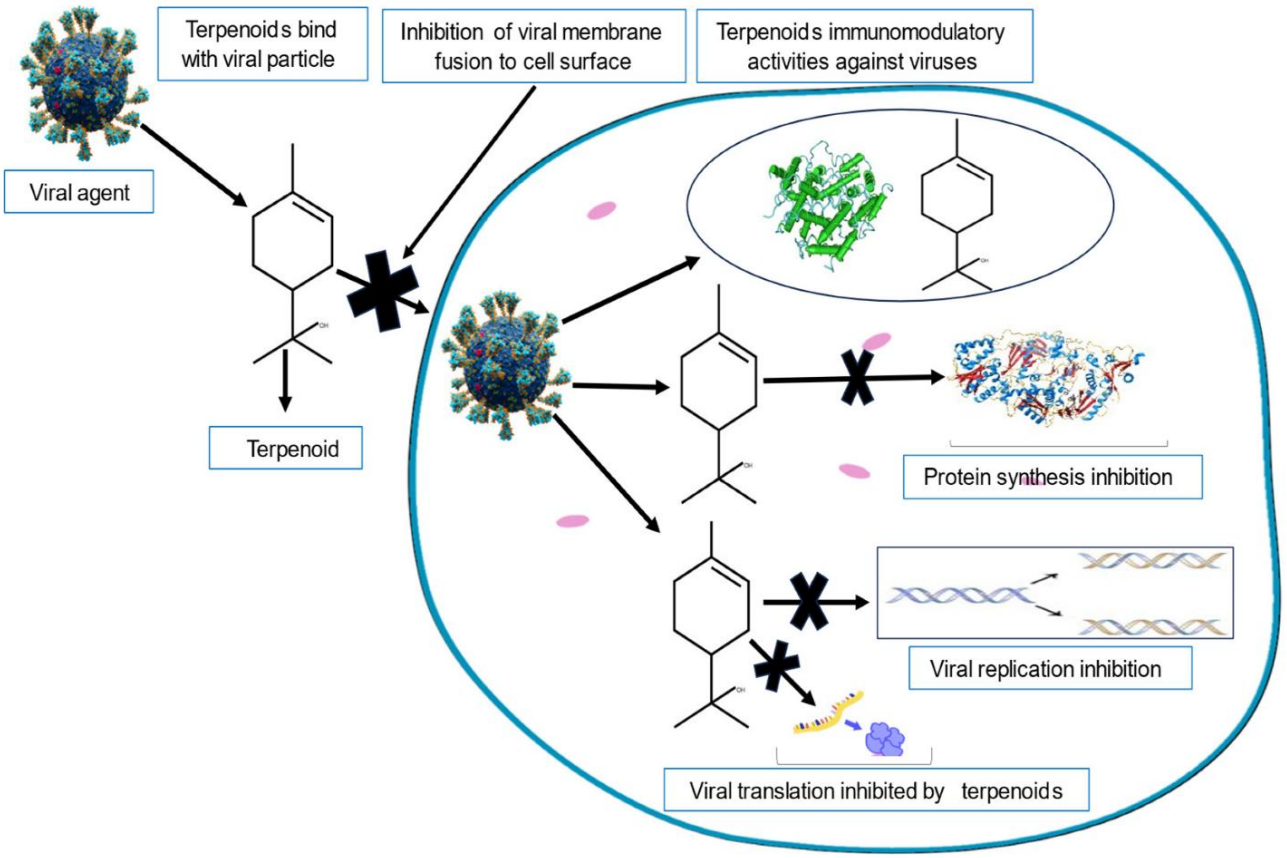


Figure 2. The mechanism of the antiviral activities of terpenoids. Source of the image: Adeosun and Loots (2024)

Suppression of viral polymerases and proteases

The replication of poultry RNA viruses primarily depends on RdRp and viral components (Phillips et al., 2023; da Fonseca et al., 2025). Inhibiting these enzymes directly prevents genome replication and protein maturation (Phillips et al., 2023; da Fonseca et al., 2025). *In silico* studies have demonstrated that flavonoids exhibit favorable docking interactions with essential NDV replication proteins, including the V protein. Quercetin and luteolin derivatives have been identified as potential inhibitors through molecular docking and molecular dynamics simulation (Sarwar et al., 2022). Piper longum extracts exhibited considerable immunomodulatory activity against NDV, enhancing HI antibody titers in broiler chickens (Karande et al., 2024).

Alkaloids such as berberine and piperine essentially interfere with nucleic acid synthesis and protease activity (Figure 3; Mohite et al., 2024; Kang et al., 2025). The ethanolic extract of Piper longum, administered orally at a dosage of 10-15 mg/kg body weight to commercial broiler chickens (strain 430-Y) from day 7 to 21, significantly enhanced HI antibody titers against NDV compared to the unvaccinated control group. This finding indicated the immunomodulatory activity of Piper longum (Sarwar et al., 2022; Karande et al., 2024). *In silico* studies showing that coumarin derivatives strongly bind to IBDV RdRp offered a plausible explanation for how coumarins inhibit viral proteases and polymerases (Sarwar et al., 2022; Karande et al., 2024). These findings indicated that phytochemical compounds can act as natural inhibitors of RdRp, similar to ribavirin but potentially with lower toxicity (Szotowska and Ledwoń, 2024; da Fonseca et al., 2025).

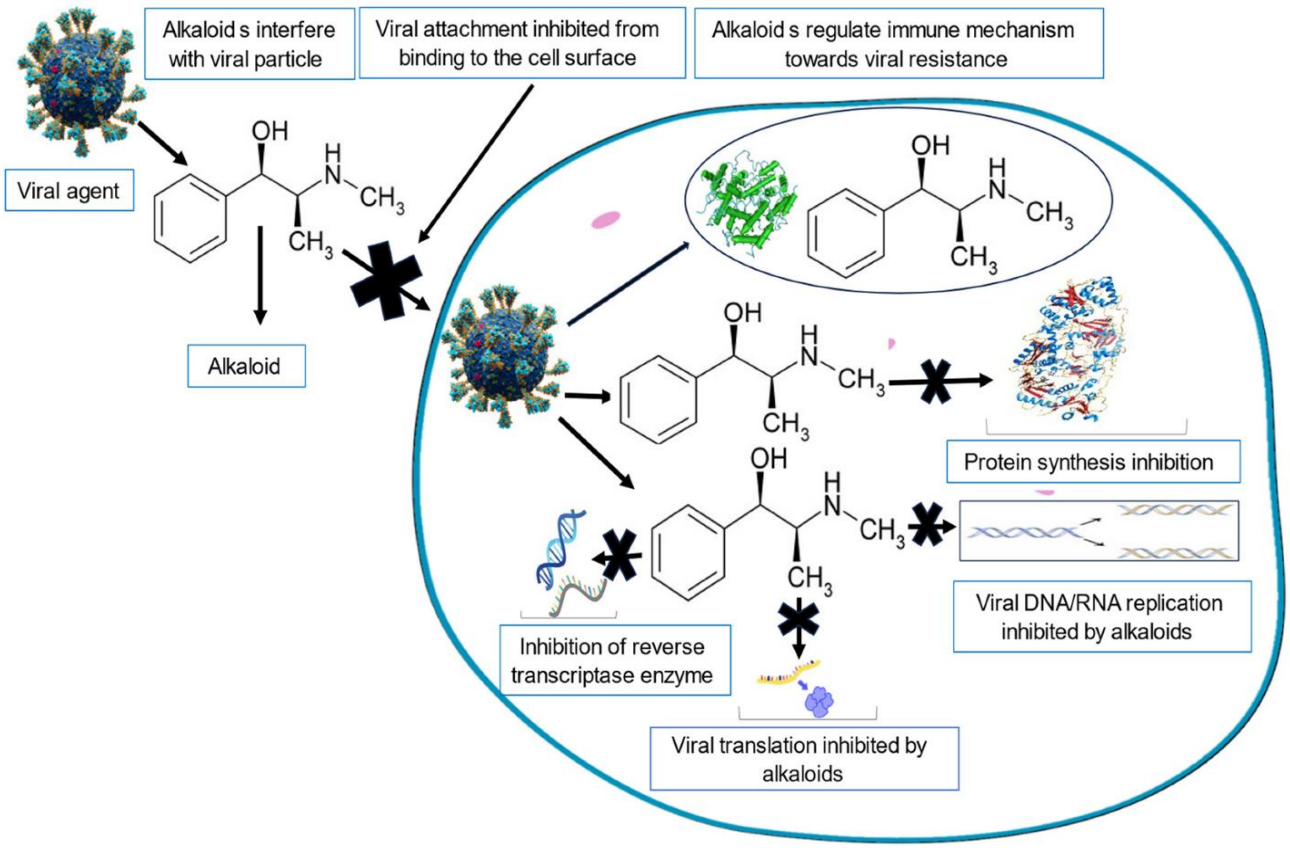


Figure 3. The mechanism of the antiviral activities of alkaloids. Source of the image: Adeosun and Loots (2024)

Modulation of host immune responses

A distinguishing characteristic of plant-based antivirals is their ability to enhance host immunity, often complementing or synergizing with vaccine-induced protection (Phillips et al., 2023; Adeosun and Loots, 2024; Tahir and Alsayeqh, 2024). Cytokine modulation by flavonoids and terpenoids mostly regulates interferon (IFN) pathways and pro-inflammatory cytokines (Intharuksa et al., 2022; da Fonseca et al., 2025). For instance, andrographolide reduces IL-6 and TNF-α production in infected tissues, mitigating immunopathology while preserving antiviral IFN-γ activity (Abd El-Ghany, 2024; El-Saadony et al., 2024; Shan et al., 2025). Adjuvant effects of saponins derived from *T. terrestris* and *Quillaja saponaria* are well-established as vaccine adjuvants in poultry, enhancing different mucosal Immunoglobulin A (IgA) and systemic Immunoglobulin G (IgG) responses against NDV and IBV (Abd El-Ghany, 2024; El-Saadony et al., 2024; Shan et al., 2025). Thus, phytochemical compounds not only inhibit viral replication but also boost both adaptive and innate immunity, which are vital for flock-level health resilience.

TRADITIONAL MEDICINAL PLANTS

***Glycyrrhiza glabra* (Licorice)**

Licorice root has actually been used in Ayurveda, Unani, and Chinese medicine for centuries (Wu et al., 2021; 2022). The primary active constituent, glycyrrhizin, has been extensively studied for its antiviral activity (Wu et al., 2021; 2022). Glycyrrhizin interferes with viral gene expression and replication, modulates host cytokine responses, and exhibits antioxidant properties (Wu et al., 2021; Abdel-Haleem et al., 2024). In mammalian systems, glycyrrhizin has demonstrated effectiveness against the hepatitis B virus and coronaviruses (Wu et al., 2021; 2022). In poultry, *in vitro* studies indicated the potential of *G. glabra* compounds to control viral diseases, particularly paramyxovirus infections (Wu et al., 2021; 2022). Glycyrrhizin has been reported to suppress paramyxovirus replication (Wu et al., 2021; 2022). *In vivo* investigations in broiler chickens have demonstrated that licorice polysaccharides enhanced macrophage phagocytic activity and specifically stimulate antibody production, thereby substantiating their application as an immunostimulant in poultry feed (Abo-Samaha et al., 2022; Abdel-Haleem et al., 2024). Given the well-established safety and traditional herbal use of *G. glabra*, it is a promising candidate for further investigation into antiviral applications in poultry (Abo-Samaha et al., 2022; Abdel-Haleem et al., 2024).

***Tephrosia purpurea* (Wild indigo)**

Tephrosia purpurea is widely used in Indian ethnomedicine, especially for its hepatoprotective, anti-inflammatory, and antioxidant properties (Athirstalaxmi et al., 2015; Choudhary et al., 2021). The phytochemical profile of *T. purpurea* root extract confirmed the presence of flavonoids, including tephrosin and rotenoid derivatives, as well as chalcones (Athirstalaxmi et al., 2015; Choudhary et al., 2021). Preliminary *in silico* studies strongly indicated that several *T. purpurea* phytochemicals, including tephrosin, rotenoid derivatives, and chalcones, have the potential to inhibit viral proteases (Youssef et al., 2023; Youssef et al., 2024). Despite a notable absence of direct poultry-virus data, particularly for IBV, the antioxidant and immunomodulatory activities observed in *T. purpurea* suggested its potential utility in studies aimed at inhibiting viral polymerases, boosting vaccine efficacy, and attenuating disease severity (Choudhary et al., 2021; Youssef et al., 2023).

***Cyperus rotundus* (nut grass)**

Cyperus rotundus is a perennial sedge used in Ayurvedic medicine to treat gastrointestinal disorders and inflammation (Samra et al., 2020; Kumar et al., 2021). Phytochemical profiling of *C. rotundus* essential oils revealed the presence of flavonoids, sesquiterpenes, polyphenols, and other bioactive compounds with antimicrobial and immunomodulatory effects (Samra et al., 2020; Ali et al., 2023). *In vitro* studies indicated that the ethanolic extract of *C. rotundus* exhibited weak to moderate antiviral activity against herpes simplex virus and SARS-CoV-2 (Kumar et al., 2021). Green-synthesized silver nanoparticles derived from *C. rotundus* extract exhibited antiviral efficacy against IBV (Abo-El-Yazid et al., 2022). Although there are few direct challenge trials against enteric poultry viruses (rotavirus, reovirus), the immunostimulatory properties and safety observed in growth studies make *C. rotundus* a promising candidate for further antiviral investigations in poultry (Abo-El-Yazid et al., 2022; Wigraiboon et al., 2024).

***Tribulus terrestris* (Puncture vine)**

Tribulus terrestris, used in Ayurvedic and Unani traditional medicine as an aphrodisiac and tonic, is notably rich in steroidal saponins, such as protodioscin and protogracillin, as well as different phytochemical compounds (Fekri Yazdi et al., 2014; Parham et al., 2020). The antiviral activity of *T. terrestris* crude extracts has been documented in non-poultry systems (Parham et al., 2020), and *in vivo* studies highlighted its effectiveness against the NDV (Malik et al., 2023). In poultry, limited studies have demonstrated that supplementation with *T. terrestris* powder significantly increases antibody titers against the NDV (Fekri Yazdi et al., 2014). The dual immunostimulatory and antiviral properties of *T. terrestris* make it a promising candidate for feed-based applications (Al-Garadi et al., 2022; Malik et al., 2023). The safety profile of *T. terrestris* requires careful evaluation, as some studies indicated that although certain doses are usually tolerated, high saponin levels may affect performance or cause histological changes (Fekri Yazdi et al., 2014; Al-Garadi et al., 2022).

***Boerhavia diffusa* (Punarnava)**

Boerhavia diffusa (Punarnava) is a widely recognized ethnomedicinal plant in India, valued for its hepatoprotective, anti-inflammatory, antioxidant, and immunostimulant properties (Manu and Kuttan, 2009; Wudali and Nagella, 2020). Phytochemical studies have confirmed that *B. diffusa* contains rotenoid compounds (boeravinones), flavonoids, lignans, and alkaloids, which are recognized in broad antiviral screens in non-poultry systems (Wudali and Nagella, 2020; Ashrafi et al., 2022). Compounds such as punarnavine (an alkaloid) have been shown to increase antibody titers, enhance

splenocyte proliferation, and lower pro-inflammatory cytokines in mice (Manu and Kuttan, 2009). In broiler chickens, adding *B. diffusa* ethanol extract at 1-10% (w/v) to drinking water, along with Costus afer, for 4 weeks enhanced hematological parameters (Nwokocha et al., 2013). The favorable safety profile and significant immunomodulatory activity of *B. diffusa* make it a promising candidate for future studies as a poultry feed supplement or vaccine adjuvant against viral diseases (Ashrafi et al., 2022; Wang et al., 2023). The phytochemical profiles, target viruses, antiviral mechanisms, and poultry health relevance of all five selected plants are collectively summarized in Table 1.

Table 1. Phytochemical composition, antiviral mechanisms, and poultry health relevance of five selected South Asian ethnomedicinal plants against poultry viral pathogens

Plant	Major compounds	Target viruses	Antiviral mechanism	Poultry relevance	Key references
Glycyrrhiza glabra	Glycyrrhizin	RNA viruses, NDV (potential)	Inhibits replication, modulates cytokines	Immunostimulant, candidate feed additive	Wu et al. (2021; 2022); Abo-Samaha et al. (2022); Abdel-Haleem et al. (2024)
Tephrosia purpurea	Flavonoids (tephrosin)	Polymerase-dependent viruses	Inhibits RNA polymerase	Potential NDV/IBV inhibitor	Athirstalaxmi et al. (2015); Choudhary et al. (2021); Youssef et al. (2023; 2024)
Cyperus rotundus	Flavonoids, terpenes	Enteric viruses	Immunomodulatory, antiviral	May protect against poultry enteric viruses	Samra et al. (2020); Abo-El-Yazid et al. (2022); Ali et al. (2023); Wigraiboon et al. (2024)
Tribulus terrestris	Steroidal saponins	Enveloped viruses, NDV, IBDV	Envelope disruption, immune adjuvant	Vaccine enhancer, prophylactic	Fekri Yazdi et al. (2014); Parham et al. (2020); Al-Garadi et al. (2022); Malik et al. (2023)
Boerhavia diffusa	Boeravinones	RNA viruses (HIV, model)	Integrase inhibition, immunostimulant	Poultry immune booster	Manu and Kuttan (2009); Nwokocha et al. (2013); Wudali and Nagella (2020); Ashrafi et al. (2022)

NDV: Newcastle disease virus, IBDV: Infectious bursal disease virus, IBV: Infectious bronchitis virus, FAdV: Fowl adenovirus, HIV: Human immunodeficiency virus, RNA: Ribonucleic acid, RdRp: RNA-dependent RNA polymerase

RELEVANCE TO POULTRY PRODUCTION AND VIRAL DISEASES

Infectious bursal disease virus

Infectious bursal disease virus targets the bursa of Fabricius, resulting in immune suppression that predisposes layers and broiler chickens to secondary infections (Kalaria et al., 2021; Toka and Geinoro, 2023). Antioxidant and immunostimulant phytochemical compounds, such as flavonoids from *Moringa oleifera* and curcuminoids from turmeric, protect lymphoid tissues by reducing oxidative stress and promoting lymphocyte recovery (Aderemi and Alabi, 2023; Morais et al., 2025). Saponins have exhibited considerable immunomodulatory potential in poultry concerning viral pathogens. Dietary supplementation with *T. terrestris* powder at 1 g/kg and 5 g/kg of feed has led to notably elevated antibody titers against NDV and avian influenza virus in broiler chickens (Fekri Yazdi et al., 2014). The crude extract of *T. terrestris*, containing 286.6 ± 3.16 µg/g of total saponins, demonstrated significant anti-NDV activity *in vitro* at 80 µg/mL. Haemagglutination assays conducted in embryonated chicken eggs revealed that pre-infection treatment decreased haemagglutinin titers from 320.0 ± 128.0 HAU (untreated controls) to 2.5 ± 1.0 HAU (Malik et al., 2018). Several studies confirmed that saponins not only enhanced IgA cell and iIEL numbers in the duodenum, jejunum, and ileum but also reduced bursal lesions in chickens challenged with IBDV, thereby supporting the observed protective effects (Saeed et al., 2024; Tahir and Alsayeqh, 2024).

Infectious bronchitis virus

The control of IBV is complicated by the emergence of variant strains and the limitations of vaccine cross-protection (Toka and Geinoro, 2023; Rafique et al., 2024). Essential oils and flavonoids with envelope-disrupting and polymerase-inhibiting activities, such as catechins and ursolic acid, may generally offer broad-spectrum antiviral protection (Yasmin et al., 2020; Tahir and Alsayeqh, 2024). Herbal feed additives such as neem (*Azadirachta indica*) and garlic (*Allium sativum*) have been reported to possess antiviral properties relevant to poultry respiratory pathogens. Neem extract

demonstrated significant antiviral activity against NDV and IBV in embryonated SPF chicken eggs, while garlic extract exhibited a dose-dependent inhibitory effect against influenza-related viruses, suggesting their potential to mitigate respiratory viral infections in poultry (Yasmin et al., 2020; Rafique et al., 2024).

Fowl adenoviruses

Fowl adenoviruses constitute a diverse group of non-enveloped DNA viruses responsible for conditions such as inclusion body hepatitis (IBH), hydropericardium syndrome (HPS), and gizzard erosion in poultry (El-Shall et al., 2022; Rashid et al., 2024). These diseases cause significant economic losses in broiler chickens and layers across Asia, with high mortality reported during outbreaks (El-Shall et al., 2022; Ishag et al., 2022). Unlike IBDV or IBV, FAdV infections often emerge in immunocompromised flocks, complicating control strategies (Ishag et al., 2022; Rashid et al., 2024). Vaccines against FAdV are neither widely accessible nor standardized, and vertical transmission complicates eradication efforts (El-Shall et al., 2022; Rashid et al., 2024). Traditional medicinal plants offer a promising supplementary approach against FAdV, particularly for their ability to improve liver function and modulate immunity (Adeosun and Loots, 2024; Tahir and Alsayeqh, 2024). *Glycyrrhiza glabra*, known for its hepatoprotective effects, might reduce liver damage caused by FAdV, as indicated by increased serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and creatine phosphokinase in FAdV-infected poultry (El-Shall et al., 2022; Abdel-Haleem et al., 2024). *Ocimum sanctum*, which is rich in ursolic acid and has documented antiviral and anti-inflammatory activity, may counteract FAdV-4-driven $CD4^+/CD8^+$ T-cell depletion and cytokine dysregulation (Rashid et al., 2024). Preliminary *in vitro* findings suggested that certain flavonoids and lignans interfere with adenoviral replication. For instance, flavonoids from *B. diffusa* inhibit RdRp activity *in vitro*. Additionally, *C. rotundus* flavonoid nanoparticles reduce IBV replication at an IC of 9.5 µg/mL in Vero cells. Moreover, podophyllotoxin-derived lignans inhibited DNA polymerase enzymes involved in adenoviral replication, but no trials have been conducted specifically with FAdV (Lawal et al., 2021; Abo-El-Yazid et al., 2022; Sarwar et al., 2022). Integrating phytogenic feed additives that target viral replication and support liver health could provide an effective strategy to reduce FAdV levels in poultry systems across South Asia (El-Shall et al., 2022; Rashid et al., 2024).

Practical applications in poultry production

Phytogenic feed additives

A primary application of medicinal plants in poultry health is their use as phytogenic feed additives (Abd El-Hack et al., 2022; Tahir and Alsayeqh, 2024). Incorporating *G. glabra* extract, *T. terrestris* powder, or *B. diffusa* leaf meal into broiler chicken and layer chickens' diets not only improved growth performance but also enhanced resistance to viral infections (Abd El-Hack et al., 2022; Jamil et al., 2022). Feed-based interventions are particularly appropriate for smallholder poultry farms in South Asia, given the irregular distribution of vaccines within the sector (Jamil et al., 2022; Pebam et al., 2022). Unlike injectable antiviral medications, feed additives provide prophylaxis for the entire flock without requiring extra labor (Abd El-Hack et al., 2022; Adeosun and Loots, 2024).

Herbal prophylactics and therapeutics

Polyherbal formulations, particularly mixtures of garlic, ginger, turmeric, onion, and Phyllanthus niruri, are commonly administered during suspected NDV or IBDV outbreaks in backyard poultry systems (Jamil et al., 2022; Pebam et al., 2022). These non-standardized, crude herbal preparations originated in culturally embedded practices but can often be refined into dependable herbal formulations (Jamil et al., 2022; Adeosun and Loots, 2024). Controlled trials have demonstrated that crude herbal preparations improved survival and performance metrics during outbreaks (Adjei-Mensah et al., 2023; Fouad et al., 2025). The immunostimulatory and hepatoprotective properties of *G. glabra* and *B. diffusa* are important for incorporation into these formulations (Wudali and Nagella, 2020; Abo-Samaha et al., 2022).

Vaccine synergy

The immunomodulatory and adjuvant effects of phytochemicals present a fundamentally practical approach to enhancing vaccine efficacy. For instance, saponin-rich extracts were utilized as adjuvants in experimental poultry vaccines, significantly enhancing both systemic and mucosal responses. This interaction is vital in areas where vaccine efficacy is compromised by inadequate cold-chain maintenance or increased pathogen variability.

Economic and sustainability considerations

From an economic perspective, plant-derived phytogenic feed additives, specifically *G. glabra* (licorice), *T. terrestris* (puncture vine), and *B. diffusa* (Punarnava), provide cost-effective interventions in comparison to synthetic medicines or recurrent vaccination campaigns (Abd El-Hack et al., 2022; Adeosun and Loots, 2024). *Glycyrrhiza glabra*,

T. terrestris, and *B. diffusa* are indeed cultivated or foraged throughout South Asia, making them cost-effective options for poultry farmers (Jamil et al., 2022; Pebam et al., 2022). The use of herbal feed additives aligns with growing consumer demand for green, antibiotic-free poultry products, thereby offering potential market advantages (Abd El-Hack et al., 2022; Tahir and Alsayeqh, 2024). Moreover, unlike synthetic antiviral medicines, plant-based products pose a lower risk of resistance development due to their multitarget mechanisms (Adeosun and Loots, 2024; da Fonseca et al., 2025).

From a sustainability perspective, medicinal plants can indeed be integrated into local agroecosystems (Jamil et al., 2022; Pebam et al., 2022). *Azadirachta indica* and *Moringa oleifera* are multipurpose tree species cultivated for food, fodder, and medicinal applications, thereby reinforcing circular farming systems (Abd El-Hack et al., 2022; Adjei-Mensah et al., 2023). This integration would reduce dependency on imported pharmaceuticals and strengthen local resilience (Pebam et al., 2022; Adeosun and Loots, 2024).

One health perspective

Plant-based antivirals are important not only for poultry health but also within the wider One Health approach (Adeosun and Loots, 2024; Tahir and Alsayeqh, 2024). Reducing viral outbreaks in poultry supports food security and economic stability in South Asia. Additionally, limiting the use of synthetic medicines decreases chemical residues in meat and eggs, reducing their potential entry into the human food chain (Abd El-Hack et al., 2022; Szotowska and Ledwoń, 2024). By mitigating immune suppression and secondary infections, phytochemicals generally reduce reliance on antibiotics, thereby addressing antimicrobial resistance (AMR), a shared global health concern (Adeosun and Loots, 2024; Tahir and Alsayeqh, 2024).

KNOWLEDGE GAPS

Limited *in vivo* studies on phytochemicals against poultry-specific viral pathogens

A significant gap exists due to the limited number of studies directly assessing antiviral activity against poultry-specific pathogens. While some plants, such as *G. glabra*, have demonstrated effectiveness against NDV *in vitro*, there are many fewer studies exploring their effects *in vivo* in poultry (Abo-Samaha et al., 2022; Wu et al., 2022). For viruses such as IBV, investigations remain limited, with most insights derived from studies conducted on non-avian model organisms, including murine and human cell lines.

Lack of standardization in plant preparations

Traditional medicinal plants are often prepared as crude extracts, decoctions, or feed additives, with inconsistent plant parts, solvents, or dosages (Jamil et al., 2022; Pebam et al., 2022). This inconsistency in choosing plant parts, extraction techniques, and dosages led to inconsistent results across studies and made reproduction difficult (Adeosun and Loots, 2024; Muñoz-Gómez et al., 2025). For instance, *G. glabra* extracts can differ significantly in glycyrrhizin content depending on whether roots, rhizomes, or aerial parts are used, with aqueous, ethanolic, or hydroalcoholic extraction yielding markedly different concentrations of bioactive constituents (Abo-Samaha et al., 2022; Wu et al., 2022). Similarly, *T. terrestris* saponin content varies considerably across growing regions, harvest stages, and formulation methods (Fekri Yazdi et al., 2014; Parham et al., 2020). Bioassay-guided fractionation and phytochemical profiling are essential for identifying active components, standardizing concentrations, and ensuring reproducibility across study groups (Adeosun and Loots, 2024; da Fonseca et al., 2025).

In vivo pharmacokinetics

No phytogenic compound can be approved for commercial poultry without first establishing safety and tissue residue data (Szotowska and Ledwoń, 2024). Yet few studies have actually examined the pharmacokinetics, tissue distribution, or excretion of antiviral phytochemicals in poultry (Adeosun and Loots, 2024; Muñoz-Gómez et al., 2025). Without safety and residue data, safe withdrawal periods for meat and eggs cannot be determined. The potential accumulation of bioactive compounds in edible tissues may pose health risks to consumers and generally complicate the export certification process (Szotowska and Ledwoń, 2024; Tahir and Alsayeqh, 2024).

Interactions with vaccines and diagnostics

Phytochemicals act as immunomodulators; however, their interactions with conventional vaccines remain poorly understood (Phillips et al., 2023). While some studies have reported increased antibody titers following phytochemical supplementation, not all compounds produce measurable enhancement of vaccine-associated humoral responses; for example, quercetin supplementation showed no significant increase in antibody titers in broilers (Abdel-Latif et al.,

2021). Furthermore, intense immune stimulation can affect diagnostic serology by elevating baseline antibody levels (Wu et al., 2021; Phillips et al., 2023).

Emerging opportunities: Nanotechnology and delivery systems

The bioavailability of phytochemicals is often compromised by rapid metabolism and instability (Phillips et al., 2023; da Fonseca et al., 2025). Nanotechnology-based delivery systems, such as liposomes, nano-emulsions, and polymeric nanoparticles, can improve solubility, stability, and targeted delivery of plant-derived antiviral agents (Chopra et al., 2021; da Fonseca et al., 2025). Curcumin nanoparticles demonstrated a tenfold increase in bioavailability compared to free curcumin. Furthermore, preclinical studies indicated that an 80 mg dose of nanocurcumin was more effective than a 500 mg dose of the free compound (Chopra et al., 2021). Additionally, nanocurcumin significantly decreased AST levels in broiler chickens at a dosage of 200 mg/kg. This finding suggested that nanotechnology-based delivery methods may facilitate the use of lower effective doses while achieving improved antiviral efficacy in poultry systems (Chopra et al., 2021). Adapting these methods for poultry could improve the delivery of phytogenic antivirals, enabling reduced, more uniform dosing with superior efficacy.

Regulatory and commercialization challenges

In many Asian countries, regulations for herbal feed additives and veterinary medicines are still evolving. In India, these herbal feed additives may be classified as Ayurvedic remedies or feed supplements; nevertheless, the regulatory framework lacks a clearly defined process for verifying claims related to viral disease control (Jamil et al., 2022; Pebam et al., 2022). In Bangladesh, there is currently no formal regulatory framework established for approving phytogenic compounds with antiviral claims for use in veterinary or poultry production. Consequently, manufacturers and farmers lacked standardized guidelines for safety, efficacy, and labelling. Without well-defined regulatory pathways for approval and standardization, investment in the commercial development and clinical validation of phytogenic antiviral agents for poultry remains severely limited.

Integrating ethnoveterinary knowledge with modern science

Ethnoveterinary knowledge provides a valuable foundation; however, its full potential can only be realized through integration with modern analytical and clinical methodologies. Future studies should use high-throughput screening and omics tools to test traditional plants against poultry viruses and to understand how these viruses affect host pathways. Molecular docking combined with artificial intelligence modelling should be used to predict plant-virus interactions and help prioritize *in vivo* experiments. Community-based participatory studies conducted in India and Bangladesh would be valuable for validating locally practiced ethnomedicinal interventions under controlled, reproducible conditions. By combining indigenous knowledge with cutting-edge science, a pipeline for novel poultry antivirals can be established.

CONCLUSION

Traditional medicinal plants of South Asia contain a rich diversity of phytochemicals, including flavonoids, terpenoids, alkaloids, saponins, and sulfur compounds, that exert broad-spectrum antiviral activity against major poultry pathogens such as NDV, IBDV, IBV, and fowl adenovirus. These effects are mediated by multiple mechanisms, including inhibition of viral entry, suppression of viral polymerases, immunomodulation, and antioxidant activity. The findings confirm that these medicinal plants can serve as affordable, residue-free feed additives for prevention or treatment, making them especially suitable for small-scale poultry farms with limited access to vaccines. Plant-based antiviral agents, confirmed through both ethnobotanical knowledge and contemporary virological methods, serve as credible options for maintaining poultry health and support the One Health approach sustainability. Future studies should focus on rigorous poultry trials that track how plant compounds move through the body and whether residues remain, while developing standardized formulations that meet safety regulations for real-world farm use. Furthermore, future studies should employ high-throughput screening and omics-based tools to systematically evaluate traditional medicinal plants against poultry viruses and to elucidate how these pathogens modulate host molecular pathways, enabling the rational development of evidence-based, plant-derived antiviral interventions.

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Authors' contributions

Rimee Dutta conducted the study, performed the data analysis, and contributed to manuscript preparation. Vijaya Sai Ayyagari provided supervision, assisted with data interpretation, and revised the manuscript. All authors have read and approved the final edition of the manuscript before publication in the World's Veterinary Journal.

Availability of data and materials

The data used and analyzed during the present study are available from the corresponding author upon reasonable request.

Competing interests

The authors declared that they have no competing interests.

Ethical considerations

Plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy have been checked by all authors. The authors also confirmed that no AI-assisted text generation tools were used in preparing this manuscript.

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Analysis of Anthropogenization and Transformation of the Human-Animal Bond in Ecuador

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ABSTRACT

The human-animal bond is currently experiencing a profound paradigm shift globally. The present study addresses the growing transformation of human–animal relationships in contemporary societies. The current study aimed to offer a critical narrative review of the anthropogenization of companion animals in Ecuador, understood as the process through which dogs, cats, and other domestic species acquire social, normative, and moral meanings that transform interspecies coexistence. Based on an interdisciplinary review of academic databases, regulatory documents, and institutional reports, the study integrates evidence from cognitive ethology, human–animal psychology, law, and urban sociology. Three central axes are analyzed. The first axis examined the impact of language, particularly ownership versus guardianship, on care practices. The second axis explores the tension between scientific evidence on animal agency and current regulations. The third axis evaluates the implications of anthropogenization for animal-welfare governance in diverse territorial contexts. The analysis identified contradictions between the biocentric constitutional framework and secondary legislation that retains possessive registers. The findings also revealed the simultaneous emergence of pathological anthropomorphization and positive anthropogenization processes. In addition, significant empirical gaps were identified for the design of coherent public policies. The study proposes an interdisciplinary research agenda and presents recommendations aimed at aligning terminology, education, and governance, with emphasis on territorial strategies and participatory approaches. Overall, the manuscript provides a conceptual framework to guide applied studies and regulatory reforms in countries characterized by sociocultural heterogeneity, such as Ecuador.

Keywords: Animal ethic, Animal right, Animal abuse, Anthropogenization, Education, Guardianship

INTRODUCTION

The relationship between humans and companion animals is undergoing a profound transformation in the 21st century, altering how societies conceive of this interspecies bond as a social, emotional, and moral relationship that cannot be reduced to traditional categories of ownership or utility (Jardat and Lansade, 2021). In high-income countries, advances in disciplines such as cognitive ethology, affective neuroscience, comparative psychology, and human-animal studies have demonstrated that species such as dogs and cats possess complex socio-emotional skills, agency, communicative competencies, and relational structures that challenge traditional conceptual frameworks based on instrumentalization or ownership (Ellis et al., 2024). The growing body of scientific knowledge has fueled international debates on the moral and legal status of companion animals, opening the door to new ways of understanding coexistence, human co-responsibility, and the role of animals as subjects with their own affective and cognitive experiences (Leconstant and Spitz, 2022). A theoretical and political reconfiguration of the human–animal relationship beyond classical anthropocentrism, prompting a critical reassessment of categories such as welfare, guardianship, agency, and animal dignity (Michel, 2023).

In parallel, multilateral organizations, professional associations, and local governments have attempted to adapt regulatory frameworks to emerging social and ethical sensitivities (Browning and Veit, 2022). Several European and North American cities have incorporated responsible pet ownership approaches, limited practices considered harmful, reproductive regulations, and evidence-based welfare policies (Contalbrigo et al., 2024). In addition, proposals have emerged to replace terms such as “owner” or “proprietor” with categories including “guardian” or “caretaker”, arguing that language shapes moral perceptions and care practices (De Castro et al., 2026). However, despite these efforts, recent literature agrees that regulatory advances have not resolved the structural tensions between patrimonial legal frameworks and contemporary sensibilities that recognize animal agency and subjectivity (Gutmann, 2024). International evidence has consistently demonstrated a mismatch between regulatory frameworks and their effective implementation, between public narratives and observed realities, and between societal expectations and the institutional capacity required to ensure comprehensive animal welfare (Villafañe-Ferrer et al., 2020).

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In Latin America, such tensions take on distinctive characteristics due to the coexistence of traditional perspectives with emerging animal rights movements, rapid urbanization, socioeconomic inequalities, and fragmented institutional systems (Yerbury and Lukey, 2021). Although several countries in Latin America have incorporated legal recognition of animals as sentient beings and updated animal protection laws, implementation remains uneven across jurisdictions, with documented deficiencies in enforcement mechanisms, limited availability of empirical data to support decision-making, insufficient institutional capacity to manage urban dog and cat populations, and the persistence of culturally rooted practices that vary significantly across territorial contexts (Zinsstag et al., 2023). Thus, the human-animal relationship in the region is characterized by profound heterogeneity, where forms of care, the meanings attributed to animals, and the state's capacity to regulate their welfare vary significantly between urban, peri-urban, and rural areas (Mota-Rojas et al., 2023).

Ecuador represents a relevant case for examining regulatory and socio-cultural tensions in the human–animal relationship. Although the country was a pioneer in recognizing nature as a subject of rights in its 2008 constitution, this biocentric framework has not been consistently translated into the domestic and urban sphere (Garate Amoroso et al., 2024). Most regulations concerning companion animals remain framed within civil-law categories of property, ownership, and control, which contrast with emerging social expectations that regard animals as affectionate members of the household (Gutmann, 2024). The coexistence of a progressive discourse on the rights of nature with a traditional treatment of domestic animals reveals a conceptual dissonance that hinders the development of coherent policies (Hiby et al., 2023). Even though several municipalities have developed ordinances to regulate coexistence, promote sterilization, and penalize mistreatment, these efforts have failed to articulate a comprehensive vision or resolve structural problems such as urban population management, the lack of inter-institutional coordination, or the absence of national animal welfare indicators (Gallo et al., 2022).

Legal and administrative language constitutes one of the domains where such dissonance becomes particularly evident. The persistence of terms such as “owner,” “holder,” or “proprietor” reflects cognitive frameworks rooted in a patrimonial conception of the human-animal bond (Helms and Bain, 2009). Although in other countries the discussion about adopting the terms “guardian” or “owner” has gained relevance, in Ecuador this debate has not yet translated into public policies or substantive regulatory reforms (Lapo-Bonilla et al., 2025). The persistence of outdated legal and conceptual frameworks limits the possibility of promoting profound cultural change and hinders the incorporation of contemporary scientific perspectives on animal agency, sentience, and subjectivity (Dawson et al., 2021). In addition, there is a lack of empirical studies that assess how terminology influences the perception of responsibilities, daily behavior, or decisions that affect the animal's well-being (Orr et al., 2023).

Understanding these challenges requires analyzing the phenomenon of anthropogenization not as a simple affective humanization, but as a multidimensional process through which companion animals acquire emotional, symbolic, cognitive, and normative meanings within the social structure (Ellis et al., 2024). Anthropogenization implies a reconfiguration of the human-animal bond that transforms animals into active participants in family, urban, and community life (Göttert and Perry, 2023). In Ecuador, this process manifests unevenly, with intense attachment practices coexisting in urban areas alongside utilitarian or functional relationships in other contexts, creating a heterogeneous landscape that has not yet been studied comprehensively (Benalcázar Jarrín et al., 2025).

Despite growing public attention to companion animals, Ecuadorian literature remains fragmented, as studies on citizen perceptions, evaluations of local ordinances, ethical reflections, and legal debates often develop along parallel paths without a unified conceptual framework to understand their interrelationships. This lack of integration makes it difficult to identify patterns, contradictions, omissions, and opportunities for more coherent animal welfare governance (Stucki, 2023). Therefore, the present study aimed to conduct an interdisciplinary critical narrative review to develop a conceptual framework for understanding the anthropogenization of companion animals and its sociocultural and normative implications in Ecuador.

MATERIALS AND METHODS

Sources and search strategy

The online search was conducted between October 2023 and November 2025 in Scopus, Web of Science, SciELO (Scientific Electronic Library Online), and Google Scholar, complemented with Ecuadorian institutional repositories, normative and legal documents, including the Constitution of the Republic of Ecuador (2008), the Organic Law of Animal Welfare (2022), municipal ordinances from different cities, and official reports such as those issued by the Council for the Protection of Rights of the Metropolitan District of Quito. Searches were conducted in English and Spanish using keyword combinations related to anthropogenization, anthropomorphization, possessive language, animal guardianship, urban animal welfare, and governance. Boolean operators (AND, OR) were used to structure queries

across titles, abstracts, and keywords. A representative search string used in Scopus and Web of Science was as follows ("companion animal" OR "pet" OR "dog" OR "cat") AND ("guardian" OR "owner" OR "proprietor" OR "holder") AND (terminolog OR language OR discourse) AND ("animal welfare" OR governance) AND (Ecuador OR "Latin America").

Inclusion criteria

To build a coherent interdisciplinary corpus, source selection included theoretical and empirical peer-reviewed articles addressing the conceptual, ethical, legal, and social dimensions of the human-animal relationship. The review also incorporated conceptual monographs, institutional reports, comparative legal analyses, and relevant Ecuadorian normative documents such as laws and municipal ordinances essential for understanding local governance and institutional practices.

Exclusion criteria

To maintain conceptual focus and methodological rigor, the review excluded medical and veterinary studies that did not explicitly address animal welfare, sentience, or the human-animal relationship. Studies focusing exclusively on commercial livestock production were also omitted to prioritize companion animals in urban contexts. Finally, documents lacking verifiable access or sufficient methodological transparency were excluded to ensure reliability.

Analytical procedure

Adapted from established methodological frameworks for thematic synthesis, the analytical procedure was structured into sequential stages starting with corpus mapping to identify and classify sources by thematic focus and discipline. The process was followed by thematic coding, which systematically organized the literature into key conceptual axes, including language and terminology, cognitive and ethological evidence, normativity and legal frameworks, territorial practices, and anthropogenization processes (Braun and Clarke, 2006). The resulting analytical structure, summarizing how these dimensions interact to shape human-animal relationships and animal welfare, is presented in Table 1. The third stage comprised interpretive integration, aimed at establishing analytical dialogues between international evidence and Ecuadorian documents. This phase emphasized the examination of similarities, divergences, and epistemological gaps, allowing for a contextualized understanding of how global debates are translated and adapted within the national framework. Finally, the fourth stage involved critical synthesis, through which a coherent narrative was constructed to conceptualize anthropogenization as a multidimensional and relational phenomenon. This synthesis highlights the interplay between cultural, legal, and affective dimensions, providing a comprehensive perspective on the transformation of the human–animal relationship.

Table 1. Key analytical axes, conceptual variables, and their implications in animal welfare

Analytical axis	Description	Key variables	Implications for animal welfare
Language and terminology	Examines the use of terms such as “owner” versus “guardian” and their cognitive effects	Ownership versus guardianship, discourse framing	Influences empathy, responsibility, and care practices
Cognitive and ethological evidence	Integrates findings on animal cognition and socio-emotional capacities	Agency, sentience, and communication abilities	Challenges objectification and supports moral consideration
Normativity and legal framework	Analyzes laws, ordinances, and institutional discourse in Ecuador	Legal status, regulatory coherence	Reveals contradictions between constitutional principles and practice
Territorial practices	Evaluates differences across urban, peri-urban, and rural contexts	Socioeconomic factors, cultural practices	Explains variability in welfare outcomes
Anthropogenization	Process of assigning social and moral meaning to animals	Emotional integration, social roles	Can improve welfare if aligned with biology
Anthropomorphization	Attribution of human traits to animals	Projection, emotional substitution	May be positive or pathological depending on context

FROM POSSESSIVE OWNERSHIP TO RELATIONAL GUARDIANSHIP

The use of the term "owner" to refer to those responsible for companion animals reflects a significant conceptual lag relative to contemporary developments in animal law and cognitive ethology (Sinclair et al., 2022). Although the category of animals as property originates from the 19th-century Spanish legal tradition, when animals were classified as movable property in pre-constitutional civil codes, the persistence of this legal classification in Ecuadorian legal and municipal discourse demonstrates the enduring legacy of an anthropocentric worldview (Garzón-Rosero and Chávez-Intriago, 2021). Legal historical studies in countries with a civil law tradition indicate that such classifications did not

emerge from ethical or bioscientific analysis, but rather from the accounting and administrative needs of agrarian and colonial societies (Hill et al., 2025). Ecuador, after the constitutional recognition of Pachamama in 2008, continues to use the same possessory registration system that was in place in 1861, which constitutes a normative inconsistency that international literature identifies as a "structural dissonance" (Chugá-Elizalde et al., 2024).

Empirical evidence demonstrates that such dissonance is not merely symbolic but performative, as language has the capacity to shape ethical attitudes (Koppel et al., 2025). Recent psycholinguistic studies indicate that the use of possessive categories when referring to sentient beings is associated with reduced empathy, increased tolerance toward harm, and the reinforcement of utilitarian cognitive frameworks (Ding and Zhang, 2023). In Ecuador, the relationship between the language used to refer to animals and empathy toward them is evident. Recent studies have shown that the use of terms that imply possession, such as "owner," can reduce empathy toward animals, while more neutral terms or those that suggest responsibility, such as "guardian," can foster more prosocial attitudes and a greater willingness to assume ethical responsibilities (Ijichi et al., 2020).

A correlation between the persistence of possessive language and adverse animal welfare outcomes is also observed at the territorial level; in the Metropolitan District of Quito, where such language remains present in community discourse and local ordinances, high rates of abandonment and mistreatment of companion animals have been recorded (Municipality of Quito, 2020). According to the final monitoring report on the implementation of public policies for the prevention of companion animal abuse, it was documented that, during the period from May 2021 to August 2022, the number of companion animals found abandoned or poisoned reached alarming figures, highlighting the need for a change in the perception and treatment of these beings (Consejo de Protección de Derechos del DMQ, 2023). This pattern coincides with international findings that identify links between linguistic objectification and animal abandonment (Fryer, 2022).

Beyond the psychological realm, animal cognitive science provides compelling evidence to question the ontological validity of the category of owner (Nematipour et al., 2022). Dogs and cats possess advanced socio-emotional skills, including recognizing human intentions, episodic memory, interpreting affective cues, and interspecies communication abilities (Pickersgill et al., 2023). Although Ecuadorian studies remain limited in methodological scope and national coverage, they corroborate previous international evidence; a literature review on animal welfare in Ecuador conducted by Villamar (2023) indicates that emotional sensitivity in mixed-breed dogs is comparable to that described in European research, highlighting the importance of considering emotional well-being in the local context. In this scenario, continuing to reproduce terms derived from the legal regime of movable property renders cognitive abilities invisible and perpetuates a classification framework incompatible with contemporary scientific evidence.

Ecuadorian jurisprudence is beginning to reflect this conceptual tension. In Ruling No. 253-20-JH, the Constitutional Court affirmed that animal welfare must be considered in the regulation of property rights, aligning itself with the Constitution's biocentric vision, which recognizes the intrinsic importance of living beings and their relationship to human and environmental well-being (Grijalva, 2025). An epistemic shift, also discussed in Anglo-Saxon literature, is reflected in the evolution of the term "owner" toward "guardian," where the figure of the guardian has been consolidated as an appropriate representative to defend the interests of animals in various jurisdictions, emphasizing responsibility and welfare rather than mere ownership (EMIRI, 2024). However, in Ecuador, this transition is still incipient, as more than 40% of the regulatory articles of the Organic Law on Animal Welfare (2022) maintain possessive language, which generates contradictions with the constitutional spirit and with the international scientific body of knowledge (Vernaza Arroyo and Cruel Preciado, 2024).

Given the persistence of possessive language in Ecuadorian regulatory frameworks, the proposed terminological alternatives, guardian and responsible keeper, are not mere semantic substitutions but rather conceptual tools with demonstrable practical implications. Comparative literature on animal welfare has shown that relational models based on guardianship lead to significant increases in caring behaviors, regulatory compliance, and adherence to health campaigns (Penaforte et al., 2024). Cities that have adopted "guardian" and "responsible ownership" models have shown increased awareness of civic responsibility toward animals (Zoanetti et al., 2024). In Ecuador, although with regional variations, progressive regulatory trends are evident, as a recent study reports that many local jurisdictions have begun adopting more inclusive terms in their ordinances since the second half of the last decade (Tănăsescu et al., 2024). These findings suggest that the transition toward more animal-centered legal terminology is occurring unevenly across the country, reflecting differences in the pace of regulatory adoption among local jurisdictions (Méndez Juez et al., 2020).

The practical effects of this terminological shift are beginning to emerge. In Latin America, numerous scientific studies have demonstrated that territorial differences in animal welfare practices and responsible pet ownership reflect structural patterns associated with educational level, degree of urbanization, and availability of cultural capital (Gallo et al., 2022). Recent studies have shown that the adoption of responsible practices (sterilization, vaccination, and registration) is significantly higher in urban areas with greater socioeconomic development and access to services, while

peri-urban and rural areas continue to experience gaps linked to education, poverty, and territorial inequality (Atero et al., 2024).

The integrated interpretation of available evidence suggests that terminology has a profound structural effect on the animal welfare ecosystem (Cornish et al., 2023). Language influences how responsibilities are distributed, how institutions design policies, and the type of affective relationship established with animals (Piermattei et al., 2024). From an ethical perspective, omitting such a shift means ignoring the growing international consensus that human–animal relationships should be based on models of shared responsibility, not rigid hierarchies of ownership (Bergmann, 2019). From a sociological perspective, maintaining the figure of the owner perpetuates colonial patterns of domination over living beings, while the transition to relational categories fosters civic practices more compatible with the Ecuadorian constitutional paradigm (Kauffman and Martin, 2018). Thus, the analysis revealed that Ecuador was at a critical juncture in which it must either deepen the conceptual coherence demanded by its Constitution by adopting language that recognizes animals as subjects of protectable interests or maintain a semantic system that reproduces legal tensions, ethical contradictions, and negative outcomes in public policy. The adoption of the concept of guardianship, or responsible guardianship, far from being a superficial gesture, constitutes a necessary strategy for achieving normative, cognitive, and cultural alignment in modernizing the country's animal welfare system.

ETHICAL, SOCIAL, AND PRACTICAL IMPLICATIONS IN ECUADOR

The ethical, social, and practical implications of replacing the term "owner" with "guardian" or "responsible caretaker" become particularly significant in the contemporary Ecuadorian context, where a biocentric constitutional framework coexists with cultural practices that reproduce historical logics of interspecies domination (Viviani, 2023). Far from being a mere linguistic flourish, recent literature argues that this terminological shift constitutes a structural variable capable of modifying moral behavior, community organization, and institutional efficiency (Henning et al., 2023). Indeed, studies in animal ethics have demonstrated that relational categories generate a greater internalization of ongoing moral obligations, given that the term "guardian" cognitively activates the notion of permanent custody and representation of the animal's interests, rather than powers of disposition and control (Kiani et al., 2022). Consistent with findings in comparative moral psychology, participants who use relational categories showed a greater willingness to anticipate the animal's potential suffering, increasing the likelihood of preventive behaviors such as regular veterinary care and sterilization (Prato-Previde et al., 2022). In other words, while the category of "owner" describes a dynamic unilateral power, the guardianship paradigm operates as a bidirectional normative and emotional bond in which the satisfaction of the animal's needs constitutes a central axis of the responsible person's moral identity.

The impact of the terminological shift extends to the social sphere as well. Several multicenter studies conducted in Latin America demonstrate that institutional linguistic frameworks shape community norms and patterns of caregiving behavior (Mota-Rojas et al., 2023; Piermattei et al., 2024). Cities that have systematically incorporated the notion of guardianship or caregiving into educational campaigns, regulations, and health programs report significant increases in citizen participation in vaccination, registration, reporting of abuse, and sterilization processes (Beach et al., 2022). Conversely, territories where discursive models based on ownership persist exhibit higher rates of functional abandonment and uncontrolled reproduction. The phenomenon is explained in the literature as the "cultural normalization of functional detachment," whereby the relationship with the animal shifts toward utilitarian logics when the linguistic category suggests availability and replaceability (Bicalho et al., 2024). In Ecuador, the relevance of this phenomenon is intensified by pronounced socioeconomic and territorial disparities, which condition access to veterinary care, responsible ownership practices, and the institutional enforcement of animal welfare regulations. Municipal observations have documented that peri-urban and urban-marginal sectors with a higher prevalence of possessive discourse are also those that exhibit a higher incidence of abandonment, violence, and unregulated breeding, suggesting that institutional language is a modulator of social behavior and not a simple neutral descriptor (Cornish et al., 2016).

The practical implications of this shift in conceptual framework are clear in public health and territorial governance. Contemporary epidemiological literature indicates that population control and zoonosis prevention programs demonstrate significantly improved outcomes when responsibility for the animal is conceptualized in terms of explicit co-responsibility between citizens and the state (Langlois et al., 2025). The term "guardian" allows for the operationalization of specific obligations (registration, vaccination, sterilization, and monitoring) and facilitates traceability in cases of abandonment or zoonotic transmission, reducing the administrative burden of municipal oversight (Balogh et al., 2023). Ecuador currently faces a critical transition in animal welfare governance due to the implementation of the Organic Law on Animal Welfare (2022), which requires greater conceptual and institutional coherence in the regulation of human–animal relationships. The situation remains hampered by the ambiguity of possessive language, which fails to clarify enforceable duties and allows for the informal transfer of animals without sanitary control, a situation widely documented in large cities (Kauffman and Martin, 2018). In this regard, incorporating

the relational category would not only align legislation with the constitutional mandate for nature protection but would also strengthen the operational capacity of local governments, particularly in jurisdictions with limited public health infrastructure (Enlund et al., 2020).

Contrary to the common belief that the guardianship model is culturally "elitist" or "restrictive", recent anthropological and ethnographic studies indicate that Indigenous and peasant communities do not resist this relational framework, provided it is grounded in notions of reciprocity and co-responsibility, which are elements present in traditional worldviews that do not regard animals as replaceable commodities, but rather as beings integrated into familial, productive, or spiritual structures (Calle Alzate, 2025). Such evidence suggests that terminological shifts have the potential to reduce territorial inequalities, rather than exacerbate them, if communication and regulatory strategies recognize the country's cultural plurality. Linguistic change, therefore, becomes a tool for epistemic and territorial justice, insofar as it promotes relational models consistent with diverse cultural rationalities, avoiding the imposition of decontextualized urban meanings.

The integrative analysis of the available evidence suggests that the conceptual shift toward responsible guardianship not only resolves a regulatory inconsistency but also acts as a social technology capable of transforming public ethics, community behaviors, and the effectiveness of animal welfare health policies in Ecuador (Calandrini and Almeida, 2026). Terminological decisions shape the distribution of responsibilities, the design of institutional policies, and the symbolic frameworks through which society evaluates the acceptability of neglect and mistreatment (van der Staay et al., 2025). From a scientific perspective, maintaining possessive terminology perpetuates a relational model associated with poorer health, ethical, and community outcomes, whereas adopting relational terminology contributes to greater coherence between legal frameworks, public health strategies, and contemporary animal welfare evidence (Meisner et al., 2024).

PATHOLOGICAL ANTHROPOMORPHIZATION AND POSITIVE ANTHROPOGENIZATION OF PETS

The contemporary trend of attributing human emotions, intentionality, and psychological traits to companion animals, termed anthropomorphization in the specialized literature, requires urgent critical review. It has traditionally been assumed that "humanizing" animals has homogeneous and generally positive effects; however, recent empirical evidence demonstrates that not all forms of anthropomorphization produce the same results in terms of animal welfare, public health, ethical responsibility, or social coexistence (Meisner et al., 2024). Thus, it is necessary to conceptually differentiate the ways of "humanizing" pets, since some strengthen animal well-being and social co-responsibility, while others generate detrimental effects that translate into behavioral, health, and regulatory dysfunctions. It is also necessary to analytically distinguish between pathological anthropomorphization and positive anthropogenization (Vezirian, 2022). The former refers to a dysregulated projection that turns the animal into a human substitute or an object of maladaptive and functionally regressive affective relationships; the second describes an informed humanization, attentive to ethological and biosocial evidence, that reconfigures ownership practices toward models of co-responsibility and care that respect the animal's biology and best interests. This distinction is not merely semantic, as it has observable causal consequences in animal and human health indicators, management practices, and policy formulation.

It is also necessary to analytically distinguish between pathological anthropomorphization and positive anthropogenization, as these processes differ significantly in their conceptual basis, practical implications, and impact on animal welfare.

As shown in Table 2, pathological anthropomorphization is primarily driven by emotional projection and may result in practices that compromise animal health and behavior, whereas positive anthropogenization is grounded in scientific evidence and promotes responsible, welfare-oriented interactions.

Table 2. Comparative framework between pathological anthropomorphization and positive anthropogenization

Dimension	Pathological anthropomorphization	Positive anthropogenization
Conceptual basis	Emotional projection of human traits	Evidence-based relational understanding
View of the animal	Human substitute or symbolic object	Sentient being with specific needs
Care practices	Overfeeding, confinement, and misinterpretation of behavior	Ethological care, proper diet, and behavioral respect
Welfare impact	Increased stress, health problems	Improved physical and emotional well-being
Human motivation	Emotional dependency or substitution	Ethical responsibility and co-responsibility
Public health impact	Increased zoonosis risk, poor management	Better compliance with vaccination and control programs
Policy relevance	Leads to ineffective or harmful practices	Supports sustainable animal welfare policies

Pathological anthropomorphization is characterized by the emotional instrumentalization of the bond, whereby the animal comes to play roles that overburden its well-being, such as an unregulated therapeutic surrogate, an exclusive commensal with inappropriate diets, or a "child" whose behavior is interpreted solely through human psychological categories. Excessive anthropomorphic interpretations may promote management practices that are inconsistent with species specific behavioral and physiological needs, including inappropriate feeding, restriction of natural behaviors, and misunderstandings of behavioral signals. Such practices have been identified as potential risks to animal welfare when human expectations supersede ethological requirements (Serpell, 2003; Bradshaw et al., 2009). Recent studies have also shown that anthropomorphism is associated with the attribution of human emotions, intentions, and social roles to companion animals (Bouma et al., 2023).

In contrast, positive anthropogenesis is a deliberate and normative process. It involves incorporating elements of human understanding, such as empathy, recognition of agency, and moral consideration, in dialogue with animal cognition and welfare rather than replacing animal cognition and welfare. A productive form of "humanizing" requires redefining ownership practices to address ethological needs (exercise, cognitive stimulation, appropriate diet, socialization) while simultaneously recognizing legitimate emotional bonds that, if managed knowledgeably, enhance adherence to preventive measures (vaccination, registration, sterilization) and lead to better public health outcomes (a reduction in the number of stray dogs and improved epidemiological traceability; Bouma et al., 2023). Multicenter literature showed that frameworks that emphasize co-responsibility rather than ownership promote higher rates of health compliance and community participation, suggesting that informed humanization can be a lever for positive intervention (Forbes, 2018).

This dual typology allows us to dismantle myths, as not all humanization is harmful and not all dehumanization is benign (Golossenko et al., 2023). From a critical perspective, pathological anthropomorphization is not an act of elevated affection, but rather the reconfiguration of old power asymmetries under a contemporary emotional aesthetic (Butterfield et al., 2012). In urban environments, this form of relationship often emerges as a compensatory mechanism for emotional voids or human social deficiencies, and in doing so, it establishes dynamics of control over the animal that ultimately erode its well-being (Bradshaw and Casey, 2007). Instead of promoting autonomy and environmental conditions compatible with its species, the relationship is organized around the human need for emotional possession, which legitimizes harmful practices such as prolonged confinement in indoor spaces, the imposition of diets geared toward appearance rather than health, or aesthetic and reproductive interventions without medical or ethological justification (Serpell and Serpell, 2002). A form of anthropomorphization, far from elevating the animal's moral status, subordinates it to the symbolic expectations of the guardian, producing an apparent well-being that masks structural suffering (Mota-Rojas et al., 2021). Furthermore, certain forms of close human–animal interaction and inappropriate management practices, including inadequate feeding, insufficient hygiene measures, and poor parasite control, may increase the risk of zoonotic disease transmission and generate challenges for public and veterinary health systems Overgaauw et al., 2020). Anthropomorphic perceptions can reinforce some of these practices when human emotional expectations take precedence over species specific biological and welfare requirements. Similar concerns have been highlighted in recent reviews examining the intersections among anthropomorphism, human health, and municipal health management (Nardi et al., 2025).

From a theoretical and methodological perspective, it is useful to approximate pathological anthropomorphization and positive anthropogenization using measurable indicators. Four evaluation axes could be proposed, encompassing ethological adjustment as the degree to which care practices respect natural behavioral repertoires and circadian rhythms, objective health including morbidity, mortality, zoonosis records, and the use of veterinary services, normative coherence understood as the correspondence between legal or municipal language ("guardian" versus "owner") and recorded practical obligations, and relational sustainability defined as the stability of the bond without pathological burdens, measured using attachment and cohabitation scales. Each axis can be operationalized using validated instruments (anthropomorphism scales, health records, local policy audits, and ethological sampling), allowing for the evaluation of whether a policy or public campaign is promoting positive anthropogenization or fostering pathological anthropomorphization. These methodological proposals connect psychometric evidence with epidemiological data and policy analysis, following a One approach Expanded Health (Prato-Previde et al., 2022).

Scientific progress involves recognizing limitations and potential unintended consequences, such as positive anthropomorphism, which, if not linked to territorial equity policies, can remain confined to urban settings and marginalize legitimate rural or community practices. Furthermore, the psychology of pet attachment can lead people to resist welfare measures when they perceive them as restrictions, for example, when access to certain spaces is limited or when new rules of coexistence are imposed. Therefore, an applied research protocol is proposed based on pilot studies conducted in heterogeneous cantons, incorporating pre- and post-intervention measurements using anthropomorphism and welfare scales, analysis of municipal health indicators, and a qualitative ethnographic evaluation to capture local

interpretations of the terminological shift. Only through robust empirical evidence can it be validated that positive anthropomorphism is a sustainable strategy and not simply a new form of anthropomorphism with a benevolent label (Whiting, 2011).

COMPANION ANIMALS IN SOCIAL, EMOTIONAL, AND WELFARE CONTEXTS

The conception of companion animals as mere objects of ownership has been profoundly questioned in recent decades (Pongrácz and Dobos, 2023). Various disciplines have documented, using different methodologies and with varying degrees of rigor, the psychosocial, emotional, and community effects that emerge from the human-animal bond (McNicholas and Collis, 2000; Purewal et al., 2017). In this context, the term "companion animal" has evolved, establishing itself not only as an emotional luxury but also as a potential agent in promoting well-being, sociability, and emotional support, particularly in urban environments where alienation may be prevalent (Holland et al., 2022).

Kretzler et al. (2022), in a systematic review on pet ownership, loneliness, and social isolation, reported that pet ownership in adults was generally associated with lower levels of perceived loneliness and social isolation, particularly during the COVID-19 pandemic. Similar findings were observed by Kogan et al. (2021), who documented lower levels of depression, anxiety, and perceived isolation among dog and cat caregivers during lockdown periods, together with improved maintenance of daily routines and psychological well-being. However, Mueller et al. (2021) did not identify a universal protective effect in adolescents, suggesting that the potential benefits of pet companionship depend on contextual and individual factors, including the quality of the human–animal bond, living conditions, emotional attachment, and social environment. Consequently, current evidence supports a conditional rather than universal effect of pet companionship on emotional well-being and social connectedness (Azkona, 2025; Li and Wong, 2025).

During the pandemic, multiple studies reported that dogs and cats were associated with lower levels of depression, anxiety, and perceived isolation, while also supporting the maintenance of daily routines and a sense of purpose, particularly among young women with a close attachment to their pets (Zachariadou and Tragantzopoulou, 2025). However, current empirical evidence remains inconsistent across populations and study designs. A recent study focused on individuals diagnosed with severe mental disorders, such as bipolar disorder and psychosis, found no significant differences in well-being, depression, anxiety, or loneliness between those who had pets and those who did not (Barr et al., 2024). The authors caution that the perceived emotional bond with pets is not enough to guarantee well-being, as factors such as material costs and the stress of care, as well as housing conditions and prior mental health, play a determining role (Northrope et al., 2025). Furthermore, a longitudinal analysis of a national cohort revealed that, in certain subgroups, cat ownership was associated with increased social isolation and loneliness, particularly among men; in contrast, dog ownership was linked to lower levels of life satisfaction in women (Hansen et al., 2024). This demonstrated that the effects of pet ownership can vary according to gender, animal species, family structure, and life trajectories, highlighting the need for caution when generalizing universal benefits (Zhao et al., 2025).

From a social perspective, companion animals have also been analyzed as facilitators of social cohesion (Ru et al., 2025). In many urban neighborhoods, dog walks generate chance encounters between neighbors, promote socialization, increase the sense of community, and, in some cases, act as catalysts for informal support networks, especially in societies where urbanization and social fragmentation erode traditional bonds (Wang, 2023). This sociocultural role has been identified as an important element for understanding the function of pets in contemporary modernity.

From the fields of ethology and animal welfare, it has also been suggested that responsible pet ownership, including vaccination, health checks, adequate space, and respect for the specific needs of each species, can be an important factor in public health (Tayde, 2025). Responsible pet ownership, coupled with education, can prevent negative effects such as abandonment, mistreatment, overpopulation, animal stress, and the transmission of zoonotic diseases (Overgaauw et al., 2020). Several recent reviews on the "human-animal relationship" propose that ethical and informed coexistence can benefit both humans and animals, contributing to a multispecies welfare model (Gonçalves and Cerqueira, 2024). However, current evidence is limited by significant methodological and contextual variability. Methodological heterogeneity (cross-sectional versus longitudinal studies, self-reporting versus representative sampling), cultural differences between countries, the scarcity of studies in diverse Latin American and socioeconomic contexts, as well as the variability among species and their contextual specificities, all affect the external validity of the findings (Gvirtz and Sabherwal, 2024). It implies that any assertion must be nuanced, as pets can function as companions, but this depends on specific conditions. Thus, when considering the figure of the “companion animal” in contexts such as Ecuador, the debate cannot be limited to an affective idealization. It is essential to adopt a critical, interdisciplinary, and empirical perspective that recognizes the complexity of the phenomenon, as individual, structural, cultural, and animal welfare variables must be an integral part of this analysis (Jacobsson and Hansson, 2014). Such an approach demands a situated analytical framework that includes representative surveys, comparative longitudinal studies, gender and territorial context analysis, public policy evaluation, health and ethological monitoring, and multidimensional (human-animal)

welfare monitoring. Only through such methods can the true dimension of the human-animal bond be rigorously assessed, especially in Ecuador and Latin America.

ANIMAL WELFARE GOVERNANCE IN ECUADOR

The shift from possession to relational guardianship and the ethical importance of language in shaping human behavior toward animals inevitably leads to a critical issue, namely, the governance of animal welfare in Ecuador. The actual effectiveness of regulations can only be understood by analyzing how institutions translate (or fail to translate) this conceptual transition into sustainable public policies. In operational terms, governance becomes the arena where terminological change, positive anthropogenesis, and public ethics must be realized (Cornish et al., 2016).

Ecuador has made rapid progress in legislative production since the 2008 Constitution, which recognized nature as a subject of rights and laid the groundwork for the creation of the Organic Law on Animal Welfare (2022), in addition to numerous municipal ordinances related to the care, registration, and prevention of animal abuse (Wuraola and Wuraola, 2025). This legal framework has placed the country in a prominent position in the region, in line with international trends in the legal protection of animals (Lostal et al., 2024). However, the contrast between legal development and practical application reveals a persistent pattern of structural inconsistency: legislation is passed quickly, but the necessary institutional and sociocultural conditions to transform citizens' practices are not in place.

Territorial policy evidence reveals a regional disparity. In the Sierra region, policies focus on controlling the population of stray dogs, while on the Coast, public health interventions and zoonotic diseases predominate (Jiménez-Quito and Durán-Ramírez, 2024). In the Amazon, regulatory initiatives show significant shortcomings, partly because they ignore traditional human-animal management models present in Indigenous worldviews (Kelsey and Gilbert, 2023). These findings suggest that the country's cultural and socioeconomic heterogeneity is not being addressed by a flexible and participatory regulatory framework. Instead, regulations are designed from a centralized perspective that assumes animal welfare is a homogeneous phenomenon, when it is configured differently in each territory (Fernandes et al., 2019). Adding to this fragmentation is another critical factor, namely the political motivations underlying the creation of regulations. Various local ordinances have been driven by agendas of political visibility rather than by in-depth empirical analyses, resulting in performative legislation. This phenomenon has already been described in environmental governance and is now being replicated in animal welfare, where laws are enacted to convey public commitment, not necessarily to address complex social challenges (Lostal et al., 2024). The result is an overproduction of regulations with low adherence and limited real impact. Such evidence aligns with the findings in Quito, where, despite having an advanced ordinance on animal welfare, abandonment rates remain critical due to insufficient internalization of the regulations, rather than a lack of regulations altogether (Consejo de Protección de Derechos del DMQ, 2023).

Comparative analysis indicates, however, that the gap between legislation and outcomes can be reduced and is not an inherent feature of regulatory systems. In Ecuador, studies on collaborative sterilization campaigns between academia and animal welfare organizations show significant improvements in participation rates and health coverage when regulatory implementation is accompanied by community education and citizen participation (Rodriguez-Pazmiño et al., 2024). This result aligns with patterns described in international studies showing that adherence to animal welfare regulations increases when citizens perceive ethical co-responsibility rather than punitive imposition (Mellor and Uldahl, 2025).

Therefore, animal welfare governance in Ecuador cannot be evaluated solely in legal terms. Its progress and limitations can only be understood when three variables are considered simultaneously, namely the ethical internalization of the new relational paradigm of guardianship, the sociocultural and territorial factors that shape the perception of what it means to “care” for an animal, and the institutional capacity to translate regulatory development into sustained, funded, and evaluated policies. Where these three dimensions align, the results are positive, whereas where they diverge, citizen resistance, weak regulatory implementation, and a decline in animal welfare indicators emerge.

Ecuador has built an ambitious legal framework but has not yet established a governance system fully consistent with it (Foundation, 2025). The transition to relational guardianship and positive anthropogenesis will only be effective if public policies incorporate territorial participation, intercultural justice, stable funding, ongoing training for municipal officials, and evaluation (Messely et al., 2013). Without processes of ethical and cultural internalization, legislation becomes a dead letter; when such processes are present, animal welfare governance can become a tool for social cohesion, public health, and multispecies justice (Szydlowski et al., 2023).

EDUCATION AS AN ALTERNATIVE IN THE FIGHT AGAINST ANIMAL ABUSE

The fight against animal abuse in Ecuador reveals a critical disconnect between the creation of regulations and the cultural transformation necessary for their effective implementation. Although regulatory policies are essential, their inadequacy is evident in the persistently high rates of animal abuse, which reflect not only the challenge of regulation but

also a crisis of values and understanding regarding the human-animal relationship. Ajzen 's theory of planned behavior (1991) suggests that behavior modification cannot be achieved solely through normative coercion; a transformation in the subjective attitudes and norms that underpin action is imperative. In this sense, the mere existence of laws, such as the Organic Law on Animal Welfare (2022), becomes an empty exercise if it is not accompanied by an educational process that fosters empathy and social responsibility.

Critiques of current legislation must be grounded in rigorous ethical analysis and empirical evidence. Many of the regulations enacted in Ecuador lack a solid ethical foundation and are often driven by political trends or the need for public visibility, rather than by empirical analysis and a deep understanding of the sociocultural dynamics that affect animal welfare. The phenomenon, known as "performative legislation," results in a regulatory framework that fails to respond to local realities, thus perpetuating animal abuse and dehumanization (Doyle et al., 2021).

Cultural resistance, especially in indigenous and rural communities, presents a significant obstacle to the implementation of external regulations, as these often conflict with local worldviews and traditional human–animal relationships. These communities possess worldviews that integrate animals into their social and spiritual structures, which contrast with regulatory approaches that tend to be imposed from an urban and Western perspective. A lack of sensitivity to these cultural realities can lead to the rejection of laws not because they oppose animal protection, but because they do not resonate with their traditional values and practices (Garcia et al., 2022).

An integrated educational approach grounded in ethical and sociocultural dimensions emerges as a critical and necessary alternative. An integrated educational approach grounded in ethical and sociocultural dimensions emerges as a critical and necessary alternative. It is not simply a matter of informing on existing laws but of cultivating a deep understanding of the ethics of care and shared responsibility towards animals (Cardilini et al., 2026). Educational programs that integrate the principles of Sumak Kawsay (Good Living), which emphasize the interconnectedness of humans, animals, and nature, can be effective in transforming attitudes and behaviors. Studies suggest that educational interventions can reduce animal abuse by 25% to 35%, underscoring the effectiveness of an approach based on empathy and respect (Arias et al., 2024). However, criticism of education should not be ignored. It is often argued that educational initiatives are expensive and require resources that are unavailable in the context of economic constraints. Nevertheless, recent studies indicate that investment in education for the prevention of animal abuse is significantly lower than the costs associated with penalties for abuse, reinforcing the economic viability of these programs (Budolfson et al., 2024). Education, therefore, is not only an ethical imperative but also an economically viable strategy that can generate long-term benefits in terms of public health and animal welfare (Zemanova, 2023).

The shift in terminology from "owner" to "guardian" or "responsible caretaker" is more than a simple linguistic change; it is a philosophical act that reflects a shift in understanding of the relationship between humans and animals. This change can influence how responsibilities are distributed and obligations toward animals are perceived. Adopting language that promotes shared responsibility can foster greater empathy and a deeper commitment to animal welfare, thus transforming the culture of pet ownership in Ecuador (Nurse, 2024).

The fight against animal abuse in Ecuador requires a critical approach that goes beyond simply creating regulations. It is imperative to recognize the importance of comprehensive education as a means of cultivating a culture of respect and responsibility toward animals. Transforming attitudes and behaviors, supported by a coherent regulatory framework sensitive to cultural realities, can be the solution for achieving a sustainable change in the perception and treatment of animals in the country. In this context, education becomes an act of social and ethical justice that can contribute to building a more compassionate society, one that is more aware of its interconnectedness with all living beings.

DISCUSSION

Understanding the anthropomorphization of companion animals in Ecuador requires situating the phenomenon within an interpretive framework that simultaneously articulates ethical, legal, sociocultural, and cognitive-ethological dimensions (Prato-Previde et al., 2022). Although the country has experienced a significant expansion of the discourse on animal welfare in recent years, particularly since the Constitution of the Republic of Ecuador (2008) and, more recently, the Organic Law on Animal Welfare (Ley Orgánica de Bienestar Animal, 2022). The conceptual discussion regarding the status of animals in Ecuadorian social life remains in its early stages. This insufficiency stems not from a lack of social awareness but rather from the absence of a systematic dialogue between international scientific evidence and local cultural particularities.

Understanding the anthropomorphization of companion animals in Ecuador requires situating the phenomenon within an interpretive framework that integrates language, cognition, legal structures, and sociocultural dynamics. Figure 1 illustrates this conceptual model, highlighting how these interacting dimensions can lead to divergent outcomes,

ranging from positive anthropogenization to pathological anthropomorphization, with corresponding effects on animal welfare, public health, and governance.

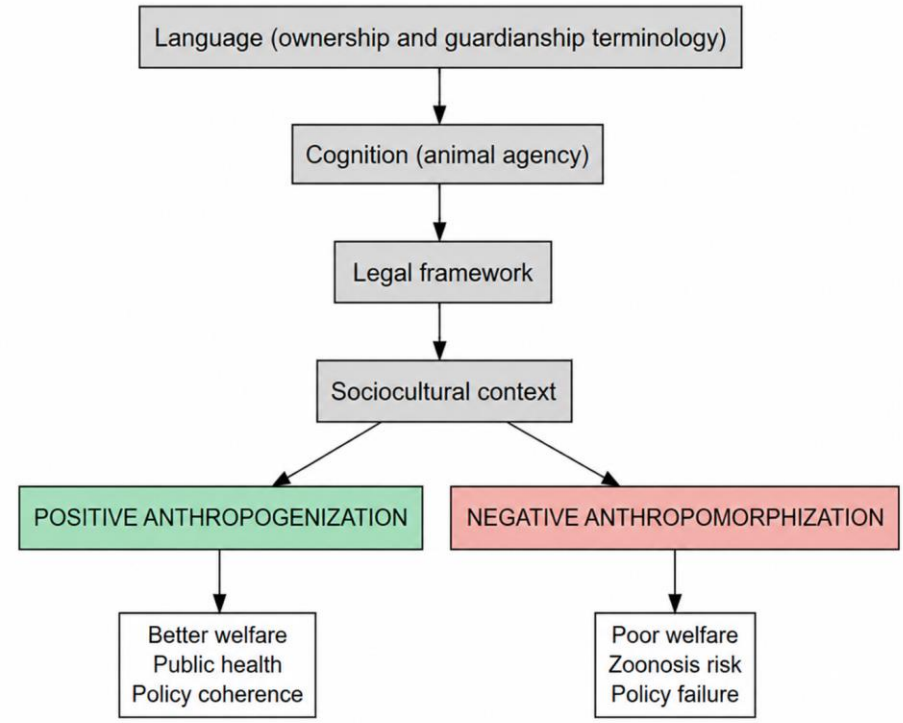


Figure 1. Conceptual model of anthropogenization and its implications for animal welfare and governance in Ecuador

One of the most consistent findings in international literature is that the moral recognition of companion animals does not emerge simply from affection or prolonged cohabitation, but from a deeper epistemological transformation, namely the understanding that dogs and cats possess cognitive, emotional, and relational capacities that position them as subjects with their own interests (Berger-Meunier et al., 2025). Although this discussion has received less attention in Ecuador, evidence such as Villamar's (2023) suggests that citizens intuitively recognize many of these attributes, even though regulations and public policies have not fully internalized their implications. The contradiction between the affective recognition of animals as family members and the persistence of legal categories that conceive of them as objects of property thus constitutes the core of a structural normative dissonance that permeates the entire animal welfare system.

Anthropogenization, understood as the process by which animals acquire social, affective, and moral meanings that transcend the logic of ownership, highlights this tension (Mota-Rojas et al., 2021). In countries such as Ecuador, where modernity has been unevenly superimposed and where traditional views coexist with emerging urban sensibilities, anthropogenization is not a homogeneous phenomenon but rather a deeply stratified one. In middle-class urban sectors, for example, the bond with animals tends to adopt characteristics of secure attachment, mutual emotional recognition, and a growing tendency toward responsible cohabitation, as Jiménez-Quito and Durán-Ramírez (2024) have pointed out. However, in urban-marginal and rural areas, the relationship is shaped by economic, territorial, and cultural dynamics that require more complex interpretations. As noted by Méndez Juez et al. (2020) and Monsalve et al. (2023), structural inequalities and material limitations condition the real possibilities of care, meaning that individual responsibility cannot be analyzed in isolation from living conditions.

The point challenges a recurring error in regulatory literature, namely the attempt to apply a universal model of responsible pet ownership that fails to consider cultural diversity or socioeconomic tensions. In Ecuador, the implementation of public policies focused exclusively on punitive regulations has proven insufficient, as documented by the Council for the Protection of Rights of the Metropolitan District of Quito (Consejo de Protección de Derechos del DMQ, 2023). The notion of "responsibility" cannot be imposed as an abstract duty, as it is contingent upon the availability of veterinary services, community education, public infrastructure, and mechanisms for citizen participation. International experience confirms that coercive approaches systematically fail when not accompanied by sustained educational and community processes (Rodriguez Ferrere, 2022). Such a pattern is particularly evident in Ecuador, where the Organic Law on Animal Welfare, despite its historical importance, still operates within a weak institutional framework, without the necessary tools to transform entrenched practices.

A central element of the discussion concerns how language shapes the conceptualization of human–animal relationships. The persistence of terms such as “custodian” or “proprietor” in Ecuadorian law is not merely a legal anachronism but a conceptual framework that organizes practices, emotions, and relational expectations. Psycholinguistic literature has conclusively shown that words not only describe reality but also produce it, as those who see themselves as “owners” tend to engage in more utilitarian and less empathetic practices, while those who define themselves as “guardians” internalize ethical and affective co-responsibility (López-Cepero et al., 2025). This poses a profound challenge for national legislation. The transition from ownership to guardianship is not only a legal matter but also a cultural one. Without a change in terminology and, therefore, in the cognitive framework that structures the relationship, any attempt at reform will be partial and incomplete.

The role of language in shaping human–animal relationships is closely linked to broader debates about the place of animals within the political community. Although the Ecuadorian Constitution has recognized the rights of nature since 2008, several authors warn that this constitutional recognition has not necessarily translated into effective protection for animals, due to normative vagueness, limitations in sub-constitutional legislation, and a still-developing body of jurisprudence (Lazo, 2023). The discussion becomes more complex when recognizing that companion animals inhabit domestic, familial, and urban environments rather than wild ecosystems, which introduces distinct ethical tensions in their regulation (Rouhiainen and Aarnio, 2025). While the theory of the rights of nature seeks to preserve ecological processes, the theory of animal welfare focuses on individual agency and the subjective experience of each animal (Peschel et al., 2025). Anthropogenization, in this sense, introduces a nuance, as it recognizes animals as participants in human social networks, which places them in a hybrid space between the biological, the emotional, and the political (Azadi et al., 2022).

Anthropogenization must incorporate the issue of pathological anthropomorphization, a phenomenon that emerges when affective recognition overflows into practices that ignore the real ethological needs of animals (Araújo et al., 2024). Adamakopoulou et al. (2023) have warned that the excessive attribution of human characteristics to animals can be as harmful as traditional objectification, as it leads to decisions that compromise the animal's well-being. In Ecuador, where the commercial supply of pet products and services has expanded rapidly, such a phenomenon is beginning to be observed, especially in middle-class urban areas. The scientific discussion, therefore, emphasizes the need for balance: recognizing the animal's subjectivity without projecting human expectations onto it that distort its well-being. Furthermore, the phenomenon of anthropogenization raises critical questions about whether current veterinary curricula in Ecuador and Latin America adequately prepare students for emerging professional challenges. Historically, veterinary education in Latin America has heavily prioritized clinical, biological, and zootechnical competencies, often providing limited training in animal welfare science, veterinary ethics, legal frameworks, and advanced communication skills (Cornish et al., 2018; Ventura et al., 2020). As the human–animal bond evolves, veterinarians increasingly encounter complex ethical dilemmas and communicative tensions with guardians, including demands for overtreatment, humanized diets, or interventions that prioritize human emotional expectations over the biological needs of animals. Therefore, modernizing veterinary curricula through stronger interdisciplinary training is essential to equip future professionals with the ethical, legal, and communicative tools required to address these emerging challenges effectively.

The anthropization of animals in Ecuador is a complex phenomenon, shaped by historical, cultural, economic, and legal tensions. Understanding it requires recognizing that animal welfare is not an isolated practice or an individual responsibility, but rather a collective process demanding profound transformations in the conceptual, institutional, and cultural frameworks that underpin interspecies coexistence. Only to the extent that these transformations converge will it be possible to construct an Ecuadorian model of responsible guardianship that responds to both scientific evidence and local realities (Lostal Becerril et al., 2024).

CONCLUSION

The analysis shows that the anthropomorphization of companion animals in Ecuador is closely linked to linguistic, legal, and sociocultural factors that shape human–animal relationships. The persistence of possessive terminology reinforces utilitarian practices, whereas emerging relational approaches grounded in guardianship are associated with greater ethical responsibility and improved welfare outcomes. At the same time, the evidence reveals a significant gap between regulatory development and its effective implementation, influenced by territorial inequalities, limited institutional capacity, and insufficient cultural internalization of animal welfare principles. These findings highlight the need to integrate language reform, public education, and context-sensitive policies in order to achieve coherent and effective animal welfare governance. The value of this analysis lies in providing an interdisciplinary framework that clarifies how terminology, governance, and social practices interact in shaping interspecies coexistence in Ecuador.

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Authors' contributions

Orlando Meneses Quelal contributed to the conceptualization of the study, data analysis, and manuscript writing. The author also conducted the research activities and ensured the integration of interdisciplinary evidence. The author has read and confirmed the last edition of the manuscript before publication in the present journal.

Availability of data and materials

This study is prepared based on the previously published literature. All data supporting the findings are included in the article and its references.

Competing interests

The authors have declared no conflict of interest.

Ethical considerations

The author declares that ChatGPT (OpenAI) was used solely for translating the original text and for minor linguistic editing. The author confirmed that the use of AI did not contribute to the scientific content, analysis, or interpretation. The author assumes full responsibility for the content of the manuscript.

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Platelet-Rich Plasma in Veterinary Wound Healing: Mechanisms, Applications, and Clinical Potential

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ABSTRACT

Wound healing is a significant challenge in both human and veterinary medicine, particularly in cases involving trauma, infection, or chronic tissue damage. Platelet-rich plasma (PRP), a product derived from the patient's own blood and enriched with growth factors and cytokines, has gained interest for its potential to promote regeneration and enhance healing. The present study aimed to evaluate current evidence on the application of PRP and its derivatives in wound healing, with a specific focus on veterinary animal models, and to identify key mechanisms, therapeutic outcomes, and existing knowledge gaps. While the present study highlighted veterinary applications, the included studies also consisted of rodent models, *in vitro* experiments, and human cell lines, providing broader mechanistic insights. A structured narrative review was conducted using 25 peer-reviewed articles published between 2024 and 2025. The selected studies included controlled trials, *in vitro* experiments, systematic reviews, and concept papers on PRP, platelet-rich fibrin, plasma rich in growth factors, and emerging formulations such as induced pluripotent stem cell-derived platelets and protein-rich PRP matrices. Key data related to study design, subjects, intervention types, outcomes, and theoretical frameworks were extracted and analyzed. All studies confirmed the positive role of PRP in promoting angiogenesis, collagen formation, epithelialization, and inflammation modulation. Evidence from veterinary models, including feline trauma cases and animal-derived PRP, supported the clinical applicability of PRP in wound healing. Several studies have demonstrated that the PRP approach is a valuable and effective treatment method in clinical practice, applicable to patients and animals. The PRP proved to be an efficacious intervention for cutaneous wounds, including surgical wounds, traumatic injuries, and chronic ulcers. However, differences in PRP preparation methods and outcome assessment criteria hinder direct comparisons across studies. Recent findings indicated new mechanisms involving telocytes and the regulation of vascular genes. It has been indicated that PRP and its derivatives are promising biological therapies for improving wound healing in veterinary practice.

Keywords: Growth factor, Platelet-rich plasma, Tissue repair, Veterinary medicine, Wound healing

INTRODUCTION

The process of wound healing is a highly coordinated and intricate physiological response that plays a vital role in restoring tissue structure and function after injury (Iacopetti et al., 2020). In veterinary practice, proper wound care is essential for ensuring animal health and well-being, particularly in situations involving traumatic injuries, burns, surgical wounds, or persistent ulcers (Accorroni et al., 2025; Li et al., 2025a). Unlike human clinical settings, veterinary wound care often faces additional challenges, including limited compliance, delayed diagnosis, and restricted access to advanced therapies, particularly in field or agricultural settings. These constraints require therapies that are accessible, cost-effective, and biologically active, capable of reliably accelerating healing and reducing complications such as infection, delayed wound closure, or excessive scarring (Accorroni et al., 2025).

One example of a biologically active treatment that is becoming increasingly popular is platelet-rich plasma (PRP). Platelet-rich plasma is a blood-derived product prepared from the patient's own blood, containing an elevated concentration of platelets in a limited volume of plasma (Farghali et al., 2024; Zhang et al., 2024; Rath et al., 2025). When platelets in PRP are activated, they release a rich combination of growth factors and cytokines that are crucial during the initial stages of tissue repair, including platelet-derived growth factor (PDGF), vascular endothelial growth

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factor (VEGF), transforming growth factor-beta (TGF-β), epidermal growth factor (EGF), and interleukins (Everts et al., 2020). These bioactive substances facilitate angiogenesis, stimulate fibroblast migration, increase collagen synthesis, and promote epithelial cell proliferation, thereby supporting and expediting tissue repair (Farghali et al., 2024; Zhang et al., 2024). Similar effects on epithelialization have been reported by Aragosa et al. (2025) using leukocyte- and platelet-rich fibrin (L-PRF). In both human and animal models, such as dogs and cats, PRP and PRF have demonstrated efficacy in managing acute and chronic wounds, cartilage and bone injuries, and post-surgical recovery (Zhang et al., 2024; Vidal-Negreira et al., 2025).

Despite encouraging clinical applications, there is significant variability across studies in PRP preparation protocols, cellular content, and therapeutic outcomes (Everts et al., 2020; Rath et al., 2025; Seidel et al., 2025). This inconsistency is particularly problematic in veterinary practice, where species-specific responses demand reliable, well-established protocols. Without consistency, veterinarians face challenges in predicting treatment efficacy, ensuring reproducible outcomes, and developing evidence-based guidelines tailored to different animal species. These challenges are particularly pronounced in veterinary medicine, where differences across species and the limited number of clinical trials make interpretation even harder. Some studies have emphasized the regenerative potential of leukocyte-rich PRP, while Carr et al. (2024) have recommended leukocyte-poor formulations to mitigate inflammation. Furthermore, Farghali et al. (2024) highlighted that PRP facilitates tissue repair in dogs by modulating the wound microenvironment through the complex delivery of growth factors and cytokines, a primary mechanism of action in clinical wound management.

In veterinary settings, recent experimental studies have explored the effects of PRP and PRF on wound healing in feline and canine trauma models, revealing improvements in collagen deposition, granulation tissue formation, and epithelial closure without significant adverse effects (Zhang et al., 2024; Wang et al., 2025a). Additionally, the use of PRP derived from alternative sources, such as induced pluripotent stem cell (iPSC)-derived megakaryocytes (iMPs) or even animal donors, such as deer, presented further opportunities and prompted questions about scalability, safety, and effectiveness across species (Kosaka et al., 2024; Jasim et al., 2025).

Given the growing yet fragmented evidence base, there is a critical need for a structured review focused specifically on PRP's role in veterinary wound healing. The present study aimed to integrate current clinical and preclinical evidence on PRP use in domestic and companion animals, elucidate its mechanisms of action in animal models, and evaluate its therapeutic potential and limitations to support the development of standardized, evidence-based protocols in veterinary medicine.

SEARCH CRITERIA

The present narrative literature review aimed to synthesize current evidence on the use of PRP in wound healing, with a focus on veterinary applications. Relevant studies were identified through searching on Scopus, PubMed, and Web of Science, conducted between January 1, 2024, and December 31, 2025, and a total of 25 peer-reviewed articles were included and studied for the present investigations. Additionally, reference lists of selected articles were manually screened to ensure comprehensive coverage. Search terms included combinations of platelet-rich plasma or PRP, platelet-rich fibrin or PRF, wound healing, tissue regeneration, skin repair, veterinary, and animal model. Only peer-reviewed articles published in English and indexed in Scopus, PubMed, and Web of Science were considered.

The inclusion criteria were applied to identify studies evaluating the effectiveness of PRP in wound healing or relevant to veterinary medicine that reported outcomes such as healing rate, histological changes, or inflammation markers. Studies involving animal models or veterinary clinical cases were included in the present study. Editorials, conference abstracts, non-peer-reviewed articles, studies involving only human subjects, and studies not related to animal models or veterinary clinical cases were excluded from the present study.

Data extracted from each study included authorship, year, design, animal model, PRP type and preparation, wound type, outcomes, and key findings. The details of the included studies, including species, PRP preparation techniques, treatment protocols, and outcomes, are summarized in Table 1. Results were categorized based on mechanisms of action, veterinary uses, technological innovations, and models. A qualitative synthesis was conducted by comparing study outcomes, identifying common patterns, and highlighting research gaps across the included studies. Figure 1 illustrates the literature review process flow diagram, depicting the main stages and sub-stages involved in article selection and review.

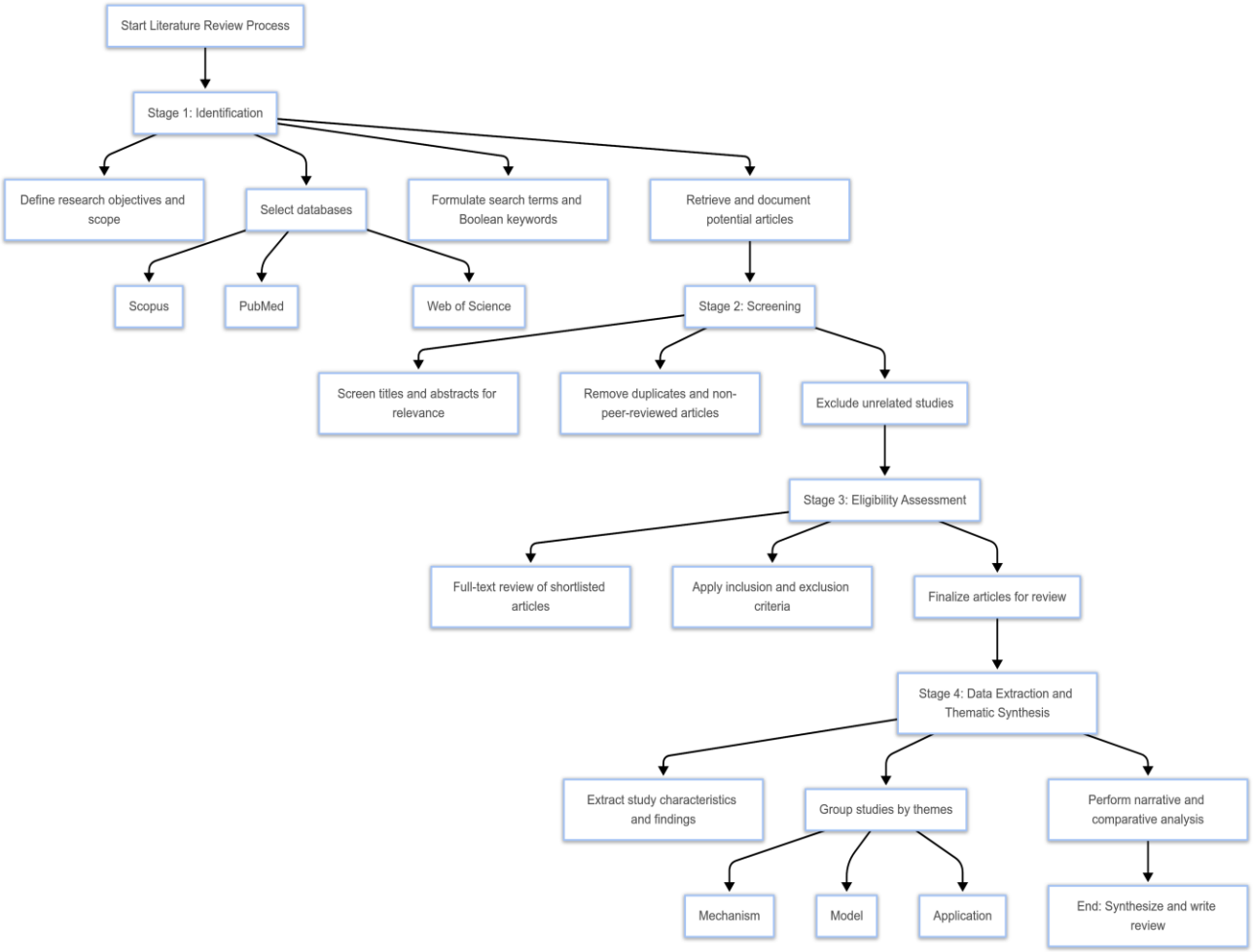


Figure 1. Process of selecting studies on platelet-rich plasma in veterinary wound healing published from 2024 to 2025. The flow diagram shows the stages and sub-stages of conducting the present review article on wound healing with platelet-rich plasma in 2024-2025. Source of the image: Authors of the present study.

MECHANISMS AND CLINICAL APPLICATIONS OF PLATELET-RICH PLASMA IN VETERINARY WOUND HEALING

Platelet-rich plasma mechanisms of action in wound repair

The reviewed studies provided robust evidence for the therapeutic potential of PRP and its derivatives to enhance wound healing in both human and veterinary contexts. Previous studies established PRP as a biologically active treatment that facilitates multiple important aspects of tissue repair, including angiogenesis, collagen synthesis, re-epithelialization, and modulation of inflammation (Figure 2; Pitafi, 2024; Jasim et al., 2025; Rath et al., 2025). The PRP applications across different animal models demonstrated enhanced wound healing, including angiogenesis, collagen synthesis, and epithelial regeneration. Variations in preparation techniques, treatment protocols, and outcomes for PRP have made it challenging to establish standardized guidelines for its use in veterinary wound management. These differences included differences in PRP preparation methods, such as single- or double-centrifugation protocols, leukocyte-rich versus leukocyte-poor PRP, and the use of activated or non-activated PRP (Everts et al., 2020; Rath et al., 2025).

Additionally, treatment protocols differed among studies, including topical application, intralesional injection, PRF gel, PRF membrane, or PRP combined with wound dressings (Pitafi, 2024; Soares et al., 2024). For instance, in feline trauma or skin wound models, platelet-rich fibrin was applied directly to the wound surface to improve re-epithelialization and wound contraction (Zhang et al., 2024). In feline skin graft models, PRP was combined with tilapia fish bio-dressing to support graft healing and tissue repair (Abellisa et al., 2024; Etriwati et al., 2025). In rat wound models, different PRP-based biomaterials were used to enhance wound repair. Hjazi (2025) reported that a PRP-loaded dermal matrix scaffold improved collagen deposition and wound healing in diabetic rats, whereas Li et al. (2025b) used a reactive oxygen species-responsive hydrogel to activate PRP and promote antibacterial activity and diabetic wound

closure in rats. Panigrahi et al. (2025) demonstrated that a PRP-functionalized iron oxide nanoparticle hydrogel accelerated healing in a rat excisional wound model, while Wang et al. (2025b) demonstrated that PRP-derived exosome-encapsulated hydrogels enhanced diabetic wound healing by reducing fibroblast ferroptosis. The translational potential of advanced PRP formulations, including iPSC-derived platelets, exosome-loaded hydrogels, and nanoparticle scaffolds, remains largely confined to experimental settings. Furthermore, the shift toward advanced regenerative medicine strategies has successfully identified specific molecular pathways that not only accelerate re-epithelialization but also minimize aesthetic scarring in complex wound models (Dutra Alves et al., 2025). Moreover, outcomes differed across studies; some studies assessed macroscopic wound closure rates, whereas others evaluated microscopic parameters, including histopathological healing scores, collagen deposition, angiogenesis, epithelialization, inflammatory cell infiltration, and granulation tissue formation.

Growth factor profiles and molecular pathways

Previous studies confirmed that platelet-derived products, such as PRP and PRF, are rich in growth factors, including PDGF and VEGF, which are crucial for wound healing (Farghali et al., 2024; Kosaka et al., 2024; Zhang et al., 2024). Furthermore, Kosaka et al. (2024) identified TGF-β and Insulin-like growth factor-1 (IGF-1) in PRP, while Zhang et al. (2024) indicated that PRF contains TGF-β1 and fibroblast growth factor 2 (FGF2) in a feline trauma model. These findings demonstrated that autologous platelet-derived products, including PRP and PRF, prepared from the patient's own blood, can accelerate wound healing in cats by improving wound closure, tissue perfusion, growth factor production, inflammation control, and collagen fiber formation, thereby highlighting their therapeutic potential in feline wound management (Figure 3; Angelou et al., 2022; Zhang et al., 2024). Similarly, Kosaka et al. (2024) found that iMPs outperformed conventional PRP in promoting angiogenesis and collagen deposition in diabetic mice, suggesting that platelet source and the standardization of wound-healing processes may influence therapeutic outcomes. The PRP facilitates wound healing by delivering different growth factors and cytokines that modulate the local microenvironment (Farghali et al., 2024). Beyond this traditionally accepted mechanism, new approaches in regenerative medicine have further proposed an expanded cellular model, suggesting that less well-characterized cellular components, such as telocytes, may act as potential mediators of PRP-induced regeneration. Everts et al. (2024) reviewed the biological properties of protein-rich platelet-rich plasma (PR-PRP) matrices and highlighted their potential for sustained growth factor release and recruitment of regenerative cells based on previous *in vitro* and *in vivo* studies. These findings indicated that platelet-derived products (PRP, PRF, and L-PRF) aid wound healing by releasing VEGF and PDGF to stimulate angiogenesis and TGF-β to enhance collagen deposition and tissue remodeling (Farghali et al., 2024; Zhang et al., 2024; Aragosa et al., 2025).

Furthermore, recent evidence indicated that PRP modulated the inflammatory stage of wound healing by regulating key pro- and anti-inflammatory cytokines, including interleukin-1 beta (IL-1β), IL-6, IL-10, and tumor necrosis factor alpha (TNF-α). Studies have indicated that PRP affects macrophage polarization and activity, thereby creating a more favorable microenvironment for tissue repair and regeneration across different animal models (Carr et al., 2024; Zhang et al., 2024; Wang et al., 2025a). Feline PRF and animal-derived PRP extract have been demonstrated to influence wound healing by regulating immune responses, promoting fibroblast proliferation, stimulating new blood vessel formation (angiogenesis), enhancing collagen deposition, and supporting epithelial regeneration (Zhang et al., 2024; Wang et al., 2025a). Additionally, these findings further supported the use of PRP as a regenerative therapy for wound management in veterinary patients, as reported by Wang et al. (2025a), who demonstrated that PRP derived from deer blood exhibited superior antioxidant capacity, wound-healing performance, and anti-inflammatory properties *in vitro*. While deer are not common laboratory models, Wang et al. (2025a) highlighted potential applications of PRP derived from wildlife species as a future therapeutic biomaterial. The study conducted by Zhang et al. (2024) provided direct experimental evidence supporting the use of PRF in cats, whereas the findings of Wang et al. (2025a) on deer-derived PRP introduced new possibilities for developing species-specific, animal-based PRP formulations. The mechanisms underlying PRP efficacy in non-conventional models, including wildlife species such as deer and novel cellular mediators such as telocytes, were poorly explored. In a comparative analysis, Watanabe et al. (2025) found that plasma rich in growth factor (PRGF), a variant of leukocyte-poor PRP, elicited higher proliferation rates in gingival epithelial cells than leukocyte-rich PRP, thereby underscoring the role of leukocyte content in shaping PRP effects.

These studies made a meaningful contribution to the understanding of PRP's regenerative capacity and support its continued exploration as a therapeutic tool in both clinical and veterinary contexts.

Table 1. Selected studies on platelet-rich plasma and its derivatives in animal wound healing (2024-2025)

Author (Year)	Design	Subjects/Model	Intervention	Outcomes	Mechanisms of action	Inconsistencies/gaps
Abellisa et al. (2024)	Controlled laboratory experimental animal study	Local cats (*Felis catus domesticus*), auto skin graft	PRP + Tilapia Mozambique skin bio-dressing, evaluating blood parameters on different days	The PRP group demonstrated significantly higher platelet dynamics and stable erythrocyte indices	PRP as a biologic adjunct modulated systemic platelet response during graft healing, and hematological parameters remain within physiological ranges	Very small sample size, no PRP-only group, outcomes limited to hematological findings, and short follow-up period
Farghali et al. (2024)	Experimental / *In vivo* study	Canine model (dogs with full-thickness skin defects)	Comparison of single-dose inactivated autologous PRP, laser therapy, and a PRP/laser combination	The combined PRP/laser therapy yielded the fastest healing potential, superior granulation tissue, and optimized remodeling	Synergistic effects occur when combining mechanical/photostimulation (laser) with autologous growth factors	Small sample size, long-term cosmetic outcomes (hair regrowth/scar texture)
Zhang et al. (2024)	Controlled animal trial	18 feline model (cats with induced skin trauma)	Topical application of autologous PRF on skin wounds	PRF improved healing, collagen formation, and reduced inflammation	Confirmed that autologous PRF acted as an effective bioscaffold that provided sustained growth factor release specifically optimized for feline skin physiology	Small sample size and no long-term follow-up
Kosaka et al. (2024)	*In vitro* + diabetic mouse model	HUVECs, fibroblasts, diabetic mice	Application of human iPSC-derived megakaryocytes and platelets	iMPs superior in wound closure, angiogenesis, and growth factor release	Transitioning from exclusively autologous donor-dependent harvesting to scalable, standardized off-the-shelf cellular therapies utilizing stem cell technology	Clinical use of iMPs is not yet established
Everts et al. (2024)	Theoretical/conceptual review	Not applicable (technical synthesis)	Analysis of advanced protein-rich, PRP matrices as multi-purpose biological platforms	Described scaffolding role, sustained GF delivery, PPP growth factors such as IGF-1, HGF	Highlights PR-PRP as a structural and biochemical scaffold	Mostly theoretical, lacks direct clinical application data
Wang et al. (2025a)	*In vitro* (animal-sourced PRP)	Keratinocytes + animal PRP (deer)	Evaluation and screening of heterologous / animal-derived PRP extracts from various animal species (with a focus on deer blood)	Deer PRP had the highest healing, antioxidant, and anti-inflammatory effects	Animal PRP is viable, deer PRP promising for veterinary/cosmetic use	Ethical concerns, translation to live models is limited
Seidel et al. (2025)	Practical review	Equine	Medium-term storage of platelet-derived orthobiologics.	Validated that medium-term storage was feasible, making platelet products more readily available for timely field application	Point-of-care limitations can be mitigated by optimizing preservation and storage protocols for biologics	Exact degradation rates of specific growth factors across different storage intervals require further molecular quantification
Vidal-Negreira et al. (2025)	Systematic review and quality evaluation	Dogs undergoing orthopedic treatments	Analysis of liquid- versus gel-based PRP therapies in veterinary orthopedic surgery.	Reported benefits in tissue repair and orthopedic recovery, but the evidence quality varied	Increasing veterinary use of PRP, heterogeneity in preparation protocols influenced outcomes	Lack of standardization in PRP preparation, dosage, and administration, and inconsistent methodological quality
Wang et al. (2025b)	Translational mechanistic experimental design (*in vitro* + controlled animal study)	Human diabetic fibroblasts, diabetic mouse wound model	PRP-derived exosome-encapsulated hydrogels applied to diabetic cutaneous wounds	Accelerated healing in mice via inhibition of fibroblast ferroptosis	Targeting cell-death pathways in chronic wounds	Complex intervention limited direct clinical scalability
Aragosa et al. (2025)	Prospective study	Veterinary cases of chronic wounds	Autologous L-PRF membranes	Marked reduction in wound surface area, control of local infection, and accelerated closure of stubborn, chronic wounds	Leukocyte inclusion provided an added antimicrobial benefit alongside the regenerative properties of fibrin	L-PRF required immediate processing and application, difficult to use in emergency/unprepared clinical settings

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Li et al. (2025a)	Controlled comparative animal infection model	Rats with infected burn wounds (dual-species biofilm)	Comparative topical application of PRP, PRF, and AG on wounds infected with a dual-species biofilm (*Pseudomonas aeruginosa* and *Staphylococcus aureus*)	Reduced bacterial burden, increased collagen, and reduced inflammation	Highlighting the powerful, innate antibacterial and antibiofilm properties of platelet concentrates, which can outperform traditional topical antimicrobials (such as silver) in complex, polymicrobial wound environments	No long-term outcomes or clinical confirmation
Rath et al. (2025)	Conceptual synthesis of mechanistic and clinical heterogeneity (methodological review design)	Multidisciplinary PRP studies	Systemic evaluation of PRP isolation methods, chemical/physical activation pathways, and diverse clinical applications	Provided a definitive structural blueprint establishing how specific processing steps alter growth factor cascades, leukocyte retention, and final therapeutic outcomes	Lack of protocol standardization was a key determinant	Did not resolve optimal dose or formulation
Carr et al. (2024)	Literature review	Canine (dogs)	PRP products are used as orthobiologic agents.	Significant support for PRP efficacy, particularly in treating canine osteoarthritis and soft tissue injuries.	Platelet-derived growth factors strongly promote tissue regeneration and reduce localized inflammation.	High variability in PRP preparation protocols (centrifugation speeds, leukocyte counts) across different studies
Li et al. (2025b)	Controlled bioengineering-animal experimental design	Diabetic infected rat wound model	ROS-responsive photo-crosslinked double-network hydrogel (HTC/MCP) loaded with PRP.	High levels of ROS at the wound site trigger the dynamic borate ester bonds to dissociate. This automatically releases on-demand antibacterial agents and activates the encapsulated PRP, reaching nearly 95% wound closure in rats in 14 days by promoting fibroblast migration and tissue microvascular density.	Precision-controlled PRP delivery	Manufacturing and regulatory barriers
Soares et al. (2024)	Review/book chapter	Dogs and cats	PRF therapy for traumatic skin wounds	The fibrin matrix allowed sustained release of growth factors, thereby accelerating dermal matrix repair	PRF was emerging as a superior, scaffold-free autologous option compared to traditional PRP for skin-specific trauma	Limited larger-scale, RCTs specifically comparing PRF directly against advanced synthetic dressings in cats
Panigrahi et al. (2025)	Controlled nanocomposite animal experiment	Rat excisional wound model	Biosynthesized iron oxide nanoparticles encapsulated within a chitosan/alginate hydrogel and functionalized with PRP	Synergistic action accelerated epithelialization and tissue cross-linking. The green-synthesized iron oxide nanoparticles provided localized antimicrobial/antioxidant defense, while the integrated PRP drove cellular proliferation and rapid dermal remodeling.	PRP-enhanced multifunctional biomaterials	Long-term biosafety unknown
Hjazi (2025)	Randomized controlled animal intervention study	Diabetic Sprague-Dawley rats	Bioactive, biodegradable 3D DDM scaffold enriched with PRP	Significantly accelerated wound closure rates by Day 14. The DDM-PRP combination enhanced extracellular matrix deposition, upregulated VEGF and TGF-β expression, and promoted structural hair follicle regeneration and epidermal thickness	PRP was the most effective treatment	While structural and histomorphometric tissue markers were highly favorable on Day 14, the long-term metabolic degradation rate of the DDM scaffold across varying degrees of diabetes severity remains uncharacterized.
Etriwati et al. (2025)	Controlled experimental animal study	9 male domestic cats (*Felis catus*), auto skin graft	Group I: moist dressing (control), Group II: Tilapia Mozambique (TM) bio-dressing, Group III: PRP applied to wound bed + TM	PRP+TM significantly improved epidermal thickness, neovascularization, hair follicles, sebaceous and sweat glands, reduced collagen density, and broader and more even caspase-3 expression	Synergistic biologic therapy enhances graft integration and tissue remodeling, apoptosis (caspase-3) as a regulator of optimal healing	Small sample size, short observation period (21 days), no PRP-only group, lack of functional/clinical outcome measures

			bio-dressing			
Jasim et al. (2025)	Controlled experimental animal study	Rabbit avulsion skin wound model	Comparison of autogenic, allogenic, and heterogenic PRP treatments	PRP treatments accelerated wound healing, with autogenic PRP generally showing superior regenerative effects	PRP effectiveness may depend on biological source compatibility, autologous PRP appears most effective	Limited sample size, immunologic responses to heterogenic PRP require further investigation, and long-term tissue quality has not been fully evaluated
Pitafi (2024)	Experimental animal study	Rabbit cutaneous wound model	Combined application of Autologous PRP with a hydrofiber dressing	Faster wound contraction, improved epithelialization, and enhanced healing quality compared to conventional management	Combination therapy using PRP and advanced dressings may synergistically improve wound healing outcomes	Lack of molecular or histopathological mechanistic analysis, applicability to naturally occurring clinical wounds remains uncertain
Accorroni et al. (2025)	Narrative review	Veterinary perspective (different species)	Topical use of sucralfate for cutaneous wound management	Protects damaged mucosa/skin, stimulates angiogenesis, and accelerates tissue remodeling	Shift toward cost-effective, easily accessible topical barriers that also promote healing	Lacks standardized clinical trial protocols specifically comparing sucralfate directly to modern bioscaffolds in small animals
Dutra Alves et al. (2025)	Systematic review	General biomedical / skin models	Advanced regenerative medicine-based approaches for skin regeneration/rejuvenation	Successfully identifies pathways to accelerate re-epithelialization and reduce aesthetic scarring	Combining biomaterials with cellular signaling molecules yields the highest-quality dermal remodeling.	Translating highly controlled lab/biotech advancements into affordable, scalable veterinary clinical therapies remains slow
Soares et al. (2025)	Clinical case series / prospective clinical application study	Domestic animals presenting traumatic skin wounds and chronic ulcers (dogs and cats)	Topical application of autologous PRP gel directly onto traumatic wounds and chronic ulcers with repeated treatment sessions	Accelerated granulation tissue formation, faster wound contraction, improved epithelialization, and successful healing in chronic ulcerative lesions	PRP gel provided concentrated growth factors, including PDGF, TGF-β, VEGF, and EGF, that stimulated angiogenesis, fibroblast proliferation, extracellular matrix deposition, and epithelial regeneration	Limited sample heterogeneity, lack of a randomized control group, absence of standardized treatment intervals, and insufficient long-term comparative outcome analysis
Watanabe et al. (2025)	*In vitro* comparative experimental study	Cultured gingival epithelial cells	Comparison between Plasma Rich in Growth Factors (PRGF) and leukocyte-containing PRP on epithelial cell culture	Both plasma preparations promoted cell proliferation and wound closure; PRGF demonstrated a more favorable epithelial migration response, while leukocyte-rich PRP produced variable inflammatory effects	Growth factors stimulated epithelial proliferation and migration, whereas leukocyte content influenced inflammatory signaling pathways that may either enhance or delay tissue repair, depending on concentration	*In vitro* design limits clinical extrapolation, the absence of an animal or *in vivo* wound model, focuses only on gingival epithelial cells, and has an unclear optimal leukocyte concentration for therapeutic use

AG: Ionized silver, DDM: Decellularized dermal matrix, ECM: Extracellular matrix, EGF: Epidermal growth factor, GF: Growth factor, HGF: Hepatocyte growth factor, HUVECs: Human umbilical vein endothelial cells, IGF-1: Insulin-like growth factor 1, iMPs: Induced pluripotent stem cell-derived megakaryocytes and platelets, iPSC: Induced pluripotent stem cell, L-PRF: Leukocyte- and platelet-rich fibrin, PDGF: Platelet-derived growth factor, PPP: Platelet-poor plasma, PRF: Platelet-rich fibrin, PRGF: Plasma rich in growth factors, PRP: Platelet-rich plasma, PR-PRP: Protein-rich platelet-rich plasma, ROS: Reactive oxygen species, TGF-β: Transforming growth factor beta, VEGF: Vascular endothelial growth factor.

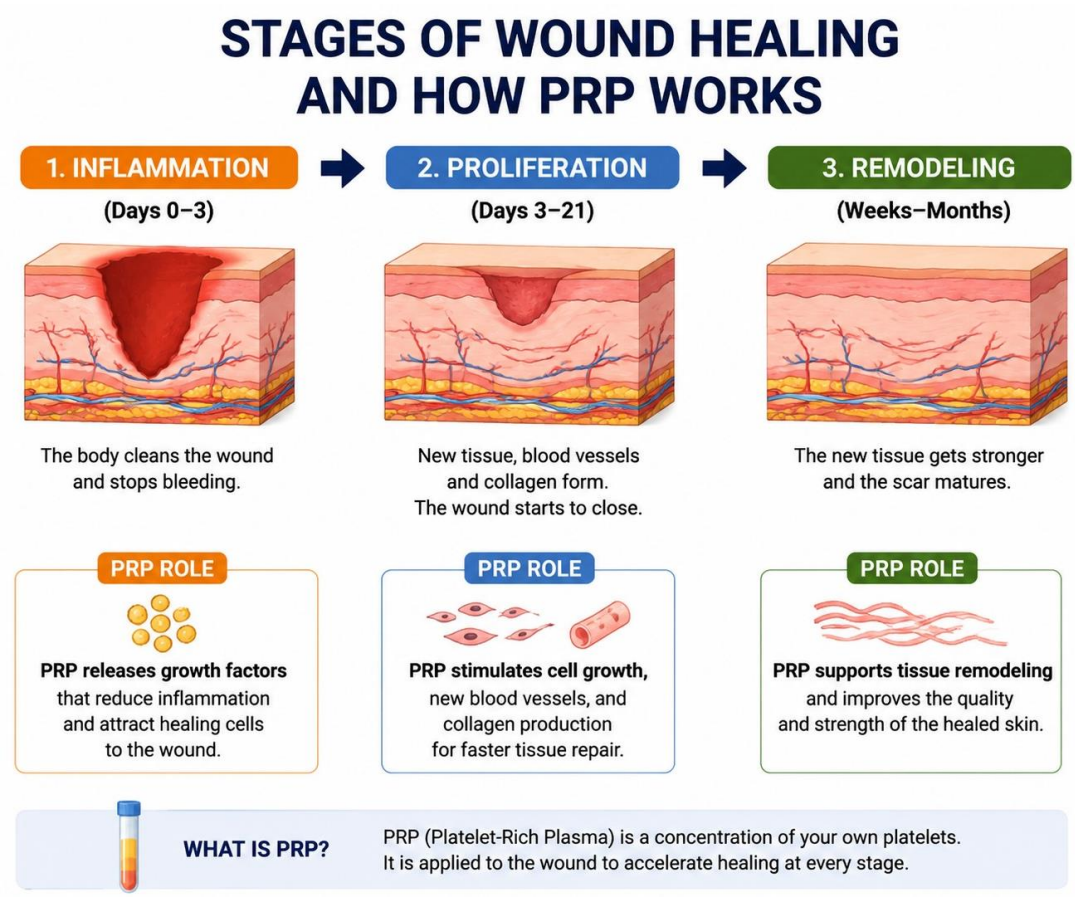


Figure 2. Stages of wound healing after using platelet-rich plasma treatment. Platelet-rich plasma is applied during the inflammatory stage to promote hemostasis and release growth factors, thereby supporting cell migration and angiogenesis in later stages. This figure was conceptualized and drafted by the authors. The initial layout was created using AI-assisted graphic tools, with all final annotations, scientific labeling, and structural integration performed manually by the authors to ensure accuracy.

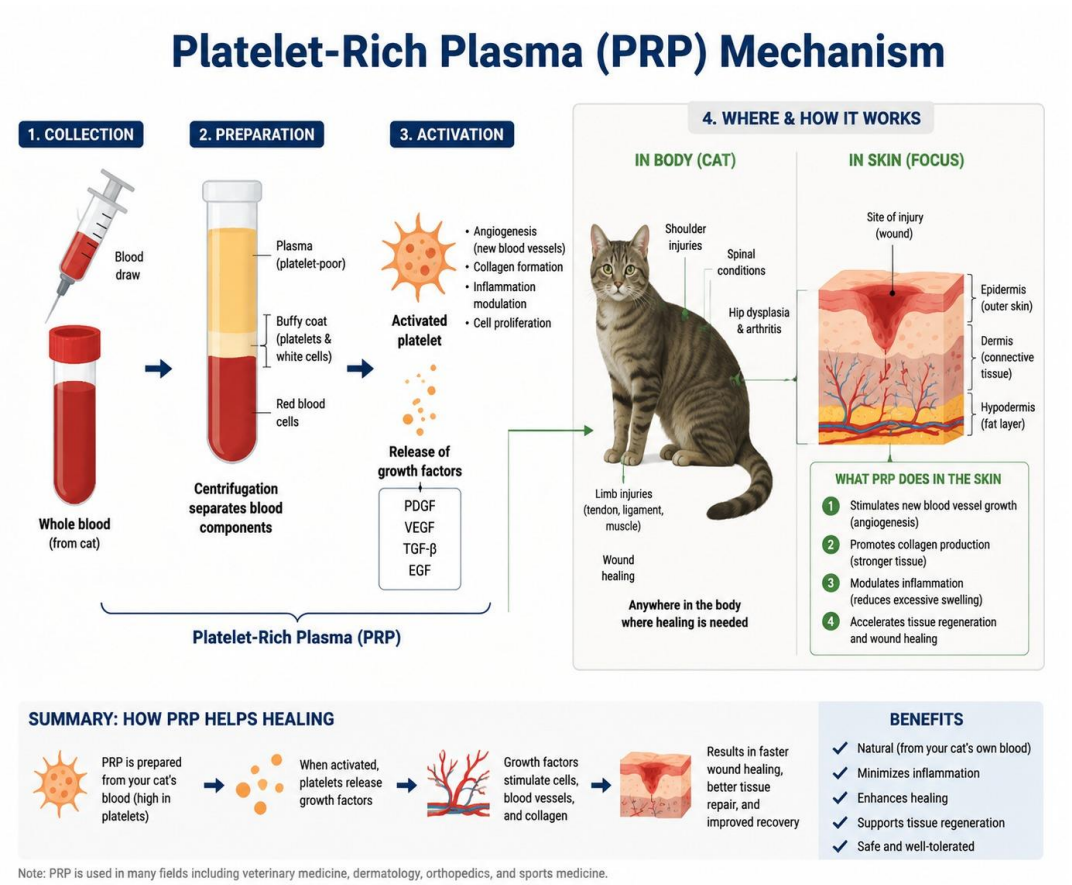


Figure 3. Platelet-rich plasma mechanism in skin wound healing in felines. Platelet-rich plasma releases growth factors that promote tissue regeneration, angiogenesis, and collagen synthesis. This figure was conceptualized and drafted by the authors. The initial layout was created using AI-assisted graphic tools, with all final annotations, scientific labeling, and structural integration performed manually by the authors to ensure accuracy.

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CHALLENGES AND STANDARDIZATION LIMITATIONS

Although there is broad agreement on the regenerative capabilities of platelet-derived products, studies have reported different outcomes. Aragosa et al. (2025) found that L-PRF facilitated complete wound re-epithelialization in chronic canine wounds. Additionally, Kosaka et al. (2024) reported promising findings with iMPs; however, their application remained confined to experimental settings, and their practicality and scalability in routine veterinary clinical practice remained uncertain. The primary challenge highlighted in several studies was the absence of established protocols for preparing PRP in animal studies. Variations in centrifugation procedures, leukocyte and platelet concentrations, and activation methods contribute to substantial inconsistencies in outcomes. The included veterinary studies differed in PRP/PRF preparation. In dogs, PRP was prepared by double centrifugation, yielding platelet concentrations of 1,050-1,550 × 10^3/µL (Iacopetti et al., 2020). In cats, PRF was prepared from 5 mL of blood by centrifugation at 400 g for 10 minutes (Zhang et al., 2024). In rabbits, PRP was prepared from 5-6 mL of blood using sequential centrifugation at 100 g and 400 g for 10 minutes (Jasim et al., 2025). These differences might account for inconsistent resulted across species and studies, complicating the replication of findings across trials and animal models (Zhang et al., 2024).

Veterinary applications

For veterinary medicine, wound treatment using PRP is particularly encouraging. The PRP offered a safe, autologous, and cost-effective solution for veterinary wound management, especially where prolonged healing or infection risk is a concern (Zhang et al., 2024; Wang et al., 2025a). These applications include the use of PRP to treat surgical incisions, traumatic skin wounds, pressure sores, and chronic non-healing ulcers (Zhang et al., 2024; Soares et al., 2025). These findings suggested that PRP could serve as a valuable adjunctive therapy to enhance wound healing in canines (Carr et al., 2024). Furthermore, Carr et al. (2024) examined the evidence for PRP in canine wound healing, highlighting its benefits in epithelialization, scar minimization, antimicrobial properties, and vascular ingrowth. However, most veterinary studies remain limited by small sample sizes, short follow-up periods, and a lack of long-term assessments of scar quality, tissue function, and potential adverse effects. The absence of standardized PRP preparation protocols across veterinary species represented a critical limitation. Species-specific differences in platelet biology, blood volume, and growth factor composition necessitate tailored centrifugation parameters, activation methods, and dosing regimens that are currently not established.

There is still no agreement on the optimal PRP formulation across species and wound types in veterinary animals. The long-term effects of PRP on tissue integrity, scar formation, and functional recovery in animals have yet to be thoroughly evaluated. The variability in preparation techniques underscored the need for established protocols to ensure consistent therapeutic efficacy. The absence of standardized preparation protocols and the inconsistent reporting of outcomes have restricted the widespread adoption of PRP in clinical practice.

CONCLUSION

The present study identified that PRP significantly accelerated wound closure, promoted neovascularization, and modulated pro-inflammatory cytokine expression in veterinary models, particularly in canines and felines. Studies in animal models have demonstrated PRP's therapeutic potential, although differences in preparation methods and outcomes have posed challenges to standardization. The present study integrated current evidence, highlighted the underlying mechanisms, and underscored the importance of further studies focused specifically on veterinary applications. Future research should emphasize standardizing protocols across different species, conducting large-scale randomized controlled trials in both companion and livestock animals, and investigating combined treatments that include PRP with biomaterials or negative-pressure wound therapy. Furthermore, continued investigation into combination therapies and advanced PRP formulations is essential to enhance the effectiveness and practical application of PRP in veterinary medicine.

DECLARATIONS

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Authors' contributions

Abellisa was responsible for the overall design and execution of the review, data collection, and analysis. Manuscript writing and ensuring all necessary revisions were made by Abellisa and Lestari Sukma Dinullah. Fikri Alsidik Ajis was instrumental in the conceptualization of the review, design, and editing of the manuscript. All authors have read and approved the final edition of the manuscript before publication in this journal.

Availability of data and materials

All data analyzed in this review are derived from previously published peer-reviewed articles. No new datasets were generated or analyzed during the current study. The datasets supporting the conclusions of this article are available within the cited literature.

Competing interests

The authors declared no conflict of interest.

Ethical considerations

The present study was originally written by the authors and has not been published elsewhere. The authors confirmed that Grammarly was used only for language editing, including grammar correction, sentence refinement, and clarity improvement. Regarding the figures, the authors affirmed that these figures were conceived and crafted to illustrate the specific research synthesis. Although Google Gemini was employed for preliminary layout and visual enhancement, all content, labels, annotations, and data presentation were meticulously curated, edited, and finalized by the authors to ensure scientific precision and alignment with the specific veterinary data. Authors have made manual revisions to ensure that the final output accurately reflects the distinct research framework and authentically depicts the PRP wound-healing stages. No AI tools were used for data analysis, interpretation, or the generation of scientific content. The authors take full responsibility for the manuscript's accuracy and integrity.

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Potential of Tropical Feed Resources for Inclusion in Commercial Livestock Diet: A Mini Review

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ABSTRACT

The demand for locally accessible, reasonably priced, and environmentally resilient alternative feed resources has increased due to rapid population growth, climate variability, land degradation, and growing competition for land between human food and animal feed. To address this problem, the utilization of emerging feed resources in the tropics is important. Therefore, the objective of this review was to identify emerging topical feed resources, nutritional potential, limitations, and integration techniques for commercial livestock production. Trees and shrubs, improved legumes and grasses such as lablab and alfalfa, insect-based proteins, aquatic feeds, root and tuber crops, agro-industrial by-products, fruit and vegetable wastes, and fermented or biotechnologically enhanced feeds are just a few of the many emerging feeds that were found to be valuable options. Emerging feeds partly fill the gap in feed supply, reduce competition for food between humans and animals, lower feed costs, and contribute to self-sufficiency in nutrients from locally available feed sources. Despite the high potential of emerging feeds, their utilization remains limited due to several interrelated constraints, including the presence of anti-nutritional factors, high fiber content and low digestibility, variable and inconsistent nutrient composition, poor palatability, high moisture content, rapid spoilage, and seasonal availability. Additional barriers include inadequate processing and preservation technologies, lack of infrastructure and equipment, limited farmer knowledge and technical skills, weak extension services, and insufficient research-based feed standards. Consequently, these factors result in inconsistent feed quality and reduced economic returns for commercial livestock producers. The review highlighted that appropriate processing techniques such as drying, chopping, grinding, ensiling, fermentation, urea or ammonia treatment, heat treatment, enzyme supplementation, and mineral fortification are required to utilize emerging feeds. In conclusion, emerging feed resources represent a viable pathway toward sustainable, cost-effective, and climate-resilient livestock production in the tropics. Strengthening studies, improving processing and preservation technologies, enhancing extension services, and building farmer capacity are essential to unlock their full potential and ensure their successful adoption in commercial livestock feeding systems.

Keywords: Diet, Emerging feed, Integration strategy, Livestock, Nutritional factor

INTRODUCTION

The demand for livestock products is estimated to increase significantly due to population growth, urbanization, and changing dietary preferences (Thornton, 2010). As a result, optimizing livestock production through improved nutritional strategies has become a pressing concern for farmers, researchers, and the scientific community (FAO, 2012; Ayobami et al., 2024). Traditional feeding practices, which often rely on fixed formulations, can lead to inefficiencies in nutrient utilization, resulting in suboptimal growth performance and increased feed costs (Akintan et al., 2024). Furthermore, conventional feeding systems contribute significantly to environmental challenges, including nutrient waste, greenhouse gas emissions, and land degradation, necessitating a shift toward more sustainable practices (Beauchemin et al., 2008).

Feeding animals with low-quality or unbalanced diets negatively impacts their production levels, health, and overall well-being. Additionally, this type of feeding leads to the inefficient use of nutrients, resulting in the emission of harmful greenhouse gases (Giri et al., 2025). On a global scale, the production, processing, and transportation of feed contribute to 45% of the total greenhouse gas emissions from the livestock industry, with enteric methane accounting for 39% of these emissions (Gerber et al., 2013). Livestock production in tropical regions faces multiple constraints, such as a lack of availability and quality feed, especially during the dry season, animal diseases, and low genetic potential of indigenous animals (Duguma and Janssens, 2021). As feed accounts for 60-70% of total production costs in commercial livestock systems, the search for affordable and sustainable alternative feed resources has become a global priority (Makkar et al., 2014).

Consequently, identifying, characterizing, and utilizing novel and alternative feeding resources that are locally available, currently underutilized, and less competitive with human food will be essential to developing sustainable livestock production (Khanal, 2024). Utilization of emerging tropical feed resources is the best option to fill the gap

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(Chisoro et al., 2023). Many emerging tropical feed resources (ETFRs) are adapted to harsh climatic conditions such as drought, poor soils, and high temperatures and possess significant nutritional potential (Khanal, 2024), making them more resilient and reliable than conventional feed crops. These resources can support commercial livestock production if appropriately processed, evaluated, and incorporated into balanced rations (Chisoro et al., 2023).

Despite their abundance, the utilization of ETFRs in commercial livestock production remains limited due to several constraints, including the presence of antinutritional factors, lack of standardized processing methods, limited research information, inadequate supply chains, and insufficient farmer awareness (Gadzama, 2025). Hence, for ETFRs to be effectively utilized, strategic approaches are necessary to incorporate them into organized commercial livestock rations (Makkar et al., 2014). Therefore, this study aimed to review the classification, nutritional potential, utilization constraints, and sustainable integration strategies of ETFRs in commercial livestock production systems.

OVERVIEW OF EMERGING TROPICAL FEED RESOURCES

Feeding strategies play a crucial role in determining the nutritional quality of animal products. The type and composition of an animal's diet directly influence the nutritional content of the products derived from them, such as meat, milk, and eggs. Various feeding strategies, including traditional and modern approaches, affect factors such as the balance of macronutrients, essential vitamins, and minerals, as well as overall product quality and safety. Alternative feed sources are being employed as agents to improve animal health and enhance the quality of animal products (Untea et al., 2023).

Emerging tropical feed resources are non-traditional, underused, or newly identified plant, animal, and microbial-based materials that are gaining recognition for their potential to supplement or replace conventional feed ingredients in animal diets. These feed materials are newly identified and are found in tropical regions such as Africa, Asia, and Latin America. Emerging feed resources can be used to feed livestock and have become increasingly important due to rising feed costs, climate change, competition for food between humans and animals, and the limited availability of traditional feed ingredients such as maize and soybean meal (Makkar et al., 2014).

The use of local novel feed substitutes or supplements, such as insect proteins, industrial by-products, and food waste, as alternatives to conventional feed materials has been researched and is still being explored extensively. This is a way for rural and small-scale farmers to feed their animals and increase production. The use of these local novel feed resources in Africa is expected to greatly support the growth of the livestock sector and help meet the current and projected demand for meat and livestock products in the region (Chisoro et al., 2023).

CLASSES OF EMERGING TROPICAL FEED RESOURCES

Multipurpose forage trees and shrubs

Woody fodder species such as *Moringa oleifera*, *Leucaena leucocephala*, *Sesbania sesban*, *Gliricidia sepium*, and *Calliandra calothyrsus* have been introduced and promoted to increase biomass for supplemental animal feed and soil fertility management (Geleto and Ayele, 2022; Amad and Zentek, 2023). The browse fodder is generally rich in protein and minerals and can be used in the dry season to supplement poor-quality natural pasture or fibrous crop residue to improve animal performance (Kemboi et al., 2017). Replacing 20% of soybean meal with *Moringa oleifera* increases milk production in dairy cows (Cohen-Zinder et al., 2016), and including 10% of *Moringa oleifera* in the diet enhances growth and immune response in broiler chickens, as reported by Jasim and Mohammed (2024). Improving the meat and bone quality of slow-growing male chickens that have access to outdoor areas (Faustin-Evaris et al., 2022). Additionally, its amino acid composition and protein digestibility are comparable to those of soybean, exceeding 92% in digestibility.

Leucaena contains high crude protein (24 - 30%), depending on the variety and time of year. The digestibility of the protein reaches 63%, and the digestibility of dry matter is between 60 and 70%, measured *in vivo* (Barros-Rodríguez et al., 2012). In this sense, the use of *Leucaena* as a protein supplement in livestock farming systems in tropical countries is widely accepted. In addition, it is a source of minerals such as sulfur, which can enhance rumen microbial populations (mainly cellulolytic fungi and bacteria; Barros-Rodríguez et al., 2014). Supplementing rice straw diets of growing native cattle with dried *Leucaena* leaves yielded positive outcomes. The sheep that were fed the diet containing 300 g/kg *Sesbania* foliage showed significantly higher average daily body weight gain (103 g/day) than the unsupplemented control group (75.6 g/day; Manaye et al., 2009).

Mulberry leaves are rich in nutrients, especially with a high crude protein content, and they provide balanced amino acids with high digestibility (Saddul et al., 2005; Yulistiani et al., 2015). When added to the diets of growing and fattening pigs at a 20% level, it successfully replaces conventional protein sources and reduces feeding costs (Peng et al., 2024).

Feeding *Acacia angustissima* as supplements to rangeland pasture hay resulted in improved nutrient intake and higher nitrogen retention compared to rangeland pasture hay alone (Hove et al., 2001). Jatropha kernel meal is a good source of nutrients with a crude protein concentration of 620 to 770 g/kg dry matter and rich in essential amino acids such as leucine, Isoleucine, methionine, and phenylalanine (Saeed et al., 2017). Phesatcha et al. (2021) reported that Flemingia hay meal supplement in dairy steers affected the nutrient digestibility and improved ruminal fermentation, especially propionate.

Tropical legumes and pasture grasses

Improved legumes and grasses are known for their high biomass production, improved crude protein content (10-18%), better digestibility, and greater persistence when grazed. They are also more resistant to drought and diseases, which helps reduce the need for imported feed, enhances livestock performance and fertility, and improves soil fertility through nitrogen fixation, especially legume species (Lelamo, 2021). Forage legumes such as Hyacinth bean (*Lablab purpureus*), stylo (*Stylosanthes guianensis*), and silver leaf desmodium (*Desmodium uncinatum*) are well-suited to areas with high temperatures and low rainfall (Missanga et al., 2023). These legumes can be used in the cut-and-carry system or as an alternative during the wet season when yields are highest, and can be conserved for feeding during the dry season (Lelamo, 2021).

A mixture of lupin, pea, faba bean, and vetch could be used to replace soybean meal in the diet of intensively reared dairy ewes without compromising milk production, reducing environmental impacts and production costs of intensive dairy sheep farms, thus helping to increase the overall sustainability of the sector (Vouraki et al., 2023). *Brachiaria brizantha* has crude protein content ranging from 11.6 and high dry matter yield (Njarui et al., 2020). Feeding *Brachiaria* increases milk production from 4 to 4.6 litres/cow per day for low-yielding animals, representing a 15% increase, and 9 to 12.6 litres/cow per day for the relatively higher-yielding dairy cattle, representing a 40% increase in Kenya (Njarui et al., 2020).

Insect-based protein sources

Growing insects is becoming a more sustainable option to address the environmental challenges of current food and feed systems, such as deforestation, land degradation, and water pollution (Chia et al., 2019). Research on various insect meals indicates that they can replace at least 50% of soymeal protein in diets for pigs and other domestic animals, including poultry and fish (Makkar et al., 2014). Black soldier fly (*Hermetia illucens*) larvae meal (containing 40-65% crude protein and 30-35% lipid, Makkar et al., 2014), housefly maggots (*Musca domestica*), mealworm (*Tenebrio molitor*), and silkworm (*Bombyx mori*) pupae meal have been widely studied for use in animal feed (Aduna, 2019).

Inclusion of black soldier fly larvae meal in the diet of broiler chickens significantly increases average body weight, promoting early growth, improved productivity, and efficiency. In addition, modify the composition of cecal microbiota (increase in beneficial *Lactobacillus* species), which indicates potential health advantages related to gut integrity and function, and contributes to meat quality (Saidani et al., 2025). Use of maggot meal in the fish feed gave higher final weight and average daily gain than the fish diet and commercial diet (Gbai et al., 2018).

Termites are a valuable source of protein (46.3%), fats, and essential amino acids. Earthworms are a natural food source for poultry kept under free-range systems and, live or dried, are highly palatable to poultry. Super worm meal can replace up to 25% of fish meal protein in tilapia juveniles (*Oreochromis niloticus*) fish diet without any adverse effect on feed utilization and body composition (Tiroesele and Moreki, 2012).

Silkworms are high in protein, low in calcium, and have a low calcium-to-phosphorus ratio (Habeanu et al., 2023). They are particularly rich in essential amino acids. Silkworm meal can fully replace fishmeal in the diets of growing and finishing pigs without affecting carcass quality, meat quality, or blood parameters (Kumar, 2017).

Aquatic feed resources

Duckweed is a good feed supplement in diets for monogastric and ruminant animals such as chickens, pigs, cattle, sheep, and goats, as well as fish in aquaculture. Its advantage over other protein sources is that it is characterized by better availability and absorption of amino acids, including lysine and methionine, as well as of vitamins (Sońta et al., 2019). Fingerlings fed on 50% commercial feed + 50% dried duckweed presented a higher weight gain than the ones fed on dried duckweed alone (Tavares et al., 2008).

Azolla (*Azolla pinnata*) is another emerging aquatic feed source that offers numerous benefits for livestock health and productivity, making it a valuable feed supplement in the livestock industry. Azolla is a nutrient-rich feed supplement for livestock that can increase feed efficiency, promote animal growth, and increase milk and meat production (Agarwal et al., 2024). About 1.5-2 Kg Azolla, when fed to milch animals after mixing with concentrate in a

1:1 ratio along with adlib sorghum fodder, has increased milk production by 20.96% in cows. The increase in milk (litres) and fat content (%) was 1-1.5 and 4.3-4.7;1-1.4 in cows, respectively (Yanshi and Anshu Rahal, 2019).

Spirulina and Chlorella microalgae contain 60-70% protein along with essential fatty acids and pigments that are beneficial for monogastric animals, and aquaculture generally compares favorably with soya bean meal and rapeseed meal (Becker, 2013). Chicken dietary supplementation of Spirulina enhanced cost efficiency, health, product quality, decreased cholesterol content, and altered the egg yolk colour, keeping in view the consumer preferences (Suryanarayana, 2024). Additionally, certain types of seaweed have the potential to lower enteric methane emissions, as reported by Li et al. (2018). Seaweeds, especially red and green macroalgae such as Ulva, Gracilaria, and Palmaria, are rich in protein (10-35%), polysaccharides, iodine, and trace minerals. These qualities make them appealing as not only supplements but also as possible substitutes for soy, fishmeal, and synthetic additives in livestock and aqua feeds, as noted by Pereira et al. (2019).

Root, tuber, and starch-based emerging feed resources

Root and tuber crops such as cassava, sweet potato, and taro offer high-energy feed options (Sanginga and Mbabu, 2015). Cassava peels contain 2-5% crude protein but are energy-dense, making them a suitable replacement for maize, according to Onyimonyi and Okeke (2021). Cassava leaves have a higher protein content, ranging from 22-30%, but they require detoxification to reduce cyanide levels. They are also high in energy, and cassava chips can replace up to 50% of maize in poultry diets, as found by Eruvbetine et al. (2003).

Supplementation of sweet potato vines silage increases dry matter intake, milk yield, and the milk components such as milk fat and solids-non-fat in dairy cows (Galla et al., 2020). It contains a moderate to high protein content (160-230 g/kg) and a reasonable fibre level (260 g/kg), according to Gakige et al. (2020). Sweet potato vines have been incorporated into goat and pig diets, in fresh or silage form, respectively, with both studies showing increased feed intake and better growth performance, indicating the potential of sweet potato vines as a viable feed supplement for livestock (Megersa et al., 2013).

Agro-industrial and fruit processing by-products

Agro-industrial by-products come from processing tropical crops and include items such as coffee husk, cocoa pod husks, banana and pineapple waste, sugarcane bagasse, molasses, cottonseed cake, sesame cake, coconut meal, and palm kernel cake (Shah et al., 2025). These are secondary products generated from agricultural processing industries and are often abundant, inexpensive, and frequently discarded, despite their nutritional value. They are affordable and available throughout the year, help in reducing waste and environmental pollution, and provide valuable sources of energy and protein, as pointed out by Shah et al (2025).

Whole cottonseed has a relatively high level of crude protein (15-25%), fat (10-33%), and cellulose (25-30%), with an average energy content of 14.52 ME MJ/kg (Charray et al., 1992). It has been safely used in ruminant diets at up to 25% of dry matter (Arieli, 1992). Whole cottonseed has an apparent digestibility of 72.4% and produces minimal methane gas due to low rumen methane production, about 4.3% (Arieli, 1998). Salting whole cottonseed can improve intake, but mixing it with other by-products gives the best results in terms of intake and growth performance (Charray et al., 1992). Whole cottonseed has been found to contain free dietary gossypol in the range of 4-17 g/kg DM (McDonald et al., 2011), which causes respiratory distress, impaired body weight gain, heart failure, anorexia, weakness, apathy, and death after several days (Gadelha et al., 2014).

Tropical fruit industries generate large amounts of nutrient-rich waste. Mango kernel meal contains 10-12% fat and 8-10% protein with high energy levels. Broiler chickens fed up to 10% mango kernel meal performed similarly to those fed maize (Ravindran and Blair, 2019). Papaya peels have higher crude protein levels, exceeding 110-130 g/kg DM, which is enough to meet the protein needs of small ruminants for maintenance and moderate growth. When included at moderate levels (10-30%) in laying hen, papaya peels improve digestion of protein and energy due to their nutrient content, supporting balanced rumen fermentation (Azevedo et al., 2011).

Pineapple waste contains a lot of soluble sugars (40-75% DM; about 70% sucrose, 20% glucose, and 10% fructose) and pectin, which is comparable to cereal grains for livestock. The high amount of fiber makes pineapple waste widely used for ruminant animal feed compared to pigs and poultry (Idayanti et al., 2021). Citrus pulp is an excellent energy source for ruminants, improving milk fat (FAO, 2018). It also improves rumen fermentation and increases butterfat yield in cows (Bampidis and Robinson, 2006). The by-products of oranges and lemons, mainly peels and rinds, are produced in large quantities from the citrus juice industry. Banana peels have high moisture content, low protein levels, and a significant amount of soluble sugars. When preserved as silage along with dry fodder, they become a suitable feed option for cattle when used with other feeds (Makwana et al., 2025).

Coffee pulp contains associated bioactive compounds, such as polyphenols with good antioxidant properties, which endow additional health benefits and anthocyanins that have potential applications as natural food colorants. The polyphenols can prevent or decrease the peroxidation of fatty acids, reduce oxidative stress in animals at critical physiological stages, and increase the shelf life of animal meat when it is included in their diets. Ensiling coffee pulp has higher crude protein content and lower values of nitrogen-free extracts and tannin (Ameca et al. 2018).

Novel and underutilized vegetation

Novel feed resources in livestock feed can be cost-effective, that is, feed costs, production costs, and increased revenue. The resources are often abundant and can be harvested easily, reducing the need for expensive commercial feed concentrates and conventional feed ingredients (Chisoro et al., 2023).

Enset by-products are rich in fiber and energy and are suitable for cattle (Tsegaye and Struik, 2002), and bamboo, often referred to as the 'world's giant grass,' has emerged as a potential solution to conventional livestock feed, known for its rapid growth, drought tolerance, high biomass production, and adaptability to diverse soil types and climates. Bamboo leaves are rich in protein, fibre, and essential minerals, and can improve livestock health and productivity, reduce methane emissions from ruminants, contributing to more sustainable and climate-resilient livestock farming practices (Zali, 2024; Sasu et al., 2025).

NUTRITIONAL POTENTIAL OF EMERGING TROPICAL FEED RESOURCES

Potential emerging feed resources include leaves and seeds of Moringa, Taro, Mango, Cassava, Pigeon pea, agro-industrial by-products such as rice bran and filter sugar cake (Ben Salem et al., 2004; Muleta, 2024). Poor-quality cellulosic roughages from farm residues such as stubbles, haulms, vines, and from other agro-industrial by-products such as slaughter-house by-products and those from the processing of sugar, cereal grains, citrus fruits, and vegetables from the processing of food for human consumption also come under the category of emerging feeds (Onte et al., 2019).

Emerging tropical feed resources often include newly commercialized feeds such as insects, algae, and hybrid tropical forages, which have shown both nutritional and economic benefits (Makkar, 2018). Non-conventional feeds such as *atella* and mill waste, which have moderate protein content and lower fibre levels, can be used as possible protein sources to improve the nutritional value of poor tropical feed materials in animal feeding systems (Gebremariam and Belay, 2021). As illustrated in Table 1, the nutritional profiles of emerging feedstuffs vary significantly depending on their constituent ingredients.

Table 1. Main source of tropical emerging livestock feeds and their chemical composition

	Chemical composition								
Source of emerging feed	**DM**	**Ash**	**CP**	**CF**	**NDF**	**ADF**	**EE**	**ME (MJ/kg DM)**	**References**
Atella	13.23	6.00	15.90	Na	40.60	19.30	Na	11.81	Gebremariam and Belay (2021)
Coffee pulps	91.13	10.82	13.24	Na	55.19	52.14	1.72	1.33	Wogderess (2016); Ameca et al. (2018)
Rice brans	89.2	Na	6.06	14.09	Na	Na	2.6	4.45	Laswai et al. (2013); Alewi et al. (2022)
Mango peel	20.8	2.6	3.21	Na	11.8	9.51	1.39	18.0 GE (MJ/kg)	Marcos et al. (2020)
Avocado peel	23.6	1.9	9.88	Na	11.5	10.4	58.9	30.5 GE (MJ/kg)	Marcos et al. (2020)
Avocado seed kernel	89.73	3.33	16				11.74	17.52	Getahun et al. (2025)
Cassava leaves	89.4	13.47	21.5	Na	62.1	49.3	6.72	3.9	Oni et al. (2011)
Cassava root chips	92.3	2.9	2.3	0.3			0.8	16.12	Muleta (2024)
Taro leaves	93	12.89	21.27	6.44			4.00	0.23	Muleta (2024)
Soya bean husk	89.55	5.20	12.32	32.54	66.10	49.72	2.70	10.07	Chellapandian (2019); Shuaib et al. (2023)
Green cabbage waste	11.00	8.75	11.85	11.85	25.55	23.14	1.89	12.17	Mahgoub et al. (2018)
Dry tomato vines	94.99	13.87	15.75	32.22	59.77	36.42	1.85	Na	Galal et al. (2020)
Dried tomato pomace	91.01	3.28	21.11	29.33	65.24	40.93	9.21	20.15 GE (MJ/kg)	Omer and Abdel-Magid (2015)
Enset corm	89.53	4.8	3.35				0.57	15.11	Getahun et al. (2025)

Fish by-product meal	87.95	41.88	43.01	Na	53.81	40.17	1.45		Samadi et al. (2023)
Poultry waste	90.38	28.82	21.34	16.09	Na	Na	2.61	5.93	Laswai et al. (2013); Usman et al. (2019)
Banana leaves	91.84	9.78	10.63	29.35	49.8	36.8	8.43	4.5	Laswai et al. (2013); Selim et al. (2022)
Bamboo leaves	61.8	11.43	8.49	35.56	54.7	38.9	4.67	8.41	Laswai et al. (2013); Selim et al. (2022)
Seaweed	18.78	14.19	10.13	6.9	16.5	9.7	3.56	Na	Selim et al. (2022)
Sugarcane bagasse	89.9	4.87	3.81	37.89	75.6	58.8	0.5	Na	Selim et al. (2022)
Pineapple peel	15.08	4.93	8.01	Na	26.48	11.97	0.35	7.34	Kiatti et al. (2023)
Pineapple pomace	20.98	2.78	8.81	Na	21.53	9.75	0.3	7.36	Kiatti et al. (2023)
Sweet potato vine	90.68	12.01	12.43	20.71	44.84	39.46	1.68	8.34	Chen et al. (2024)
Mealworm	97.02	6.99	53.83	7.53	Na	Na	28.03	Na	Sajid et al. (2023)
Earthworm	94.23	10.07	38.99	0	18.26	5.93	7.5	Na	Samadi et al. (2023)
Moringa leaves	77.4	14.1	26.3	8.8	Na	Na	5.7	10.0	Laswai et al. (2013)
Banana pseudostem hay	93	12.4	3.5	Na	64.6	36.2	1.3	5.63	Laswai et al. (2013); Oliveira et al. (2014)
Banana kocho	93.59	20.34	10.04	Na	Na	Na	0.88	7.20	Getahun et al. (2025)
Mango seeds	89.04	2.85	8.93	21.93	Na	Na	3.5	Na	El-Sanafawy et al. (2023)
Mango seed kernel	58.13	2.61	5.95	2.32	Na	Na	11.54	13.97 (ME MJ/kg)	Elgindy (2017); Beriso and Tesfaye (2024)

ADF: Acid detergent fibre, CF: Crude fibre, CP: Crude protein, EE: Ether extract, GE: Gross energy, ME: Metabolizable energy, Na: Not available, NDF: Neutral detergent fibre.

CONSTRAINTS OF EMERGING TROPICAL FEED RESOURCES

Anti-nutritional factors

Some new feed materials are known to contain high levels of lignin, silica, toxins, and various anti-nutritional compounds, while offering low energy and protein content (Beriso, 2022). A major problem in using legume plants as feed for ruminant animals is the presence of tannins and other anti-nutritional substances such as saponins, cyanogens, mimosine, and coumarins, which reduce the absorption and use of nutrients (Andhale, 2024).

Processing and technological limitations

Non-conventional feed resources often require specific processing and preservation techniques to enhance their nutritional value and decrease any potential anti-nutritional factors. However, there is a lack of proper processing and preservation techniques for these resources, which limits their utilization (Muleta, 2024).

Mechanical treatments such as grinding fibrous crop residues, pelletizing, extrusion, and dehydration need costly and energy-consuming machines, which are rare or expensive in tropical regions (Sundstøl and Owen, 1985). Techniques such as grinding, pelletizing, steaming, or heating need fuel or electricity, but many rural areas lack reliable electricity to run feed mills in Ethiopia (Mengistu et al., 2017).

Seasonality, availability, and perishability

The availability of non-conventional feed resources can be limited, especially during dry seasons or in areas with low agricultural productivity. Additionally, the composition and quality of these resources may also vary seasonally, making it difficult to formulate consistent and balanced diets (Muleta, 2024). Tropical crop residues and fruit waste are typically available only during harvest seasons (Devendra and Leng, 2011). Research has shown that drying and ensiling non-traditional feeds such as fruit and vegetable waste are effective preservation techniques that help overcome seasonal supply problems (Wadhwa and Bakshi, 2013).

The high moisture content of wet brewery grain (80 to 85%) makes the by-product particularly susceptible to microbial growth and subsequent spoilage within 7 to 10 days (Chanie and Fievez, 2017). This issue limits their strategic use unless suitable preservation methods are available. Treatments such as drying, grinding, and storage can improve the use of these resources, but adequate research and development are needed to optimize processing methods (Andhale, 2024).

Lack of knowledge, limited research, and extension services

Producers often lack knowledge of the optimal timing for crop harvest, processing techniques (such as silage, drying, and ensiling), detoxification approaches, and feed formulation standards (Tolera et al., 2012). Many small-scale farmers may lack the knowledge, skills, and resources necessary to effectively incorporate these feed sources into their feeding practices (Sime and Duguma, 2025). Inadequate data on the nutritional value and digestibility of novel feedstuffs remains a primary barrier to their widespread use in animal production (Muleta, 2024). According to FAO (2012), many tropical feed resources remain poorly studied, thereby restricting their adoption and limiting the availability of accurate nutrient composition data. As noted by Devendra and Leng (2011), insufficient extension support for safe inclusion levels, processing procedures, and ration formulation is an additional barrier to the use of emerging feed sources.

PROCESSING AND INTEGRATION STRATEGIES OF EMERGING TROPICAL LIVESTOCK FEED INTO COMMERCIAL DIET

Many ETFRs require significant processing before they can be used. Various processing techniques, such as fermentation, boiling, sun-drying, ensiling, autoclaving, milling, soaking, sprouting, germination, gamma radiation, and genomic technology, effectively reduce certain anti-nutritional factors, such as cyanogenic glycosides, oxalate, and trypsin inhibitors (Olarinre et al., 2022; Lahutiya and Yadav, 2023). Insects need drying, grinding, and safe handling of the substrate (Chia et al., 2021). Cassava requires sun-drying, fermentation, or boiling to eliminate cyanide (Egboduku et al., 2024). Duckweed and Azolla should be dried or partially sun-cured to prevent spoilage (Chaji and Pormhammad, 2025).

Methods such as ferrous sulphate, chopping, de-huling, autoclaving, grinding, lactic acid fermentation (reduces tannins by 60% (Zhang et al., 2026), alkaline treatment up to 90% in agro-industrial by-products and tree leaves (Olarinre et al., 2022). At 60°C for 6 hours, it effectively reduced anti-nutrient content in tiger nut tannins by 61%, polyphenols by 48%, phytate by 44%, oxalate by 58%, and alkaloids by 13%, as well as improving the nutritional value (Kemboi et al., 2023).

Main methods to reduce hydrogen cyanide levels and improve cassava peels' consumption by ruminants involve soaking, boiling, sun-drying, ensiling, and sulphur addition (Padmaja and Steinkraus, 1995). Heat treatment and fermentation are effective in reducing protease inhibitor activity in plant-based feed (Olukomaiya et al., 2020).

Goitrogens, commonly found in avocado peels, can interfere with iodine metabolism, potentially leading to thyroid enlargement and reduced metabolic efficiency (Tripathi and Mishra, 2007). Iodine supplementation in poultry diets is recommended to counteract the effects of goitrogens (Surai et al., 2019). Lectins, also found in high levels in avocado peels, can bind to the intestinal lining, impairing nutrient absorption and causing digestive issues (Vasconcelos and Oliveira, 2004). Thermal processing and enzymatic treatments are effective ways to reduce lectin activity (Zhang et al., 2026).

When phytase supplements are added to broiler diets, they improve their overall growth and development, and enhance calcium and phosphorus uptake by bones (Rizwanuddin et al., 2023). The crude protein (CP) content of the treated rice straw increased as urea-molasses treatment increased from 0 to 4% (Akinfemi et al., 2020). For aflatoxin-contaminated oilseeds used in animal feed, ammonization is considered the preferred processing method (Kemboi et al., 2023).

CONCLUSION

Emerging tropical feed resources offer a promising approach to address the ongoing challenges of feed scarcity, high feed prices, and the growing competition between human and animal nutrition in tropical areas. These resources are valuable because they are nutritionally rich, widely available, and can thrive in difficult environmental conditions. They can be used as substitutes for or as supplementary materials to traditional feed ingredients such as maize, soybean meal, and alfalfa. ETFRs include various types such as multi-purpose forage trees and legumes, insect-based protein sources, aquatic plants, improved grasses, fruit and vegetable by-products, and feeds that have been fermented or enhanced through biotechnology. These feeds have shown great potential to boost commercial livestock production while reducing environmental impacts and feed costs.

Despite these benefits, several factors hinder the use of ETFRs in livestock systems. These include issues such as high water content, short shelf life, and the presence of antinutritional substances, including tannins, cyanogenic glycosides, oxalates, and mimosine. There is also an inconsistency in nutrient levels, a lack of standardized processing methods, poor storage facilities, limited knowledge among farmers, and insufficient support from extension services. The

availability of these feeds can vary with the seasons, and the reliability of agro-industrial supply chains is uncertain, which complicates their year-round use. Advanced processing techniques such as ensiling, fermentation, extrusion, pelleting, and heat treatment are not widely used due to high costs, limited equipment, a lack of technical skills, and weak infrastructure. To make ETFRs valuable, there should be collaboration among researchers, government officials, extension workers, and feed producers. Improving farmer education, developing affordable processing methods, supporting research on detoxification and enhancing nutritional content, and establishing effective supply chains can greatly increase the use of these feeds. To properly use these new feed resources, it is advisable to conduct further research to evaluate their nutritional value and economic viability for livestock.

DECLARATIONS

Authors' contributions

Mule Demlie, Nestanet Beyero, and Awoke Kefyalew designed the review. Nestanet Beyero constructed the title and offered a constructive idea for revising the manuscript. Mulu Demlie conducted the conceptualization, literature search, writing, and original draft. Awoke Kefyalew contributed to the literature search, visualization (tables), drafting the review, and editing. All authors read and approved the final version of the manuscript to be published.

Availability of data and materials

The data that support the findings of this study are available in the present study.

Competing interests

The authors declared no conflicts of interest.

Ethical considerations

The authors declare that this manuscript is original and is not being considered elsewhere for publication. Ethical issues, including consent to publish, misconduct, fabrication of data, and redundancy, have been checked by all authors. The authors did not use any AI applications for writing and preparing this article.

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Challenges and Perspectives of Cryopreservation in Ram Semen of Indigenous Sheep: A Review

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ABSTRACT

Artificial insemination (AI) is a widely used technique to enhance genetic diversity and breeding efficiency in livestock such as cattle, sheep, goats, and pigs. Sheep are important in developing the livelihoods of poor farmers by reducing poverty and promoting sustainable agriculture and food security. Therefore, the present study aimed to explore the challenges and perspectives associated with cryopreserving ram semen to conserve indigenous sheep ecotypes. Indigenous sheep populations are vital for meat production in challenging environmental conditions, where feed is limited and land is scarce. However, concerns have emerged about the decline in indigenous sheep populations, mainly due to the growth of composite breed production. Composite breeds, which are preferred for their faster growth rates and larger body size, reduce the capacity of indigenous sheep to survive in environments affected by climate change. This decline highlighted the importance of assisted reproductive technologies, such as AI and semen cryopreservation, in preserving the genetic resources of indigenous sheep before they become extinct. While AI and semen cryopreservation have been successful in livestock species such as cattle and pigs for genetic preservation, results in sheep have been less successful. Ram semen quality and fertility are often compromised by the freezing and thawing process. Ram spermatozoa produce high levels of reactive oxygen species (ROS) and are sensitive to cold shock, leading to oxidative stress, cellular damage, and conception rates of 30-50%. Adding antioxidants, such as polyunsaturated fatty acids and plant extracts, to semen extenders can reduce ROS production and mitigate oxidative damage, thereby improving sperm quality and functionality during cryopreservation. Despite these advances, optimizing freezing and thawing protocols and addressing knowledge gaps are necessary to enhance semen preservation outcomes.

Keywords: Antioxidant, Artificial insemination, Extender, Nanoparticle, Sheep, Semen cryopreservation

INTRODUCTION

Semen cryopreservation has been shown to improve reproductive efficiency following the use of artificial insemination (AI) by facilitating the exchange of superior genetic material worldwide (Yimer et al., 2016; Kurmi et al., 2018; Maksimovic et al., 2018). Semen cryopreservation is an important technique for enhancing global food security, as it allows the use of superior genetic material to improve livestock performance (Ngcobo et al., 2023). However, the use of semen cryopreservation posed significant challenges at the farm level, especially for rural farmers in underdeveloped and developing nations, due to resource and educational constraints. The use of semen cryopreservation remains in the research phase. In developing countries, although research facilities are available, they are often located far from smallholder farmers, making cryopreservation of semen impractical. Thus, food insecurity is increasing because the indigenous sheep population cannot be preserved, having been diminished by uncontrolled crossbreeding and climate change (Ngcobo et al., 2022). To address the food insecurity crisis in developing and underdeveloped countries, the United Nations has taken action to support farmers, enhance their livelihoods, and protect the global community by implementing the sustainable development goals targets for zero hunger and no poverty. Mitigation strategies included educating farmers on the use of assisted reproductive technologies (ARTs) to improve the reproductive efficiency of their livestock (Sithole et al. 2025). However, there has been limited reporting in developing and underdeveloped countries on advancements in ART applications, particularly in semen cryopreservation. Even in developed countries with adequate resources and education, ram semen cryopreservation continues to pose challenges. Conflicting results have been reported in studies evaluating the use of different semen diluents during ram semen characterization and cryopreservation.

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Current studies on ram spermatozoa during and after thawing primarily examine how sperm parameters respond physically across different environments and time points (Yimer et al., 2016; Zhang et al., 2024). To understand sperm responses to cold shock and oxidizing stress induced by reactive oxygen species (ROS), scientists used different semen extenders, antioxidants, and nanoparticles to balance cytoplasmic function and increase the number of natural antioxidants on the sperm plasma membrane during cryopreservation. The addition of some of these diluents and antioxidants has improved semen quality following AI (Yimer et al., 2016; Abbasi et al., 2021; Arruda et al., 2021). However, challenges associated with sperm viability during cryopreservation and thawing affect the success of AI. According to the previous studies, Kurmi and Naqvi (2014) documented low conception rates of 20% and 26.6% following single and double insemination with frozen-thawed semen. Furthermore, Halawa et al. (2025) reported a conception rate of 45.4%, while Najafi et al. (2024) observed a rate of 83.3%. Bernia and Benamora achieved the highest conception rate of 90.47% following the utilization of fresh semen. Gibbon et al. (2019) reported a satisfactory pregnancy rate of 50-70% after using fresh semen. According to Yimer et al. (2016), cryopreservation not only lowers sperm quality, increases DNA damage, decreases mitochondrial function, and increases oxidative stress, but also these damages subsequently elevate the risks of abortion and apoptosis in ewes (Yimer et al., 2016). Consequently, low conception and lambing rates associated with cryopreserved semen have increased the use of liquid preserved semen to achieve optimal pregnancy rates (Najafi et al., 2014). Furthermore, factors including oxidative stress, cryoinjuries, and reduced sperm viability have been identified as major limitations affecting the success of ram semen cryopreservation. Therefore, the current study aimed to address the challenges and perspectives associated with cryopreservation of ram semen to ensure the successful application of AI in conserving indigenous sheep ecotypes.

OVERVIEW OF DIFFERENT SHEEP CONSERVATION METHODS

The ARTs, such as semen cryopreservation and AI, have been used in previous years to conserve sheep biodiversity. However, there are still challenges related to their application. These challenges include limited access to resources and knowledge for rural farmers and limited success with ram semen cryopreservation, which is associated with increased embryonic loss and impacts sheep production (Yimer et al., 2016). According to Ngcobo et al. (2022), the two principal conservation strategies employed were *in situ* (extensive farming) and *ex situ* (intensive farming and the use of ARTs). So far, there has been criticism of the implementation of the *in*-situ approach. Approximately 80% of emerging farmers raised their indigenous breeds under extensive management for communal grazing, with limited monitoring. This situation led to challenges, including uncontrollable crossbreeding and inbreeding, which contributed to the decline in indigenous breed populations. A genetic cross of Dorper in Zulu diversity demonstrated evidence of uncontrolled crossbreeding (Selepe et al., 2018). As Ngcobo et al. (2022) stated, *ex situ* conservation (*in vivo* and *in vitro*) remains a crucial technique for conserving indigenous breeds. To prevent crossbreeding, it is recommended to keep distinct breeds separated in designated areas, such as those at the Agricultural Research Council (ARC), utilizing the *in vivo* conservation method. Additionally, *in vivo* conservation has been successful, as animals were continuously monitored throughout the process. However, challenges arise when applying ARTs *in vitro*.

Semen cryopreservation plays a significant role in breeding and conservation programs, particularly for small ruminants (Kurmi et al., 2018). This method preserves genetic material from superior sires, allowing the widespread transfer of desirable traits to many female sheep and enhancing genetic progress (Kurmi et al., 2018; Selepe et al., 2018). Azizunnesa et al. (2014) observed that semen preservation facilitated the frequent use of AI, the conservation of endangered species, and the international exchange of genetic material. A report highlighting the severe decline in indigenous sheep breeds has caused concern among farmers, since these breeds are vital for wool production and play a crucial role in eradicating poverty and hunger (Ngcobo et al., 2022). Genes associated with high adaptability and disease resistance enable farmers to maintain indigenous breeds at minimal cost. It is important to highlight that around 80% of farmers who depend on indigenous breeds are smallholders (Kunene and Fossey, 2006). However, commercial farmers crossbreed indigenous sheep with exotic breeds to produce a composite breed with improved genetic material (Ngcobo et al., 2019). Therefore, these composite breeds meet market demand, which, in turn, impoverishes rural farmers.

Over 500,000 sheep are artificially inseminated annually in Australia, 300,000 in France, 60,000 in Spain, and 50,000 in Canada, as reported by Gibbons et al. (2019). According to Falch et al. (2022), the production of sheep embryos, *in vivo*-derived and *in vitro*-produced (IVP), increased by 20% between 2018 and 2019. Viana et al. (2022) indicated that although no frozen-thawed embryo transfers were recorded, the study did find a significant increase in fresh IVP embryo transfers. Ngcobo et al. (2022) noted that advanced reproductive biotechnologies, including estrus synchronization, were applied in South African indigenous sheep. Conversely, limited information is available regarding the outcomes of AI using post-thawed semen. Given the decline in indigenous sheep breeds in South Africa, there is an imperative to enhance the application of *ex situ* conservation methods to preserve the remaining genetic resources of these breeds.

However, *ex situ* conservation methods are more effective in developed countries than in underdeveloped or developing nations, as evidenced by the lower number of reports on AI follow-up using frozen semen from these regions. The limited report on AI follow-up studies using frozen-thawed semen in developing countries heightened concerns that ineffective sheep production management could put developing countries such as South Africa at risk of hunger and poverty (Ngcobo et al., 2022). Farming with indigenous sheep is increasingly essential for rural farmers, as these breeds are at risk of extinction and should be conserved (Kunene et al., 2011).

Artificial insemination in sheep has achieved limited success, often depending on chilled semen to improve fertility (Najafi et al., 2014). Laparoscopic AI can help overcome the lower fertility rates usually associated with frozen-thawed semen, which result from the ewe's complex reproductive anatomy, particularly the cervix (Kumar and Naqvi, 2014). Rizkallah et al. (2022) reported a 20-30% pregnancy rate following cervical AI with frozen-thawed semen, making sheep breeders reluctant to use AI due to an inability to achieve positive results, particularly following the use of cryopreserved semen. Laparoscopic AI enables direct semen deposition into the uterus, which enhances the likelihood of fertilization compared to cervical and vaginal AI. Nonetheless, laparoscopic AI applications remain controversial due to factors such as cost, required expertise, and ethical considerations related to surgery and anesthesia (Rizkallah et al. 2022). This approach encourages the use of cervical AI and discourages the use of vaginal AI (Akhter et al. 2023). Vaginal AI is cheaper and simpler than cervical AI. However, imprecise sperm placement during vaginal AI and issues with the cervical barrier may hinder sperm from reaching the site of fertilization, thereby reducing the probability of conception (Masoudi et al., 2017).

Kumar and Naqvi (2014) indicated that controlled internal drug release and equine chorionic gonadotropin are frequently employed exogenous agents at the end of the progesterone therapy during estrus synchronization, with the purpose of promoting cervical relaxation. Several studies have reported that the use of these exogenous substances can effectively modify estrus responses in many mammals, although they do not guarantee optimal fertilization (Najafi et al., 2014; Gibbons et al., 2019; Benia and Benamora, 2023). Fierro et al. (2013) found that the effectiveness of this exogenous substance on the estrus response was influenced by a longer interval between doses, which negatively impacted the fertilization rate. Low fertilization rates in ewes with an optimal body condition score of 3-4 are associated with increased oxidative stress in male germ cells, which is a recognized negative effect of semen cryopreservation (Yimer et al., 2016; Thompson et al., 2024).

The number of services per insemination had a significant effect on lambing rate after trans-cervical AI with frozen semen (Table 1). Double inseminations conducted between 14 and 22 hours following estrus detection yielded higher conception and lambing rates (26.40% and 19.3%) compared to a single insemination performed at 10 hours (20% and 10%; Kumar and Naqvi, 2014). According to Bergstein-Galan et al. (2022), the semen preservation method affected the success of laparoscopic AI in Dorper, white Dorper, and crossbred ewes. A higher conception rate was observed with fresh semen (53.84) than with frozen-thawed semen (30.23). Masoudi et al. (2017) reported the best fertility results with laparoscopic AI compared to the vaginal and trans-cervical AI in Zandi sheep. These results are aligned with the findings reported by Najafi et al. (2014). However, both semen types had lower fertility rates than those reported by Halawa et al. (2025). According to Benia and Benamor (2023), the reproductive performance of ewes utilizing cervical AI was superior in ewes aged three years and above that were inseminated with semen from mature rams, compared to those that were inseminated with semen from younger rams. Regarding the use of vaginal AI, Madrigali et al. (2021) found no substantial difference in fertility results among Assaf, Awassi, and the Assaf × Awassi crossbreed.

Semen cryopreservation

Semen cryopreservation is the long-term storage of animal germ cells at cryogenic temperatures (-150 °C or lower) to support the application of ARTs (Kurmi et al., 2018). This method can be used in countries such as South Africa, where smallholder farms are mostly found in distant areas with no access to research facilities and government services. Despite the advantages of semen cryopreservation, this method has been reported to cause a high level of oxidative stress, which damages spermatozoa and reduces their post-thaw survival (Yimer et al., 2016; Bustani and Baiee, 2021; Sun et al., 2024). Therefore, a decrease of about 40-50% in sperm viability following semen cryopreservation has been documented (Mafolo, 2018). Moreover, poor semen quality has been found to result in poor embryonic development during early gestation and to be associated with higher embryonic losses (Falchi and Yimer et al., 2016). Among the many factors that lead to poor frozen-thawed semen quality, the heightened production of ROS by non-viable spermatozoa and the acidic pH were especially notable (Riesco et al., 2021; Mehdipour et al., 2025). Therefore, enhancing semen extenders with antibiotics and antioxidants is crucial for preserving semen quality, minimizing oxidative stress, and ultimately improving cryopreservation and conception rate (Mphaphathi et al., 2016; Yimer et al., 2016).

Table 1. Effects of different artificial insemination methods on sheep fertility

Breed	AI method	Semen type	Number of females inseminated	Conception rate (%)	Lambing rate (%)	Litter size rate (%)	Twinning rate (%)	References
Bharat merino	Trans-cervical	Frozen	Group 1 100	20	10	-	-	Kumar and Naqvi (2014)
Bharat merino	Trans-cervical	Frozen	Group 2 140	26.4	19.3	-	-	Kumar and Naqvi (2014)
Zandi	Vaginal	Fresh	50	62	62	-	10.0	Masoudi et al. (2017)
Zandi	Laparoscopic	Fresh	50	66	64	-	6.0	Masoudi et al. (2017)
Zandi	Trans-cervical	Fresh	50	64	62	-	6.0	Masoudi et al. (2017)
Ghezel	Laparoscopic	Fresh	30	83.3	76.6	1.30	28.0	Najafi et al. (2024)
Ghezel	Cervical	Fresh	30	60	60	1.28	34.6	Najafi et al. (2014)
Ghezel	Natural mating with PSMG injection	Fresh	30	90	86.6	1.35	18.5	Najafi et al. (2014)
Ghezel	natural mating without PSMG injection	Fresh	30	70	66.7	1.10	10.0	Najafi et al. (2014)
Palestinian Assaf	-	Fresh	37	61.1	-	-	-	Halawa et al. (2025)
Palestinian Assaf	-	Frozen	50	45.4	-	-	-	Halawa et al. (2025)
Palestinian	Natural mating		36	71.9	-	-	-	Halawa et al. (2025)
Bangladesh	Laparoscopic	Frozen	-	72.2	100		-	Kumar and Naqvi (2014)
Rembi	Cervical	Fresh	Group 1 42	61.90	-	-	-	Benia and Benamora (2023)
Rembi	Cervical	Fresh	Group 2 42	78.58	-	-	-	Benia and Benamora (2023)
Rembi	Cervical	Fresh	Group 3 42	90.47	-	-	-	Benia and Benamora (2023)
Awassi	Vaginal	Fresh	94	45.7	56.4	1.23	-	Madrigali et al. (2021)
Assaf	Vaginal	Fresh	228	47.4	67.1	1.47	-	Madrigali et al. (2021)
Crossbreed (Awassi x Assaf)	Vaginal	Fresh	170	46.8	63.9	1.40	-	Madrigali et al. (2021)

Semen extenders

Semen extenders are specialized cryopreservation media designed to protect sperm against damage from freezing, osmotic stress, oxidative damage, and ice crystal formation, thereby preserving sperm viability (Bustani and Baiee, 2021). Notably, semen extenders differ in their chemical and physical properties due to their diverse roles in sperm functionality (Yimer et al., 2016). These roles include protecting sperm cells against cold shock, maintaining optimal pH, supplying essential nutrients, and controlling microbial growth. During semen cryopreservation, sperm survival largely depends on protective semen extenders, such as egg yolk, skim milk, AndroMed®, soybean lectin, and antioxidants, including moringa extracts, ascorbic acid, nanoparticles, and curcumin (Kulaksiz et al., 2010; Mafolo, 2018; Arruda et al., 2021). However, variation in sperm motility and morphometry after dilution during semen cryopreservation is influenced by the extender's chemical composition, concentration, and dilution method.

Since extenders are usually not chemically identical, they can originate from either plants or animals. Materials from the same source can differ in chemical composition. Egg yolk is often used as a semen extender and protein source because of its low levels of low-density lipoproteins, yet its composition can vary between batches (Swelum et al., 2023). However, the content of fatty acids differs among different bird species, which demonstrates significant effects on semen viability (Table 2; Kulaksiz et al., 2016; Swelum et al., 2023).

Table 2. Influence of different semen extenders on ram semen quality

Breeds	Extender	Findings	References
Karayaka	Chucker egg yolk	It demonstrated the most effective cryoprotection, with the highest sperm motility at 54.0%, outperforming the other five avian egg yolks. Frozen sperm had a higher viability rate (59%) than sperm preserved in other species.	Kulaksiz et al. (2010)
Karayaka	Chicken egg yolk	Recorded a lower percentage of sperm viability, motility, membrane integrity, and acrosomal integrity.	Kulaksiz et al. (2010)
INRA 180	Skim milk-based	Sperm quality and seminal plasma composition were superior in low-frequency ejaculation compared with high-frequency ejaculation, and earlier ejaculators exhibited greater quality than later ones. Furthermore, seminal plasma components were correlated with sperm quality after 24 hours of storage.	Benmoula et al. (2022)
Santa Ines	8% Low-Density Lipoprotein -Tris –glucose extender	Improved velocity parameters, motility, and curvilinear velocity	Pereria et al. (2023)
Leccese	Milk lactose egg yolk (Milk- LY)	Milk-LY extender outperforms Tris-Fructose egg yolk in preserving sperm quality at intermediate concentrations (100-500 × 10^6 spermatozoa/mL). However, increasing sperm concentration to 800 × 10^6 spermatozoa/mL negatively impacts sperm characteristics, potentially reducing lambing rates despite higher sperm numbers. For laparoscopic insemination, semen doses of 20-40 × 10^6 spermatozoa per ewe were effective.	D'Alessandro et al. (2021)
Suffolk	Ovipro	Improved sperm motility, viability, and low morphological abnormalities within the short term, from 24 to 48 hours.	Hegedusova et al. (2012)
Suffolk	Triladyl	Demonstrated effective stabilizing properties, yielding consistent results over extended storage periods (24-96 hours).	Hegedusova et al. (2012)
Duolang	Soybean lecithin in Tri's fructose extender	Ram sperm retained their fertilizing ability when preserved at 0°C in a Tris-based extender containing 0.5% soybean lecithin instead of egg yolk, resulting in normal offspring after insemination.	Zhao et al. (2021)
Bangladeshi indigenous	Tris fructose egg yolk	Exhibited less improvement in semen parameters compared to Triladyl.	Rekha et al. (2016)
Bangladeshi indigenous	Triladyl	Triladyl has high sperm motility, viability, and functional integrity	Rekha et al. (2016)
Crossbred- pirot prameka Wurttemberg and lle de France	Soybean lecithin (AndroMed®-based extender)	Improved sperm morphology and motility	Maksimovic et al. (2018)
Dorper	AndroMed®	The percentage of live sperm with intact acrosomes was highest in fresh semen (97.8%) and decreased with refrigeration and freezing.	Quintero-Elisea et al. (2024)
Dorper	OviXcell	Sperm viability rates were 87% in fresh semen, 72% in refrigerated semen, and 55% in post-thawed semen, with a slightly higher rate of 68% observed in a specific post-thawed semen context.	Quintero-Elisea et al. (2024)
Kail	Tris-based extender	Tris-based extender outperformed skim-milk and sodium citrate extenders, yielding higher percentages of motile, progressive motile, rapid progressive, and medium progressive motile sperm	Hameed et al. (2024)
Wrzosówka rams	milk extender containing 5% egg yolk	Replacing egg yolk with soybean lecithin worked well in milk extender but was ineffective in Tris extender.	Gogol et al. (2019)

According to Yimer et al. (2016) and Zhang et al. (2024) excessive dilution can reduce sperm density and effective sperm count, even when the volume of insemination is increased. Given the ewe's complex reproductive anatomy, an increased semen volume with reduced viscosity can worsen reflux of spermatozoa from the cervix, thereby adversely affecting conception and pregnancy rates (D'Alessandro et al., 2021). Conversely, insufficient dilution can threaten sperm survival because it lacks the necessary nutrients, buffers, and antioxidants that maintain stable pH and redox

balance and reduce seminal plasma levels (Zhang et al., 2024). Therefore, choosing the correct semen dilution ratio and method is essential for enhancing and preserving sperm viability and motility (Table 3).

The present results indicated that the milk skim lactose egg yolk extender was more effective than Tris-fructose egg yolk at preserving semen quality when diluted with intermediate sperm concentration (Table 2; 100 to 500 × 10^6 spermatozoa/mL). A rapid increase in the sperm concentration led to a decrease in sperm viability, thus reducing the lambing rate (D'Alessandro et al., 2021). Benmoula et al. (2022) observed positive outcomes with low-frequency ejaculation compared with high-frequency ejaculation when using a skim milk-based extender in INFRA180 rams. The use of Ovipro and Triladyl on Suffolk ram semen improved semen quality, but their capacity was affected by hours during freezing. Ovipro extender improved sperm motility and viability and reduced morphological abnormalities in the short term (24 to 48 hours), whereas Triladyl lasted longer (24 to 96 hours). Consequently, Rekha et al. (2016) reported Triladyl as a superior semen extender compared to tris fructose egg yolk extender. According to Kulaksiz et al. (2010), the use of egg yolk from different avian species indicated that chukar egg yolk may serve as an effective substitute for chicken egg yolk in the cryopreservation of semen. Zhao et al. (2021) reported that soybean lectin in a tris-based extender can be used to achieve successful AI and increase lambing rate following semen cryopreservation. Maksimovic et al. (2018) reported that soybean lectin enhanced semen parameters across different breeds. Quintero-Elisea et al. (2024) compared OviXcell and AndroMed® semen extenders, and the results indicated that both extenders can be used for the cryopreservation of ram semen, as they improved semen quality.

Table 3. Effects of egg yolk from different avian species on rams' semen quality

Avian species	Concentration	Findings	Reference
Japanese quail	15%	The best results were obtained with quail egg yolk compared with chicken egg yolk EY. Thus, quail EY can replace chicken EY.	Swelum et al. (2023)
Chicken, pigeon, duck, Goose and turkey	15%	None of these three avian EYs were suitable substitutes for chicken egg yolk in ram semen extenders, except for quail EY, which resulted in lower post-thaw semen quality.	Swelum et al. (2023)
Pigeon, turkey, Japanese quail, Guinea fowl, and domestic chicken	10%	Sperm motility was best preserved after dilution with Japanese quail and chicken egg yolks, outperforming the other avian egg yolks in terms of cryoprotective effects.	Taha et al. (2020)
Chucker, domestic chicken, goose, turkey, and Japanese quail	15%	Chucker egg yolk could replace chicken egg yolk as a suitable component in semen extenders for cryopreservation.	Kulaksiz et al. (2010)
Chicken	0, 5, 10, 15, and 20%	A 10% egg yolk concentration yielded better membrane integrity and sperm motility in cryopreserved semen, whereas 20% egg yolk concentration resulted in higher acrosome integrity.	Mafolo (2018)
	5, 10, 15, and 20%	Sperm motility, viability, and functional integrity were significantly enhanced during preservation when using 10% egg yolk compared to other concentrations.	Azizunnesa et al. (2014)
Turkey, partridge, ostrich, duck, tortoise, or chicken egg yolk	20%	Semen diluents with partridge egg yolks enhanced cleavage rates and early embryonic development. In contrast, ostrich, turkey, and duck egg yolks yielded fertilization and embryonic development rates comparable to those of traditional diluents containing 20% chicken egg yolk.	Ali et al. (2013)

EY: Egg yolk

Impact of egg yolk from different bird species on ram sperm quality

Different egg yolks from different bird species have been used to preserve ram semen, yielding varying results. A study by Swelum et al. (2023) has explored the relationship between the fatty acid composition of egg yolks from different bird species and the quality of ram semen after thawing. As mentioned previously, egg yolk is the most commonly used semen extender to improve semen quality during storage. The protective effects of egg yolk on sperm during cooling and freezing can be attributed to its rich phospholipid, cholesterol, and low-density lipoprotein composition, which help maintain sperm membrane integrity, prevent cold shock, and improve sperm viability (Azizunnesa et al., 2014). However, several studies have indicated that nutrition, breed, and age of the egg also influence egg yolk fatty acids.

Zhang et al. (2024) reported that as birds grow older, their egg yolks experience notable changes, such as reduced moisture, higher crude fat levels, and increased diversity in fatty acids (Zhang et al., 2024). Since mid-laying eggs have higher fatty acid content than early laying eggs, it is recommended to extract the yolk from eggs collected or hatched during the mid-laying stage. Furthermore, fatty acid profiling has been performed on egg yolk from various avian species, regardless of egg age (Kulaksiz et al., 2010; Swelum et al., 2023). According to Swelum et al. (2023), the egg yolks of turkeys, pigeons, and chickens contain significantly higher percentages of polyunsaturated fatty acids (PUFAs), with levels two to three times higher than those found in duck and goose egg yolks. Consequently, differences in the proportion of PUFAs in avian egg yolk may explain the variation in the results observed when comparing the effectiveness of egg yolk from avian species on ram semen quality (Swelum et al., 2023). Kulaksiz et al. (2010) reported that, when comparing EY across different bird species, chucker EY demonstrated superior semen quality to chicken EY and other species' EY. Furthermore, Taha et al. (2016) disapproved of the use of pigeon, turkey, and guinea fowl EY and suggested replacing chicken EY with Japanese quail EY because of the higher fatty acid level in quail egg yolk compared to chicken egg yolk. Consequently, examining the impact of each EY species on ram semen revealed that each species had a unique EY composition, warranting further investigation. Perumal (2018) noted that differences in EY among species were affected by their habitat. Wild birds, such as quail and chukars, have easy access to omega-3 sources, such as fish, seeds, and insects. In contrast, domestic chickens rely entirely on commercial feed for their omega-3 intake. Thus, omega-3 supplementation in the diet of domestic avian species is critical for improving the nutritional profile and health benefits of egg yolks (Ngcobo et al., 2024; Yaung et al., 2023).

Several studies have indicated that egg yolk levels affect semen preservation, with high concentrations potentially being toxic to semen (Zhang et al., 2024). Since egg yolk comes from animals, it may pose a risk of transmitting diseases through unidentified pathogens. Therefore, understanding the specific impact of each egg yolk and determining the best dilution concentration for semen are essential. According to Azizunnesa et al. (2014), the ideal egg yolk concentration required for semen dilution depends on the preservation method, such as cryopreservation, liquid storage, or freeze-drying. Azizunnesa et al. (2014) found that prolonged preservation time negatively impacted sperm quality, reducing motility, viability, functional integrity, and normal morphology. Reduced sperm quality indicated that, whether egg yolk is used or not, semen freezing reduces motility, damages morphological integrity, and raises embryonic mortality. Fatty acid profiling indicated that the absence of butyric and docosahexaenoic acids in egg yolk reduces its ability to protect sperm from cold shock and maintain the quality of cryopreserved semen (Azizunnesa et al., 2014). Supplementation or advancement of EY fatty acids content is necessary for successful semen cryopreservation. As a result, antioxidants are extensively used in PUFAs to improve EY functionality in preserved semen.

The effects of EY from different bird species are presented in Table 3. According to Swelum et al. (2023), 15% of Japanese quail EY was found to be effective in comparison with other avian EY. Thus, it was recommended to use Japanese quail EY to replace chicken EY on cryopreserved ram semen. These findings were consistent with those of Taha et al. (2016), who observed that the egg yolks of chicken and quail offered superior cryoprotective effects compared to those of other avian species, thereby enhancing sperm motility. Taha et al. (2016) further recommended the inclusion of a 10% concentration of chicken and quail EY. Consequently, Azizunnesa et al. (2014) and Mafolo (2018) found that EY at 10% concentration yielded optimal semen quality compared with 0%, 5%, 15%, and 20% concentrations. Kulaksiz et al. (2010) documented that chucker EY yielded superior results in ram semen compared to chicken EY. In the study conducted by Kulaksiz et al. (2010), a 15% egg yolk concentration was used for all avian species. These results were consistent with those reported by Swelum et al. (2023), who showed that a 15% EY concentration improved ram semen quality. with those of Swelum et al. (2023), who demonstrated that 15% EY enhanced ram semen quality. Furthermore, according to Ali and Floris (2013), using 20% partridge EY yielded higher cleavage rates and enhanced early embryonic development in ewes, compared to EY derived from ostrich, turkey, duck, tortoise, and chicken.

Antioxidants rich in long-chain polyunsaturated fatty acids

There is growing interest in using long-chain polyunsaturated fatty acids (LCPUFAs) as antioxidants to combat oxidative stress and reduce the harmful effects of lipid peroxidation on sperm fertility (Rizkallah et al., 2022). Ram sperm membranes should be altered or improved to enhance ram semen efficiency, since ram sperm membranes have a low phospholipid/cholesterol ratio and a high unsaturated-to-saturated fatty acid ratio (Yimer et al., 2016). As noted by Yimer et al. (2016), antioxidants are classified as either enzymatic, which neutralize excess ROS and prevent cellular damage, or non-enzymatic (dietary supplements), which should be incorporated into ruminant feed owing to the animals' inability to produce specific fatty acids. Additionally, Oppedisano et al. (2020) noted that LCPUFAs, comprising 18, 20, or 22 carbon atoms, can support sperm function and offer therapeutic benefits in different conditions, including cardiovascular disease and hypertension. Omega-3 (alpha-linoleic acid [ALA], docosahexaenoic acid, docosapentaenoic

acid, and eicosatetraenoic acid [EPA]), omega-6 (arachidonic acid, gamma-linoleic acid, and linoleic acid), and omega-9 (oleic acid) are the three categories for LCPUFAs (Ngcobo et al., 2024). When these fatty acids are supplemented in animal diets, mammals convert ALA to EPA, which is essential for the synthesis of reproductive hormones, such as luteinizing hormone (LH), testosterone, and follicle-stimulating hormone (FSH). These reproductive hormones play a vital role in maintaining sperm membrane fluidity, mediating cellular responses, supporting protein synthesis, and sustaining astrocytes and retinal pigment epithelial cells in the testicles (Yaun et al., 2023; Ngcobo et al., 2024). The interactions between antioxidants and sperm function are still poorly understood. However, previous studies have noted that ram sperm membranes have a high ratio of unsaturated to saturated fatty acids and low lipoprotein levels, along with the absence of a robust antioxidant system, unlike those in other ruminants (Azizunnesa et al., 2014; Yimer et al., 2016; Akther et al., 2023).

According to Yimer et al. (2016), freezing and dilution of sperm decreased the levels of natural antioxidants, thereby increasing ROS and impairing sperm quality and fertilization potential. As a result, several studies indicated that using PUFAs can help resolve sperm quality abnormalities (Table 4; Zarei et al., 2018; Ngcobo et al., 2024; Abdollahzadeh et al., 2025). Flaxseed oil, a plant-based source of omega-3, can be supplemented to the diet to improve sperm's low phospholipid concentration and lower malondialdehyde (MDA) levels during lipid peroxidation (Ngcobo et al., 2024). Additionally, vitamin E can enhance reproductive processes without reducing sperm efficiency (Saini et al., 2021; Yuan et al., 2023). Moreover, Masoudi and Dadashpour-Davachi (2021) reported that fish oil improved semen quality and ewe fertility parameters. An optimal hydrophobic tail of fatty acids was reported to help preserve sperm cell membrane integrity and improve fluidity, thereby supporting sperm motility and function (Yimer et al., 2016). Additionally, fatty acids can integrate into the lipid bilayer of the sperm membrane, protect sperm from oxidative damage, and reduce ROS. The addition of these hydrophobic antioxidants can help balance lipid, antioxidant, and ROS levels in the sperm cytoplasm. Therefore, it is crucial to sustain a balanced intake of LPUFAS to replace the utilization of hydrophilic antioxidants that are not absorbed by ROS during semen cryopreservation.

Table 4. Effects of different semen antioxidants on rams' semen quality

Breed	Type of antioxidant	Level used/concentration	Findings	References
Hu	Alpha-lipoic acid (ALA)	0, 0.025, 0.05, 0.1, 0.5, 1 mM	Alpha-lipoic acid (ALA) supplementation at 0.1 mM effectively mitigates oxidative stress in sperm and improves semen quality during preservation at 4°C.	Sun et al. (2024)
Zandi	Fish oil	-	Fish oil (FO) supplementation significantly improved all aspects of sperm quality and quantity compared to the control diet.	Alizadeh et al. (2014)
Afshari	Flaxseed oil	5%	Improved semen during liquid storage	Zarei et al. (2018)
Bapedi, Zulu, Damara, and Namaqua	Flaxseed oil	FLO - 5%	A combination of flaxseed oil and ascorbic acid (FLO and ASA) improved semen quality, in vitro fertilization, and cleavage rate.	Ngcobo et al. (2024)
Bapedi, Zulu, Damara, and Namaqua	ascorbic oil	4%	Adding ascorbic acid improved semen quality compared to the negative and positive controls	Ngcobo et al. (2024)
	Docosahexaenoic (DHA)	0.15, 0.30, or 0.45 g	Adding Docosahexaenoic acid to ram semen extenders enhances semen quality and fertility after thawing.	Abdi-Benemar et al. (2015)
Moghani	Persia fat	-	Adding fish oil or Persia fat to the diet enhanced the resilience of sperm to freezing and thawing, resulting in better quality Adding fish oil or Persia fat to the diet enhanced the resilience of sperm to freeze and thawing, resulting in better quality.	Hedayat-Evrigh et al. (2019)
Shall	Omega-6 to omega-3 fatty acids	Low ratio 4:1	Dietary supplementation of a low ration improved semen parameters and reduced DNA damage compared to a high ration	Abdollahzadeh et al. (2025)
Iranian Zandi	Fish oil		Fish oil supplementation enhanced semen parameters and reproductive performance, leading to better pregnancy and lambing rates	Masoudi et al. (2021)
Moghani	Sunflower oil + vitamin C	diet without SFO and VC	Rams fed with SFO and VC supplements showed improved semen quality and higher fertility rates	Ezazi et al. (2019)

Antioxidants play different vital roles in ram semen quality during cryopreservation. For instance, Abdi-Benemar et al. (2013) and Sun et al. (2024) used ALA and DHA (precursors of LCPUFAs) and found that 0.1 mM at 40°C noticeably reduced sperm damage from oxidative stress and improved semen quality. Moreover, dietary supplementation of fish oil has been demonstrated to improve sperm quality and quantity (Alizadeh et al., 2014; Hedayat-Evrigh et al., 2019; Abdollahi et al., 2021). Given that the sperm dilution method did not harm sperm function, it instead led to an increase in pregnancy and lambing rates in ewes supplemented with fish oil compared with palm oil. However, Hedayat-Evrigh et al. (2019) observed no significant difference in the quality of ram semen when comparing Persian oil to fish oil after feeding. *In vitro* fertilization, cleavage rate, and semen quality shelf life were all improved in sheep by dietary supplementation with 5% flaxseed oil and ascorbic acid (Zarei et al., 2018; Ngcobo et al., 2024). Additionally, Alizadeh et al. (2014) found that adding fish oil to a vitamin E diet resulted in beneficial effects compared with a control diet lacking fish oil. Recent findings by Abdollahzadeh et al. (2025) revealed that semen samples with a lower ratio (4:1) significantly improved semen quality parameters and exhibited reduced DNA damage. Finally, Ezazi et al. (2019) found that the quality of semen has increased, maintained sperm health and fertility with a synergetic effect following the use of sunflower oil and vitamin C.

Nanotechnology and cryopreservation

Nanoparticles (NPs) are tiny particles measuring 1-100 nm (Khan et al., 2017; Abbasi et al., 2021; Arruda et al., 2021). According to Khan et al. (2017), NPs are divided into four groups, namely, metal, ceramic, fullerene, and polymeric. Because of their large surface area and nanoscale size, NPs offer diverse applications, including transgenesis, targeted delivery to sperm cells, and antimicrobial and antioxidant effects (Khan et al., 2017; Khalil et al., 2024a). Several studies have investigated the potential of NPs to improve the physical and chemical properties of cryopreserved semen. According to Arruda et al. (2021), there was an improvement in semen quality in bulls, boars, camels, mice, rams, bucks, and other species following the use of NPs. Khalil et al. (2024a) reported that adding 100 µg/mL of zinc oxide NPs, selenium NPs, silver NPs, and copper NPs to semen extender improved the cryotolerance and reproductive potential of buffalo bull sperm. The small particle sizes of NPs can penetrate the sperm membrane and sperm nucleus without causing toxicity to the whole sperm cell. However, an excessive increase in the NPs concentration on the sperm during semen storage was reported to cause toxicity and reduce sperm viability. The toxicity was discovered following the use of 1000 µg/mL of zinc oxide on cryopreserved semen. In contrast, Arruda et al. (2021) observed that zinc oxide nanoparticles at 10-100 µg/mL had no cytotoxic effects on ram sperm motility; rather, motility was enhanced, attributed to zinc's involvement in ATP production and its regulatory function in phospholipid energy metabolism. Abbasi et al. (2021) reported that magnetic oxide NPs improved the vitrification of immature mouse oocytes and altered the expression of pluripotent genes. According to Arruda et al. (2021) and Khalil et al. (2024), Fe_3O_4 NPs minimized recrystallisation and devitrification during warming, protected immature oocytes from cryodamage, improved vitrification results, and increased the pluripotency of resultant embryos. Kanwar et al. (2023) evaluated the antibacterial properties of silver NPs and reported notable improvements in both pre- and post-freezing motility, along with increased antibacterial activity. The microbial load in frozen-thawed semen from the control and supplemented groups remained within acceptable limits. Furthermore, compared with the control group, Khalil et al. (2024) observed a decline in the overall numbers of bacteria, fungi, and yeast in the semen of the group that received zinc oxide NPs. The effects of adding iron oxide and silver NPs to ram semen on bacterial growth control and other sperm quality and function parameters were examined by Tsakmakidis et al. (2021). The findings of Tsakmakidis et al. (2021) demonstrated that while silver NPs offered more antibacterial qualities as well as cytotoxicity, iron NPs had stronger antibacterial impacts. Therefore, these findings suggested that NPs have antibacterial properties on semen.

The results obtained in Table 5 by Moradi et al. (2022) when comparing semen treated with magnetic NPs and untreated semen (control group). There were notable differences in several sperm characteristics, including viability, membrane function, abnormalities, lipid peroxidation (LPO) levels, and DNA integrity among magnetic NPs treated semen and control groups. Moreover, Arruda et al. (2021) indicated that application of zinc oxide NPs increased the mitochondrial membrane potential without affecting the ram spermatozoa's kinetics following the freezing-thawing procedure. Improved sperm motility post semen dilution with zinc oxide was reported to be associated with zinc chemical properties. The use of selenium NPs was reported to improve motility, viability index, and membrane integrity and reduce acrosome deficiencies (Hozyen et al., 2019). In addition, Hozyen et al. (2019) reported that the addition of selenium NPs to extenders resulted in reduced DNA damage. Murawski et al. (2014) reported that deionized water can be used on semen extenders to improve the fertilizing ability of frozen-thawed semen and further improve the lambing rate following AI.

Table 5. Effects of different nanoparticles on rams' semen quality

Breed	Type of nanoparticles	Level used/concentration	Findings	References
Unknown	Magnetic nanoparticles	50 µg/ml MNPs	Adding magnetic nanoparticles (MNPs) to ram semen extenders didn't significantly impact key semen quality parameters, including viability, membrane function, abnormality, oxidative stress, or DNA integrity, compared with the control group.	Moradi et al. (2021)
Santa Inês	Zinc oxide nanoparticles)	0, 10, 50, 100, or 200 µg/mL	The addition of all concentrations improved semen quality without altering kinetics and protecting spermatozoa membranes during freezing and thawing.	Arruda et al. (2021)
Lacunae	Iron oxide nanoparticles	3.072 mg Fe3O4/mL	Iron oxide possesses antibacterial effects.	Tsakmakidis et al. (2021)
Lacunae	Silver (Ag) nanoparticles	2.048 mg Ag-Fe/mL	Silver nanoparticles exhibited strong antibacterial effects but also showed toxic effects on cells.	Tsakmakidis et al. (2021)
Barki	Selenium nanoparticles	1 µg/ml	Improved semen parameters while also reducing DNA deterioration.	Hozyen et al. (2019)
synthetic breed: Berrichon, Charolaise, and PON	Non-water (Deionized water)	1 µg/ml	Improved semen quality and ewe fertility following artificial insemination.	Murawski (2014)

Plant extracts to preserve sheep semen

Choosing semen diluents, whether plant- or animal-derived, is essential for successful sperm cryopreservation. However, the use of animal-based sources was poorly investigated. According to Ngcobo et al. (2024), the use of animal-derived diluents was unsustainable due to increasing competition for food between humans and animals. Consequently, the widespread use of animal products worsened the living conditions of poor people. However, given the rising demand for resources to sustain a growing human population, the use of animal-derived diluents is unsustainable (Ngcobo et al., 2024). Therefore, this limitation recommended the use of plant-based extracts as a viable alternative to animal-origin sources for semen cryopreservation, reducing reliance on animal products and mitigating competition for food resources. Plant extracts possess natural antioxidant, antimicrobial, and anti-inflammatory qualities, making them a promising, low-cost, and natural additive for preserving and enhancing sperm function during storage (Ros-Santaella et al., 2021). The application of plant extract to preserve sheep semen quality is a promising field of study (Berean et al., 2024). According to Ros-Santaella et al. (2021), plant extracts have emerged as promising, cost-effective, and natural additives for preserving and enhancing sperm function during semen storage. In addition, plant extracts presented an environmentally sustainable, non-animal-based method of preserving animal genetics, thereby reducing the potential transfer of hormonal and antibiotic-resistant genes to humans via the food chain (Ngcobo et al., 2024). The inclusion of plant extracts in ram and other male semen depends on their ability to minimize oxidative damage and scavenge ROS. Moreover, according to Mphaphathi et al. (2024), plant extracts are an integral natural antioxidant source with lower cytotoxicity than synthetic therapeutic antioxidants. Their antimicrobial and antioxidant properties enabled them to reduce oxidative stress and to scavenge ROS.

Published findings indicate that the method of preparing and extracting plant extracts, together with the concentration of the semen extender, influences both their interaction with semen extenders and the resulting semen quality. The ability of plant extracts to interact with sperm suggested that plant extracts can be used to treat semen as an antioxidant. Adding 20 µmol/L of curcumin to refrigerated semen increased the levels of multi-antioxidant enzymes and notably reduced ROS production during freezing (Table 6; Ji et al., 2024). Rosemary aqueous extract (Motlagh et al., 2018), wild marjoram (Alenezy et al., 2019), and quinoa seed extract (Khalil et al., 2024) have been reported to enhance the antioxidant capacity of ram semen during cryopreservation. Vahedi et al. (2018) observed improvements in sperm parameters following thymus V supplementation after freezing and thawing. According to Shorky et al. (2021), the use of *Moringa oleifera* leaf extract improved post-thaw ram semen quality and fertility by boosting antioxidant enzyme activity, reducing DNA fragmentation, and minimizing lipid peroxidation. In addition, Alenezy et al. (2019) observed improvements in ram sperm parameters after treatment with *Origanum vulgare* (oregano) extract in a capacitation medium. El-Harairy et al. (2018) indicated that there was no negative impact on semen after using soybean lectin, and soybean lectin was recommended to replace the use of EY in preserved semen. Furthermore, Allai et al. (2016) noted that ram semen quality can be improved by supplementing skim milk and egg yolks with 1% acetone extract ACTEX.

Table 6. Effects of different plant extracts on preserving ram semen

Breed	Plant extract	Concentration	Findings	References
Hu ram	Curcumin	20 µmol/L.	Increased level of multi-antioxidant enzymes and significantly reduced production of ROS during freezing.	Ji et al. (2024)
Rahmani ram	Quinoa seed extracts	0, 250, 500, 750, and 1000	Promoted sperm antioxidants-related genes and reduced apoptosis-related genes.	Khalil et al. (2024)
Moghani ram	Thymus vulgaris	2, 4, 8, 12, and 16Ml/Dl	Improved sperm parameters were observed after supplementation with thymus V following freezing and thawing. However, only the addition of 16Ml/dl extract was criticized.	Vahedi et al. (2018)
Barki ram	*Moringa oleifera* leaves extract (MOLE)	Control-0%, group 2 (MOLE) - 300mg/mL, group 3 (MOLE)- 600mg/mL, group 4 (vit and selenium)- 2.5mg/mL, and group 5 (Vit and selenium) 5mg/mL	Improved ram semen and reduced spermatozoa DNA fragmentation and MDA.	Shorky et al. (2021)
Santa Inês ram	Aqueous extract of noni	0, 24, 72, and 120 µg/mL	The addition of tris-egg-yolk-glycerol-based did not improve semen parameters. Only the aqueous extract of noni improved and maintained sperm functioning and membrane integrity.	Nascimento et al. (2018)
Rahmani ram	Propolis ethanolic extract	0, 0.1, 0.5, and 1.0 mg/ml	No detrimental effect on semen was reported following the use of soybean lectin. Thus, concluded that soybean lectin can replace EY on preserved semen.	El-Harairy et al. (2018)
Chal-ram	Rosemary aqueous extract	0, 2, 4, 6, and 8%	Supplementation of SL extender with rosemary aqueous extract influences post-thawed ram sperm quality in a dose-dependent manner.	Motlagh et al. (2014)
	Wild Marjoram (Origanum vulgare)	0.3, 0.6, 1.2 µg/ml and 25.0, 50.0, 100.0 µg/ml	Origanum vulgare extract had a positive impact on ram sperm quality when added to the capacitation medium.	Alenezy et al. (2019)
Boujaâd rams	*Opuntia ficus*-indica extract (ACTEX)	0%, 1%, 2%, 4%, and 8%	The addition of 1% of ACTEX on skim milk and Tris-based EY improved semen parameters.	Allai et al. (2016)

CHALLENGES IN CRYOPRESERVING

Cryoprotectant toxicity and different dilution rates

Although semen storage techniques have improved, finding an ideal extender that maintains sperm characteristics while minimizing damage remains a challenge (Kameni et al., 2021). The diluent is crucial for sperm viability, providing essential nutrients, maintaining a stable environment, and extending survival during storage (Zhang et al., 2023). Different cryoprotectants can affect sperm quality differently, depending on factors such as concentration, composition, storage time, and temperature. Zhang et al. (2024) evaluated different dilutions (1:1, 1:2, 1:3, and 1:4) of diluent I (Tris-based with egg yolk) on Hu ram semen, while maintaining diluent II (diluent I with glycerol) at a fixed 1:1 ratio. The findings of Zhang et al. (2024) indicated that a two-step dilution (1:3, 1:2) was the most suitable method and ratio for diluting Hu ram semen after cryopreservation. The 1:1 and 1:4 ratios compromised the semen quality. Therefore, a specific concentration is necessary for improving semen quality. However, sperm viability may be compromised by improper diluent addition. The quality of the semen diluent and its capacity to preserve semen quality during lipid peroxidation are linked to semen toxicity. Anel-Lopez et al. (2021) raised concerns about the use of gentamicin, lincomycin, and spectinomycin in semen, indicating their harmful effects and the risk of developing antibiotic-resistant bacteria. These medications had no antibacterial activity and were ineffective on semen quality. Therefore, one of the main problems leading to the failure of semen cryopreservation in many species is the lack of standardized dilution and concentration ratios.

Unimproved protocols for semen cryopreservation

Kameni et al. (2021) observed that prolonged storage of semen consistently led to a decline in quality, reflected in reduced intracellular enzymatic activity, viability, and motility, independent of diluent composition, storage temperature, collection method, or dilution factor. Ari et al. (2011) reported that collecting semen daily (with 1 day of rest) improved post-thaw quality, motility, viability, and membrane integrity, and reduced abnormalities and acrosomal damage, compared with collecting semen every 4 days. D'Alessandro et al. (2021) evaluated sperm motility and function at different preservation times and found improvements in semen quality six hours post-storage. D'Alessandro et al. (2021) observed that the extender's initial impact on motility and function from 3 to 6 hours was reversible. Since these early changes may result from sub-lethal damage, it is recommended that ram sperm adapt to the extender for three to six hours before conducting the quality assessment. Preliminary details, such as storage temperature, duration, dilution rate,

and storage conditions, are crucial and should align with optimal-quality diluents. Da Silva Passarelli et al. (2020) found that a 2-hour equilibration time provided superior cryotolerance than a 4-hour period at 5°C, suggesting that a simplified freezing protocol enhanced the success of semen cryopreservation. Therefore, it is essential to perform proper dilution steps, allowing spermatozoa enough time to osmotically adapt to their changing environment and prevent osmotic stress during freezing.

Ejaculation frequency

The frequency of semen collection, whether through electroejaculation (EE) or artificial vagina (AV), affects semen quality. Bearden and Fuquay (1980) found that EE and AV produced similar total sperm counts and fertility rates, though sperm collected via AV exhibited greater resistance to cold shock. Regular collection intervals are essential for preserving semen quality, as Mahmuda (2014) emphasized that consistent collection promoted sperm maturity during spermatogenesis. Nevertheless, repeated ejaculations may decrease semen volume, concentration, and motility (Jennings and Mcweeney, 1976).

Effects of an imbalanced temperature level during semen freezing

Sheep semen is frequently preserved at low temperatures (around 4°C), and determining an appropriate diluent is essential for successful preservation (Zhang et al., 2018). Compared to frozen or chilled storage, ram spermatozoa kept in liquid at 23°C did not have a standardized concentration (Rizkallah et al., 2022). Ram semen is extremely vulnerable to freezing and cold shock, which can reduce sperm fertility and viability (Kanwar et al., 2018). Extreme ROS production from cryopreservation damages sperm functioning (Kameni et al., 2021). Post-thaw semen quality can be compromised when equilibration conditions are changed (Benmoula et al., 2019; Rizkallah et al., 2022). Compromised semen results could be justified by increased bacterial proliferation, such as *Staphylococcus* species and *Escherichia coli* (*E. coli*). Consequently, semen extenders were supplemented with antibiotics and NPs to reduce oxidative stress, provide protection against cold shock, and inhibit microbial growth (Larbi Allai et al., 2018). However, the use of some antibiotics and antioxidants has been challenged by bacterial resistance.

Bacterial contamination

As noted by Ahmen et al. (2018), semen collection is not a sterile procedure, which can lead to bacterial contamination. *Escherichia coli*, *Staphylococcus* species, and *Bacillus* species are the most common bacteria found in ram semen and are transmitted from the surface of the penis and prepuce, collection area, equipment, and people during semen handling. Cross-contamination extensively affects females' reproductive tracts during AI. However, a higher bacterial load in sperm count is associated with decreased sperm viability. Ahmen et al. (2018) found that antibiotics in semen extenders inhibited bacterial growth and helped prevent bacterial transmission during AI. Yániz et al. (2010) found that enterobacterial contamination affected ram semen quality during storage at 15°C, while antibiotics such as gentamicin and ceftiofur demonstrated strong antimicrobial effects. Yániz et al. (2010) reported bacterial resistance to a wide range of antibiotics, recording resistance rates of 20% for ampicillin, 53% for penicillin, 15% for streptomycin, 4% for spectinomycin, 47% for erythromycin, 33% for oxytetracycline, 13% for polymyxin B, and 15% for co-trimoxazole. The bacterial resistance rates for these antibiotics exhibited that some of the antibiotics were ineffective against microbial growth, leading to resistance among the bacteria. Moreover, Anel-Lopez et al. (2021) expressed concerns regarding the use of gentamycin, lincomycin, and spectinomycin due to their toxic effects on sperm quality.

PROSPECTS TO IMPROVE THE PRESERVABILITY OF RAM SEMEN

Standardization of semen freezing protocols

Developing established protocols is essential for semen storage, offering specific guidelines on storage duration, temperature, collection procedures aligned with the method, dilution rates, and storage conditions. These protocols help maintain sperm quality during freezing, ensuring consistency and optimal sperm health (Zhang et al., 2024). Unimproved protocols and imbalanced temperatures negatively impacted semen quality. Improving these protocols is expected to help farmers follow reliable guidelines, thereby enhancing the reproductive efficiency of their sheep and other breeds (Kameni et al., 2021).

Optimal concentration and standardization of plant extracts

Optimal levels of plant extracts and antioxidants are essential for semen preservation, as they protect sperm cells and help maintain their viability and function (Yimer et al., 2016). This process begins by analyzing the chemical composition of the selected plants, enabling scientists to establish safe and effective concentrations that do not

compromise semen quality. Plant extracts differ from synthetic extracts, because plant extracts are natural substances whose nutritional profiles depend on climate and extraction methods. The optimal concentrations for improving ram semen quality were 20 µmol/L of curcumin and 1% *Opuntia ficus-indica* extract (Allai et al., 2016; Ji et al., 2024). Additionally, studying the bioactive compounds in plant extracts can further enhance semen preservation and increase the success of AI procedures (Zhang et al., 2024).

The development of established protocols for the extraction and processing of plant-derived additives used in semen extenders is crucial (Alenezy et al., 2019). To effectively use plant extracts in semen cryopreservation, it is essential to maintain the integrity of bioactive compounds and preserve the quality of the extracted materials (Mphaphathi et al., 2025). A comprehensive analysis of the chemical compositions of plant extracts, including fatty acid profiles, is necessary to understand their impact on semen quality and identify potential sperm shortages (Ngcobo et al., 2024). Additionally, toxicological evaluations are essential to determine the safety of plant extracts for use in semen cryopreservation. Plant extracts should demonstrate sufficient antioxidant activity to protect spermatozoa from oxidative stress during cryopreservation (Alenezy et al., 2019; Shorky et al., 2021). The antioxidant properties of plant extracts play a vital role in preserving sperm cells from oxidative damage. Phenolic-rich extracts and flavonoid-rich extracts, known for their high antioxidant activity, might be beneficial in protecting sperm cells (Allai et al., 2016). Advanced technologies, such as automated semen analyzers and nanomechanical sensors, can aid in assessing sperm parameters and evaluating the effects of plant extracts on sperm function. By optimizing extraction methods, standardizing plant extracts, and conducting thorough toxicological evaluations, scientists can develop effective and safe solutions for semen cryopreservation. The integration of advanced technologies and a thorough understanding of the effects of plant extracts on semen quality can ultimately improve the success rates of cryopreservation protocols (El-Harairy et al., 2018).

In vivo studies for validating the efficacy of plant extracts

Although plant extracts have demonstrated potential to enhance the quality of cryopreserved semen, additional in *vivo* studies are needed to verify their role in improving ovine reproductive efficiency. These studies should investigate the impact of plant extracts on semen quality parameters, such as motility, viability, and fertility, and their potential to optimize AI protocols. Additionally, although efforts have been made to enhance semen quality, the impact of plant extracts on ewe reproductive efficiency remains insufficiently understood. Direct dietary supplementation of plant extracts to ewes may improve reproductive performance, and further studies are needed to explore the potential benefits of different plant extracts on both male and female fertility.

CONCLUSION

Studies have underscored the importance of conserving indigenous sheep ecotypes. The limited success of AI highlighted the urgent need to improve semen cryopreservation methods to protect the genetic diversity of indigenous sheep ecotypes before they become extinct. Despite considerable advances in developed countries in oxidative damage and other factors influencing semen cryopreservation, the inability to enhance the effectiveness of frozen semen for AI could worsen food insecurity and deepen poverty in underdeveloped and developing countries. To improve the success of sheep semen cryopreservation, further studies are required to establish a standardized freezing protocol for ram semen that effectively reduces freezing-related stressors. This freezing protocol should thoroughly assess the effects of plant extracts on semen quality, fertility, and reproductive performance in ewes. Furthermore, conducting *in vivo* studies and exploring the advantages of directly incorporating plant extracts into diets can provide cost-effective, evidence-based methods to improve sheep fertility.

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Author's contributions

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Availability of data and materials

The data used and analyzed in the present study were obtained from publicly accessible publications cited in this manuscript.

Competing interests

The authors declared no conflict of interest.

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All authors have reviewed ethical considerations, including plagiarism, consent to publish, misconduct, data fabrication and falsification, duplicate publication and submission, and redundancy. Furthermore, the authors did not utilize any artificial intelligence tools in the data preparation, writing, or revision of the manuscript.

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Artificial Intelligence Applications in Small Animal Medicine: The Current Status, Key Challenges, and Future Prospects with Special Emphasis on Blockchain Innovation

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ABSTRACT

Artificial intelligence (AI) and machine learning are rapidly transforming companion animal veterinary medicine. The present narrative review examined current applications, challenges, and future directions for AI in the care of dogs, cats, and other companion animals. The current study is based on a structured literature search across PubMed/MEDLINE, Scopus, and IEEE Xplore from January 2015 to April 2026, with clearly defined inclusion and exclusion criteria to ensure transparency and reproducibility. In diagnostic imaging, computer vision algorithms improve the speed and accuracy of interpreting radiographs, ultrasound, and dermatoscopic images, helping detect conditions such as hip dislocation, osteoarthritis, foreign bodies, skin lesions, and parasites. In digital pathology, AI aids in identifying abnormal cells from blood and histology slides, supporting diagnoses such as lymphoma and anemia. Wearable devices with AI algorithms continuously monitor physiological data, including activity, sleep, and early signs of pain. Natural language processing (NLP) extracts information from electronic health records to facilitate epidemiological studies. Despite this promise, several challenges remain. Technical hurdles include the need for large, high-quality annotated datasets, which are harder and more costly to obtain than in human medicine, and the high genetic diversity among dog and cat breeds, which makes it difficult to develop generalizable algorithms. Ethical and legal issues involve liability for diagnostic errors, data privacy, and algorithmic bias, requiring new regulatory frameworks. Practical barriers include high costs, integration into clinical workflows, and the need to train veterinarians to effectively use and critically evaluate AI outputs. Future progress depends on close collaboration among AI specialists, veterinarians, biologists, and industry. Priorities include developing cost-effective, user-friendly tools; creating internationally shared, standardized databases; and establishing ethical and professional guidelines. Blockchain may serve as a trust layer for recording and verifying data access events, rather than as a primary repository for large-scale medical data. Ultimately, AI is expected to act as a powerful assistant, not a replacement for veterinary expertise, enabling earlier diagnoses, personalized treatments, improved preventive care, and a better quality of life for companion animals and their owners.

Keywords: Artificial intelligence, Companion animal, Diagnostic imaging, Machine learning, Veterinary medicine

INTRODUCTION

The ability of Artificial Intelligence (AI) to learn from data, identify intricate patterns, and carry out cognitive functions is pushing the limits of what's possible in diagnosis and treatment (Gill et al., 2023; Kulkarni and Singh, 2023; Amer and Amer, 2025). Artificial intelligence is one of the most important technologies of the 21st century, and is not confined to human medicine; it has also entered veterinary medicine, particularly in the treatment of companion and small animals, including dogs and cats (Pereira et al., 2023; Albadrani et al., 2024; Arshad et al., 2025; Sadr et al., 2025a). Pets nowadays are regarded not just for their usefulness, but as beloved family members and treasured friends. As a result, the psychological and social significance of their health and well-being is considerable (McConnell et al., 2019).

The field of veterinary care for small animals continues to face a range of obstacles, such as the high cost of advanced diagnostic equipment, a shortage of specialized veterinarians in rural areas, and limited access to emergency care, despite substantial progress over the past several decades (Quain et al., 2021). One of these problems is the significant genetic variability across breeds of dogs and cats, which determines how diseases manifest, how they respond to therapy, and even their anatomy (Kurhaluk and Tkaczenko, 2025). Another issue is that the variety of paraclinical tests, including radiographic imaging, ultrasonography, and pathology, is often used in conjunction with a physical examination, the owner's medical history, and other diagnostic procedures (Abdisa, 2017). There is a significant amount of skill, extensive experience, and often a great deal of time required to interpret this data, which may be problematic in today's hectic clinical environment (Clarke and Knights, 2018; Petrovski and Kirkwood, 2025). In this context, AI can

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automate quantitative analysis, provide diagnostic recommendations, and unearth hidden links within massive volumes of health data (Akinsulie et al., 2024; Figure 1).

In the field of medical image analysis, computer vision algorithms can analyze radiographs to identify hip dislocations, osteoarthritis, or foreign bodies with high accuracy (Franco-Gonçalo et al., 2024). Machine learning algorithms can also analyze ultrasound images to examine internal viscera and dermatoscopic images to detect skin lesions (Abunadi and Senan, 2021). An AI system may be used in digital pathology to scan blood or histology slides to detect aberrant cells and assist in diagnosing diseases such as lymphoma and anemia (Zuraw and Aeffner, 2022; Piccione et al., 2025). Beyond diagnosis, wearable devices paired with AI algorithms can continuously collect and analyze physiological data in animal care. Data in animal care may include activity levels, sleep patterns, and even early indicators of discomfort (Xiao et al., 2025). By automatically extracting information from computerized animal health records, natural language processing (NLP) also enables more extensive epidemiological studies and more effective management of animal populations (Ezanno et al., 2021; Figure 2).

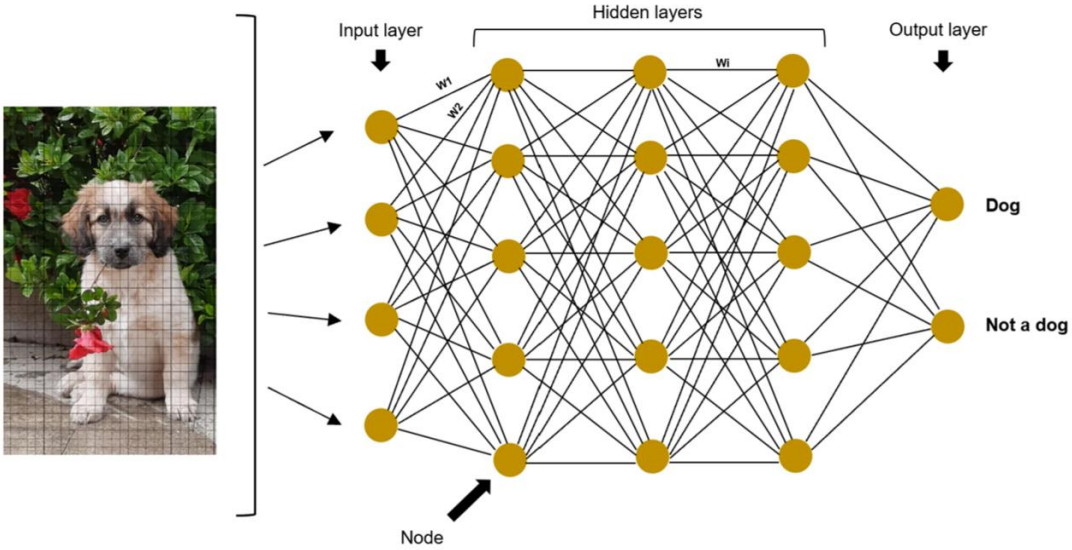


Figure 1. Architecture of an artificial neural network, in which the pixels of a digital image of a dog serve as input. There are four hidden layers and two possible outputs: Dog or not dog. The nodes are arranged in layers and joined by connections. The weights are represented by the letter **W** (**W1**, **W2**, and **Wi** in the figure; MDPI Copyright, Source of image adapted from Pereira et al., 2023). **Wi** typically represents a general or individual weight within the network.

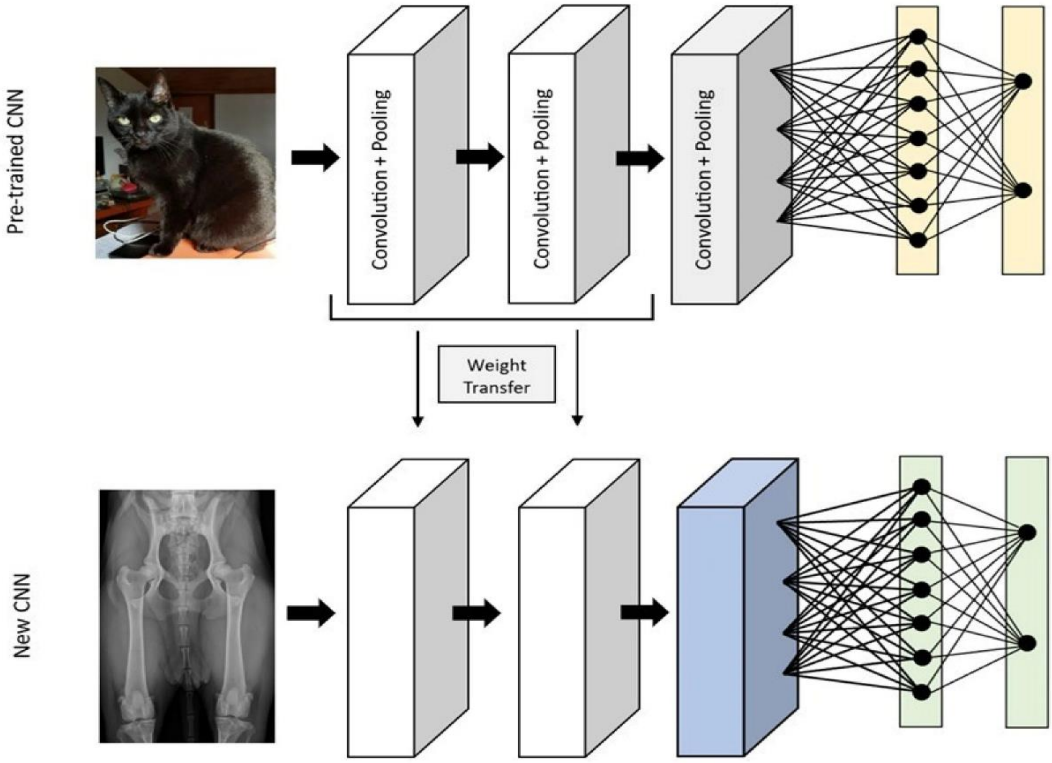


Figure 2. The transfer learning process involves using part of the weights from a convolutional neural network trained to evaluate non-medical images to classify radiographs. (MDPI Copyright, Source of image adapted from Pereira et al., 2023).

AI in veterinary treatment for small animals is not straightforward and confronts several challenges (Zhang et al., 2024; Ranjbar Kamrani et al., 2026). Technical difficulties are perhaps the first and most significant hurdle (Jia et al., 2025). Training effective AI models requires access to vast, high-quality, well-annotated datasets (Zha et al., 2025). It is more difficult and expensive to collect such data than in human medicine because treatment facilities are spread out across the country, prices are higher, and permission from veterinary specialists is required (Ezanno et al., 2021; Akbarein et al., 2025). Moreover, the substantial genetic variation across breeds might introduce bias into algorithms and diminish their generalizability. Hence, a model trained on data from one breed would not apply to another breed.

AI scientists, veterinarians, biologists, engineers, and industry professionals will need to collaborate more closely and in an interdisciplinary manner to fully grasp the promise of AI to improve the health and well-being of companion animals (Ezanno et al., 2021). Several key projects are included in the present approach, including developing user-friendly, cost-effective technologies, building standardized, global, shared databases, and adopting professional and ethical guidelines (Talebi and Nezhad, 2024; Zhang et al., 2024; Sun, 2025). AI will not replace the skill, experience, and clinical judgment of veterinarians; rather, it will serve as a strong complement to help veterinarians. AI has the potential to lead to earlier diagnoses, more individualized therapies, more efficient preventative monitoring, and eventually, an increase in the quality of life for companion animals and the people who own them. The aim of the present review was to investigate the current state of AI applications in small-animal veterinary medicine, to analyze the most significant technical, ethical, and practical challenges that this field is currently facing, and to outline future perspectives and pathways for the development and deployment of these transformative technologies in a manner that is both effective and responsible.

METHODS

The current study is presented as a narrative review to provide a comprehensive overview of the landscape, challenges, and future directions of AI and blockchain applications in small animal medicine. Given the narrative nature of the present review, a systematic, PRISMA-style methodology was not employed. However, to ensure transparency, rigor, and reproducibility of the literature synthesis, the following methodological framework was adopted regarding the scope of literature search, sources, timeframe, and selection criteria. A structured literature search was conducted to identify relevant peer-reviewed articles, conference proceedings, and industry white papers. The search was performed across three major electronic databases, such as PubMed/MEDLINE, Scopus, and IEEE Xplore, supplemented by manual searches of Google Scholar and the reference lists of retrieved articles to identify additional pertinent studies. The search strategy was designed to capture the intersection of veterinary medicine, artificial intelligence, and blockchain technology. The search was limited to literature published between January 2015 and April 2026. This timeframe was selected to focus on the period during which AI applications, particularly deep learning, gained significant traction in clinical medicine, aligning with the emergence of blockchain discussions in healthcare data management. The search utilized combinations of the following keywords and MeSH terms including of AI ("artificial intelligence" OR "machine learning" OR "deep learning" OR "neural networks" OR "computer vision" OR "large language models") AND ("veterinary" OR "small animal" OR "companion animal" OR "canine" OR "feline") and For Blockchain ("blockchain" OR "distributed ledger" OR "smart contracts") AND ("veterinary" OR "small animal medicine" OR "medical records").

Inclusion criteria and exclusion criteria

Inclusion criteria were articles were included if they described the development, validation, or clinical implementation of AI algorithms (diagnostic imaging, predictive analytics, wearable sensors) specifically in small animal (canine and feline) practice; discussed the architectural, ethical, or regulatory frameworks for blockchain integration in veterinary data management; were original research, systematic reviews, or authoritative consensus statements published in English; and addressed key challenges such as data standardization, interoperability, or data security relevant to the convergence of AI and blockchain.

Exclusion criteria were articles were excluded if they focused exclusively on large animal, exotic, or laboratory animal medicine without relevance to small animal practice; described AI applications only in a non-medical context (agricultural management or basic biological research) without clinical translational relevance; were editorials, opinion pieces, or non-peer-reviewed preprints lacking substantive technical detail; were published prior to 2015, unless they represented seminal foundational work directly cited to contextualize a historical concept; or were not available in English.

The selected literature was synthesized thematically to construct the narrative. The review was structured to first present the current status of AI across diagnostic, therapeutic, and predictive domains in small animal medicine. Subsequently, key challenges related to data silos, privacy, and algorithmic bias were analyzed. Finally, the prospective role of blockchain technology was explored as a potential solution to these challenges, with a focus on enhancing data integrity, security, and multi-institutional collaboration.

ARTIFICIAL INTELLIGENCE: CONCEPTS, ALGORITHMS, AND CAPABILITIES

Systems capable of performing activities that would normally require human intellect, such as visual perception, voice recognition, decision-making, and language translation, fall under the umbrella of AI, a large field within computer science (Buiten, 2019; Zhu et al., 2023). In recent decades, AI has transitioned from a purely academic field into an applied transformative technology across a variety of industries (Gruetzemacher and Whittlestone, 2022). This transformation has occurred as a result of the explosion in the volume of digital data, also known as big data, as well as the increase in computing power, particularly with graphics processing units, or GPUs, and advancements in algorithmic development. AI can be divided into two broad categories, including narrow AI, which is designed to perform a specific task, such as facial recognition or playing chess, and general AI, a hypothetical concept referring to a

machine with human-level cognitive capabilities capable of performing a wide range of tasks (Dellermann et al., 2021; Korteling et al., 2021). All current applications of AI, including those in medical and veterinary medicine, are considered narrow AI (Akbarein et al., 2025).

One of the most important and commonly used subfields in AI is machine learning (Sarker, 2021). Machine learning is the study of computer systems that, rather than being explicitly programmed to perform a task, learn from data and improve their performance over time through experience (Alzubi et al., 2018). A model trained on data is the fundamental component of machine learning (Sarker, 2021). Machine learning is a complex mathematical function that, after training, can be used to generate predictions or judgments when applied to new data, called test data (Uçar et al., 2020). The process of machine learning may be broken down into different categories (Taye, 2023). One of the process categories is supervised learning, which involves training the model on labeled data, for example, radiographic images (Nguyen and Patrick, 2014). Learning without supervision, in which the model uncovers previously unknown patterns and structures in unlabeled data, and reinforcement learning, a form of learning in which an agent interacts with its environment and adjusts its behavior to maximize total reward or avoid a penalty (Morales and Escalante, 2022; Figure 3).

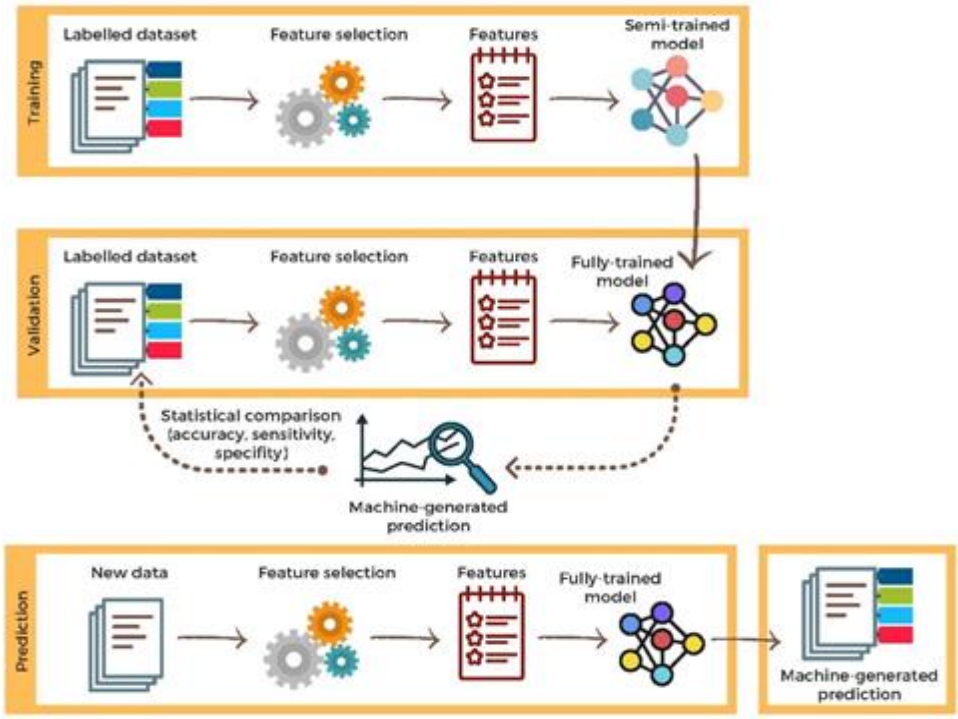


Figure 3. A brief description of the supervised learning flowchart, including training, validation, and prediction. (MDPI Copyright, Source of image adapted from Bouhali et al., 2022).

Deep learning, a subfield of machine learning, has emerged as a driving force behind the current artificial intelligence revolution (Srinivas et al., 2022). It employs artificial neural networks with complex, layered architectures referred to as deep that are inspired by the structural organization of the human brain (Taye, 2023). Deep learning networks consist of multiple layers of artificial neurons, also known as processing nodes. Each layer of these networks extracts information from the input data at a different level of abstraction (Qamar and Zardari, 2023). When processing a radiographic image, for instance, the earliest layers may be responsible for identifying edges and flanks, the intermediate layers for recognizing basic geometric patterns, and the deeper layers for extracting intricate information, such as the precise pattern of a bone fracture or a lung opacity (Mijwil et al., 2022). The remarkable achievements of AI in fields such as computer vision and NLP can be attributed, in large part, to the development of specific deep learning architectures (Chai et al., 2021). Deep learning architectures include convolutional neural networks for processing image data, as well as recurrent neural networks and transformers for processing sequential data such as transcription and speech (Xu et al., 2021; Taye, 2023).

Additionally, AI comprises a wide variety of algorithms and methods beyond deep learning (Gupta et al., 2021). The NLP, which enables machines to understand, interpret, and generate human language, can be used in veterinary medicine to analyze text-based clinical notes, automatically extract information from electronic health records, and even develop chatbots that answer questions posed by pet owners (Davies et al., 2024; Stimmer et al., 2025). The analysis of more structured data, such as blood test results, is often performed using traditional machine learning techniques (Khan et al., 2020). Machine learning techniques include algorithms such as random forests, support vector machines, and logistic regression (Boateng et al., 2020). When selecting an algorithm, it is important to consider the characteristics of the data (photos, text, or numbers), the amount of readily accessible data, and the eventual objective (classification, diagnosis, or prediction; Saturi, 2023).

Having a solid understanding of their capabilities and constraints is necessary for successful implementation in veterinary care. Artificial intelligence, and deep learning in particular, has the capacity to uncover intricate and nuanced patterns in high-dimensional

data, such as pixel data (Wilson and Anwar, 2024). Deep learning patterns may be concealed from even the most knowledgeable specialists (Rane et al., 2024). Because deep learning models can handle diverse data volumes at high speed and with a high degree of consistency, these systems serve as relentless assistants (Archana and Jeevaraj, 2024). On the other hand, deep learning patterns do not possess the reasoning capability, profound conceptual knowledge, clinical intuition, or the ability to consider the broader context of a case, such as the animal's full history or environmental circumstances, as a veterinarian does. The quality and quantity of the training data are also significant factors in determining their effectiveness, and they can learn and amplify biases present in the data (Mehrabi et al., 2021). Consequently, AI in veterinary medicine should be seen as a potent auxiliary tool, with the experienced veterinarian remaining responsible for making final decisions and carrying out clinical responsibilities (Basran and Appleby, 2024; Akbarein et al., 2025).

APPLICATIONS OF AI IN SMALL ANIMAL MEDICINE

Radiology and diagnostic imaging

The use of AI, particularly deep learning-based computer vision, in veterinary radiography for small animals is one of the most sophisticated and promising areas (Sharun et al., 2024). In the future, deep learning-based computer vision has the potential to serve as a digital assistant to radiologists and revolutionize the interpretation of a wide variety of diagnostic images (Akbarein et al., 2025). Diagnostic images include conventional radiography (X-ray), computed tomography (CT), magnetic resonance imaging (MRI), and ultrasound (Hussain et al., 2022). AI-powered algorithms can quickly scan medical images and recognize patterns associated with diseases (Hespel et al., 2022). For instance, in human chest radiography, deep learning-based computer vision can automatically segment the heart, lungs, diaphragm, and major arteries (Mittal et al., 2017). Additionally, deep learning-based computer vision can identify anomalies such as pleural effusion, alveolarization, cardiomegaly, and foreign bodies with high accuracy in humans (Zhou et al., 2021). The application of AI in musculoskeletal imaging enables screening and grading of osteoarthritis in the hip and elbow joints, detecting minor fractures or dislocations, and even evaluating bone density (Akbarein et al., 2025). In addition to accelerating the diagnostic process, this automation can enhance the quality and consistency of interpretations by reducing errors caused by human fatigue or inattention (Panahi, 2025).

The development of similar systems for veterinary care, on the other hand, is fraught with significant obstacles, primarily because of the different biological heterogeneity among patients (Kurhaluk and Tkaczenko, 2025). The training dataset should be representative of a wide range of breeds, ages, and weights to be considered complete. In addition, AI is most effective in this context when it is used as a facilitator and a warning system (Tiggeloven et al., 2025). For instance, it may prioritize photos, highlighting those at high risk of being pathogenic for prompt veterinary examination, mark questionable regions with antonyms, or provide accurate quantitative measures, such as heart size relative to the chest or joint angles (Maqbool et al., 2025). AI enables general veterinarians to assess photos with greater confidence while freeing radiologists' time for the most difficult cases (Rubini et al., 2025). It is the development of integrated, user-friendly technologies that connect directly with diagnostic imaging software such as Picture Archiving and Communication System (PACS) and provide preliminary results that will hold the key to the future of this industry (Appleby and Basran, 2024).

Clinical and anatomical pathology

The field of pathology is now experiencing a great change in AI, with the potential to fundamentally shift how tissue and cell samples are analyzed and interpreted (Khandia et al., 2026). In clinical pathology or hematology, systems that are based on deep learning can automatically scan peripheral blood slides and conduct white cell differentiation, such as counting and categorizing neutrophils, lymphocytes, and eosinophils, with a level of accuracy and speed that surpasses that of conventional approaches (Rahmoun et al., 2025). Deep learning algorithms can recognize aberrant cell morphologies, such as leukemic blasts or atypical serous cells, and can even evaluate micromorphological characteristics, such as cytoplasmic granulation and nuclear divisions (Maqbool et al., 2025). Deep learning can recognize and categorize casts, crystals, and epithelial cells through urinalysis (Suhail and Brindha, 2021). This results in higher data uniformity, reduced variation between operators, and increased time that experienced technicians can devote to more sophisticated analytical tasks (Liou et al., 2024). Digitalized tissue slides, also known as whole slide images, may be analyzed by AI algorithms, which can then quantify important diagnostic characteristics (Zuraw and Aeffner, 2022).

In histology, AI can perform tasks with high accuracy, such as identifying, categorizing, and grading neoplasms (Fitzke et al., 2021; Rahmoun et al., 2025). Pathologists may benefit from this quantitative analysis, as it can provide more objective information and reduce the subjective variability among assessors (Battazza et al., 2024; Piccione et al., 2025). In addition, AI can identify prognostic biomarkers in tissues (Lancellotti et al., 2021). Prognostic biomarkers include specific patterns of protein expression or histological characteristics related to therapy response (Webster et al., 2011). The need for large datasets of pathologist-annotated slides with extensive experience is the primary obstacle in this field (Balachandran, 2025). Additionally, the veterinary pathologist remains responsible for the final interpretation and diagnosis of the patient. This is because AI does not fully comprehend the patient's history and clinical symptoms, nor does it have comprehensive knowledge of pathology (Fadhail, 2025; Vickram et al., 2025).

Dermatology

The diagnosis of skin diseases can be difficult because many conditions share similar clinical signs such as erythema, papules, pustules, and alopecia (Smith et al., 2024). Skin disorders are among the most prevalent causes for visits to small-animal veterinary clinics (Alizadeh et al., 2024). Artificial intelligence is providing an effective tool for the differential diagnosis and treatment of skin disorders (Patel and Vishwakarma, 2025). Using AI, the properties of the lesion, such as its form, border, color, and size, may be

extracted and compared with a database containing thousands of photos of known diseases (Langon et al., 2025). Early lesions that are caused by environmental or food allergies, bacterial infections such as pyoderma, fungal infections such as dermatophytosis, external parasites such as *Sarcoptes* or *Demodex* mites, and even skin neoplasia such as mast cell tumors or melanoma may be identified with the use of AI (Apostolopoulos et al., 2025).

The use of AI for analyzing dermatoscopic images is a more specific application. One type of portable light microscope is a dermatoscope, designed to provide a more detailed view of the structures of the skin and hair (Burroughs and Diaz, 2023). Dermatoscope high-magnification images can be analyzed by algorithms that detect specific microscopic patterns essential for diagnosing disorders such as mange (caused by the mite *Sarcoptes scabiei*) and demodicosis, as well as the vascular characteristics of skin moles (Alanazi, 2025). Particularly for general veterinarians with limited dermatoscopy expertise, this technique has the potential to improve diagnostic accuracy (Owens et al., 2023). Not only can AI assist with diagnosis, but it can also work to monitor how well therapy is working (Appleby and Basran, 2022). By comparing serial photos from multiple visits, the system can objectively evaluate the lesion's improvement or spread (Burroughs and Diaz, 2023; Sri et al., 2025).

Microbiology and diagnosis of infectious diseases

The field of microbiology is on the verge of a revolution driven by AI (Smith and Kirby, 2020; Pillai et al., 2022). Although most published studies focus on human medicine, the same AI principles have shown promising results in small animal veterinary microbiology, including automated antibiogram reading for canine urinary tract infections and metagenomic detection of enteric pathogens in cats with chronic diarrhea (Shan et al., 2024). The automated examination of bacterial cultures and the assessment of antibiotic susceptibility are two of the most important applications in human and veterinary medicine (Marletta et al., 2023; Wong et al., 2023). In the field of human clinical microbiology, advanced imaging devices can capture images of culture plates at predetermined intervals, and AI algorithms can monitor and even forecast bacterial colony growth (Burns et al., 2023). Advanced imaging devices can assist technicians in the initial identification of significant pathogens, such as *Staphylococcus*, *Escherichia coli*, and *Pseudomonas*, by providing preliminary colony classification based on morphology, including form, color, size, and edges (Alsulimani et al., 2024). In disk diffusion tests, also known as antibiograms, AI can estimate the zone of inhibition diameter with high precision and interpret the susceptibility result (susceptible, intermediate, or resistant) in accordance with internationally recognized recommendations, such as the Clinical and Laboratory Standards Institute (CLSI) VET01-S (Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals; CLSI, 2024) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoint tables (Toutain et al., 2017; Nguyen et al., 2025). Both the expediency of detecting the spread of antimicrobial resistance and the granularity of measurement are consequently enhanced (Miglietta et al., 2025).

A more advanced application of AI in microbiology is in metagenomics and next-generation sequencing (Chisompola et al., 2025; Syed et al., 2025). Given the enormous amounts of sequencing data generated from clinical samples, such as blood, urine, and tissue, AI and machine learning have emerged as powerful adjuncts for analyzing this complex data (Islam et al., 2025). From a wide range of host- and natural microbiome-derived sequences, the convolutional neural networks and random forest classifiers algorithms can recognize and categorize the genetic sequences of bacterial, viral, fungal, and parasitic pathogens (Miglietta et al., 2025). A further capability of AI is the ability to recognize genetic patterns associated with antibiotic resistance in sequencing data. The molecular patterns include the presence of specific resistance genes and may also predict which drugs may not be effective (Jia et al., 2025). This might facilitate more focused therapy and reduce inappropriate antibiotic use. On the other hand, due to the high cost of sequencing equipment and the need for sophisticated computing infrastructure, the general adoption of these technologies is now mostly restricted to research and reference labs (An et al., 2025).

The behavioural observations in animals

Beyond the analysis of molecular and imaging data, artificial intelligence is increasingly capable of detecting disease through the interpretation of behavioural observations, specifically vocalizations and movement patterns. These modalities offer non-invasive, continuous, and objective means of health assessment in small animal medicine. In the domain of acoustic analysis, deep learning models have demonstrated considerable utility. Convolutional neural networks (CNNs) can classify canine vocalizations such as barks, whines, and howls with high accuracy, and can differentiate between playful and aggressive contexts based solely on acoustic features (Gómez-Armenta et al., 2024). More importantly, machine learning has been successfully applied to detect respiratory pathologies from vocal sounds. The "BrachySound" study employed ML models to analyze respiratory audio samples from brachycephalic dogs, achieving 85% accuracy in classifying Brachycephalic Obstructive Airway Syndrome (BOAS), thereby offering a rapid, objective alternative to subjective auscultation (Oren et al., 2023). Furthermore, recurrent neural networks have been developed to automatically detect and grade stertor (snoring sounds) in laryngeal recordings, enabling widespread screening for BOAS (McDonald et al., 2024). In parallel, AI-powered movement analysis provides a window into musculoskeletal and neurological health. Markerless deep neural networks can track canine joint range of motion from standard video footage, detecting reductions as subtle as 6.1 degrees in dogs with elbow osteoarthritis (Gordon et al., 2025). Artificial neural networks analyzing inertial sensor data have also proven capable of discriminating between sound and lame dogs with approximately 86% accuracy, integrating these assessments into user-friendly clinical applications (Figueirinhas et al., 2022). Wearable sensors equipped with machine learning pipelines can even detect trained behavioral alerts for seizure events (Brady et al., 2025). Collectively, these approaches, including vocalization analysis and movement tracking, enable earlier, more objective disease detection and continuous remote monitoring. When combined with blockchain technology for secure, immutable storage of behavioral health data, such systems could facilitate multi-institutional collaboration and personalized, preventive veterinary care.

BLOCKCHAIN SYSTEM: TRANSPARENCY, SECURITY, AND DATA INTEGRITY

Blockchain, as a distributed, decentralized ledger, has the potential to address some of the fundamental challenges in pet health data management. This potential stems from the fact that it inherently provides a transparent and secure platform for recording and validating events and transactions (Chattu, 2021). One of the main advantages of this technology is that every data record is verifiable and cannot be easily changed (Giri and Dash, 2023). The blockchain structure is based on a chain of blocks, each containing a timestamp and a cryptographic hash of the previous block, and is collaboratively maintained and updated by a set of nodes (Zheng et al., 2018). Any changes to the recorded data or the addition of a new record require a majority of the network to reach a consensus; this requirement for a majority consensus mechanism makes data manipulation practically costly and difficult (Zheng et al., 2018). In the veterinary context, this immutability and the requirement for majority consensus could revolutionize the way animal records are managed (Quy et al., 2023). Data such as test results, treatment reports, and diagnostic images can be captured and referenced as reliable, immutable records (Hickman et al., 2020). In addition, information such as birth certificates, vaccination records, and disease histories can be defined as a unified digital identity for each animal; an identity that becomes more complete over the animal's lifetime, allowing for authentication and validation of information (Hickman et al., 2020). Access to this unified digital identity and its associated records can be provided in a controlled manner to stakeholders, including the animal's owner, treating veterinarian, animal shelters, insurance companies, and regulatory agencies. In such a model, reliance on centralized databases and time-consuming paper-based processes is reduced, and security risks associated with data centralization are also mitigated (Figure 4; Sharma et al., 2021).

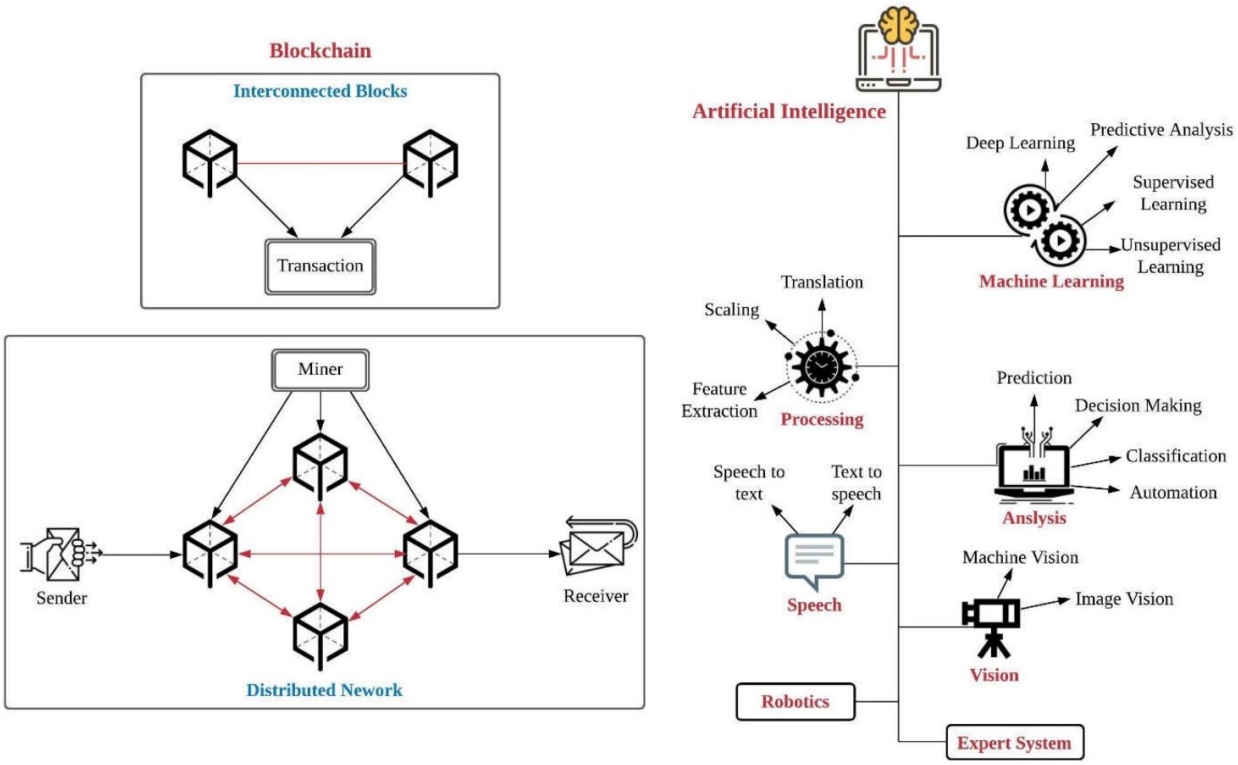


Figure 4. Overview of artificial intelligence and blockchain. (MDPI Copyright, Source of image adapted from Sharma et al. (2021).

Before detailing the applications of blockchain, it is worth explicitly stating how it concretely tackles the three major AI challenges in veterinary medicine, namely data integrity by immutably hashing training datasets and access logs, transparency via cryptographically verifiable audit trails for every data contribution and model update, and mitigation of bias by documenting data provenance, preprocessing steps, and demographic variables, making hidden biases detectable and correctable (Iqbal et al., 2021). This trust layer thus directly complements AI without acting as a primary data repository.

One of the most serious and practical applications of blockchain in pet veterinary medicine is supply chain management and the authentication of drugs, vaccines, and biological products (Makkar and Costa, 2020). Issues such as mislabeling, fraud, and the distribution of expired or counterfeit drugs pose direct threats to animal health and are especially prevalent in the online veterinary drug market (Iqbal et al., 2021). In this context, assigning a unique digital identifier to each product unit in the production line (scannable codes) creates a public, tamper-proof record of each product's movement (Iqbal et al., 2021). A unique identifier is then recorded and updated throughout the product life cycle, from production and quality control to distribution, storage, and finally supply to the pharmacy or clinic (Makkar and Costa, 2020). With a simple scan, the veterinarian or pet owner can access the product's complete history, verify storage conditions, and confirm that the drug was obtained from official, reliable sources (Makkar and Costa, 2020). Implementing such a mechanism not only increases the safety of drug treatment but also helps to combat the informal market and protect reputable brands (Iqbal et al., 2021). In addition, in the event of an adverse drug reaction, it enables rapid, accurate traceability back to the source of production, making the recall process for defective products more targeted and effective (Thakur et al., 2024).

Compared with many existing digital solutions, blockchain is a more advanced and future-proof option, as it enables large-scale sharing of research and epidemiological data without compromising data security (Sai et al., 2022). To study emerging diseases, breed genetics, treatment efficacy, and long-term outcomes, access to large datasets from animal populations is essential, and, naturally, these databases should cover a diverse range of data (Sai et al., 2022). By leveraging blockchain, researchers and institutions can securely and efficiently exchange data, including anonymized clinical data, genetic information, medical images, and results from subsequent studies (Patel, 2019; Tagde et al., 2021). Since blockchain can act as a trusted platform for recording access, every time data is recorded or accessed, the event remains recorded and verifiable in the ledger (Tagde et al., 2021). Furthermore, ledger-based mechanisms can clarify data ownership and usage restrictions and specify frameworks for data access and exploitation via smart contracts (Sai et al., 2024). In practice, such a model can facilitate international collaboration, reduce research duplication, and accelerate the production of new knowledge (Sai et al., 2024). In particular, rapid, decentralized, and reliable reporting of disease cases on the blockchain platform has the potential to enhance the surveillance and management of infectious diseases with pandemic potential, such as canine influenza or zoonotic diseases, and help animal health and public health agencies monitor outbreaks in near real-time and implement more targeted and timely interventions (Sai et al., 2024).

Beyond these applications, the convergence of blockchain with artificial intelligence creates synergistic opportunities that directly address core challenges in veterinary AI (Choi et al., 2024). First, blockchain can secure the training data lifecycle for AI models (Zhang et al., 2024). High-quality, trustworthy labeled data are essential for developing robust diagnostic algorithms; blockchain provides an immutable record of data provenance, including the origin of each image or clinical record, any preprocessing steps applied, and the identity of annotating veterinarians (Kasralikar et al., 2025). This transparency reduces the risk of data tampering or undetected bias, ultimately improving the reliability of AI models (Parlak et al., 2025). Second, smart contracts can govern AI algorithm updates (Iqbal et al., 2021). When an AI model requires retraining or a new version is released, smart contracts can automate validation, approval workflows, and deployment across participating clinics, ensuring that all users operate on the same validated version while maintaining an auditable change log (Hawashin et al., 2026). Third, distributed ledgers can track AI decision-making for auditability. In clinical practice, when an AI system recommends a diagnosis or treatment, recording the input features, model version, and output confidence score on a blockchain creates a tamper-proof audit trail. This is particularly valuable for post-hoc analysis, medicolegal inquiries, or adverse event investigations, as clinicians can verify whether the AI's recommendation was correctly interpreted and whether the model performed as expected (Hossain et al., 2025; Musamih et al., 2026). By integrating these mechanisms, blockchain not only complements AI but also addresses concerns about transparency, reproducibility, and accountability issues that have historically limited clinical adoption of AI in veterinary medicine (Choi et al., 2024).

Although blockchain-based systems offer potential advantages, several serious challenges should be addressed before they can be fully implemented in veterinary medicine (Makkar and Costa, 2020). Many public blockchains experience significant processing latency and energy consumption under high transaction volumes, such as during continuous data collection from wearables (Hawashin et al., 2026). Continuous data collection from wearables limitations largely stem from the architecture of these networks and the way transactions are designed, and they challenge scalability and efficiency (Hawashin et al., 2026). One solution proposed in this context is the use of private or consortium blockchains with stricter access controls, sometimes referred to as layer-2 solutions. In addition to technical challenges, coordination and standardization are also very important (Musamih et al., 2026). For a blockchain system to be truly effective at the industry level, it is necessary for different actors in the field, from veterinarians and laboratories to pharmaceutical companies, breed registries, and government agencies, to agree on a set of common protocols, data formats, and access models; a process that is naturally time-consuming and costly (Musamih et al., 2026). In addition, legal and governance issues should be clarified, including determining the legal ownership of data recorded on the chain, the legal status of smart contracts, and compliance with data protection requirements such as the European General Data Protection Regulation (Jabbar et al., 2021). Finally, it is essential to educate end users, especially technicians and veterinarians, to understand and use these infrastructures properly, so that these systems do not remain merely on paper (Jabbar et al., 2021; Kha et al., 2023).

Limitations and ethical rules for using AI in clinical evaluation

The conceptual and regulatory frameworks governing AI in clinical decision-making remain markedly underdeveloped in veterinary medicine compared to human healthcare, creating a landscape defined by principle-based guidance and significant regulatory lacunae, especially outside Europe (Busch et al., 2025). Professional bodies have issued foundational ethical position statements: the joint (American College of Veterinary Radiology) ACVR and (European College of Veterinary Diagnostic Imaging) ECVDI 2025 statement, emphasizing patient safety anchored in a veterinarian-in-the-loop principle and transparency, warns that currently, no commercially available veterinary diagnostic imaging AI product meets the required standards for transparency, validation, or safety (Appleby et al., 2025). However, a systematic audit of 71 commercial veterinary AI products found a mean transparency score of only 6.4%, with 63.3% of vendors failing to disclose even a single performance metric (School of Veterinary Medicine, University of Wisconsin-Madison, 2026). This transparency gap forces individual practitioners to bear the legal and ethical burden of validating AI tools without access to essential performance data (School of Veterinary Medicine, University of Wisconsin-Madison, 2026). In the FDA-regulated US market, the Center for Veterinary Medicine has not categorized veterinary AI diagnostic tools as pre-market approved medical devices similar to human AI counterparts, effectively leaving clinical validation to individual veterinarians (Duggirala et al., 2025). Conversely, the European Union offers the first legally binding framework in the form of the 2024 EU AI Act, which classifies medical AI as "high-risk" and mandates strict compliance for human health AI systems, though its direct application to veterinary medicine remains ambiguous (Busch et al., 2025; Parlak, 2025). Meanwhile, 47.2% of countries globally have no AI-specific healthcare framework (Busch et al., 2025). Given this fragmented regulatory terrain, professional consensus has coalesced around core principles, including that AI must augment rather than replace veterinary clinical

judgment, with ultimate accountability resting with the attending veterinarian (SAVC, 2024; Appleby et al., 2025). This principle-based approach mandates transparent data handling, explicit disclosure of AI use to clients, and continuous post-market surveillance.

FUTURE PROSPECT

It is not going to be discrete, standalone instruments that will be the future of AI in veterinary treatment for small animals; rather, it will be an interconnected, intelligent ecosystem (Pereira et al., 2023). Imagine a system that gathers information from a variety of sources, including the animal's extensive electronic health history; real-time findings from wearable sensors measuring heart rate, activity, sleep quality, and even aberrant feeding patterns; medical photographs; pathology reports; and genetic data (Pereira et al., 2023). A central AI-based platform will consolidate and evaluate this large stream of data across several facets using sophisticated algorithms (Wilson and Anwar, 2024). The system will be able to uncover complex patterns and hidden connections that are hidden from the view of a human expert (Wilson and Anwar, 2024). For instance, it may detect a subtle correlation between a specific pattern on an echocardiogram, an inflammatory marker in a blood test, and a behavioral change recorded by the pet owner, and it may also warn of the possibility of developing a specific cardiorenal disease at an earlier stage than the beginning (Balachandran, 2025). By using a holistic approach, the veterinarian can transition from a reactive to a proactive, predictive stance, enabling them to intervene at the preclinical stage of diseases (Amer and Amer, 2025). Artificial intelligence will serve as an auxiliary "digital brain" within the proposed blockchain-based health data management system, continuously monitoring the animal's health and delivering deep insights to support tailored decisions (Figure 5; Aguilar-Lazcano et al., 2023).

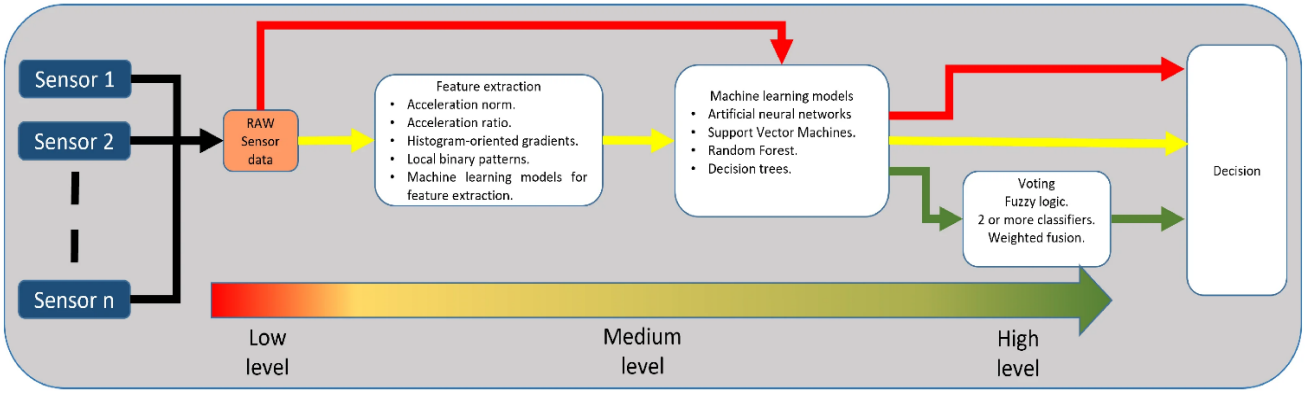


Figure 5. Representation of the three levels of sensor fusion. (MDPI, Copyright. Source of image adapted from Aguilar-Lazcano et al. (2023).

The transition toward Precision Veterinary Medicine or Personalized Veterinary Medicine will be one of the most significant effects AI will have in the future (Weiderman et al., 2025). AI algorithms will be able to predict the likelihood of developing specific diseases, such as cancer or hereditary diseases, by analyzing genomic, metabolomic, and microbiome data, as well as an animal's individual history of responses to previous treatments (Wilson and Anwar, 2024). Additionally, machine learning algorithms will be able to predict responses to specific drugs (pharmacogenomics) and design fully customized diets and treatments (Sarker, 2021). By leveraging the tumor's genetic profile and the animal's immunological history, for instance, the system may provide a recommendation for the chemotherapy regimen most beneficial for a dog diagnosed with lymphoma (Koo et al., 2021; Alshammari et al., 2025). Moreover, AI will serve as a powerful engine for discovering new information in animal biology and disease research (Alshammari et al., 2025). Using AI, it is possible to identify previously unknown risk factors for disease, discover new disease subtypes with different prognoses and treatment responses, and even create predictive models for infectious disease outbreaks, such as canine influenza or parvovirus, based on spatial and epidemiological data (Saturi, 2023). AI capabilities are achieved through the analysis of massive datasets shared across multiple centers, containing millions of animal records (Uçar et al., 2020).

AI in the future can help make healthcare knowledge more accessible to the general public (Gruetzemacher and Whittlestone, 2022). Diagnostic tools and even nanobiosensors that are powered by AI and can be placed on mobile phones or other low-cost devices might make it possible for ordinary veterinarians working in rural locations or tiny clinics to get access to a level of specialized analysis, such as radiography, pathology, or dermatology to detect even zoonotic diseases such as helminths in animals that were previously only accessible in major professional centers (Nagamori et al., 2021; Ward et al., 2022; Akbarein et al., 2025). AI has the potential to improve the overall quality of services while also reducing the care gap across areas (Sadr et al., 2025b). Nevertheless, this promising future will not be devoid of difficulties and will bring new obstacles that are more difficult to address from professional, ethical, and legal standpoints. When a system offers a therapy suggestion, the veterinarian should be able to understand the logic behind it; this is the dispute between the black box and the transparent box (Islam et al., 2025). When information from a variety of sources is combined, concerns over data privacy grow more complex and controversial (Sadr et al., 2025b). Certain legal frameworks should be explicitly stated to determine accountability for system errors (Islam et al., 2025). Legal frameworks should be established between the software developer, the user veterinarian, and the service provider (Islam et al., 2025). Additionally, the education of the next generation of veterinarians should be rethought to include digital literacy, the capacity to engage critically with AI outputs, and the important clinical skills that should be maintained alongside their use. This educational reorientation also ushers in a new era of precision, predictability, and personalized care in veterinary medicine.

CONCLUSION

The field of small-animal veterinary care is poised for a paradigm shift in diagnostics, therapeutics, and management, driven by AI. The ability of deep learning and computer vision algorithms to increase the speed, accuracy, and objectivity of interpreting complex clinical data is demonstrated by their practical, promising applications in specialized fields such as radiology, pathology, dermatology, and microbiology. AI applications demonstrate the unparalleled ability of machine learning algorithms to help improve the interpretation of clinical data. However, the successful development of these technologies depends heavily on overcoming fundamental challenges. The most important of these challenges include the absence of large datasets that are of high quality and diverse, as well as accurate annotations; the extensive genetic and anatomical variation among breeds that hinders the generalizability of models; and the absence of clear ethical, legal, and professional frameworks regarding accountability, data privacy, and the reduction of algorithmic bias. In conclusion, it is essential to emphasize that AI in veterinary medicine will not replace human judgment, experience, and communication; rather, it will serve as an intelligent assistant and a distinctive supplement to these aspects of the veterinarian's practice. A balanced approach that focuses on the tremendous potential of AI and wisely addresses the challenges ahead is required to realize the ultimate vision of improving the health and well-being of companion animals through earlier diagnosis, personalized treatments, and effective preventive care. Alongside AI, it is important to consider the infrastructure layers of trust and data governance, such as blockchain, to record verifiable events and manage data access. These layers complement AI, and their practical success will depend on solving technical, legal, and adoption challenges. Key limitations include the algorithm's sensitivity to image quality and lighting angle, as well as its inability to eliminate the need for additional diagnostic procedures such as cytology, culture, or biopsy. Moreover, AI cannot replace a comprehensive clinical evaluation and history-taking; its utility is therefore confined to screening and to serving as an initial guidance tool. To strengthen the technical basis of the systems by creating internationally shared and standardized databases, and to facilitate adoption by the veterinary community by designing user-friendly tools and integrating them seamlessly into clinical workflows, the future of this field depends on the establishment of sustainable interdisciplinary collaborations between veterinarians, AI experts, regulators, and industry. Collaborations between veterinarians, AI experts, regulators, and industry are necessary for the future of this field.

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Authors' contributions

Pouria Ahmadi Simab and Fatemeh Shiri were responsible for the conception and design of the study, acquisition and analysis of the data, interpretation of the findings, and drafting and critical revision of the article. Amin Shahinzadeh, Arya Heidari, and Amirreza Safaie contributed to the study's design and the interpretation of the results, and critically revised the manuscript. Sina Ghanbari and Pouria Ahmadi Simab were involved in drafting the article and critically revising its intellectual content. Amir Hossein Atazadeh designed and wrote the blockchain section of the manuscript. All authors have read and approved the final edition of the manuscript before publication.

Availability of data and materials

All reviewed datasets during the current study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

Ethical considerations

This manuscript is free of plagiarism, fabrication, falsification of data, duplication of publications, or other violations of scientific ethics. All presented information was compiled honestly, accurately, and responsibly. The authors confirmed that no AI applications were used for writing and preparing this article.

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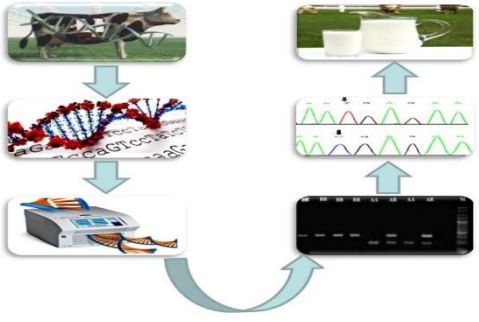
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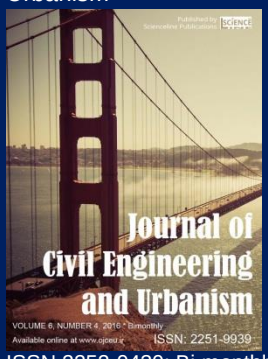
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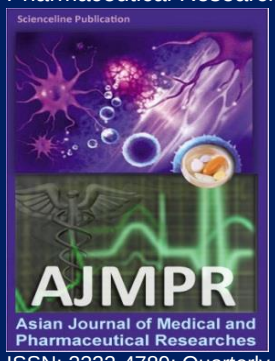
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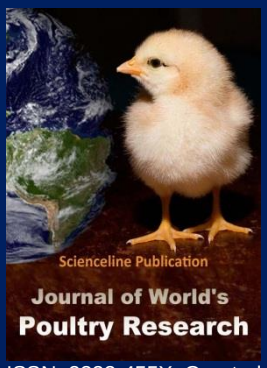
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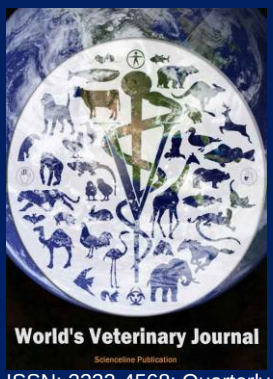
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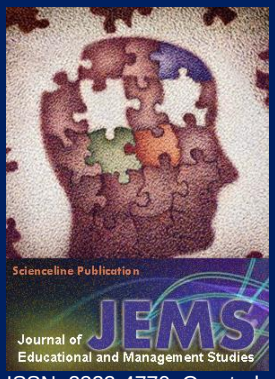
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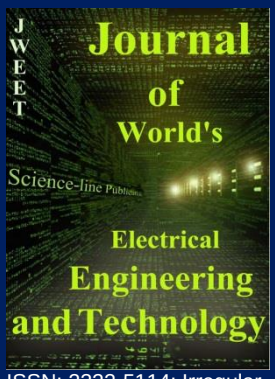
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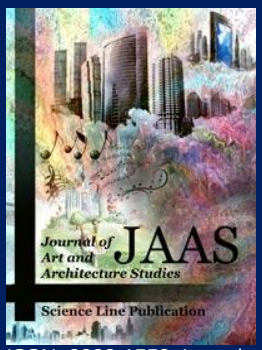
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