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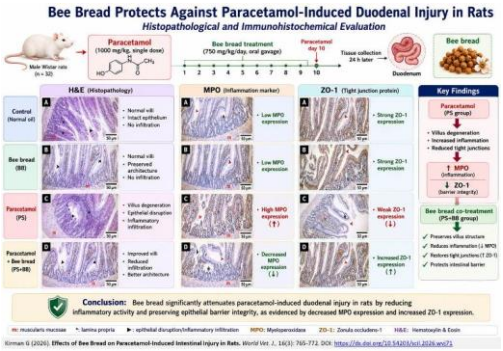
Research Paper

Effects of Bee Bread on Paracetamol-Induced Intestinal Injury in Rats

Kirman G.

World Vet. J. 16(3): 765-772, 2026; pii:S232245682600071-16

DOI: https://dx.doi.org/10.54203/scil.2026.wvj71



ABSTRACT: Although paracetamol (PS) is widely used as a safe analgesic and antipyretic agent in both humans and animals at therapeutic doses, high-dose administration may cause damage not only to the liver and kidneys but also to the gastrointestinal system. The present study aimed to evaluate the protective effects of bee bread (BB) on intestinal integrity and inflammation in a paracetamol-induced intestinal injury model in Wistar rats using histopathological and immunohistochemical methods. The expression levels of the tight junction protein *zonula occludens-1* (*ZO-1*) and the inflammatory marker myeloperoxidase (MPO) were evaluated by immunohistochemistry. A total of 32 male rats (8-10 weeks old, 250-300 g) were randomly assigned to four groups (n = 8 per group), consisting of the control group, the BB group (750 mg/kg/day orally for 10 days), the PS group (1000 mg/kg administered as a single oral dose on day 10), and the PS plus BB group. The BB group exhibited normal intestinal histological architecture, preserved *ZO-1* expression, and low MPO immunoreactivity, comparable to those observed in the control group. In contrast, the PS group showed marked mucosal irregularities, villus degeneration, increased inflammatory cell infiltration, decreased *ZO-1* expression, and increased MPO immunoreactivity. Compared with the PS group, BB treatment alleviated paracetamol-induced intestinal injury, increased *ZO-1* expression, and reduced MPO immunoreactivity in the PS plus BB group. In conclusion, BB administration alone did not adversely affect intestinal morphology or the evaluated immunohistochemical markers and partially protected against paracetamol-induced intestinal injury by preserving intestinal barrier integrity and attenuating inflammatory responses.
Keywords: Bee bread, Histology, Paracetamol, *Zonula occludens-1*

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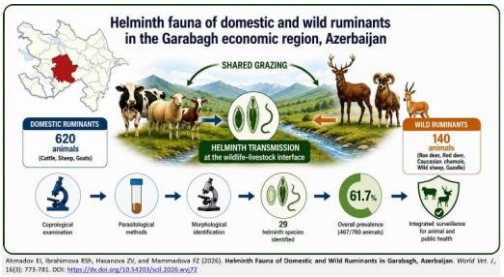
Research Paper

Helminth Fauna of Domestic and Wild Ruminants in Garabagh, Azerbaijan

Ahmadov EI, Ibrahimova RSh, Hasanova ZV, and Mammadova FZ.

World Vet. J. 16(3): 773-781, 2026; pii:S232245682600072-16

DOI: https://dx.doi.org/10.54203/scil.2026.wvj72



ABSTRACT: Helminth infections represent a significant constraint impacting the health and productivity of ruminants, with their distribution closely associated with ecological and management factors. The present study aimed to investigate the species composition, prevalence, and biocenological characteristics of helminths in domestic and wild ruminants in the Garabakh Economic Region of Azerbaijan. Between 2022 and 2025, a total of 760 animals were examined. Out of these, 620 were domestic ruminants such as cattle, sheep, and goats, whereas the other 140 included wild ruminants such as European roe deer, red deer, Caucasian chamois, wild sheep, and goitered gazelle. Helminth infections were evaluated through standard coprological techniques such as flotation, sedimentation, and larval recovery methods. Species identification was conducted based on morphological characteristics, and faunistic similarity was assessed using the Jaccard index. A total of 29 helminth species were identified, with 18 presented in both domestic and wild hosts. The overall prevalence was 61.7%, with goats demonstrating the highest infection rate at 74.2%. The most frequently detected species across the total examined population of domestic and wild

ruminants were *Fasciola hepatica, Moniezia expansa, Moniezia benedeni, Haemonchus contortus*, and *Trichostrongylus axei*. The high faunistic similarity between cattle and wild sheep, and between goats and Caucasian chamois (Ij = 0.51), indicated active circulation of parasites through shared grazing areas and water sources. Out of the 16 helminth species identified in domestic ruminants, 15 were found in wild hosts. Wild sheep and chamois exhibited the most similar faunal composition. The overall prevalence did not differ significantly between domestic (63.1%) and wild (55.7%) ruminants, with *Fasciola hepatica* detected in both groups. These quantitative results indicated that wild sheep and chamois played an active role in maintaining helminths alongside livestock, highlighting the importance of ecologically integrated surveillance in shared grazing regions.
Keywords: Economic region, Grazing, Helminth, Ruminant

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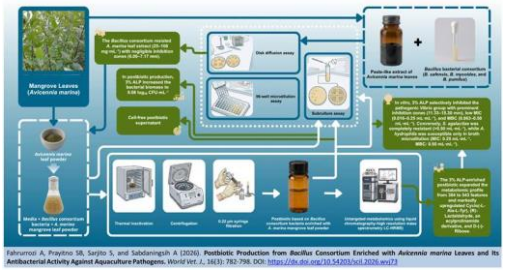
Research Paper

Postbiotic Production from *Bacillus* Consortium Enriched with *Avicennia marina* Leaves and Its Antibacterial Activity Against Aquaculture Pathogens

Fahrurrozi A, Prayitno SB, Sarjito S, and Sabdaningsih A.

World Vet. J. 16(3): 782-798, 2026; pii:S232245682600073-16

DOI: https://dx.doi.org/10.54203/scil.2026.wvj73



ABSTRACT: Bacterial infections pose a critical challenge in aquaculture, causing mass mortality and financial losses. The present study aimed to formulate postbiotics from a *Bacillus* (*B.*) consortium (*B. safensis*, *B. mycoides*, and *B. pumilus*) enriched with *Avicennia marina* (*A. marina*) leaf powder (ALP) and evaluate their activity against *Streptococcus agalactiae* (*S. agalactiae*), *Aeromonas hydrophila* (*A. hydrophila*), *Vibrio* (*V.*) *harveyi*, *V. parahaemolyticus*, and *V. alginolyticus*. The postbiotics' antibacterial efficacy was assessed using broth microdilution to determine minimum inhibitory concentrations (MICs), followed by subculturing to determine minimum bactericidal concentrations (MBCs). The disk diffusion assay assessed the tolerance of *Bacillus* consortium to *A. marina* leaf extracts at 25, 50, 75, and 100 mg mL^{-1}, with ethanol as the negative and ciprofloxacin as the positive controls. Postbiotic production used the medium as the control and three graded concentrations of ALP at 1%, 2%, and 3%, followed by thermal inactivation, centrifugation, and filtration to ensure the absence of live cells. The postbiotic was examined using liquid chromatography-high-resolution mass spectrometry to determine its secondary metabolite profiles. The 3% ALP-enriched postbiotic was selected for final antibacterial efficacy analysis against aquaculture pathogens. The results demonstrated that the *Bacillus* consortium was resistant to leaf extracts at 25-100 mg mL^{-1}, with only minimal inhibition zones of 6.00 to 7.17 mm. In postbiotic production, 3% ALP enhanced bacterial viability to 9.08 log_{10} CFU mL^{-1}. The 3% ALP-enriched postbiotic increased the number of metabolites from 384 (baseline control) to 543, accompanied by upregulation of Cyclo(-L-Ala-L-Tyr), (R)-Lactaldehyde, an acylprolinamide derivative, and D-(-)-Ribose. *In vitro*, 3% ALP selectively inhibited *Vibrio* spp., demonstrating significant inhibition zones (11.33-15.30 mm), low MIC values (1.6-25% v/v), and low MBC values (6.3-50% v/v). Although disk diffusion produced no inhibition zones for either *A. hydrophila* or *S. agalactiae*, *A. hydrophila* exhibited susceptibility at 25% v/v in the MIC and at 50% v/v in the MBC. In contrast, *S. agalactiae* remained resistant at the highest concentration, with MIC and MBC values exceeding 50% v/v. Enriching the *Bacillus* consortium with ALP increased the active metabolites in *Bacillus* postbiotics, offering a promising control against *Vibrio* spp. in aquaculture.
Keywords: Aquaculture, *Avicennia marina*, *Bacillus* consortium, Metabolite, Postbiotic, Vibriosis

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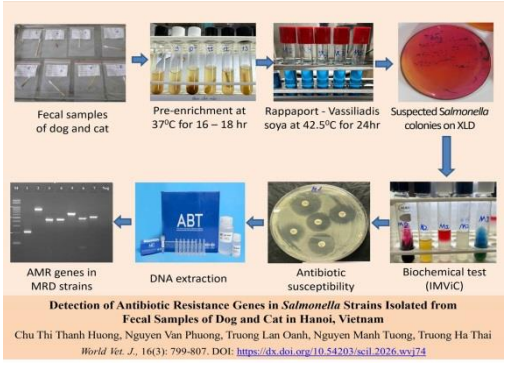
Research Paper

Detection of Antibiotic Resistance Genes in *Salmonella* Strains Isolated from Fecal Samples of Dogs and Cats in Hanoi, Vietnam

Huong CTT, Van Phuong N, Oanh TL, Tuong NM, and Ha Thai T.

World Vet. J. 16(3): 799-807, 2026; pii:S232245682600074-16

DOI: https://dx.doi.org/10.54203/scil.2026.wvj74



ABSTRACT: *Salmonella* belongs to the family *Enterobacteriaceae* and is a common pathogen that causes disease in humans and animals. Dogs and cats are potential sources of zoonotic diseases, including those caused by *Salmonella* spp. The current study aimed to investigate the prevalence and

antibiotic resistance of *Salmonella* strains in fecal samples from dogs and cats collected at veterinary clinics in Hanoi, Vietnam. From November 2024 to December 2025, 161 fecal samples from dogs and cats were collected from 12 veterinary clinics in Hanoi, Vietnam, for *Salmonella* isolation. Fecal samples were cultured in peptone buffer water (PBW) and on selective media such as Rappaport-Vassiliadis soybean (RVS) broth and xylose lysine deoxycholate (XLD) agar; suspected *Salmonella* colonies were confirmed by biochemical tests, including sugar fermentation, H_2S, and IMViC reactions. Antibiotic susceptibility was tested by agar diffusion, and genes implicated in antimicrobial resistance were detected by PCR amplification. The results showed that 11 (6.8%) of 161 fecal samples were *Salmonella*-positive; *Salmonella* was detected in 9 of 92 (9.8%) dog fecal samples and 2 of 69 (2.9%) cat fecal samples. The isolates exhibited the highest resistance to tetracycline (90.0%), followed by streptomycin (81.2%). Resistance to chloramphenicol and trimethoprim/sulfamethoxazole was 45.5%; for nalidixic acid, amoxicillin/clavulanic acid, doxycycline, gentamicin, and cefotaxime, resistance rates ranged from 27.3% to 36.4%. The isolates showed similar resistance rates to neomycin, kanamycin, and ciprofloxacin at 18.2%. Twelve antibiotic resistance genes, including *bla*$_{TEM}$, *aadA1*, *aadA2*, *aphA1-1AB*, *aac (3')-IV*, *sul1*, *cmlA1*, *floR*, *tetA*, *tetB*, *qnrA*, and *qnrS*, were amplified in the antibiotic-resistant *Salmonella* strains, whereas *bla*$_{PSE-1}$, *bla*$_{OXA-1}$, *tetG*, *qnrB*, and *aac (6')-ib-cr* were not detected. The results of the current study underscore the importance of monitoring antibiotic resistance in *Salmonella* spp. in companion animals to protect public health.
Keywords: Antibiotic resistance gene, Cat, Dog, *Salmonella*

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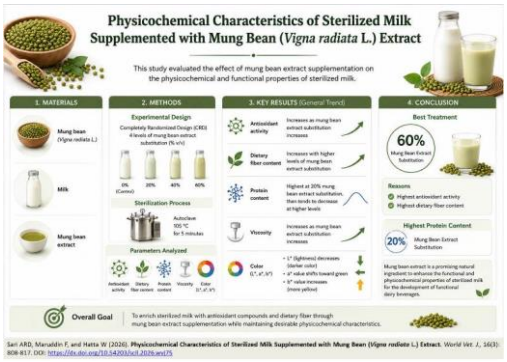
Research Paper

Physicochemical Characteristics of Sterilized Milk Supplemented with Mung Bean (*Vigna radiata* L.) Extract

Sari ARD, Maruddin F, and Hatta W.

World Vet. J. 16(3): 808-817, 2026; pii:S232245682600075-16

DOI: https://dx.doi.org/10.54203/scil.2026.wvj75



ABSTRACT: Sterilized milk is a nutritious dairy product; however, its dietary fiber and antioxidant contents are relatively low. Investigating the potential of sterilized milk is important to support the development of nutritionally enhanced and high-quality functional dairy beverages. The present study aimed to investigate the effects of incorporating mung bean (*Vigna radiata* L.) extract on the physicochemical properties, antioxidant activity, and dietary fiber content of sterilized milk. The present study followed a completely randomized design and consisted of four treatment groups with three replications per treatment. The first group was the control, with 100% milk and 0% mung bean extract (A1), the second group consisted of 80% milk and 20% mung bean extract (A2), the third group included 60% milk and 40% mung bean extract (A3), and the fourth group consisted of 40% milk and 60% mung bean extract (A4). Parameters analyzed included antioxidant activity, dietary fiber content, protein content, viscosity, and color (L*, a*, b*). The current results indicated that mung bean substitution had significant effects on all observed parameters. Increasing substitution levels increased antioxidant activity from 18.54% (A1) to 29.14% (A4), and dietary fiber content increased from 0.25% (A1) to 3.22% (A4). The highest protein content (2.28%) was achieved using a 20% mung bean extract substitution. Higher mung bean substitution increased the viscosity from 1.43 centipoise (cP) in the control group to 1.84 cP in Group A4. Compared to the control group, Group A4 exhibited a decrease in lightness (L*) from 74.80 to 66.90 and redness (a) from 5.61 to 0.58, alongside an increase in yellowness (b*) from 2.44 to 3.67. Substituting with 60% mung bean extract yielded the highest antioxidant activity and dietary fiber levels, while 20% mung bean extract led to the highest protein content.
Keywords: Antioxidant activity, Color value, Dietary fiber, Mung bean, Sterilized milk, Viscosity

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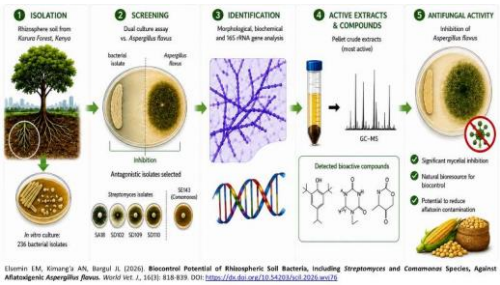
Research Paper

Biocontrol Potential of Rhizospheric Soil Bacteria, Including *Streptomyces* and *Comamonas* Species, Against Aflatoxigenic *Aspergillus flavus*

Elsemin EM, Kimang'a AN, Bargul JL.

World Vet. J. 16(3): 818-838, 2026;
pii:S232245682600076-16

DOI: https://dx.doi.org/10.54203/scil.2026.wvj76



ABSTRACT: After decades of extensive exploration of bioactive compounds from *Streptomyces* spp., the discovery of new bioactive chemicals from routinely studied strains has been declining progressively.

To tackle this challenge, recent studies have focused on *Actinobacteria* from under-explored ecological niches with significant microbial diversity. The present study aimed to assess the possible antifungal activity of *Streptomyces* spp. isolated from rhizospheric soils of Karura Forest, Nairobi, Kenya, against *Aspergillus flavus* (*A. flavus*), a fungal species known to produce aflatoxin. A total of 236 bacteria were isolated through *in vitro* cultures and characterized using macroscopic, microscopic, and biochemical tests to identify *Streptomyces*-like isolates, which were identified by dry, powdery colonies, filamentous branching hyphae, aerial mycelia, and the formation of spore chains, with Gram staining carried out later on isolates advanced through dual culture screening for antifungal activity. Dual culture assays, with *A. flavus* grown alone as the negative control, were performed to select the promising candidates for further extraction, gas chromatography-mass spectrometry (GC-MS) metabolite profiling, and antifungal testing by the disc diffusion method against PBS and nystatin as the negative and positive controls, respectively. The selected isolates also underwent molecular identification by PCR amplification and Sanger sequencing of the 16S rRNA gene. The analysis revealed that 190 isolates exhibited *Streptomyces*-like characteristics. Dual culture assays revealed that certain isolates inhibited the mycelial growth of *A. flavus*, and GC-MS profiling of the corresponding extracts confirmed the presence of bioactive metabolite classes. The pellet crude extracts of the six selected isolates (SA18, SD102, SD109, SD110, SE143, and SE194), exhibited the highest antifungal efficacy, with inhibitory zones of up to 31.60 ± 2.80 mm, relative to the positive control (nystatin); five of the isolates belonged to the genus *Streptomyces*, while SE143 was identified as *Comamonas*. Supernatant extracts and crude filtrates revealed lower and more variable inhibition across most of the isolates. The present findings indicated that rhizosphere-derived *Streptomyces* and *Comamonas* isolates from Karura Forest are potential natural candidates for biological control of *A. flavus*.
Keywords: Antifungal activity, *Aspergillus flavus*, Biocontrol, *Comamonas* sp., *Streptomyces* spp.

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Research Paper

Optimizing Fermented Water Hyacinth Supplementation with *Neurospora crassa* in Ducks: Implications for Performance and Small Intestine Morphology

Maslami V, Wiryawan IKG, and Noesidiq A.

World Vet. J. 16(3): 839-855, 2026; pii:S232245682600077-16

DOI: https://dx.doi.org/10.54203/scil.2026.wvj77



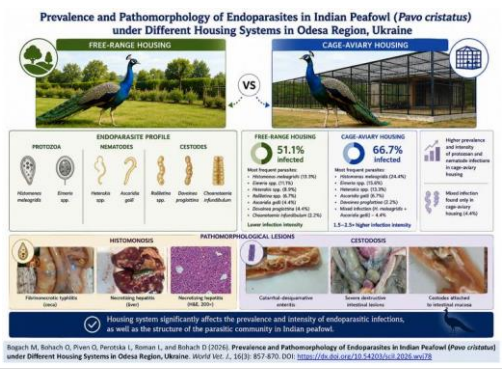
ABSTRACT: Water hyacinth is a potential alternative feed ingredient for ducks, but its utilization is limited by high fiber content and low digestibility. The present study aimed to evaluate the effects diets containing water hyacinth fermented with *Neurospora crassa* on feed intake, nutrient intake, growth performance, carcass traits, and intestinal morphology in Pekin ducks. A total of 100 ducks with an average initial body weight of 116 g were used in a completely randomized design with five treatments and four replications. Each replicate consisted of five ducks, and the experiment was conducted from 7 to 35 days of age. The treatments consisted of 0% water hyacinth (P1), 5% non-fermented water hyacinth (P2), 5% fermented water hyacinth (P3), 10% fermented water hyacinth (P4), and 15% fermented water hyacinth (P5). The feeding trial was conducted for four weeks. The analyzed parameters included feed intake, crude protein intake, crude fiber intake, metabolizable energy, feed conversion ratio, final body weight, weight gain, carcass percentage, and intestinal morphology. The results revealed that fermented water hyacinth inclusion had no significant effect on feed intake, metabolizable energy, feed conversion ratio, final body weight, weight gain, carcass percentage, breast percentage, thigh percentage, back percentage, and wing percentage among all treatment groups. No significant differences were observed in intestinal morphology parameters, including duodenal length, jejunal length, ileal length, and intestinal villus length among groups P1 to P5. However, ducks fed 15% fermented water hyacinth (P5) demonstrated significantly higher crude protein and crude fiber intakes compared to other groups. Water hyacinth fermented with *Neurospora crassa* can be included in Pekin duck diets at up to 15% without negatively affecting feed intake, growth performance, carcass traits, or intestinal morphology, while increasing crude protein and crude fiber intakes.
Keywords: Carcass trait, Duck, Fermentation, Intestinal morphology, *Neurospora crassa*, Water hyacinth
[Full text-PDF] [Crossref Metadata] [Scopus] [Export from ePrint]

Research Paper

Prevalence and Pathomorphology of Endoparasites in Indian Peafowl (*Pavo cristatus*) under Different Housing Systems in Odesa Region, Ukraine

Bogach M, Bohach O, Piven O, Perotska L, Roman L, and Bohach D.

World Vet. J. 16(3): 856-869, 2026; pii:S232245682600078-16



DOI: https://dx.doi.org/10.54203/scil.2026.wvj78

ABSTRACT: Parasitic infections represent a major health concern in ornamental and exotic poultry, adversely affecting the health, productivity, and survival of Indian peafowl (*Pavo cristatus*). The present study aimed to determine the prevalence and species composition of endoparasites in Indian peafowl maintained under free-range and aviary housing systems, and to characterize the gross and pathomorphological lesions associated with histomonosis and cestodosis. The present study was conducted in 2020-2025 on private farms and bioparks in the Odesa Region, Ukraine, and included 90 Indian peafowl, 44 males and 46 females, aged 1-2 years. Fecal samples were collected in all seasons, with 45 peafowl kept under free-range housing and 45 under cage-aviary housing. Fecal samples were analyzed by direct smear, flotation, and the McMaster method. The pathomorphological manifestations of histomonosis were examined through a retrospective analysis of 17 cases in Indian peafowl aged 6 weeks to 16 months. Meanwhile, gross lesions of cestodosis were assessed during postmortem examinations of three naturally infected peafowl. The overall prevalence of endoparasitic infections in Indian peafowl was 51.1% under free-range housing and 66.7% under cage-aviary housing. Under free-range housing, Indian peafowl were most frequently infected with *Histomonas meleagridis* (*H. meleagridis*; 13.3%), *Eimeria* spp. (11.1%), *Heterakis* spp. (8.9%), *Raillietina* spp. (6.7%), *Ascaridia galli* (4.4%), *Davainea proglottina* (4.4%), and *Choanotaenia infundibulum* (2.2%). In cage-aviary housing, the most common parasites were *H. meleagridis* (24.4%), *Eimeria* spp. (15.6%), *Heterakis* spp. (13.3%), *Ascaridia galli* (6.7%), and *Davainea proglottina* (2.2%). A mixed infection of *H. meleagridis* and *Ascaridia galli* was found exclusively in cage-aviary housing (4.4%). No apparent sex-related differences in susceptibility to protozoan infections were observed. The intensity of protozoan and nematode infections was generally higher in Indian peafowl housed in cage-aviaries than in those under free-range housing. Histomonosis was characterized by fibrinonecrotic typhlitis and characteristic necrotizing hepatitis, whereas cestodosis was associated with catarrhal-desquamative enteritis and severe destructive intestinal lesions. The current results indicated that the housing system affected the prevalence and intensity of parasitic infections, as well as the structure of the parasitic community, in Indian peafowl.
Keywords: Cestodosis, Hepatitis, Histomonosis, Indian peafowl, Protozoan infection
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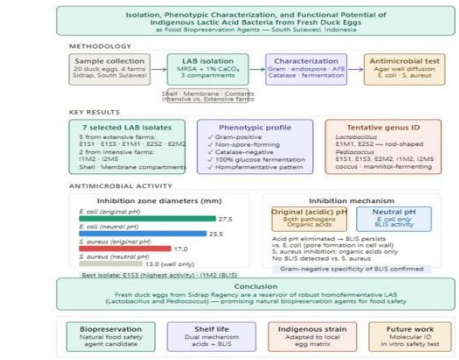
Research Paper

Isolation, Phenotypic Characterization, and Functional Potential of Indigenous Lactic Acid Bacteria from Fresh Duck Eggs

Irham M, Hatta W, and Maruddin F.

World Vet. J. 16(3): 870-879, 2026; pii:S232245682600079-16

DOI: https://dx.doi.org/10.54203/scil.2026.wvj79



Irham M, Hatta W, and Maruddin F (2026). **Isolation, Phenotypic Characterization, and Functional Potential of Indigenous Lactic Acid Bacteria from Fresh Duck Eggs.** *World Vet. J.*, 16(3): 871-880. DOI: https://dx.doi.org/10.54203/scil.2026.wvj79

ABSTRACT: Duck eggs are a highly nutritious source of animal protein; however, they are particularly susceptible to microbial contamination. Indigenous lactic acid bacteria (LAB) capable of colonizing fresh egg matrices may possess adaptive traits, making them effective natural biopreservation agents. The present study aimed to isolate, phenotypically characterize, and evaluate the antimicrobial activity of LAB derived from different compartments of fresh duck eggs, specifically the inner eggshell, egg membrane, and egg contents. Duck eggs were obtained from two intensive farms (I1 and I2) and two extensive farms (E1 and E2) located in Sidrap Regency, South Sulawesi, Indonesia, during 2024. The LAB isolates were obtained using a selective culture method employing De Man, Rogosa and Sharpe agar enriched with 1% $CaCO_2$. Twenty fresh duck eggs (five per location) were sampled, yielding 35 isolates, of which seven were confirmed as LAB based on morphological and biochemical analyses. Characterization included Gram, endospore, and acid-fast staining, catalase testing, and carbohydrate fermentation profiling. Antimicrobial activity against *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*) was evaluated using agar well diffusion method at the original (acidic) and neutral pH levels to identify bacteriocin-like inhibitory substances (BLIS). Seven selected LAB isolates were obtained, including five from extensive farms (E1S1, E1S3, E1M1, E2S2, E2M2) and two from intensive farms (I1M2, I2M5). All isolates demonstrated complete glucose fermentation and a homofermentative pattern. Isolates E2S2, E2M2, and I1M2 fermented glucose, lactose, fructose, and mannitol, whereas E1S1, E1S3, E1M1, and I2M5 fermented glucose exclusively. Based on phenotypic data, the isolates were preliminarily classified as *Lactobacillus* and *Pediococcus* species. Antimicrobial activity testing demonstrated significant inhibitory potential, with maximum inhibition zones of 27.5 mm against *E. coli* and 17.0 mm against *S. aureus*. Although inhibition zone diameters decreased at neutral pH, the persistence of a clear zone against *E. coli* indicated BLIS production. The disappearance of inhibitory activity against *S. aureus* at neutral pH indicated that organic acids caused the pathogen's inhibition. The current findings indicated that fresh duck eggs contain a high abundance of homofermentative LAB, which could serve as effective natural biopreservation agents to improve food safety and prolong shelf life.
Keywords: Antimicrobial activity, Duck egg, Lactic acid bacteria, *Lactobacillus*, *Pediococcus*

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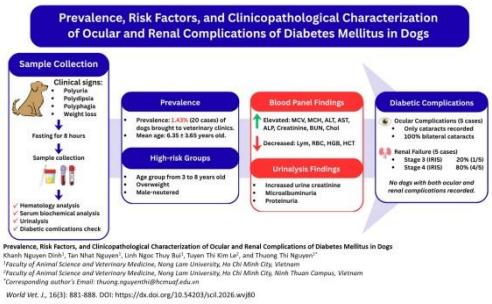
Research Paper

Prevalence, Risk Factors, and Clinicopathological Characterization of Ocular and Renal Complications of Diabetes Mellitus in Dogs

Dinh KN, Nguyen TN, Thuy Bui LN, Kim Le TT, and Nguyen TT.

World Vet. J. 16(3): 880-887, 2026; pii:S232245682600080-16

DOI: https://dx.doi.org/10.54203/scil.2026.wvj80



ABSTRACT: Canine diabetes mellitus (DM) is an endocrinopathy in dogs, which can lead to diabetic complications in the patient's eyes and kidneys. The present study aimed to describe the prevalence, risk factors, hematologic and serum biochemical findings, and incidence of ocular and renal complications of DM in dogs at veterinary clinics in Ho Chi Minh City, Vietnam. Dogs brought to veterinary clinics with clinical signs of polyuria, polydipsia, polyphagia, and weight loss were examined for DM, including hematology, serum biochemical, and urine analyses, from January to December 2025. Dogs confirmed with DM had hyperglycemia after at least 8 hours of fasting and concomitant glycosuria. The incidence of ocular and renal complications of diabetic dogs was recorded by using slit-lamp and urine parameters (elevated serum creatinine, blood urea nitrogen, microalbuminuria, and urine protein-to-creatinine ratio), respectively. Twenty canine DM cases were identified, accounting for 1.43% of the total dogs brought to veterinary clinics during the study. The mean age of diabetic dogs was 6.35 ± 3.65 years. The highest prevalence of DM was in the 3-8 years old group, the overweight group, and the male-neutered group among male dogs; however, female-entire dogs had the highest overall prevalence (3.36%). Diabetic dogs demonstrated elevated mean corpuscular volume, mean corpuscular hemoglobin, alanine aminotransferase, aspartate transaminase, alkaline phosphatase, creatinine, blood urea nitrogen, and cholesterol, and decreased Lymphocytes, red blood cells, hemoglobin, and hematocrit in hematology and serum biochemical analyses. Urinalysis results of diabetic dogs demonstrated an increase in creatinine, microalbuminuria, and proteinuria, which could be used to diagnose diabetic renal complications in dogs. Cataract formation was the only ocular complication recorded in the present study, which was recorded in 5 diabetic dogs, and all diagnosed dogs had cataracts in both eyes. Renal failure was observed in 5 diabetic dogs, including 4 dogs with stage 4 renal failure and 1 dog with stage 3 renal failure. No dogs had both ocular and renal complications at the same time. In the current study, dogs that were older, overweight, or male-neutered had higher DM prevalence, with cataracts and stage 4 renal failure being the most common diabetic complications.
Keywords: Diabetes mellitus, Dog, Hematology, Ocular complication, Renal complication

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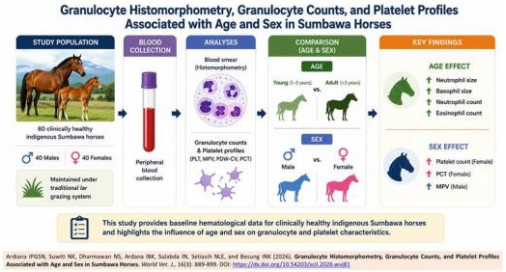
Research Paper

Granulocyte Histomorphometry, Granulocyte Counts, and Platelet Profiles Associated with Age and Sex in Sumbawa Horses

Ardiana IPGSN, Suwiti NK, Dharmawan NS, Ardana IBK, Sulabda IN, Setiasih NLE, and Besung INK.

World Vet. J. 16(3): 888-898, 2026; pii:S232245682600081-16

DOI: https://dx.doi.org/10.54203/scil.2026.wvj81



ABSTRACT: The Sumbawa horse (*Equus caballus*) is an indigenous Indonesian horse breed traditionally maintained under an extensive grazing system, characterized by unrestricted grazing and exposure to natural climatic conditions. This study aimed to evaluate granulocyte histomorphometry, granulocyte counts, and platelet profiles in clinically healthy indigenous Sumbawa horses in association with age and sex, providing baseline hematological data for this indigenous horse breed. A total of 80 clinically healthy indigenous Sumbawa horses meeting the predefined inclusion criteria were selected using purposive sampling and enrolled in this cross-sectional study. Peripheral blood smears were used for the histomorphometry evaluation of neutrophils, eosinophils, and basophils, whereas granulocyte counts, platelet counts, and platelet indices, including mean platelet volume (MPV), platelet distribution width (PDW-CV), and plateletcrit (PCT), were analyzed with an automated hematology analyzer. Sex had no significant effect on granulocyte histomorphometry or granulocyte counts. However, female horses exhibited significantly higher platelet counts and PCT values, whereas MPV was significantly higher in

males. Adult horses exhibited significantly larger neutrophils and basophils, together with higher neutrophil and eosinophil counts. In contrast, platelet counts and platelet indices did not differ significantly between age groups. These findings indicated that granulocyte structural and quantitative characteristics are more responsive to age-related physiological adaptation, whereas platelet variation is predominantly associated with sex.
Keywords: Granulocyte count, Granulocyte histomorphometry, Platelet indices, Sumbawa horse, Sex

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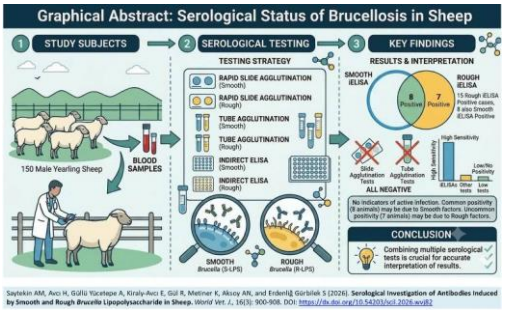
Research Paper

Serological Investigation of Antibodies Induced by Smooth and Rough *Brucella* Lipopolysaccharide in Sheep

Saytekin AM, Avcı H, Güllü Yücetepe A, Kiraly-Avcı E, Gül R, Metiner K, Aksoy AN, and Erdenliğ Gürbilek S.

World Vet. J. 16(3): 899-907, 2026; pii:S232245682600082-16

DOI: https://dx.doi.org/10.54203/scil.2026.wvj82



ABSTRACT: Different *Brucella* species, characterized by smooth and rough cell walls, are recognized as etiological agents of ovine brucellosis. While serological studies in adult and older sheep are common, those on the serological status of young animals are limited. The present study aimed to investigate brucellosis in sheep caused by smooth- and rough-walled *Brucella* species at two slaughterhouses in the Marmara Region of Turkey using serological methods. Three different analyses, including the rapid slide test, the tube agglutination test, and the indirect enzyme-linked immunosorbent assay (iELISA), each included smooth- and rough-structured *Brucella* antigens, resulting in a total of six separate tests. Blood sera were randomly obtained from 150 male sheep, approximately 1 year old and weighing about 70 kilograms, from the Merino, Kıvırcık, Lacauné, and Tahirova breeds. These sheep were brought to slaughterhouses from several provinces, including 25 from Tekirdağ, 45 from İstanbul, 43 from Kırklareli, 17 from Çanakkale, and 20 from Kocaeli. Using the rough iELISA, 15 positive cases (10%) were identified, of which eight were concurrently positive with the smooth iELISA. The low positivity rate and negative results from tube agglutination and rapid slide tests decreased the probability that the sheep had active brucellosis. Positive results for the same sample in both S-iELISA and R-iELISA tests may be linked to smooth LPSs, while positivity only in R-iELISA could be attributed to rough LPSs. Although no *Brucella ovis* or *Brucella melitensis* were found in the animals examined in the present study, the positive ELISA results suggested that brucellosis could have important epidemiological implications. Using two iELISA methods with smooth and rough LPS antigens concurrently enhanced the diagnostic sensitivity of indirect serological diagnosis of brucellosis.
Keywords: Brucellosis, Sheep, Slaughterhouse, Rough lipopolysaccharide, Smooth lipopolysaccharide

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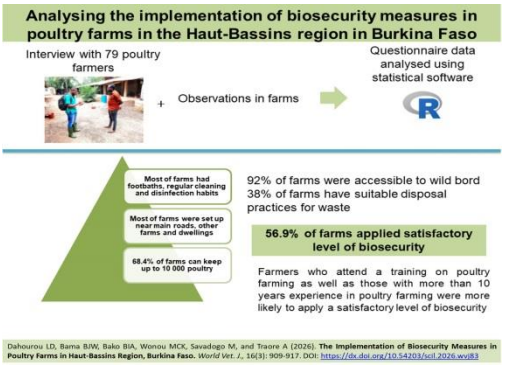
Research Paper

The Implementation of Biosecurity Measures in Poultry Farms in Haut-Bassins Region, Burkina Faso

Dahourou LD, Bama BJW, Bako BIA, Wonou MCK, Savadogo M, and Traore A.

World Vet. J. 16(3): 908-916, 2026; pii:S232245682600083-16

DOI: https://dx.doi.org/10.54203/scil.2026.wvj83



ABSTRACT: Poultry farming is an important socio-economic sector in Burkina Faso. However, disease outbreaks can substantially impact livestock production, so appropriate preventive measures should be implemented. The present study aimed to assess the implementation of biosecurity measures in poultry farms in the Haut-Bassins Region, Burkina Faso. The present cross-sectional study included 79 poultry farms and used a validated questionnaire. Questions were asked of each poultry farmer through interviews, and farms were observed to verify the implementation of certain biosecurity measures, including the presence and quality of foot baths, the presence of stagnant water, the orientation of the buildings, and the presence of gates. The current results indicated that the majority of farms were managed by men (86.1%), and 45.6% of farmers had more than 10 years' experience. Regarding farm capacity, 68.4% could house up to 10,000 poultry, and 64.6% of the reported farms had facilities that could accommodate more than 1,000 poultry. Over 59.5% of farms were located more than 300 meters away from the

main road; however, 64.6% were located less than 500 meters from the nearest poultry farm. More than 97.5% of farms were enclosed by fences, and 87.3% of these fences were made from waterproof materials. On farms, 60.8% had functional footbaths, 11.4% produced poultry feed, and 92.4% were not protected against wild birds entering their airspaces. Most farms (97.5%) regularly cleaned and disinfected materials and equipment, but only 38% had suitable waste disposal practices, and 22.8% kept water waste outside the farm. The present study reported that 56.9% of farms implemented a satisfactory level of biosecurity. Farmers who attended poultry farming training were more likely to apply biosecurity practices on their farms. The present study delineated the application of biosecurity measures on farms and emphasized the importance of raising awareness about biosecurity among poultry farmers in the Haut-Bassins Region of Burkina Faso.
Keywords: Biosecurity, Implementation, Poultry farm, Survey, Questionnaire

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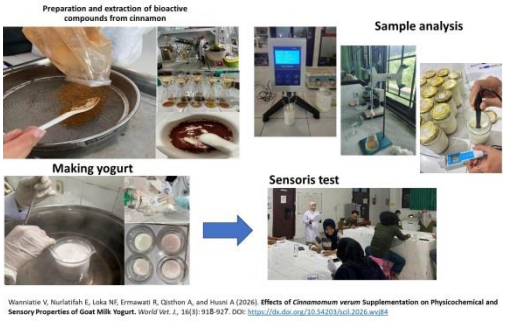
Research Paper

Effects of *Cinnamomum verum* Supplementation on Physicochemical and Sensory Properties of Goat Milk Yogurt

Wanniatie V, Nurlatifah E, Loka NF, Ermawati R, Qisthon A, and Husni A.

World Vet. J. 16(3): 917-926, 2026; pii:S232245682600084-16

DOI: https://dx.doi.org/10.54203/scil.2026.wvj84



ABSTRACT: Yogurt made from goat's milk has a pungent odor that persists in the product. Cinnamon is high in cinnamaldehyde, a compound responsible for its sweet, slightly spicy flavor, distinctive aroma, and warming sensation, all of which can enhance the flavor of goat milk yogurt. The present study aimed to evaluate the physicochemical and sensory properties of goat milk yogurt products supplemented with cinnamon. A completely randomized design was employed, with five treatment groups and four replications for each. The control group included goat milk yogurt without cinnamon, and the remaining treatment groups included goat milk yogurt with cinnamon at concentrations of 0.1%, 0.2%, and 0.3%. The physicochemical characteristics, including fat content, moisture content, viscosity, pH, and acidity, as well as sensory attributes (color, aroma, taste, texture, and overall acceptability) were evaluated. The present results indicated that different cinnamon concentrations influenced the fat content, moisture level, and pH of the yogurt, but did not affect its acidity or viscosity. Sensory evaluation of the goat milk yogurt demonstrated that cinnamon addition impacted the yogurt's color, aroma, taste, texture, and overall acceptability. The 0.2% cinnamon increased fat and pH levels, decreased moisture, and did not alter acidity or viscosity. The current findings suggest that 0.1% cinnamon enhanced the aroma of goat milk yogurt without reducing acceptability, whereas higher concentrations of cinnamon may be less favored.
Keywords: Cinnamon, Goat milk, Physicochemical, Sensory, Yogurt

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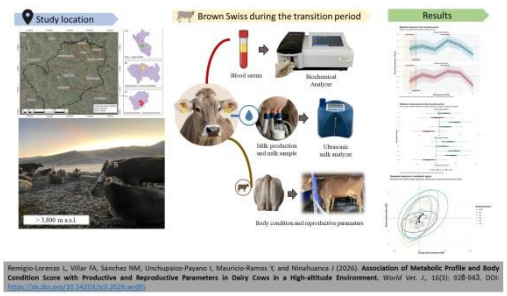
Research Paper

Association of Metabolic Profile and Body Condition Score with Productive and Reproductive Parameters in Dairy Cows in a High-altitude Environment

Remigio-Lorenzo L, Villar FA, Sánchez NM, Unchupaico-Payano I, Mauricio-Ramos Y, and Ninahuanca J.

World Vet. J. 16(3): 927-943, 2026; pii:S232245682600085-16

DOI: https://dx.doi.org/10.54203/scil.2026.wvj85



ABSTRACT: High-altitude environments impact metabolic adaptation during the transition from late gestation to early lactation, thereby affecting milk production, metabolic health, and reproductive recovery. The present study aimed to evaluate the associations between metabolic status and body condition with milk yield and composition, metabolic disorders, and reproductive intervals in Brown Swiss cows grazing in high-altitude areas. A prospective longitudinal observational study was conducted at a single production facility in Peru, located at an altitude of 3,829 meters above sea level. Sixty-one multiparous cows, approximately 5.5 to 7 years old, with 4 to 5 recorded calvings, had an average prepartum body weight of 692.4 ± 123.6 kilograms. These cows were monitored from 4 weeks before calving

until 9 weeks postpartum. Metabolic adaptation during the transition period was evaluated prepartum and at postpartum weeks 1, 3, 5, 7, and 9. Levels of β-hydroxybutyrate, glucose, cholesterol, triglycerides, albumin, total protein, ionized calcium, body condition score, estimated body weight, milk yield and composition, and reproductive intervals were measured. Energy-lipid imbalance was determined by measuring β-hydroxybutyrate, glucose, cholesterol, and triglycerides, whereas serum protein levels were assessed by measuring albumin and total protein. Both integrated indicators, energy–lipid imbalance and serum protein status, demonstrated non-linear patterns, peaking at their highest posterior levels around postpartum week 5. Postpartum week had a greater impact on milk production and composition than the animals' metabolic status. The body condition score demonstrated the strongest association with ketosis, where a 1-standard-deviation increase was associated with lower odds of ketosis. Ketosis prevalence was highest at week 5 (19.6%), whereas hypocalcemia persisted as a common condition throughout the follow-up period. Higher serum protein levels were associated with a shorter calving-to-conception interval, although this finding was imprecise. The postpartum period was more strongly associated with milk production, whereas body condition was the most indicative measure of ketosis risk.
Keywords: Body condition score, Dairy cow, Ketosis, Milk yield, Postpartum period, Transition period

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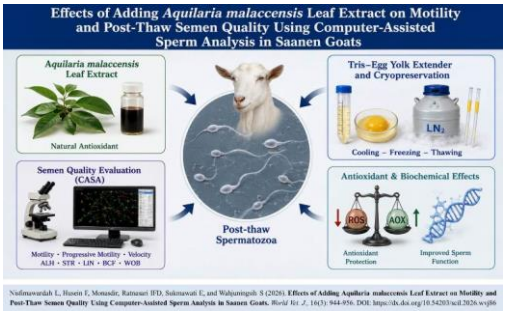
Research Paper

Effects of Adding *Aquilaria malaccensis* Leaf Extract on Motility and Post-Thaw Semen Quality Using Computer-Assisted Sperm Analysis in Saanen Goats

Nisfimawardah L, Husein F, Monasdir, Ratnasari IFD, Sukmawati E, and Wahjuningsih S.

World Vet. J. 16(3): 944-956, 2026; pii:S232245682600086-16

DOI: https://dx.doi.org/10.54203/scil.2026.wvj86



ABSTRACT: The quality of frozen semen from Saanen goats plays a vital role in the success of artificial insemination. The present study aimed to evaluate the effects of different semen diluents on post-thaw semen quality and sperm kinematic parameters using computer-assisted sperm analysis (CASA). Fresh semen samples were obtained from two 3-4-year-old Saanen goats, weighing 64 kilograms and 92 kilograms, with an average fresh motility of 80%. Semen quality was assessed under a microscope, focusing on motility, viability, abnormalities, concentration, and total motile spermatozoa. In the current laboratory experiment, five treatment groups were used with 10 replicates. The first group served as the negative control and consisted of tris-egg yolk without *Aquilaria malaccensis* leaf extract, the second group contained tris-egg yolk with 0.10 mg of *Aquilaria malaccensis* leaf extract, the third group consisted of tris-egg yolk with 0.15 mg of *Aquilaria malaccensis* leaf extract, the fourth group contained tris-egg yolk with 0.20 mg of *Aquilaria malaccensis* leaf extract, and the fifth group received the AndroMed® (positive control). The present results indicated that the use of different diluents had a significant effect on post-thawing semen quality, including motility, progressive motility, average path distance, curved line distance, straight line distance, average path velocity, straight line velocity, curvilinear velocity, amplitude of lateral head, beatcross frequency, and wobble. However, there was no significant difference in post-thawing semen quality in terms of individual motility, viability, abnormalities, concentration, total motile spermatozoa, straightness, and linearity. The use of tris-egg yolk diluents supplemented with *Aquilaria malaccensis* leaf extract at a concentration of 0.10 mg and AndroMed® indicated that the highest results among all treatments were demonstrated by the highest post-thaw semen quality.
Keywords: Antioxidant, *Aquilaria malaccensis*, Sperm kinematic, Saanen goat

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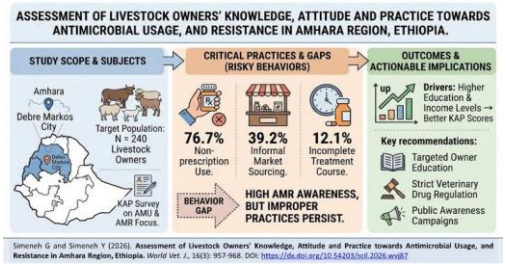
Research Paper

Assessment of Livestock Owners' Knowledge, Attitude and Practice towards Antimicrobial Usage, and Resistance in Amhara Region, Ethiopia

Simeneh G and Simeneh Y.

World Vet. J. 16(3): 957-968, 2026;
pii:S232245682600087-16

DOI: https://dx.doi.org/10.54203/scil.2026.wvj87



ABSTRACT: Antibiotic resistance is a major concern for humans and animals, especially in less developed countries such as Ethiopia. Livestock owners often misuse antibiotics, use incorrect doses, and ignore instructions for proper medication use. The present study aimed to evaluate the knowledge, attitudes, and practices regarding antimicrobial use (AMU) and antimicrobial

resistance (AMR) in Ethiopia. A cross-sectional study was conducted to assess livestock owners' knowledge, attitudes, and practices (KAP) regarding antibiotic use, antibiotic residues, and antibiotic resistance in Ethiopia. Structured interviews were conducted with eligible participants to gather insights on their perceptions and self-reported practices related to AMU and AMR. A survey involving 240 livestock owners assessed their backgrounds, knowledge, attitudes, and practices. Among the livestock owners, 84.6% were men, 36.2% had only completed primary education, and 88.8% were aware of AMR and AMU. Antimicrobial use and AMR were not comprehended by 58.8% of respondents. A significant number of respondents mistakenly believed that antibiotics were effective against viral infections. Additionally, 76.7% of respondents reported consuming animal-derived products at home from animals treated with over-the-counter medications without observing the recommended withdrawal periods, even though 75% of respondents were aware of the antibiotic withdrawal periods for ill animals. In the present study, 47.9% of respondents reported giving antibiotics to their animals in the past month, primarily for illness prevention or treatment. There was a significant association across KAP scores and gender, education, income, and farming experience. Although livestock owners have adequate knowledge and positive attitudes toward antibiotic use, many still engage in practices that may contribute to AMR.
Keywords: Antimicrobial use, Antimicrobial resistance, Drug residue, Livestock owner, Veterinary

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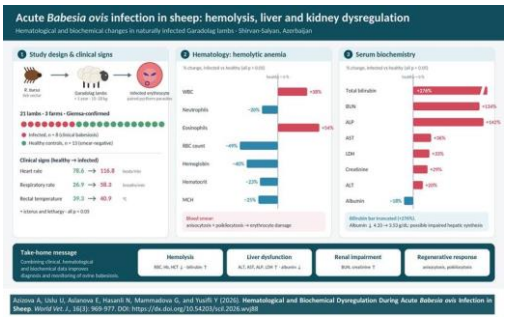
Research Paper

Hematological and Biochemical Dysregulation During Acute *Babesia ovis* Infection in Sheep

Azizova A, Uslu U, Aslanova E, Hasanli N, Mammadova G, and Yusifli Y.

World Vet. J. 16(3): 969-977, 2026; pii:S232245682600088-16

DOI: https://dx.doi.org/10.54203/scil.2026.wvj88



ABSTRACT: Babesiosis, caused by *Babesia ovis*, is a significant tick-borne disease affecting small ruminants, characterized by hemolytic anemia, liver dysfunction, and metabolic imbalances. The current study aimed to investigate the clinical, hematological, and biochemical alterations linked to naturally occurring ovine babesiosis in the Shirvan-Salyan region of Azerbaijan. A total of 21 Garadolag-breed lambs, comprising 11 females and 10 males, all under 1 year old and weighing 15-18 kg, were purposively sampled from three farms. The present study included animals exhibiting clinical signs such as hyperthermia and anemia, as well as healthy animals from the same flocks for comparison. A total of 21 Garadolag sheep were examined, consisting of eight with severe clinical signs of babesiosis and thirteen that appeared healthy. Because clinical signs were observed in the flock, all animals underwent a thorough examination. Babesiosis was confirmed by microscopic examination of Giemsa-stained blood smears, and hematological as well as serum biochemical parameters were determined using automated analyzers. The infected sheep exhibited significant reductions in red blood cell count, hemoglobin concentration, hematocrit, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration, alongside leukocytosis, eosinophilia, and monocytosis, indicating severe hemolytic anemia. Blood smear examination revealed anisocytosis and poikilocytosis, indicating erythrocyte destruction and a regenerative response. Serum biochemical evaluation revealed elevated levels of bilirubin, blood urea nitrogen, creatinine, total cholesterol, and triglycerides, as well as increased levels of alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, and lactate dehydrogenase. Conversely, the levels of total protein, albumin, and globulin decreased, indicating compromised liver synthetic function and changes in protein metabolism. The present study demonstrated that acute *Babesia ovis* infection in Garadolag lambs was characterized by distinct clinical signs, including primarily hyperthermia, icterus, and significant parasitemia, alongside specific systemic disturbances.
Keywords: *Babesia ovis*, Biochemical parameter, Hemolytic anemia, Liver dysfunction, Metabolic disorder

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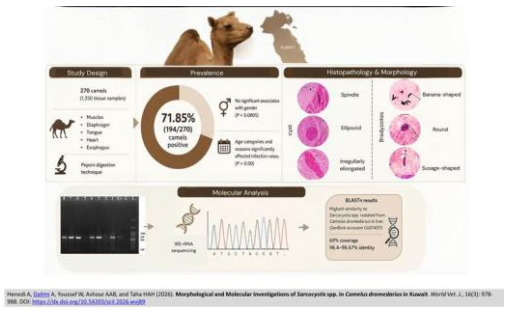
Research Paper

Morphological and Molecular Investigations of *Sarcocystis* spp. in *Camelus dromedarius* in Kuwait

Henedi A, Dalimi A, Youssef W, Ashour AAB, and Taha HAH.

World Vet. J. 16(3): 978-988, 2026; pii:S232245682600089-16

DOI: https://dx.doi.org/10.54203/scil.2026.wvj89



ABSTRACT: Sarcocystosis is a disease caused by *Sarcocystis* parasites, which are widely distributed among mammals, birds, and reptiles and may cause myositis and encephalitis. The present study aimed to investigate the prevalence, morphological and histopathological characteristics, and molecular identification of *Sarcocystis* infection in dromedary camels (*Camelus dromedarius*) in Kuwait using pepsin digestion, histopathological examination, and molecular characterization based on the 18S rRNA gene. A total of 1,350 tissue samples from muscles, diaphragm, tongue, heart, and esophagus were collected from 270 camels slaughtered in Kuwait. The camels comprised 214 males and 56 females and were categorized into three age groups, including younger than 8 months (n = 99), between 8 months and 3 years old (n = 113), and older than 3 years old (n = 58). Microscopic examination revealed *Sarcocystis* infection in 194 of 270 camels (71.85%). Infection was not significantly associated with sex, whereas age and season were significantly associated with *Sarcocystis* prevalence. *Sarcocystis* prevalence was 63.3% in muscle, 63.0% in diaphragm, 62.2% in tongue, 62.0% in heart, and 56.3% in esophagus. Histopathological examination revealed three cyst morphologies, including spindle-shaped, ellipsoidal, and irregularly elongated. Pepsin digestion revealed three distinct bradyzoite morphologies, including banana-shaped, sausage-shaped, and round forms. Five tissue samples were selected for molecular analysis using 18S rRNA sequencing. The BLASTn analysis demonstrated the highest similarity to *Sarcocystis* spp. isolated from *Camelus dromedarius* in Iran (GenBank accession no. GU074011), with 69% query coverage and a sequence identity of 98.4-98.67%. The present study reported a high prevalence of *Sarcocystis* infection (71.85%) among dromedary camels in Kuwait, with infection rates significantly influenced by age and season. Three distinct cyst and bradyzoite morphologies, along with molecular data showing high sequence similarity to Iranian dromedary isolates, strongly suggested that *Sarcocystis* infections are widespread and diverse among camel populations in Kuwait.
Keywords: *Camelus dromedarius,* Molecular analysis, *Sarcocystis* spp., Season

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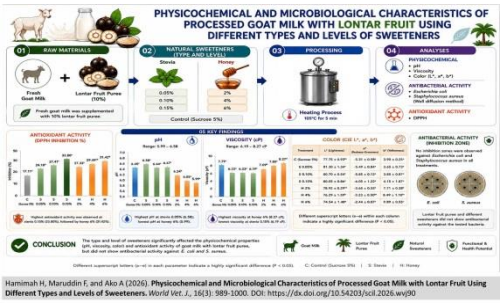
Research Paper

Physicochemical and Microbiological Characteristics of Processed Goat Milk with Lontar Fruit Using Different Types and Levels of Sweeteners

Hamimah H, Maruddin F, and Ako A.

World Vet. J. 16(3): 989-1000, 2026; pii:S232245682600090-16

DOI: https://dx.doi.org/10.54203/scil.2026.wvj90



ABSTRACT: Fresh goat milk is perishable and has a distinctive flavor that may reduce consumer acceptance. Incorporating lontar fruit and sweeteners such as honey and stevia may improve its physicochemical properties. The present study aimed to investigate the effects of different sweetener types and concentrations on the characteristics of goat milk supplemented with lontar fruit puree. A completely randomized design was employed, comprising seven treatments, including a control group with 5% sucrose, honey at three concentrations (2%, 4%, 6%), and stevia at three concentrations (0.05%, 0.1%, 0.15%), each with four replicates. Antioxidant activity, antibacterial activity, degree of acidity (pH), viscosity, and color values, including brightness (L*), redness (a*), and yellowness (b*), were evaluated. Honey-containing treatments demonstrated higher viscosity and lower pH than stevia-containing treatments, while the highest antioxidant activity was observed in the 0.15% stevia treatment (33.80%). No microbiological inhibition was observed against *Escherichia coli* and *Staphylococcus aureus*. The inclusion of honey in goat milk supplemented with lontar fruit puree resulted in higher viscosity than treatments containing stevia. Formulations with milk and honey had a lower pH than those with stevia. The 0.05% stevia treatment had the highest L* value (brightest color) and a negative a* value (a greener hue), while honey increased the intensity of red and yellow coloration with increasing concentration. The 0.15% stevia treatment demonstrated the highest antioxidant activity and favorable physicochemical characteristics.
Keywords: Goat milk, Lontar fruit, Microbiological characteristic, Physicochemical characteristic, Sweetener

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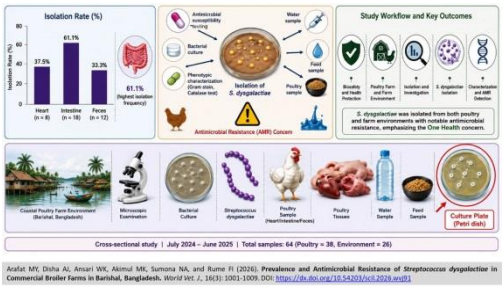
Research Paper

Prevalence and Antimicrobial Resistance of *Streptococcus dysgalactiae* in Commercial Broiler Farms in Barishal, Bangladesh

Arafat MY, Disha AJ, Ansari WK, Akimul MK, Sumona NA, and Rume FI.

World Vet. J. 16(3): 1001-1009, 2026; pii:S232245682600091-16

DOI: https://dx.doi.org/10.54203/scil.2026.wvj91



ABSTRACT: *Streptococcus dysgalactiae* (*S. dysgalactiae*) is a significant

pathogen affecting veterinary and public health. However, data on its presence and antimicrobial resistance in poultry production in Bangladesh are scarce. The present study aimed to investigate the prevalence, identification methods, and antibiotic susceptibility profiles of *S. dysgalactiae* in poultry, water, and feed samples collected from poultry farms in the coastal regions of Barishal, Bangladesh. A total of 64 samples were randomly collected, including poultry samples (heart, intestine, feces) and farm-associated sources (water and feed). These samples were analyzed using conventional phenotypic methods, including culture characteristics, hemolytic activity on blood agar, Gram staining, and catalase testing. Antimicrobial susceptibility was determined by the Kirby-Bauer disk diffusion method. Molecular confirmation by polymerase chain reaction (PCR) was not performed. *S. dysgalactiae* was found in 26 (40.62%) samples, with a significantly higher occurrence in poultry samples (52.63%) compared to environmental samples (23.08%). Among poultry samples, intestinal specimens exhibited the highest isolation frequency. All isolates were resistant to ampicillin and penicillin, while moderate resistance was observed to amoxicillin, azithromycin, streptomycin, doxycycline, and oxytetracycline. Comparatively lower resistance rates were detected for ciprofloxacin, levofloxacin, and gentamicin. Multidrug resistance was identified among isolates collected from poultry and environmental samples. The current findings demonstrated the presence of multidrug-resistant *S. dysgalactiae* in poultry production systems in coastal Barishal and provided baseline epidemiological data for Bangladesh. The study highlighted the need for prudent antimicrobial use, stronger farm biosecurity, and continuous surveillance.
Keywords: Antibiotic resistance, Broiler chicken, Poultry, Prevalence, *Streptococcus dysgalactiae*

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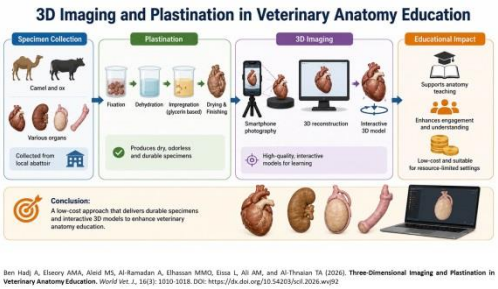
Research Paper

Three-Dimensional Imaging and Plastination in Veterinary Anatomy Education

Ben Hadj A, Elseory AMA, Aleid MS, Al-Ramadan A, Elhassan MMO, Eissa L, Ali AM, and Al-Thnaian TA.

World Vet. J. 16(3): 1010-1018, 2026; pii:S232245682600092-16

DOI: https://dx.doi.org/10.54203/scil.2026.wvj92



ABSTRACT: Veterinary anatomy plays a crucial role in veterinary education. However, cadaveric dissection encounters significant challenges such as high costs, ethical concerns, and limited accessibility, particularly in developing countries. The present study aimed to develop supplementary, cost-effective educational tools using smartphone-based three-dimensional (3D) imaging and a modified Elnady plastination technique applied to ox and camel organs to enhance gross anatomy teaching. Digital photogrammetry was employed to create 3D models of the axis, skull, femur, lumbar vertebrae, and soft-tissue specimens of the head, including the nasal and oral cavities of an adult bull, utilizing a smartphone camera. Subsequently, the 3D models were processed and refined using the KIRI Engine 3D Scanner application. Bull specimens were obtained from the Anatomy Laboratory at the College of Veterinary Medicine, King Faisal University, Al-Ahsa, Saudi Arabia, from the existing collection. Meanwhile, anatomical specimens from five adult male camels (5 hearts, 10 kidneys, 5 penises, and 5 testes) and six adult bulls (6 testes) were plastinated using a modified version of the Elnady protocol to create long-lasting teaching tools. The 3D imaging produced precise, high-resolution, and interactive models of the ox skeleton, which clearly exhibited anatomical structures and spatial relationships with adjacent bones. The ox skeletal and soft-tissue 3D models demonstrated excellent detail, allowing clear visualization of anatomical structures. Plastinated specimens of the adult male camel preserved their anatomical integrity, exhibited minimal shrinkage, had adequate structural clarity, remained dry, odorless, and suitable for repeated handling. Among the ox specimens, shrinkage was observed in the testicular parenchyma following plastination. Using 3D imaging alongside plastination enhanced visual and tactile learning. The current findings indicated that smartphone-based 3D photogrammetry and the modified Elnady plastination technique offered a practical, scalable approach to modernizing anatomy teaching, especially in limited-resource settings.
Keywords: Anatomy, Education, Ox, Plastination, Veterinary, 3D imaging

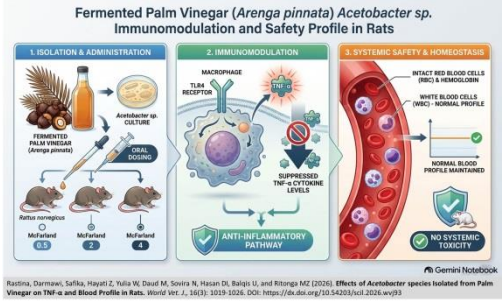
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Research Paper

Effects of *Acetobacter* species Isolated from Palm Vinegar on TNF-α and Blood Profile in Rats

Rastina, Darmawi, Safika, Hayati Z, Yulia W, Daud M, Sovira N, Hasan DI, Balqis U, and Ritonga MZ.

World Vet. J. 16(3): 1019-1026, 2026; pii:S232245682600093-16



DOI: https://dx.doi.org/10.54203/scil.2026.wvj93

ABSTRACT: Palm vinegar (*Arenga pinnata*), a traditional fermented product, contains acetic acid bacteria, particularly *Acetobacter* sp., which may serve as a natural immunomodulatory agent. The current study aimed to evaluate the effects of *Acetobacter* sp. isolated from palm vinegar on the immune response of white rats. A total of 24 male rats, aged 6-8 weeks and weighing 150-200 g, were randomly assigned to four groups of six rats each. The study included a control group that received normal saline and three treatment groups that received McFarland at doses of 0.5 (1.5×10^8 CFU/mL), 2 (6.0×10^8 CFU/mL), and 4 (1.2×10^9 CFU/mL) orally over 21 days. *Acetobacter* sp. was administered once a day for 14 days, and effects were monitored through blood samples collected weekly. Serum samples were collected during the first and second weeks of observation to assess tumor necrosis factor-alpha (TNFα) levels by enzyme-linked immunosorbent assay (ELISA) and for hematological examination. The results showed no significant difference in TNF-α levels between the first and second weeks. In the first week, mean TNF-α decreased in a dose-dependent manner, with the lowest value in the McFarland 4 group. During the second week of observation, measured parameters fluctuated across the treatment groups. Levels in the control group and McFarland 2 increased, while the McFarland 0.5 and 4 groups continued to decrease. Red blood cell (RBC) and WBC analysis showed no significant differences between observation times and between treatment groups. Descriptively, in the first week, the control group had the highest score and McFarland's 0.5 the lowest, but the values at doses 2 and 4 remained within the normal physiological range. In the second week, most groups showed an upward trend, except McFarland 0.5, which remained low. It can be concluded that *Acetobacter* sp. isolated from palm vinegar is effective as an immunomodulatory agent through a decrease in TNF-α levels. The stability of RBC and WBC parameters within normal limits confirms the safety of these bacteria, making them functional probiotic candidates that do not trigger systemic inflammation or blood balance disorders.
Keywords: *Acetobacter*, Palm vinegar, Probiotic, Tumor necrosis factor-alpha

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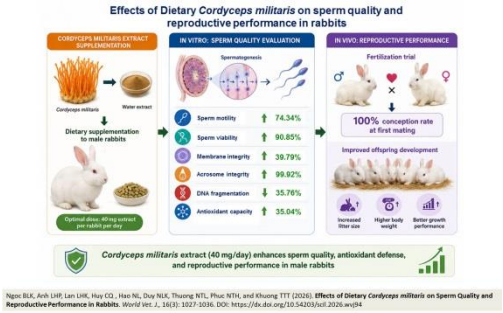
Research Paper

Effects of Dietary *Cordyceps militaris* on Sperm Quality and Reproductive Performance in Rabbits

Ngoc BLK, Anh LHP, Lan LHK, Huy CQ , Hao NL, Duy NLK, Thuong NTL, Phuc NTH, and Khuong TTT.

World Vet. J. 16(3): 1027-1036, 2026; pii:S232245682600094-16

DOI: https://dx.doi.org/10.54203/scil.2026.wvj94



ABSTRACT: *Cordyceps militaris* is a medicinal mushroom rich in bioactive compounds and has been investigated for its potential antioxidant properties. The present study aimed to evaluate the effects of dietary supplementation with *Cordyceps militaris* extract on semen quality and reproductive performance in male rabbits. A total of 15 adult male rabbits, with an average body weight of 3.56 ± 0.34 kg, were randomly assigned to five treatments receiving 0 (control), 20, 40, 80 and 160 mg of *Cordyceps militaris* extract daily for 14 weeks. Semen was collected at weeks 6, 8, 10, 12, and 14, with an average volume of 0.4 ± 0.1 mL per collection, and evaluated for sperm viability, motility, acrosome and membrane integrity, DNA fragmentation, and antioxidant capacity. A total of 15 adult female rabbits, with an average body weight of 3.37 ± 0.26 kg, were used for mating and were managed under the same experimental conditions. After eight weeks of supplementation, treated males were naturally mated with female rabbits to evaluate fertility and litter performance. Compared with the control group, *Cordyceps militaris* supplementation improved sperm motility, viability, membrane integrity, acrosome integrity, and antioxidant capacity, while reducing DNA fragmentation. The 40 mg/day treatment showed the most favourable overall effects on semen quality across the evaluation period. At week 8, rabbits receiving 40 mg/day showed higher sperm motility (74.34%), viability (90.85%), acrosome integrity (99.92%), membrane integrity (39.79%), and seminal antioxidant capacity (35.04%), together with lower DNA fragmentation (35.76%), compared with the control group. The 40 mg/day group showed a significantly higher pregnancy rate at first mating than the control group, reaching 100%. Rabbits that received 40 mg/day achieved a 100% first-mating pregnancy rate, while litter traits and offspring growth did not differ significantly among treatments. Overall, dietary supplementation with *Cordyceps militaris* improved semen quality and reproductive performance in male rabbits, with 40 mg/day providing the most favourable overall response among the tested supplementation levels.
Keywords: Antioxidant capacity, Dietary supplementation, DNA fragmentation, Sperm, Reproductive performance

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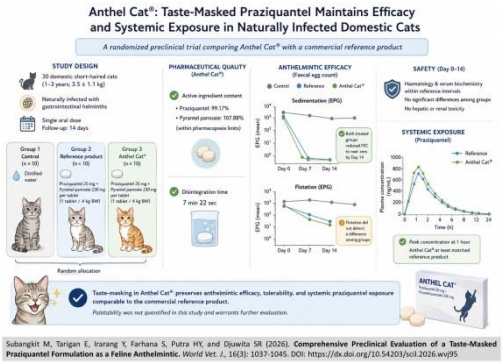
Research Paper

Comprehensive Preclinical Evaluation of a Taste-Masked Praziquantel Formulation as a Feline Anthelmintic

Subangkit M, Tarigan E, Irarang Y, Farhana S, Putra HY, and Djuwita SR.

World Vet. J. 16(3): 1037-1045, 2026; pii:S232245682600095-16

DOI: https://dx.doi.org/10.54203/scil.2026.wvj95



Subangkit M, Tarigan E, Irarang Y, Farhana S, Putra HY, and Djuwita SR (2026). **Comprehensive Preclinical Evaluation of a Taste-Masked Praziquantel Formulation as a Feline Anthelmintic.** *World Vet. J.*, 16(3): 1037-1045. DOI: https://dx.doi.org/10.54203/scil.2026.wvj95

ABSTRACT: Helminthiasis is common in domestic cats, compromising their welfare and posing a zoonotic risk. Praziquantel is a highly effective agent against cestodes; however, its bitter taste may cause hypersalivation and resistance to oral administration. The present randomized, controlled trial aimed to evaluate the pharmaceutical qualities, anthelmintic efficacy, tolerability, and systemic exposure to praziquantel (Anthel Cat), formulated with taste-masking and solubilizing excipients, compared with a commercially available product. A total of 30 domestic short-haired cats (*Felis catus*), aged 1-3 years and weighing 3.5 ± 1.1 kilograms (kg), comprising 20 males and 10 females, all of which had naturally acquired gastrointestinal helminth infection confirmed by fecal examination, were randomly allocated into three groups of ten. The control group received distilled water, the commercial group received a commercially available product, and the Anthel group received Anthel Cat tablets. Both active treatments contained 20 mg of praziquantel and 230 mg of pyrantel pamoate per tablet and were administered at the recommended dose of 1 tablet per 4 kg of body weight, as a single oral dose. Cats were monitored for 14 days. Active ingredient content, disintegration time, fecal egg counts determined by flotation and sedimentation, hematology, serum biochemistry, taste acceptance, and plasma praziquantel concentration were assessed. Anthel Cat active ingredient contents were 107.88% of pyrantel pamoate and 99.17% of praziquantel, both within pharmacopoeial limits, with a mean disintegration time of 7 minutes 22 seconds. By day 14, fecal egg counts in both treated groups declined to near zero by sedimentation, whereas flotation did not demonstrate a difference among groups. Hematological and serum biochemical parameters remained within reference ranges and did not differ among groups, which indicated no hepatic or renal toxicity. Peak plasma praziquantel levels occurred 1 hour after dosing in both treated groups, and Anthel Cat did not differ from the commercial product. For taste acceptance, Anthel Cat produced no measurable saliva volume. Salivation duration was elevated in both treated groups. The result demonstrated that Anthel Cat has the same quality as the commercial product in pharmaceutical quality, anthelmintic efficacy, tolerability, and systemic exposure. However, limiting the salivary aversion response, particularly saliva volume.
Keywords: Anthelmintic, Cat, Efficacy, Praziquantel, Pyrantel pamoate

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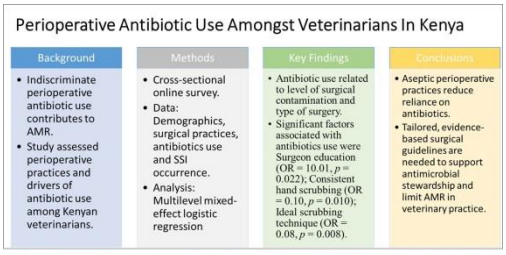
Research Paper

Antibiotic Use in Veterinary Surgery: Perioperative Practices and Associated Factors in Kenya

Moffat N, Edwin MW and Demesi MJ.

World Vet. J. 16(3): 1046-1059, 2026; pii:S232245682600096-16

DOI: https://dx.doi.org/10.54203/scil.2026.wvj96



Moffat N, Edwin MW and Demesi MJ (2026). **Antibiotic Use in Veterinary Surgery: Perioperative Practices and Associated Factors in Kenya.** *World Vet. J.*, 16(3): 1046-1059. DOI: https://dx.doi.org/10.54203/scil.2026.wvj96

ABSTRACT: Antibiotics are extensively used in animals undergoing surgery to prevent surgical site infections (SSIs) and improve surgery outcomes. The current study aimed to evaluate perioperative practices among Kenyan veterinarians and determine their correlation with perioperative antibiotic administration. The present study employed a cross-sectional survey design, in which a structured questionnaire was distributed to veterinarians in Kenya who were registered with the Kenya Veterinary Board. Detailed information on respondents' demographics, types of surgical procedures, perioperative practices, antibiotic usage patterns, and the incidence of SSI were collected. Eighty-three veterinarians participated in the study from January 1, 2023, to June 30, 2023, comprising 48 (57.8%) males and 35 (42.2%) females. The average number of surgeries performed monthly was 8.7, primarily elective and reconstructive procedures. Hand scrubbing before surgery was widely practiced (86.7%); However, only 42.2% adhered to recommended techniques, which include using a scrub solution followed by 70% ethyl alcohol disinfection. The primary sterilization methods were chemical immersion (49.4%) and autoclaving (37.5%). The majority of respondents (85.5%) considered antibiotic use optimal, primarily based on their personal experience (74.7%). Antibiotics were predominantly administered postoperatively (67.5%) and were highly likely to be used in contaminated, dirty, and emergency surgeries. Penicillins and cephalosporins were the most commonly used antibiotic classes perioperatively. Antibiotic usage was notably correlated with the surgeon's educational background, routine hand scrubbing, and adherence to ideal scrubbing protocols. The present study highlighted the existing perioperative antibiotic practices and identified main risk factors for antimicrobial misuse among veterinarians in Kenya.
Keywords: Antibiotic use, Infection, Antimicrobial resistance, Surgery, Veterinarian

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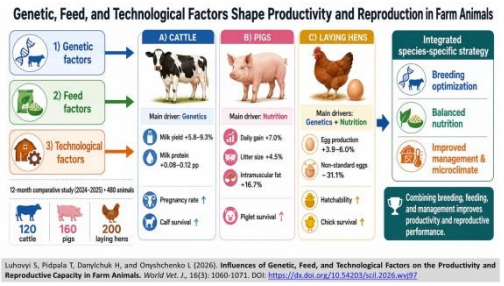
Review

Influences of Genetic, Feed, and Technological Factors on the Productivity and Reproductive Capacity in Farm Animals

Luhovyi S, Pidpala T, Danylchuk H, and Onyshchenko L.

World Vet. J. 16(3): 1060-1071, 2026; pii:S232245682600097-16

DOI: https://dx.doi.org/10.54203/scil.2026.wvj97



ABSTRACT: Productive and reproductive performance in farm animals is shaped by the combined effects of genetic potential, nutritional conditions, and technological management, although the relative importance of these factors differs among species. The present study aimed to identify the main genetic, nutritional, and technological factors influencing the productive and reproductive performance of farm animals and to assess their relative contributions and interactions. A 12-month controlled comparative study included 480 clinically healthy animals, including 120 cattle, 160 pigs, and 200 laying hens of the selected breeds. Within each species and breed, eligible animals were grouped according to age, sex, physiological status, and body condition score. Animals with similar values for these variables were then matched and non-randomly allocated to control and experimental groups to minimize pre-intervention differences between the groups. In cattle, the assessed parameters included milk yield, milk fat and protein content, liveweight gain, pregnancy rate after first insemination, calf birth weight, and calf survival to weaning. In pigs, average daily gain, slaughter yield, intramuscular fat content, litter size, piglet birth weight, and pre-weaning survival were evaluated. In laying hens, egg production, egg weight, the proportion of eggs with shell or shape defects, hatchability, and chick survival were assessed. Measurements were obtained from July 2024 to June 2025 from production records, electronic weighing, infrared milk analysis, ultrasonography, Soxhlet extraction, and automated egg analysis. In cattle, genetic factors, particularly breed and breeding improvement, had the greatest influence in increasing milk yield by 5.8-9.3% and milk protein content by 0.08-0.12 percentage points. In pigs, nutritional factors made the greatest contribution, including optimization of the dietary protein-to-energy ratio, which increased average daily gain by 7.0%, litter size by 4.5%, and intramuscular fat content by 16.7%. In laying hens, combined nutritional and genetic factors increased egg production by 3.9-6.0% and reduced the proportion of non-standard eggs by 31.1%. Technological optimization of microclimate, stocking density, reproductive management, and stress reduction stabilized performance and increased piglet survival by 1.5-2.8%. The findings support species-specific integration of breeding, nutritional, and management strategies.
Keywords: Breeding, Microclimatic condition, Optimization of feeding, Product quality, Reproduction indicator

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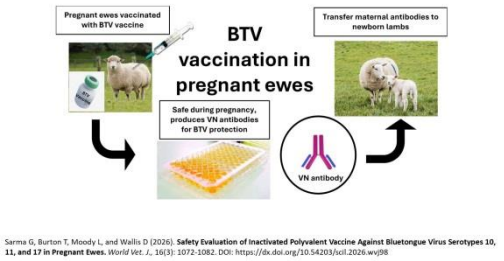
Research Paper

Safety Evaluation of Inactivated Polyvalent Vaccine Against Bluetongue Virus Serotypes 10, 11, and 17 in Pregnant Ewes

Sarma G, Burton T, Moody L, and Wallis D.

World Vet. J. 16(3): 1072-1082, 2026; pii:S232245682600098-16

DOI: https://dx.doi.org/10.54203/scil.2026.wvj98



ABSTRACT: Bluetongue is a noncontagious, vector-borne Orbivirus-induced disease of domestic and wild ruminants. Both live and inactivated monovalent vaccines are used to control bluetongue disease worldwide. The objective of this study was to evaluate the safety and post-vaccination seroconversion of an inactivated polyvalent bluetongue virus (BTV) vaccine containing serotypes 10, 11 and 17 in pregnant ewes. The vaccine was evaluated by vaccinating 66 pregnant ewes (33 for each study group). The ewes enrolled for this study represented 24 dairy, 30 meat, and 12 crossbreds and were aged 1-9 years. The average body weight of the animals ranged from 73 to 82 kg. Pregnant ewes representing all three trimesters of pregnancy were used in this study. Each animal was vaccinated with a 2.0 ml dose of the vaccine subcutaneously. All ewes were revaccinated similarly after one month. Local injection site reactions after both vaccinations, systemic and other adverse reactions, effects on pregnancy, fetal development, delivery of the lambs, and post-vaccination virus-neutralizing (VN) antibody titers were monitored after each vaccination. Mild to moderate injection site reactions such as swelling or lump formation were seen in 29 injection sites (22.0%). All injection site reactions were resolved completely within 2 – 7 weeks without further complications and treatment. Mild to moderate post-vaccination systemic reactions such as anorexia/lethargy/laminitis were seen only in two ewes, which lasted for four days. Bluetongue vaccination in the pregnant ewes did not affect the pregnancy, fetal development, and delivery of the lambs. There were no premature abortions or any form of teratogenic deformities in any of the lambs delivered. The vaccine in the pregnant ewes induced post-vaccination VN antibody titers against all three serotypes of BTV. Lambs born to the vaccinated ewes also showed a protective level of VN antibody titers against all three serotypes of

the virus following passive transfer of maternal antibodies. The levels of VN antibody titers seen in the ewes and lambs were considered sufficient to provide protection against field virus challenge. The results of the current study indicated that inactivated polyvalent bluetongue virus vaccine had no adverse effects on pregnancy and lamb delivery.
Keywords: Bluetongue, Inactivated vaccine, Maternal antibody, Pregnant ewe, Safety, Virus-neutralizing antibody

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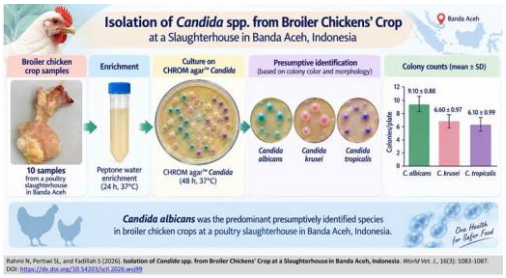
Short Communication

Isolation of *Candida* spp. from Broiler Chickens' Crop at a Slaughterhouse in Banda Aceh, Indonesia

Rahmi N, Pertiwi SL, and Fadillah S.

World Vet. J. 16(3): 1083-1087, 2026; pii:S232245682600099-16

DOI: https://dx.doi.org/10.54203/scil.2026.wvj99



ABSTRACT: *Candida* spp. are opportunistic yeasts commonly found in the gastrointestinal tract of poultry. The present study aimed to determine the distribution and compare the colony counts of presumptively identified *Candida* spp. recovered from the crop of broiler chickens at a poultry slaughterhouse in Banda Aceh, Indonesia. Ten crop samples collected from individual broiler chickens were enriched in peptone water and subsequently cultured on CHROM agar™ Candida. Presumptive identification of *Candida* spp. was based on characteristic colony color and morphology. The results revealed marked differences in colony counts among the three presumptively identified *Candida* species. *Candida albicans* (*C. albicans*) exhibited the highest mean colony count (9.10 ± 0.88 colonies/plate), followed by *C. krusei* (6.60 ± 0.97 colonies/plate) and *C. tropicalis* (6.10 ± 0.99 colonies/plate). Pairwise comparisons revealed that *C. albicans* had significantly higher colony counts than both *C. krusei* and *C. tropicalis*, whereas no significant difference was observed between *C. krusei* and *C. tropicalis*. The present findings indicated that *C. albicans* was the predominant presumptively identified *Candida* species in broiler chicken crops and highlight the need to further investigate their occurrence, epidemiology, and potential public health implications in poultry production and processing systems.
Keywords: Broiler chicken, *Candida albicans*, *Candida krusei*, *Candida tropicalis*, Crop

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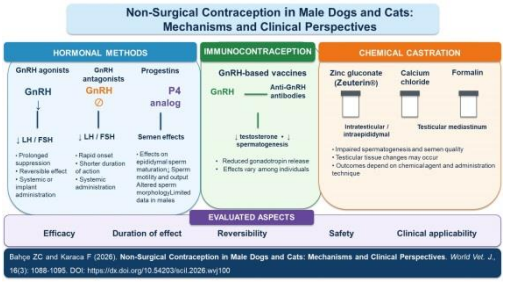
Review

Non-Surgical Contraception in Male Dogs and Cats: Mechanisms and Clinical Perspectives

Bahçe ZC and Karaca F.

World Vet. J. 16(3): 1088-1095, 2026; pii:S232245682600100-16

DOI: https://dx.doi.org/10.54203/scil.2026.wvj100



ABSTRACT: Surgical sterilization is widely used for population control and reproductive management in dogs and cats; however, its irreversible nature has increased interest in non-surgical contraceptive methods. Hormonal approaches, including progestins, GnRH agonists, and GnRH antagonists, as well as immunocontraceptive and chemical methods, have been investigated for fertility control in male dogs and cats. The present review summarizes the current literature on non-surgical contraceptive methods in male dogs and cats, focusing on their mechanisms of action, effectiveness, reversibility, duration of effect, and reported side effects. Non-surgical approaches include GnRH agonists and antagonists administered systemically or via implants, immunocontraceptive methods, and chemical castration agents administered by intratesticular or intraepididymal injection. GnRH agonists have been reported to provide prolonged and reversible suppression of reproductive function, whereas GnRH antagonists generally have a more rapid onset but a shorter duration of action. Immunocontraceptive methods can suppress testosterone concentrations and spermatogenesis, although their effects may vary among individuals. Chemical castration can impair spermatogenesis and semen quality, with outcomes depending on the chemical agent and administration technique. In conclusion, non-surgical contraceptive methods revealed potential for fertility control in male dogs and cats.
Keywords: Cat, Chemical castration, Dog, Gonadotropin-releasing hormone, Immunocontraception

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Review

An Update on Canine Parvovirus in Ecuador and Worldwide: Pathogenesis, Diagnosis, Epidemiology, Treatment, and Control

Huera EPR and Matamoros DFM.

World Vet. J. 16(3): 1096-1111, 2026; pii:S232245682600101-16

DOI: https://dx.doi.org/10.54203/scil.2026.wvj101



ABSTRACT: Canine parvovirus (CPV) remains one of the most relevant viral pathogens affecting domestic dogs worldwide, particularly in young and unvaccinated populations, due to its high environmental resistance and rapid transmission. The present study aimed to provide an updated and integrative overview of epidemiology, pathogenesis, diagnosis, and control of CPV through a systematic qualitative review. A comprehensive literature search was carried out using major academic databases, including PubMed, SciELO, and Scopus, considering studies published between 2020 and 2026. A total of 312 articles were initially identified, of which 70 met the inclusion criteria and were selected for article review. The results confirmed the global circulation of multiple variants (CPV-2a, CPV-2b, and CPV-2c), including the presence of CPV-2b in Ecuador, as well as the persistence of the virus in diverse environments and its impact on susceptible populations such as puppies between 6 weeks and 6 months of age and susceptibility, and some breeds such as Doberman Pinschers, Rottweilers, and American Pit Bull Terriers. Molecular diagnostic techniques, particularly PCR and qPCR, remain the most reliable tools for early detection. Despite advances in supportive therapy and emerging antiviral approaches, vaccination continues to represent the most effective preventive strategy. Continuous surveillance and improvements in diagnostic and immunization strategies are essential to reduce the impact of CPV infections.
Keywords: Canine parvovirus, Genoma, Pathogenesis, Transmission

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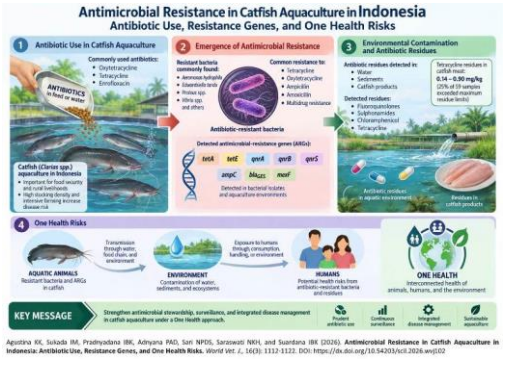
Review

Antimicrobial Resistance in Catfish Aquaculture in Indonesia: Antibiotic Use, Resistance Genes, and One Health Risks

Agustina KK, Sukada IM, Pradnyadana IBK, Adnyana PAD, Sari NPDS, Saraswati NKH, and Suardana IBK.

World Vet. J. 16(3): 1112-1122, 2026; pii:S232245682600102-16

DOI: https://dx.doi.org/10.54203/scil.2026.wvj102



ABSTRACT: Catfish (*Clarias* spp.) aquaculture plays a crucial role in food security and rural livelihoods in Indonesia. Inappropriate antimicrobial use (AMU) can increase antimicrobial resistance (AMR), posing risks to aquaculture, environmental health, and food safety. The present study aimed to review AMR associated with catfish aquaculture in Indonesia within a One Health framework. A review was carried out according to structured literature review guidelines. Relevant peer-reviewed articles published between 2020 and 2026 were found in Scopus, Web of Science, PubMed, ScienceDirect, and Google Scholar. The searches employed specific combinations of keywords concerning catfish aquaculture, antimicrobial usage, antimicrobial resistance, and Indonesia. Data on AMU, resistant bacteria, antimicrobial-resistance genes (ARGs), and antibiotic residues were extracted from the included studies and categorized into these four subjects descriptively. Findings across studies were compared to identify commonly reported antimicrobials, patterns of bacterial resistance, detected ARGs, and the occurrence of antibiotic residues. Due to differences in study designs, sampling methods, bacterial species, and antimicrobial susceptibility testing methods, the findings were synthesized narratively rather than pooled statistically. The current findings indicated that oxytetracycline, tetracycline, and enrofloxacin were the most commonly used antibiotics. Resistant bacteria, particularly *Aeromonas hydrophila*, frequently exhibited resistance to tetracycline, oxytetracycline, ampicillin, and amoxicillin. Other pathogens, including *Edwardsiella tarda* and *Proteus* spp., also demonstrated multidrug resistance to tetracycline, oxytetracycline, ampicillin, and amoxicillin. The ARGs such as *tetA*, *tetE*, *qnrA*, *qnrB*, *qnrS*, *ampC*, *blaGES*, and *mexF* were detected in bacterial isolates and aquaculture environments. Antibiotic residues, including fluoroquinolones, sulphonamides, chloramphenicol, and tetracycline, were detected in aquatic environments and catfish products. Oxytetracycline residues ranging from 0.14 to 0.90 mg/kg were detected in catfish meat, with 25% of 59 samples exceeding the reported

maximum residue limits. The current findings suggested that catfish aquaculture in Indonesia may contribute to the emergence and spread of AMR. The co-occurrence of antibiotic use, resistant bacteria, ARGs, and environmental contamination highlighted the need for strengthened antimicrobial stewardship, surveillance, and integrated disease management under a One Health approach.
Keywords: Antimicrobial resistance, Catfish aquaculture, Gene, One Health

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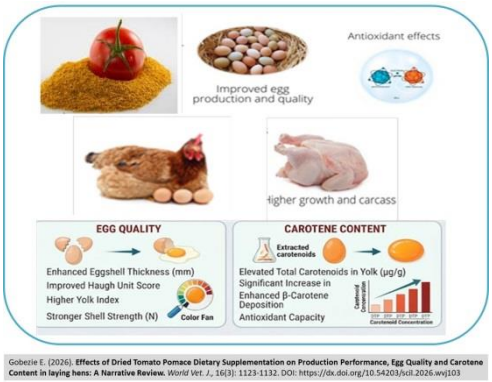
Review

Effects of Dried Tomato Pomace Dietary Supplementation on Production Performance, Egg Quality and Carotene Content in laying hens: A Narrative Review

Gobezie E.

World Vet. J. 16(3): 1123-1132, 2026; pii:S232245682600103-16

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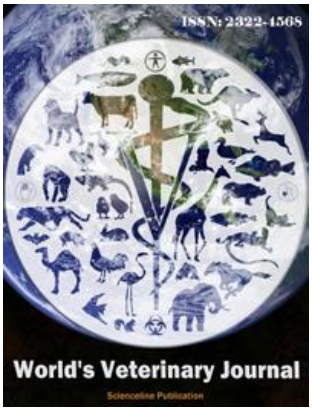


ABSTRACT: Tomato pomace is a rich source of protein, fiber, carbohydrates, and carotenoids for layer hens. Adding a moderate level of dried tomato pomace to laying hen diets is considered safe and has no adverse effects on performance metrics such as egg production, feed intake, egg weight, or shell thickness. The present study aimed to evaluate the effects of nutritional supplementation with dried tomato pomace on the productivity, egg quality, and carotene content of laying hens. A narrative literature search was conducted using scientific databases, including Scopus, PubMed, and Web of Science. The search primarily focused on peer-reviewed studies published from 2019 to 2026. Relevant previous studies were included to provide important background and context for the current study. Although tomatoes are rich in nutrients, such as carotenoids, fiber, vitamins, and bioactive compounds, processing industries often discard tomato pomace as waste. Improper disposal of tomato waste also poses environmental issues. Due to its nutritional content, dried tomato pomace could serve as a dietary supplement for chickens, especially in laying hens, where dietary carotenoids can influence egg yolk coloration and nutritional quality. Dietary tomato pomace, a useful delivery system for improving human health, positively affected lycopene deposition in egg yolk. Dried tomato pomace can be added to laying hens' diets at up to 5-12% (50-120 g/kg feed) to improve egg yolk color and carotenoid content without adversely affecting laying hens' productivity, while also lowering production costs and egg cholesterol content. Including dried tomato pomace at levels up to 12% (120 g/kg feed) can improve egg nutrition by increasing natural antioxidants such as lycopene and β-carotene, making it an economical and sustainable feed supplement for poultry.
Keywords: Carotene, Egg quality, Laying hen, Production performance, Tomato pomace

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Effects of Bee Bread on Paracetamol-Induced Intestinal Injury in Rats

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ORIGINAL ARTICLE
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ABSTRACT

Although paracetamol (PS) is widely used as a safe analgesic and antipyretic agent in both humans and animals at therapeutic doses, high-dose administration may cause damage not only to the liver and kidneys but also to the gastrointestinal system. The present study aimed to evaluate the protective effects of bee bread (BB) on intestinal integrity and inflammation in a paracetamol-induced intestinal injury model in Wistar rats using histopathological and immunohistochemical methods. The expression levels of the tight junction protein *zonula occludens-1* (*ZO-1*) and the inflammatory marker myeloperoxidase (MPO) were evaluated by immunohistochemistry. A total of 32 male rats (8-10 weeks old, 250-300 g) were randomly assigned to four groups (n = 8 per group), consisting of the control group, the BB group (750 mg/kg/day orally for 10 days), the PS group (1000 mg/kg administered as a single oral dose on day 10), and the PS plus BB group. The BB group exhibited normal intestinal histological architecture, preserved *ZO-1* expression, and low MPO immunoreactivity, comparable to those observed in the control group. In contrast, the PS group showed marked mucosal irregularities, villus degeneration, increased inflammatory cell infiltration, decreased *ZO-1* expression, and increased MPO immunoreactivity. Compared with the PS group, BB treatment alleviated paracetamol-induced intestinal injury, increased *ZO-1* expression, and reduced MPO immunoreactivity in the PS plus BB group. In conclusion, BB administration alone did not adversely affect intestinal morphology or the evaluated immunohistochemical markers and partially protected against paracetamol-induced intestinal injury by preserving intestinal barrier integrity and attenuating inflammatory responses.

Keywords: Bee bread, Histology, Paracetamol, *Zonula occludens-1*

INTRODUCTION

Paracetamol is a widely used antipyretic and analgesic agent and is considered safe at therapeutic doses (Jaeschke and Ramachandran, 2024). However, overdose may induce toxicity primarily affecting the liver, while the kidneys, gastrointestinal tract, and, in severe cases, the central nervous and cardiovascular systems may also be involved (Jaeschke and Ramachandran, 2024). During paracetamol metabolism, the reactive metabolite N-acetyl-p-benzoquinone imine (NAPQI) is generated, leading to glutathione depletion, mitochondrial oxidative stress, and subsequent cellular injury (Jaeschke and Ramachandran, 2024).

Emerging evidence indicates that paracetamol overdose may impair intestinal integrity by disrupting the epithelial barrier, increasing intestinal permeability, and promoting mucosal injury (Perananthan et al., 2024). The intestinal mucosa serves as a dynamic physical and immunological barrier that prevents the translocation of luminal microorganisms, toxins, and antigens into the systemic circulation (Horowitz et al., 2023). Intercellular tight junctions are essential for regulating paracellular permeability and preserving the integrity of the intestinal epithelial barrier (Arumugam et al., 2025). *Zonula occludens-1* (*ZO-1*) is a cytoplasmic scaffolding protein that anchors transmembrane tight junction proteins, including claudins and occludin, to the actin cytoskeleton, thereby ensuring tight junction assembly and epithelial barrier stability (Horowitz et al., 2023). In mice, experimental loss of intestinal epithelial *ZO-1* has been shown to modestly increase tight junction permeability and impair mucosal repair (Kuo et al., 2021).

Myeloperoxidase (MPO), a neutrophil-derived heme enzyme predominantly stored in neutrophil granules and released upon neutrophil activation, is widely recognized as a reliable biomarker of tissue inflammation and neutrophil infiltration (Cartwright et al., 2024; Clough et al., 2024). Furthermore, oxidative stress can increase tight junction permeability and alter the localization of tight junction proteins in intestinal epithelial cells, thereby impairing epithelial barrier function (Hasegawa et al., 2021).

Bee bread (BB) is a naturally fermented bee product containing abundant flavonols (quercetin, kaempferol, and isorhamnetin) together with phenolic acids including caffeic, ferulic, p-coumaric, gallic, and protocatechuic acids, all of which are recognized for their antioxidant and anti-inflammatory properties (Bakour et al., 2022). Simulated gastrointestinal digestion studies demonstrated that BB exhibits higher intestinal bioaccessibility of phenolic compounds than bee pollen, suggesting improved delivery of these bioactive molecules to the intestinal epithelium (Aylanc et al., 2021).

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Although direct in vivo evidence regarding the protective effects of BB against paracetamol-induced intestinal injury remains limited, its high intestinal bioaccessibility together with its well-documented antioxidant and anti-inflammatory properties provides a strong rationale for investigating its therapeutic potential (Aylanc et al., 2021; Bakour et al., 2022). Therefore, the present study aimed to investigate the potential protective effects of bee bread against paracetamol-induced duodenal injury in rats by evaluating histopathological changes and the immunohistochemical expression of *ZO-1* and MPO.

MATERIALS AND METHODS

Ethical approval

The present study was approved by the Local Animal Ethics Committee of Siirt University, Siirt, Türkiye (Approval No. 2026.03.23). All procedures involving tissue collection and histopathological and immunohistochemical evaluations were conducted in accordance with the approval granted by the committee and the institutional guidelines for the care and use of laboratory animals. No additional experimental animals were used, in accordance with the principles of the 3Rs (Replacement, Reduction, and Refinement).

Study design and tissue collection

The present study was a randomized controlled experiment using animals. A total of 32 male Wistar albino rats were randomly assigned to four experimental groups (8 per group), including a control, a BB group, paracetamol (PS group), and paracetamol with bee bread (PS + BB group). The control group received 0.5 mL sterile saline (0.9% NaCl) by oral gavage once daily for 10 consecutive days. The second group received 750 mg/kg/day of bee bread by oral gavage for 10 consecutive days (BB group). The BB, a naturally fermented bee-pollen product, was stored in its original sealed container at room temperature (approximately 22°C), protected from direct sunlight, according to the manufacturer's instructions until use. The selected dose was based on previous experimental studies demonstrating the antioxidant and anti-inflammatory efficacy of BB in rats (Suleiman et al., 2021; Bakour et al., 2022). In the third group, rats received sterile saline by oral gavage for 10 days, followed by a single oral administration of paracetamol (1000 mg/kg) on day 10 (PS group). This dose was selected because it is a well-established hepatotoxic dose in rats and has been widely used to induce acute oxidative tissue injury (Jaeschke and Ramachandran, 2024). In the fourth group, rats received BB (750 mg/kg/day) by oral gavage for 10 consecutive days; two hours after the final BB administration on day 10, a single oral dose of paracetamol (1000 mg/kg) was administered (PS + BB group). The 2-hour interval was chosen to ensure gastrointestinal absorption of BB prior to giving paracetamol, aligning with prophylactic pretreatment strategies used in experimental studies (Suleiman et al., 2021).

Animals were fasted for 12 hours before paracetamol administration and tissue collection, while water was provided *ad libitum*. Duodenal tissues were collected 24 hours after paracetamol administration for histopathological and immunohistochemical analyses. Throughout the experimental period, all animals had free access to tap water and a standard commercial pellet diet (Arden Feed Industry, Turkey).

Histopathological examination

Histopathological examination was performed using standard hematoxylin and eosin staining procedures as previously described by Bancroft and Gamble (2008). Paraffin sections were stained with Harris hematoxylin solution and alcoholic eosin Y solution (Ref. No. BS-009, Histomed, Türkiye) and examined using a light microscope (Olympus CX23, Japan). Histopathological assessment included evaluation of the overall architecture of the intestinal mucosa, epithelial integrity, villus morphology, inflammatory cell infiltration, and evidence of mucosal injury. Histopathological and immunohistochemical evaluations were performed by an expert investigator blinded to the experimental groups.

Immunohistochemical examination

Immunohistochemical examination was performed as previously described by Ramos-Vara and Miller (2014), with minor modifications. Paraffin-embedded tissue sections (4-6 μm thick) were placed on poly-L-lysine-coated glass slides. The sections were subsequently deparaffinized in xylene and rehydrated through graded ethanol solutions. Heat-induced antigen retrieval was carried out in ethylenediaminetetraacetic acid (EDTA) buffer. To eliminate endogenous peroxidase activity, the sections were incubated with 3% hydrogen peroxide, after which nonspecific protein binding was blocked using Ultra V Block. Sections were incubated overnight at +4°C with primary antibodies against MPO (STJA0010464, St John's Laboratory, UK; 1:500) and *ZO-1* (21773-1-AP, Proteintech, USA; 1:500). After washing with PBS, biotinylated secondary antibody and streptavidin–peroxidase were applied. Immunoreactivity was detected using 3,3′-diaminobenzidine (DAB) as the chromogenic substrate, after which the sections were counterstained with Harris

hematoxylin. The stained slides were subsequently evaluated and digitally photographed using a light microscope (Olympus CX23, Japan; Ramos-Vara and Miller, 2014). MPO immunoreactivity was used to assess intestinal inflammation, whereas *ZO-1* immunoreactivity was used to evaluate intestinal barrier integrity.

Statistical analysis

Since immunohistochemical scores represented ordinal data, results were expressed as median (minimum–maximum). Statistical analyses were performed using Jamovi software (Version 2.6.44). The normality of data distribution was assessed using the Shapiro–Wilk test. As the data were not normally distributed, differences among the four experimental groups were analyzed using the Kruskal–Wallis test. When a significant overall difference was detected, pairwise comparisons were performed using the Dwass–Steel–Critchlow–Fligner (DSCF) multiple comparison test. Data are presented as median (minimum–maximum), and a P-value less than 0.05 was considered statistically significant.

RESULTS

Histopathology

Histopathological examination demonstrated preserved duodenal architecture in the control and BB groups. Paracetamol administration resulted in marked intestinal mucosal injury, whereas BB treatment substantially attenuated these pathological changes. Although the PS plus BB group showed clear histological improvement compared with the PS group, the duodenal morphology was not completely restored to that observed in the control group (Figure 1).

Immunohistochemical results of myeloperoxidase

Low MPO immunoreactivity was observed in the control and BB groups, whereas the PS group exhibited markedly increased *MPO* expression. Post hoc DSCF analysis demonstrated significantly higher MPO immunoreactivity in the PS group than in the control ($p = 0.003$) and BB ($p = 0.003$) groups. Bee bread treatment significantly reduced MPO expression compared with the PS group (median: 2 [2-3] versus 4 [3-4]; $p = 0.018$). However, MPO immunoreactivity in the PS plus BB group remained significantly higher than that in the control ($p = 0.049$) and BB ($p = 0.049$) groups (Figure 2).

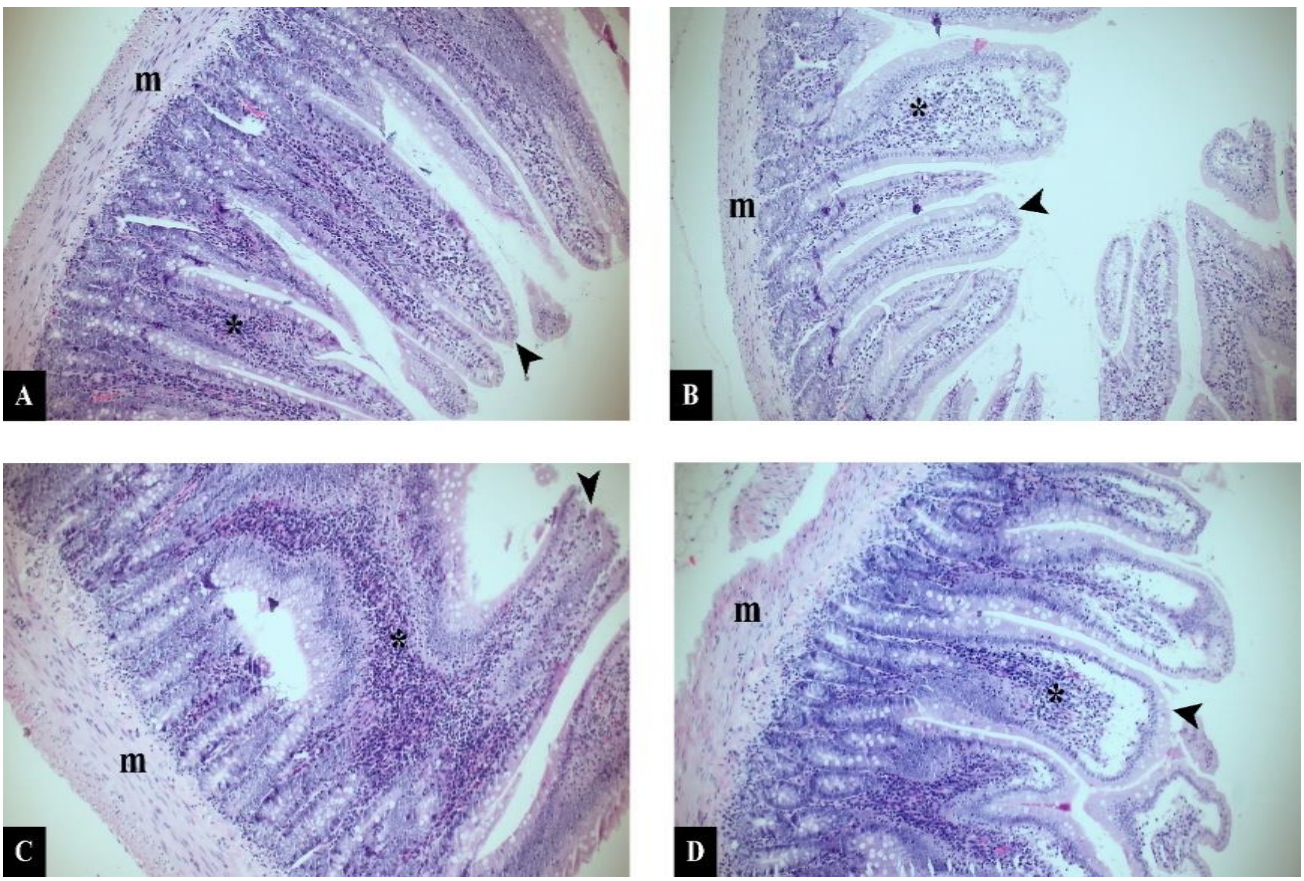


Figure 1. Representative hematoxylin and eosin (H&E)-stained sections of the duodenum in male rats. **A:** Control, **B:** Bee bread, **C:** Paracetamol, and **D:** Paracetamol plus bee bread. Black arrowheads: Epithelial alterations, *: Inflammatory cell infiltration, m: Muscularis layer, original magnification ×20. Scale bar: 100 µm.

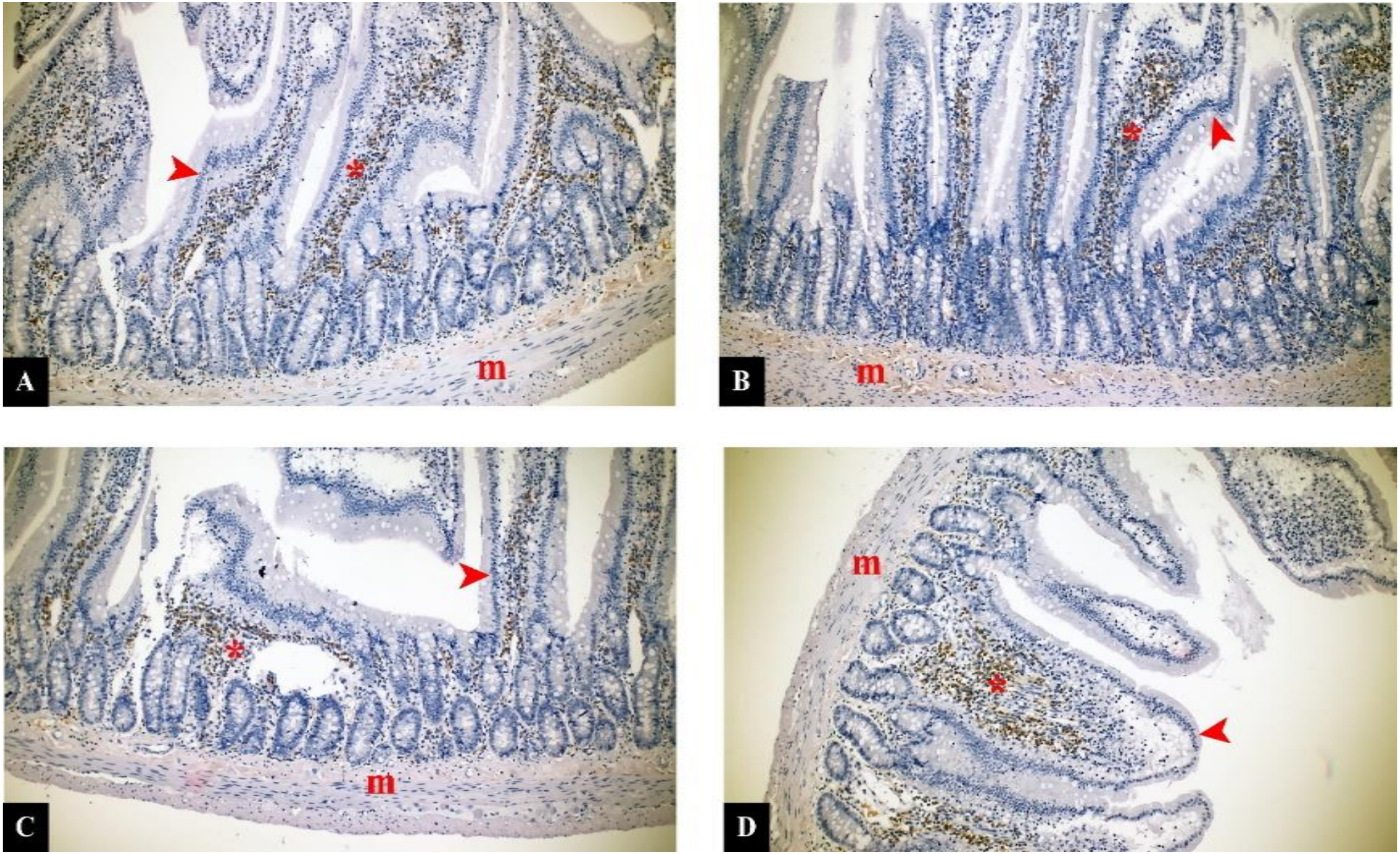


Figure 2. Immunohistochemical staining of myeloperoxidase in the duodenum of male rats. **A**: Control, **B**: Bee bread, **C**: Paracetamol, and **D**: Paracetamol plus bee bread. *: Inflammatory cell infiltration, m: Muscularis layer, DAB chromogen with Harris hematoxylin counterstaining, original magnification ×20. Scale bar: 100 μm.

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Immunohistochemical results of *Zonula occludens-1*

Strong and continuous membranous *ZO-1* immunoreactivity was observed along the villus epithelium in the control and BB groups. In contrast, the PS group exhibited weak and discontinuous epithelial staining. Overall differences among the experimental groups were statistically significant ($p = 0.002$). Post hoc DSCF analysis demonstrated significantly lower *ZO-1* immunoreactivity in the PS group than in the control ($p = 0.012$) and BB ($p = 0.012$) groups. Bee bread treatment significantly increased *ZO-1* immunoreactivity in the PS plus BB group compared with the PS group. No significant differences were detected between the PS plus BB group and either the control or BB groups (both $p = 0.941$; Figure 3).

Collectively, the findings of the present study indicated that BB attenuates paracetamol-induced intestinal injury and inflammation while partially restoring intestinal barrier integrity through reduced MPO expression and improved *ZO-1* immunoreactivity.

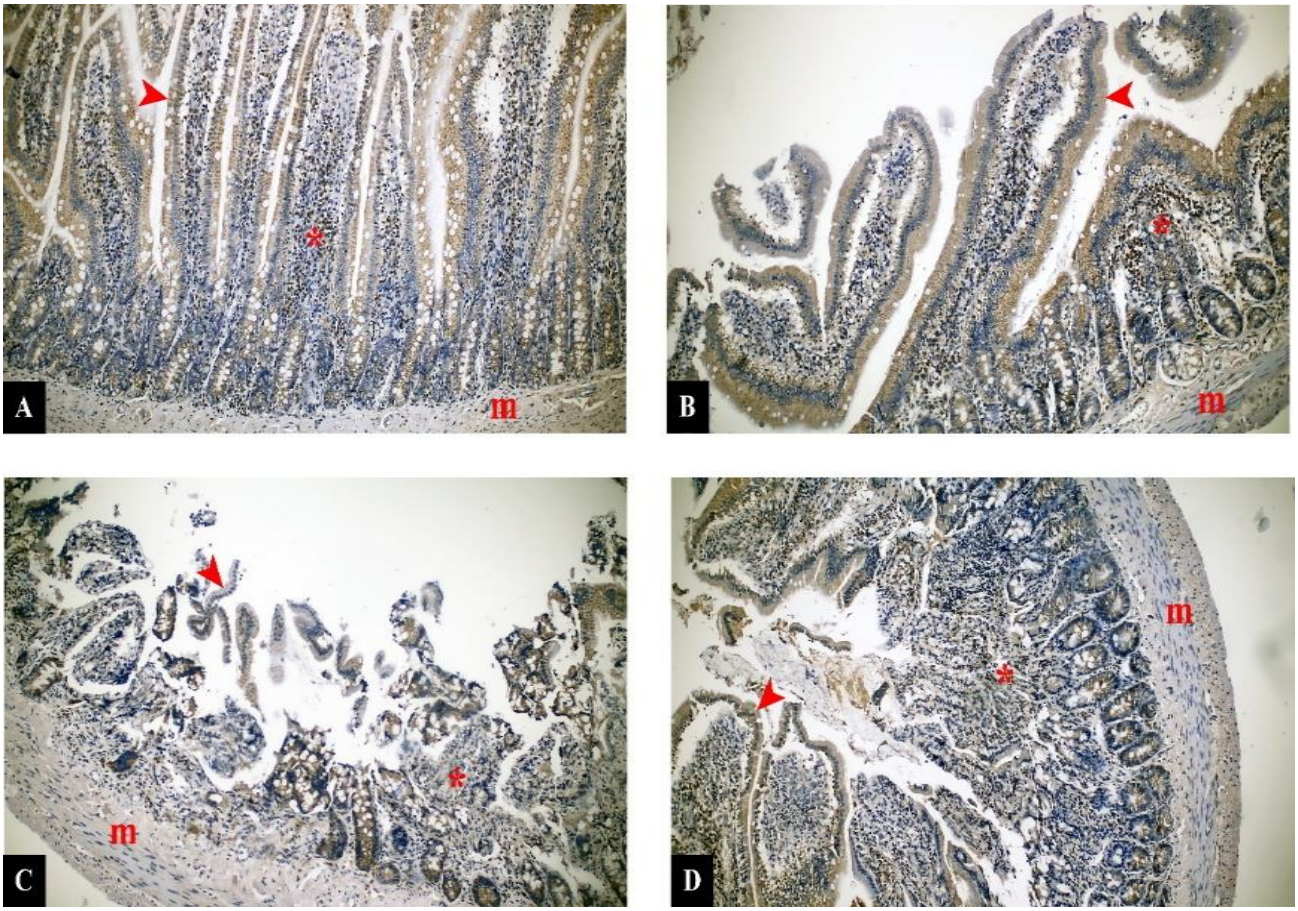


Figure 3. Immunohistochemical staining of *zonula occludens-1* (*ZO-1*) in the duodenum of male rats. **A**: Control, **B**: Bee bread, **C**: Paracetamol, **D**: Paracetamol plus bee bread. Arrowheads indicate *ZO-1*-positive epithelial cells; m: Muscularis layer. *: Inflammatory cell infiltration, DAB chromogen with Harris hematoxylin counterstaining. Original magnification ×20, Scale bar: 100 µm.

Table 1. Semi-quantitative myeloperoxidase and *zonula occludens-1* immunoreactivity scores in the duodenum of 8–10-week-old male rats

Parameter	C	BB	PS	PS + BB	P-value
MPO	1.5 (1-2)^{a}	2 (1-2)^{a}	4 (3-4)^{c}	2 (2-3)^{b}	< 0.001
ZO-1	3 (2-4)^{a}	3 (2-4)^{a}	1 (1-2)^{b}	2.5 (2-3)^{a}	0.002

C: Untreated control rats, BB: Bee bread-treated rats, PS: Paracetamol-treated rats, PS + BB: Paracetamol plus bee bread-treated rats, MPO: Myeloperoxidase, *ZO-1*: *Zonula occludens-1*. ^{abc}Different superscript letters in the same row were significantly different at $p < 0.05$.

DISCUSSION

In the present study, paracetamol administration produced marked duodenal injury characterized by villus degeneration, inflammatory cell infiltration, increased myeloperoxidase immunoreactivity, and reduced *ZO-1* expression. These findings are consistent with those of Alabbas et al. (2023), who found that acetaminophen induced intestinal injury, increased intestinal permeability, and reduced *ZO-1* expression in the colonic epithelium of mice. Furthermore, Alabbas et al. (2023) demonstrated bacterial translocation to the liver and spleen, emphasizing the role of intestinal barrier

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dysfunction in acetaminophen-related systemic toxicity. Unlike the current results indicating the duodenum as a direct target of paracetamol-induced injury, Alabbas et al. (2023) mainly concentrated on the colon.

The present findings were further supported by recent clinical evidence demonstrating intestinal epithelial injury following acute paracetamol overdose. Perananthan et al. (2024) demonstrated that patients with acute paracetamol overdose exhibited increased circulating biomarkers of enterocyte injury, indicating that intestinal epithelial damage also occurs in humans. Therefore, the decreased *ZO-1* expression observed in the present study provides histopathological and immunohistochemical evidence that complements these clinical observations.

The marked increase in MPO immunoreactivity in the PS group indicates enhanced neutrophil-associated inflammation within the duodenal mucosa. The present findings extend the observations of Alabbas et al. (2023) by demonstrating that acetaminophen-induced intestinal injury is not limited to the colon but also involves the proximal small intestine. Importantly, BB administration significantly reduced MPO immunoreactivity while restoring *ZO-1* expression toward control levels, suggesting attenuation of intestinal inflammation together with preservation of epithelial barrier integrity.

Paracetamol-induced intestinal injury shares several pathogenic mechanisms with paracetamol-induced hepatotoxicity, including oxidative stress, inflammatory responses, mitochondrial dysfunction, and disruption of the intestinal epithelial barrier (Alabbas et al., 2023; Jaeschke and Ramachandran, 2024; Perananthan et al., 2024). Following overdose, increased metabolism of paracetamol by cytochrome P450 enzymes leads to the excessive formation of the highly reactive metabolite N-acetyl-p-benzoquinone imine (NAPQI; McGill and Jaeschke, 2013; Jaeschke and Ramachandran, 2024). Although hepatocytes are the primary site of NAPQI generation, intestinal epithelial cells also express cytochrome P450 enzymes capable of producing this reactive metabolite, thereby contributing to local intestinal injury (Alabbas et al., 2023; Jaeschke and Ramachandran, 2024; Perananthan et al., 2024). Excessive NAPQI formation depletes intracellular glutathione, promotes mitochondrial oxidative stress, induces enterocyte apoptosis, and disrupts epithelial tight junctions, ultimately increasing intestinal permeability (Horowitz et al., 2023; Jaeschke and Ramachandran, 2024; Arumugam et al., 2025). Impairment of the intestinal barrier facilitates bacterial translocation and amplifies local inflammatory responses through activation of the gut–liver axis (McGill and Jaeschke, 2013; Alabbas et al., 2023).

Oxidative stress and inflammation act synergistically to impair intestinal barrier function and promote mucosal injury (Bhattacharyya et al., 2014). Bee bread is a natural fermented bee product rich in polyphenols, flavonoids, phenolic acids, amino acids, vitamins, minerals, and antioxidant enzymes, including quercetin, kaempferol, caffeic acid, gallic acid, and *p*-coumaric acid (Bakour et al., 2022). Bioactive compounds in BB enhance endogenous antioxidant defense through activation of the Nrf2/HO-1 pathway while suppressing NF-κB and MAPK-mediated inflammatory signaling, thereby reducing oxidative stress, limiting pro-inflammatory cytokine production, and preserving epithelial tight-junction proteins (Bakour et al., 2022). The observed effects of BB were consistent with the proposed mechanisms, as evidenced by significantly reduced MPO immunoreactivity and restored *ZO-1* expression in the present study. Similar protective effects on intestinal epithelial barrier integrity and inflammatory responses have been reported for fermented bee pollen, which enhances tight-junction protein expression and suppresses NF-κB-mediated inflammatory signaling (Zhang et al., 2024).

The concordance between the histopathological and immunohistochemical findings supports the close relationship between preservation of intestinal morphology and maintenance of epithelial barrier integrity. Alabbas et al. (2023) demonstrated that acetaminophen-induced intestinal injury was accompanied by disruption of tight-junction proteins, increased intestinal permeability, and inflammatory alterations. Likewise, Zhang et al. (2024) reported that fermented bee pollen enhanced epithelial barrier integrity by increasing tight-junction protein expression through inhibition of the NF-κB-mediated MLCK-MLC signaling pathway. The present findings support the hypothesis that BB protects against paracetamol-induced intestinal injury through coordinated preservation of epithelial barrier integrity and attenuation of inflammatory responses.

CONCLUSION

Paracetamol administration induced marked duodenal injury characterized by mucosal alterations, increased MPO immunoreactivity, and decreased *ZO-1* expression. Bee bread administration attenuated these changes by reducing MPO immunoreactivity and improving *ZO-1* expression, together with partial preservation of intestinal morphology. The findings of the present study indicated that BB exerts a partial protective effect against paracetamol-induced intestinal injury by attenuating inflammation and supporting epithelial barrier integrity. The main limitation of the current study was the lack of biochemical and molecular analyses of oxidative stress and inflammatory pathways. Future studies

incorporating biochemical, molecular, and intestinal permeability analyses are recommended to further clarify the mechanisms underlying the protective effects of BB.

DECLARATIONS

Author contributions

Günsel Kirman conceptualized and designed the study, developed the methodology, conducted the investigation, curated the data, performed the formal statistical analyses, prepared the figures and visualizations, wrote the original draft of the manuscript, and reviewed and edited the final version of the manuscript. The author has read and approved the final edition of the manuscript before publication in the present journal.

Availability of the data and materials

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

Competing interests

The author declares that there are no competing interests.

Ethical considerations

Ethical issues, including plagiarism, consent to publish, scientific misconduct, data fabrication and/or falsification, duplicate publication and/or submission, and redundancy, have been carefully considered and checked by the author. The author used ChatGPT (OpenAI) solely to improve the language, grammar, and readability of the manuscript. The AI tool was not used for data collection, data analysis, interpretation of results, or generation of scientific conclusions. The author reviewed, edited, and approved all content and accepts full responsibility for the accuracy and integrity of the manuscript before submission, revision, and publication.

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Helminth Fauna of Domestic and Wild Ruminants in Garabagh, Azerbaijan

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ABSTRACT

Helminth infections represent a significant constraint impacting the health and productivity of ruminants, with their distribution closely associated with ecological and management factors. The present study aimed to investigate the species composition, prevalence, and biocenological characteristics of helminths in domestic and wild ruminants in the Garabakh Economic Region of Azerbaijan. Between 2022 and 2025, a total of 760 animals were examined. Out of these, 620 were domestic ruminants such as cattle, sheep, and goats, whereas the other 140 included wild ruminants such as European roe deer, red deer, Caucasian chamois, wild sheep, and goitered gazelle. Helminth infections were evaluated through standard coprological techniques such as flotation, sedimentation, and larval recovery methods. Species identification was conducted based on morphological characteristics, and faunistic similarity was assessed using the Jaccard index. A total of 29 helminth species were identified, with 18 presented in both domestic and wild hosts. The overall prevalence was 61.7%, with goats demonstrating the highest infection rate at 74.2%. The most frequently detected species across the total examined population of domestic and wild ruminants were *Fasciola hepatica*, *Moniezia expansa*, *Moniezia benedeni*, *Haemonchus contortus*, and *Trichostrongylus axei*. The high faunistic similarity between cattle and wild sheep, and between goats and Caucasian chamois (Ij = 0.51), indicated active circulation of parasites through shared grazing areas and water sources. Out of the 16 helminth species identified in domestic ruminants, 15 were found in wild hosts. Wild sheep and chamois exhibited the most similar faunal composition. The overall prevalence did not differ significantly between domestic (63.1%) and wild (55.7%) ruminants, with *Fasciola hepatica* detected in both groups. These quantitative results indicated that wild sheep and chamois played an active role in maintaining helminths alongside livestock, highlighting the importance of ecologically integrated surveillance in shared grazing regions.

Keywords: Economic region, Grazing, Helminth, Ruminant

INTRODUCTION

Wildlife populations serve as important reservoirs for a wide range of infectious agents, including zoonotic parasites. These zoonotic parasites associated with wildlife belonged to different taxonomic groups, including nematodes, cestodes, trematodes, and protozoa (Polley, 2005). Moreover, the infective stages of parasites, such as eggs and larvae, can persist for prolonged periods under suitable environmental conditions, thereby increasing the likelihood of transmission to susceptible hosts (Soulsby, 2012). The role of domestic and wild ruminants in sustaining helminth reservoirs depends on several factors, such as the types and numbers of hosts, environmental conditions, the biological traits of the habitat, and human activities. The epizootic patterns of many diseases, such as helminthiases, are affected by the natural climatic and economic-ecological factors specific to particular regions (Almaksudov et al., 2010; Atayev et al., 2022; Klimova, 2022).

Helminth infections are widespread in domestic and farm animals, causing significant economic damage to the livestock industry (de Araújo, 2023). Helminth infections can modulate host immune responses, often leading to chronic infections that negatively impact animal health and productivity (Maizels and Yazdanbakhsh, 2003; Hoerauf et al., 2005; Elliott et al., 2007).

Recent parasitological studies indicated that helminthiasis remains a major factor undermining the health and productivity of ruminants worldwide (Charlier et al., 2020; de Araújo, 2023; Mendoza-de Gives et al., 2023). Epidemiological and epizootiological screenings conducted across diverse geographical regions confirmed a high prevalence of gastrointestinal helminths in domestic and wild ruminants. A recent meta-analysis examining the global distribution dynamics of *Haemonchus contortus* (*H. contortus*) observed that this nematode exhibited considerable ecological plasticity across small (sheep, goats) and large ruminants and was a predominant species in numerous regions (Ma et al., 2025).

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The higher prevalence of helminth infections observed among juvenile animals compared to adults is generally attributed to the undeveloped acquired immunity. As hosts age and are repeatedly exposed to parasites, their immune responses become stronger, reducing the ability of infective larvae to establish and grow, and ultimately decreasing parasite survival and reproduction (Efremov, 2016). Recent monitoring of domestic ruminants highlighted the substantial economic and epizootic importance of helminthiasis. A large-scale screening in Qazvin Province, Iran, from 2024 to 2025, found helminth infections in 75.35% of sheep, 65.73% of goats, and 24.50% of cattle. The main parasites identified were *Fasciola hepatica* (*F. hepatica*), *Dicrocoelium dendriticum*, and *Nematodirus spathiger* (Hoseini et al., 2025). These findings demonstrated that helminths can cause significant economic losses in modern livestock production systems and restrict animals' ability to reach their full genetic potential. Studies of wild ruminants have demonstrated that the incidence of parasitic infestations differs significantly by host species and ecological and regional characteristics. For instance, in Sub-Saharan African countries, the prevalence rates of gastrointestinal helminths ranged from 1.4% to 100% for nematodes, 0.8% to 100% for trematodes, and 1.4% to 50% for cestodes (Phetla et al., 2024). Conversely, the prevalence of rumen trematodes in wild ruminants ranged from 0.8% to 100%, nematodes from 1.4% to 100%, and cestodes from 1.4% to 50%, indicating that wild fauna play a significant epizootiological role as natural reservoirs of helminth infections (Sibula et al., 2024).

In natural ecosystems, helminths are a key part of parasitic communities and often coexist within intricate parasite populations. Wild ruminants function as significant reservoir hosts, sustaining the circulation of helminth populations and facilitating the transmission of shared parasite species to domestic ruminants within overlapping grazing areas (Winter et al., 2018; Rossi et al., 2019). When wild and domestic ruminants share grazing areas and watering sources, the transmission and interspecific circulation of helminths are facilitated and accelerated. Epidemiological investigations confirmed that several gastrointestinal and pulmonary helminths, including *Ostertagia*, *Trichostrongylus*, and *Haemonchus* species, can be transmitted between domestic and wild ruminants in Austria, with wild ruminants acting as reservoirs within natural sites (Winter et al., 2018).

It has been established that helminth transmission can occur between wild ungulates (European bison, moose, roe deer, red deer, and sika deer) and domestic ruminants (cattle, sheep, and goats; Muromtsev, 2016). Given the shared susceptibility of domestic and wild ruminants to different helminth species and their overlapping grazing environments, comprehensive studies of host-parasite dynamics are essential. Such investigations are particularly critical as ecological and human-related factors can influence transmission patterns, host relationships, and the composition of helminth communities (Morgan and Wall, 2009; Winter et al., 2018). Based on the current study and existing literature, the species composition, prevalence, and biosenological characteristics of helminths were examined in domestic ruminants such as cattle (*Bos taurus taurus*), sheep (*Ovis aries*), and goats (*Capra hircus*), and wild ruminants, including European roe deer (*Capreolus capreolus*), red deer (*Cervus elaphus*), Caucasian chamois (*Rupicapra rupicapra*), wild sheep (*Ovis orientalis*), and goitered gazelle (*Gazella subgutturosa*) in the Garabagh economic region of Azerbaijan. The present study aimed to examine the species composition, distributional characteristics, and biocenotic relationships of helminths in domestic and wild ruminants in the Garabagh economic region of Azerbaijan, identify shared helminth species between domestic and wild hosts, and analyze the mechanisms of helminth circulation in shared grazing regions.

MATERIALS AND METHODS

Ethical approval

This study involved only the collection and analysis of fecal samples obtained from domestic and wild ruminants under standard management conditions. No invasive procedures, surgical interventions, experimental treatments, or therapeutic interventions were performed. The Ethics Committee of the Institute for Living Systems Research determined that the study is exempt from formal ethical approval requirements and issued an Ethics Committee Exemption Letter (Decision/Protocol No. EC-AZ-2026-002).

Study location

The study was conducted between 2022 and 2025 in the Garabagh economic region of Azerbaijan (Figure 1), encompassing natural and anthropogenic landscapes. The region is characterized by a highly diverse landscape structure, including lowland semi-deserts, steppe ecosystems, foothill zones, broad-leaved forests, subalpine meadows, and alpine grazing areas. The altitudinal gradient ranges from approximately 100 to 3500 meters above sea level (coordinates of 39°59′21″N 46°54′00″E), creating diverse ecological conditions favorable for the circulation of numerous helminth species and their intermediate hosts. Climate conditions differ from a mild, warm, semi-arid environment in the plains to a cold mountain climate in the high-altitude areas. The annual precipitation ranges from 250 to 800 mm, while the average annual temperature varies between 6°C and 14°C.



Figure 1. The study area of the Garabagh economic region in Azerbaijan (Designed by authors)

Animals

The study included 760 ruminants, consisting of 620 domestic animals sampled from farms, herds, or villages within the study area, and 140 wild animals. The domestic ruminants consisted of 220 cattle (*Bos taurus taurus*), 280 sheep (*Ovis aries*), and 120 goats (*Capra hircus*). Fecal samples from domestic and wild ruminants were collected non-invasively from freshly deposited feces in the environment. Fresh fecal samples were collected from cattle on a convenience sampling basis during visits to a total of 55 privately owned farms. Samples were collected immediately after spontaneous defecation from the first eligible animals observed, whenever possible, to minimize environmental contamination, and were transferred into sterile containers for laboratory analysis. No animals were captured, physically restrained, handled, or sedated during the sampling process.

Among the cattle, 65 were less than 1 year old, 72 were 1 to 3 years old, and 83 were over 3 years old, including 88 males and 132 females. Among the sheep, there were 90 young, 95 subadults, and 95 adults; of these, 102 were males and 178 females. The goat population included 32 young, 39 subadults, and 49 adults, with 41 males and 79 females.

The study also included 140 wild ruminants representing five species naturally occurring in the Garabagh region. These animals were represented by fecal samples collected non-invasively from naturally occurring populations during field surveys, rather than from captured or handled animals. They comprised 35 European roe deer (*Capreolus capreolus*), 20 red deer (*Cervus elaphus*), 25 Caucasian chamois (*Rupicapra rupicapra*), 30 wild sheep (*Ovis orientalis*), and 30 goitered gazelles (*Gazella subgutturosa*). Data on wild animals were obtained through field surveys and non-invasive sampling methods (fecal collection). This methodology facilitated a more comprehensive evaluation of the species composition and distribution patterns of the helminth fauna within wild ruminant populations.

Sample collection and parasitological examination

Field surveys were conducted across grazing areas, mountain pastures, watering points, migration routes, forest edges, and natural shelters used by domestic and wild ruminants. Fresh fecal samples from domestic ruminants were collected immediately after defecation from freshly deposited feces. For wild ruminants, samples were obtained non-invasively from freshly deposited feces identified in the field by their fresh appearance and additional field indicators (Schilling et al., 2022). Approximately 20-30 g of fecal material was placed into sterile, labeled plastic containers. Samples were transported under refrigerated conditions to the Parasitology Laboratory, Institute for the Study of Living Systems, Baku, Azerbaijan, and examined within 24-48 hours after collection.

Parasitological examinations were performed using standard qualitative and quantitative helminthological techniques widely applied in veterinary parasitology. During the initial processing of samples, sequential washing procedures were employed, followed by quantitative flotation examinations using the McMaster technique, the most widely used method for estimating parasite egg counts in feces (Gordon and Whitlock, 1939; Cringoli et al., 2004). Larval recovery was performed using the Baermann technique, a widely applied method for the isolation of nematode larvae from fecal and herbage samples based on the original description by Baermann (1917) as modified by Persson

(1974). Sedimentation procedures were conducted according to the formalin-ethyl acetate sedimentation technique, a standard concentration method in clinical parasitology first validated by Truant et al. (1981). Additionally, the pasture helminthological assessment method developed by Kotelnikov (1984) was employed to evaluate environmental contamination and the circulation of infective stages in grazing areas. Helminth eggs, larvae, and other diagnostic stages were identified under a light microscope (Carl Zeiss Axio Lab.A1, Germany) at 10x and 40x magnifications based on their morphological characteristics. Identification was performed according to established parasitological keys and published descriptions (Taylor et al., 2016).

The prevalence and abundance of helminth infections were calculated using established epidemiological indices commonly applied in parasitological studies. The faunistic similarity between helminth communities of domestic and wild ruminants was assessed using the Jaccard similarity index (Poulin, 2003).

Statistical analysis

The prevalence (%) and abundance indices of helminths in the examined animals were calculated using standard parasitological formulas, where prevalence was defined as the proportion of infected hosts and abundance as the average number of parasites per examined host, according to Bush et al. (1997) and Rózsa et al. (2000). The similarity between the helminth faunas of domestic and wild ruminants was evaluated using the Jaccard similarity index. Differences were considered statistically significant at a p-value less than 5% ($p < 0.05$). The Wilson score method was used to calculate the 95% confidence intervals for prevalence. All statistical calculations were performed using the IBM SPSS Statistics software package (version 26, IBM Corp., Armonk, NY, USA).

RESULTS AND DISCUSSION

In the present study, 29 helminth species were identified in domestic and wild ruminants, of which 18 species were common to both host groups (Table 1).

Table 1. Prevalence of helminth infections in domestic and wild ruminants examined in the Garabagh economic region of Azerbaijan (2022-2025)

Host species	Examined (number)	Infected (number)	Prevalence (%)
Cattle	220	118	53.6
Sheep	280	184	65.7
Goats	120	89	74.2
Roe deer	35	18	51.4
Red deer	20	11	55.0
Chamois	25	17	68.0
Wild sheep	30	20	66.7
Gazelle	30	12	40.0
Total	760	469	61.7

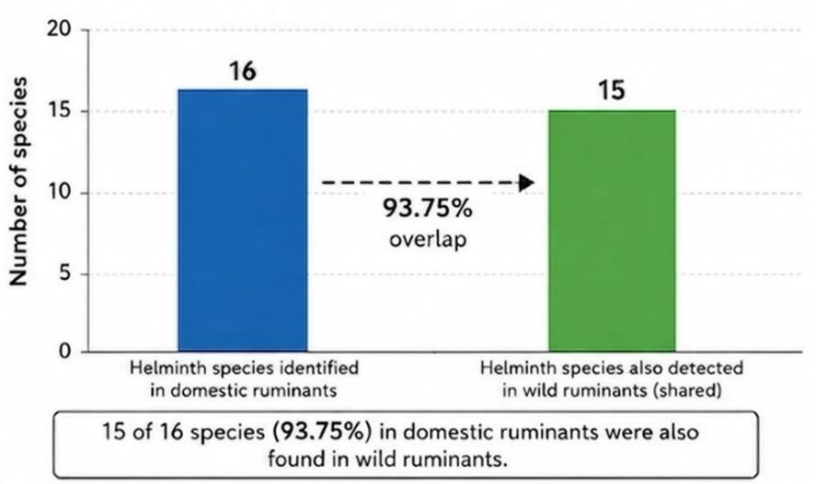


Figure 2. Comparison of helminth species abundance and shared taxa between domestic and wild ruminants

The high proportion of shared parasite species, given the use of common grazing areas and water sources, indicated a persistent circulation of helminths between domestic and wild ruminants. In the current study, 15 of the 16 helminth species identified in domestic ruminants were also found in wild ruminants (Figure 2), indicating a considerable level of faunistic overlap. The current findings indicated that shared grazing environments played an important role in the transmission of helminths among different host groups, as supported by Winter et al. (2018) and Rossi et al. (2019).

A total of 16 helminth species were identified in domestic ruminants and 15 in wild ruminants. Of these, 15 species were common to both domestic and wild ruminants, representing 93.75% species overlap, while one species was found exclusively in domestic ruminants. Faunistic similarity indices indicated significant overlap in helminth communities between domestic and wild ruminants, particularly between goats and Caucasian chamois (Ij = 0.51) and between cattle and wild sheep (Ij = 0.57), highlighting the importance of shared habitats in parasite transmission (Table 2).

Table 2. Jaccard similarity index results between helminth communities of domestic and wild ruminants in Garabagh economic region of Azerbaijan (2022-2025)

Host pair	Shared species (number)	Jaccard index (Ij)
Cattle – Wild sheep	8	0.57
Goat – Caucasian chamois	7	0.51
Sheep – Roe deer	6	0.43
Sheep – Red deer	5	0.38
Goat – Gazelle	4	0.31
Cattle – Roe deer	5	0.36

In total, 469 of the 760 examined ruminants (61.7%) were infected with at least one helminth species. The prevalence differed notably among host species, from 40.0% in goitered gazelles (*Gazella subgutturosa*) to 74.2% in domestic goats (*Capra hircus*). Host species and infection status were found to be highly significantly associated (χ^2 = 24.546, df = 7, $p < 0.001$), indicating that helminth prevalence differed across domestic and wild ruminants within the Garabagh economic region of Azerbaijan. Pairwise comparisons, employing the Bonferroni correction, demonstrated that the prevalence in domestic goats (74.2%) was significantly greater than that observed in gazelles (40.0%; χ^2 = 12.737, df = 1, $p < 0.001$). No other pairwise differences were statistically significant after correction. Notably, there was no significant difference in overall prevalence between domestic ruminants (391/620, 63.1%) and wild ruminants (78/140, 55.7%; χ^2 = 2.614, df = 1, $p > 0.05$), indicating similar helminth circulation across the wildlife-livestock interface. The Jaccard similarity indices ranged from 0.31 to 0.57 (Table 2). A moderate to substantial similarity was observed between cattle and wild sheep (Ij = 0.57), and between domestic goats and Caucasian chamois (Ij = 0.51). These findings indicated that active helminth distribution occurred in shared grazing regions and at water sources.

In the current study, 16 helminth species were identified in domestic ruminants, comprising one trematode species (*F. hepatica*), two cestode species, including *Moniezia expansa* (M. *expansa*) and *Moniezia benedeni* (*M. benedeni*), and 13 nematode species. The majority of the identified species were Nematoda, including *Chabertia ovina*, *Bunostomum trigonocephalum*, *Oesophagostomum venulosum*, and *Trichostrongylus axei*, which were detected in both domestic and wild ruminants, highlighting the dominance of gastrointestinal nematodes within the studied host populations. These findings were consistent with previous reports indicating that nematodes constitute the largest component of the regional helminth fauna (Kolesnichenko, 1969). The present study indicated that many gastrointestinal nematodes were not host-specific and could circulate between wild and domestic ruminants. Similar host associations have previously been reported for ruminants in Azerbaijan, indicating that common grazing areas could promote the spread and persistence of these parasites between wildlife and livestock (Ahmedov et al., 2025). Several helminth species identified during the survey exhibited host-specific distributions and were recorded only in domestic ruminants. In contrast, most of the detected helminths were shared between domestic and wild host populations. This pattern indicated that although some host-associated species were present, the helminth communities of domestic and wild ruminants in the Garabagh region of Azerbaijan were primarily interconnected. Consistent with the current findings, Rossi et al. (2019) identified several shared parasite species, indicating that common grazing habitats facilitate parasite circulation at the wildlife-livestock interface.

In the 1960s, Kolesnichenko (1969) carried out comprehensive helminthological examinations of 75 sheep from diverse districts within the Mountainous Garabagh region of Azerbaijan, and recorded 52 helminth species, which included three trematodes, seven cestodes, and 42 nematodes. Recent helminthological investigations conducted within the Garabagh economic zone of Azerbaijan, including farms in Agjabadi and Barda districts, have identified 16 helminth species in domestic ruminants such as sheep, goats, and cattle for the first time. The identified parasite species included

F. hepatica (Linnaeus, 1758), *M. expansa* (Rudolphi, 1810) Blanchard, 1891, *M. benedeni* (Moniez, 1879) Blanchard, 1891, *Chabertia ovina* (Fabricius, 1788), *Strongyloides papillosus* (Wedl, 1856), *Bunostomum trigonocephalum* (Rudolphi, 1808) Railliet, 1902, *Oesophagostomum venulosum* (Rudolphi, 1809) Railliet and Henry, 1913, *Trichostrongylus axei* (Cobb, 1879), *Trichostrongylus carpicola* Ransom, 1907, *Ostertagia ostertagi* (Stiles, 1892), *Ostertagia circumcincta* (Stadelmann, 1894) Ransom, 1907, *Ostertagia occidentalis* Ransom, 1907, *H. contortus* (Rudolphi, 1803), *Nematodirus spathiger* (Railliet, 1896), *Trichocephalus* (*Trichuris*) *ovis* (Abildgaard, 1795), and *Trichocephalus* (*Trichuris*) *skrjabini* (Baskakow, 1924). Among the examined domestic ruminants, cattle demonstrated the greatest helminth species diversity, hosting all 16 species identified in the present study.

According to the helminthological investigations in the Agjabadi and Barda districts of Azerbaijan, the following species were identified in cattle, including *F. hepatica*, *M. expansa*, *M. benedeni*, *Strongyloides papillosus*, *Ostertagia ostertagi*, *Nematodirus spathiger*, *Chabertia ovina*, and *Trichostrongylus axei*. In the present study, *F. hepatica*, *Strongyloides papillosus*, *M. expansa*, *M. benedeni*, *Trichostrongylus capricola*, *H. contortus*, *Trichocephalus* (*Trichuris*) *skrjabini*, and *Trichocephalus* (*Trichuris*) ovis were recorded in sheep. In goats, *Ostertagia occidentalis*, *Oesophagostomum venulosum*, *Chabertia ovina*, *Bunostomum trigonocephalum*, *Ostertagia circumcincta*, *H. contortus*, and *Trichostrongylus axei* were detected during the present study.

Fasciola hepatica, a zoonotic parasite associated with human fascioliasis, was among the helminths identified in the present study, thereby posing a considerable public health concern. Its detection in both domestic and wild ruminants confirmed that these host populations served as vital reservoirs for the maintenance and distribution of *F. hepatica* within the study area. Additionally, *Strongyloides papillosus* was detected in the present study, a parasite with a broad host range that could have implications for veterinary and public health. The presence of these parasites in domestic ruminants that share grazing areas with wild ruminants underscored the potential epidemiological importance of the wildlife–livestock interface in maintaining parasite transmission cycles (Viney and Lok, 2015; Moreira et al., 2023).

Different helminth groups exhibited distinct transmission strategies, ecological requirements, and distribution patterns determined by host specificity, life-cycle characteristics, and environmental factors (Taylor et al., 2016). However, many helminth species have free-living larval stages that are strongly influenced by climate factors, particularly temperature and humidity, which affect their development, survival, and transmission (O'Connor et al., 2006; Taylor et al., 2016). Consequently, seasonal patterns of helminth infection are commonly observed in grazing animals. The spatial and temporal distribution of these parasites may be shaped by the abundance and dispersal of intermediate hosts and vertebrate reservoir hosts (Morgan and Wall, 2009). Elevations in ambient temperature are expected to alter ecological conditions and accelerate the development of environmentally transmitted parasite stages (Kutz et al., 2005; Fischlin et al., 2007). These changes may promote parasite transmission, ultimately leading to increased infection and reduced productivity in grazing livestock.

The environment in extensively grazed regions could have influenced the *Moniezia* spp. life cycle, thereby sustaining parasite populations in local ruminants. This finding indicated that grazing areas and ecosystems play a vital role in maintaining cestode circulation among domestic and wild ruminant species. The relatively high densities of oribatid mites recorded in the present study, which ranged from 170-350 and 550-1200 individuals/m², indicated that suitable intermediate hosts for *Moniezia* spp. were readily available in the Garabagh economic region of Azerbaijan. The concurrent detection of *M. expansa* and *M. benedeni* in grazing ruminants supported the importance of pasture ecosystems in maintaining parasite transmission. These results indicated that environmental factors and host overlap contributed to the circulation of monieziosis within the Garabagh economic region of Azerbaijan.

In the present study, *M. expansa* and *M. benedeni* were identified in domestic ruminants (sheep, goats, and cattle) from the Garabagh economic region of Azerbaijan. Additionally, these cestodes were identified in wild ruminants, including *Ovis orientalis*, indicating that wild herbivores served as potential reservoirs for *Moniezia* spp. within natural ecosystems. A comparative analysis of host–parasite relationships indicated that wild herbivores, including wild sheep and gazelles, helped maintain helminth transmission in shared grazing areas (Modabbernia et al., 2021). The results of the present study demonstrated that helminth transmission in the Garabagh economic region occurred in mixed grazing areas that included both domestic and wild ruminants. Furthermore, the study of helminth fauna in the Garabagh economic region of Azerbaijan identified 87 helminth species, comprising 19 biohelminths and 68 geohelminths (Asadov, 1960; Kolesnichenko, 1969), reflecting the diversity of parasite transmission strategies in the region. The relatively high prevalence of trematodes and nematodes in domestic ruminants, compared with wild hosts, indicated that livestock played a critical role in maintaining parasite populations in grazing regions.

In domestic ruminants, helminth communities were recorded, ranging from three-species and five-species to multi-species associations. Similarity analysis revealed notable overlap in helminth fauna between goats and Caucasian chamois and between cattle and wild sheep, indicating ecological relatedness among parasite communities across host groups. Lower helminth prevalence was observed in wild ruminants compared with domestic sheep and goats, reflecting

differences in host density, grazing intensity, and exposure to contaminated pasture environments. The co-occurrence of domestic and wild ruminants in shared high-mountain summer grazing regions created favorable conditions for parasite transmission and helped maintain helminth circulation in both natural and semi-natural ecosystems. The present findings revealed substantial overlap in helminth species between domestic and wild ruminants, highlighting ecological connectivity among host populations within shared grazing ecosystems.

CONCLUSION

Among the 16 helminth species recorded in domestic ruminants, 15 were recorded in wild ruminants as well. The most prevalent parasites identified in the present study included *F. hepatica*, *M. expansa*, *M. benedeni*, *H. contortus*, and *Trichostrongylus axei*. The current findings demonstrated that wild ruminants could serve as significant reservoirs of helminths in natural biosemiotic systems, thereby facilitating their transmission to domestic animals. Since ecological and climatic factors greatly influence helminth distribution in biocenoses, it is recommended to regularly monitor helminth infections in both wild and domestic ruminants to effectively manage these infections levels. Additionally, further studies focusing on long-term seasonal patterns and pathways of parasite transmission between domestic and wild ruminants are recommended.

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Authors' contributions

Elshad Ilyas Ahmadov conceptualized and designed the study, conducted the field investigations, analyzed the data, and drafted the manuscript. Reyhan Shoxlat Ibrahimova contributed to sample collection, laboratory examinations, and the literature review. Zhala Vasif Hasanova participated in laboratory analyses, data processing, and manuscript editing. Farida Zohrab Mammadova assisted with field investigations, data collection, and manuscript editing. All authors have read, reviewed, and approved the final edition of the manuscript.

Availability of data and materials

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

Competing interests

The authors declare no financial or non-financial competing interests regarding the publication of this article.

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. The authors accept full responsibility for the originality of the study before submission and before publication.

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Postbiotic Production from *Bacillus* Consortium Enriched with *Avicennia marina* Leaves and Its Antibacterial Activity Against Aquaculture Pathogens

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ABSTRACT

Bacterial infections pose a critical challenge in aquaculture, causing mass mortality and financial losses. The present study aimed to formulate postbiotics from a *Bacillus* (*B.*) consortium (*B. safensis*, *B. mycoides*, and *B. pumilus*) enriched with *Avicennia marina* (*A. marina*) leaf powder (ALP) and evaluate their activity against *Streptococcus agalactiae* (*S. agalactiae*), *Aeromonas hydrophila* (*A. hydrophila*), *Vibrio* (*V.*) *harveyi*, *V. parahaemolyticus*, and *V. alginolyticus*. The postbiotics' antibacterial efficacy was assessed using broth microdilution to determine minimum inhibitory concentrations (MICs), followed by subculturing to determine minimum bactericidal concentrations (MBCs). The disk diffusion assay assessed the tolerance of *Bacillus* consortium to *A. marina* leaf extracts at 25, 50, 75, and 100 mg mL^{-1}, with ethanol as the negative and ciprofloxacin as the positive controls. Postbiotic production used the medium as the control and three graded concentrations of ALP at 1%, 2%, and 3%, followed by thermal inactivation, centrifugation, and filtration to ensure the absence of live cells. The postbiotic was examined using liquid chromatography-high-resolution mass spectrometry to determine its secondary metabolite profiles. The 3% ALP-enriched postbiotic was selected for final antibacterial efficacy analysis against aquaculture pathogens. The results demonstrated that the *Bacillus* consortium was resistant to leaf extracts at 25-100 mg mL^{-1}, with only minimal inhibition zones of 6.00 to 7.17 mm. In postbiotic production, 3% ALP enhanced bacterial viability to 9.08 log_{10} CFU mL^{-1}. The 3% ALP-enriched postbiotic increased the number of metabolites from 384 (baseline control) to 543, accompanied by upregulation of Cyclo(-L-Ala-L-Tyr), (R)-Lactaldehyde, an acylprolinamide derivative, and D-(-)-Ribose. *In vitro*, 3% ALP selectively inhibited *Vibrio* spp., demonstrating significant inhibition zones (11.33-15.30 mm), low MIC values (1.6-25% v/v), and low MBC values (6.3-50% v/v). Although disk diffusion produced no inhibition zones for either *A. hydrophila* or *S. agalactiae*, *A. hydrophila* exhibited susceptibility at 25% v/v in the MIC and at 50% v/v in the MBC. In contrast, *S. agalactiae* remained resistant at the highest concentration, with MIC and MBC values exceeding 50% v/v. Enriching the *Bacillus* consortium with ALP increased the active metabolites in *Bacillus* postbiotics, offering a promising control against *Vibrio* spp. in aquaculture.

Keywords: Aquaculture, *Avicennia marina*, *Bacillus* consortium, Metabolite, Postbiotic, Vibriosis

INTRODUCTION

Mangrove ecosystems contain several bioactive compounds, such as flavonoids, which act as antimicrobial, antioxidant, and immunomodulatory agents (Rahmania et al., 2025). One of the most promising mangrove species for the extraction of bioactive compounds is *Avicennia marina* (*A. marina*), due to its prevalence and natural abundance along tropical and subtropical coastlines, including the coastal regions of Indonesia (Rahmawati et al., 2019; Cerri et al., 2025). The interactions between *A. marina* and non-pathogenic bacteria have not been widely reported in the context of fermentation, largely because most of the bioactive compounds in *A. marina* have been documented primarily for their direct antimicrobial effects against pathogenic bacteria (Rahmania et al., 2025). Therefore, combination formulations that use plant-derived substrates and produce postbiotics hold great potential to enhance functional properties and health benefits (Sravya et al., 2025). Probiotics produced by *Bacillus* (*B.*) species, including *B. safensis*, *B. mycoides*, and *B. pumilus*, exhibit significant potential within the aquaculture and functional food industries owing to the enzymes and bioactive compounds they synthesize (Foysal et al., 2020; Wu et al., 2021; Raman et al., 2025). However, the integration and metabolic responses of *Bacillus* spp. with compounds derived from *A. marina* leaves have not been extensively studied (Saxena et al., 2020).

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Postbiotics are metabolic products that do not contain live microorganisms, making them safer and more stable than probiotics, which contain live cells. Nevertheless, postbiotics can support the immune response and protect aquatic animals against pathogens (Thakur et al., 2025). The use of *A. marina* leaf powder (ALP) as a substrate can support *Bacillus* spp. in secreting hydrolytic enzymes (cellulase) for degrading coarse fiber structures (Kurniawan et al., 2017). This biotransformation process broadens the range of metabolites through the production of functional organic acids and bacteriocins, thereby supporting the development of postbiotic formulations for health in the aquaculture sector (Rahman et al., 2020; Salminen et al., 2021; Rahman et al., 2023). The leaves of *A. marina* naturally contain several bioactive compounds, including phenolics, saponins, flavonoids, terpenoids, and alkaloids (Rozirwan et al., 2022). Plant-derived active compounds, such as phenolics at sub-inhibitory concentrations, act as mild stressors on *Bacillus* spp., thereby activating secondary metabolism and promoting the production of a broader diversity of bioactive compounds during fermentation (Wang et al., 2018; Hurtado-Navarro et al., 2025). Other plant-derived compounds from *A. marina* leaves, such as saponins, which are biosurfactants with amphiphilic and physicochemical properties, are functionally similar to surfactin or microbial-based biosurfactants (Karnwal et al., 2023). When these triterpenoid saponins are exposed to *Bacillus* spp. at sub-lethal concentrations, the bacteria undergo adaptive physiological changes rather than die, reprogramming the lipid composition of their membranes to survive (Uttlová et al., 2016). Furthermore, flavonoids at concentrations below the inhibitory threshold can cause subtle changes in cellular signaling and enzyme activity, driven by mild physiological stress rather than by lethal cellular damage (Patil et al., 2024). In addition, it has been reported that exposure to terpenoid and alkaloid groups can disrupt the structural integrity of *Bacillus* spp. (Huang et al., 2022a). However, at sub-inhibitory levels, these terpenoid and alkaloid compounds interfere only with the bacterial target site, without stopping its growth (Radeck et al., 2017).

Pathogenic bacterial infections are a major challenge in aquaculture, causing high mortality and significant economic losses due to reduced production (Sarjito et al., 2022). *Streptococcus agalactiae* (*S. agalactiae*) is a pathogen of concern in many cultured fish in aquaculture (Sapugahawatte et al., 2022). This concern emerges in the aquaculture sector due to high mortality rates, with *S. agalactiae* becoming more prevalent in many aquaculture-producing countries, including Indonesia (Lusiastuti et al., 2024). Furthermore, *Aeromonas hydrophila* (*A. hydrophila*) is a freshwater opportunistic bacterium responsible for motile aeromonas septicemia (MAS) and has been identified as one of the most pathogenic bacteria contributing to losses in different farmed fish species, such as *Oreochromis niloticus*, as well as crustaceans such as *Litopenaeus vannamei* (Tao et al., 2026). In brackish and marine aquaculture, *Vibrio* species such as *Vibrio harveyi* (*V. harveyi*), *Vibrio alginolyticus* (*V. alginolyticus*), and *Vibrio parahaemolyticus* (*V. parahaemolyticus*) are the primary agents of vibriosis, which frequently leads to disease outbreaks (Sarjito and Sabdono, 2021). *Vibrio* spp. are also known to resist multiple antibiotics, posing a challenge in aquaculture (Loo et al., 2020). The variety of bacterial threats in aquaculture systems necessitates safer and more stable alternatives for pathogen control. Therefore, the use of postbiotics is considered more efficient than the use of live cells or probiotics (Thakur et al., 2025). Significant knowledge gaps persist regarding the specific metabolic synergies within a *Bacillus* consortium in response to *A. marina* compounds, as well as the overall antimicrobial efficacy of the derived postbiotics against a broad range of aquaculture pathogens. The use of postbiotics based on the genus *Bacillus*, enriched with *A. marina* leaf substrate, has the potential to be a promising alternative for suppressing the growth of major pathogenic bacteria. The current study aimed to develop a postbiotic formulation by assessing the resistance of a *Bacillus* consortium, comprising *B. safensis*, *B. mycoides*, and *B. pumilus*, against *A. marina* leaf extract, monitoring postbiotic production, including bacterial viability and metabolite profiling through liquid chromatography-high-resolution mass spectrometry (LC-HRMS), as well as evaluating the efficacy of the postbiotic against prevalent aquaculture pathogens.

MATERIALS AND METHODS

Ethical approval

The procedures conducted in the present study adhered to the guidelines established by the Microbiology and Chemistry Laboratory of Ahli Usaha Perikanan (AUP) Polytechnic, Serang, Banten, Indonesia, the Laboratory Guidelines of the Fish and Environmental Health Testing Center, Serang, Banten, Indonesia, the Laboratory Guidelines for Fisheries Product Quality Testing and Implementation, Serang, Banten, Indonesia, and the Research Laboratory Guidelines of Corpora Science, Yogyakarta, Special Region of Yogyakarta, Indonesia.

Avicennia marina leaves preparation

Avicennia marina leaves were obtained from the Northern coastal area of Serang City, Banten Province, Indonesia. Mature leaves were randomly collected from 20 healthy trees, with a strict restriction of less than ten percent of total foliage per tree (300 g tree^{-1}). This restriction was implemented to prevent physiological stress and habitat disturbance,

as artificial defoliation up to 25% did not significantly impact mangrove growth (Tong et al., 2003). The implementation of the randomized, non-destructive harvesting protocol yielded a total wet mass of 6 kg of leaf samples for extraction. The ethanol extract of *A. marina* leaves was prepared according to the methodology of Bitwell et al. (2023), in which mature mangrove leaves were cleaned and dried at 23 °C for 72-96 hours until the moisture content reached ≤ 10%, then ground into powder. A total of 100 g of the powder was macerated in 96% food-grade ethanol (1:10; Malindo Raya Industrial Ltd., Indonesia) for 72 hours, with periodic stirring using a magnetic stirrer (without heating) at 150 rpm for five minutes every eight hours in two replicates. All filtrates were combined in an evaporator at 40-50 °C and 100 mBar, followed by final drying in a universal laboratory oven (Memmert, Germany) at 45 °C for two hours, yielding a paste-like crude extract of *A. marina*.

Bacillus consortium bacterial culture

The *Bacillus* consortium, comprising three strains previously isolated from mangrove sediments and identified via a molecular approach, included *B. safensis* LC849759.1, *B. mycoides* MT509769.1, and *B. pumilus* LC852608.1, was sourced from the Microbiology Laboratory at the Faculty of Fisheries and Marine Sciences, Diponegoro University, Indonesia (Chrisnawati et al., 2023; Fadhila et al., 2023; Widya et al., 2024). The prepared bacterial consortium was a powder formulation coated with calcium carbonate ($CaCO_3$) at a 1:1:1 ratio and a density of 10^8 CFU g^{-1}. For the initial plating suspension, 0.1 g of the *Bacillus* consortium powder was empirically weighed, suspended in sterile 0.85% sodium chloride (NaCl) solution to a final volume of 10 mL, and homogenized with a vortex mixer. A total of 100 µL of the homogenized bacterial suspension was spread onto sterile nutrient agar (Merck KGaA, Germany) and incubated for approximately 24 hours at 30°C. After the incubation period, colonies were harvested using a sterile loop needle based on their specific morphological characteristics to ensure that the three bacterial species were collected. *Bacillus mycoides* was identified macroscopically and confirmed with a biological microscope at 40x magnification (Nikon Corporation, Japan) by rhizoid-shaped colonies resembling circular root filaments, grayish-white in color, and dull in surface texture (Di Franco et al., 2002). *Bacillus pumilus* was identified by its circular, undulate (wavy-edged) colonies, whitish to cream color, and matte, raised appearance (Irkitova et al., 2021). *Bacillus safensis* was characterized by small, round colonies with flat to slightly wavy edges, milky white or shiny cream in color, and smooth in texture (Satomi et al., 2006). The entire colony biomass was then suspended in a sterile NaCl solution, and cell density was adjusted by adding colonies or diluting until the turbidity reached the 0.5 McFarland standard (Feliatra et al., 2021).

Disk diffusion assay of _Avicennia marina_ extract against _Bacillus_ consortium

The disk diffusion test was conducted according to CLSI (2020) guidelines, using a sterile cotton swab dipped into the bacterial consortium suspension at a density of 0.5 McFarland. After swirling the swab against the tube wall to remove excess liquid, the inoculum was spread in three directions across the Mueller-Hinton agar (MHA, pH 7; Merck KGaA, Germany) to ensure a uniform layer. The paste-like crude extract of *A. marina* was dissolved in 96% food-grade ethanol and subjected to vigorous vortex mixing (Thermolyne, United States) at approximately 2500 rpm for three minutes to ensure complete, particle-free solubilization, yielding four treatment groups (Azis et al., 2022). The treatment groups included 25 mg mL^{-1} of *A. marina* was dissolved in l mL 96% food-grade ethanol or 2.5% w/v (Group B), 50 mg mL^{-1} or 5% w/v (Group C), 75 mg mL^{-1} or 7.5% w/v (Group D), and 100 mg mL^{-1} or 10% w/v (Group E), respectively (Ibrahim et al., 2022; Priambodo et al., 2024). Prior to impregnation, the laminar airflow cabinet (Medfuture Biotech Co., Ltd., China) was sterilized using UV radiation. Immediately prior to impregnation, each treatment solution was shortly vortexed, after which sterile paper discs (6 mm diameter, MN 827 ATD, Macherey-Nagel GmbH & Co. KG, Germany) were impregnated with 10 µL of *A. marina* leaf extract and then dried under aseptic conditions for approximately 30 minutes with the airflow blower on and the UV lamp off, ensuring complete evaporation of the ethanol vehicle (Mohideen et al., 2026). The prepared discs containing different concentrations (groups B, C, D, and E) were placed on the consortium bacterial inoculum medium, with approximately 24 mm distances between discs. Next, the treatment medium was incubated upside down at approximately 30°C for 24 hours. For comparison, ciprofloxacin (Oxoid Ltd., England) at a concentration of 5 µg was used as a positive control (Group F) because of its activity against *Bacillus* spp. (Adewumi et al., 2009; Zhai et al., 2023). Meanwhile, a 96% food-grade ethanol solution, dried under conditions identical to those used for the treatment groups, served as a negative control (Group A). After incubation, the inhibition zone was measured using a digital caliper with an accuracy of 0.01 mm.

Postbiotics production with _Bacillus_ consortium enriched with _Avicennia marina_ leaf powder

The postbiotic production procedure begins with preparing a basal medium. All medium ingredients, including peptone (1.0% w/v), sodium hydrogen phosphate (Na2HPO4, 0.4% w/v; Merck KGaA, Germany), sodium dihydrogen phosphate (NaH2PO4, 0.4% w/v; Merck KGaA, Germany), magnesium sulfate heptahydrate (MgSO4.7H2O, 0.02%

w/v; Merck KGaA, Germany), and Tween 80 (0.10% w/v; Merck KGaA, Germany), were dissolved in 100 mL of sterile water, homogenized on a magnetic stirrer and hotplate at 45°C for 10 minutes, and sterilized in an autoclave at 121°C for 15 minutes, following standard basal media for Bacillus cultivation (Atlas, 2010). After sterilization, the liquid medium was cooled to 30°C, and 1.5% (w/v) glucose was added to all treatments. After the glucose dissolved with magnetic stirring, sterile water was added to bring the final volume to 198 mL before inoculation. All treatments were carried out in three replicates. For postbiotic production, dried ALP, prepared as previously described, was used directly as a substrate without prior solvent extraction. The basal medium without any ALP addition was designated as the baseline control group (BC). In the treatment groups, ALP was added at concentrations of 1.0%, 2.0%, and 3.0% (w/v). All groups, including the BC and graded ALP treatments, were provided with a standard 1.5% (w/v) glucose concentration as the main carbon source. The pH was adjusted to 7.0, and sterile water was added to bring the final volume to 198 mL before inoculation. The same 0.5 McFarland standard suspension (approximately 1.5×10^8 CFU mL was used as the inoculum. All treatments were inoculated with 2.0 mL of the *Bacillus* consortium to achieve a final concentration of 1% (v/v) in a total volume of 200 mL, then incubated at 30°C for 24 to 48 hours (Tong et al., 2023; Chen et al., 2026).

Bacterial viability analysis

Bacterial viability assessment was conducted in two stages, with three replications. The initial analysis was performed on liquid cultures obtained prior to the inactivation steps of postbiotic production, by counting bacteria (30-300 colonies) in serial dilutions (10^{-1} to 10^{-5}), expressed as CFU mL^{-1}. Postbiotic production was carried out through inactivation (heating) steps using a universal laboratory oven at 65°C (Memmert, Germany) for 10 minutes, two centrifugations at 10,000 and 12,000 rpm for 10 minutes each, and two filtrations using 25 mm hydrophilic polyethersulfone syringe filters (0.22 µm membrane; OneMed, Indonesia), with the final product being a postbiotic suspension (Paul Beulah and Rajasekar, 2024). The second test, using the same counting method as the initial test, was conducted on the postbiotic suspension to enumerate bacterial colonies.

Liquid chromatography-high-resolution mass spectrometry

The LC-HRMS was employed for untargeted metabolomic analysis following the protocols outlined by Susanto et al. (2026). Metabolomic analysis began with sample preparation. A 200 µL aliquot of the postbiotic suspension sample was immediately mixed with a precooled extracting solution of methanol, acetonitrile, and an aqueous solution (2:2:1, v/v) to induce rapid cooling and quenching, then homogenized by vortexing. The separation of chemical components was carried out using the Vanquish Horizon UHPLC System (Thermo Scientific™, United States). This separation process used an Accucore™ C18 column with dimensions of 100 mm × 2.1 mm (internal diameter) and a particle size of 2.6 µm. For molecular mass identification, an Orbitrap Exploris 240 HRMS instrument (Thermo Scientific™, United States) was operated in full-scan mass spectrometry (Full MS) and data-dependent tandem mass spectrometry (dd-MS2) modes. The resolution levels applied to these measurements were 60,000 full width at half maximum (FWHM) for Full MS and 22,500 FWHM for MS2, respectively. Data processing was performed using Compound Discoverer version 3.3 software (Thermo Scientific™, United States). Potential metabolite annotation was performed using an automated workflow that leveraged multiple structural repositories. High-resolution MS/MS spectra were matched against the mzCloud (Jansen et al., 2022; HighChem LLC, 2025), LIPID MAPS (Sud et al., 2007), the Arita Lab Flavonoid Database (Arita and Suwa, 2008), the ChEBI database (Hastings et al., 2012), KEGG (Kanehisa and Goto, 2000), the Natural Product Atlas (van Santen et al., 2022), and PubMed (Sayers et al., 2022) references for metabolite structure verification.

Antibacterial activity test of postbiotics against aquaculture pathogenic bacteria

Antibacterial activity was assessed against five types of pathogenic bacteria in aquaculture using postbiotic samples derived from an ALP-enriched *Bacillus* consortium. The antibacterial activity analysis employed disc diffusion, broth microdilution, and subculture techniques. *Streptococcus agalactiae* ATCC 12386, *A. hydrophila* ATCC 7966, *V. harveyi* ATCC 14126, *V. parahaemolyticus* ATCC 17802, and *V. alginolyticus* ATCC 17749 were obtained from the collections of the laboratories of the Center for Brackish Water Aquaculture (Jepara, Indonesia), the Center for Fish Health and Environmental Testing (Serang, Indonesia), and the Technical Implementation Unit for Testing and Application of Fishery Product Quality (Serang, Indonesia). The bacterial suspension was adjusted to a 0.5 McFarland turbidity standard, which corresponded to approximately 1.5×10^8 CFU mL^{-1}. The actual CFU count was then confirmed by serial dilution and plate counting.

Disk diffusion assay of postbiotic against aquaculture pathogenic bacteria

The disc diffusion assay using postbiotic samples followed the CLSI (2020) guidelines and was performed on MHA for *A. hydrophila*, on MHA supplemented with 5% (0.05 mL mL^{-1}) sterile lysed horse blood for *S. agalactiae*, and on

MHA supplemented with 1% (10 mg mL^{-1}) analytical-grade sodium chloride (NaCl) for the three *Vibrio* species. The inoculation was performed by dipping a sterile cotton swab into the standardized bacterial suspension and rotating it firmly against the inside wall of the tube to remove excess liquid. The swab was then streaked evenly in three directions across the entire agar surface to ensure a uniform bacterial load and avoid liquid pooling. Following inoculation, sterile 6-mm paper discs pre-impregnated with 10 µL of undiluted postbiotic (100% v/v) were placed onto the inoculated agar medium at approximately 24 mm apart. The treatment medium was then inverted and incubated at approximately 28°C for approximately 24 hours.

Minimum inhibitory concentration of postbiotic against aquaculture pathogenic bacteria

The broth microdilution method was performed to determine the minimum inhibitory concentration (MIC) in accordance with CLSI (2020) guidelines, using twofold serial dilutions with three replicates. Standard Cation-Adjusted Mueller-Hinton Broth (CAMHB; Merck KGaA, Germany) was used for *A. hydrophila*, whereas for *S. agalactiae*, the CAMHB medium was supplemented with 5% sterile lysed horse blood. Additionally, CAMHB supplemented with 1% NaCl was utilized for the three *Vibrio* species. In the initial stage, 50 µL of sterile medium was added to all wells of a 96-well microplate (well rows 2 to 12). Then, 100 µL of undiluted postbiotic stock (100% v/v) was added to well column 1. A total of 50 µL of stock extract was transferred to well column 2, which already contained 50 µL of media, and homogenized using a micropipette with the up-and-down pipetting technique. This dilution process (1:2) was repeated sequentially up to the tenth well column, until a uniform volume (50 µL) was obtained in all well columns. The initial concentration ranged from well columns 1 to 10, from 100% v/v to 0.19% v/v. In the final step, 50 µL of bacterial suspension was prepared in media at a density of 1×10^6 CFU mL^{-1} and inoculated into each well (except well 11), resulting in a concentration in wells 1 to 10 of 50% to 0.09% v/v while achieving a final standard inoculum concentration of 5×10^5 CFU mL^{-1}. Well 11 served as the growth control (bacteria without postbiotic). The 96-well microtiter plates containing the treatments were then incubated at 28°C for 24 hours. MIC values were considered valid only when the growth control exhibited turbidity.

Minimum bactericidal concentration of postbiotic against aquaculture pathogenic bacteria

A subculture assay was performed to determine the minimum bactericidal concentration (MBC), with three replicates. To determine the MBC value, 50 µL was taken from the well with a concentration equal to double the MIC (2× MIC) and from the growth control well. This suspension was inoculated onto standard MHA for *A. hydrophila*, MHA with 5% sterile lysed horse blood for *S. agalactiae*, and MHA with 1% NaCl for the three *Vibrio* species. The agar plate containing the inoculum was incubated at 28°C for 24 hours. After the incubation period, the number of live colonies (30-300) was counted, expressed as CFU mL^{-1}.

Data analysis

Experimental data, including quantitative bioassays, semi-quantitative evaluations, and untargeted chemical profiling, were analyzed using IBM SPSS Statistics version 26.0 (IBM Corp., USA). Quantitative zones of inhibition for extract-treated *Bacillus* consortium (six treatments, three replicates) and postbiotic-treated aquaculture pathogens (five treatments, three replicates) were assessed using the Kruskal-Wallis H-test to determine the overall effects of the treatments and presented as mean ± SD. Subsequently, Dunn's pairwise comparisons ($p < 0.05$) were performed, with Bonferroni-adjusted p-values applied solely to the extract-treated groups. Categorical postbiotic data from broth microdilution and subculture outcomes for pathogens were analyzed using Fisher's exact test followed by pairwise Z-tests. Bacterial viability counts (4 treatments, 3 replicates) were subjected to Log_{10} transformation and subsequently analyzed using a one-way ANOVA with Tukey's post-hoc test, following verification of normality (Shapiro-Wilk) and homogeneity of variances (Levene's) assumptions. Finally, the chemical profiles, which included one BC group and three postbiotic dosage treatment groups containing ALP at 1%, 2%, and 3% (w/v), were filtered by peak area using a stringent fold-change (FC) threshold. Confident identified metabolites exhibiting a two-fold or greater enrichment ($FC \geq 2.0$) compared to the BC group were highlighted as dose-responsive indicators to capture prominent biological changes (Smith et al., 2006). However, due to the single-replicate design of the metabolomics analysis, a more conservative FC threshold of ≥ 5.0 was subsequently applied to filter confidently identified metabolites and minimize false positives. Subsequently, the antibacterial potency of the identified metabolites was predicted *in silico* via PASS Online by submitting the canonical Simplified Molecular-Input Line-Entry System (SMILES) string for each metabolite, retrieved from the PubChem database. Compounds with a probability of being active (*Pa*) greater than the probability of being inactive (*Pi*) were considered to have potential antibacterial activity (Ks et al., 2026).

RESULTS

Bacillus consortium resistance against *Avicennia marina* leaf extract

The present results revealed a significant overall difference in inhibition zone diameters among the treatment groups ($p < 0.05$; Figure 1). Further comparative analysis confirmed that the negative control (Group A) and the 25 mg mL^{-1} *A. marina* leaf extract treatment (Group B) had the same mean diameter (6.00 ± 0.00 mm), indicating no inhibitory activity. A gradual increase in inhibition zone diameters was observed in groups C (6.42 ± 0.01 mm), D (6.83 ± 0.02 mm), and E (7.17 ± 0.04 mm), resulting in minor inhibition zones. The present results indicated that groups C and D did not differ significantly from Group A ($p > 0.05$). Group E differed significantly from groups A and B ($p < 0.05$), while no significant differences were observed among groups C, D, and E ($p > 0.05$). The current results indicated no significant difference among groups C, D, and E ($p > 0.05$). The positive control produced significantly larger inhibition zones (23.78 ± 0.16 mm) compared to all extract-treated groups ($p < 0.05$). All extract treatment groups (B to E) produced inhibition zones below the 10 mm resistance threshold, categorizing the bacterial consortium as resistant to the *A. marina* leaf extract (Burhanuddin et al., 2019; Irkitova et al., 2021).

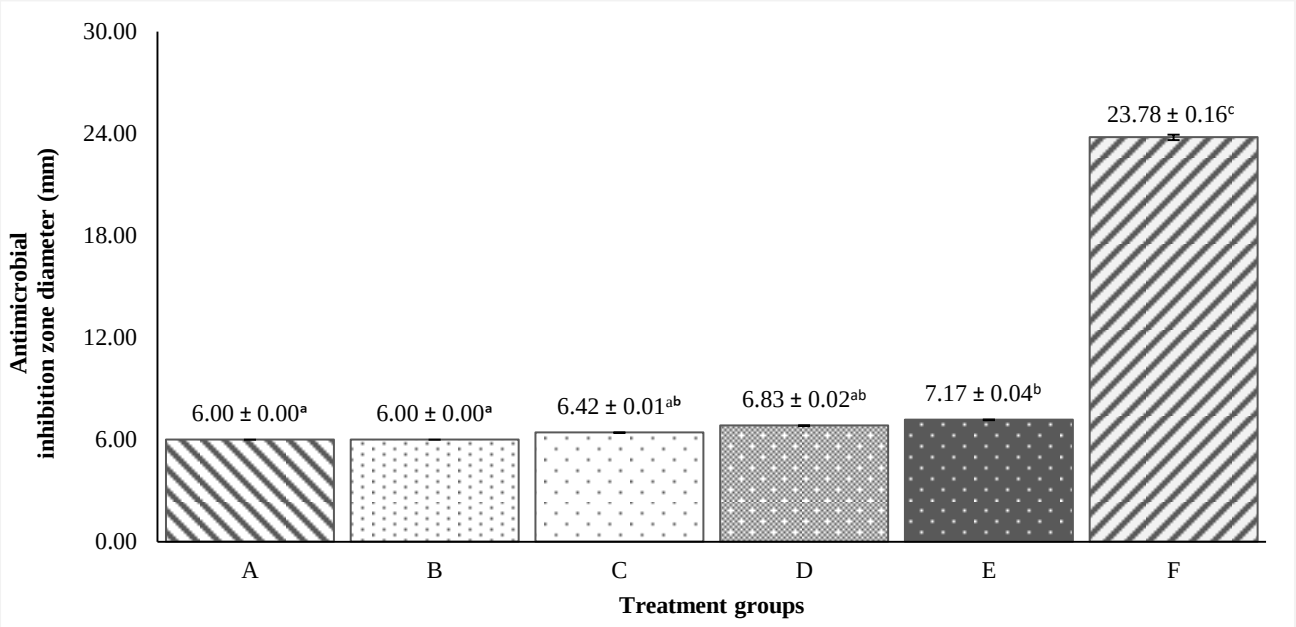


Figure 1. Antimicrobial inhibition zone diameters in the disk diffusion assay of *Bacillus* consortium enriched with *Avicennia marina* leaf extract. [a,b,c] Mean different superscript letters indicate significant differences across the groups ($p < 0.05$). **A:** Negative control group (ethanol), **B:** 25 mg mL^{-1} of *A. marina* leaf extract, **C:** 50 mg mL^{-1} of *A. marina* leaf extract, **D:** 75 mg mL^{-1} of *A. marina* leaf extract, **E:** 100 mg mL^{-1} concentration of *A. marina* leaf extract, **F:** Positive control group (Ciprofloxacin). Data are presented as mean ± SD.

Postbiotic bacterial viability

Bacterial viability was assessed to determine the effects of different ALP concentrations (1%, 2%, and 3%) and the BC group before and after thermal inactivation, centrifugation, and filtration (Figure 2). The viability of the *Bacillus* consortium in the BC group was 7.86 ± 0.012 $\log_{10}$ CFU mL^{-1}. Prior to inactivation, centrifugation, and filtration, the addition of ALP resulted in a significant, concentration-dependent increase in bacterial viability compared to the BC group ($p < 0.05$). Specifically, the bacterial viability increased to 8.80 ± 0.007 $\log_{10}$ CFU mL^{-1} in the 1% ALP group, 8.98 ± 0.009 $\log_{10}$ CFU mL^{-1} in the 2% ALP group, and 9.08 ± 0.010 $\log_{10}$ CFU mL^{-1} in the 3% ALP group, with all treatment groups being significantly different from both the control and each other ($p < 0.05$). In contrast, the viability of the *Bacillus* consortium at all postbiotic concentrations (including the control) after inactivation, centrifugation, and filtration demonstrated no significant differences ($p > 0.05$). The bacterial viability values across all treatment groups, including the BC group, were zero, indicating that the postbiotic suspension contained no live bacteria. Comparison of these findings indicated that adding a substrate (prebiotic) before inactivation increased bacterial viability in culture, with higher ALP concentration promoting greater bacterial growth. Meanwhile, the bacterial viability values after inactivation, centrifugation, and filtration indicated compliance with the scope of postbiotics, which defines postbiotics as preparations without live microorganisms (Thakur et al., 2025).

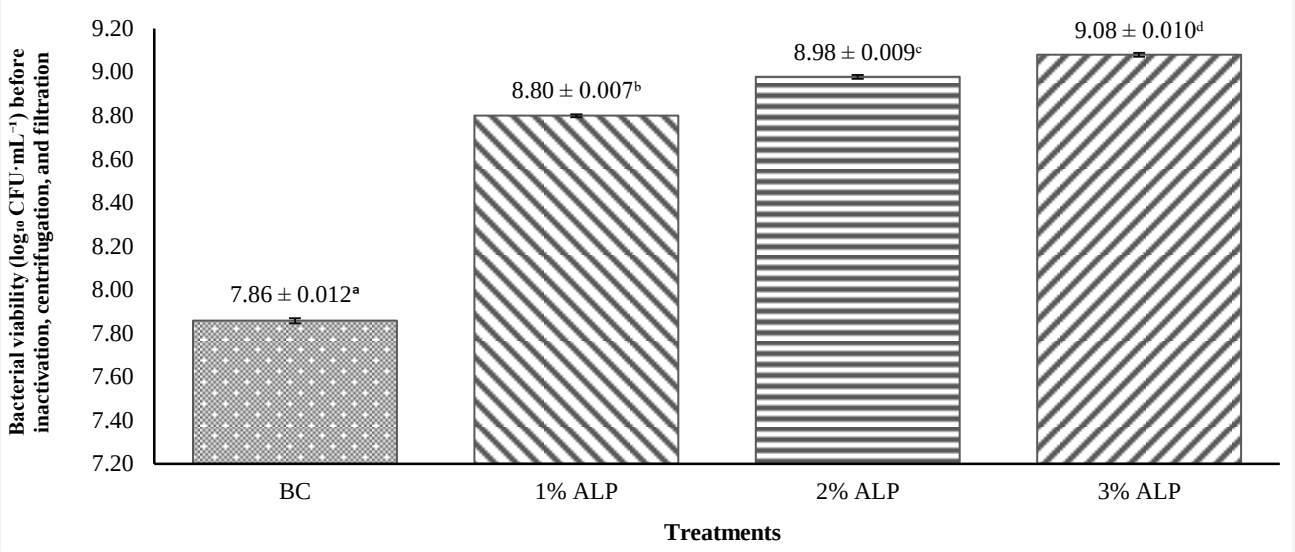


Figure 2. Bacterial viability values before inactivation, centrifugation, and filtration of *Avicennia marina* leaf powder. [a,b,c,d] Mean different superscript letters indicate significant differences across the treatment groups ($p < 0.05$). BC: Baseline control group, 1% ALP: *A. marina* leaf powder at 1%, 2% ALP: *A. marina* leaf powder at 2%, 3% ALP: *A. marina* leaf powder at 3%. Data are presented as mean ± SD.

Metabolomics profiling and *in silico* screening of antibacterial potency of postbiotic

Initial untargeted metabolomics screening via LC-HRMS revealed a concentration-dependent increase in the total number of identified metabolites. In the BC group, 384 metabolites were detected, increasing to 493 in the 1% ALP group, 515 in the 2% ALP group, and peaking at 543 in the 3% ALP group. The peak area of each identified metabolite was normalized to its relative abundance (constant-sum normalization). Initial screening using a fold-change threshold of FC > 2.0 compared to the BC group yielded 21 candidate metabolites with four accumulation profile trends (Table 1). According to Table 1, Profile 1, the kinetic pattern demonstrated a linear increase and was characterized by three main metabolites, with the largest increase in peak area observed in the 3% ALP group for Cyclo(-L-Ala-L-Tyr), corresponding to an FC of 8.00. The Versicomide D metabolite exhibited the largest increase in the 2% ALP group, with an FC of 12.70, followed by (R)-Lactaldehyde (FC = 10.34; Table 1, Profile 2). In contrast to the increasing trend observed in Profile 2, Profile 3 demonstrated a consistent decrease in metabolite accumulation as ALP concentration increased. The sharpest decrease in the 3% ALP group was for Tenacibactin B metabolite, with an FC of 4.73. Finally, Table 1, Profile 4 exhibited a biphasic trend dominated by D-(-)-Ribose, which had a sharp increase in the 1% ALP group (FC = 40.99), followed by a sharp decline in the 2% ALP group (FC = 20.56), and a subsequent increase in the 3% ALP group (FC = 20.66).

In addition to the *in vitro* kinetic profile evaluation, an *in silico* analysis using the online PASS tool was conducted to predict the likelihood of metabolite antibacterial activity. The PASS software predicted antibacterial activity when the *Pa* value is higher than *Pi*. A *Pa* value greater than 0.3 indicated moderate activity with high structural novelty, and a value greater than 0.7 suggested a high probability of structural similarity to existing medicines (Ks et al., 2026). In general, the resulting exposure parameters indicated that most identified secondary metabolite candidates had *Pa* values greater than *Pi* (*Pa* > *Pi*), suggesting strong experimental potential for antibacterial activity. Among the main metabolites identified in Profile 1, the metabolite Cyclo-(L-Ala-L-Tyr) demonstrated the highest probability of antibacterial activity, with a *Pa* value of 0.325 and a very low *Pi* value of 0.051. Several key metabolites identified in the present study exhibited notable antibacterial potential, including (R)-Lactaldehyde, Cereusitin A, and FL6DDAGI0001. Conversely, 3-(Hexanoylamino)-2-(pentyloxy)propyl 2-(trimethylammonio)ethyl phosphate was detected as having no antibacterial activity, with *Pa* and *Pi* values of zero. In the present study, based on the FC value (FC > 2) and interpretation criteria (*Pa* > 0.3), the addition of ALP to the basic growth medium of *Bacillus* consortium bacteria indicated successful enrichment of 10 antibacterial metabolites. However, because this study used a single-replicate design for metabolomics analysis, biological variance could not be distinguished from technical artifacts. Therefore, a highly conservative FC threshold (FC > 5) was subsequently applied to filter confidently identified metabolites and minimize false positives. Applying a FC limit, MS/dd-MS2 fragmentation analysis detected the accumulation of five key compounds, including Cyclo-(L-Ala-L-Tyr), (R)-Lactaldehyde, (+)-Flavipucine, 1-[(2R)-3-Cyclopentyl-2-{[formyl(hydroxy)amino]methyl}propanoyl]-N-(ethylcarbamoyl)-L-prolinamide, and D-(-)-Ribose. Prioritizing metabolites with FC > 5 provided a strong indication that the substantial accumulation of these compounds resulted from ALP supplementation.

Table 1. Metabolomic profiling, graded fold-change kinetics, and *in silico* antibacterial potency scores (*Pa*/*Pi*) of the 21 selected candidate secondary metabolites from the postbiotic made by the *Bacillus* consortium enriched with *Avicennia marina* leaf powder

Identified metabolite name	Molecular formula	ADM (ppm)	Calc.MW	m/z	RT (minutes)	Reference ion	Peak area (BC)	Fold-change (FC) > 2.0			*Pa*	*Pi*
								1% ALP	2% ALP	3% ALP		
								Kinetic profile 1				
Cyclo-(L-Ala-L-Tyr)	$C_{12}H_{14}N_2O_3$	1.55	234.1008	235.10807	2.406	[M+H]+1	1478946.4	6.53	7.88	8.00	0.325	0.051
leu-gly-leu	$C_{14}H_{27}N_3O_4$	0.84	301.20041	302.20768	3.905	[M+H]+1	4520442.6	3.87	5.59	7.47	0.188	0.130
Tenacibactin A	$C_{15}H_{28}N_2O_5$	-0.33	316.19972	315.19267	5.518	[M-H]-1	1330985.2	4.33	5.60	6.32	0.251	0.082
								Kinetic profile 2				
Thymidine	$C_{10}H_{14}N_2O_5$	0.47	242.09038	243.09769	0.889	[M+H]+1	14654097.8	2.08	2.41	2.26	0.432	0.024
3-(Hexanoylamino)-2-(pentyloxy)propyl 2-(trimethylammonio)ethyl phosphate	$C_{19}H_{41}N_2O_6P$	-3.79	424.26862	425.27591	3.991	[M+H]+1	269221675.7	2.48	2.75	2.19	0.000	0.000
met-pro	$C_{10}H_{18}N_2O_3S$	1.27	246.10413	247.1114	1.878	[M+H]+1	17140378.3	2.28	2.94	2.60	0.178	0.027
N-Acetyl-D-alloisoleucine	$C_8H_{15}NO_3$	0.55	173.10529	172.098	3.54	[M-H]-1	9238325.8	2.84	3.97	2.70	0.277	0.069
Succinic acid	$C_4H_6O_4$	0.25	118.02664	117.01936	0.902	[M-H]-1	417138243.6	5.07	9.02	8.11	0.288	0.065
(R)-Lactaldehyde	$C_3H_6O_2$	-0.03	74.03678	73.0295	0.902	[M-H]-1	50343018.9	5.34	10.34	9.02	0.390	0.033
trans-Cinnamic acid	$C_9H_8O_2$	0.45	148.0525	147.04522	3.912	[M-H]-1	26890389.2	2.41	4.38	2.71	0.312	0.056
TRILAURYLAMINE	$C_{36}H_{75}N$	0.08	521.58999	522.59727	18.761	[M+H]+1	15551314.7	2.83	3.03	2.83	0.224	0.099
Versicomide D	$C_{19}H_{25}N_3O_4$	1.61	359.18508	360.19236	3.438	[M+H]+1	11940373.3	10.31	12.70	11.22	0.193	0.124
								Kinetic profile 3				
Cereusitin A	$C_{25}H_{34}N_4O_6$	0.42	486.24804	485.24051	5.899	[M-H]-1	2251035.9	2.59	2.40	2.05	0.345	0.044
Indole-3-lactic acid	$C_{11}H_{11}NO_3$	0.13	205.07392	238.10739	4.353	[M+H+MeOH]+1	6855156.2	6.20	5.30	4.15	0.127	0.108
Tenacibactin B	$C_{14}H_{26}N_2O_5$	0.41	302.1843	301.17706	4.547	[M-H]-1	1408088.6	9.03	5.48	4.73	0.276	0.069
								Kinetic profile 4				
(+)-Flavipucine	$C_{12}H_{15}NO_4$	0.59	237.10025	236.09297	4.346	[M-H]-1	8444567.6	5.81	4.94	5.35	0.439	0.023
1-[(2R)-3-Cyclopentyl-2-{[formyl(hydroxy)amino]methyl}propanoyl]-N-(ethylcarbamoyl)-L-prolinamide	$C_{18}H_{30}N_4O_5$	1.12	382.22205	381.21385	3.097	[M-H]-1	4423280.0	7.87	7.02	7.13	0.452	0.021
Acetyl-L-carnitine	$C_9H_{17}NO_4$	0.32	203.11582	202.10855	3.823	[M-H]-1	36399307.2	3.27	2.33	2.94	0.186	0.131
D-(-)-Ribose	$C_5H_{10}O_5$	0.23	150.05286	195.05106	0.761	[M+FA-H]-1	11045182.7	40.99	20.56	20.66	0.378	0.036
FL6DDAGI0001	$C_{22}H_{26}O_6$	0.03	386.17295	387.18023	8.846	[M+H]+1	5282553.8	3.06	2.23	4.16	0.464	0.020
Streptodiketopiperazine A	$C_{13}H_{14}N_2O_2$	1.03	230.10577	231.11304	2.999	[M+H]+1	5412984.0	4.79	4.14	5.86	0.358	0.041

ADM: Annotated deltamass (ppm), Calc. MW: Calculated molecular weight, m/z: Mass-to-charge ratio, RT: Retention time (minutes), ALP: *A. marina* leaf powder, *Pa*/*Pi*: *In silico* antibacterial potency scores, *Pa*: Probability of activity, *Pi*: Probability of inactivity, BC: Baseline control group, 1% ALP: *A. marina* leaf powder at 1%, 2% ALP: *A. marina* leaf powder at 2%, 3% ALP: *A. marina* leaf powder at 3%.

Antibacterial activity of postbiotics against pathogenic bacteria

To assess the broad-spectrum antimicrobial effectiveness of postbiotics, 3% ALP was selected for its highest pre-inactivation cell biomass and greatest chemical diversity. The antimicrobial efficacy of postbiotics was evaluated by measuring the inhibition zone diameter against five target aquaculture bacterial pathogens (Figure 3). There were significant differences in the distribution of antimicrobial inhibition zones among the evaluated pathogenic bacteria ($p < 0.05$). The current results demonstrated that *V. parahaemolyticus* had the largest inhibition zone diameter, at 15.30 ± 0.53 mm, which was not significantly different from that of *V. alginolyticus*, at 14.85 ± 0.22 mm ($p > 0.05$). A moderate inhibitory effect was observed against *V. harveyi*, which indicated an average clear zone diameter of 11.33 ± 0.58 mm. No inhibition zone was observed against *S. agalactiae* or *A. hydrophila* (disc diameter remained at 6.00 mm). The lack of variation for *S. agalactiae* and *A. hydrophila* reflected the absence of inhibition beyond the disc diameter. This negative result indicated a limitation of the current study, suggesting that the antimicrobial efficacy of this postbiotic might have been selective or strain-specific.

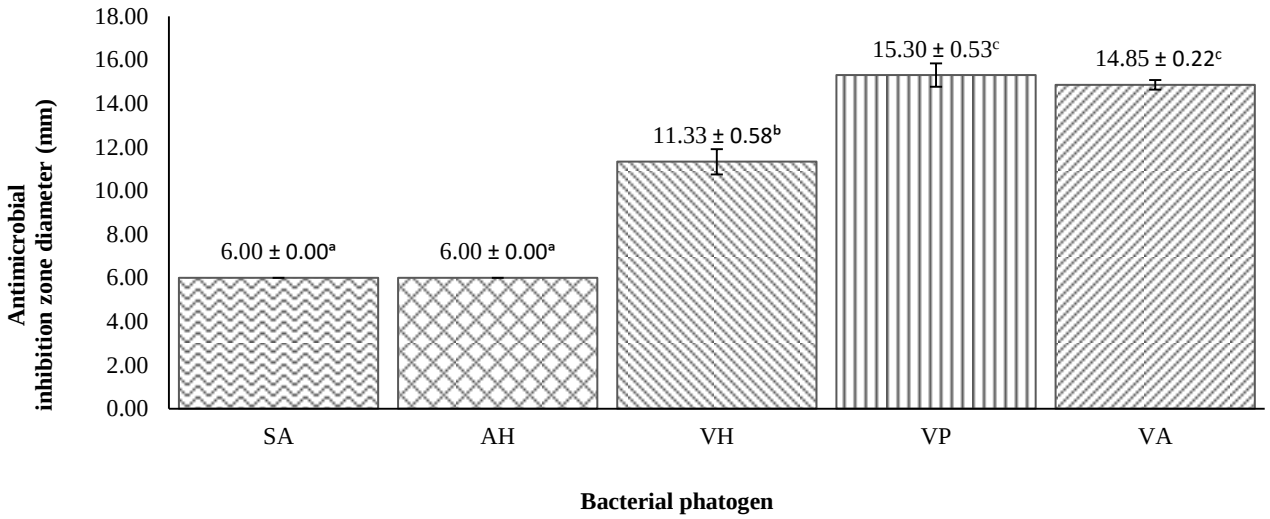


Figure 3. Antimicrobial inhibition zones against aquaculture pathogenic bacteria in the disk diffusion assay, using a 100% (v/v) concentration of the postbiotic derived from a *Bacillus* consortium enriched with 3% *Avicennia marina* leaf powder. [a,b] Mean different superscript letters indicated significant differences among the five evaluated aquaculture pathogens ($p < 0.05$). SA: *Streptococcus agalactiae*, AH: *Aeromonas hydrophila*, VH: *Vibrio harveyi*, VP: *Vibrio parahaemolyticus*, and VA: *Vibrio alginolyticus*. Data are presented as mean ± SD.

The antibacterial potency of postbiotics was further determined by evaluating the MIC and classifying the aquaculture pathogens into susceptibility categories (Table 2). There was a significant relationship between bacterial species and their respective susceptibility profiles ($p < 0.05$). The analysis results divided the five pathogens into two statistically distinct groups based on their sensitivity patterns. *Streptococcus agalactiae* was classified into a separate subgroup, exhibiting complete resistance to the postbiotic, with MIC values surpassing the highest concentration tested (>50% v/v; $p < 0.05$). Compared to the absolute resistance of *S. agalactiae*, all three biological replicates of the other four species (*A. hydrophila, V. harveyi, V. parahaemolyticus*, and *V. alginolyticus*) demonstrated significantly complete susceptibility to the postbiotic at concentrations ≤ 50% v/v ($p < 0.05$). Among the susceptible strains, different degrees of postbiotic sensitivity were observed based on actual MIC values. *Vibrio harveyi* indicated the highest sensitivity, requiring only a very low concentration of the postbiotic (1.6% v/v) to inhibit its growth. Moderate susceptibility was observed in *V. parahaemolyticus* at an MIC value of 12.5% v/v, followed by *A. hydrophila* and *V. alginolyticus*, both of which required a higher concentration of the postbiotic (25 % v/v) to inhibit growth. Negative disc diffusion results but positive MIC results for *A. hydrophila* highlighted the limitations of the present study. This discrepancy between negative disk diffusion results and positive MIC results for *A. hydrophila* might be due to poor diffusion of compounds in the agar and indicated that the antimicrobial efficacy of this specific postbiotic may be selective, highly strain-dependent, or influenced by the test medium.

To determine the bactericidal capacity of postbiotics, the MBC was evaluated, and pathogens were classified into the appropriate susceptibility categories (Table 3). There was a significant relationship between bacterial species and their bactericidal susceptibility profiles ($p < 0.05$). Further analysis divided the five aquaculture pathogens into two statistically distinct groups, consistent with the trend observed in the MIC analysis. *Streptococcus agalactiae* was isolated into a separate (resistant) sub-group, whereas *A. hydrophila*, *V. harveyi*, *V. parahaemolyticus*, and *V. alginolyticus* formed the susceptible group. Compared with the susceptible group, the actual MBC values for *S. agalactiae* at concentrations exceeding 50% v/v indicated that the postbiotic had no bactericidal activity against this species within the tested concentration range ($p < 0.05$). Conversely, all three biological replicates for these four species

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demonstrated a significant susceptibility to the postbiotic treatment at concentrations ≤ 50% v/v ($p < 0.05$). Examination of the actual volumetric concentrations within the susceptible groups revealed different degrees of bactericidal tolerance. *Vibrio harveyi* was the most susceptible pathogen, with the lowest MBC of 6.3% v/v. A moderate bactericidal susceptibility was observed for *V. parahaemolyticus* at 25% v/v. Meanwhile, *A. hydrophila* and *V. alginolyticus* indicated higher tolerance, requiring a postbiotic concentration of 50% v/v to achieve complete bacterial eradication. Based on the inhibition zone, MIC, and MBC values, the postbiotic exhibited selective antibacterial activity against aquaculture pathogens, with greatest efficacy against *Vibrio* species.

Table 2. Minimum inhibitory values and susceptibility categorization of the postbiotic made by the *Bacillus* consortium enriched with *Avicennia marina* leaf powder against aquaculture bacterial pathogens

Bacterial pathogen	Actual MIC value (% v/v)	Susceptible (≤ 50% v/v)	Resistant (> 50% v/v)	Z-Test grouping
	Statistical model	n (%)	n (%)	
Streptococcus agalactiae	> 50	0 (0.0%)	3 (100.0%)	a
Aeromonas hydrophila	25	3 (100.0%)	0 (0.0%)	b
Vibrio harveyi	1.6	3 (100.0%)	0 (0.0%)	b
Vibrio parahaemolyticus	12.5	3 (100.0%)	0 (0.0%)	b
Vibrio alginolyticus	25	3 (100.0%)	0 (0.0%)	b

Statistical model: The most frequently observed inhibitory concentration value among the biological replicates. Z-Test grouping: Different letters indicate statistically significant differences among the bacterial pathogens ($p < 0.05$). a: Resistant pathogen, b: Susceptible pathogens. n: Total number of replicates

Table 3. Minimum bactericidal values and susceptibility categorization of the postbiotic made by the *Bacillus* consortium enriched with *Avicennia marina* leaf powder against aquaculture bacterial pathogens

Bacterial pathogen	Actual MBC Value (% v/v)	Susceptible (≤ 50% v/v)	Resistant (> 50% v/v)	Z-Test grouping
	Statistical model	n (%)	n (%)	
Streptococcus agalactiae	> 50	0 (0.0%)	3 (100.0%)	a
Aeromonas hydrophila	50	3 (100.0%)	0 (0.0%)	b
Vibrio harveyi	6.3	3 (100.0%)	0 (0.0%)	b
Vibrio parahaemolyticus	25	3 (100.0%)	0 (0.0%)	b
Vibrio alginolyticus	50	3 (100.0%)	0 (0.0%)	b

Statistical model: The most frequently observed inhibitory concentration value among the biological replicates. Z-Test grouping: Different letters in the same column indicated statistically significant differences among the bacterial pathogens ($p < 0.05$). a: Resistant pathogen, b: Susceptible pathogens. n: Total number of replicates

DISCUSSION

The preliminary experiment demonstrated that the *Bacillus* consortium, comprising *B. safensis*, *B. mycoides*, and *B. pumilus*, exhibited a notably high level of tolerance to *A. marina* leaf extract. The inhibition zone observed in the disc diffusion assay was deemed biologically insignificant because its size was below the resistance threshold (<10 mm; Burhanuddin et al., 2019; Irkitova et al., 2021). The disc diffusion assay demonstrated that the *A. marina* extract exhibited negligible inhibitory activity against the *Bacillus* consortium in the present study. Previous studies have reported that *A. marina* leaf extract had direct antimicrobial activity against several Gram-positive bacteria (Okla et al., 2021; Sravya et al., 2025). However, the absence of an inhibitory zone in the present study indicated the biological compatibility between the halophyte plant substrate and the targeted bacterial isolate (Seukep et al., 2020). The present study was consistent with the findings of Ibrahim et al. (2022), who reported that ethanol, acetone, and ethyl acetate extracts of *A. marina* (100 mg mL^{-1} in dimethyl sulfoxide) exhibited small inhibition zones (< 7.6 mm) against *B. subtilis*. Species of the genus *Bacillus* are known to have robust adaptive mechanisms to environmental stressors and phytochemical compounds, primarily through alterations in the lipid composition of cell membranes and the activation of efflux systems (Diomandé et al., 2015; Seukep et al., 2020). Moreover, metabolic interactions within a multi-strain consortium often have greater collective protection than single cultures, due to extracellular matrix secretion or metabolic coordination (Azeem et al., 2025). Therefore, the negligible inhibition zones observed at concentrations of 50 to 100 mg mL^{-1}, along with the absence of an inhibition zone at 25 mg mL^{-1}, justified the utilization of ALP as a safe functional substrate in postbiotic production processes.

The incorporation of ALP was shown to notably enhance the viability of the bacterial consortium prior to the inactivation phase in a dose-dependent manner. The increase in bacterial viability from 7.86 ± 0.012 $\log_{10}$ CFU mL^{-1} (baseline control) to 9.08 ± 0.10 $\log_{10}$ CFU mL^{-1} in the 3% ALP group indicated the functional role of the mangrove leaf extract as a potential prebiotic carbon source. This finding is consistent with Goh et al. (2022), who reported that structural polysaccharides and plant-derived natural fibers can serve as non-digestible prebiotics. These compounds are selectively fermented by *Bacillus* spp. and provide a primary energy source for cell replication. The improved growth kinetics can be attributed to the effective use of complex sugars present in mangrove leaves, which are broken down into simple glucose by the innate extracellular hydrolytic enzymes of the *Bacillus* consortium (Sam-on et al., 2023). In addition, the nutrient profile of coastal halophyte plants has been reported to be rich in osmoprotectant compounds and specific micronutrients that synergistically trigger microbial anabolic metabolism, thereby enabling a massive increase in bacterial population density before entering the stationary phase (Rudnyckyj and Thomsen, 2026). In contrast to this massive pre-inactivation growth, after inactivation by heating, centrifugation, and double filtration, the residual viability of all postbiotic suspensions dropped to zero in the present experiment. The complete absence of viable cells confirmed that the final preparations were completely free of living microbial cells and met the International definition of a safe postbiotic preparation (Thakur et al., 2025).

Metabolomics analysis via LC-HRMS revealed potential changes in secondary metabolite profiles across different ALP concentrations. The 3% ALP treatment increased metabolites, and five key compounds were detected, including Cyclo(-L-Ala-L-Tyr), (R)-Lactaldehyde, (+)-Flavipucine, D-(-)-Ribose, and 1-[(2R)-3-Cyclopentyl-2-{[formyl(hydroxy)amino]methyl}propanoyl]-N-(ethylcarbamoyl)-L-prolinamide, which were classified as an Acylprolinamide derivative according to Axten et al. (2012) and NCBI (2026a). Based on the results of *in silico* screening, the majority of the detected compounds indicated *Pa* values greater than 0.3, suggesting potential antibacterial activity.

The presence of Cyclo(L-Ala-L-Tyr) in the BC group, according to the mechanism supported by Ryan et al. (2009), indicated that Cyclo(L-Ala-L-Tyr) originated from the utilization of free amino acids in the peptone component as the sole source of organic nitrogen in the base medium. Diketopiperazines are secondary metabolites that have been confirmed to be synthesized by *Bacillus* spp. and function as potential antimicrobial agents against target microbes, as well as serve as intercellular communication signaling molecules (Lu et al., 2009; Bofinger et al., 2017). The high level of Cyclo(L-Ala-L-Tyr) following the addition of ALP was likely due to an influx of plant-derived amino acids, which increased the nutrient reservoir in the fermentation medium, as evidenced by the findings of Hinokidani et al. (2020). Additionally, NATURAL phenolic compounds in the mangrove leaf powder act as organic elicitors, serving as low-level environmental stressors. According to the findings of Bofinger et al. (2017) and Patil et al. (2024), the environmental stress induced by the phenolic compounds triggers a bacterial defense response that upregulates gene transcription, resulting in much higher excretion levels of defense peptides such as Cyclo(L-Ala-L-Tyr).

Furthermore, other compounds, such as (R)-Lactaldehyde in the BC group, were derived from the catabolism of glucose, the main carbon source in the baseline medium. This observation was consistent with the findings of Wang et al. (2024), who described that lactaldehyde served as a key biochemical intermediate in the methylglyoxal pathway during glucose catabolism. The notable rise in (R)-Lactaldehyde levels following ALP addition may have resulted from increased glycolytic flux, driven by additional glucose released from mangrove fiber degradation, consistent with the findings of Mehta et al. (2024). The accumulation of (R)-Lactaldehyde contributed to the antibacterial potential of the postbiotic prepared in the present study, which was aligned with the findings of Aljaafari et al. (2022), who reported that (R)-Lactaldehyde could function as a reactive metabolite compound possessing natural inhibitory properties against the growth of pathogenic bacteria.

The initial LC-HRMS annotation identified (+)-Flavipucine, yet manual investigations revealed that this metabolite was taxonomically and biologically irrelevant to the candidate compounds in the *Bacillus* spp. expression system and their antibacterial potential. (+)-Flavipucine is unlikely to produce a dominant signal in negative ionization mass spectrometry (Loesgen et al., 2011; Kruve et al., 2014; NCBI, 2026b). Therefore, the $[M-H]^-$ signal indicates a higher likelihood of N-Lactoylphenylalanine (Lac-Phe), a pseudo-dipeptide that possesses a similar molecular formula and molecular weight (NCBI, 2026c). In the present study, conducting manual investigations of these structural and taxonomic metabolites was crucial for eliminating automated misannotations and directly guiding subsequent antibacterial screening towards a biologically plausible metabolite produced by the *Bacillus* spp. system. In the present study, the detected $[M-H]^-$ signal yielded an experimental m/z value of 236.09297, with a high mass accuracy of 0.59 ppm, perfectly matching the exact theoretical mass of Lac-Phe (Hoene et al., 2022). From a chemotaxonomic perspective, N-Lactoylphenylalanine (Lac-Phe) is a metabolite more likely to be synthesized by *Bacillus* spp. in the medium (Udrea et al., 2026). However, the *in silico* prediction screening results for Lac-Phe indicated *Pa* (0.180) and *Pi* (0.137) values that did not meet the criteria for antibacterial activity. Manual selection confirmed that the metabolite was

N-Lactoylphenylalanine (Lac-Phe) rather than the database-annotated (+)-Flavipucine. However, Lac-Phe was excluded as an antibacterial candidate due to a low predicted activity score (*Pa* < 0.3).

The presence of acylprolinamide derivatives in the control group indicated that the *Bacillus* consortium likely synthesized acyl-amide-based secondary metabolites naturally, using the growth medium to support their basal metabolic activity (Stecker et al., 2022; Shiri et al., 2023). The increase in compound production following the addition of mangrove leaf powder might be linked to environmental stress-elicitation mechanisms (Razzaq et al., 2025). A 1% ALP treatment was believed to induce a hormetic effect strong enough to activate a bacterial defense response. Meanwhile, fluctuations in the results of 2% and 3% ALP treatments suggested that exposure to mangrove secondary metabolites at higher doses induced environmental stress, potentially shifting cellular energy from secondary biosynthesis to essential survival processes. The accumulation of acylprolinamide derivatives was believed to contribute to antibacterial activity, as reported by Axten et al. (2012), that the acylprolinamide group has the potential for broad-spectrum antibacterial activity. The detection of D-(-)-Ribose in the BC group might be attributed to pentose phosphate pathway metabolism, which occurred during glucose oxidation in the basal media (Liu et al., 2023). However, the D-(-)-Ribose peak area increased following the addition of ALP, likely because the inherent hemicellulosic components of *A. marina* leaves (Almardeai et al., 2017) provided the *Bacillus* consortium with additional precursors for pentose sugar accumulation. The D-(-)-Ribose peak demonstrated a notable increase in the overall group of pentose sugars. The accumulation of D-(-)-Ribose in postbiotics in the present study was an important finding because it has been shown to be effective as an adjuvant that restores the sensitivity of resistant bacteria to standard antibiotics such as gentamicin (Zhou et al., 2022).

The present study focused exclusively on four key compounds found in both the control and treatment groups, namely Cyclo(-L-Ala-L-Tyr), (R)-Lactaldehyde, an acylprolinamide derivative, and D-(-)-Ribose. The focus on these four metabolites was driven by the specific objective of assessing the impact of ALP supplementation, as a quantitative stimulant, on the abundance of metabolites identified in the *Bacillus* expression system.

The antibacterial efficacy of the postbiotic, based on a *Bacillus* consortium enriched with 3% ALP, showed a distinctive selectivity pattern, with the highest inhibitory activity against marine pathogenic *Vibrio* bacteria. Based on the diameter of the inhibition zone, the most extensive response was recorded against *V. parahaemolyticus* and *V. harveyi*. The Current findings strongly indicate the underlying functional capacity of key compounds that exhibited increased abundance following ALP supplementation. According to Leyton et al. (2012), the genus *Bacillus* produces diketopiperazine compounds that act as quorum-sensing antagonists against *Vibrio* spp., strongly reducing virulence and biofilm formation. Furthermore, the increased abundance of D-(-)-Ribose in the postbiotic formulation enriched with 3% ALP was believed to provide a unique synergistic effect. Consistent with the present study, Niazy (2021) reported that at specific concentrations, D-ribose can interfere with the quorum-sensing pathway (AI-2) and downregulate luxS expression, thereby remarkably inhibiting biofilm maturation in *Vibrio* species.

In the microdilution evaluation assays, *V. harveyi* was identified as the most sensitive strain with an MIC of 1.6% v/v and an MBC of 6.3% v/v. The observed high sensitivity indicated that the outer membrane of *V. harveyi* was especially susceptible to the enzyme-targeting small molecules present within the postbiotic. The physiological susceptibility of *V. harveyi* aligned with the ability of acylprolinamide derivatives to penetrate the Gram-negative membrane and competitively inhibit peptide deformylase (PDF), thereby preventing the post-translational maturation of essential proteins (Mamelli et al., 2009; Axten et al., 2012). The observed intracellular enzymatic inhibition was indicated to act in conjunction with (R)-Lactaldehyde (Jabri et al., 2023). In the present study, these reactions likely facilitated the detoxification of methylglyoxal and maintained the overall efficacy of the postbiotic matrix against pathogenic bacteria.

In contrast to the susceptibility observed in the Gram-negative strain (*V. harveyi*), the 3% ALP postbiotic exhibited no meaningful antibacterial efficacy against the Gram-positive pathogen, *S. agalactiae*, indicating absolute resistance, with MIC and MBC values exceeding the maximum tested concentration (> 50% v/v). This result contradicted some previous studies that found *A. marina* leaf extracts generally exhibit broad-spectrum activity against Gram-positive bacteria due to the absence of a selective outer membrane (Okla et al., 2021; Sravya et al., 2025). Nevertheless, the observed resistance of *S. agalactiae* was consistent with previous findings in which Gram-positive bacteria exhibited lower susceptibility to *A. marina* extracts, which are characterized by predominantly polar antimicrobial agents (Ibrahim et al., 2022). This disparity indicated that the antibacterial activity of the 3% ALP postbiotic formulation was not caused by a general membrane disruption, as observed in typical organic solvent extracts, but instead resulted from specific molecular interactions. The resistance of S. agalactiae could be due to a structural barrier, such as a thick peptidoglycan layer, or the lack of specific molecular targets that Cyclo(L-Ala-L-Tyr) compounds and acylprolinamide derivatives can recognize (Fieulaine et al., 2016). Meanwhile, the moderate response of *A. hydrophila* to the 3% ALP postbiotic indicates differential susceptibility, likely due to variations in its lipopolysaccharide structure or efflux pump system.

These differences may necessitate a higher concentration of the active compound to effectively disrupt its cellular defense mechanisms (Gonzalez-Avila et al., 2021; Huang et al., 2022b).

CONCLUSION

The postbiotic formulation produced by the *Bacillus* consortium demonstrated favorable compatibility with the *A. marina* leaf substrate. The absence of an inhibition zone at 25 mg mL^{-1} of *A. marina* leaf extract, along with minimal zones (6.42 ± 0.02 mm to 7.17 ± 0.02 mm) exhibited at 50 to 100 mg mL^{-1}, indicated that *A. marina* leaf extract effectively acted as a growth-supporting factor. The 3% ALP treatment increased bacterial viability before the inactivation phase and enriched the identified metabolite profile, from 384 metabolites (baseline control) to 543 metabolites. Consequently, this process resulted in the accumulation of four key antibacterial candidate compounds, namely Cyclo(-L-Ala-L-Tyr), (R)-Lactaldehyde, an acylprolinamide derivative, and D-(-)-Ribose. *In vitro*, the postbiotic derived from the *Bacillus* consortium enriched with 3% ALP demonstrated selective antibacterial efficacy against the pathogenic *Vibrio* group, as evidenced by prominent inhibition zones ranging from 11.33 to 15.30 mm, low MIC (1.6-25% v/v), and low MBC (6.3-50% v/v) values. While both *S. agalactiae* and *A. hydrophila* indicated no inhibition zones in the disk diffusion assay, *A. hydrophila* demonstrated susceptibility in the broth microdilution method, in contrast to the complete resistance of *S. agalactiae* up to the highest tested concentration (MIC > 50% v/v). Although enhanced bacterial viability and enriched metabolite profiles indicated the postbiotic's antibacterial potential, the metabolomic screening was performed on a single biological replicate per treatment group; therefore, future studies should include biological replicates to strengthen the metabolomic data analysis.

DECLARATIONS

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Authors' contributions

Ashari Fahrurrozi conducted visualization, methodology, data collection, formal analysis, data management, conceptualization, and manuscript drafting. Slamet Budi Prayitno and Sarjito Sarjito contributed to the study design, conceptualization, methodology, supervision, and manuscript drafting. Aninditia Sabdaningsih managed supervision, administration, data processing, and drafted the manuscript. All authors have read and approved the final edition 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 declared that they have no conflicts of interest or personal relationships that could affect this article.

Ethical considerations

The authors declared that the manuscript is original, has not been published elsewhere, and that the latest edition was confirmed before publication. The authors declared the data's originality prior to submission and reaffirmed it during the peer-review process, assuming full responsibility for its originality. The authors declared that no AI tools were used for writing and preparing the article.

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Detection of Antibiotic Resistance Genes in *Salmonella* Strains Isolated from Fecal Samples of Dogs and Cats in Hanoi, Vietnam

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ABSTRACT

Salmonella belongs to the family *Enterobacteriaceae* and is a common pathogen that causes disease in humans and animals. Dogs and cats are potential sources of zoonotic diseases, including those caused by *Salmonella* spp. The current study aimed to investigate the prevalence and antibiotic resistance of *Salmonella* strains in fecal samples from dogs and cats collected at veterinary clinics in Hanoi, Vietnam. From November 2024 to December 2025, 161 fecal samples from dogs and cats were collected from 12 veterinary clinics in Hanoi, Vietnam, for *Salmonella* isolation. Fecal samples were cultured in peptone buffer water (PBW) and on selective media such as Rappaport-Vassiliadis soybean (RVS) broth and xylose lysine deoxycholate (XLD) agar; suspected *Salmonella* colonies were confirmed by biochemical tests, including sugar fermentation, H_2S, and IMViC reactions. Antibiotic susceptibility was tested by agar diffusion, and genes implicated in antimicrobial resistance were detected by PCR amplification. The results showed that 11 (6.8%) of 161 fecal samples were *Salmonella*-positive; *Salmonella* was detected in 9 of 92 (9.8%) dog fecal samples and 2 of 69 (2.9%) cat fecal samples. The isolates exhibited the highest resistance to tetracycline (90.0%), followed by streptomycin (81.2%). Resistance to chloramphenicol and trimethoprim/sulfamethoxazole was 45.5%; for nalidixic acid, amoxicillin/clavulanic acid, doxycycline, gentamicin, and cefotaxime, resistance rates ranged from 27.3% to 36.4%. The isolates showed similar resistance rates to neomycin, kanamycin, and ciprofloxacin at 18.2%. Twelve antibiotic resistance genes, including bla_{TEM}, *aadA1*, *aadA2*, *aphA1-1AB*, *aac (3')-IV*, *sul1*, *cmlA1*, *floR*, *tetA*, *tetB*, *qnrA*, and *qnrS*, were amplified in the antibiotic-resistant *Salmonella* strains, whereas bla_{PSE-1}, bla_{OXA-1}, *tetG*, *qnrB*, and *aac (6')-ib-cr* were not detected. The results of the current study underscore the importance of monitoring antibiotic resistance in *Salmonella* spp. in companion animals to protect public health.

Keywords: Antibiotic resistance gene, Cat, Dog, *Salmonella*

INTRODUCTION

Salmonella belongs to the family *Enterobacteriaceae*, and to date, more than 2500 *Salmonella* serovars have been identified across different hosts. These serovars are common pathogens that cause disease in humans and animals (Majowicz et al., 2010; Knodler et al., 2019). Dogs and cats are potential sources of zoonotic diseases, including those caused by *Salmonella* spp. (Leonard et al., 2012). However, transmission of *Salmonella* from dogs and cats to humans, in particular, has not received adequate attention (Bataller et al., 2020). Furthermore, studies on antibiotic and multidrug resistance, as well as the mechanisms underlying resistance in bacteria of dog and cat origin, are very limited (Leonard et al., 2012; Srisanga et al., 2017). In addition, the emergence and increase of multidrug-resistant *Salmonella* are significantly impacting treatment efficacy and leading to high mortality rates (WHO, 2016; Divek et al., 2018). Antibiotic resistance is projected to cause approximately 10 million deaths per year by 2050 and cost over $100 trillion worldwide (WHO, 2016).

In Vietnam, approximately 6.48 million dogs and 5.58 million cats are kept for various purposes, many of which are considered pets (Kirin Capital, 2023). Pet dogs and cats often live in the same area as their owners, and close interaction between pets and owners creates a potential pathway for disease transmission to humans (Pedersen et al., 2007; Leonard et al., 2012). However, studies on diseases in dogs and cats in Vietnam often focus on viral diseases such as rabies virus (RABV; Nguyen et al., 2011; Harada et al., 2026), canine distemper virus (CDV; Truong et al., 2022; Van et al., 2025), canine parvovirus type 2 (CPV-2; Hoang et al., 2019; Bui et al., 2023), and feline leukemia virus (FPV; Minh et al.,

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2023), and have not yet focused on bacterial infections such as *Escherichia coli* and *Salmonella* spp., as well as antibiotic resistance and the mechanisms of antibiotic resistance in these bacteria.

Numerous studies conducted in many countries have shown that *Salmonella* strains isolated from dogs and cats are resistant to many antibiotics and carry multiple antibiotic resistance genes (Kiflu et al., 2017; Srisanga et al., 2017; Yildiz and Demirbilek, 2024; Zhao et al., 2024). Currently, information on antibiotic resistance in bacteria, including *Salmonella*, isolated from dogs and cats in Vietnam is limited, highlighting the need for studies of pet-derived bacteria to support strategies to control zoonotic transmission. The present study aimed to determine the prevalence and antibiotic susceptibility of *Salmonella* strains isolated from dogs and cats in Northern Vietnam and to identify antibiotic resistance genes in antibiotic-resistant isolates.

MATERIALS AND METHODS

Ethical approval

The present study was conducted by collecting fecal samples from dogs and cats at veterinary clinics, in accordance with the animal welfare and safety procedures of the Committee on Animal Research and Ethics (CARE) at the Faculty of Veterinary Medicine, Vietnam National University of Agriculture, Vietnam (Approval No. CARE-2024/06). Before the study, ethical considerations were addressed, and pet owners' consent was obtained.

Sampling

From November 2024 to December 2025, 161 fecal samples from dogs (n = 92) and cats (n = 69) were randomly collected from twelve veterinary clinics in Hanoi, Vietnam. Healthy dogs and cats were those brought to the clinics for vaccinations, grooming, and deworming. Unhealthy dogs and cats were included in the study based on typical gastrointestinal symptoms such as diarrhea and were being treated at the clinics. The samples were collected according to the guidelines of QCVN 01-83:2011/BNNPTNT of the Ministry of Agriculture and Rural Development (MARD, 2011). Briefly, fresh fecal samples were carefully collected from each animal using rectal swabs with sterile cotton swabs. Each sample was placed in a separate sample bag and labeled with information such as age, gender, history of diarrhea, and history of antibiotic treatment. The samples were then placed in an ice box and transported to the Department of Veterinary Microbiology and Infectious Diseases, Faculty of Veterinary Medicine, Vietnam National University of Agriculture, for analysis within 24 hours.

Isolation of *Salmonella* spp.

Salmonella isolation from fecal samples followed the method described in the previous study (Bataller et al., 2020), with some modifications. In the laboratory, each fecal sample was homogenized with buffer peptone water (BPW; Merck, Germany) at a 1:9 ratio and incubated at 37°C for 18-24 hours for pre-enrichment. Next, 0.1 mL of the pre-enriched culture in BPW was added to 10 mL of Rappaport-Vassiliadis soya broth (RVS; Merck, Germany) and incubated at 42.5°C for 24-48 hours. Then, a loopful of RVS broth was streaked onto xylose lysine deoxycholate agar (XLD; Merck, Germany) and incubated at 37°C for 24 hours. Black colonies suspected of being *Salmonella* were selected from each plate and cultured on nutrient agar slants (NA; Merck, Germany). A single colony was randomly selected and subjected to biochemical tests, including sugar fermentation, H2S test, IMViC reactions in triple sugar iron (TSI) agar, Tryptone broth (TB), Methyl-red Voges-Proskauer (VP-MR) broth, and Simmons citrate agar (SCA, Merck, Germany). All isolates were stored in brain heart infusion broth (BHI; Merck, Germany) supplemented with 50% glycerol at -80°C for subsequent experiments.

Antibiotic susceptibility test

The antibiotic susceptibility test was performed according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2020). The agar diffusion method was performed on Mueller-Hinton agar (MHA, Merck, Germany) following Bauer et al. (1966). A total of 12 antibiotic agents (Oxoid, UK) from seven classes were used, including penicillins (amoxicillin/clavulanic acid [AMC, 20/10µg]), cephalosporins (cefotaxime [CTX, 30µg]), quinolones (ciprofloxacin [CIP, 5µg], nalidixic acid [NAL, 30µg]), tetracyclines (doxycycline [DOX, 30µg], tetracycline [TET, 30µg]), aminoglycosides (gentamicin [GEN, 10µg], kanamycin [KAN, 30µg], neomycin [NEO, 30µg], streptomycin [STM, 10µg]), phenicols (chloramphenicol [CHL, 30µg]), and sulfonamides (trimethoprim/sulfamethoxazole [STX, 1.25/23.75µg]). Briefly, a standardized suspension of the isolates was applied to MHA plates. Antibiotic disks were then placed on the inoculated agar surface and incubated at 37°C for 16 to 18 hours. *Escherichia coli* ATCC 25922 was used for quality control. An isolate was classified as resistant or multidrug-resistant to antibiotics based on the definition of Magiorakos et al. (2012).

Detection of antibiotic-resistant genes

DNA was extracted using the TopPURE® Genomic DNA Extraction Kit (ABT, Vietnam) according to the manufacturer's instructions. The following genes implicated in antimicrobial resistance [bla_{PSE-1}, bla_{OXA-1}, and bla_{TEM} for β-*lactam*-resistant strains; *aadA1*, *aadA2, aac(3')-IV,* and *aphA-1AB* for aminoglycoside-resistant strains; *catA1*, *cmlA1*, and *floR* for chloramphenicol-resistant strains; *sul1* for sulfonamide-resistant strains; *tetA*, *tetB*, and *tetG* for tetracyclines-resistant strains; and the plasmid-mediated quinolone resistance (PMQR) including *qnrA*, *qnrB*, *qnrS*, and *aac(6')-ib-cr* for quinolone-resistant strains] were detected by PCR amplification. The specific primers are presented in Table 1, and PCR reaction conditions were based on previous studies (Ng et al., 1999; Guerra et al., 2004; Robicsek et al., 2006; Platell et al., 2011). The reaction components included 12.5 µl of GoTag® Green Master Mix (Promega, USA), 1 µl each of the forward and reverse primers (10 µM), 8.5 µl of purified water, and 2 µl of template DNA. PCR products were electrophoresed with a 100 bp DNA ladder on a 1.5% agarose gel supplemented with RedSafe™ Nucleic Acid Staining Solution (Intron, Korea). The desired PCR product was visualized and photographed using the Gelpic100 system (Phusa Biochem, Vietnam).

Data analysis

The data were entered and calculated in Microsoft Excel 2016.

Table 1. Primers used in the present study to detect antibiotic-resistant genes

Antibiotic group	Primers		Sequences (5' ⟶ 3')	PCR products (bp)	Reference
β-lactam	bla_{TEM}	F	TTGGGTGCACGAGTGGGT	503	Guerra et al. (2004)
		R	TAATTGTTGCCGGGAAGC		
	bla_{OXA-1}	F	AGCAGCGCCAGTGCATCA	708	Guerra et al. (2004)
		R	ATTCGACCCCAAGTTTCC		
	bla_{PSE-1}	F	CGCTTCCCGTTAACAAGTAC	419	Guerra et al. (2004)
		R	CTGGTTCATTTCAGATAGCG		
Aminoglycosides	*aadA1-like*	F	GTGGATGGCGGCCTGAAGCC	526	Guerra et al. (2004)
		R	ATTGCCCAGTCGGCAGCG		
	aadA2	F	TGTTGGTTACTGTGGCCGTA	381	Ng et al. (1999)
		R	GCTGCGAGTTCCATAGCTTC		
	aac(3')-IV	F	GTTACACCGGACCTTGGA	674	Guerra et al. (2004)
		R	AACGGCATTGAGCGTCAG		
	aphA1-1AB	F	AAACGTCTTGCTTTCGAGGC	461	Guerra et al. (2004)
		R	CAAACCGTTATTCATTCGTGA		
Sulphonamides Chloramphenicol	*sul1*	F	CTTCGATGAGAGCCGGCGGC	436	Guerra et al. (2004)
		R	GCAAGGCGGAAACCCGCGCC		
	cmlA1	F	TGTCATTTACGGCATACTCG	435	Guerra et al. (2004)
		R	ATCAGGCATCCCATTCCCAT		
	floR	F	CACGTTGAGCCTCTATAT	868	Guerra et al. (2004)
		R	ATGCAGAAGTAGAACGCG		
Tetracyclines	*tetA*	F	GCTACATCCTGCTTGCCT	210	Guerra et al. (2004)
		R	CATAGATCGCCGTGAAGA		
	tetB	F	TTGGTTAGGGGCAAGTTTTG	600	Guerra et al. (2004)
		R	GTAATGGGCCAATAACACCG		
	tetG	F	GCTCGGTGGTATCTCTGC	500	Guerra et al. (2004)
		R	AGCAACAGAATCGGGAAC		
Quinolones	*qnrA*	F	ATTTCTCACGCCAGGATTTG	516	Robicsek et al. (2006)
		R	GATCGGCAAAGGTTAGGTCA		
	qnrB	F	GATCGTGAAAGCCAGAAAGG	469	Robicsek et al. (2006)
		R	ACGATGCCTGGTAGTTGTCC		
	qnrS	F	ACGACATTCGTCAACTGCAA	417	Robicsek et al. (2006)
		R	TAAATTGGCACCCTGTAGGC		
	aac(6')-Ib-cr	F	ATGACTGAGCATGACCTTGC	519	Platell et al. (2011)
		R	TTAGGCATCACTGCGTGTTC		

RESULTS

Isolation of *Salmonella* species

After biochemical testing, 11 of 161 samples (6.8%) were *Salmonella*-positive. Of these, the *Salmonella* isolation rates from dog and cat fecal samples were 9.8% and 2.9%, respectively (Table 2). For fecal samples from dogs and cats with diarrhea and from healthy dogs and cats, the rates were 6 (8.2%) of 73 samples and 5 (5.7%) of 88 samples, respectively.

Antibiotic resistance of *Salmonella* species

The antibiotic resistance rates of eleven *Salmonella* strains are shown in Table 3. Tetracycline resistance was the most common (90.0%), followed by streptomycin (81.2%). Resistance to chloramphenicol and trimethoprim/sulfamethoxazole was also recorded at 45.5% for each antibiotic. Resistance to other antibiotics, including nalidixic acid, amoxicillin/clavulanic acid, doxycycline, gentamicin, and cefotaxime, ranged from 27.3% to 36.4%. The isolates were resistant to neomycin, kanamycin, and ciprofloxacin at a similar rate of 18.2%.

Detection of antibiotic resistance genes

The results of detecting antibiotic resistance genes in *Salmonella* strains are presented in Table 4 and Figures 1 and 2. Twelve antibiotic resistance genes [bla_{TEM}, *aadA1, aadA2, aphA1-1AB, aac (3')-IV, sul1, cmlA1, floR, tetA, tetB, qnrA,* and *qnrS*] were amplified from the antibiotic-resistant *Salmonella* strains, whereas bla_{PSE-1}, bla_{OXA-1}, *tetG, qnrB,* and *aac (6')-ib-cr* were not detected. The *sul1* gene was found in all (100%) of the five trimethoprim/sulfamethoxazole-resistant isolates. The *tetA* and *tetB* genes were detected in 7 (70.0%) and 3 (30.0%) of the ten tetracycline-resistant isolates, respectively. Aminoglycoside resistance genes such as *aadA1* and *aadA2* were detected in 8 (88.9%) and 4 (44.4%) of the nine streptomycin-resistant isolates, respectively; three (33.3%) of the streptomycin-resistant isolates carried both genes simultaneously. The *aphA1-1AB* and *aac (3')-IV* genes were amplified in one (50.0%) of the two kanamycin-resistant isolates and in two (50.0%) of the four gentamicin-resistant isolates, respectively. All chloramphenicol-resistant isolates carried at least one resistance gene, with 4 (80.0%) and 3 (60.0%) of the five isolates carrying the *cmlA1* and *floR* genes, respectively; of these, two (40.0%) isolates carried both genes simultaneously. The *qnrS* and *qnrA* genes were detected in two (66.7%) and one (33.3%) of the three quinolone-resistant isolates. The bla_{TEM} gene was found in all (100%) of the five β-*lactam*-resistant isolates, including three strains co-resistant to amoxicillin/clavulanic acid and cefotaxime, and two strains resistant to one of these antibiotics.

Table 2. Prevalence of *Salmonella* spp. in dog and cat fecal samples collected at veterinary clinics in Hanoi, Vietnam, during November 2024-December 2025

Oder	Type of samples	Cat fecal samples		Dog fecal samples	
		Number	Positive number (%)	Number	Positive number (%)
1	Healthy	38	1 (2.6)	50	4 (8.0)
2	Diarrhea	31	1 (3.2)	42	5 (11.9)
3	Total	69	2 (2.9)	92	9 (9.8)

Table 3. Antibiotic resistance of *Salmonella* strains isolated from dog and cat fecal samples collected at veterinary clinics in Hanoi, Vietnam, during November 2024-December 2025

Order	Antibiotics	Sensitive number (%)	Intermediate number (%)	Resistance number (%)
1	Amoxicillin / clavulanic acid	7 (63.6)	0 (0.0)	4 (36.4)
2	Cefotaxime	6 (54.5)	1 (9.1)	4 (36.4)
3	Ciprofloxacin	8 (72.7)	1 (9.1)	2 (18.2)
4	Doxycycline	5 (45.5)	2 (18.2)	4 (36.4)
5	Kanamycin	7 (63.6)	2 (18.2)	2 (18.2)
6	Gentamicin	5 (45.5)	2 (18.2)	4 (36.4)
7	Nalidixic acid	7 (63.6)	1 (9.1)	3 (27.3)
8	Neomycin	8 (72.7)	1 (9.1)	2 (18.2)
9	Chloramphenicol	5 (45.5)	1 (9.1)	5 (45.5)
10	Streptomycin	2 (18.2)	0 (0.0)	9 (81.8)
11	Tetracycline	1 (9.1)	0 (0.0)	10 (90.9)
12	Trimethoprim / Sulfamethoxazole	4 (36.4)	2 (18.2)	5 (45.5)

Table 4. Antibiotic resistance phenotypes and associated resistance genes in *Salmonella* strains isolated from dog and cat fecal samples collected at veterinary clinics in Hanoi, Vietnam, from November 2024 to December 2025

Order	Code	Antibiotic resistant phenotypes	Antibiotic resistant genotypes
1	D14-061024	STM	-
2	D55-101224	STM, TET	*aadA1*, *tetA*
3	D43-120125	STM, DOX, TET	*aadA2*, *tetA*
4	D71-150325	CHL, STM, TET	*cmlA1*, *aadA1*, *tetA*
5	D08-110125	CHL, STM, TET	*cmlA1*, *aadA1*, *tetA*
6	D58-101224	NAL, GEN, STM, TET, STX	*qnrS*, *aadA1*, *tetB*, *sul1*
7	M02-161125	AMC, CTX, GEN, KAN, DOX, TET	bla_{TEM}, *aphA1-1AB*, *tetB*
8	D31-171124	CTX, CHL, GEN, STM, DOX, TET, STX	bla_{TEM}, *cmlA1*, *floR*, *aac(3)-IV*, *aadA1*, *tetA*, *sul1*
9	D23-090325	AMC, CTX, NEO, STM, DOX, TET, STX	bla_{TEM}, *aadA1*, *aadA2*, *tetB*, *sul1*
10	M14-111024	AMC, CIP, NAL, CHL, NEO, STM, TET, STX	bla_{TEM}, *qnrS*, *floR*, *aadA1*, *aadA2*, *tetA*, *sul1*
11	D62-110225	AMC, CTX, CIP, NAL, CHL, KAN, GEN, STM, TET, STX	bla_{TEM}, *qnrA*, *cmlA1*, *floR*, *aadA1*, *aadA2*, *aac(3)-IV*, *tetA*, *sul1*

AMC: Amoxycillin/clavulanic acid, CTX: Cefotaxime, CIP: Ciprofloxacin, DOX: Doxycycline, KAN: Kanamycin, GEN: Gentamicin, NAL: Nalidixic acid, NEO: Neomycin, CHL: Chloramphenicol, STM: Streptomycin, TET: Tetracycline, STX: Trimethoprim/Sulfamethoxazole; '-': Not detected

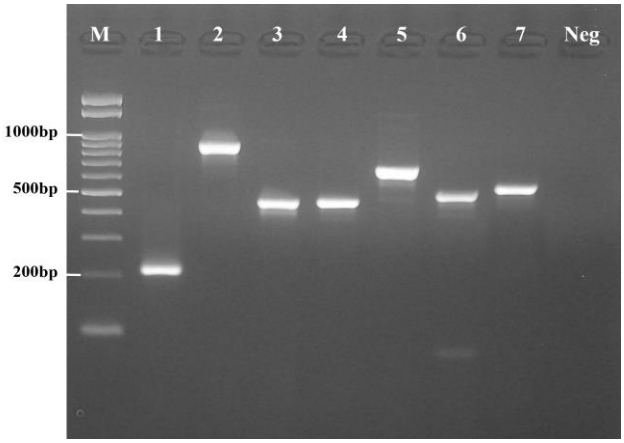


Figure 1. Antibiotic-resistant genes in the *Salmonella* strain D31-171124. M: Marker (100 bp), Lanes 1-7: *tetA* (210 bp), *floR* (868 bp), *cmlA1* (435 bp), *sul1* (436 bp), *aac(3)-IV* (674 bp), bla_{TEM} (503 bp), *aadA1* (526 bp), Neg: Negative control

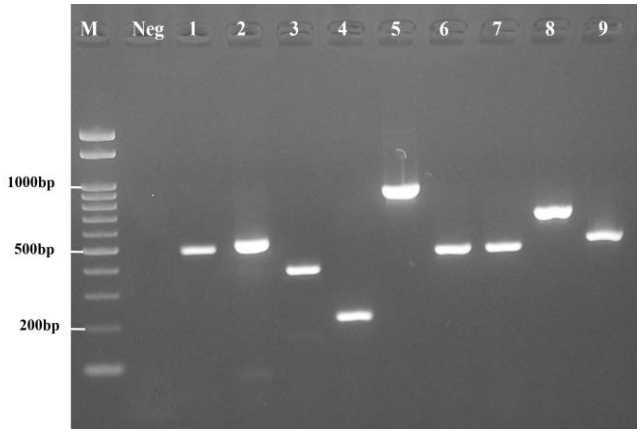


Figure 2. Antibiotic-resistant genes in the *Salmonella* strain D62-110225. M: Marker (100 bp), Neg: Negative control, Lanes 1-9: bla_{TEM} (503 bp), *aadA1* (526 bp), *aadA2* (381 bp), *tetA* (210 bp), *floR* (868 bp), *cmlA1* (435 bp), *sul1* (436 bp), *aac(3)-IV* (674 bp), *qnrA* (516 bp)

DISCUSSION

The higher *Salmonella* isolation rate from dog fecal samples than from cat fecal samples aligns with previous studies in the United States (Reimschuessel et al., 2017) and China (Wei et al., 2020), which reported isolation rates of 2.5-9.47% and 0.6–1.77%, respectively. The isolation rate from dog fecal samples in the current study was similar to rates of 9.47% in China (Wei et al., 2020), 10.5% in Iran (Salehi et al., 2012), and 11.7% in Ethiopia (Kiflu et al., 2017). However, these rates were lower than the 23.0% *Salmonella*-positive rate in dog fecal samples reported in a previous study conducted in Canada (Leonard et al., 2011). Notably, the prevalence of *Salmonella* in dog feces was only 0.23% in the UK (Lowden et al., 2015), 1.0% in Turkey (Bagcigil et al., 2007), and 2.5% in the United States (Reimschuessel et al., 2017). The isolation rates in cat fecal samples in the current study are consistent with positive rates of 0.5%, 0.6%, and 1.77-3.7% in studies conducted in the United States (Reimschuessel et al., 2017), South Africa (Gelaw et al., 2018), and China (Wei et al., 2020; Zhao et al., 2024), respectively. Overall, the isolation rates of *Salmonella* in both dogs and cats vary across countries and regions, which can be explained by differences in sample size, sampling methods and strategies, feeding habits, pet hygiene conditions, sampling season, and geographical and climatic differences between study areas (Sanchez et al., 2002; Seepersadsingh et al., 2010; Wei et al., 2020).

The tetracycline resistance rate among *Salmonella* strains from dogs and cats was similar to the 92.0% reported in a previous study conducted in China (Wei et al., 2020). However, lower resistance rates, such as 11.1%, 32.0%, 59.5%,

and 60.0%, have been reported in previous studies from Turkey, Thailand, Ethiopia, and Iraq (Kiflu et al., 2017; Srisanga et al., 2017; Yildiz and Demirbilek, 2024; Najim et al., 2025). The doxycycline resistance rate was similar to the 30.9% recorded in a study conducted in Ethiopia (Kiflu et al., 2017). However, in a similar study conducted in Turkey, the rate was reported to be 11.1% (Yildiz and Demirbilek, 2024). In the present study, resistance to streptomycin, trimethoprim/sulfamethoxazole, chloramphenicol, gentamicin, and kanamycin in *Salmonella* strains generally matched the rates of 44.6-76.0%, 38.9-41.5%, 44.7-51.7%, 29.6-64.0%, and 32.1-38.9%, respectively, observed in previous studies conducted in China (Wei et al., 2020; Zhao et al., 2024). However, lower rates of resistance to streptomycin (25.4-38.1%), trimethoprim/sulfamethoxazole (9.5-28.0%), chloramphenicol (7.1-17.2%), gentamicin (2.4-20.0%), and kanamycin (0.0-2.4%) have also been observed in dog- and cat-derived *Salmonella* strains in previous studies conducted in Ethiopia (Kiflu et al., 2017), Thailand (Srisanga et al., 2017), Turkey (Yildiz and Demirbilek, 2024), and Iraq (Najim et al., 2025). The neomycin resistance rate (18.6%) was lower than the rate (50.0%) recorded in a study conducted in Turkey (Yildiz and Demirbilek, 2024).

Resistance to amoxicillin/clavulanic acid aligns with the 26.2% rate reported in a study from Ethiopia (Kiflu et al., 2017). However, a previous study in Iraq reported a 48.0% resistance rate among dog-derived *Salmonella* strains (Najim et al., 2025). Notably, in previous studies in China (Wei et al., 2020) and Turkey (Yildiz and Demirbilek, 2024), all dog- and cat-derived *Salmonella* strains were susceptible to amoxicillin. The rate of cefotaxime resistance was lower than the 44.0-52.0% observed in another study conducted in China (Zhao et al., 2024). In the present study, the isolates showed resistance to ciprofloxacin and nalidixic acid at rates of 18.2% and 27.3%, respectively. Resistance to these antibiotics among dog- and cat-derived *Salmonella* strains varied across countries, ranging from susceptibility to 2.4% in Ethiopia and Thailand (Kiflu et al., 2017; Srisanga et al., 2017), 22.2-44.4% in Turkey (Yildiz and Demirbilek, 2024), and 25.8-80.0% in China (Wei et al., 2020; Zhao et al., 2024). Antibiotic resistance in *Salmonella* strains can depend on location, region, season, antibiotic susceptibility testing method, and source of isolation (Aga et al., 2025; Zhang et al., 2026). The varying rates of resistance across localities and regions highlight the role of antibiotic management and use policies in reducing antibiotic resistance in livestock (Osivand et al., 2025).

The amplification rates of tetracycline and sulfonamide resistance genes in the current study are consistent with previous research in China (Zhao et al., 2024), in which the *tetA* and *sul1* genes were found in 45.9-53.3% and 52.2–83.6% of tetracycline- and sulfonamide-resistant *Salmonella* strains isolated from dogs and cats, respectively. However, in a similar study conducted in Thailand, only 20.0% of sulfonamide-resistant strains carried the *sul1* gene, and 51.1% of tetracycline-resistant strains carried either the *tetA* or *tetB* gene, with 21.3% carrying both the *tetA* and *tetB* genes (Srisanga et al., 2017). The widespread distribution of sulfonamide (*sul1*, *sul2*, *sul3*) and tetracycline (*tetA*, *tetB*, *tetC*, *tetG*) resistance genes in antibiotic-resistant *Salmonella* strains from various sources has been noted worldwide, including Bangladesh, China, and Thailand (Zhu et al., 2017; Das et al., 2022; Puangseree et al., 2025). These findings may explain the high rates of resistance to these antibiotic classes in *Salmonella* globally (Zhao et al., 2024). Furthermore, the screening rates for aminoglycoside and chloramphenicol resistance genes are consistent with the study by Srisanga et al. (2017) conducted in Thailand, which reported that aminoglycoside-resistant *Salmonella* strains carried the *aadA1* gene alone and both *aadA1* and *aadA2* genes simultaneously at rates of 80.0% and 20.0%, respectively; the *cmlA1* gene was detected at a rate of 60.7% in chloramphenicol-resistant strains.

The prevalence of plasmid-mediated quinolone resistance (PMQR) genes in quinolone-resistant *Salmonella* strains aligns with a study from China (Zhao et al., 2024), which reported that 17.6%, 60.3-67.4%, and 38.2-44.1% of quinolone-resistant strains carried the *qnrA*, *qnrS*, and *aac(6')-Ib-cr* genes, respectively. In contrast, in Thailand, only 4.9% of quinolone-resistant *Salmonella* strains carried the *qnrS* gene, and other PMQR genes were not detected (Srisanga et al., 2017). The amplification of the bla_{TEM} gene in β-l*actam*-resistant *Salmonella* strains was higher than the 40.0-55.4% rates detected in drug-resistant strains in previous studies in China and Thailand (Srisanga et al., 2017; Zhao et al., 2024). In the present study, the absence of the bla_{OXA-1} and bla_{PSE-1} genes may be due to the small sample size. However, in the aforementioned studies by Srisanga et al. (2017) and Zhao et al. (2024), the bla_{PSE-1} and bla_{OXA} genes were also amplified in only two (3.2%) of 62 ampicillin-resistant strains and ten (3.0%) of 334 β-*lactam*-resistant strains.

CONCLUSION

The present study provided initial insights into the prevalence and antibiotic resistance of *Salmonella* spp. in dog and cat feces collected from veterinary clinics in Northern Vietnam. Despite limitations, including a small sample size, a narrow sampling scope, and an unclear link between infection rates and variables such as breed and age, the findings indicate that, even with low fecal infection levels, dogs and cats can still transmit *Salmonella* to humans through close contact. Some isolated strains may also show resistance to multiple antibiotics and harbor various resistance genes, increasing the

risk of spreading these genes within the community. Larger studies are necessary to identify resistance genes, including plasmids and mutations, and to examine their connection to resistance patterns and multidrug-resistant strains. Additionally, monitoring antibiotic use in animals at veterinary clinics is crucial to reducing antibiotic resistance.

DECLARATIONS

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Authors' contributions

Chu Thi Thanh Huong designed the investigation and methodology and contributed to writing the manuscript. Nguyen Van Phuong, Nguyen Manh Tuong, and Truong Lan Oanh collected the samples and participated in the experiments. Truong Ha Thai provided supervision and methodological support and contributed primarily to writing. All authors revised and approved the final edition of the manuscript.

Availability of data and materials

All data are included in the submitted paper and will be available upon reasonable request from the corresponding author.

Competing interests

The authors declare no conflicts of interest.

Ethical considerations

The present study was originally written without copying any data from published studies or books. No AI tools were used to prepare the current study. All authors accept responsibility for the originality of the data and the manuscript, both at the time of submission and after final revision for publication.

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Physicochemical Characteristics of Sterilized Milk Supplemented with Mung Bean (*Vigna radiata* L.) Extract

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ABSTRACT

Sterilized milk is a nutritious dairy product; however, its dietary fiber and antioxidant contents are relatively low. Investigating the potential of sterilized milk is important to support the development of nutritionally enhanced and high-quality functional dairy beverages. The present study aimed to investigate the effects of incorporating mung bean (*Vigna radiata* L.) extract on the physicochemical properties, antioxidant activity, and dietary fiber content of sterilized milk. The present study followed a completely randomized design and consisted of four treatment groups with three replications per treatment. The first group was the control, with 100% milk and 0% mung bean extract (A1), the second group consisted of 80% milk and 20% mung bean extract (A2), the third group included 60% milk and 40% mung bean extract (A3), and the fourth group consisted of 40% milk and 60% mung bean extract (A4). Parameters analyzed included antioxidant activity, dietary fiber content, protein content, viscosity, and color (L*, a*, b*). The current results indicated that mung bean substitution had significant effects on all observed parameters. Increasing substitution levels increased antioxidant activity from 18.54% (A1) to 29.14% (A4), and dietary fiber content increased from 0.25% (A1) to 3.22% (A4). The highest protein content (2.28%) was achieved using a 20% mung bean extract substitution. Higher mung bean substitution increased the viscosity from 1.43 centipoise (cP) in the control group to 1.84 cP in Group A4. Compared to the control group, Group A4 exhibited a decrease in lightness (L*) from 74.80 to 66.90 and redness (a) from 5.61 to 0.58, alongside an increase in yellowness (b*) from 2.44 to 3.67. Substituting with 60% mung bean extract yielded the highest antioxidant activity and dietary fiber levels, while 20% mung bean extract led to the highest protein content.

Keywords: Antioxidant activity, Color value, Dietary fiber, Mung bean, Sterilized milk, Viscosity

INTRODUCTION

The demand for functional and nutritious food has risen as consumers have become increasingly aware of the correlation between diet and chronic health conditions (Żurek et al., 2026). Milk is a nutrient-dense food containing a wide range of essential nutrients; however, high-temperature sterilization processes can reduce some of its bioactive components. Bioactive components in milk are biologically active compounds that produce beneficial physiological effects as essential nutrients, such as proteins, fats, vitamins, and lactose (Kilic-Akyilmaz et al., 2022). Additionally, while cow's milk is high in protein and minerals, it naturally lacks dietary fiber (Antunes et al., 2022). Milk contains endogenous antioxidants that contribute to its nutritional and functional attributes. Moreover, the antioxidant capacity of milk can be improved by integrating natural plant-derived ingredients during milk processing (Stobiecka et al., 2022). Although the health advantages of dietary fiber are well-known, many adults worldwide still do not reach the recommended intake (Ioniță-Mîndrican et al., 2022). A recent systematic review and meta-analysis study revealed that adults in Indonesia and Malaysia consumed only 10.7%-72.7% of the advised dietary fiber intake, highlighting a significant gap compared to current dietary recommendations (Agustina et al., 2025). This condition underscores the importance of developing innovative food products enriched with dietary fiber to enhance nutrient intake and promote public health.

Mung bean (*Vigna radiata* L.) is regarded as a promising ingredient due to its high protein content (20.97%-32.6%; Tarahi, 2024), crude fiber levels ranging from 3.8% to 6.15% depending on the cultivar and geographical origin (Mehta et al., 2021), and the presence of bioactive compounds such as flavonoids, including vitexin and isovitexin, which demonstrate antioxidant activity (Desta et al., 2024). In addition, mung beans contain dietary fiber ranging from 14.57% to 24.5% (Yucel et al., 2025). Adding mung bean extract to sterilized milk is expected to enhance its nutritional profile by increasing protein content, antioxidant capacity (Zheng et al., 2020), and dietary fiber. However, this addition may affect different physicochemical properties of milk (Liu et al., 2025).

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Previous studies have demonstrated the potential of plant-based ingredients to improve the functional quality of dairy products. Enriching goat milk yogurt with mung bean extract improved its physicochemical properties (Firmansyah et al., 2025), while the addition of *Moringa oleifera* extract enhanced the nutritional quality of cow's milk (Lisak Jakopović et al., 2022). Likewise, spinach powder has been successfully used as a functional ingredient in soft cheese production (El-Sayed, 2020). Overall, these studies indicated that plant-derived materials rich in phenolic compounds, flavonoids, and dietary fiber can enhance the functional properties of dairy products while influencing their physicochemical characteristics. However, limited information is available concerning the incorporation of mung bean extract into sterilized milk, specifically regarding its impact on antioxidant activity, dietary fiber, protein content, viscosity, and color subsequent to thermal sterilization. Accordingly, the present study aimed to assess the impact of mung bean substitution on the physicochemical characteristics of sterilized milk to develop a functional dairy beverage with improved nutritional and functional properties.

MATERIALS AND METHODS

Ethical approval

This study did not involve human participants or animals; therefore, ethical approval was not required. Also, the final product was not consumed by live humans, animals, or organisms.

Materials

The materials employed in the current study included Dancow® full-cream milk powder (PT. Nestle, Indonesia), distilled water (PT. Sumber Rejeki Berkat Abadi, Makassar, Indonesia), Lemovita® stevia (Lemovita, Indonesia), porang flour (Ikarie Organic®, Indonesia), mung beans purchased from a local market in Makassar, Indonesia, and Le Minerale® bottled drinking water from PT Tirta Fresindo Jaya, Indonesia. The equipment utilized included a stainless steel pan, thermometer, measuring cup, stirrer, sieve, autoclave (Tomy SX-500, Japan), stove, blender, spoon, scale, and Ostwald viscometer (Pyrex®, USA).

Experimental design

The present study utilized a completely randomized design (CRD) with four levels of mung bean extract substitution in sterilized milk formulations. The control group comprised 100% milk without any mung bean extract (A1), the second group consisted of 80% milk and 20% mung bean extract (A2), the third group consisted of 60% milk and 40% mung bean extract (A3), and the fourth group contained 40% milk and 60% mung bean extract (A4). Each treatment group was replicated three times, resulting in a total of 12 experimental units. The parameters observed in the present study included antioxidant activity, dietary fiber content, protein content, viscosity, and color characteristics such as L*, a*, and b* of milk.

Mung bean extract preparation

The mung bean extract was prepared following a modified procedure based on the study of Huang et al. (2024). Initially, the mung beans were sorted, after which 112.5 g were weighed and soaked in water at room temperature for eight hours. Following soaking, the beans were drained and boiled in water (12.5%, w/v) at 100°C for 15 minutes. The cooked beans were then blended until a homogeneous consistency was achieved and subsequently filtered through a filter cloth to obtain the mung bean extract. Finally, 0.1% (w/v) stevia was added and thoroughly mixed. Prior to formulation, the mung bean extract was characterized through laboratory analyses. The protein and dietary fiber contents were analyzed at the Makassar Public Health Laboratory (Labkesmas Makassar, Indonesia), while antioxidant activity was determined at the Laboratory of Animal Product Technology, Faculty of Animal Science, Universitas Hasanuddin, Makassar, Indonesia. The analysis revealed that the mung bean extract contained 1.18% protein, 2.27% dietary fiber, and exhibited 23.40% antioxidant activity.

Sterilized mung bean-fortified milk formulation

The sterilized milk substitute with mung bean extract was prepared by dissolving 13.5% (w/v) full-cream milk powder in drinking water according to the manufacturer's instructions, along with 0.1% (w/v) stevia, followed by homogenization. Mung bean extract was then added at 0%, 20%, 40%, and 60%, and the mixture was homogenized again. Porang flour was added at a constant concentration of 0.05% (w/v) in all formulations, followed by further homogenization to obtain a uniform mixture. The mixtures were then subjected to heat treatment in an autoclave at 105°C for five minutes. The chosen processing temperature was in accordance with the range specified for in-bottle milk sterilization guidelines (105-120°C) as documented by Melini et al. (2017), although the current study employed a

shorter heating duration. This temperature-time combination was selected based on preliminary laboratory trials conducted prior to the main experiment, encompassing microbiological analysis by the Microbiology Faculty of Animal Science at Hasanuddin University, Makassar, Indonesia. This analysis confirmed that the treatment was adequate to ensure product safety under the conditions implemented in the present study.

Following thermal processing, the samples were cooled at room temperature for approximately 30 minutes prior to conducting physicochemical analyses (Yuan et al., 2023). The evaluated parameters included antioxidant activity, crude fiber, protein content, viscosity, and color attributes (L, a, and b*).

Antioxidant activity

Antioxidant activity was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical-scavenging method. The DPPH reagent (0.2 mM) was prepared by dissolving 4 mg of DPPH in methanol and diluting to a final volume of 50 mL. A total of 1 mL of the DPPH reagent was combined with 1 mL of the sample solution at the respective concentrations. The mixture was incubated in the dark at room temperature for 30 minutes, after which absorbance was recorded at 517 nm using a UV-Vis spectrophotometer. The percentage of DPPH radical-scavenging activity was calculated according to de Menezes et al. (2021) using Formula 1. Antioxidant activity was expressed as the half-maximal inhibitory concentration (IC_{50}, µg/mL). The IC_{50} value was determined by linear regression of sample concentration against the percentage of DPPH radical inhibition. The IC_{50} value indicated the concentration of the sample necessary to attain 50% scavenging of DPPH radicals as well as 50% inhibition.

$$\text{Free radical scavenging activity (\%)} = \frac{\text{Absorbance of reagent blank-Absorbance of sample}}{\text{Absorbance of reagent blank}} \times 100\% \quad \text{(Formula 1)}$$

Crude fiber content

Crude fiber content was determined using the gravimetric method following sequential acid-alkali digestion according to the AOAC official method (978.10; Thiex, 2009; Formula 2).

$$\text{Crude fibre (\%)} = \frac{W2-W1}{W} \times 100\% \quad \text{(Formula 2)}$$

Where W1 was the weight of the empty filter paper (g), W2 was the weight of the filter paper containing the residue after oven drying (g), and W was the weight of the sample used for analysis (g).

Protein content

Protein content was analyzed according to the Kjeldahl method as described by Shivanna et al. (2025). The total nitrogen content was subsequently calculated using the following equation (Shivanna et al., 2025, Formulas 3 and 4).

$$\text{N (\%)} = \frac{14.007 \times (\text{V s - V b}) \times \text{Normality of sulphuric acid}}{\text{weight of sample (g))}} \times 100 \quad \text{(Formula 3)}$$

$$\text{Protein content (\%)} = \text{N (\%)} \times 6.38 \quad \text{(Formula 4)}$$

Where Vs was the volume (mL) of 0.1 N H_2SO_4 titrant used for the test portion, and Vb was the volume (mL) of 0.1 N H_2SO_4 titrant used for the blank determination.

Viscosity

Viscosity was measured using an Ostwald viscometer in accordance with the procedures reported by Lewis (1990), as cited in Shivanna et al. (2025). The viscosity of each sample was expressed as absolute viscosity (cP) and determined at 27°C. Sample density was measured independently using a pycnometer at a consistent temperature, with a thermostatically controlled water bath employed to maintain a constant temperature of 27°C. Specific gravity (Formula 5), relative viscosity (Formula 6), and absolute viscosity (Formula 7) were determined using the following equations.

$$\text{Specific gravity} = \frac{\text{Weight of pycnometer fill with sample -Weight of empty pycnometer}}{\text{Weight of pycnometer fill with water -Weight of empty pycnometer}} \quad \text{(Formula 5)}$$

$$\text{Relative viscosity} = \frac{A}{B} \times \text{specific gravity} \quad \text{(Formula 6)}$$

Where A represented the flow time of the sample from the upper tip to the lower mark of the Ostwald viscometer, and B represented the corresponding flow time of water measured under the same conditions.

Absolute viscosity = Relative viscosity × viscosity of water at 27 °C (Formula 7)

Color

Color characteristics were assessed utilizing the Hunter Lab color system. Within this system, the L* value indicates lightness, ranging from 0 (black) to 100 (white), whereas the a* value describes the red (+) to green (−) axis, and the b* value corresponds to the yellow (+) to blue (−) axis (Hasnul Hadi et al., 2021).

Statistical analysis

The data were analyzed using analysis of variance (ANOVA) within a completely randomized design (CRD) to assess the impact of mung bean extract substitution on various milk parameters. In cases where significant differences ($p < 0.05$) were identified, Duncan's multiple range test was utilized for mean comparison. All statistical analyses were conducted using SPSS software (version 24).

RESULTS AND DISCUSSION

Antioxidant activity

The current results indicated that mung bean extract substitution had a significant effect on the antioxidant activity of sterilized milk ($p < 0.05$; Table 1). The antioxidant activity increased proportionally with the incorporation of mung bean extract, reaching its highest value in Group A4. The observed increase in antioxidant activity indicated that mung bean extract effectively enhanced the antioxidant capacity of sterilized milk. The increase in antioxidant activity was attributed to bioactive compounds in mung bean extract, particularly polyphenols and flavonoids (An et al., 2024). Polyphenols and flavonoids are known to act as free radical scavengers and electron donors, thereby reducing oxidative reactions and neutralizing reactive oxygen species (ROS; Zhang and Huang, 2024). Furthermore, mung bean comprises bioactive compounds, including polysaccharides, which enhance its antioxidant activity (Hou et al., 2019). Therefore, increasing the proportion of mung bean extract increased the concentration of antioxidant compounds in the sterilized milk formulations, resulting in higher antioxidant activity. The observed improvement in antioxidant activity additionally indicated a synergistic effect between the antioxidant compounds inherently present in milk and those present in mung bean extract. Milk comprises different antioxidant components, including casein, whey proteins, and antioxidant vitamins such as vitamins A, C, and E (Stobiecka et al., 2022). The combination of milk antioxidant components with the phenolic compounds of mung bean extract may have contributed to the improved antioxidant potential of the sterilized milk. Previous studies have demonstrated that incorporating mung bean-derived ingredients enhanced antioxidant activity, primarily due to the high levels of phenolic and flavonoid compounds (Villanueva-Bermejo et al., 2024). These findings highlighted the suitability of mung bean extract as a functional ingredient for producing antioxidant-enriched sterilized milk.

Table 1. Antioxidant activity of sterilized milk substituted with mung bean extract

Mung bean extract substitution (%)	Antioxidant
0	18.54 ± 0.73^{a}
20	20.26 ± 0.45^{a}
40	22.96 ± 1.30^{b}
60	29.14 ± 1.90^{c}

Data are presented as mean ± standard deviation. ^{a,b,c} Mean different superscript letters in the same column indicate significant differences ($p < 0.05$).

Fiber content

The present results indicated that dietary fiber content increased significantly with increasing levels of mung bean extract substitution ($p < 0.05$; Table 2). Higher substitution levels resulted in greater fiber content, with the highest value observed in Group A4. The increase in dietary fiber level was primarily attributed to the naturally high dietary fiber content of mung beans (Sudhakaran and Bukkan, 2021). Previous studies have reported that mung beans are a rich source of dietary fiber, including soluble fiber, insoluble fiber, and resistant starch (Zafar et al., 2023; Chauhan et al., 2024). Consequently, increasing the proportion of mung bean extract added more indigestible carbohydrate components to the product, resulting in a progressive increase in fiber content. Besides dietary fiber, mung beans have high levels of resistant starch, which acts similarly to fiber by resisting digestion in the small intestine and undergoing fermentation in the colon (Chauhan et al., 2024). Resistant starch may improve the functional qualities of sterilized milk by providing more fermentable substrates for beneficial gut bacteria, thereby supporting digestive health. The higher dietary fiber content achieved through mung bean substitution highlighted its potential as a functional ingredient to enhance the nutritional profile of sterilized milk. Previous studies have indicated that dietary fiber contributes to several health-

promoting effects, such as improved glycemic control, reduced serum cholesterol, greater satiety, and improved gastrointestinal function (Alahmari, 2024).

Table 2. Fiber content of sterilized milk substituted with mung bean extract

Mung bean extract substitution (%)	Fiber content
0	0.25 ± 0.09^{a}
20	1.32 ± 0.67^{b}
40	2.04 ± 0.15^{c}
60	3.22 ± 0.25^{d}

Data are presented as mean ± standard deviation. ^{a,b,c,d} Means with different superscript letters in the same column indicate significant differences ($p < 0.05$).

Protein content

Mung bean extract substitution had a highly significant effect on the protein content of sterilized milk ($p < 0.05$; Table 3). Protein content demonstrated a non-linear response to increasing substitution levels, reaching its highest value in Group A2. This finding indicated that a moderate mung bean extract substitution level provided a favorable balance between the milk and plant proteins, whereas excessive substitution altered the protein composition of the final product.

Mung beans are known for their high protein content, ranging from 22-28%, along with a well-balanced essential amino acid profile (Yi-Shen et al., 2018). However, elevated substitution levels of mung bean extract did not correspondingly enhance the protein content of sterilized milk, which was likely due to the extract's greater water content and lower protein solids compared to whole milk. Plant proteins differ structurally from milk proteins, notably caseins and whey proteins, leading to variations in their functional properties, such as solubility (Tang et al., 2023). Consistent findings were reported by Firmansyah et al. (2025), who observed a progressive decrease in protein content from 3.46% (control) to 3.07% in goat milk yogurt substituted with 15-45% mung bean extract, an effect that was attributed to dilution due to the extract's lower protein concentration. The present results indicated that increasing mung bean extract substitution reduced protein content in dairy products, which was consistent with the findings of Firmansyah et al. (2025), although fermented yogurt was used in their study. When the mung bean substitution exceeds 20%, the reduction in protein content might be linked to physicochemical interactions that occurred during thermal processing. Mung bean extract contains phenolic compounds, polysaccharides, and dietary fiber that can interact with milk proteins. Protein-phenolic interactions occur via hydrogen bonds, hydrophobic interactions, and, under thermal conditions, covalent bonding following phenolic oxidation. Protein-phenolic interactions can cause conformational changes, promote aggregation, and decrease protein solubility, thereby modifying proteins' functional properties (Zhang et al., 2021a). In addition, sterilization at high temperatures promotes protein denaturation and aggregation, further intensifying these structural modifications (Huang et al., 2022).

Table 3. Protein content of the sterilized milk substituted with mung bean extract

Mung bean extract substitution (%)	Protein content
0	2.17 ± 0.05^{b}
20	2.28 ± 0.30^{b}
40	1.71 ± 0.17^{a}
60	1.69 ± 0.35^{a}

Data are presented as mean ± standard deviation. ^{a,b} Means with different superscript letters in the same column indicate significant differences ($p < 0.05$).

Viscosity

The present results indicated that mung bean extract substitution significantly affected the viscosity of sterilized milk and produced a thicker product ($p < 0.05$; Table 4). Viscosity increased progressively with increasing levels of mung bean extract substitution, with the highest value observed in Group A4.

The increase in milk viscosity was primarily associated with the higher concentration of total solids supplied by mung bean extract, including dietary fiber, starch, and other polysaccharides (Tarahi et al., 2024). These macromolecular components have a high water-binding capacity, allowing them to absorb and hold water within the system, which increases resistance to flow (Petronilho et al., 2021; Ariyarathna et al., 2025). As the level of mung bean extract increased, the amount of suspended and dissolved solids in the product increased, reducing the mobility of water molecules and consequently increasing viscosity.

Table 4. Viscosity of sterilized milk substituted with mung bean extract

Mung bean extract substitution (%)	Viscosity
0	1.43 ± 0.06^{a}
20	1.64 ± 0.05^{b}

40	1.72 ± 0.05^{bc}
60	1.84 ± 0.09^{c}

Data are presented as mean ± standard deviation. [a,b,c] Means with different superscript letters in the same column indicate significant differences ($p < 0.05$).

The observed increase in viscosity might be attributed to the thermal trait of starch during the sterilization process. Exposure to high temperatures can induce starch swelling and partial gelatinization, resulting in increased water retention and the development of a more structured system (Ariyarathna et al., 2025). Furthermore, interactions among milk proteins, polysaccharides, and dietary fiber may facilitate the formation of a more stable colloidal network, thereby improving the product's consistency and viscosity. The current findings suggested that mung bean extract can improve not only the nutritional value of sterilized milk but also its physical properties. The increase in viscosity may be beneficial for developing functional dairy beverages by improving body, texture, and product stability. Similar observations have been documented in previous studies, where the integration of plant-derived components rich in starch and dietary fiber led to an increase in the viscosity of milk-based beverages (Petronilho et al., 2021; Ariyarathna et al., 2025).

Color attributes

L* value

Mung bean extract substitution had a highly significant effect on the lightness (L*) of sterilized milk ($p < 0.05$; Table 5). The L* value decreased progressively with increasing levels of mung bean extract substitution, indicating a gradual reduction in product brightness. The highest L* value was observed in the control group, whereas the lowest L* value occurred in Group A4. These results demonstrated that increasing the proportion of mung bean extract produced a darker sterilized milk.

The reduction in lightness was primarily associated with the inherent color characteristics of mung bean extract, influenced by natural pigments and phenolic compounds present in the extract (Dahiya et al., 2015). The bright appearance of milk is largely attributed to light scattering by casein micelles and fat globules, which contribute to its whiteness and opacity characteristics (Serna-Hernandez et al., 2021). In contrast, mung bean extract contains several plant-derived compounds, including phenolic compounds and flavonoids, which may contribute to darker color development through the formation of dark-brown polymers and a reduction in lightness (L; Huang et al., 2022). Consequently, increasing the concentration of mung bean extract resulted in lower lightness. Thermal processing during sterilization may have further contributed to the decrease in lightness by promoting Maillard reactions and the formation of brown-colored compounds (Zhang et al., 2021b). High-temperature treatment can promote non-enzymatic browning reactions, particularly the Maillard reaction between reducing sugars and amino compounds, leading to the formation of brown-colored pigments (Huang et al., 2022). In addition, phenolic compounds from mung bean extract may undergo oxidation, degradation, and other heat-induced transformations during processing, which may further influence the product's color characteristics (Zhang et al., 2022).

The decline in L* values observed in the present study indicated that mung bean extract substitution not only enhanced the functional properties of sterilized milk but also modified its visual appearance. Similar reductions in lightness have been reported in milk-based beverages containing legume-derived ingredients, where increasing proportions of plant extracts altered the color characteristics of the products due to the presence of natural pigments (Kurbanova et al., 2024). The observed alterations in color parameters suggest that these color changes were a natural result of integrating mung bean extract into sterilized milk formulations. This finding reflected the contribution of plant-derived pigments and highlighted the successful incorporation of mung bean bioactive compounds into the dairy matrix.

Table 5. L* value of sterilized milk substituted with mung bean extract

Mung bean extract substitution (%)	Color attribute L
0	74.80 ± 0.79^{c}
20	72.85 ± 1.27^{bc}
40	72.29 ± 0.58^{b}
60	66.90 ± 1.39^{a}

Data are presented as mean ± standard deviation. [a,b,c] Means with different superscript letters in the same column indicate significant differences ($p < 0.05$).

a* value

These findings demonstrated that incorporating mung bean extract significantly affected the value, resulting in a greener appearance of the sterilized milk ($P < 0.05$; Table 6). The a* value decreased progressively with increasing levels of mung bean extract substitution, indicating a shift toward greener color tones.

The decrease in a* values may be attributed to the contribution of natural pigments in mung bean extract, notably chlorophyll and its derivatives, which influence the green-red color balance of plant-derived materials (Huang et al., 2022). Chlorophyll absorbs light in the red and blue regions of the visible spectrum while reflecting green wavelengths, thereby contributing to a greener appearance and reducing redness. Consequently, increasing the proportion of mung bean extract resulted in lower a* values and a more pronounced green hue in the sterilized milk. Thermal processing during sterilization further affected color by causing chlorophyll degradation and pigment transformations, as observed in heat-treated mung bean materials (Huang et al., 2022). High temperatures can convert chlorophyll into pheophytins and other pigments, affecting color expression while maintaining greenish or olive-green hues (Huang et al., 2022). In addition, interactions between chlorophyll compounds and other plant constituents may have affected the overall color stability of the product during processing. The shift toward greener color tones observed in the present study was related to the increasing concentration of mung bean-derived pigments in the formulation. Similar pigment-related color changes have been reported in mung bean-based products, where chlorophyll-derived pigments contributed to the green coloration and influenced the overall color characteristics during processing (Huang et al., 2022). These findings confirmed that mung bean extract not only enhanced the functional properties of sterilized milk but also produced a distinctive color profile that can serve as a visual indicator of successful plant-based fortification.

Table 6. *a value of sterilized milk substituted with mung bean extract

Mung bean extract substitution (%)	Color attribute *a
0	5.61 ± 0.27^{d}
20	3.91 ± 0.17^{c}
40	2.24 ± 0.16^{b}
60	0.58 ± 0.93^{a}

Data are presented as mean ± standard deviation. ^{a,b,c,d} Means with different superscript letters in the same column indicate significant differences ($p < 0.05$).

b* value

Mung bean extract substitution had a highly significant effect on the b* value of sterilized milk ($p < 0.05$; Table 7). Increasing the proportion of mung bean extract resulted in progressively higher b values, reflecting a greater degree of yellowness. Increasing the level of mung bean extract contributed to a progressive rise in the b* value, which was likely attributable to the carotenoid pigments naturally present in the extract (Zulueta et al., 2007). Mung bean contains carotenoid compounds, such as β-carotene, which are natural pigments that display yellow to orange hues (Maoka, 2020; Cheng et al., 2023). As a result, adding more mung bean extract led to a higher concentration of yellow pigments in the product, which increased the b* value.

Thermal processing during sterilization may have further contributed to the increase in yellowness. High-temperature treatment can promote pigment transformation and non-enzymatic browning reactions, leading to the formation of colored compounds that enhance yellow and brown color tones (Huang et al., 2022). In addition, the degradation of chlorophyll pigments during heating may reduce the intensity of green coloration and increase the visual prominence of yellow pigments, thereby contributing to a higher b* value.

The observed increase in yellowness (b*), coupled with decreased lightness (L*) and reduced redness (a*), indicated that mung bean extract significantly modified the visual appearance of sterilized milk. The current findings confirmed that mung bean extract not only improved the functional properties of sterilized milk but also established a color profile that can serve as a visual indicator of plant-based fortification and may increase product appeal to consumers in pursuit of functional dairy products.

Table 7. b* value of sterilized milk substituted with mung bean extract

Mung bean extract substitution (%)	Color attribute *b
0	2.44 ± 0.22^{a}
20	2.88 ± 0.47^{b}
40	3.28 ± 0.98^{c}
60	3.67 ± 0.70^{d}

Data are presented as mean ± standard deviation. ^{a,b,c,d} Means with different superscript letters in the same column indicate significant differences ($p < 0.05$).

CONCLUSION

Substituting mung bean extract with sterilized milk enhanced its functional quality and influenced its physicochemical

characteristics. The highest level of mung bean substitution (60%) resulted in the greatest antioxidant activity, dietary fiber content, viscosity, and yellowness (b* value), while producing the lowest lightness (L* value) and the lowest redness (a* value), indicating a shift toward a darker, greener, and more yellow appearance. The highest protein content was obtained with 20% mung bean extract substitution. The current study demonstrated that adjusting the proportion of mung bean extract can be employed to achieve specific nutritional and functional characteristics in sterilized milk. Accordingly, mung bean extract can be used as an effective functional ingredient in the formulation of nutritionally enriched sterilized milk. Nevertheless, the present study was confined to the assessment of physicochemical characteristics and did not encompass an evaluation of shelf life, microbiological stability, or the interactions between calcium derived from milk and iron obtained from mung bean extract, including their retention and bioavailability. Consequently, further investigations are necessary to examine these parameters in order to provide a more comprehensive assessment of the product's nutritional value and storage stability.

DECLARATIONS

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Authors' contributions

Andi Ratna Dewi Sari conceived the study, conducted the experiments, collected and analyzed the data, and prepared the original manuscript. Fatma Maruddin supervised the study, contributed to the study design, and critically reviewed and revised the manuscript. Wahniyathi Hatta contributed to data interpretation and critically reviewed the manuscript. All authors read and approved the final edition of the manuscript and agreed to be accountable for all aspects of the study.

Availability of data and materials

All data generated and analyzed during this study are presented in this manuscript, and no supplementary materials are associated with this article. Additional data are available from the corresponding author upon reasonable request.

Competing 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. All authors accept full responsibility for the originality of the data and the final edited version of the manuscript before submission and after the final proof prior to publication. The authors confirmed that no AI tools were used to prepare this study.

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Biocontrol Potential of Rhizospheric Soil Bacteria, Including *Streptomyces* and *Comamonas* Species, Against Aflatoxigenic *Aspergillus flavus*

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ABSTRACT

After decades of extensive exploration of bioactive compounds from *Streptomyces* spp., the discovery of new bioactive chemicals from routinely studied strains has been declining progressively. To tackle this challenge, recent studies have focused on *Actinobacteria* from under-explored ecological niches with significant microbial diversity. The present study aimed to assess the possible antifungal activity of *Streptomyces* spp. isolated from rhizospheric soils of Karura Forest, Nairobi, Kenya, against *Aspergillus flavus* (*A. flavus*), a fungal species known to produce aflatoxin. A total of 236 bacteria were isolated through *in vitro* cultures and characterized using macroscopic, microscopic, and biochemical tests to identify *Streptomyces*-like isolates, which were identified by dry, powdery colonies, filamentous branching hyphae, aerial mycelia, and the formation of spore chains, with Gram staining carried out later on isolates advanced through dual culture screening for antifungal activity. Dual culture assays, with *A. flavus* grown alone as the negative control, were performed to select the promising candidates for further extraction, gas chromatography-mass spectrometry (GC-MS) metabolite profiling, and antifungal testing by the disc diffusion method against PBS and nystatin as the negative and positive controls, respectively. The selected isolates also underwent molecular identification by PCR amplification and Sanger sequencing of the 16S rRNA gene. The analysis revealed that 190 isolates exhibited *Streptomyces*-like characteristics. Dual culture assays revealed that certain isolates inhibited the mycelial growth of *A. flavus*, and GC-MS profiling of the corresponding extracts confirmed the presence of bioactive metabolite classes. The pellet crude extracts of the six selected isolates (SA18, SD102, SD109, SD110, SE143, and SE194), exhibited the highest antifungal efficacy, with inhibitory zones of up to 31.60 ± 2.80 mm, relative to the positive control (nystatin); five of the isolates belonged to the genus *Streptomyces*, while SE143 was identified as *Comamonas*. Supernatant extracts and crude filtrates revealed lower and more variable inhibition across most of the isolates. The present findings indicated that rhizosphere-derived *Streptomyces* and *Comamonas* isolates from Karura Forest are potential natural candidates for biological control of *A. flavus*.

Keywords: Antifungal activity, *Aspergillus flavus*, Biocontrol, *Comamonas* sp., *Streptomyces* spp.

INTRODUCTION

Poverty and food insecurity remain major, interconnected problems in many nations, affecting millions of households and communities (FAO/IFAD/UNICEF/WFP/WHO, 2025). Food insecurity, characterized by unstable access to sufficient, safe, and nutritious food, has directly motivated large-scale crop intensification projects worldwide (FAO, 2010; Godfray et al., 2010). The objective of the crop intensification project was to enhance the production rate of multiple agricultural crops (FAO, 2011; Pretty et al., 2018), including wheat, corn, and rice (FAO, 2016); cassava (FAO, 2013); bananas (Ronner et al., 2023); and soybeans and beans (Nassary et al., 2020). In food crop production, mycotoxin emergence poses a significant health risk to consumers, surpassing the dangers associated with pesticide and synthetic residue presence, in addition to yield losses from fungal infections (Oliveira et al., 2013). The concept of mycotoxins was initially utilized in the 1960s during the investigation of the fatalities of many turkeys (genus *Meleagris*) in England due to the ingestion of groundnut meal (Bennett and Klich, 2003). The harmful compound was subsequently recognized as B1 aflatoxin (McCormick, 2013). Aflatoxin B1 (AFB1) is the most potent and toxic aflatoxin, produced mainly by fungi such as *Aspergillus flavus* and *Aspergillus parasiticus* (Frisvad et al., 2019). AFB1 is carcinogenic, mutagenic, and suppressive of immunity, regarded as the most deleterious mycotoxin (Riba et al., 2010; Yu, 2012).

Mycotoxins, including aflatoxins, infect a substantial amount of the world's food supply, including cereals such as maize, groundnuts, and tree nuts (Eskola et al., 2020). Regulations have been put in place in different countries to shield consumers from the adverse effects of mycotoxins (Van Egmond et al., 2007). Aflatoxins are complex aromatic

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compounds that contaminate a range of staple foods such as groundnuts and maize (Payne and Brown, 1998; Wild and Gong, 2010). In low-income countries, aflatoxins (AFB1, AFB2, AFG1, and AFG2) are naturally occurring compounds and have been shown to be strong carcinogens in humans (WHO, 2023). AFB1 is the predominant secondary metabolic form of aflatoxin (Ismail et al., 2018; Frisvad et al., 2019).

Aflatoxin contamination of food products contributes markedly to the global risk of hepatocellular carcinoma (HCC), one of the most common causes of cancer-related death worldwide (Liu and Wu, 2010; McGlynn et al., 2021). Reducing this risk requires effective control of the fungi responsible for aflatoxin contamination in food crops.

Biocontrol is one of the options to restrict disease spread because it utilizes naturally occurring antagonistic microbes (Khan et al., 2023). Soil-dwelling actinomycetes such as *Streptomyces* are of interest as biocontrol agents because they produce a variety of antifungal metabolites, including lytic enzymes, chitinases, and antibiotics (Shakeel et al., 2018). Antimicrobial resistance has become a major global public-health concern (Murray et al., 2022), intensifying interest in discovering novel bioactive compounds from environmental microorganisms. The increasing occurrence of antifungal resistance and difficult-to-treat invasive fungal infections further demonstrates the need to identify new antifungal compounds, particularly for the protection of vulnerable and immunocompromised populations (Fisher et al., 2022). Only a few antifungal agents are commercially available compared with antibacterial agents because the similarity in cellular characteristics between fungi and human cells complicates the creation of selective antifungal agents (Perfect, 2017; Fisher et al., 2022). The limited availability of antifungal agents has stimulated the exploration of novel bioactive chemicals across diverse biological systems through various methodologies, for instance, microbial genome mining as well as metagenomics (Mendes et al., 2013).

The bulk of *Actinobacteria* species that have been researched commercially were isolated from soil (Barka et al., 2015). After decades of bioprospecting, novel bioactive chemicals from soil *Actinobacteria* are getting more difficult to strain-isolate (Goodfellow and Fiedler, 2010). Therefore, many researchers have turned their search for microbial sources of putative antifungal chemicals to a variety of biological habitats, such as tropical rainforests, marine environments, hydrothermal vents, and microbiological symbionts associated with animal and plant species (Kim et al., 2006; Choi et al., 2009).

Actinobacteria are a cost-effective and promising natural method for efficient detoxification of mycotoxins (Oliveira et al., 2013). *Actinobacteria* are Gram-positive, filamentous bacteria found in a wide range of aquatic and terrestrial settings (Barka et al., 2015). *Streptomyces* are well studied for their ability to produce different useful compounds, such as antifungal drugs, antibiotics, and enzymes like chitinases, which give considerable antagonistic capabilities against the growth of fungi (Barka et al., 2015). The majority of natural-based antimicrobial agents used in veterinary and human medicine are produced by *Streptomyces* spp. (Barka et al., 2015). For instance, strains like *Streptomyces yanglinensis* 3-10, *roseolus*, *philanthi* RL-1-178, and *alboflavus* TD-1 were found to produce antifungal agents against *Aspergillus flavus* (Caceres et al., 2018; Shakeel et al., 2018; Yang et al., 2019; Boukaew and Prasertsan, 2020). Microbes in the soil were studied to identify beneficial microbes to control aflatoxin production in food and animal feed (Uasoontornnop et al., 2022). Current strategies used to combat AFB1 include both chemical and physical methods (Sipos et al., 2021; Hamad et al., 2023). Their practical use is, however, constrained by nutrient loss, reduced sensory quality, and higher equipment costs (Sipos et al., 2021; Hamad et al., 2023). Synthetic fungicides such as triazoles and acylalanines are commonly used in agriculture to control fungal diseases (Pánek et al., 2022; Szczygieł et al., 2024), yet their prolonged use has driven the emergence of fungicide-resistant fungal strains and raised concerns about harm to human health and beneficial microbiota (Emmert and Handelsman, 1999; Verweij et al., 2009). The standard fungicide Bavistin, for example, indicated markedly lower activity against agriculturally important fungi than antifungal compounds isolated from bacteria such as *Bacillus cereus* subsp. *thuringiensis* and *Streptomyces* sp. (Kumar et al., 2014; Kaur et al., 2019; Uasoontornnop et al., 2022). Biological control has therefore become a priority as a dependable and secure technique that reduces the need for chemical pesticides (Khan et al., 2023).

Kenyan bioprospecting for antifungal *Actinobacteria* has so far centred on the country's national parks (Nonoh et al., 2010); Karura Forest, one of the largest remaining tracts of indigenous forest in greater Nairobi, has not been examined for this purpose (Njeru, 2010). Its rhizospheric *Actinobacteria* remain untested against *Aspergillus flavus* (*A. flavus*), making it a promising site for identifying locally adapted antifungal isolates.

While *Streptomyces* is the most extensively documented genus for this purpose, antifungal activity among soil bacteria is not restricted to this genus alone. Other genera such as *Comamonas*, though less commonly studied for this purpose, have also demonstrated antifungal activity through mechanisms such as competition for nutrients and space, production of iron-chelating siderophores, and release of antifungal volatile compounds (El-Banna, 2007; Timofeeva et al., 2022; Zhao et al., 2022). Given this broader potential, the present study set out to isolate and characterize bioactive soil bacterial isolates, with *Streptomyces* spp. as the primary target genus, from rhizospheric soil collected in Karura Forest, Kenya, and to evaluate their antifungal activity as candidate biocontrol agents against *A. flavus*.

MATERIALS AND METHODS

Ethical considerations

Prior to conducting field sampling, authorization was sought from the National Commission for Science, Technology and Innovation (NACOSTI) under license Number NACOSTI/P/25/4177513.

Samples source

Bacterial isolates used in this study were obtained from soil collected at a depth of 5-10 cm and from the plant rhizosphere (root zone) in Karura Forest in Northern Central Nairobi, Kenya, a forest known for its diverse indigenous vegetation (Nyambane et al., 2016). After purification and identification, the active isolates were found to belong to two genera, *Streptomyces* and *Comamonas*. The *Aspergillus flavus* strain used in the present study was provided by the Kenya Seed Company, from an infected maize sample exhibiting visible mould growth. The maize had not been treated with chemical pesticides and was sourced from Kitale County, Kenya's maize-growing region.

Field sampling

Rhizosphere soil samples were collected from the root zones of plants at a depth of 5-10 cm from 10 sampling sites within Karura Forest. Approximately 50 g of soil was collected in duplicate from each site, placed in sterile polyethylene bags, properly labelled, and tightly sealed to prevent spillage, before being transported to the laboratory in a cool box containing ice packs at approximately 4˚C. Samples were then air-dried at room temperature 25˚C for 7 days, following an established protocol for selective *Streptomyces* isolation (Antido and Climacosa, 2022); this step exploits the greater desiccation resistance of actinomycete spores relative to vegetative bacterial cells, reducing non-spore-forming bacteria and other microorganisms that were not *Streptomyces* spp. (Antido and Climacosa, 2022).

Large debris was then removed by manually crushing and sieving the air-dried soil with a sterile mortar and pestle and passing it through a 0.8-mm stainless-steel mesh sieve, sterilized by autoclaving at 121˚C for 20 minutes before use and between samples to minimize cross-contamination (Njenga et al., 2017; Khadayat et al., 2020).

Soil from each predefined pair of sampling sites was then pooled to produce five composite samples representing the ten original sites, in order to maximize the recovery of potentially useful *Streptomyces*-like isolates across the sampled area. Sites KE1 and KE2 were combined to form composite sample SA; sites KE3 and KE4 to form SB; sites KA1 and KA2 to form SC; sites KA3 and KA4 to form SD; and sites KA5 and KA6 to form SE; and these SA to SE, KE1 to KE4 and KA1 to KA6 labels are used throughout the present study to refer to the pooled samples. The locations of all ten individual sampling sites within Karura Forest are shown in Figure 1.

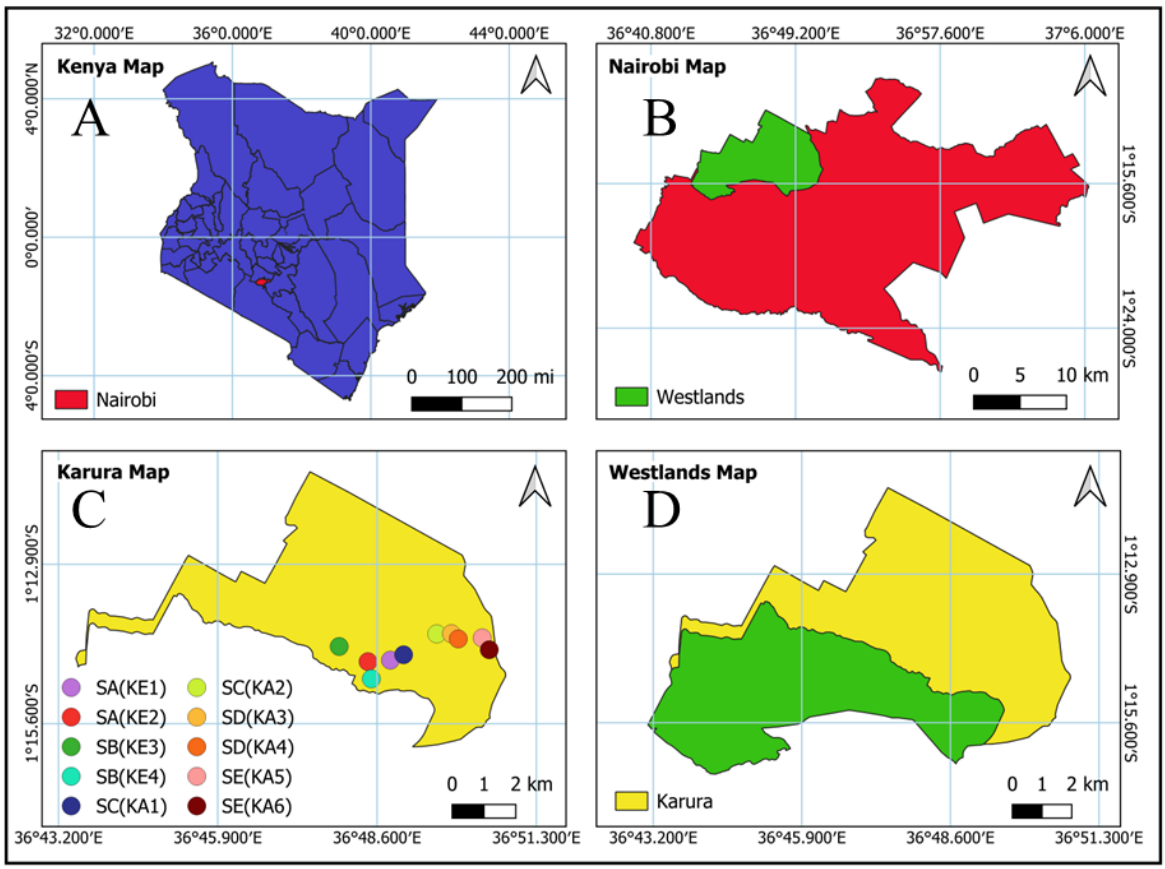


Figure 1. Location of the study area. **A:** Kenya, showing Nairobi County, **B:** Nairobi County, showing Westlands sub-county, **C:** Karura Forest, showing the ten sampling sites (KE1-KE4, KA1-KA6) color-coded by composite sample (SA-SE), **D:** Westlands sub-county, showing the boundary of Karura Forest.

Isolation of *Streptomyces*-like bacteria

Streptomyces-like bacteria were isolated from soil samples using a conventional serial dilution plate method on Yeast Extract-Malt Extract Agar (ISP2), prepared according to the formulation described by Shirling and Gottlieb (1966), including 4 g/L yeast extract (Loba Chemie Pvt. Ltd., India), 10 g/L malt extract (Loba Chemie Pvt. Ltd., India), 4 g/L dextrose (Loba Chemie Pvt. Ltd., India), and 20 g/L agar (Griffchem Fine Chemicals, India), with the pH adjusted to 7.2. No antifungal or antibacterial supplements were added to the medium, in order to avoid suppressing antibiotic-sensitive *Streptomyces* morphotypes; selectivity was instead achieved through soil pretreatment, morphological screening, and repeated purification. 3 g of soil was taken and subsequently mixed with 27 mL of sterile distilled water and vortex-mixed until visibly homogeneous (approximately 1 minute) to prepare a 10^{-1} suspension, with each subsequent tenfold dilution vortex-mixed in turn up to 10^{-6}. An aliquot of 0.1 mL from each dilution (10^{-1} to 10^{-6}) was plated in triplicate onto ISP2 agar plates (Kharel et al., 2010; Caceres et al., 2018). Plates were checked daily for the appearance of chalky, powdery, or leathery colonies typical of *Streptomyces* spp. and incubated for 5-7 days at 28˚C (Saraswathi et al., 2020; Kazi et al., 2022). Between 2 and 8 presumptive colonies were selected from each plate, depending on colony abundance, and purified by repeated streaking onto fresh ISP2 plates until visually pure cultures were obtained. The purified isolates were subsequently used for characterization and co-culture assays. Working cultures were maintained on ISP2 slants at 4˚C for use throughout the study, while backup stocks were preserved in 20% glycerol at -20˚C or -80˚C for future use (Njenga et al., 2017).

Characterization of *Streptomyces*-like isolates

Identification of *Streptomyces*-like isolates relied on cultural and morphological features, assessed according to the *Actinobacteria* criteria outlined in *Bergey's Manual of Systematic Bacteriology* (Kämpfer, 2012). Colony color and texture were recorded for each isolate, with color classified according to the scheme described by Uasoontornnop et al. (2022); isolates displaying colony morphology characteristic of *Streptomyces* spp., such as chalky, powdery, or leathery texture, were retained for further study. Gram reaction, along with catalase and indole tests as supportive biochemical checks, was determined afterward on isolates carried forward through dual culture screening, following standard procedures (MacFaddin, 2000; Njenga et al., 2017).

Initial screening of *Aspergillus flavus*-inhibiting bacterial isolates through co-culture

The possible antagonistic effects of *Streptomyces*-like species against *A. flavus* were assessed using dual culture. All isolates displaying *Streptomyces*-like colony morphology, regardless of Gram reaction, were screened for antagonistic activity against *A. flavus* by dual culture. A loopful of the bacterial isolate was streaked onto one side of the Potato Dextrose Agar (PDA; Oxoid, United Kingdom) plate, no less than 3 cm from the center, and pre-incubated for 3-5 days at 28˚C to allow the streak to establish before the fungus was introduced (Sangdee et al., 2016; Campos-Avelar et al., 2021). A 6-mm-diameter agar plug was then removed from the leading edge of a 7-day-old *A. flavus* culture and positioned on the opposite side of the plate, no more than 3 cm from the center; the dual-culture plates were incubated for a further 5 days at 28˚C, with three replicates per isolate. Negative-control plates contained only the *A. flavus* plug on PDA, with no bacterial streak, and were incubated under the same conditions. At this stage, inhibition was assessed qualitatively, based on the presence or absence of a visible zone of reduced fungal growth; isolates showing clear inhibition were selected as candidate antagonists for further investigation (Sangdee et al., 2016; Shakeel et al., 2018).

Extraction of the bioactive compounds from the bacterial isolates

A loopful of each bacterial isolate was inoculated into 250 mL of ISP2 broth and incubated at 28˚C for 7 days on a rotary shaker at 150 rpm to promote production of antifungal substances (Shakeel et al., 2018). Following incubation, the fermentation broth was centrifuged at 2,000 rpm for 5 minutes at 20˚C to separate the bacterial biomass from the culture supernatant. The supernatant was passed through a 0.22 µm membrane filter and designated as the crude filtrate (CF), used directly for antifungal activity testing. The remaining supernatant was extracted with an equal volume of ethyl acetate in a separating funnel, shaken overnight at 28˚C, and left to separate into two distinct phases; the upper ethyl acetate layer was collected as the supernatant crude extract (SCE). The biomass pellet was likewise extracted overnight at 28˚C, using 150 mL of methanol (Laboklin Chimique, France), to yield the pellet crude extract (PCE). Both extracts were concentrated under reduced pressure using a rotary evaporator (Labtech EV400-L, Italy) at 45˚C, and the final crude extracts (SCE and PCE) were stored at 4˚C until use in antifungal activity assays (Shakeel et al., 2018).

***In vitro* antifungal activity using the disc diffusion assay**

The crude extracts (PCE, SCE, and CF) were dissolved in dimethyl sulfoxide (DMSO; Griffchem, India) to a stock concentration of 15 mg/mL (w/v) and stored at 4˚C until required. Working solutions at a final concentration of 1.5

mg/mL were prepared by diluting the stock in sterile distilled water. Antifungal activity was evaluated by disc diffusion, including sterile paper discs (Whatman, 12.5 cm, U.K.), 6 mm in diameter, were placed onto PDA plates inoculated with a fungal spore suspension (10^6 spores/mL) using a sterile cotton swab, in line with the concentration used by Abdel-Nasser et al. (2024), followed by application of a 20 µL aliquot of each extract (PCE, SCE, or CF) to the discs, alongside a positive control disc (Nystatin, SD025-1CT, HiMedia Laboratories, India) at 100 µg/mL and phosphate-buffered saline (PBS) as the negative control. Plates were incubated at 28˚C for 72 hours, after which inhibition zones were measured, with the 6 mm disc diameter subtracted from each reading to obtain the true zone of inhibition. The assay was conducted in three replicates (Shakeel et al., 2018).

Chemical profiling using gas chromatography-mass spectrometry

The crude extracts of the six active bacterial isolates were analyzed by gas chromatography-mass spectrometry (GC-MS), a technique suitable for the detection of volatile and semi-volatile organic compounds in complex biological samples, to identify bioactive metabolites potentially responsible for the antifungal activity observed in the *in vitro* screening assay. The pellet crude extract (PCE) of each isolate was analyzed directly in absolute methanol, prior to injection. Profiling of SA18, SD102, SD109, SD110, SE143, and SE194 was performed on a Shimadzu GC-MS (QP-2010SE system, Japan), and detected compounds were tentatively identified by comparing their mass spectra against the NIST 14 Mass Spectral Library.

Molecular characterization based on 16S rRNA sequencing

DNA was extracted from isolates showing activity in the *in vitro* antifungal assays, using the Quick-DNA Fungal/Bacterial Miniprep kit (D6005, Zymo Research, USA) according to ZYMO Research's protocol. The 16S rRNA gene was then amplified by PCR in a Biometra TRIO Thermal Cycler (Analytik Jena, Germany), using the universal primers fD1 (27F: AGAGTTTGATCMTGGCTCAG) and rP3 (1492R: TACGGYTACCTTGTTACGACTT; Weisburg et al., 1991). The cycling program comprised an initial denaturation at 94˚C for 5 minutes, followed by 31 cycles of denaturation at 95˚C for 30 seconds, annealing at 54˚C for 90 seconds, and extension at 72˚C for 120 seconds, with a final extension step at 72˚C for 5 minutes. Under these conditions, the primer pair yields a product of approximately 1.4 to 1.5 kb (Frank et al., 2008). Amplicons were resolved on a 1% (w/v) agarose gel in 1× TAE buffer, alongside an Accuris SmartCheck 50 bp DNA Ladder (Accuris Instruments, USA), stained with SafeView (ABM G108), and visualized on an E-Box Gel Documentation System (Vilber, France). The products were subsequently purified and sent for Sanger sequencing at Macrogen, Europe. Background noise was removed from the resulting sequences, which were then edited in BioEdit software version 7.0.5.2. The edited sequences were compared against the NCBI database using BLASTn for identification (Abiola and Oyetayo, 2016; Antido and Climacosa, 2022; Seidu et al., 2024). The sequences were then aligned using MUSCLE in MEGA11, and a phylogenetic tree was inferred by the Neighbor-Joining method, with node support assessed through 1,000 bootstrap replicates (Tamura et al., 2021).

Statistical analysis

OriginPro® (2024b) software was utilized for graphical analysis and data processing, with each experiment performed at least three times. Data were expressed as the mean ± standard deviation and analysed using one-way analysis of variance (ANOVA) with Tukey's test. Statistically significant differences across different groups were identified at $p < 0.05$.

Accession numbers

The bacterial isolates identified in the present study have been deposited in NCBI GenBank under the following accession numbers, including PZ675387, PZ675345, PZ675385, PZ675346, PZ675374, and PZ675373.

RESULTS AND DISCUSSION

***Streptomyces*-like isolate characterization**

After subculturing, presumptive *Streptomyces*-like colonies collectively displayed aerial mycelia ranging from white to grey, brown, and black, with dry, chalky, raised, and wrinkled colony surfaces (Figure 2). A total of 190 isolates displaying *Streptomyces*-like colony morphology were selected for a dual-culture screening assay against *A. flavus*, with Gram staining and biochemical characterization carried out afterward, including on isolates that indicated antifungal activity in the screening. All six active isolates, SA18, SD102, SD109, SD110, SE143, and SE194, were catalase-positive and indole-negative. Five active isolates, SA18, SD102, SD109, SD110, and SE194, were Gram-positive and filamentous, consistent with the phenotypic characteristics of *Streptomyces* spp., in agreement with previous studies

(Law et al., 2017; Khan et al., 2023). *Streptomyces* species have also been reported to produce secondary metabolites capable of inhibiting *A. flavus* (Managamuri et al., 2017; Alam et al., 2022). Isolate SE143 was Gram-negative and was subsequently identified by molecular analysis as *Comamonas* sp. rather than *Streptomyces*.

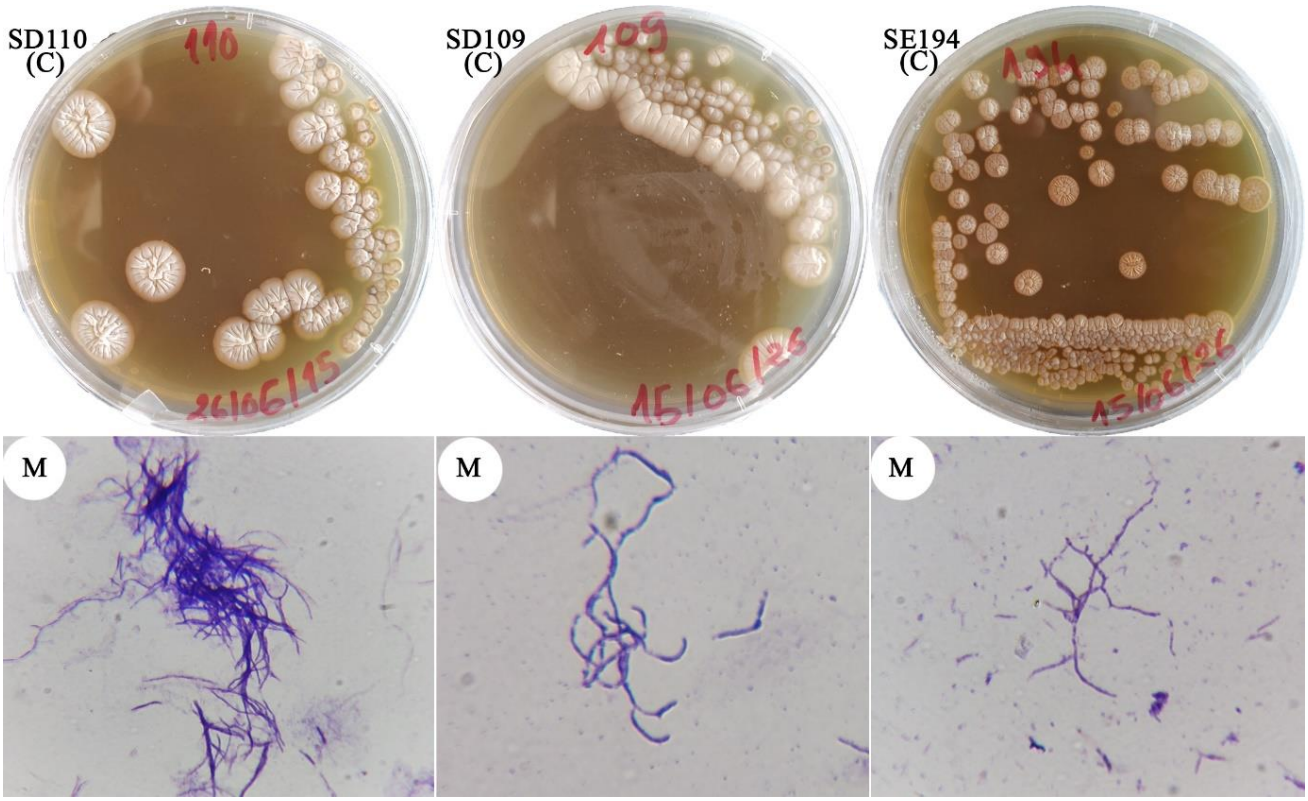


Figure 2. Morphological characterization of representative *Streptomyces* isolates SD110, SD109, and SE194. The upper panels correspond to the macroscopic colony **(C)** morphology, such as dry, raised, wrinkled, and chalky colonies, which are typical of *Streptomyces* spp. On Yeast Extract-Malt Extract Agar plates at 7 days. The lower panels correspond to the microscopic morphology **(M)**, showing Gram-positive, filamentous, and branching structures, consistent with Actinobacterial characteristics.

Characterization of *Aspergillus flavus*

The *A. flavus* strain was examined macroscopically and microscopically on PDA. The gross colony morphology, including its yellowish-green to olive-green color, and the microscopic features in lactophenol cotton blue-stained wet mounts were consistent with *A. flavus* morphology (Hedayati et al., 2007; Leema et al., 2010), as shown in Figure 3.

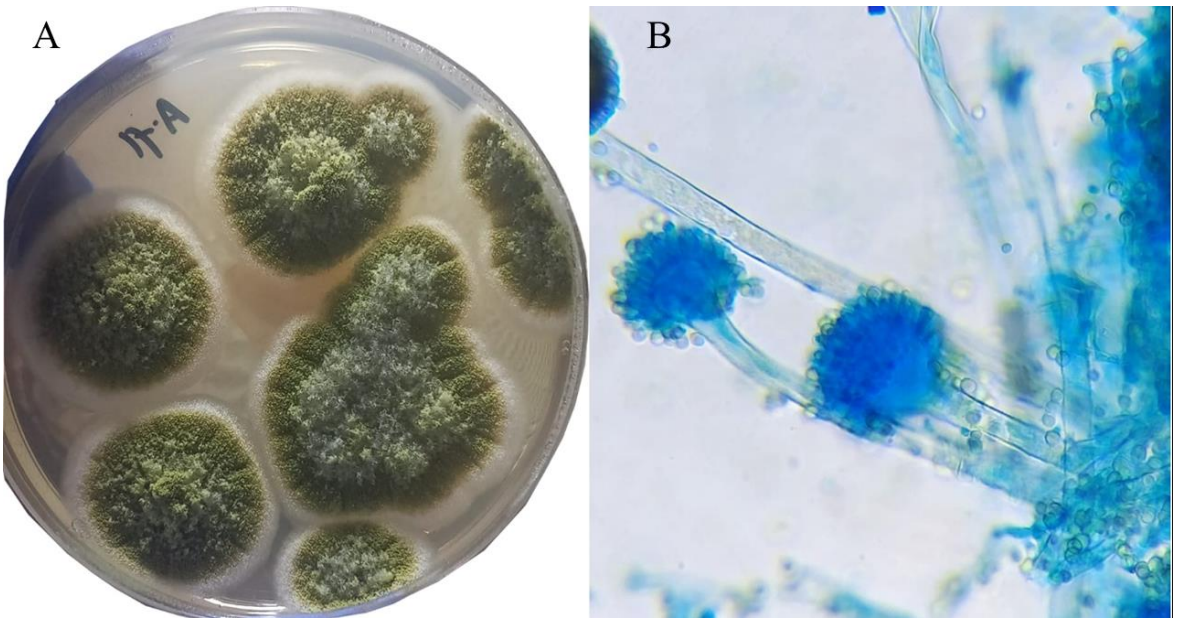


Figure 3. The colony and microscopic features of *Aspergillus flavus*. **A:** The 7-day-old colonies on Potato Dextrose Agar appear powdery, yellowish-green to olive-green with white margins, indicating active growth and spore production. **B:** Microscopically, septate hyphae, upright conidiophores, swollen vesicles, and chains of round conidia are observed, which support the identification of the isolate as *Aspergillus flavus*.

Initial screening of *Aspergillus flavus*-inhibiting bacterial isolates through co-culture

Six of the isolates tested in the initial dual-culture screening, SA18, SD102, SD109, SD110, SE143, and SE194, revealed potential antagonistic activity against *A. flavus*. All six isolates produced large inhibition zones, while the negative control indicated no inhibition. The isolates were therefore selected for extraction and the subsequent antifungal assay (Figure 4). The pattern of inhibition zones observed in the co-culture screening, contrasted with the lack of inhibition in the negative control, suggested that the isolates possess the ability to produce secondary metabolites capable of inhibiting *A. flavus*, consistent with antagonistic behavior reported for other soil bacterial isolates against *A. flavus* in co-culture (Sangdee et al., 2016; Shakeel et al., 2018; Campos-Avelar et al., 2021).

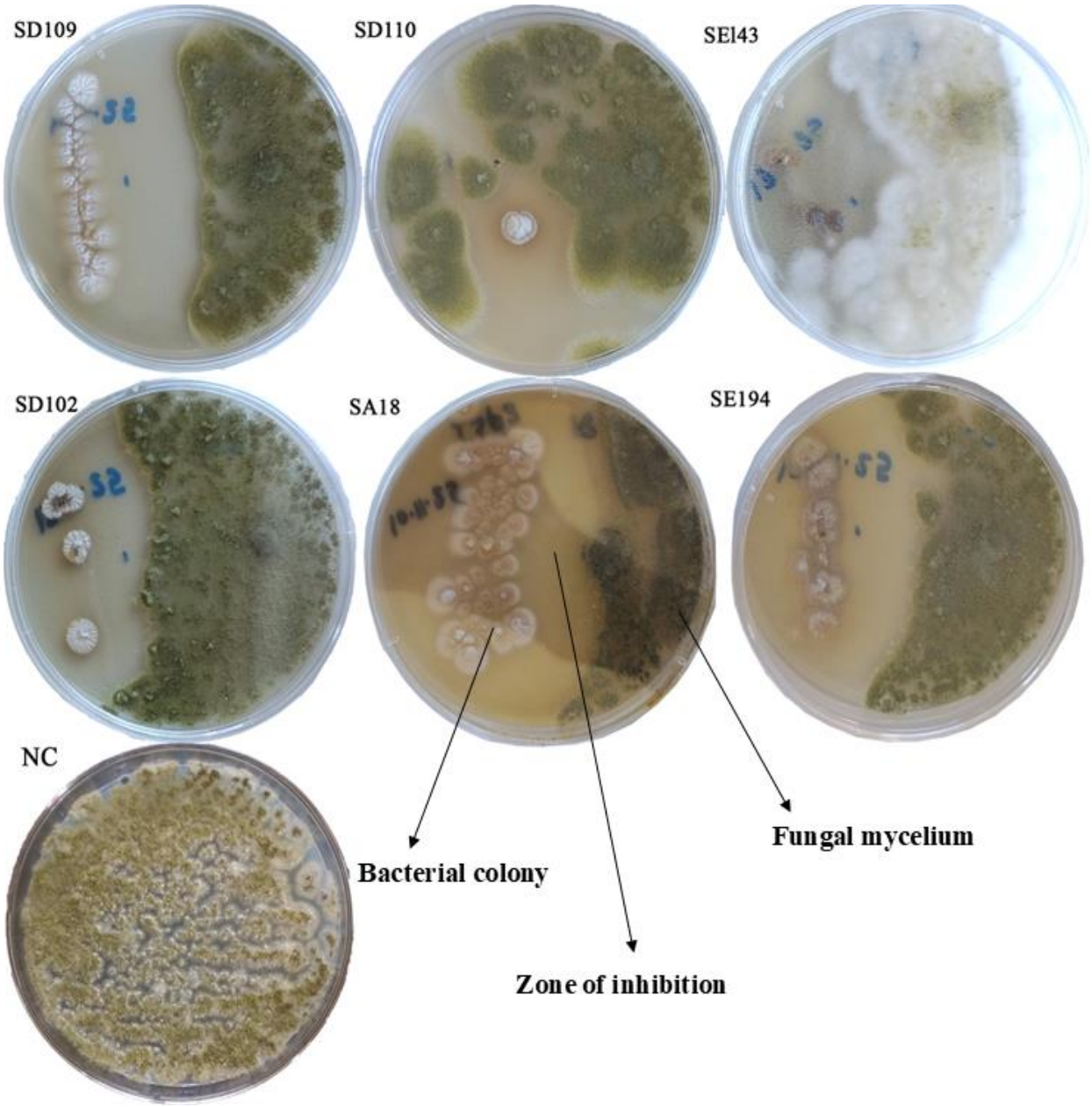


Figure 4. Dual culture assay revealing the antagonistic activity of selected *Streptomyces*-like isolates against *Aspergillus flavus* on Potato Dextrose Agar plates. The isolates SA18, SD102, SD109, SD110, SE143, and SE194 indicated inhibition of growth of *A. flavus*, as evidenced by the clear zone of inhibition observed between the bacterial colonies and the fungal mycelium.

***In vitro* antifungal assay**

As illustrated in Figure 5A, the negative control (PBS) indicated no inhibition of *A. flavus*; however, the inhibitory zone was clearly observable across all isolates subjected to a concentration of 1.5 mg/mL CE for each of the PCE, SCE, and CF.

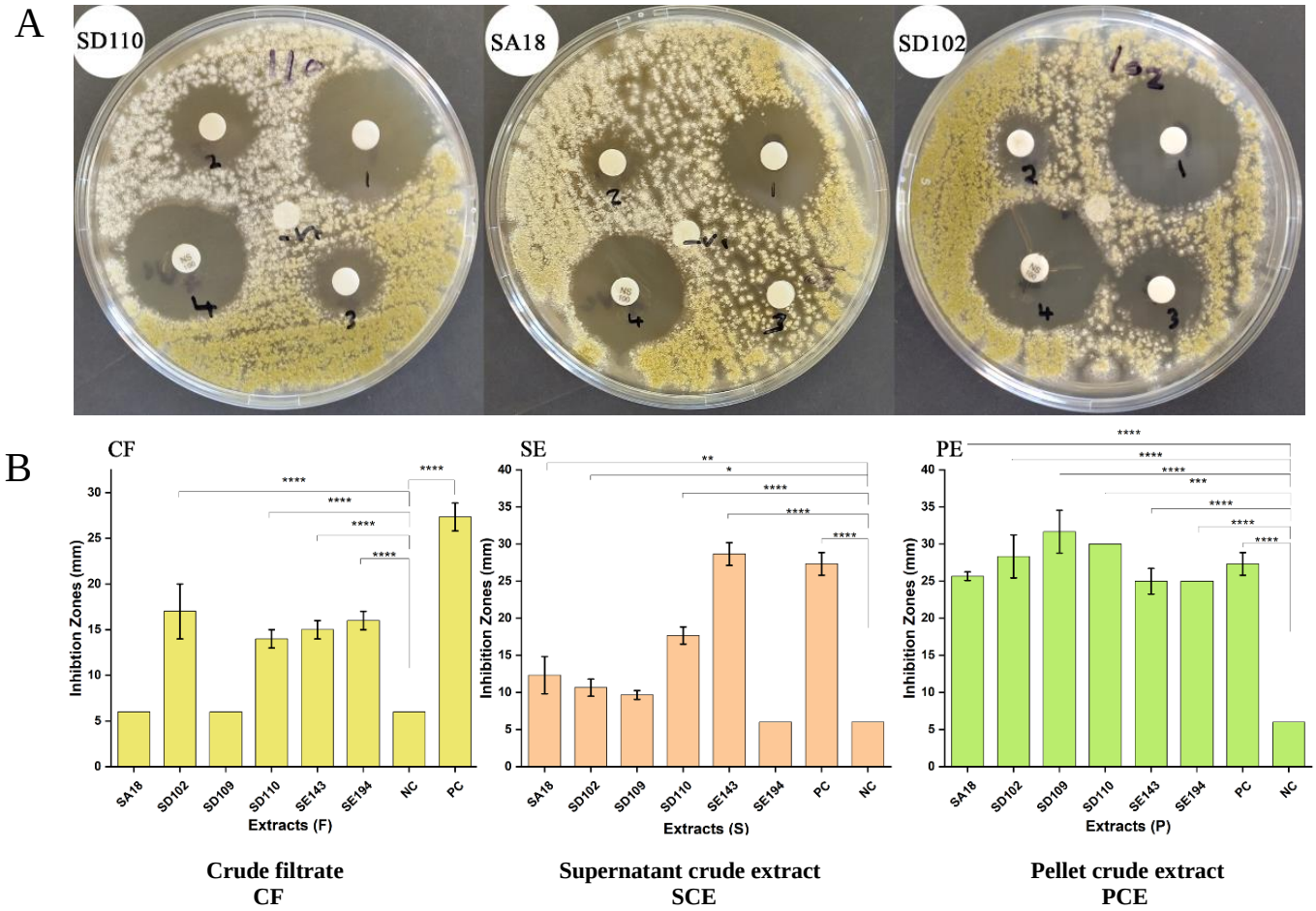


Figure 5. Antifungal activity of bacterial crude extracts against *A. flavus* in the disc diffusion assay. Inhibition zones produced by the extracts and controls for three of the six selected isolates against *A. flavus*. Discs 1, 2, 3, and 4 represent the pellet extract, supernatant extract, crude filtrate, and positive control, respectively. **A:** The negative control, PBS, was located in the center of each plate. **B:** Statistical analysis of the zones' diameters of the extracts against the negative control at the 0.05 level. The extracts significantly inhibited the growth of *A. flavus* compared with the negative control ($p < 0.05$).

The PCE indicated inhibition zones of 25.60 ± 0.50, 28.30 ± 2.80, 31.60 ± 2.80, 30.00 ± 0.00, 25.00 ± 1.70, and 25.00 ± 0.00 mm, corresponding to SA18, SD102, SD109, SD110, SE143, and SE194, respectively. The SCE, tested at the same concentration, indicated inhibition zones of 12.30 ± 2.50, 10.60 ± 1.10, 9.60 ± 0.50, 17.60 ± 1.10, 28.60 ± 1.50, and 0.00 mm for the same six isolates, respectively. Notably, SE143 was the one isolate for which the SCE (28.60 ± 1.50 mm) outperformed the PCE (25.00 ± 1.70 mm), the reverse of the pattern seen in the other five isolates, indicating that its antifungal metabolites were released into the culture supernatant rather than remaining concentrated in the biomass. The CF produced inhibition zones of 0.00, 17.00 ± 3.00, 0.00, 14.00 ± 1.00, 15.00 ± 1.00, and 16.00 ± 1.00 mm for SA18, SD102, SD109, SD110, SE143, and SE194, respectively, while the positive control produced an inhibition zone of (27.30 ± 1.50 mm) (Figure 5B). Several PCE values, most notably SD109 at 31.60 ± 2.80 mm, exceeded the positive control, while others, including SA18, SD102, and SE143, remained below it. The inhibition zones of all PCE at the tested concentration were significantly different compared to the negative control ($P < 0.05$); meanwhile, the zones of inhibition for the SCE for all isolates were significantly different except for SD109 and SE194 isolates (Figure 5B). Furthermore, the CF indicated significant differences in most of the isolates, including SD102, SD110, SE143, and SE194, without any difference in the SA18 and SD109 isolates (Figure 5B).

Taken together, the data indicated that the PCE consistently outperformed the supernatant extract and crude filtrate across most isolates, a pattern consistent with reports that many *Streptomyces* secondary metabolites remain associated with the mycelium or adsorbed onto the cell surface rather than being released into the surrounding medium, generally yielding more bioactive material from biomass extracts than from extracellular fractions (Manteca et al., 2008; Van Wezel and McDowall, 2011; Barka et al., 2015). Extracellular activity was not entirely absent, however: the supernatant crude extract inhibited *A. flavus* growth for five of the six isolates, and the crude filtrate inhibited growth for four of the six isolates, generally at lower potency than the pellet extract. Only SE194's supernatant extract, along with the crude filtrates of SA18 and SD109, revealed no detectable inhibition, which may reflect that extracellular metabolites were not produced at that growth stage, were synthesized below the inhibitory threshold, or were degraded during extraction and cultivation (Djinni et al., 2014). Similar observations have been reported for other *Streptomyces* species, in which antifungal activity was mostly shown in mycelial extracts, indicating the relevance of intracellular or cell-related metabolites in fungal proliferation suppression (Manteca et al., 2008; Prapagdee et al., 2008).

Molecular identification of isolates using 16S rRNA sequencing

Molecular analysis confirmed the morphological identification of five of the six soil isolates from Karura Forest as *Streptomyces*, while SE143 was reclassified as *Comamonas*, correcting its earlier morphology-based identification as a *Streptomyces*-like colony. Isolates were originally selected based on colony traits typical of *Streptomyces*. Amplification was clearly visible in all six lanes on the agarose gel, including SA18, SD102, SD109, SD110, SE143, and SE194, with PCR products of approximately 1.5 kb (Figure 6).

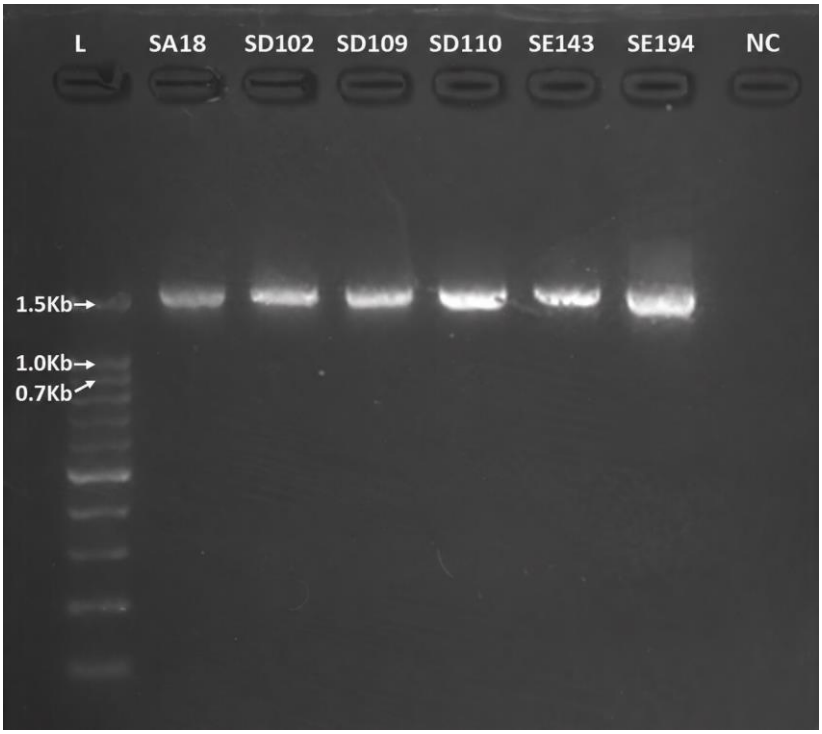


Figure 6. Electrophoresis of 16S rRNA PCR amplicons from antifungal bacterial isolates. Lane L represents the 50 bp DNA ladder, while lanes SA18, SD102, SD109, SD110, SE143, and SE194 indicate PCR products from isolates that exhibited antifungal activity, and lane NC represents the no-template negative control. The discrete bands observed at approximately 1.5 kb confirm successful amplification of the bacterial 16S rRNA gene, with no amplification in the negative control.

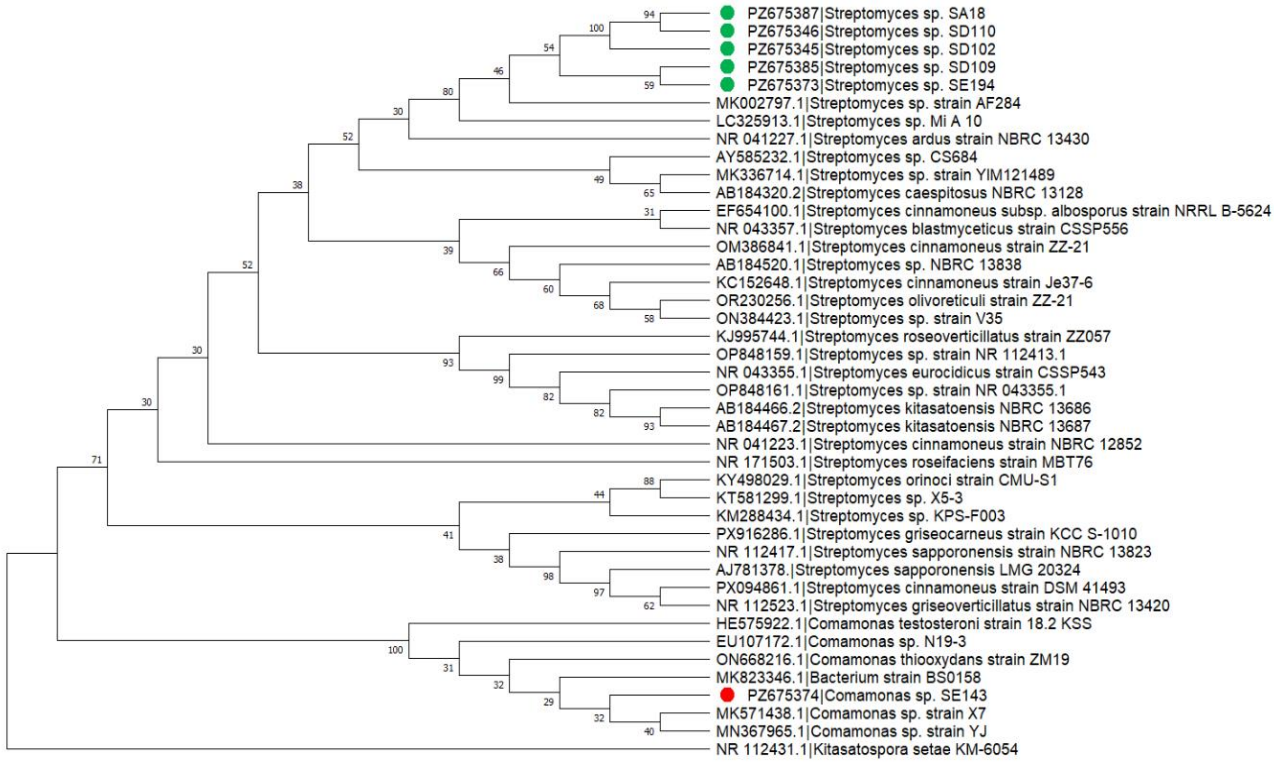


Figure 7. Phylogenetic tree based on partial 16S rRNA gene sequences showing the relationship between the selected isolates SA18, SD110, SD102, SD109, SE194, and SE143 and closely related reference strains obtained from GenBank. The clustering pattern places SA18, SD102, SD109, SD110, and SE194 within the genus *Streptomyces*, while SE143 clusters separately within the genus *Comamonas*.

Analysis of 1,634 aligned 16S rRNA nucleotide positions enabled the resolution of six bioactive Karura Forest soil isolates into two independent bacterial lineages; a dominant *Streptomyces* lineage and a separate branch of *Comamonas*-like bacteria. Five of the isolates (SA18, SD102, SD109, SD110, and SE194) were definitively clustered within the *Streptomyces* genus. The five *Streptomyces* isolates are indicated by green markers, while SE143 is shown by a red marker. Bootstrap values are displayed at the internal nodes, and *Kitasatospora setae* KM-6054 was included as the outgroup (Figure 7).

The phylogenetic placement demonstrated that most of the active antifungal strains belong to the phylum *Actinomycetota*, specifically the genus *Streptomyces*. The predominance of *Streptomyces* among the antifungal-active isolates aligned with earlier reports describing *Streptomyces* as a ubiquitous and readily culturable soil inhabitant capable of producing a wide range of bioactive secondary metabolites, a combination of traits that likely explains why *Streptomyces* dominated the pool of antifungal isolates recovered from Karura Forest soil in the present study (Dow et al., 2023; Manhas et al., 2023).

Further analysis of the topology of this genus identified a sibling-taxa pair (94% bootstrap value) between isolates SA18 and SD110, suggesting a close phylogenetic relationship in the forest ecosystem. SD102 diverged closest to this pair with strong bootstrap support of 100%, and SD109 and SE194 formed a neighboring sister subclade (Figure 7). All five isolates cluster closely within the same clade, but their different branch lines revealed that they are distinct wild-type strains. However, the 16S rRNA gene is highly conserved, and single-gene alignments generally lack sufficient resolution to confidently resolve *Streptomyces* to the species level. Consistent with a recent study by Kiepas et al. (2024) that 16S rRNA identity is a poor surrogate for multi-locus or whole-genome species classifications in *Streptomyces* genus, and the isolates have therefore been cautiously categorized as *Streptomyces* sp; the clear isolation of these bacteria from the standard database reference strains clearly suggests that Karura Forest harbors a may harbor locally adapted *Streptomyces* varieties of locally adapted *Streptomyces* varieties.

The *in vitro* inhibitory activity of the six indigenous isolates against *A. flavus* growth points to a potential tool for addressing a key ecological and agricultural concern in Kenya, aflatoxin contamination, although the present results reflect growth inhibition under laboratory conditions rather than confirmed suppression of aflatoxin biosynthesis or performance in field or storage settings. The growth inhibition of *A. flavus* observed here is consistent with earlier reports indicating that environmental *Streptomyces* strains can suppress *A. flavus* mycelial growth, with some also shown to reduce aflatoxin B1 biosynthesis, supporting the broader potential of *Streptomyces* as a biocontrol resource (Shakeel et al., 2018; Campos-Avelar et al., 2021).

Interestingly, Isolate SE143 was completely different from the actinobacterial clusters and clustered closely within the genus *Comamonas* with the reference strains X7 and YJ. The divergent placement of SE143 raises the possibility that antifungal potential within the Karura Forest soil microbiome is not limited to *Actinobacteria*, though that pattern rests on a single non-*Streptomyces* isolate and would need confirmation from additional *Comamonas* or other non-actinobacterial isolates before it can be generalised. *Comamonas* species are not as commonly used as *Streptomyces* for direct antifungal uses, but their opportunistic metabolic flexibility is gaining recognition (El-Banna, 2007). Other *Comamonas* strains have been reported to inhibit fungal pathogens through mechanisms such as competition for space and nutrients, iron-chelating siderophore production, and the release of antifungal volatile organic compounds (El-Banna, 2007; Timofeeva et al., 2022; Zhao et al., 2022). None of the proposed mechanisms were investigated for SE143 in the present study, so their relevance to SE143's antifungal activity remains to be tested.

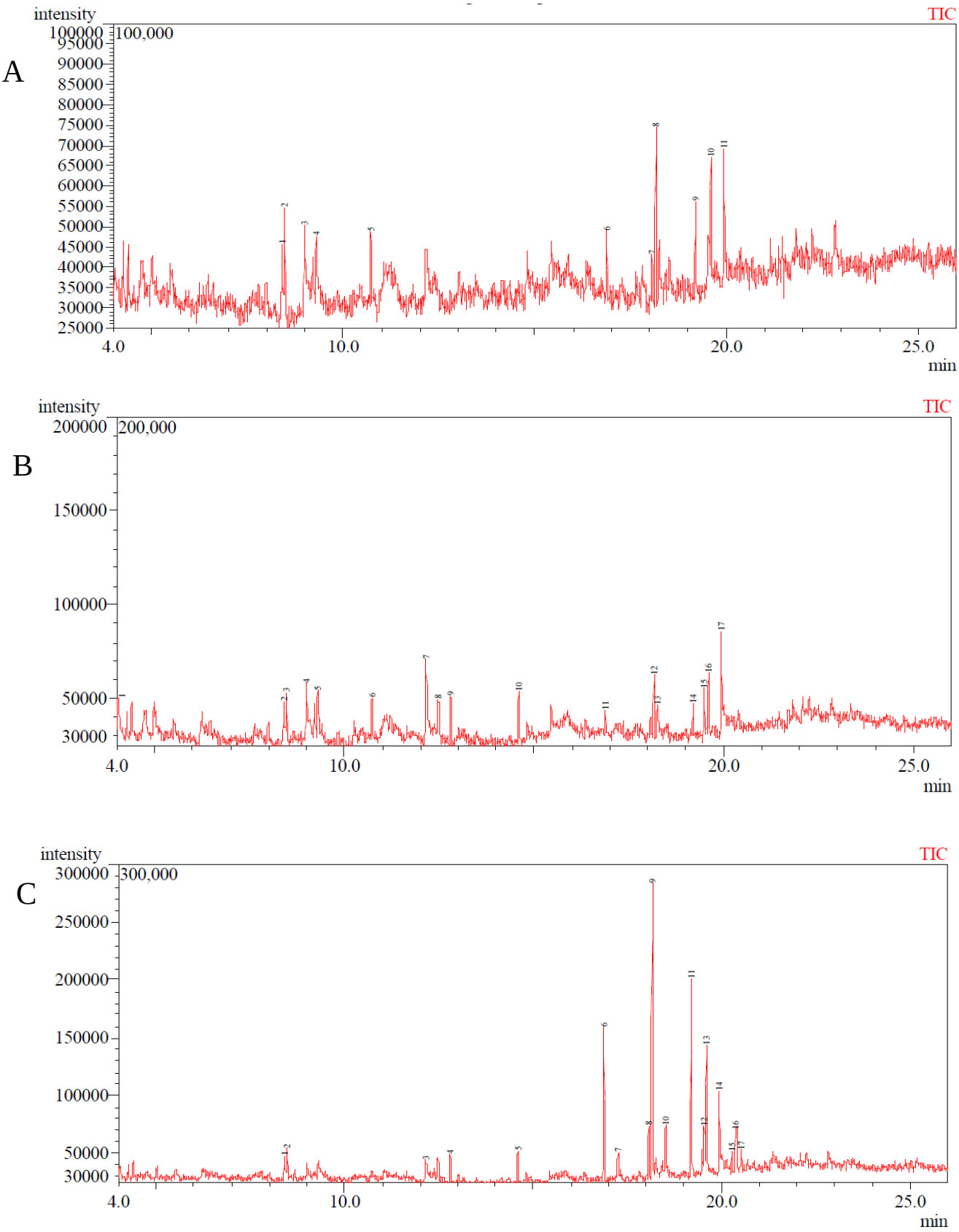
Gas chromatography-coupled mass spectrometry analysis

Chemical components of the PCE of the bacterial isolates SA18, SD102, SD109, SD110, SE143, and SE194, as determined by GC-MS, are shown in Figure 8A-F and Tables 1 to 6.

GC-MS analysis of the six bioactive isolates produced chromatograms of varying complexity, with the number of peaks ranging from 11 (SD102 and SE194) to 19 (SD110) across an overlapping retention window of roughly 4.0 to 21.4 minutes (Figure 8A-F, Tables 1 to 6). Fatty acids and their methyl esters, particularly tetradecanoic, pentadecanoic, and hexadecanoic acid derivatives, formed the leading peak class in SD102, SD109, and SE143. SA18, SD110, and SE194 instead revealed a more oxygenated profile, led by butanedioic acid derivatives, dl-glyceraldehyde dimer, and 2(3H)-furanone, dihydro-4-hydroxy-. Notably, SE143, which was identified as *Comamonas* rather than *Streptomyces*, still produced a lipid-rich profile comparable to the *Streptomyces* isolates, dominated by pentadecanoic acid, 14-methyl-, methyl ester and related fatty acid methyl esters.

Across all six isolates, the chromatograms converged on a shared core of chemical classes fatty acids and fatty acid methyl esters; organic acid derivatives, such as substituted butanedioic acids; furanone derivatives; and glyceraldehyde-related oxygenated compounds. Two isolates, SD110 and SE194, additionally carried the nitrogen-containing heterocycle pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-, a metabolite previously reported from *Streptomyces* extracts

(Ser et al., 2015), while the phenolic compound 2,4-di-tert-butylphenol was restricted to SA18 and SD109. Minor peaks were recorded for every isolate, including cyclopropyl carbinol, short-chain hydrocarbons, and additional fatty acid methyl ester isomers. The isolates therefore differed less in the broad chemical classes present than in the relative abundance and the specific fatty acid or organic acid derivatives that dominated each extract.



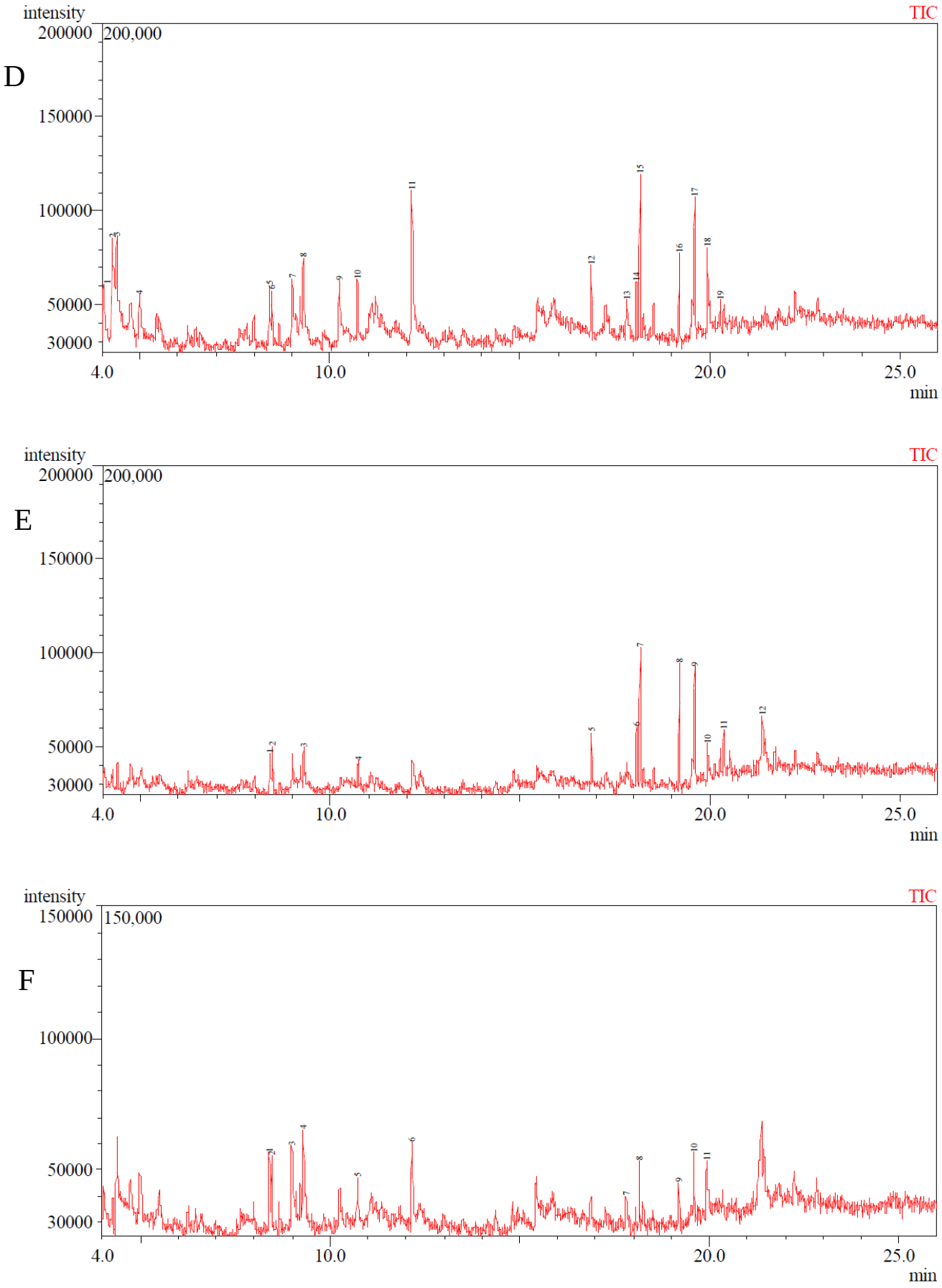


Figure 8. Gas chromatography-mass spectrometry total ion chromatogram of isolates. **A:** SA18, **B:** SD102, **C:** SD109, **D:** SD110, **E:** SE143, and **F:** SE194.

Table 1. Gas chromatography-mass spectrometry profile of bioactive compounds identified in SA18 isolate

SA18 Peak number	Retention time (minute)	Integration time	Peak area (%)	Name of identified compound	Molecular weight (g/mol)	Molecular formula	PubChem compound identifier
1	4.035	4.006	4.37	Glyceraldehyde	90.08	$C_3H_6O_3$	751
2	8.413	8.346	5.56	Cyclopropyl carbinol	72.11	C_4H_8O	75644
3	8.472	8.445	4.81	Tridecane, 2-methyl-	198.39	$C_{14}H_{30}$	15269
4	9.000	8.958	8.65	Butanedioic acid, 2,3-bis(acetyloxy)-, [R-(R*,R*)]-	234.16	$C_8H_{10}O_8$	103496
5	9.297	9.262	7.75	2(3H)-Furanone, dihydro-4-hydroxy-	102.09	$C_4H_6O_3$	95652
6	10.729	10.691	4.89	1,2,3-Propanetriol, 1-acetate	102.09	$C_4H_6O_3$	33510
7	12.160	12.114	12.44	Butanedioic acid, 2-hydroxy-2-methyl-, (S)-	148.11	$C_5H_8O_5$	441696
8	12.472	12.447	4.43	Propanoic acid, 2-methyl-, 3-hydroxy-2,2,4-trimethylpentyl ester	216.32	$C_{12}H_{24}O_3$	6490
9	12.796	12.756	4.98	Butanoic acid, butyl ester	144.21	$C_8H_{16}O_2$	7983
10	14.601	14.564	5.09	2,4-Di-tert-butylphenol	206.32	$C_{14}H_{22}O$	7311
11	16.880	16.857	1.79	Tridecanoic acid, 12-methyl-, methyl ester	242.40	$C_{15}H_{30}O_2$	21204
12	18.159	18.128	5.26	Tetradecanoic acid, 12-methyl-, methyl ester; (S)-	256.42	$C_{16}H_{32}O_2$	23618376
13	18.249	18.210	3.27	3-Hydroxy-4,5-dimethoxybenzoic acid	198.17	$C_9H_{10}O_5$	74709
14	19.193	19.167	2.41	Hexadecanoic acid, methyl ester	270.45	$C_{17}H_{34}O_2$	8181
15	19.476	19.447	4.91	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	282.33	$C_{17}H_{24}O_3$	545303
16	19.597	19.534	6.29	Methyl tetradecanoate	242.40	$C_{15}H_{30}O_2$	31284
17	19.932	19.896	13.07	Pentadecanoic acid	242.40	$C_{15}H_{30}O_2$	13849

Table 2. Gas chromatography-mass spectrometry profile of bioactive compounds identified in isolate SD102

SD102 Peak number	Retention time (minute)	Integration time	Peak area (%)	Name of identified compound	Molecular weight (g/mol)	Molecular formula	PubChem compound identifier
1	8.414	8.369	6.75	Cyclopropyl carbinol	72.11	C_4H_8O	75644
2	8.470	8.439	8.80	Tridecane, 2-methyl-	198.39	$C_{14}H_{30}$	15269
3	8.994	8.958	9.23	dl-Glyceraldehyde dimer	180.16	$C_6H_{12}O_6$	251615
4	9.299	9.262	9.22	2(3H)-Furanone, dihydro-4-hydroxy-	102.09	$C_4H_6O_3$	95652
5	10.726	10.697	6.62	1,2,3-Propanetriol, 1-acetate	134.13	$C_5H_{10}O_4$	33510
6	16.878	16.810	6.20	Tridecanoic acid, 12-methyl-, methyl ester	242.40	$C_{15}H_{30}O_2$	21204
7	18.059	18.047	3.59	Pentadecanoic acid, methyl ester	256.42	$C_{16}H_{32}O_2$	23518
8	18.157	18.087	14.07	Tetradecanoic acid, 12-methyl-, methyl ester, (S)-	256.42	$C_{16}H_{32}O_2$	23618376
9	19.196	19.143	8.39	Methyl tetradecanoate	242.40	$C_{15}H_{30}O_2$	31284
10	19.597	19.558	11.43	Hexadecanoic acid, methyl ester	270.45	$C_{17}H_{34}O_2$	8181
11	19.932	19.896	15.71	Tetradecanoic acid	228.37	$C_{14}H_{28}O_2$	11005

Table 3. Gas chromatography-mass spectrometry profile of bioactive compounds identified in isolate SD109

SD109 Peak number	Retention time (minute)	Integration time	Peak area (%)	Name of identified compound	Molecular weight (g/mol)	Molecular formula	PubChem compound identifier
1	8.411	8.375	2.34	Cyclopropyl carbinol	72.11	C_4H_8O	75644
2	8.470	8.439	2.69	Tridecane	184.36	$C_{13}H_{28}$	12388
3	12.160	12.114	2.38	Butanedioic acid, 2-hydroxy-2-methyl-, (S)-	148.11	$C_5H_8O_5$	441696
4	12.796	12.768	2.69	Propanoic acid, 2-methyl-, 3-hydroxy-2,2,4-trimethylpentyl ester	216.32	$C_{12}H_{24}O_3$	6490
5	14.602	14.570	2.36	2,4-Di-tert-butylphenol	206.32	$C_{14}H_{22}O$	7311
6	16.878	16.839	11.97	Tridecanoic acid, 12-methyl-, methyl ester	242.40	$C_{15}H_{30}O_2$	21204
7	17.254	17.230	1.64	Tetradecanoic acid	228.37	$C_{14}H_{28}O_2$	11005
8	18.065	18.029	4.06	Pentadecanoic acid, methyl ester	256.42	$C_{16}H_{32}O_2$	23518
9	18.159	18.105	22.70	Methyl tetradecanoate	242.40	$C_{15}H_{30}O_2$	31284
10	18.517	18.484	3.78	Eicosanoic acid	312.53	$C_{20}H_{40}O_2$	10467
11	19.195	19.161	15.21	Hexadecanoic acid, methyl ester	270.45	$C_{17}H_{34}O_2$	8181
12	19.538	19.505	3.78	Eicosanoic acid	312.53	$C_{20}H_{40}O_2$	10467
13	19.597	19.563	10.26	Nonadecanoic acid, methyl ester	312.53	$C_{20}H_{40}O_2$	15610
14	19.935	19.896	7.92	Octadecanoic acid	284.48	$C_{18}H_{36}O_2$	5281
15	20.270	20.252	1.26	Hexadecanoic acid, 14-methyl-, methyl ester	284.48	$C_{18}H_{36}O_2$	520159
16	20.363	20.328	3.40	Heptadecanoic acid, methyl ester	284.48	$C_{18}H_{36}O_2$	15609
17	20.514	20.485	1.57	10-Octadecenoic acid, methyl ester	296.49	$C_{19}H_{36}O_2$	12830109

Table 4. Gas chromatography-mass spectrometry profile of bioactive compounds identified in isolate SD110

SD110 Peak number	Retention time (minute)	Integration time	Peak area (%)	Name of identified compound	Molecular weight (g/mol)	Molecular formula	PubChem compound identifier
1	4.035	4.012	4.24	Glyceraldehyde	90.08	$C_3H_6O_3$	751
2	4.277	4.251	8.43	Dimethyl Sulfoxide	78.13	C_2H_6OS	679
3	4.391	4.362	4.48	2-Furanmethanol	98.10	$C_5H_6O_2$	7361
4	4.985	4.957	5.41	Dihydroxyacetone	90.08	$C_3H_6O_3$	670
5	8.411	8.375	3.72	Cyclopropyl carbinol	72.11	C_4H_8O	75644
6	8.470	8.445	2.43	Undecane	156.31	$C_{11}H_{24}$	14257
7	9.008	8.958	8.11	dl-Glyceraldehyde dimer	180.16	$C_6H_{12}O_6$	251615
8	9.300	9.256	4.41	2(3H)-Furanone, dihydro-4-hydroxy-	102.09	$C_4H_6O_3$	95652
9	10.251	10.218	4.81	Tetrahydrofuran-5-on-2-methanol, .alpha.-[.alpha.-methoxy-(tetrahydrofuran-5-on-2-ylmethoxy)]-	260.24	$C_{11}H_{16}O_7$	558876
10	10.727	10.691	4.01	1,2,3-Propanetriol, 1-acetate	134.13	$C_5H_{10}O_4$	33510
11	12.158	12.114	12.88	Butanedioic acid, 2-hydroxy-2-methyl-, (S)-	148.11	$C_5H_8O_5$	441696
12	16.876	16.845	3.78	Methyl 9-methyltetradecanoate	256.42	$C_{16}H_{32}O_2$	554137
13	17.815	17.790	1.29	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-	154.17	$C_7H_{10}N_2O_2$	193540
14	18.061	18.029	3.61	Pentadecanoic acid, methyl ester	256.42	$C_{16}H_{32}O_2$	23518
15	18.159	18.099	9.31	Tetradecanoic acid, 12-methyl-, methyl ester, (S)-	256.42	$C_{16}H_{32}O_2$	23618376
16	19.194	19.167	4.52	Nonadecanoic acid, methyl ester	312.53	$C_{20}H_{40}O_2$	15610
17	19.597	19.563	7.04	Methyl tetradecanoate	242.40	$C_{15}H_{30}O_2$	31284
18	19.934	19.896	5.92	Tridecanoic acid	214.34	$C_{13}H_{26}O_2$	12530
19	20.269	20.240	1.60	Heptadecanoic acid, methyl ester	284.48	$C_{18}H_{36}O_2$	15609

Table 5. Gas chromatography-mass spectrometry profile of bioactive compounds identified in isolate SE143

SE143 Peak number	Retention time (minute)	Integration time	Peak area (%)	Name of identified compound	Molecular weight (g/mol)	Molecular formula	PubChem compound identifier
1	8.409	8.381	4.36	Cyclopropyl carbinol	72.11	C_4H_8O	75644
2	8.470	8.445	4.89	Undecane	156.31	$C_{11}H_{24}$	14257
3	9.302	9.268	5.26	2(3H)-Furanone, dihydro-4-hydroxy-	102.09	$C_4H_6O_3$	95652
4	10.737	10.702	3.50	1,2,3-Propanetriol, 1-acetate	134.13	$C_5H_{10}O_4$	33510
5	16.876	16.851	5.81	Tridecanoic acid, 12-methyl-, methyl ester	242.40	$C_{15}H_{30}O_2$	21204
6	18.064	18.035	5.79	Pentadecanoic acid, methyl ester	256.42	$C_{16}H_{32}O_2$	23518
7	18.157	18.122	14.94	Tetradecanoic acid, 12-methyl-, methyl ester, (S)-	256.42	$C_{16}H_{32}O_2$	23618376
8	19.194	19.155	13.40	Hexadecanoic acid, methyl ester	270.45	$C_{17}H_{34}O_2$	8181
9	19.595	19.563	16.23	Pentadecanoic acid, 14-methyl-, methyl ester	270.45	$C_{17}H_{34}O_2$	21205
10	19.932	19.902	5.55	l-(+)-Ascorbic acid 2,6-dihexadecanoate	652.94	$C_{38}H_{68}O_8$	54722209
11	20.358	20.293	6.57	Methyl 18-methylnonadecanoate	326.56	$C_{21}H_{42}O_2$	530340
12	21.375	21.272	13.69	9,12-Octadecadienoic acid, methyl ester	294.47	$C_{19}H_{34}O_2$	5284421

Table 6. Gas chromatography-mass spectrometry profile of bioactive compounds identified in isolate SE194

SE194 Peak number	Retention time (minute)	Integration time	Peak area (%)	Name of identified compound	Molecular weight (g/mol)	Molecular formula	PubChem compound identifier
1	8.406	8.375	10.05	Cyclopropyl carbinol	72.11	C_4H_8O	75644
2	8.469	8.445	7.61	Undecane	156.31	$C_{11}H_{24}$	14257
3	8.994	8.958	16.39	dl-Glyceraldehyde dimer	180.16	$C_6H_{12}O_6$	251615
4	9.298	9.268	8.88	2(3H)-Furanone, dihydro-4-hydroxy-	102.09	$C_4H_6O_3$	95652
5	10.728	10.697	5.42	1,2,3-Propanetriol, 1-acetate	134.13	$C_5H_{10}O_4$	33510
6	12.161	12.120	13.36	Butanedioic acid, 2-hydroxy-2-methyl-, (S)-	148.11	$C_5H_8O_5$	441696
7	17.811	17.772	7.12	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-	154.17	$C_7H_{10}N_2O_2$	193540
8	18.156	18.134	8.28	Tridecanoic acid, 12-methyl-, methyl ester	242.40	$C_{15}H_{30}O_2$	21204
9	19.193	19.161	5.31	Pentadecanoic acid, 14-methyl-, methyl ester	270.45	$C_{17}H_{34}O_2$	21205
10	19.593	19.558	7.55	Hexadecanoic acid, methyl ester	270.45	$C_{17}H_{34}O_2$	8181
11	19.937	19.902	10.04	Tridecanoic acid	214.34	$C_{13}H_{26}O_2$	12530

The GC-MS profiling of the selected bioactive isolates revealed a diverse metabolite pattern, reinforcing the antifungal activity observed in the *in vitro* disc diffusion assay. In general, the chromatograms were mainly characterized by fatty acids, fatty acid methyl esters, derivatives of organic acids, oxygenated metabolites, phenolic compounds, compounds related to furanone, and nitrogen-containing heterocyclic metabolites. The presence of such chemical compounds is common in bacterial extracts, as fatty acids and derived compounds are known to impede the propagation of fungi by affecting cellular integrity, altering permeability properties, and disrupting fungal growth (Zhao et al., 2020; Guimarães and Venâncio, 2022). Consequently, the presence of fatty acid, organic acid, and furanone-related compounds is consistent with the antifungal effects observed. In particular, the GC-MS profile of SA18 (Figure 8A, Table 1) indicated a broad chemical composition dominated by organic acids, furanone derivatives, fatty acid derivatives, and phenolic compounds. The major compound was pentadecanoic acid, followed by butanedioic acid, 2-hydroxy-2-methyl-, (S)-, butanedioic acid, 2,3-bis(acetyloxy)-, and 2(3H)-furanone, dihydro-4-hydroxy-. The co-occurrence of 2,4-di-tert-butylphenol, methyl tetradecanoate, and hexadecanoic acid methyl ester in the SA18 extract points to metabolite classes that other studies have linked to membrane-active and oxidative stress-related antifungal mechanisms (Zhao et al., 2020; Guimarães and Venâncio, 2022), although neither mechanism was tested directly in the present study and no single compound from the extract was isolated for individual bioassay against *A. flavus*. Bioactive metabolites such as phenolic compounds, e.g., 2,4-di-tert-butylphenol, were reported from different biological sources, while fatty acid derivatives may destabilize fungal lipid membranes and increase cellular leakage (Zhao et al., 2020; Guimarães and Venâncio, 2022). The mixed metabolite profile most likely contributed to the antifungal inhibition recorded for SA18.

SD102 (Figure 8B, Table 2) exhibited a more lipid-related chemical composition, with tetradecanoic acid, 12-methyl-, methyl ester, and hexadecanoic acid methyl ester as the main components. Other identified metabolites, such as dl-glyceraldehyde dimer, 2(3H)-furanone, dihydro-4-hydroxy-, and methyl tetradecanoate, also imply that the isolate produced oxygenated and furanone-type compounds. Fatty acids and fatty acid methyl esters were present, indicating that the main mechanism of antifungal activity of SD102 could be the disruption of the membrane and alteration of permeability (Bhattacharyya et al., 2020). Fungal cell membranes are very sensitive to chemicals interacting with lipids, and this has been associated with antifungal action in similar fatty acid profiles (Guimarães and Venâncio, 2022). In the case of SD109, the GC-MS profile (Figure 8C, Table 3) was dominated by lipid-derived metabolites. The main compounds were methyl tetradecanoate, hexadecanoic acid methyl ester, tridecanoic acid, 12-methyl-, methyl ester, nonadecanoic acid methyl ester, and octadecanoic acid. Medium- and long-chain fatty acids and their methyl esters made up most of the detected peaks in the SD109 extract, a pattern consistent with membrane-targeting antifungal activity reported in other fatty acid-rich extracts (Prasath et al., 2019; Bhattacharyya et al., 2020; Lee et al., 2021). The additional detection of 2,4-di-tert-butylphenol adds further strength to the bioactive profile of SD109, as phenolic metabolites can also increase antimicrobial action through the induction of oxidative stress, interference with enzymatic activity, and membrane damage (Zhao et al., 2020). Thus, the antifungal activity of SD109 is probably due to synergistic effects of lipid-derived metabolites and phenolic compounds. The GC-MS profile of SD110 (Figure 8D, Table 4) revealed a more balanced chemical composition with oxygenated metabolites, organic acid derivatives, fatty acid methyl esters, furanone-related compounds, and nitrogen-containing heterocycles. The major compounds were butanedioic acid, 2-hydroxy-2-methyl-, (S)-, tetradecanoic acid, 12-methyl-, methyl ester, dimethyl sulfoxide, dl-glyceraldehyde dimer, and methyl tetradecanoate. SD110 additionally contained the heterocyclic metabolite pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-, a compound previously reported from *Streptomyces* extracts and linked to bioactive secondary metabolism (Ser et al., 2015). The combined presence of fatty acid methyl esters, organic acid derivatives, and the heterocyclic compound suggested that SD110 may function via more than one antifungal pathway, potentially including membrane destabilization (Bhattacharyya et al., 2020; Guimarães and Venâncio, 2022) as well as metabolic interference and oxidative stress-related effects (Yu et al., 2025).

Although SE143 has been identified as a *Comamonas* and not a *Streptomyces*, its GC-MS profile (Table 5) also supports its antifungal activity. The chromatogram (Figure 8E) was dominated by fatty acid methyl esters, especially pentadecanoic acid, 14-methyl-, methyl ester, tetradecanoic acid, 12-methyl-, methyl ester, 9,12-octadecadienoic acid methyl ester, and hexadecanoic acid methyl ester. Both saturated and unsaturated fatty acid methyl esters were present, a combination linked in other bacterial extracts to lipid membrane interaction as a possible antifungal route (Bhattacharyya et al., 2020). The presence of 9,12-octadecadienoic acid methyl ester, a linoleic acid methyl ester derivative, is especially relevant because unsaturated fatty acids and their derivatives are often discussed as antifungal compounds, capable of altering membrane function and fungal development (Prasath et al., 2019; Bhattacharyya et al., 2020; Guimarães and Venâncio, 2022). Thus, the activity of SE143 appears to be explained more by its fatty acid-rich chemical profile than by taxonomic identity.

The GC-MS profile of SE194 (Figure 8F, Table 6) differed from the more lipid-dominant isolates by revealing a stronger contribution from oxygenated and heterocyclic compounds. The major compounds included dl-glyceraldehyde

dimer, butanedioic acid, 2-hydroxy-2-methyl-, (S)-, cyclopropyl carbinol, tridecanoic acid, and 2(3H)-furanone, dihydro-4-hydroxy-. Hexahydro-pyrrolo[1,2-a]pyrazine-1,4-dione was detected at a higher relative abundance in SE194 than in SD110, the only other isolate in which the compound occurred; it has been reported from *Streptomyces* extracts and associated with biologically active secondary metabolism (Ser et al., 2015). The antifungal activity observed for SE194 can be attributed to the presence of a heterocyclic molecule, organic acids, and furanone derivatives, a combination consistent with the broader capacity of *Streptomyces* species to generate diverse secondary metabolites (Alam et al., 2022). Overall, the GC-MS data revealed that the active isolates contain a variety of metabolite classes that have been reported. The chemical profiles of the *Streptomyces* isolates SA18, SD102, SD109, SD110, and SE194 were in agreement with the ability of *Streptomyces* to produce a wide range of beneficial secondary metabolites (Alam et al., 2022). SE143, identified taxonomically as *Comamonas* rather than *Streptomyces*, nonetheless indicated antifungal activity in the disc diffusion assay, consistent with its high abundance of fatty acid methyl esters and with reports of antifungal activity in *Comamonas* species (El-Banna, 2007; Guimarães and Venâncio, 2022). The observed *in vitro* antifungal activity is therefore unlikely to result from a single compound. A more plausible explanation is the combined presence of several metabolite classes, fatty acid derivatives, phenolic compounds, organic acids, furanone derivatives, and nitrogen-containing heterocyclic metabolites, acting together rather than individually, a pattern consistent with reports that environmental bacteria, including *Streptomyces*, commonly co-produce multiple antimicrobial compounds that function synergistically (Meyer and Nodwell, 2021).

CONCLUSION

The current findings demonstrated that Karura Forest soil is a valuable source of bioactive bacterial isolates with antifungal potential. Selective isolation, preliminary characterization, 16S rRNA gene amplification, and antifungal screening resulted in the successful isolation and confirmation of five active *Streptomyces* isolates (SA18, SD102, SD109, SD110, and SE194) and one active *Comamonas* isolate (SE143) as promising candidates for further biological evaluation. The isolates demonstrated consistent antifungal activity in the *in vitro* disc diffusion assay, thus suggesting that their inhibitory effects were associated with the biologically active metabolites produced by the bacteria. GC-MS profiling of the bioactive extracts revealed diverse metabolite classes, including fatty acids, fatty acid methyl esters, organic acid derivatives, furanone-related compounds, phenolic metabolites, and nitrogen-containing heterocyclic compounds such as 2,4-di-tert-butylphenol and pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro. The presence of the compound classes across both *Streptomyces* and *Comamonas* extracts indicates that useful antifungal metabolites in the collection were not limited to *Actinobacteria*. Overall, Karura Forest soil represents a promising reservoir of indigenous, beneficial microorganisms for biocontrol development. The six isolates identified here are attractive candidates for bio-inoculant development, with potential to help manage *A. flavus* contamination and support food safety in Kenyan agricultural systems, pending further evaluation under crop-based, storage, and field conditions as candidates for future biocontrol against *A. flavus* and other postharvest fungal pathogens. Different limitations should be addressed in future work. Quantitative measurement of aflatoxin B1 levels, by high-performance liquid chromatography (HPLC) or ELISA, following treatment with the bioactive extracts is needed to confirm whether the observed suppression of *A. flavus* growth translates into reduced aflatoxin production, since only fungal growth inhibition was assessed in the present study. *In vivo* validation on crop and stored-grain substrates under realistic storage and field conditions is also required before the isolates can be considered practical biocontrol candidates, given that efficacy was demonstrated only under controlled *in vitro* conditions.

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Authors' contributions

Esraa Mohammady Abdelzaher Elsemin conceived the study idea, designed the methodology, carried out the data collection and statistical analysis, and composed the initial draft of the manuscript. Andrew Nyerere Kimang'a proposed Karura Forest as the soil sampling location and, together with Joel Ltilitan Bargul, contributed to the critical revision and editing of the manuscript. All authors reviewed and approved the final version of the manuscript prior to its publication in the current journal.

Availability of data and materials

All data generated and analyzed in the current study are incorporated within the publication, with no additional files provided. Inquiries for supplementary data produced in this work should be addressed to the corresponding author.

Competing interests

The authors declared no conflicts of interest.

Ethical considerations

The authors declared that the current study is original, has not been submitted for publication or under review elsewhere, and complies with ethical norms concerning data integrity and the prevention of plagiarism. The authors confirmed that no artificial intelligence tools were employed in the composition and production of the manuscript. The authors confirm that the results and underlying data presented in this manuscript are original and have been independently verified by the authors for accuracy and integrity prior to submission and acceptance for publication.

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Optimizing Fermented Water Hyacinth Supplementation with *Neurospora crassa* in Ducks: Implications for Performance and Small Intestine Morphology

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ABSTRACT

Water hyacinth is a potential alternative feed ingredient for ducks, but its utilization is limited by high fiber content and low digestibility. The present study aimed to evaluate the effects of diets containing water hyacinth fermented with *Neurospora crassa* on feed intake, nutrient intake, growth performance, carcass traits, and intestinal morphology in Pekin ducks. A total of 100 ducks with an average initial body weight of 116 g were used in a completely randomized design with five treatments and four replications. Each replicate consisted of five ducks, and the experiment was conducted from 7 to 35 days of age. The treatments consisted of 0% water hyacinth (P1), 5% non-fermented water hyacinth (P2), 5% fermented water hyacinth (P3), 10% fermented water hyacinth (P4), and 15% fermented water hyacinth (P5). The feeding trial was conducted for four weeks. The analyzed parameters included feed intake, crude protein intake, crude fiber intake, metabolizable energy, feed conversion ratio, final body weight, weight gain, carcass percentage, and intestinal morphology. The results revealed that fermented water hyacinth inclusion had no significant effect on feed intake, metabolizable energy, feed conversion ratio, final body weight, weight gain, carcass percentage, breast percentage, thigh percentage, back percentage, and wing percentage among all treatment groups. No significant differences were observed in intestinal morphology parameters, including duodenal length, jejunal length, ileal length, and intestinal villus length among groups P1 to P5. However, ducks fed 15% fermented water hyacinth (P5) demonstrated significantly higher crude protein and crude fiber intakes compared to other groups. Water hyacinth fermented with *Neurospora crassa* can be included in Pekin duck diets at up to 15% without negatively affecting feed intake, growth performance, carcass traits, or intestinal morphology, while increasing crude protein and crude fiber intakes.

Keywords: Carcass trait, Duck, Fermentation, Intestinal morphology, *Neurospora crassa*, Water hyacinth

INTRODUCTION

Duck farming plays a vital role in supporting food security and the local economy (Adzitey and Adzitey, 2011). Small-scale duck farming contributes to food security and rural economic development by improving household access to animal-source foods, increasing duck consumption and sales, and providing an important source of income for smallholder farmers (Hossain et al., 2021; Herlina et al., 2023; Priyanto et al., 2025). In addition to producing eggs and meat, ducks also possess significant economic value, as duck farming provides income for farmers, supports household livelihoods, and contributes to the economic sustainability of smallholder farming systems (Ali et al., 2025). In 2020, the global duck population was estimated at 1.15 billion, of which approximately 1.0 billion, or 89%, were located in Asia, with the highest population concentrations found in China, Vietnam, Bangladesh, and Indonesia (FAO, 2025). According to Statistics Indonesia (2026), the duck population in Indonesia was recorded at 45,330,236. The large duck population may partly reflect ducks' resilience to environmental conditions and their capacity to adapt to locally available feed (Rosario and Secondez, 2025). The relatively large duck population may be linked to their tolerance of local environmental conditions and their capacity to convert available local and alternative feed resources into productive outputs (Rosario and Secondez, 2025). However, duck productivity is often constrained by the quality and availability of feed. Poor feed quality negatively affects growth performance, feed efficiency, and overall production performance in duck farming systems (Linh et al., 2022). Feed quality remains an important constraint in duck production, as inadequate or nutritionally imbalanced diets may reduce production performance and overall productivity in duck farming systems (Susanti et al., 2025).

The high cost of feed ingredients may limit farmers' ability to provide high-quality diets for Pekin ducks, leading to the use of lower-cost alternative feed ingredients with variable nutritional quality (Ding et al., 2022). Rising feed costs often force producers to compromise on ingredient quality and nutritional balance in feed formulation (Mottet et al., 2017). Constraints in feed availability and high feed costs may result in the formulation of nutritionally inadequate diets,

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thereby reducing production performance and farm efficiency (Gunawan et al., 2024). Feed costs represent the most substantial component of duck production expenses. Because feed cost represents a major proportion of poultry production expenses, the use of locally available alternative feed ingredients is needed to reduce dependence on commercial feed (Thirumalaisamy et al., 2016; Alhotan, 2021).

Water hyacinth is a promising low-cost feed resource because it grows abundantly in freshwater ecosystems, is widely available in tropical areas, and can be processed into poultry feed ingredients, with its rapid growth under favorable environmental conditions enabling the production of substantial biomass and strengthening its potential as an alternative feed ingredient (Chala et al., 2026). Water hyacinth (*Eichhornia crassipes*), an invasive aquatic weed with rapid growth and high biomass production, has potential as an alternative feed ingredient for ducks due to its abundant availability and low utilization cost. However, its high fiber content limits its direct application in poultry diets, requiring appropriate processing methods such as fermentation to improve its nutritional value and utilization efficiency (Harun et al., 2021; Antonio et al., 2025). The high biomass production, extensive distribution, and relatively low economic value of water hyacinth provide opportunities for its utilization as a feed ingredient, particularly after appropriate processing to enhance nutrient availability and utilization (Harun et al., 2021; Nandiyanto et al., 2024). Different studies have reported that water hyacinth (*Eichhornia crassipes*) has potential as an alternative feed ingredient for livestock and poultry due to its availability, low cost, and nutritional composition (Gunnarsson and Petersen, 2007; Hamiyanti et al., 2025). Although water hyacinth is commonly classified as an invasive aquatic weed, its high availability in freshwater environments provides an opportunity for its utilization as an inexpensive alternative feed ingredient in duck production systems (Rajan et al., 2023; Hamiyanti et al., 2025). Muazzasari et al. (2023) reported that water hyacinth infestation in the Batujai Reservoir, Lombok Island, covers approximately 267 hectares. Despite its abundance, water hyacinth cannot be optimally used as a poultry feed ingredient without prior processing. However, its application in poultry diets should be considered carefully, as its relatively high fiber content and potential antinutritional compounds may reduce nutrient digestibility and utilization in poultry (Ayoola et al., 2024; Nhara et al., 2026). Fermentation was therefore applied to improve its feeding value by modifying the fiber structure and increasing the availability of nutrients. *Neurospora crassa* was selected as the fermenting agent in the present study based on previous evidence showing that this fungus can produce fiber-degrading enzymes that break down complex plant materials, thereby improving the suitability of water hyacinth for inclusion in duck diets (Ciptaan et al., 2022). However, the high crude fiber content of water hyacinth, which is difficult for livestock to digest, poses a significant challenge to its utilization as feed. High levels of fiber and antinutritional factors in this feed ingredient may reduce digestibility and nutrient utilization, thereby posing a significant challenge to its use as a feed ingredient (Hossain et al., 2015). Water hyacinth contains 28.10% crude fiber, 8.7% crude protein, and 3.32% crude fat (Saud et al., 2023).

One microorganism that has been used for feed fermentation is *Neurospora crassa*, recognized for its ability to produce hydrolytic enzymes, such as cellulase, that facilitate the degradation of complex organic substrates (Yu et al., 2023). Previous studies have demonstrated that fermentation using *Neurospora crassa* can reduce the crude fiber content of water hyacinth and enhance its nutritional value as a potential feed ingredient (Nuraini et al., 2009; Maslami et al., 2024). *Neurospora crassa* is a filamentous fungus commonly used as a fermenting microorganism due to its ability to produce hydrolytic enzymes that degrade complex organic materials, including fiber components, which supports its application in feed fermentation by reducing crude fiber content and improving the nutritional quality of fibrous plant materials such as water hyacinth (Chen et al., 2025). *Neurospora crassa* produces cellulase enzymes and has an advantage over other molds because it also produces β-carotene (Bayram et al., 2019; Toma et al., 2023). The utilization of water hyacinth fermented with *Neurospora crassa* has the potential to enhance feed digestibility and nutritional value in local ducks, as previous findings have demonstrated that *Neurospora crassa* fermentation can reduce the crude fiber content of water hyacinth, improve its value as a feed ingredient, and support growth performance when included in duck diets (Maslami et al., 2024).

The incorporation of *Neurospora crassa*-fermented feed ingredients has been reported as a promising strategy to improve feed utilization and maintain productive performance, as previous studies demonstrated that *Neurospora crassa*-fermented ingredients can be included in broiler diets without negatively affecting production performance and carcass weight, while also contributing to improved carcass characteristics through the utilization of fermented plant-based feed materials (Mirnawati et al., 2018; Fenita et al., 2024). Mirnawati et al. (2018) and Fenita et al. (2024) are among the previous studies reporting that Neurospora crassa-fermented plant-based feed ingredients can be included in broiler diets, with no negative effects on productive performance or carcass quality. Furthermore, Nuraini et al. (2009) reported that the inclusion of *Neurospora crassa*-fermented feed ingredients in poultry diets improved nutrient utilization and supported growth performance, indicating the potential of fermented feed products as alternative feed sources for poultry. A previous study by Maslami et al. (2024) demonstrated that fermentation of water hyacinth using *Neurospora crassa* improved its nutritional characteristics as an alternative feed ingredient by reducing crude fiber content and

enhancing its potential utilization as an energy source. In addition, fermented water hyacinth with *Neurospora crassa* has been evaluated in poultry diets and was reported to be usable at appropriate inclusion levels without negative effects on digestive organ development (Al-Aboudi and Hamodi, 2023; Maslami et al., 2025). The present study aimed to assess the effects of dietary inclusion of water hyacinth fermented with *Neurospora crassa* on growth performance, carcass traits, and small intestinal morphology in ducks.

MATERIALS AND METHODS

Ethical approval

The present study was conducted in accordance with the approved ethical procedures of the Ethics Committee of the University of Mataram, Mataram, Indonesia. All procedures involving Pekin ducks were carried out in accordance with the institutional guidelines for animal care and use, and ethical approval was obtained prior to the commencement of the experiment.

Study time and location

The present study was conducted from February to July 2025. Duck rearing was conducted at the Teaching Farm Laboratory, Faculty of Animal Husbandry, University of Mataram, Indonesia.

Study design

The experiment was conducted using a completely randomized design with five treatment groups and four replications, yielding a total of 20 experimental pens. Allocation of treatments to pens was carried out using a randomization process to minimize potential allocation bias. The treatment groups included 0% water hyacinth (P1), 5% water hyacinth (P2), 5% fermented water hyacinth (P3), 10% fermented water hyacinth (P4), and 15% fermented water hyacinth (P5). Each replicate within each treatment consisted of five ducks serving as experimental units. All pens were maintained under uniform management practices and comparable environmental conditions throughout the study period. A total of 100 local commercial Pekin ducks were used in the present study. The ducks were purchased from a local market in West Lombok, Indonesia, and consisted of mixed-sex, one-day-old ducklings with an average initial body weight of 50 g. After an adaptation period, the experimental treatments were started in the second week when the ducks reached an average initial body weight of 116 g.

The ducks were housed in pens measuring 1 × 1 × 0.7 m with a stocking density of 5 ducks/m². Each pen was equipped with a tube feeder and a manual drinker, and the floor was covered with rice husk bedding, which was replaced or maintained regularly to ensure hygiene. During the experimental period, the house temperature was maintained at approximately 22-30˚C, with relative humidity ranging from 72%-89%. A continuous lighting program of 24 h light and 0 h dark was applied throughout the experimental period based on previously reported lighting management protocols for broiler chickens (Bayram and Özkan, 2010). Before the dietary treatments began at 7 days of age, the ducks were reared from day 1 to day 7 in the same treatment pens and were provided with commercial starter feed. During the initial period, all ducks were managed under uniform housing conditions, with free access to feed and drinking water. The experimental diets were introduced after the ducks reached 7 days of age. The experimental diets were formulated using corn, rice bran, soybean meal, commercial feed, mineral premix containing calcium and phosphorus, and fermented water hyacinth as feed ingredients. The dietary formulation was adjusted according to the inclusion levels of fermented water hyacinth in each treatment while meeting the nutrient requirements of ducks according to the Indonesian National Standardization Agency (BSN, 2015). The composition and nutrient content of the experimental diets are presented in Table 1, while the calculated nutrient composition and metabolizable energy values (kcal/kg) of the experimental diets are presented in Table 2.

Water hyacinth fermentation

Fresh water hyacinth used in this study was obtained from Batu Jai Dam, West Lombok, Indonesia. The material was cleaned and dried under sunlight before being processed for fermentation. The dried water hyacinth was then ground using a hammer mill equipped with a 2 mm sieve to produce uniformly ground material. The ground water hyacinth material obtained after drying and grinding was used as the substrate for fermentation with *Neurospora crassa*. Water hyacinth fermentation was conducted using *Neurospora crassa* for five days at a temperature of 30˚C. Before fermentation, the substrate moisture content was adjusted to approximately 50%, and the initial pH of the water hyacinth substrate was 7.0, which provided suitable conditions for the growth of *Neurospora crassa*. The fermentation process was performed under aerobic conditions with adequate air exchange. After incubation, the fermented water hyacinth was dried and used as an ingredient in the experimental diets. Following grinding, the water hyacinth was subjected to

fermentation, which was initiated by adding groundwater to achieve a moisture content of 50%. Before fermentation, the water hyacinth substrate was sterilized in an autoclave using hot steam at 121˚C for 15 minutes. After sterilization, the substrate was allowed to cool to 40˚C before being inoculated with *Neurospora crassa*. The sterilized water hyacinth substrate was inoculated with 2% *Neurospora crassa* starter based on the substrate weight and mixed thoroughly until the starter was evenly distributed. The inoculated substrate was then placed in clean fermentation containers and incubated for five days at room temperature under aerobic conditions. Following this step, the water hyacinth was dried and prepared for the subsequent fermentation process (Maslami et al., 2024).

Table 1. Nutrient content of feed ingredients and metabolizable energy for Pekin ducks during a 28-day rearing period

Feed ingredients	Crude protein	Crude fat	Crude fiber	ME (kcal/kg)	Ca	P
Corn	8.04	4.52	3.64	2730.68	0.01	0.3
Rice bran	11.3	8.56	14.19	2794.67	0.03	1.07
Commercial feed*	20.5	2	8	2900	3.5	0.5
Soybean meal	36.5	9	3	2760	0.2	0.6
Mineral (Ca and P)**	0	0	0	0	32.5	1
Fermented water hyacinth	14.06	0.09	22.17	3332.38	1.3	0.3
Water hyacinth	11	0.023	28	3000	1.3	0.3

ME: Energy metabolism, Ca: Calcium, P: Phosphorus. *Commercial feed: CP511 PT, Japfa Cemfeed, Indonesia. **Minerals (Ca and P), Japfa Cemfeed, Indonesia

Table 2. Composition of experimental diets fed to Pekin ducks for 28 days (from 7 to 35 days of age)

Feed ingredients	P1	P2	P3	P4	P5
Corn	30.00	35.00	32.00	25.00	22.00
Rice bran	7.50	4.50	4.50	4.50	4.50
Commercial feed	36.00	34.00	35.00	35.00	35.00
Soybean meal	25.00	20.00	22.00	24.00	22.00
Mineral (Ca and P)	1.50	1.50	1.50	1.50	1.50
Fermented water hyacinth	0.00	0.00	5.00	10.00	15.00
Water hyacinth	0.00	5.00	0.00	0.00	0.00
Total (%)	100.00	100.00	100.00	100.00	100.00
Corn	19.76	18.14	18.99	19.86	21.09
Crude fat	6.05	5.47	5.57	5.43	5.12
Crude fiber	4.71	5.61	5.32	6.24	7.17
Ca (%)	0.94	0.97	0.99	1.05	1.12
P (%)	0.52	0.64	0.82	0.49	0.48
Metabolizable energy (Kcal /kg)	2870.80	2871.50	2893.40	2924.07	2953.57

P1: 0% water hyacinth, P2: 5% water hyacinth, P3: 5% fermented water hyacinth, P4: 10% fermented water hyacinth, P5: 15% fermented water hyacinth, Ca: Calcium, P: Phosphorus

Preparation of the cage

The ducks were housed in partitioned floor pens during the experimental period. Each pen was covered with rice husk bedding and contained five ducks, representing one experimental unit. The rice husk bedding was maintained regularly to keep the pen clean and reduce moisture accumulation. Each pen was equipped with a feeder and drinker, and all pens were managed under similar housing and environmental conditions throughout the study. Before the arrival of the one-day-old Pekin ducklings, the housing area and partitioned floor pens were cleaned and whitewashed as part of the cage preparation procedure. Upon arrival, the ducklings were weighed to determine their average initial body weight, which was 50 g. The ducklings were then placed in the prepared pens and managed under uniform housing conditions before the dietary treatments began. Following a three-day whitewashing process, the cages were treated with a disinfectant solution (Rodalon). Prior to the arrival of the one-day-old Pekin ducklings, each pen was equipped with a 100-watt lamp, drinking and feeding facilities, a black plastic tray for fecal collection, and a weighing scale to support the management and data collection procedures. Each partitioned floor pen was assigned a serial number and served as one experimental unit. At 7 days of age, the ducklings were allocated to their respective pens, with five ducks housed in each pen. Thus, the stocking density was five ducks per pen, equivalent to 5 ducks/m² based on the pen area. Before being placed in their respective enclosures, the ducklings were weighed.

Management and housing

As a disease prevention measure, the ducks were vaccinated against Newcastle disease (ND) on day 1 and day 14. The cages were numbered 1-20, and the treatments were randomly assigned to the cages. At 7 days of age, all ducks were weighed individually to determine their baseline body weight before being allocated to their respective treatment groups. Ducks with body weights below or above the reference value were then selected and distributed evenly across the treatment groups, so that each experimental unit had a relatively similar initial body weight. The ducks were subsequently distributed into the numbered cage units. Each cage unit measured 100 × 80 × 75 cm (length × width × height) and accommodated 5 ducks.

Experimental diet

The experimental diets were formulated and balanced based on the Indonesian National Standard for meat-type duck grower-finisher feed (BSN, 2018). The diets were prepared according to the predetermined feed composition for each treatment and were provided *ad libitum* throughout the experimental period. The remaining feed was weighed on a daily basis. Water was provided *ad libitum* and replaced every morning and evening. Body weight was recorded weekly after the ducks had been fasted for 8 hours. The treatment was concluded on day 35.

Measurement of parameters

Feed and nutrient intake

The total treatment period is from 7 days to 35 days, with weekly measurements of feed intake. The intake of crude protein and crude fat was determined by calculating the product of the crude protein and crude fat content of the feed and the feed intake. The intake of crude protein and crude fiber was determined by multiplying the crude protein and crude fiber contents of the diet by feed intake (Mirza et al., 2016). The crude protein and crude fiber contents of the experimental diets were calculated from the feed formulation using the nutrient composition of each feed ingredient, which had previously been analyzed in the laboratory. Metabolizable energy was calculated by multiplying the amount of feed consumed by the metabolizable energy content of each experimental diet.

Performance and carcass traits

Weekly body weight gain was calculated from the difference between body weights recorded at the beginning and end of each observation week. Feed conversion ratio (FCR) was determined by dividing feed intake by body weight gain, following Gous (2010), who described FCR as feed intake per unit of weight gain. Before slaughter, live body weight was recorded. Carcass percentage was calculated as the ratio of carcass weight to live body weight multiplied by 100%, following Soeparno (2015).

Morphology of the small intestine

The small intestine was first cleared of digesta and then weighed. Histological evaluation was performed using one duck from each replicate, resulting in four ducks per treatment. For each duck, four jejunal sections per treatment were examined. Intact and well-oriented villi were selected from each section, and their morphological characteristics were measured. Segment lengths were determined anatomically, with the duodenum measured from the ampulla to the bile duct, the jejunum from the bile duct to Meckel's diverticulum, and the ileum from Meckel's diverticulum to the ileocecal junction (Han et al., 2016; Kimiaeitalab et al., 2017). Intestinal villus length was measured using a 1.5-cm jejunal sample carefully obtained from the region midway between the bile duct opening and Meckel's diverticulum. The jejunal sample was thoroughly rinsed with 0.9% NaCl physiological saline solution to maintain its integrity and prepare it for subsequent fixation (Sadeghi et al., 2015). The fixation process involved the use of a 100 g/L buffered formalin solution at pH 7.0, which served as a stabilizing agent during the procedure. After fixation, the intestinal samples were prepared for histological analysis by embedding them in paraffin, slicing them into 5-µm-thick sections, and applying hematoxylin and eosin staining (Sadeghi et al., 2015). Intestinal villus height was measured using a light microscope (Olympus, Japan) following hematoxylin and eosin (H&E) staining. Intact and well-oriented villi were selected from each jejunal section, and villus height was measured using image analysis software. The mean value of villus measurements from each duck was used for statistical analysis to ensure consistency and reproducibility of the histological data.

Statistical analysis

The data were analyzed using Analysis of Variance (ANOVA), followed by Duncan's Multiple Range Test (DMRT) when significant differences among treatments were detected. The level of significance was set at p less than 0.05. All statistical analyses were performed using SAS software (Statistical Analysis System) version 9.4.

RESULTS AND DISCUSSION

Feed and nutrient intake

The results indicated that dietary inclusion levels of fermented water hyacinth had no significant effect ($p > 0.05$) on feed intake, metabolizable energy, or FCR. The metabolizable energy values of the experimental diets were estimated based on their proximate nutrient composition. In contrast, dietary supplementation with fermented water hyacinth significantly affected ($p < 0.05$) crude protein and crude fiber intake. Crude fiber intake was quantified by first determining the fiber content of each experimental diet through proximate analysis. The effects of dietary treatments containing fermented water hyacinth with *Neurospora crassa* on feed intake, nutrient intake, metabolizable energy, and FCR of Pekin ducks during the 35-day feeding trial are presented in Table 3.

Table 3. Effects of diets containing fermented water hyacinth with *Neurospora crassa* on feed and nutrient intake, metabolizable energy, and feed conversion ratio in Pekin ducks over a 35-day feeding trial

Parameters	Treatment groups					P-value
	P1	P2	P3	P4	P5	
Feed intake (g)	2328.40 ± 26.53	2446.90 ± 146.61	2406.50 ± 46.53	2353.90 ± 20.27	2425.30 ± 23.85	0.20
Crude protein intake (g)	450.23 ± 11.551^{b}	438.43 ± 26.59^{b}	444.65 ± 16.35^{b}	453.38 ± 9.54^{b}	516.77 ± 28.45^{a}	< 0.05
Crude fiber intake (g)	107.35 ± 2.75^{d}	135.59 ± 8.22^{bc}	124.57 ± 4.58^{c}	142.45 ± 3.00^{b}	175.69 ± 9.67^{a}	< 0.05
Metabolizable energy (kcal/kg)	6541.12 ± 193.82	6940.13 ± 486.11	6774.36 ± 287.58	6675.35 ± 162.18	7237.13 ± 460.11	0.07
Feed conversion ratio (%)	2.49 ± 0.04	2.42 ± 0.03	2.44 ± 0.05	2.45 ± 0.07	2.43 ± 0.05	0.44

$^{a, b, c}$ Mean different superscript letters in the row indicate significant differences across the treatments ($p < 0.05$). P1: 0% water hyacinth, P2: 5% water hyacinth, P3: 5% fermented water hyacinth, P4: 10% fermented water hyacinth, P5: 15% fermented water hyacinth.

Feed intake

The inclusion of fermented water hyacinth up to 15% did not affect feed intake in meat ducks during the experimental period, indicating that the fermented ingredient was well accepted by the ducks without adverse effects on feed consumption. The comparable feed intake among treatments suggested that fermentation with *Neurospora crassa* improved the suitability of water hyacinth as a feed ingredient by enhancing its nutritional characteristics and reducing the limitations associated with its high fiber content. Similar responses have been reported in ducks fed fermented water hyacinth-based diets, where appropriate inclusion levels maintained feed acceptance and production performance (Rompas et al., 2016; Pangeran et al., 2021; Hamiyanti et al., 2025). Furthermore, the similar metabolizable energy content among dietary treatments may have contributed to the consistent feed intake observed in this study, as dietary energy density plays an important role in regulating voluntary feed consumption in poultry (Hong et al., 2022; Lim et al., 2024; Wu et al., 2026).

In the present study, the crude fiber content of the experimental diets ranged from 4.71% to 7.17%. Although this range should not necessarily be interpreted as optimal, it can be regarded as acceptable because it does not exceed the maximum crude fiber limit of 5% specified for grower meat duck feed in SNI 8508:2018 (BSN, 2018). The crude fiber values observed in the present study were therefore unlikely to cause a marked reduction in feed intake, particularly because the metabolizable energy content of the diets remained relatively adequate and comparable among treatments**.** A recent study by Hao et al. (2021) revealed that dietary fiber can contribute to gastrointestinal development and gut microbial balance in ducks when supplied at an appropriate level. In a broader poultry context, moderate fiber inclusion may support gut function and nutrient utilization, although its effect depends on the fiber source, diet composition, and bird species (Jha and Mishra, 2021; Zhang et al., 2023a). Thus, the crude fiber level in the present diets was more appropriately interpreted as being within an acceptable range for duck feed, rather than as a universal optimal level.

As illustrated in Figure 1, the weekly feed intake of ducks increased with age. The increase may be associated with the greater nutrient and energy requirements required to support body growth and development during the growing period, as previously reported in growing White Pekin ducks (Hong et al., 2022). Based on the results of the present study, feed intake tended to increase after the first week of rearing. The increase in feed intake may be associated with the increasing nutrient and energy requirements required to support body weight gain and growth during the growing period, as dietary metabolizable energy plays an important role in determining growth performance and nutrient utilization in ducks (Wu et al., 2019; Guo et al., 2023). The higher energy requirements associated with increasing body weight may support muscle development, bone formation, and other metabolic processes during the growth period. Consequently, ducks tend to increase feed consumption to meet their nutritional requirements for growth and

development (Hong et al., 2022). According to the findings of Wang et al. (2024), the amount of feed consumed by poultry increases in direct proportion to their body weight and the stage of their physiological development. Moreover, the increase in feed intake observed as ducks grew older may be related to their higher energy requirements for maintenance and body tissue development. In duck diets, dietary energy level is an important factor influencing growth performance and feed utilization, as reported in studies evaluating different metabolizable energy levels in White Pekin ducks and BAU Black and White ducks (Barman et al., 2025). The average feed intake recorded in the present study was within a range similar to the values reported by Rusfidra et al. (2025), who observed feed intake in meat ducks ranging from 81.21 to 84.07 g/bird.

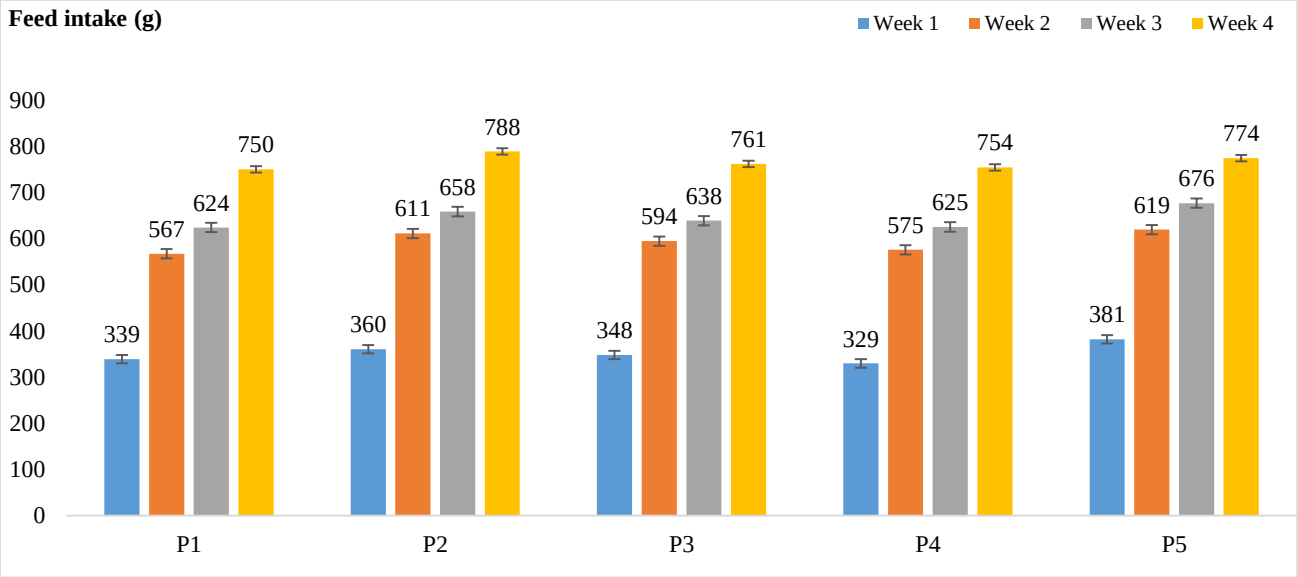


Figure 1. Weekly feed intake of mixed-sex Pekin ducks aged 7 days fed diets containing fermented water hyacinth with *Neurospora crassa* over 28 days. P1: 0% water hyacinth, P2: 5% water hyacinth, P3: 5% fermented water hyacinth, P4: 10% fermented water hyacinth, P5: 15% fermented water hyacinth

Crude protein intake

The differences in crude protein intake among treatment groups can be explained by the variation in feed intake and the crude protein content of each experimental diet. In the present study, crude protein intake was not measured as a separate nutrient consumed directly, but was calculated from the amount of feed consumed by the ducks and the crude protein concentration of the corresponding diet. Therefore, treatment groups with higher feed intake and higher dietary crude protein content tended to display greater crude protein intake. Crude protein intake was influenced by both the quantity of feed consumed and the nutrient composition of the diet.

Fermented water hyacinth inclusion significantly affected crude protein intake among treatment groups, with the highest intake observed in ducks fed the 15% fermented water hyacinth diet. The higher crude protein intake observed in Pekin ducks fed this diet was likely associated with the increased crude protein concentration of the fermented water hyacinth-based ration, as dietary protein content directly influences nutrient intake and protein supply for growth performance in poultry (Musigwa et al., 2023). Differences in dietary composition, particularly crude protein concentration, may contribute to variations in protein intake among Pekin ducks, as dietary protein levels influence nutrient intake and growth performance in growing ducks (Shen et al., 2026). Similar observations were reported by Wu et al. (2024), who demonstrated that diets with higher protein content increased protein consumption in Pekin ducks. Variations in crude protein intake compared with those in previous studies may be related to differences in duck strain, age, body weight, feed intake, dietary composition, and experimental conditions (Zeng et al., 2015; Xie et al., 2017). Therefore, comparisons with previous reports should be interpreted within the context of different experimental designs. The relationship between dietary crude protein concentration, feed intake, and crude protein intake is presented in Figure 2.

Adequate crude protein intake is essential for supporting growth and physiological functions in ducks because dietary protein provides amino acids required for tissue deposition, protein synthesis, and efficient nutrient utilization (Wu et al., 2026). In Pekin ducks, crude protein level has been shown to influence growth performance and feed efficiency, indicating the importance of sufficient protein supply during the growing period (Cho et al., 2020). A recent study by Wu et al. (2024) reported that dietary crude protein level and amino acid balance are closely related to growth performance, carcass traits, nutrient digestibility, and nitrogen utilization in growing Pekin ducks.

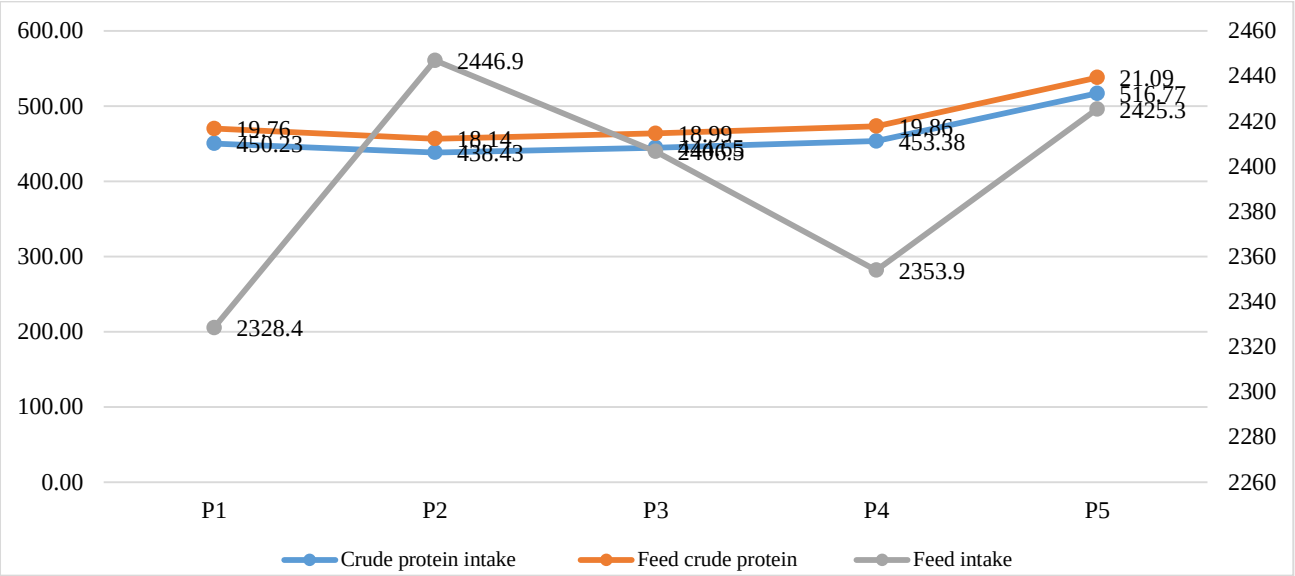


Figure 2. Interaction between feed intake, dietary crude protein content, and crude protein consumption in mixed-sex Pekin ducks fed diets containing fermented water hyacinth with *Neurospora crassa* from day 7 to 35 days of age. P1: 0% water hyacinth, P2: 5% water hyacinth, P3: 5% fermented water hyacinth, P4: 10% fermented water hyacinth, P5: 15% fermented water hyacinth

Crude fiber intake

The differences in crude fiber intake among treatment groups can be attributed to variations in feed intake and the crude fiber content of each experimental diet. In the present study, crude fiber intake was not measured as a separate component consumed directly, but was calculated based on the amount of feed consumed and the crude fiber concentration of the corresponding diet. Therefore, treatment groups with higher feed intake or higher dietary crude fiber content tended to indicate greater crude fiber intake. Crude fiber intake was influenced by both the inclusion level of fermented water hyacinth in the diet and the total amount of feed consumed by the ducks.

The results of the present study indicated a significant difference in crude fiber intake among the treatment groups ($p < 0.05$). Crude fiber intake was higher in P5 compared with the other groups, whereas P1 indicated the lowest crude fiber intake, indicating that dietary treatments influenced the amount of crude fiber consumed by the ducks. The highest crude fiber intake was observed in treatment P5, reaching 175.69 g per bird (6.27 g per bird per day). The greater crude fiber intake in treatment group P5 was related to the higher crude fiber concentration of the diet, which reached 7.17%. The crude fiber level observed in the treatment diet required careful interpretation because it exceeded the maximum crude fiber level of 5% recommended for meat duck grower feed according to SNI 8508:2018 (BSN, 2018). The response of ducks to dietary fiber may also differ according to the fiber source and feed formulation. In the present study, fermented water hyacinth was used as the main fiber source, whereas El-Katcha et al. (2021) used soybean hulls and reported that higher dietary fiber did not significantly reduce body weight, although it affected feed efficiency and intestinal morphology. Therefore, the increased crude fiber intake in P5 mainly reflected the higher fiber content of the diet and feed consumption, rather than indicating that the diet remained within the recommended limit. The increase in crude fiber intake corresponding to feed fiber content is illustrated in Figure 3.

In the present study, crude fiber intake differed significantly among treatment groups compared to the control group ($p < 0.05$). The variation in crude fiber intake among treatments was mainly associated with differences in dietary crude fiber concentration and feed consumption. Although digestive function was not directly measured in the present study, dietary fiber has been reported to influence gastrointestinal function, nutrient utilization, and intestinal morphology in poultry, depending on the fiber source, inclusion level, and diet formulation (Singh et al., 2021; Zhang et al., 2023b). Therefore, the digestive responses discussed in the present study should be considered as supportive interpretations based on previous literature rather than direct experimental observations, as dietary crude fiber has been reported to influence gastrointestinal function by enhancing intestinal motility (peristalsis), which may facilitate digestive processes and gastric emptying (Singh and Kim, 2021). The promotion of peristalsis by crude fiber facilitates feed processing, reduces the risk of constipation, and supports gastric emptying, as dietary fiber has been reported to modulate gastrointestinal activity, digestive organ development, nutrient utilization, and gut health in poultry, with its effects depending on the type, source, and inclusion level of fiber (Tejeda and Kim, 2021). Furthermore, the increased crude fiber content may contribute to improvements in intestinal morphology, as reflected by the morphological changes observed in the present study. Short-chain fatty acids (SCFAs) act as an energy source for intestinal cells, thereby contributing to overall digestive health (Zhang et al., 2023b).

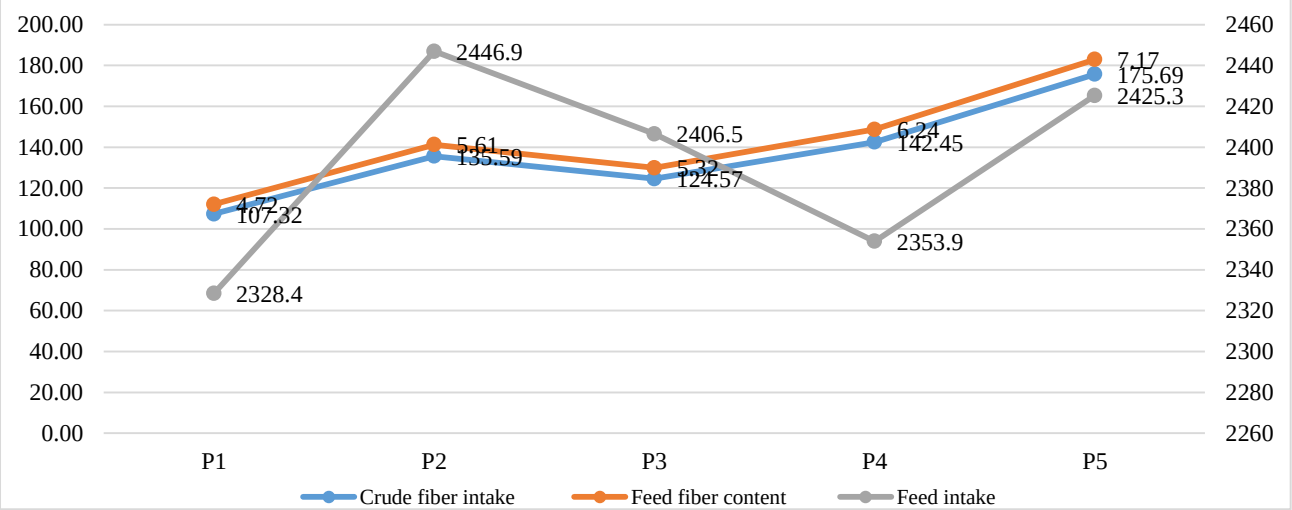


Figure 3. Interaction between feed intake, crude fiber content of feed, and crude fiber consumption in mixed-sex Pekin ducks fed diets containing fermented water hyacinth with *Neurospora crassa* from day 7 to 35 days of age. P1: 0% water hyacinth, P2: 5% water hyacinth, P3: 5% fermented water hyacinth, P4: 10% fermented water hyacinth, P5: 15% fermented water hyacinth

Metabolizable energy

The results of the present study revealed that there was no significant difference in metabolizable energy among the treatment groups, namely P1, P2, P3, P4, and P5, which were fed diets containing different levels of fermented water hyacinth ($p > 0.05$). The absence of a significant difference in energy intake can be attributed to the similar energy content of the diets across the various treatments, which did not influence the amount of energy consumed by the ducks ($P > 0.05$). The pattern of metabolizable energy among treatments is presented in Figure 4. The metabolizable energy content of the rations ranged from 2870.80 to 2953.57 kcal/kg, with P5 slightly exceeding the recommended upper limit of 2900 kcal/kg. The metabolizable energy range of the experimental diets was generally consistent with the levels recommended by BSN (2019) and NRC (1994), which specify 2700-2900 kcal/kg for poultry diets. During the 28-day treatment period, daily metabolizable energy ranged from 233.60 kcal/kg per bird per day in P1 to 258.47 kcal/kg per bird per day in P5. In contrast, El-Katcha et al. (2021) reported lower daily metabolizable energy in Pekin ducks, ranging from 163.23 to 167.03 kcal/duck/day; however, differences in experimental design, feed ingredients, and ration composition should be considered when comparing these results with the present study. Differences in daily metabolizable energy may be related to feed type, ration composition, duck breed, and age, as these factors influence nutrient density, palatability, metabolic requirements, and feed consumption capacity (Yang et al., 2025). It is important to recognize the pivotal role of metabolizable energy in supporting duck growth. The energy consumed by ducks is utilized for different metabolic processes, including body growth, thermoregulation, and physical activity (Fouad et al., 2018). When ducks consume sufficient energy, it is directed toward supporting the formation of body tissues, such as muscles and bones, which are essential for optimal growth (Zeng et al., 2015).

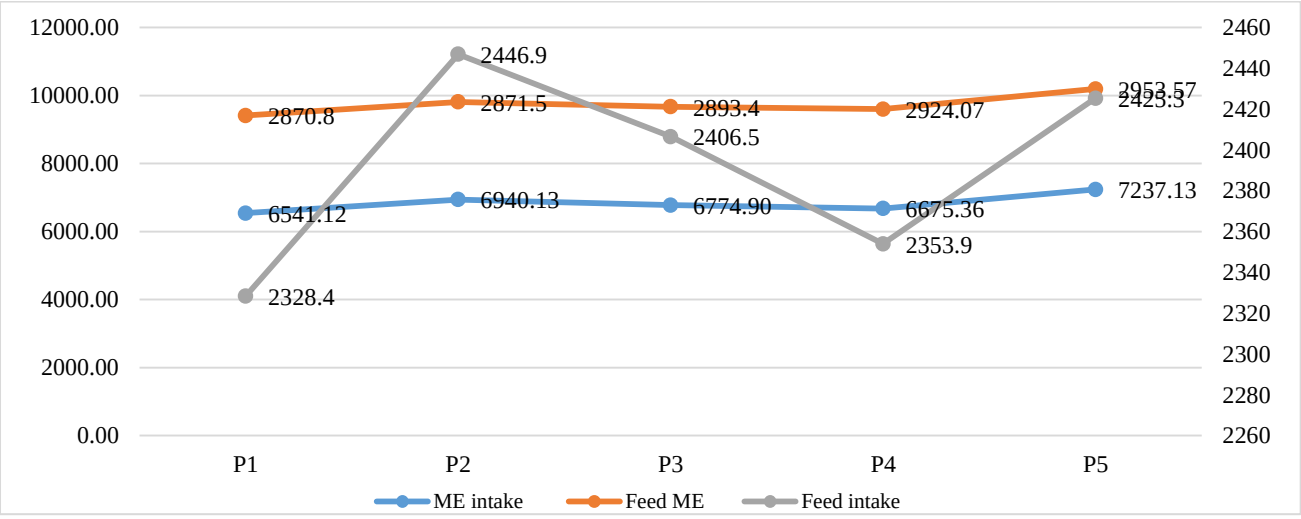


Figure 4. Relationship between feed intake, metabolizable energy content of feed, and energy consumption in mixed-sex Pekin ducks fed diets containing fermented water hyacinth with *Neurospora crassa* from day 7 to 35 days of age. P1: 0% water hyacinth, P2: 5% water hyacinth, P3: 5% fermented water hyacinth, P4: 10% fermented water hyacinth, P5: 15% fermented water hyacinth

Feed conversion ratio

The results indicated that dietary supplementation with different levels of water hyacinth had no significant effect on the FCR of ducks ($p > 0.05$). The absence of a significant difference in FCR may be related to the relatively comparable nutrient intake among treatments, allowing ducks to utilize the diets with similar efficiency. In the present study, the crude protein content of the experimental diets ranged from 18.14% to 21.09%, while the metabolizable energy was formulated at 2,900 kcal/kg. As described by Hong et al. (2022), the balance between protein and energy in the diet exerts a significant influence on body weight, which, in turn, modulates FCR. The FCR values obtained in the present study ranged from 2.42 to 2.49, which were lower than those reported in previous studies, including 5.48 in Muscovy ducks fed commercial diets (Sabrina et al., 2025) and 3.04 in Magelang meat ducks (Listyowati et al., 2020). The lower FCR recorded in the present study suggests that the ducks were able to convert feed into body weight more efficiently. Differences in breed, feed formulation, nutrient composition, management practices, and experimental conditions may explain the variation in FCR values among studies. The absence of significant variations in FCR can be ascribed to the comparable feed intake observed among the treatment groups. Despite the variation in the quantity of water hyacinth present in the diets, the ducks exhibited no significant difference in their feed consumption, resulting in comparable body weight gains ($p > 0.05$). The relationship among feed intake, body weight gain, and FCR is presented in Figure 5. The figure revealed that changes in feed intake and body weight gain are closely associated with the resulting FCR values. In Pekin ducks, Li et al. (2020) found that sufficient overall feed consumption and daily weight gain were important factors contributing to efficient feed conversion. Although a direct relationship between higher feed intake and improved FCR has not been consistently demonstrated, the amount of feed consumed plays an important role in determining the efficiency of feed conversion into body weight gain. In this context, ducks that consume adequate feed to support their growth tend to have lower FCR values, indicating better feed efficiency (Zhao et al., 202

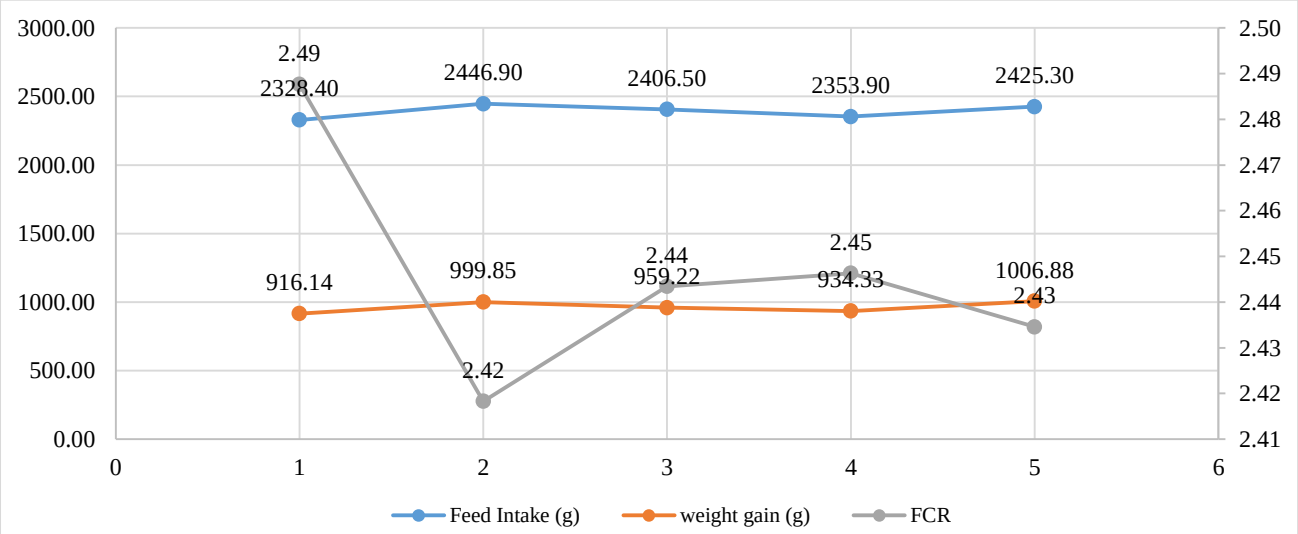


Figure 5. Relationship between feed intake, body weight gain, and feed conversion ratio in mixed-sex Pekin ducks fed diets containing fermented water hyacinth with *Neurospora crassa* from day 7 to 35 days of age. P1: 0% water hyacinth, P2: 5% water hyacinth, P3: 5% fermented water hyacinth, P4: 10% fermented water hyacinth, P5: 15% fermented water hyacinth

Performance and carcass traits

The results of the present study indicated that supplementation with fermented water hyacinth at different levels did not significantly affect final body weight, body weight gain, carcass percentage, breast percentage, thigh percentage, back percentage, or wing percentage ($p > 0.05$). The average values of growth performance and carcass characteristics are shown in Table 4.

Table 4. Mean growth performance and carcass traits of Pekin ducks fed diets containing fermented water hyacinth with *Neurospora crassa* reared for 28 days

Parameters	Treatment groups					P-value
	P1	P2	P3	P4	P5	
Final body weight (g)	1029.69 ± 27.02	1122.50 ± 70.84	1080.27 ± 52.69	1049.83 ± 45.68	1114.58 ± 55.94	0.43
Weight gain (g)	916.14 ± 26.62	999.85 ± 65.39	959.22 ± 50.83	934.33 ± 43.17	1006.88 ± 59.99	0.338
Carcass percentage	61.20 ± 1.91	58.91 ± 0.82	61.36 ± 1.51	60.97 ± 1.31	60.11 ± 1.29	0.55
Breast percentage	33.25 ± 0.62	32.86 ± 1.05	33.09 ± 0.86	33.16 ± 0.81	32.76 ± 0.62	0.97
Thigh percentage	31.70 ± 0.41	31.12 ± 1.25	31.68 ± 0.96	31.00 ± 0.54	31.80 ± 1.45	0.95
Back percentage	20.98 ± 0.77	21.47 ± 0.85	21.40 ± 1.49	21.75 ± 0.85	22.16 ± 1.03	0.82
Wing percentage	14.51 ± 0.47	14.54 ± 0.61	13.83 ± 0.64	14.09 ± 0.51	13.32 ± 0.78	0.06

P1: 0% water hyacinth, P2: 5% water hyacinth, P3: 5% fermented water hyacinth, P4: 10% fermented water hyacinth, P5: 15% fermented water hyacinth.

Final body weight

The results of the present study revealed that different levels of water hyacinth inclusion in the diet did not significantly affect the final body weight of ducks among the treatment groups (P1-P5; $p > 0.05$), as presented in Table 4. The final body weight of ducks at 35 days of age ranged from 1,029.69 g in P1 to 1,122.50 g in P2, with values of 1,080.27 g in P3, 1,049.83 g in P4, and 1,114.58 g in P5. The absence of a significant difference among treatments may be related to the adequacy of dietary nutrients and the relatively comparable feed intake across treatment groups. Although the level of water hyacinth included in the diets varied, the available nutrients appeared sufficient to support duck growth during the experimental period. The comparable final body weight among treatments is consistent with previous reports showing that adequate dietary protein and metabolizable energy are key factors in maintaining duck growth performance, even when feed ingredients or diet composition differ among treatments (Li et al., 2020). The protein content of the feed plays a crucial role, and maintaining a balance between protein and energy is essential to optimize growth. Excessive protein and imbalanced energy in the diet may lead to fat accumulation, which hinders the development of muscle mass in ducks (Starčević et al., 2021).

The absence of a significant difference in final body weight can be ascribed to the comparable feed intake observed across treatment groups. Feed intake is a pivotal factor influencing growth and final body weight in Pekin ducks. The amount of feed consumed is directly related to the energy and nutrients required to support body development. Shu et al. (2025) indicated that discrepancies in final body weight are predominantly dictated by feed intake. The final body weight of ducks in the present study ranged from 1,029.69 to 1,122.50 g at 35 days of age. The final body weight observed in the present study was lower than that reported by Gunawan et al. (2023), who recorded a body weight of 1,500 g in Pekin ducks at 42 days of age fed diets containing 22% crude protein. The difference may be related to the older age of ducks and the higher dietary crude protein level used in their study. The final body weight observed in the present study was higher than the 769.84 g reported by Wen et al. (2015) for Pekin ducks at 35 days of age.

Weight gain

As demonstrated in Table 4, the results indicate that there is no statistically significant difference ($p > 0.05$) in body weight gain among ducks fed diets with varying levels of water hyacinth. Body weight gain of Pekin ducks across the treatments was recorded at 916.14 g-1006.88 g. The body weight gain observed in the present study was greater than that reported for Kamang ducks aged 3-7 weeks (616.75 g), male Bayang ducks aged 3-8 weeks (521.86 g), and male Tegal ducks raised from 2-8 weeks (480.83 g; Rafian et al., 2023; Triani et al., 2024).

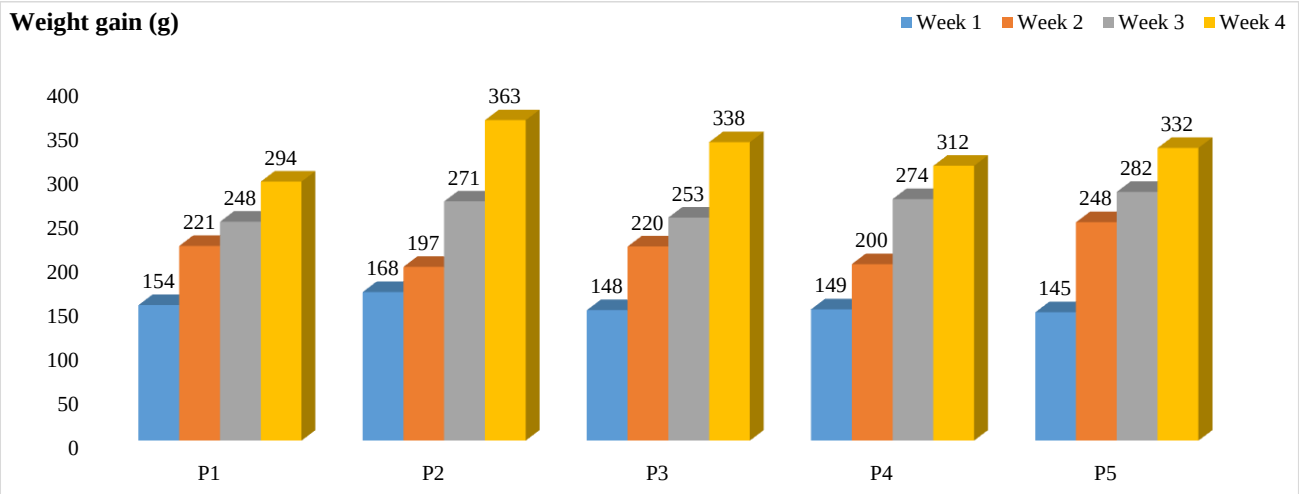


Figure 6. Weight gain of mixed-sex Pekin ducks fed diets containing fermented water hyacinth with *Neurospora crassa* from day 7 to 35 days of age. P1: 0% water hyacinth, P2: 5% water hyacinth, P3: 5% fermented water hyacinth, P4: 10% fermented water hyacinth, P5: 15% fermented water hyacinth

The absence of a significant difference in body weight gain can be attributed to the similarity in final body weight, feed intake, and nutrient content across treatments. The comparable body weight gain among treatments may be associated with similar feed intake and adequate nutrient availability in the experimental diets, which allowed ducks to maintain relatively similar growth responses (Shu et al., 2025). In addition, the study by Downing et al. (2022) indicated that the primary factors influencing duck growth are the consumption, quality, and quantity of feed provided.

The weekly body weight gain increased with the age of the Pekin ducks, as illustrated in Figure 6. Observations of Pekin duck weight gain revealed a consistent weekly increase as the ducks aged. The body weight gain recorded in the

present study, which reached 916.14 g, indicates that Pekin ducks continued to grow during the experimental period. The progressive body weight gain observed in the present study is consistent with Zhou et al. (2024), who reported that Pekin ducks gain body weight progressively as they mature. Progressive growth in ducks is influenced by physiological factors, including metabolism, nutritional requirements, and environmental conditions (Inayat et al., 2024).

Carcass percentage

A thorough examination of the data revealed that the carcass percentage of Pekin ducks did not differ significantly ($p > 0.05$), with values ranging from 58.91% to 61.36%, as illustrated in Table 4. The carcass percentages recorded in the present study among the treatment groups (P1-P5) were comparable to the reported eviscerated carcass yield of Pekin ducks, which ranges from 58.9% to 62% after the removal of feathers, head, feet, and internal organs (Kokoszyński et al., 2019). The inclusion of fermented water hyacinth at different dietary levels did not significantly influence carcass yield or carcass part proportions among treatments (P1-P5; $p > 0.05$). Based on Table 4, carcass percentage varied from 58.91% in P2 to 61.36% in P3. The breast proportion ranged from 32.76% in P5 to 33.16% in P4, thigh proportion from 31.00% in P4 to 31.80% in P5, back proportion from 20.98% in P1 to 22.16% in P5, and wing proportion from 13.32% in P5 to 14.54% in P2. In the present study, the crude protein content of the experimental diets ranged from 18.14% to 21.09%, indicating that the diets still provided sufficient protein to support carcass development. Therefore, the absence of significant differences in carcass percentages among treatments may be related to the relatively adequate crude protein content across the experimental diets. The relationship between dietary nutrient levels, production performance, and carcass yield is supported by Sritiawthai et al. (2013), who reported that dietary protein and energy levels influence production performance and carcass yield in meat ducks. Crude protein constitutes a substantial component of Pekin duck feed and plays a pivotal role in promoting muscle growth and carcass development. Dietary protein concentration has been reported to improve growth performance in Pekin ducklings, while adequate amino acid supply, particularly methionine, contributes to better carcass and breast meat yield in growing Pekin ducks (Chen et al., 2016; Wu et al., 2019).

Intestinal morphology characteristics

The findings of the present study indicated that supplementation with fermented water hyacinth at different levels did not significantly influence the length of the duodenum, jejunum, ileum, or intestinal villi in Pekin ducks ($p > 0.05$). The average values of intestinal morphology parameters are shown in Table 5.

Table 5. Effects of diets containing fermented water hyacinth with *Neurospora crassa* on intestinal morphology of Pekin ducks reared for 28 days

Parameter / Treatment groups	Duodenum (cm)	Jejunum (cm)	Ileum (cm)	Intestinal villi (µm)
P1	40.5 ± 2.18	57.5 ± 1.12	27 ± 1.41	288.16 ± 24.47
P2	40.25 ± 1.48	60.75 ± 2.95	26.75 ± 1.30	244.18 ± 14.29
P3	40.25 ± 1.09	60.5 ± 5.77	27.25 ± 1.79	308.19 ± 39.14
P4	41.75 ± 1.30	63.25 ± 5.40	27 ± 1.58	263.91 ± 31.76
P5	40.5 ± 1.12	63.25 ± 2.86	27 ± 2.23	258.29 ± 29.28
P-value	0.67	0.50	0.94	0.10

P1: 0% water hyacinth, P2: 5% water hyacinth, P3: 5% fermented water hyacinth, P4: 10% fermented water hyacinth, P5: 15% fermented water hyacinth.

Preliminary analysis of the results in Table 5 indicated that there was no statistically significant difference ($p > 0.05$) in the lengths of the duodenum, jejunum, ileum, or intestinal villi. The non-significant differences among all treatment groups (P1, P2, P3, P4, and P5) in duodenal length, jejunal length, ileal length, and intestinal villus length indicated that fermented water hyacinth supplementation did not markedly affect small intestinal morphology in Pekin ducks. Based on Table 5, duodenal length ranged from 40.25 cm in P2 and P3 to 41.75 cm in P4, while jejunal length ranged from 57.50 cm in P1 to 63.25 cm in P4 and P5. Ileal length ranged from 26.75 cm in P2 to 27.25 cm in P3, and intestinal villus length ranged from 244.18 µm in P2 to 308.19 µm in P3. The uniformity in measurements is likely attributable to the comparable feed intake across treatments, which ensured a consistent supply of nutrients for intestinal growth. The relatively similar intestinal morphology observed among the treatment groups in the present study may be related to comparable feed intake and adequate nutrient availability across the experimental diets. Nutrient intake is important for maintaining intestinal epithelial development and villus structure because intestinal villi are associated with nutrient absorption capacity. Similar to the present study, Sritiawthai et al. (2013) reported

that variation in dietary energy did not significantly alter villus height or crypt depth in the duodenum, jejunum, and ileum of meat ducks, although dietary protein affected jejunal villus height. In addition, Jiang et al. (2012) reported that dietary fiber sources such as alfalfa meal could influence gastrointestinal development and villus morphology in growing ducks, indicating that feed composition may affect intestinal structure depending on nutrient balance and ingredient characteristics.

The treatments did not significantly affect the small-intestinal morphology of Pekin ducks ($p > 0.05$). In the present study, the crude fiber content of the experimental diets ranged from 4.71% to 7.17%. Therefore, the absence of significant differences in intestinal morphology among all treatment groups (P1, P2, P3, P4, and P5) may be associated with the crude fiber levels provided by the diets, which remained within a range that could still be tolerated by Pekin ducks. According to Hao et al. (2021), the range of crude fiber to which ducks can adapt is relatively wide, ranging from 3.09% to 7.52%. Physiologically, crude fiber contributes to increased intestinal motility and overall digestive health. However, at low to moderate crude fiber levels, dietary fiber may have a limited effect on intestinal villus morphology when the diets still provide adequate nutrients. In the present study, the crude fiber content ranged from 4.71% to 7.17%, which was close to the adaptable fiber range reported by Han et al. (2017) in meat ducks. The absence of significant changes in intestinal morphology in the present study may indicate that the crude fiber level in the diet remained within a tolerable range for ducks. Previous studies have shown that ducks can adapt to a relatively wide range of dietary fiber levels, although intestinal responses may vary depending on fiber level, fiber source, and dietary supplementation strategy (El-Katcha et al., 2021). A similar response was reported by Kim et al. (2019), who found that different dietary energy levels in Pekin ducks did not significantly change jejunal villus height or crypt depth. Consequently, even with differences in fiber composition among treatments, adequate nutrient availability and metabolizable energy support normal intestinal morphology development.

CONCLUSION

Water hyacinth fermented with *Neurospora crassa* can be included in Pekin duck diets at up to 15% without negatively affecting feed intake, growth performance, carcass traits, or intestinal morphology under the conditions of the present study, while increasing crude protein and crude fiber intakes. The present study had limitations, including the relatively short experimental period and the use of only one duck breed, which may limit the broader application of the findings. Further studies are suggested to investigate the long-term effects of fermented water hyacinth utilization, evaluate different fermentation methods, and examine its application in different duck breeds and production conditions

DECLARATIONS

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Authors' contributions

Vebera Maslami was responsible for conceptualizing the study, experimental implementation, data collection, and initial manuscript drafting. I Ketut Gede Wiryawan contributed to data processing, statistical analysis, interpretation of the results, and manuscript revision. Azhary Noersidiq contributed to study supervision, methodological guidance, interpretation of findings, and manuscript editing. All authors read and approved the final edition of the manuscript.

Availability of data and materials

The data generated in this study are relevant and presented in this published article. Supporting data related to the findings are available from the corresponding author upon reasonable request.

Competing interests

The authors declared no conflict of interest.

Ethical considerations

The authors confirmed that the manuscript is original, has not been published elsewhere, and represents the final edition approved prior to publication. The authors stated that no AI-based tools were utilized in conducting the study

or preparation of the article. All authors confirmed the originality and authenticity of the data presented in this study before submitting and after accepting the manuscript.

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Prevalence and Pathomorphology of Endoparasites in Indian Peafowl (*Pavo cristatus*) under Different Housing Systems in Odesa Region, Ukraine

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ABSTRACT

Parasitic infections represent a major health concern in ornamental and exotic poultry, adversely affecting the health, productivity, and survival of Indian peafowl (*Pavo cristatus*). The present study aimed to determine the prevalence and species composition of endoparasites in Indian peafowl maintained under free-range and aviary housing systems, and to characterize the gross and pathomorphological lesions associated with histomonosis and cestodosis. The present study was conducted in 2020-2025 on private farms and bioparks in the Odesa Region, Ukraine, and included 90 Indian peafowl, 44 males and 46 females, aged 1-2 years. Fecal samples were collected in all seasons, with 45 peafowl kept under free-range housing and 45 under cage-aviary housing. Fecal samples were analyzed by direct smear, flotation, and the McMaster method. The pathomorphological manifestations of histomonosis were examined through a retrospective analysis of 17 cases in Indian peafowl aged 6 weeks to 16 months. Meanwhile, gross lesions of cestodosis were assessed during postmortem examinations of three naturally infected peafowl. The overall prevalence of endoparasitic infections in Indian peafowl was 51.1% under free-range housing and 66.7% under cage-aviary housing. Under free-range housing, Indian peafowl were most frequently infected with *Histomonas meleagridis* (*H. meleagridis*; 13.3%), *Eimeria* spp. (11.1%), *Heterakis* spp. (8.9%), *Raillietina* spp. (6.7%), *Ascaridia galli* (4.4%), *Davainea proglottina* (4.4%), and *Choanotaenia infundibulum* (2.2%). In cage-aviary housing, the most common parasites were *H. meleagridis* (24.4%), *Eimeria* spp. (15.6%), *Heterakis* spp. (13.3%), *Ascaridia galli* (6.7%), and *Davainea proglottina* (2.2%). A mixed infection of *H. meleagridis* and *Ascaridia galli* was found exclusively in cage-aviary housing (4.4%). No apparent sex-related differences in susceptibility to protozoan infections were observed. The intensity of protozoan and nematode infections was generally higher in Indian peafowl housed in cage-aviaries than in those under free-range housing. Histomonosis was characterized by fibrinonecrotic typhlitis and characteristic necrotizing hepatitis, whereas cestodosis was associated with catarrhal-desquamative enteritis and severe destructive intestinal lesions. The current results indicated that the housing system affected the prevalence and intensity of parasitic infections, as well as the structure of the parasitic community, in Indian peafowl.

Keywords: Cestodosis, Hepatitis, Histomonosis, Indian peafowl, Protozoan infection

INTRODUCTION

The Indian peafowl (*Pavo cristatus* Linnaeus, 1758) is a member of the family *Phasianidae* in the order *Galliformes* (Kushwaha and Kumar, 2016). The natural distribution of the Indian peafowl spans South Asia, including India, Sri Lanka, Myanmar, and parts of the Malay Peninsula. Owing to its remarkable ornamental appeal, the Indian peafowl has been extensively introduced and is frequently maintained in zoological gardens, private breeding facilities, and park collections worldwide (Rajashekara et al., 2019). Captive housing conditions, including high stocking density, restricted movement, and limited opportunities for natural foraging, may increase the risk of gastrointestinal parasite transmission in Indian peafowl (Rodrigues et al., 2020). Furthermore, even with proper veterinary and sanitary management in zoological collections, poultry can be exposed to infectious parasite stages through contaminated feed, water, and soil, as well as the presence of intermediate hosts within aviaries (Dutta et al., 2013; Ombugadu et al., 2019).

Despite their relatively high tolerance to adverse environmental conditions, Indian peafowl remain vulnerable to a broad spectrum of parasitic diseases, among which gastrointestinal endoparasites are of primary importance (Kathiravan et al., 2017). Helminths and protozoa are frequently reported in the gastrointestinal tracts of poultry (Ruhnke et al., 2021). Among helminths, the classes *Nematoda*, *Cestoda*, and *Trematoda* are most commonly reported in domestic and wild poultry. Nematodes are considered the most prevalent and pathogenic group due to their widespread presence and

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significant negative impact on host health (McJunkin et al., 2003; Udoh et al., 2014). Unlike nematodes, the diversity of cestode species that infect poultry is relatively limited (Abd El-Ghany, 2022). Nevertheless, several cestode species are of considerable epizootiological importance and may cause significant financial losses (Bogach et al., 2020).

Histomonosis is a major protozoan disease in poultry that can lead to significant economic losses and sometimes result in high-mortality outbreaks (Liebhart et al., 2017). Nematodes and cestodes infect the gastrointestinal tract of poultry, leading to damage to the intestinal mucosa, enteritis, and reduced nutrient absorption. These effects cause growth impairment, reduced productivity, higher risk of other infections, emaciation, and, in cases of severe infestation, mortality in young poultry. Poultry reared on deep litter or in backyard systems show the highest rates of helminth infections compared to those kept in cage housing systems (Basit et al., 2014; Abd El-Ghany, 2022).

Under captive conditions, peafowls exhibit increased susceptibility to endoparasitic infections, largely owing to inappropriate anthelmintic use and inadequate or delayed assessment of health status (Pradeep et al., 2017). In peafowl, endoparasitic infections frequently remain subclinical; however, heavy parasite burdens may result in clinical disease and even mortality (Lemos et al., 2024).

Parasites have multiple adverse effects on the host, including competing for nutrients and vitamins, disrupting digestion and absorption, mechanically damaging the intestinal tract, and releasing toxic metabolites. These effects result in progressive weight loss and worsening physiological health in infected poultry (Kruchynenko, 2021). Parasitic infections are prevalent among wild poultry; however, scientific investigations have predominantly focused on infections that threaten livestock production or public health (Daszak et al., 2000). Therefore, investigating the prevalence and species composition of endoparasites in peafowl maintained under free-range and aviary housing systems is important for improving strategies to prevent and control parasitic infections in ornamental and wild poultry. The present study aimed to investigate the prevalence and species diversity of endoparasites in Indian peafowl and to characterize the gross and pathomorphological lesions caused by histomonosis and cestodosis.

MATERIALS AND METHODS

Ethical approval

The present study was conducted in accordance with the current legislation of Ukraine (Article 26 of the law of Ukraine No. 3447-VI of 21 February 2006, On Protection of Animals from Cruelty), the General Ethical Principles for Animal Experiments (Reznikov, 2003), and International bioethical standards, including the provisions of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Strasbourg, 1986; Simmonds, 2018; Kabene and Baadel, 2019). The bioethics protocol No. 4/25, dated 8 October 8.10.2025, was reviewed and approved by the Bioethics Commission of the National Scientific Center, Institute of Experimental and Clinical Veterinary Medicine, Kharkiv, Ukraine, in accordance with established procedures.

Study design

The prevalence of gastrointestinal parasitic infections in Indian peafowl was investigated during 2020-2025 in private households operating under free-range conditions and in bioparks of the Odesa region, Ukraine, using a cage-aviary housing system. Under the free-range housing system, Indian peafowl were kept together with chickens (*Gallus gallus domesticus*), ducks (*Anas platyrhynchos domesticus*), and turkeys (*Meleagris gallopavo*), sharing common feeding and watering facilities. Indian peafowl had unrestricted access to outdoor areas, where a grain mixture and green forage were provided, while natural foraging for insects, earthworms, and other invertebrates was also possible. The outdoor areas were cleaned of manure twice a year. No anthelmintic treatment had been administered to the Indian peafowl during the six months preceding sample collection. Indian peafowl were kept in fenced wire-mesh aviaries under the cage-aviary system, providing suitable space for free movement.

Sample collection

The study was conducted on 90 Indian peafowl, including 44 males and 46 females aged 1-2 years. Sample collection was conducted throughout 2020-2025 across all seasons. Of the total number of peafowl, 45 were reared under a free-range housing system, whereas the remaining 45 were kept in bioparks under a cage-aviary housing system. For parasitological examination, one fecal sample was collected directly from the rectum of each Indian peafowl using a sterile cotton swab, yielding a total of 90 samples. The collected material was placed into sterile vials containing 10% formalin, labeled with individual identification numbers, transported to the laboratory, and stored at +4°C until analysis. During sample collection, the macroscopic characteristics of the feces, including color, consistency, and the presence of blood or mucus were evaluated (Keatts et al., 2016; Kathiravan et al., 2017). For the pathomorphological examination, necropsies were performed on 20 Indian peafowl from the same group of 90 individuals that died during the study period.

Coprological examinations were performed using standard parasitological methods (Zajac et al., 2021). Fecal samples were thoroughly homogenized with a 50% glycerol-water solution at a 1:1 ratio. A drop of the resulting suspension was placed on a glass slide, covered with a coverslip, and examined under a light microscope (Carl Zeiss, Germany) at 400x magnification. Protozoa and coccidian oocysts were detected using the direct smear technique, followed by microscopic identification based on their morphological characteristics (Hodgson, 1970). Fecal samples were examined for the presence of nematode eggs, and infection intensity was determined using the McMaster technique, which calculates the number of eggs per gram of feces (EPG; Titilincu et al., 2009).

Eggs of the genera *Ascaridia* and *Heterakis* were distinguished by their morphometric and morphological characteristics. Identification was based on egg shape, size, and shell morphology. *Ascaridia* spp. eggs were oval, measuring 75-80 × 40-50 μm, whereas *Heterakis* spp. eggs were slightly smaller, measuring 63-75 × 36-48 μm (Tarbiat et al., 2021). *Histomonas meleagridis* (*H. meleagridis*) was detected by microscopic examination of impression smears prepared from affected organs and fecal smears obtained from Indian peafowl. Impression smears were prepared from grossly altered areas of the liver and ceca, fixed in methanol for 3-5 minutes, and stained using the Romanowsky-Giemsa method. For fecal examination, direct smears were prepared on clean, grease-free glass slides, air-dried, fixed in ethanol for 5-10 minutes, and stained with Romanowsky-Giemsa stain. The stained slides were observed under a light microscope at 630x magnification (90x objective and 7x eyepiece). *Histomonas meleagridis* trophozoites were identified by their characteristic morphology, with blue cytoplasm and a reddish-purple nucleus, enabling clear visualization of the parasite (Liebhart et al., 2017). The oncospheres of cestodes belonging to the genera *Davainea* and *Raillietina* were differentiated by staining the eggs with a 1:10,000 brilliant green solution. Following incubation, the preparations were examined under a light microscope. The oncospheres of *Davainea proglottina* acquired a light green coloration within 3-5 minutes, whereas those of *Raillietina* spp. remained unstained during the same period, enabling reliable differentiation between the two genera (Bogach et al., 2015). *Choanotaenia infundibulum* was identified through morphological examination of adult cestodes found during postmortem analysis of Indian peafowl, following the diagnostic criteria outlined by Permin and Hansen (1998).

Postmortem and helminthological examinations

Postmortem examinations and helminthological investigations were performed on 20 Indian peafowl obtained from the Laboratory of Parasitology at the Odesa Research Station of the National Scientific Center, Institute of Experimental and Clinical Veterinary Medicine, Odesa, Ukraine. Carcasses were diagnosed with histomonosis, mixed ascariasis-histomonosis infection, and cestode infections caused by *Raillietina* spp., *Choanotaenia infundibulum*, and *Davainea proglottina*. Complete postmortem examinations were performed using the standard evisceration technique according to the established avian necropsy procedures. Gross examination included a systematic assessment of the internal organs, with particular emphasis on the digestive tract. The intestinal mucosa was evaluated for the presence and severity of inflammatory, degenerative, hemorrhagic, and necrotic lesions, and helminths present within the intestinal lumen were recovered and recorded (Swayne et al., 2020). Recovered cestodes were collected, rinsed in physiological saline, and identified based on their morphological characteristics. Species identification was based on the total length and width of the strobila, the morphology of the scolex, the shape and size of the proglottids, and the morphological features of mature segments, following established parasitological identification keys (Soulsby, 1982). Based on a combination of morphological characteristics, the parasites were identified as *Raillietina* spp., *Choanotaenia infundibulum*, and *Davainea proglottina*.

A retrospective analysis was conducted on 17 fatal cases of Indian peafowl, aged 6 weeks to 16 months, submitted to the laboratory during the study period to investigate the pathomorphological features of histomonosis. The diagnosis was established through an integrated approach that incorporated epizootiological data, characteristic gross lesions in the ceca and liver, and microscopic examination of impression smears and tissue scrapings from affected organs. Romanowsky-Giemsa staining was used to identify vegetative forms (trophozoites) of *H. meleagridis*. The observed pathological lesions were documented using a digital camera (Canon EOS 90D, Japan). In order to facilitate systematic presentation and demonstrate the progression of the disease, photographs of the ceca and liver were categorized according to lesion severity. The retrospective reconstruction of histomonosis stages was conducted based on peafowls' history, the extent of cecal lesions, and the severity of degenerative and necrotic changes in the liver.

Data analysis

Statistical analysis was performed using Microsoft Excel 2007 and STATA 13 (StataCorp, College Station, TX, USA). Prior to analysis, all data were checked for completeness and accuracy of entry. Descriptive statistics were used to characterize the prevalence of parasitic infections. Results were presented as absolute mean values with 95% confidence intervals (95% CI). Differences in prevalence among the study groups were evaluated using the Pearson chi-

square (χ^2) test. When expected cell frequencies were less than 5, Fisher's exact test was applied. Differences were considered statistically significant at a P-value less than 0.05. Mixed infections were treated as a separate analytical category and were not included in the corresponding single-parasite groups. Owing to the small number of mixed infections (n = 2), no additional statistical analyses beyond comparing prevalence between housing systems were performed.

RESULTS

The overall prevalence of gastrointestinal parasitic infections in Indian peafowl differed according to the housing system (Table 1). The overall prevalence of gastrointestinal parasitic infections in Indian peafowl was 58.9%. Under the free-range housing system, the overall prevalence was 51.1%, whereas it increased to 66.7% under the cage-aviary housing system. Although gastrointestinal parasitic infections were more frequently detected in Indian peafowl maintained under cage-aviary housing, the difference between the two housing systems was not statistically significant ($p > 0.05$). Figures 1A, 1B, and 1C illustrated the most prevalent protozoan parasites of Indian peafowl.

Table 1. Prevalence of gastrointestinal parasitic infections in Indian peafowl under different housing systems in Odesa Region, Ukraine during 2020-2025

Gastrointestinal parasites		Overall (n = 90)		Free-range housing (n = 45)		Cage-aviary housing (n = 45)		P-value
		Infected n (%)	95% CI	Infected n (%)	95% CI	Infected n (%)	95% CI	
Protozoa	*Eimeria* spp.	12 (13.3)	8.0-21.3	5 (11.1)	4.8-23.5	7 (15.6)	7.7-28.8	0.535
	Histomonas meleagridis	17 (18.9)	12.2-28.1	6 (13.3)	6.3-26.2	11 (24.4)	14.2-38.7	0.178
Nematodes	*Ascaridia galli*	5 (5.6)	2.4-12.4	2 (4.4)	1.2-14.8	3 (6.7)	2.3-17.9	1.000
	Heterakis spp.	10 (11.1)	6.2-19.1	4 (8.9)	3.5-20.7	6 (13.3)	6.3-26.2	0.502
Cestodes	*Raillietina* spp.	3 (3.3)	1.1-9.3	3 (6.7)	2.3-17.9	0 (0.0)	0.0-7.9	0.242
	Choanotaenia infundibulum	1 (1.1)	0.2-6.0	1 (2.2)	0.4-11.6	0 (0.0)	0.0-7.9	1.000
	Davainea proglottina	3 (3.3)	1.1-9.3	2 (4.4)	1.2-14.8	1 (2.2)	0.4-11.6	1.000
Mixed infection	*Histomonas meleagridis* plus *Ascaridia galli*	2 (2.2)	0.6-7.7	0 (0.0)	0.0-7.9	2 (4.4)	1.2-14.8	0.494

n: Number of Indian peafowl examined, CI: 95% confidence interval, $p < 0.05$ was considered statistically significant.

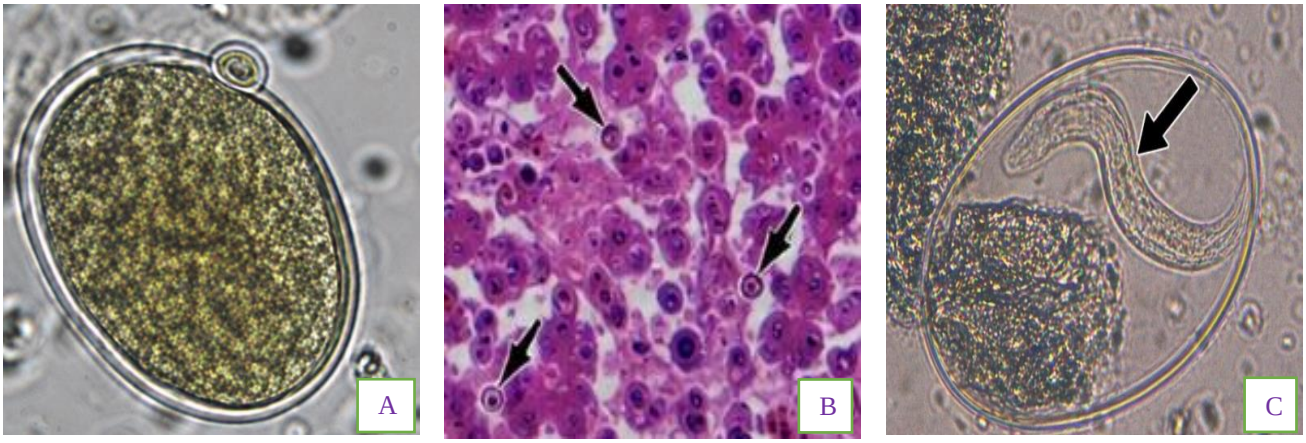


Figure 1. Parasites detected from Indian peafowl in Odesa Region, Ukraine during 2020-2025. **A**: Unsporulated oocyst of *Eimeria* spp. (400x), **B:** Trophozoites of *Histomonas meleagridis* (630x), **C**: *Heterakis* spp. egg containing a larva (400x)

The overall prevalence of protozoan infections was higher in Indian peafowl maintained under the cage-aviary housing system (40.0%) than in those kept under free-range housing (24.4%). *Eimeria* spp. and *H. meleagridis* were the predominant protozoan parasites, accounting for the majority of the detected protozoan infections. Nematode infections were more common in Indian peafowl kept in cage-aviary housing (20.0%) than in those kept in free-range housing (13.3%). This difference was likely attributable to housing-related factors, including higher stocking density and the accumulation of infective stages in the environment. In contrast, cestode infections were more common in Indian peafowl kept in free-range housing, with a prevalence of 13.3%. However, only 2.2% of Indian peafowl maintained under cage-aviary housing were infected. The higher prevalence of cestode infections in free-ranging Indian peafowl was linked to natural intermediate hosts and other potential sources of infection in the outdoor environment.

Mixed parasitic infections were detected only sporadically and were recorded exclusively in a single Indian peafowl maintained under the cage-aviary housing system. Overall, the distribution of gastrointestinal parasites indicated differences in parasite community composition between the two housing systems; however, no statistically significant differences were found between the study groups ($p > 0.05$). In the free-range housing system, there were no significant sex-related differences in the prevalence or types of gastrointestinal parasitic infections in Indian peafowl ($p > 0.05$). Additionally, the parasite species composition was comparable between males and females (Table 2).

The parasite community was mainly composed of protozoan infections, particularly *H. meleagridis* and *Eimeria* spp. Nematode infections from *Ascaridia galli* and *Heterakis* spp. were present in both sexes, indicating no significant differences in prevalence ($p > 0.05$). Cestode infections, including *Raillietina* spp., *Choanotaenia infundibulum*, and *Davainea proglottina*, were detected in both sexes, with no significant sex-related differences in prevalence ($p > 0.05$). No mixed infections were detected among Indian peafowl housed in free-range systems, indicating that single-species infections predominated. Under the cage-aviary housing system, parasitic infections were detected in both male and female peafowl. The parasite species composition was similar between males and females, but female Indian peafowl tended to have a higher prevalence of some parasite species (Table 3).

Under the cage-aviary housing system, the parasite community was dominated by protozoan infections. *Histomonas meleagridis* and *Eimeria* spp. were the most prevalent parasites and were detected more frequently than the other endoparasites identified in the present study.

Nematode infections caused by *Ascaridia galli* and *Heterakis* spp. were detected in both sexes, with comparable prevalence rates ($p > 0.05$). Cestode infections were rare under cage-aviary housing. Only one female Indian peafowl kept under cage-aviary housing was infected with *Davainea proglottina*, and no other cestode species were detected in the cage-aviary group. Mixed infection with *H. meleagridis* and *Ascaridia galli* was observed only under the cage-aviary housing system, suggesting that this housing system may facilitate the simultaneous circulation of multiple parasitic agents. Analysis of parasites related to housing system and sex revealed higher infection intensity in Indian peafowl housed in cage-aviary systems than in free-range systems (Table 4).

Table 2. Prevalence of gastrointestinal parasitic infections in Indian peafowl under free-range housing system according to sex in Odesa Region, Ukraine during 2020-2025

Gastrointestinal parasites	Males (n = 22)		Females (n = 23)		P-value
	Infected n (%)	95% CI	Infected n (%)	95% CI	
Eimeria spp.	2 (9.1)	2.5-28.0	3 (13.0)	4.5-32.1	1.000
Histomonas meleagridis	3 (13.6)	4.8-34.2	3 (13.0)	4.5-32.1	1.000
Ascaridia galli	1 (4.5)	0.8-21.8	1 (4.3)	0.8-21.9	1.000
Heterakis spp.	2 (9.1)	2.5-28.0	2 (8.7)	2.4-27.8	1.000
Raillietina spp.	2 (9.1)	2.5-28.0	1 (4.3)	0.8-21.9	1.000
Choanotaenia infundibulum	1 (4.5)	0.8-21.8	0 (0.0)	0.0-13.8	0.489
Davainea proglottina	1 (4.5)	0.8-21.8	1 (4.3)	0.8-21.9	1.000
Histomonas meleagridis plus *Ascaridia galli*	0 (0.0)	0.0-13.8	0 (0.0)	0.0-13.8	1.000

n: Number of Indian peafowl examined, CI: 95% confidence interval, $p < 0.05$ was considered statistically significant.

Table 3. Prevalence of gastrointestinal parasitic infections in Indian peafowl under cage-aviary housing according to sex in Odesa Region, Ukraine during 2020-2025

Gastrointestinal parasites	Males (n = 22)		Females (n = 23)		P-value
	Infected n (%)	95% CI	Infected n (%)	95% CI	
Eimeria spp.	3 (13.6)	4.8-34.2	4 (17.4)	7.1-36.8	1.000
Histomonas meleagridis	5 (22.7)	10.0-43.6	6 (26.1)	12.6-45.7	1.000
Ascaridia galli	1 (4.5)	0.8-21.8	2 (8.7)	2.4-27.8	1.000
Heterakis spp.	3 (13.6)	4.8-34.2	3 (13.0)	4.5-32.1	1.000
Raillietina spp.	0 (0.0)	0.0-13.8	0 (0.0)	0.0-13.8	1.000
Choanotaenia infundibulum	0 (0.0)	0.0-13.8	0 (0.0)	0.0-13.8	1.000
Davainea proglottina	0 (0.0)	0.0-13.8	1 (4.3)	0.8-21.9	1.000
Histomonas meleagridis plus *Ascaridia galli*	1 (4.5)	0.8-21.8	1 (4.3)	0.8-21.9	1.000

n: Number of Indian peafowl examined, CI: 95% confidence interval, $p < 0.05$ was considered statistically significant.

On average, the intensity of parasitic infection was 1.5-2.5-fold higher under the cage-aviary housing system than under free-range housing. Higher parasite infection rates were observed in both males and females, with females generally demonstrating higher rates. Among the protozoa, *Eimeria* spp. and *H. meleagridis* indicated the highest intensity. In the cage-aviary housing system, the levels of *Eimeria* spp. exceeded those observed in free-range housing by more than twofold, whereas *H. meleagridis* demonstrated an approximately twofold increase. The intensity of *Ascaridia galli* and *Heterakis* spp. was comparatively lower than that of the protozoa; however, it was 1.5 to 2.0 times greater in cage-aviary housing than in the free-range housing system. Among the nematodes, the highest values were recorded for *Heterakis* spp. Cestodes were detected at relatively low infection intensity and were mainly found in the free-range housing system, with only a single case of *Davainea proglottina* recorded in a female under cage-aviary housing. Mixed infection of *H. meleagridis* and *Ascaridia galli* was observed only under cage-aviary housing, with higher values in females. Overall, the current results indicated higher parasite levels in Indian peafowl maintained under a cage-aviary housing system than under free-range housing. The present results demonstrated differences in the prevalence, intensity, and species composition of gastrointestinal parasites between the two housing systems. Because *H. meleagridis* was among the most frequently detected parasites, the subsequent stage of the study focused on the pathomorphological changes in the internal organs of Indian peafowl at different stages of histomonosis.

In Indian peafowl, the initial gross lesions of histomonosis appeared as early as 3-5 days after clinical signs began, while severe organ deformation in the final stage typically developed within 12-21 days.

At the early stage of acute histomonosis, which corresponded to 3-5 days after the onset of clinical signs in peafowls, pathological changes were predominantly attributable to *H. meleagridis* infection in the cecal mucosa and the development of an initial inflammatory response. Postmortem examination during the acute stage revealed marked vascular congestion and hyperemia of the mesentery. The loops of the small and large intestines were edematous, and the serosal surface was thickened and moderately hyperemic. In the caudal part of the intestine, early areas of tissue damage exhibiting yellow fibrinous deposits were observed, indicative of acute catarrhal-hemorrhagic or serofibrinous inflammation (Figure 2).

As the disease progressed, approximately 5-7 days after the onset of clinical signs, marked focal thickening of the walls of both ceca was observed. The serosal surface was intensely hyperemic, with a prominent vascular pattern and bluish-red discoloration due to vascular congestion. As a result of exudative inflammation and cellular infiltration of the submucosa, the cecal walls became markedly thickened and firm, while the lumen gradually narrowed due to the accumulation of fibrinonecrotic exudate (Figure 3).

Table 4. Intensity of parasitic infection in Indian peafowl according to housing system and sex in Odesa Region, Ukraine during 2020-2025

Gastrointestinal parasites	Free-range housing (n = 45)		Cage-aviary housing (n = 45)	
	Males (n = 22)	Females (n = 23)	Males (n = 22)	Females (n = 23)
Eimeria spp. (OPG)	320 ± 110 (n = 2)	360 ± 130 (n = 3)	740 ± 230 (n = 3)	820 ± 270 (n = 4)
Histomonas meleagridis (trophozoites per field of view)	6.8 ± 2.5 (n = 3)	7.4 ± 3.0 (n = 3)	14.5 ± 4.8 (n = 5)	18.2 ± 5.9 (n = 6)
Ascaridia galli (EPG)	5.0 (n = 1)	6.0 (n = 1)	9.0 (n = 1)	10.4 ± 4.1 (n = 2)
Heterakis spp. (EPG)	7.5 ± 2.8 (n = 2)	9.2 ± 3.5 (n = 2)	15.1 ± 5.0 (n = 3)	18.2 ± 5.8 (n = 3)
Raillietina spp. (worms/poultry)	2.1 ± 0.8 (n = 2)	3.0 (n = 1)	–	–
Choanotaenia infundibulum (worms/poultry)	6.0 (n = 1)	–	–	–
Davainea proglottina (worms/poultry)	1.5 (n = 1)	2.0 (n = 1)	–	1.0 (n = 1)
Histomonas meleagridis (trophozoites per field of view) plus *Ascaridia galli* (EPG)	–	–	8.0 7.0	9.0 11.0

n: Number of Indian peafowl examined, OPG: Oocysts per gram of feces, EPG: Eggs per gram of feces. Data are presented as mean ± SD.



Figure 2. Gross pathological changes in the internal organs of an Indian peafowl during the early stage of histomonosis (3-5 days after the onset of clinical signs), reared in Odesa Region, Ukraine. The black arrow indicates the cecum, which shows early pathological changes.



Figure 3. Gross appearance of the ceca with early typhlitis (5-7 days after the onset of clinical signs) in an Indian peafowl, reared in the Odesa Region, Ukraine. The black arrow indicates the pathologically altered cecum with early typhlitis.

At the stage of severe diffuse typhlitis, approximately 7-12 days after the onset of clinical signs, profound destructive changes in the cecal walls were observed. As a result of the intensive progression of the pathological process caused by *H. meleagridis*, the cecal walls became diffusely and irregularly thickened, while the lumen contained large amounts of fibrinonecrotic (caseous) material. Through the thinned and hyperemic serosa, multiple yellowish-gray parts of necrotic lesions were visible. In some areas of the mucosa and muscular layer, erosive and ulcerative lesions were detected, resulting from pathogen-induced tissue destruction, indicating the development of a destructive form of the disease (Figure 4).

In the terminal (chronic) stage of the disease, typically corresponding to 12-15 days of the pathological process, marked organ deformation was observed, along with the formation of large fibrinonecrotic conglomerates and a risk of peritonitis development. At this chronic stage, the ceca of Indian peafowl had lost their normal anatomical structure and appeared as firm, nodular masses. Large nodular areas of yellow and dirty-white coloration were observed on the surface. The cecal lumen was filled with dry, dense caseous (cheesy) fibrinonecrotic masses of layered structure, causing pronounced dilatation of the lumen and stretching of the organ walls (Figure 5).

At the final stage of histomonosis development, approximately 15-21 days after the onset of clinical signs, marked accumulation of fibrinonecrotic masses was observed, which largely replaced the structural components of the cecal wall. In areas with the most extensive caseous deposits, the intestinal wall became markedly thinned and lost its structural integrity. Marked pathological changes created conditions for possible intestinal perforation, leakage of infected contents into the abdominal cavity, and the development of diffuse fibrinous peritonitis (Figure 6).

Parallel to the destructive changes in the ceca, a specific necrotizing hepatitis developed due to the spread of *H. meleagridis* via the portal circulation to the liver. The severity and rate of macroscopic lesion development in the hepatic parenchyma depended on the severity of typhlitis and were characterized by a sequential progression of pathological changes. In the early phase of the widespread invasive process, identified through history and clinical-pathological data indicating initial signs of catarrhal-hemorrhagic typhlitis at 5-7 days into the disease, hepatomegaly and hepatic congestion were observed. The liver exhibited a moderately firm consistency, with slightly rounded lobe margins. Against a dark cherry-red or brown parenchyma background, single small, irregularly distributed, round, grayish-yellow parts were detected, predominantly located subcapsularly (Figure 7).

During the peak of the disease, approximately 7-12 days after clinical signs appear, which corresponds to the stage of diffuse diphtheritic typhlitis, significant destructive and necrotic changes occur in the liver, typical of histomonosis.

Necrotic regions were markedly enlarged and assumed round or disc-shaped forms with clearly defined margins. Crater-like depressions in the central region of the lesions, resulting from coagulative necrosis and subsequent tissue destruction, were observed. The peripheral zone of the lesions remained marginally elevated above the surface of the parenchyma and displayed a yellowish-white or dirty gray coloration (Figure 8).

As the infection progressed to the chronic and terminal stages (after 12-15 days of the pathological process), pronounced dystrophic and necrotic lesions of the liver were observed. The hepatic parenchyma lost its normal color and developed a diffuse clay-brown or dirty-yellow appearance, indicating severe degenerative changes, including fatty and granular degeneration. Numerous necrotic foci enlarged, expanded peripherally, and coalesced to form large fibrinonecrotic conglomerates that extended through a substantial portion of the hepatic lobes (Figure 9).

The hepatic parenchyma was diffusely altered, exhibiting a clay-brown coloration, indicating severe degenerative processes, including fatty and granular degeneration. Numerous necrotic foci expanded in size, coalesced, and formed large conglomerates extending through a substantial thickness of hepatic tissue. In some cases, at the terminal stages of infection, extensive areas of deep tissue destruction were observed, involving the serosal membranes of adjacent organs in the pathological process (Figure 10).

The described extensive and severe lesions of the hepatic parenchyma were associated with marked impairment of liver function, development of pronounced endogenous intoxication, and represented one of the key factors contributing to Indian peafowl mortality.

In addition to cases of monoinfection with histomonosis, mixed parasitic infections were recorded during the study. Postmortem examination of dead juvenile Indian peafowl infected with *H. meleagridis* and *Ascaridia galli* revealed complex destructive and inflammatory changes in the digestive tract. The pathological findings were characterized by a combination of lesions typical of histomonosis and ascariasis, manifested by inflammatory, dystrophic, and necrotic changes in the intestine, accompanied by impaired functional status of the gastrointestinal tract (Figure 11).

In the small intestine, a high intensity of infection with adult *Ascaridia galli* nematodes was observed. Pale yellow, spindle-shaped helminths were observed within the intestinal lumen, occasionally forming dense aggregates. The mucosa of the small intestine in the areas of parasite localization exhibited acute catarrhal-hemorrhagic inflammation, characterized by diffuse hyperemia, edema, thickening of the intestinal folds, and multiple small petechial hemorrhages at sites of mechanical damage caused by the parasites.

Damage to the intestinal mucosa by ascarids contributed to disruption of the intestinal barrier, development of secondary inflammatory processes, and created conditions for the progression of pathological changes, particularly cecal involvement in histomonosis.

Simultaneously, characteristic lesions of the ceca caused by intensive development of *H. meleagridis* were observed. Both cecal pouches were markedly enlarged, with diffusely thickened and firm walls. The serosal surface appeared dull, intensely hyperemic, and exhibited a pronounced vascular pattern. The cecal lumina were dilated and filled with dense fibrinous-caseous (croupous) masses of pale gray or yellowish coloration, which adhered tightly to areas of mucosal damage.

During the necropsy of dead Indian peafowl and examination of the gastrointestinal tract, the small intestine exhibited pronounced diffuse destructive and inflammatory changes associated with parasitism by cestodes of the genera *Raillietina* spp. and *Choanotaenia infundibulum* (Figures 12, 13).

In the longitudinal section of the intestine, a high intensity of cestode invasion was observed. The parasites were long, ribbon-like strobilae of pale cream or whitish-yellow coloration, predominantly localized in the middle third of the small intestine, where they occasionally formed dense, intertwined aggregates. A large number of helminths led to pronounced narrowing of the intestinal lumen and, in some areas, partial obstruction of the intestinal tube. The intestinal mucosa at the sites of parasite localization demonstrated marked catarrhal-desquamative enteritis, characterized by hyperemia, edema, thickening, and flattening of the mucosal folds. The surface was covered with a substantial layer of thick, translucent to turbid mucus, caused by mechanical irritation and tissue damage from the parasites' attachment structures. In infections caused by *Choanotaenia infundibulum*, the strobila exhibited characteristic morphological features, including triangular or bell-shaped proglottids, with the posterior part of each segment wider than the anterior and partially overlapping the subsequent segment, resulting in an irregular, wavy outline. The parasites were mainly localized in the anterior segments of the small intestine and induced focal inflammatory and destructive changes in the mucosa. At the sites of helminth attachment to the intestinal mucosa, thick mucus exudate, vascular hyperemia, focal petechial hemorrhages, and superficial necrosis of the intestinal villi were observed. Pathomorphological examination revealed extensive damage to the intestinal villi, mucosal hyperemia and edema, and catarrhal-desquamative enteritis. During necropsy, macroscopic changes consistent with Davaineosis-related toxic duodenitis were noted in the anterior part of the small intestine (duodenum). A large number of small tapeworms identified as *Davainea proglottina* were found in the lumen of the opened intestine (Figure 14).

The parasites were characterized by a short strobila and were mainly localized on the mucosal surface and within its deeper layers. In the sites of parasite localization, pronounced pathomorphological changes of the intestinal wall were observed. The mucosa was diffusely edematous and hyperemic, exhibiting signs of catarrhal-desquamative inflammation. Attachment of the parasites via the scolex and mechanical damage caused by hooks led to injury of the epithelial lining and villi, accompanied by small focal hemorrhages and necrobiotic tissue changes.

On gross examination, these changes appeared as grayish and dark brown discoloration due to the accumulation of mucous exudate, desquamated epithelium, and blood admixtures. Pathomorphological examination of the duodenum in Indian peafowl infected with *Davainea proglottina* revealed disruption of the intestinal epithelium, mucosal hyperemia, edema, and focal destructive lesions.

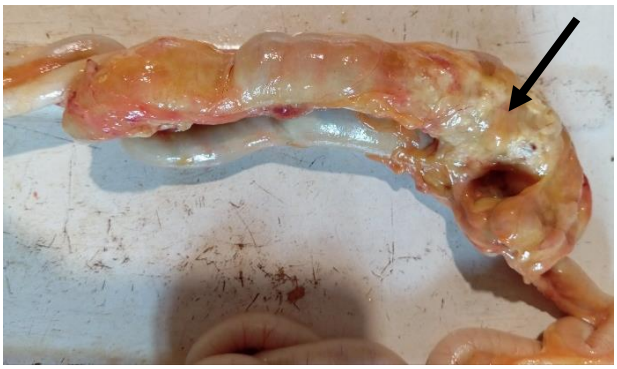


Figure 4. Diffuse necrotizing typhlitis at the peak of infection (7-12 days after the onset of clinical signs) in an Indian peafowl reared in Odesa Region, Ukraine. The black arrow indicates the area of extensive necrotic damage to the cecal wall.

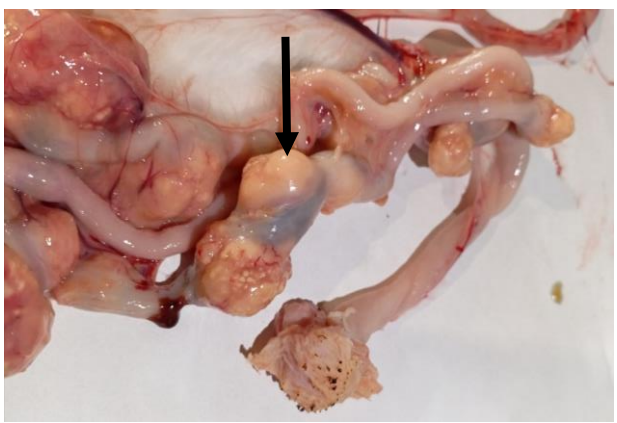


Figure 5. Chronic stage of histomonosis (12-15 days after the onset of clinical signs) in an Indian peafowl with deformation of the ceca, reared in Odesa Region, Ukraine. The black arrow indicates the inflamed and pathologically altered cecum.

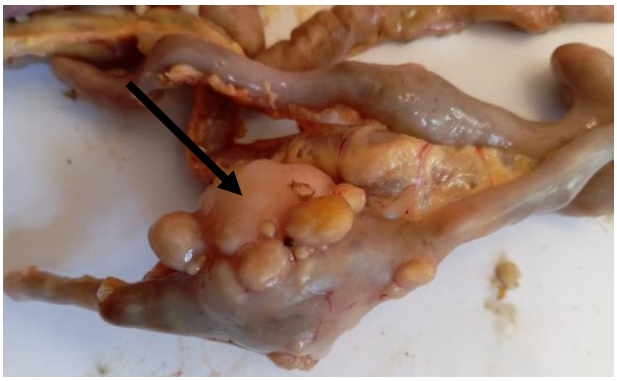


Figure 6. Extensive fibrinonecrotic process (15-21 days after the onset of clinical signs) in the ceca of an Indian peafowl reared in Odesa Region, Ukraine. The black arrow indicates marked thickening and nodular alteration of the cecal wall.

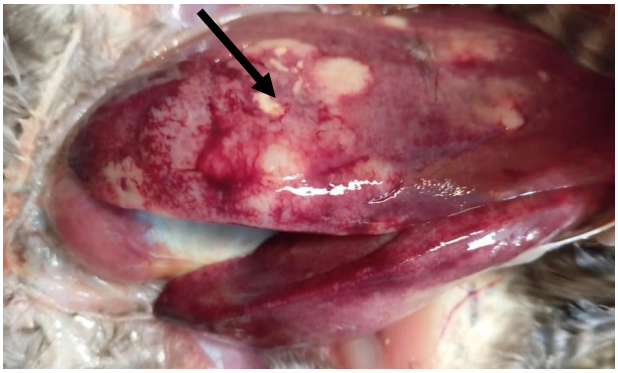


Figure 7. Gross lesions in the liver of an Indian peafowl at the early stage of histomonosis (5-7 days after the onset of clinical signs), reared in Odesa Region, Ukraine. The black arrow indicates multifocal necrotic lesions in the hepatic parenchyma.



Figure 8. Gross appearance of specific necrotizing hepatitis in Indian peafowl at the peak of infection (7-12 days after the onset of clinical signs), reared in Odesa Region, Ukraine. The black arrow indicates multifocal necrotic lesions in the hepatic parenchyma.

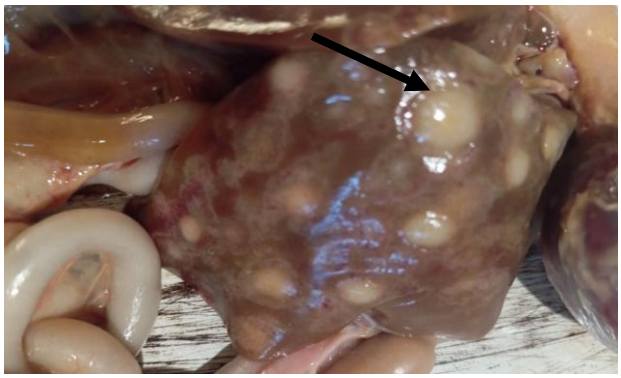


Figure 9. Progressive multifocal dystrophic and necrotizing lesions in the liver of an Indian peafowl at the advanced stage of the pathological process (12-15 days after the onset of clinical signs), reared in Odesa Region, Ukraine. The black arrow indicates extensive multifocal necrotic lesions in the hepatic parenchyma.

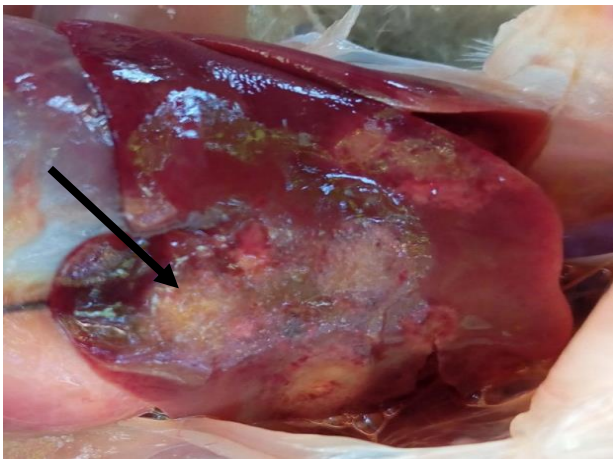


Figure 10. Deep focal necrosis of the hepatic parenchyma at the terminal stage of histomonosis (15-21 days after the onset of clinical signs) in an Indian peafowl reared in Odesa Region, Ukraine. The black arrow indicates extensive multifocal necrotic lesions involving large areas of the hepatic parenchyma.



Figure 11. Gross lesions of the gastrointestinal tract in an Indian peafowl with mixed histomonosis-ascariasis invasion reared in Odesa Region, Ukraine. The black arrows indicate pathological changes in the intestine associated with histomonosis and the presence of *Ascaridia galli*.



Figure 12. Gross lesions in the intestinal lumen of an Indian peafowl during heavy infection with *Raillietina* spp. in Odesa Region, Ukraine. The black arrow indicates a cestode in the intestinal lumen.



Figure 13. Gross lesions of the small intestine and general appearance of cestode strobilae during heavy invasion by *Choanotaenia infundibulum* in an Indian peafowl reared in Odesa Region, Ukraine. The black arrow indicates cestodes of *Choanotaenia infundibulum* in the intestinal lumen.

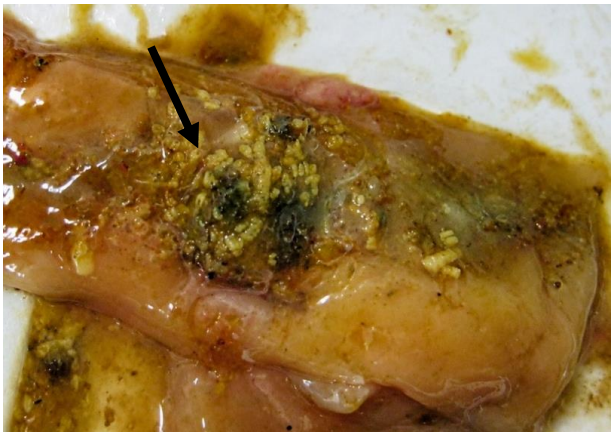


Figure 14. Gross lesions of the duodenum and presence of *Davainea proglottina* in the mucous exudate of an Indian peafowl reared in Odesa Region, Ukraine. The black arrow indicates *Davainea proglottina* cestodes in the mucous exudate.

DISCUSSION

According to previous studies, a wide spectrum of gastrointestinal parasites has been reported in Indian peafowl, including *Hymenolepis* spp., *Davainea proglottina*, *Strongyloides* spp., *Heterakis gallinarum*, *Ascaridia galli*, *Capillaria columbae*, *Acuaria spiralis*, and protozoan parasites such as *Cryptosporidium meleagridis* and *Eimeria* spp. (Kathiravan et al., 2017). Most gastrointestinal parasites are localized in the intestine, where they reproduce, leading to environmental contamination of the same or other poultry, either directly through fecal contamination or via intermediate hosts (Salavati et al., 2023).

When poultry are kept in aviaries, even with proper sanitary management, the observed level of invasion may be linked to contamination of soil, feed, and water with parasite eggs, the introduction of pathogens by free-living wild poultry, as well as factors such as high stocking density, stress, immunosuppression, and concurrent diseases. Under these circumstances, even low-pathogenic parasites may induce notable clinical manifestations (Carrera-Játiva et al., 2020).

In free-range Indian peafowl that interacted with other domestic poultry, the detected level of invasion may result from direct interspecies contact, as well as from shared grazing and feeding areas, which enabled parasitic agents to circulate among different bird types (Penczykowski et al., 2016). Among parasitic diseases, protozoan infections, particularly coccidiosis, were reported as predominant in peafowl worldwide (Zhang et al., 2022). Although parasites typically cause little or no clinical impact in healthy free-living individuals, parasitic infections constitute a major health concern in captive poultry, particularly under high-density housing systems (Jaiswal et al., 2013).

The current results demonstrated a high prevalence of gastrointestinal parasitosis in Indian peafowl, regardless of the housing system. The overall prevalence was 51.1% in free-range housing and 66.7% in cage-aviary housing, indicating a tendency toward higher parasite levels under confined conditions. According to Carrera-Játiva et al. (2018), under zoo conditions with strictly managed sanitation and daily cleaning of artificial enclosures, the prevalence of gastrointestinal parasites was 31.0% in captive poultry and 65.5% in free-ranging poultry.

Protozoan infections predominated in free-range housing, whereas cestode infections were more frequent in cage-aviary housing. The highest prevalence rates were recorded for histomonosis, particularly under cage-aviary housing (24.4%), while coccidiosis was detected in 11.1-15.6% of Indian peafowl. Mixed infections were rare and occurred only sporadically. The obtained prevalence rates were consistent with coprological monitoring data from different geographic regions. In India, the prevalence of endoparasitoses in peafowl ranged from 46.6% to 66.7%, with *Capillaria* (34.0-58.33%), *Ascaridia* (16.66-29.0%), and *Heterakis* (13.4%) as the dominant parasites. Protozoa such as *Eimeria* spp. (12.5-25.0%) and *Giardia* spp. (7.31%) were also recorded. Mixed infections were detected in 34.0% of Indian peafowl in India (Farooq et al., 2020; Manjunatha et al., 2023). In Brazil, nematode invasions in the form of mono- and mixed infections were identified in 66.6% of examined peafowl, with *Ascaridia* eggs being the most frequently detected (Teixeira et al., 2012).

In Nigeria, the highest prevalence among parasites was recorded for *Ascaridia galli* (100%), while *Raillietina* sp. were detected in 28.6% of examined peafowl (Bekeh et al., 2021). The highest overall prevalence (70.0%) was reported in peafowl from Pakistan, where *Eimeria* spp. dominated (66.33%), while the prevalence of *Hymenolepis* spp., *Ascaris* spp., *Ascaridia* spp., *Strongyloides* spp., and *Heterakis* spp. ranged from 3.33% to 10.0% (Naz, 2021).

Previous studies indicated that Indian peafowl exhibited greater susceptibility to *H. meleagridis* infection compared to chickens and pheasants. Conversely, the clinical and pathological manifestations of naturally occurring histomonosis were similar to those observed in chickens and turkeys (Lund and Chute, 1972; Clarke et al., 2017). Particular attention

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was given to the prevalence of *H. meleagridis*, as histomonosis is regarded as one of the most severe parasitic diseases affecting poultry (Hauck et al., 2013). The identification of histomonad trophozoites in 13.3% of peafowl housed in free-range systems and 24.4% in cage-aviary settings indicated active circulation of the pathogen within the studied populations.

It is well established that *Heterakis* spp. play an important role in maintaining the epizootic cycle, functioning as reservoirs for *H. meleagridis* and facilitating the long-term survival and dissemination of the pathogen within poultry populations (Beer et al., 2022). In the present study, *Heterakis* spp. were identified in 8.9-13.3% of peafowl, supporting the possibility of a sustained natural cycle of histomonosis infection.

Clinical signs of histomonosis typically appear 7-14 days after infection and include depression, reduced feed intake, impaired coordination, ruffled plumage, and changes in fecal consistency and coloration (Hauck et al., 2013). One of the most characteristic signs is the appearance of yellow-gray or sulfur-yellow droppings, traditionally regarded as a typical clinical manifestation of blackhead disease (Spencer, 2010; Majidi et al., 2025).

Pathomorphological changes in histomonosis in galliform poultry were characterized by progressive diphtheritic-necrotizing typhlitis and specific necrotizing hepatitis, with the formation of characteristic round necrotic parts in the liver (Clarke et al., 2017; Michelazzo et al., 2017). Similar lesions were observed in turkeys by Grafl et al. (2015), who considered necrotic lesions of the ceca and liver to be pathognomonic for histomonosis. According to Grabensteiner et al. (2006), hepatic lesions may range from portal heterophilic granulomas to multifocal or coalescing necrotic foci with different severity. The pathomorphological changes observed in the present study fully corresponded to classical descriptions of histomoniasis. The current results indicated pathological progression, from initial catarrhal-hemorrhagic lesions in the ceca to the formation of large caseonecrotic conglomerates and multiple necrotic foci in the liver, consistent with the current understanding of the pathogenesis of histomonosis in galliform Indian peafowl.

Similar lesions were reported by De Sousa et al. (2021) during a postmortem examination of a three-month-old male peafowl kept in mixed-species poultry housing. The Indian peafowl exhibited severe bilateral diffuse necrotizing typhlitis and multifocal coalescing necrotizing hepatitis. Histological analysis of the ceca revealed transmural necrotizing inflammation of the intestinal wall, with many trophozoites identified as *H. meleagridis*, further confirming the pathological changes noted in the present study.

Nematodoses were represented by *Ascaridia galli* and *Heterakis* spp., which were more frequently recorded under cage-aviary housing. The intensity of invasion by these parasites was higher than in peafowl under free-range housing. Similar results were reported by Titilincu et al. (2009), who observed a high prevalence of ascariasis and heterakidosis in captive peafowl. The mixed infection of *H. meleagridis* and *Ascaridia galli* observed in the present study was notable, as it caused severe intestinal pathology. The presence of catarrhal-hemorrhagic enteritis combined with croupous necrotizing typhlitis led to more extensive damage to the intestinal wall than single infections, suggesting that co-infection may synergistically worsen parasitic conditions.

Cestodoses were predominantly detected in peafowl under free-range housing, where *Raillietina* spp., *Choanotaenia infundibulum*, and *Davainea proglottina* were recorded. The overall prevalence of cestode infections was lower than that of protozoan and nematode infections; however, their presence indicated the involvement of intermediate hosts in maintaining the epizootic cycle. The higher infection level in free-range Indian peafowl was likely associated with access to insects, mollusks, and other invertebrates acting as intermediate hosts for cestodes. A similar relationship between housing system and cestode prevalence has been reported by Dutta et al. (2013) and Abd El-Ghany (2022).

The intensity of parasitic infection was 1.5-2.5-fold higher in Indian peafowl maintained under cage-aviary housing than in those kept under free-range housing. The most pronounced differences were observed in *Eimeria* spp., *H. meleagridis*, and *Heterakis* spp., thereby indicating the critical influence of housing conditions in determining the level of parasitic infection in peafowl. Ombugadu et al. (2019) documented a notable prevalence of gastrointestinal parasites in poultry maintained in zoological gardens, with infection rates reaching up to 100% in some areas, and identified five parasite taxa, including *coccidia*, *Balantidium* spp., *Capillaria* spp., *Ascaris* spp., and *Strongyloides* spp. Among these parasites, *Capillaria* spp. was the most prevalent (14.1%), while mixed infections were observed in 10.1% of poultry cases. Infection intensity ranged from 63.0 ± 16.67 to 101.98 ± 10.36 EPG for nematodes and from 297.89 ± 20.41 to 332.47 ± 16.67 oocysts per gram for coccidia. Increased stocking density and accumulation of infective parasite stages in the environment may facilitate parasite transmission among poultry (Heckendorn et al., 2009).

The current results aligned with previous findings, as both the prevalence and severity of parasitic infections were elevated in the cage-aviary housing system, indicating an increased risk of parasite accumulation and transmission under these conditions. Thus, the present study confirmed that peafowl were susceptible to a wide range of protozoan, nematode, and cestode infections. *Histomonas meleagridis*, *Eimeria* spp., and *Heterakis* spp. were frequently observed in parasitic pathology, while the housing system notably influenced parasite abundance.

CONCLUSION

The present study demonstrated that the housing system influenced the prevalence and intensity of parasitic infections in Indian peafowl, with higher parasite levels observed under the cage-aviary system. Histomonosis was identified as one of the most clinically significant parasitic infections, characterized by necrotizing lesions of the ceca and liver, while cestode infections were associated with catarrhal-desquamative enteritis and impaired intestinal function. A limitation of the study was the inability to determine the exact time course of pathological changes in naturally occurring histomonosis cases, due to the retrospective nature of the analysis. Further studies involving larger populations and molecular characterization of parasite species are recommended to improve understanding of parasite transmission dynamics and develop effective control strategies in Indian peafowl.

DECLARATIONS

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Authors' contributions

Mykola Bogach conceptualized the study and participated in parasitological and postmortem examinations, data analysis, and manuscript revision. Olena Bohach participated in sample collection, laboratory investigations, statistical analysis, and manuscript preparation. Olha Piven contributed to sample collection and parasitological examinations. Liudmyla Perotska participated in data analysis and scientific revision of the manuscript. Liliya Roman participated in postmortem examinations and documentation of pathomorphological findings. Denys Bohach contributed to laboratory investigations and data organization. All authors reviewed and approved the final edition of the manuscript for publication.

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.

Competing interests

The authors declared no conflicts of interest.

Ethical considerations

This manuscript is original, has not been previously published, and has not been submitted simultaneously to any other scientific journal. The authors confirmed that the manuscript has been screened for academic integrity using plagiarism-detection software and that the reported findings are based exclusively on their own original scientific research. The authors confirmed that no artificial intelligence tools were used in the preparation or writing of the manuscript. The authors take full responsibility for the accuracy, originality, and integrity of the final content and affirmed that they are solely responsible for the originality of the manuscript throughout the submission, revision, and publication processes.

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Isolation, Phenotypic Characterization, and Functional Potential of Indigenous Lactic Acid Bacteria from Fresh Duck Eggs

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ABSTRACT

Duck eggs are a highly nutritious source of animal protein; however, they are particularly susceptible to microbial contamination. Indigenous lactic acid bacteria (LAB) capable of colonizing fresh egg matrices may possess adaptive traits, making them effective natural biopreservation agents. The present study aimed to isolate, phenotypically characterize, and evaluate the antimicrobial activity of LAB derived from different compartments of fresh duck eggs, specifically the inner eggshell, egg membrane, and egg contents. Duck eggs were obtained from two intensive farms (I1 and I2) and two extensive farms (E1 and E2) located in Sidrap Regency, South Sulawesi, Indonesia, during 2024. The LAB isolates were obtained using a selective culture method employing De Man, Rogosa and Sharpe agar enriched with 1% $CaCO_2$. Twenty fresh duck eggs (five per location) were sampled, yielding 35 isolates, of which seven were confirmed as LAB based on morphological and biochemical analyses. Characterization included Gram, endospore, and acid-fast staining, catalase testing, and carbohydrate fermentation profiling. Antimicrobial activity against *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*) was evaluated using agar well diffusion method at the original (acidic) and neutral pH levels to identify bacteriocin-like inhibitory substances (BLIS). Seven selected LAB isolates were obtained, including five from extensive farms (E1S1, E1S3, E1M1, E2S2, E2M2) and two from intensive farms (I1M2, I2M5). All isolates demonstrated complete glucose fermentation and a homofermentative pattern. Isolates E2S2, E2M2, and I1M2 fermented glucose, lactose, fructose, and mannitol, whereas E1S1, E1S3, E1M1, and I2M5 fermented glucose exclusively. Based on phenotypic data, the isolates were preliminarily classified as *Lactobacillus* and *Pediococcus* species. Antimicrobial activity testing demonstrated significant inhibitory potential, with maximum inhibition zones of 27.5 mm against *E. coli* and 17.0 mm against *S. aureus*. Although inhibition zone diameters decreased at neutral pH, the persistence of a clear zone against *E. coli* indicated BLIS production. The disappearance of inhibitory activity against *S. aureus* at neutral pH indicated that organic acids caused the pathogen's inhibition. The current findings indicated that fresh duck eggs contain a high abundance of homofermentative LAB, which could serve as effective natural biopreservation agents to improve food safety and prolong shelf life.

Keywords: Antimicrobial activity, Duck egg, Lactic acid bacteria, *Lactobacillus*, *Pediococcus*

INTRODUCTION

Duck eggs (*Anas platyrhynchos*) are a highly valuable local poultry product, known for their rich nutritional content and significant economic role within the national animal protein supply chain. In many regions across Indonesia, duck eggs have been served as a significant source of animal protein for local communities. Data from the Central Bureau of Statistics indicated that total duck egg production in Indonesia reached 358,000 tonnes in 2023, up from 349,000 tonnes in 2022 and 334,000 tonnes in 2021 (Statistics Indonesia, 2024). In Indonesia, South Sulawesi was ranked third among provinces in duck egg production in 2023. Several districts, including Sidrap and Wajo, have become prominent centers of duck farming, significantly contributing to local and regional egg supplies (Statistics Indonesia, 2024).

Duck eggs are widely consumed fresh and in processed forms, such as salted eggs, particularly in Asian countries, including Indonesia. Compared with chicken eggs, duck eggs are larger, have a deeper-colored yolk, and contain more protein and fat (Krismiyanto et al., 2020). In addition, duck eggs contain natural bioactive compounds, including avidin, ovotransferrin, and lysozyme, that have antimicrobial activity (Nian et al., 2022). Eggs play an important role in microbiological studies because they maintain internal sterility while serving as nutrient-rich substrates for microorganisms that penetrate their defenses (Wellman-Labadie et al., 2010). Similar to other avian eggs, duck eggs

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have a complex structure comprising an outer shell, membrane, and egg contents (albumen and yolk), with each compartment providing distinct nutrient profiles and inherent defense mechanisms (Guyot et al., 2016). Nevertheless, the eggshell and membrane are not entirely sterile and serve as primary biological barriers vulnerable to microflora from feed, feces, and the barn environment (Wierup, 2017). The nutrient-rich environment on and within the egg makes it a potential habitat for various microorganisms, including lactic acid bacteria (LAB; Saputra and Rahayu, 2013).

Lactic acid bacteria are a group of Gram-positive, non-spore-forming, facultatively anaerobic bacteria widely recognized for their important roles in food preservation and safety (Badis et al., 2022). The antimicrobial activity of LAB is primarily attributed to the production of lactic acid, which lowers the environmental pH and creates unfavorable conditions for the proliferation of spoilage microorganisms and pathogenic bacteria (Badis et al., 2022). Furthermore, LAB can produce additional antimicrobial substances, such as hydrogen peroxide, diacetyl, and bacteriocins, which enhance their inhibitory effects against foodborne pathogens (Dabiza et al., 2023). The potential of LAB in different livestock-derived food products has been demonstrated, with Murtari et al. (2020) successfully isolating *Lactobacillus plantarum* and *Lactobacillus fermentum*, and Sugata et al. (2024) identifying *Streptococcus* and *Lactobacillus* genera from fresh cow milk. These results verified the presence of active indigenous microorganisms, which could be further studied in other livestock products.

Although LAB have been found in several food products, investigations specifically targeting the different compartments of fresh duck eggs, including the inner shell, membrane, and egg contents, remain very limited. Previous studies mainly focused on fermented dairy products and processed egg products such as salted eggs. Saputra and Rahayu (2013) documented the presence of *Lactobacillus plantarum*, *Lactobacillus casei* subsp. *rhamnosus*, *Enterococcus gallinarum*, and *Pediococcus acidilactici* from salted eggs. Meanwhile, Surya and Nugroho (2025) reported isolating *Lactobacillus* and *Weissella* from egg white and egg yolk. The present study aimed to isolate and characterize indigenous LAB from fresh duck eggs to obtain locally adapted strains capable of colonizing the egg and demonstrating potential as natural biopreservation agents.

MATERIALS AND METHODS

Ethical approval

The present study was conducted in accordance with the approved ethical procedures of the Hasanuddin University, Makassar, Indonesia, ethics committee. Since no live animals were used, handled, or subjected to any experimental interventions, formal institutional ethical approval was not required.

Study design

Fresh duck egg samples were collected directly from local commercial farmers after production. The study focused exclusively on isolating and characterizing LAB from these eggs. After isolating LAB from the eggshell, membrane, and interior, the culture was purified and characterized using different methods, including Gram, endospore, acid-fast, and cell-shape staining, as well as biochemical assays such as catalase activity and carbohydrate fermentation tests. The final step involved assessing antimicrobial activity against *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*) by the agar well diffusion method. Isolations were performed in duplicate, and antimicrobial assays were repeated three times. The study was conducted at the Laboratory of Microbiology and Animal Health, Faculty of Animal Science, and the Laboratory of Microbiology, Faculty of Medicine, Hasanuddin University, Makassar, Indonesia.

Sample collection

Fresh duck eggs were collected directly from four farms in Sidrap Regency, South Sulawesi, Indonesia, during 2024, comprising two intensive and two extensive farming systems. The flock size at each location ranged from 500 to 1,000 ducks. A total of 20 fresh eggs (five per farm) were collected and labeled according to origin and management system. Sampling criteria included collection within 3 to 5 hours of laying, maintaining integrity without cracking, and remaining unwashed. Samples were transported to the laboratory in a cool box maintained at approximately 4°C and placed on sterile racks to prevent cross-contamination.

Isolation and preliminary selection of lactic acid bacteria

The LAB isolation procedure adhered to the modified methodology established by Sugata et al. (2024). Samples from each egg compartment, including the inner shell, membrane, and contents, were prepared under aseptic conditions and subsequently inoculated into De Man, Rogosa, and Sharpe (MRS) broth for pre-enrichment, using the swab method for the shell and membrane, and 1 g for the egg contents. After anaerobic incubation at 37°C for 24 hours, cultures were streaked onto MRS agar enriched with 1% calcium carbonate ($CaCO_3$). The addition of $CaCO_3$ served as a visual

indicator, with the formation of a clear zone around the colonies signifying acid production, a hallmark of LAB (Kurnia et al., 2020). Single colonies were identified by their morphological features, such as a white or yellowish color, a circular shape, a convex or flat elevation, and entire margins, as well as the presence of a clear zone. These colonies were purified through at least three rounds of streaking before being stored.

Microscopic identification and differential characterization of isolates

Cell morphology and Gram reaction were confirmed following the method of Sugata et al. (2019), with some modifications, using crystal violet, Lugol's iodine, 96% alcohol, and safranin. Gram-positive isolates with bacillus or coccus morphology under 1000x oil-immersion microscopy (Olympus, Japan) were identified as LAB candidates (Kurnia et al., 2020). Endospore staining verified that the organism did not form spores (Vasantha et al., 2021). Acid-fast staining was included as a supplementary step to exclude acid-fast genera such as *Mycobacterium*, although this test was not strictly essential for the identification of LAB (Tjayadi et al., 2017).

Catalase test

The catalase test was performed to determine whether the isolates produced catalase, following the method outlined by Hidayat et al. (2018). This test was critically important because LAB do not synthesize catalase and, as a result, were expected to yield a negative result. One loopful of the LAB isolate was smeared onto a clean glass slide, and 2-3 drops of 3% hydrogen peroxide (H_2O_2) were applied to the smear. The absence of bubble formation indicated a catalase-negative result, consistent with LAB identity.

Carbohydrate fermentation analysis

The metabolic pattern of isolates was determined through sugar fermentation tests covering glucose, lactose, sucrose, and mannitol, following the method of Liang et al. (2018). Phenol red carbohydrate broth (Oxoid, UK), supplemented with an inverted Durham tube, was used to detect gas production. The medium composition per liter of distilled water consisted of 10 g of peptone, 5 g of NaCl, 10 g of test sugar, 1 g of beef extract, and 180 mg of phenol red, serving as a pH indicator. After sterilization and inoculation, cultures were incubated microaerophilically at 37°C for 24 hours. Positive fermentation results were indicated by a color change from red to yellow (acid production), while gas bubbles in the Durham tube indicated heterofermentative LAB characteristics.

Antimicrobial activity testing

Antimicrobial potential was tested against *E. coli* and *S. aureus* using the agar well diffusion method (Rizal et al., 2019). Pathogen suspensions were adjusted to the 0.5 McFarland standard before inoculation onto Mueller-Hinton agar (Septian et al., 2020). Cell-free supernatant (CFS) was obtained by centrifugation at 6,000 × g for 15 minutes, followed by filtration through a sterile 0.2 µm membrane (Murtari et al., 2020). A volume of 100 µL of CFS was added to each well. The CFS was evaluated at its original acidic pH and at a neutral pH (6.5-7.0), adjusted with NaOH, to distinguish the activity of organic acids from that of bacteriocin-like inhibitory substances (BLIS). After incubation at 37°C for 24 hours, inhibition zone diameters (mm) were measured.

Data analysis

Data were analyzed descriptively using qualitative and quantitative approaches. Qualitative observations included colony morphology, Gram reaction, endospore staining, acid-fast staining, catalase activity, and carbohydrate fermentation profiles to characterize isolates. Quantitative data were obtained from inhibition zone diameter measurements against *E. coli* and *S. aureus* under original and neutral pH conditions. Tentative genus identification was conducted according to Bergey's Manual of Systematic Bacteriology (De Vos et al., 2009).

RESULTS AND DISCUSSION

Lactic acid bacteria isolation

Indigenous LAB isolates from different compartments of fresh duck eggs are presented in Table 1. The selected isolates demonstrated distinct acidogenic capabilities, as evidenced by clear zones around their colonies. The majority of isolates exhibited colony characteristics consistent with those of LAB, namely circular shape, white color, entire margin, convex/flat elevation, and glossy surface, with diameters of 0.1-0.4 mm (Table 1). However, selection analyses demonstrated that not all growing colonies produced a transparent zone. Isolates such as E1S4, E1M2, E1C2, and I1M1 did not create clear zones, suggesting they were non-acidogenic microbes that simply tolerated the MRSA medium.

Table 1. Colony morphology characteristics of isolates obtained from different compartments of fresh duck eggs in Sidrap Regency, South Sulawesi, Indonesia

Sample code	Shape	Color	Margin	Elevation	Surface	Size	Clear zone ($CaCO_3$)
E1S1	Circular	White	Entire	Convex	Glossy	0.2 mm	Present
E1S2	Circular	White	Entire	Convex	Glossy	0.2 mm	Present
E1S3	Circular	White	Entire	Convex	Glossy	0.2 mm	Present
E1S4	Circular	White	Entire	Convex	Glossy	0.2 mm	Absent
E1S5	Circular	White	Entire	Convex	Glossy	0.1 mm	Present
E1 M1	Circular	White	Entire	Convex	Glossy	0.2 mm	Present
E1M2	Circular	White	Entire	Convex	Non-glossy	0.2 mm	Absent
E1M3	Circular	White	Entire	Convex	Glossy	0.2 mm	Present
E1M4	Circular	White	Entire	Convex	Glossy	0.1 mm	Absent
E1M5	Circular	White	Entire	Convex	Glossy	0.3 mm	Present
E1C1	Circular	White	Entire	Convex	Glossy	0.2 mm	Present
E1C2	Circular	White	Entire	Convex	Glossy	0.2 mm	Absent
E1C3	Circular	White	Entire	Convex	Non-glossy	0.2 mm	Absent
E1C4	Circular	White	Entire	Convex	Glossy	0.4 mm	Absent
E1C5	Circular	White	Entire	Convex	Non-glossy	0.2 mm	Absent
E2S1	Circular	White	Entire	Convex	Glossy	0.2 mm	Absent
E2S2	Circular	White	Entire	Convex	Glossy	0.3 mm	Present
E2S3	Circular	White	Entire	Convex	Glossy	0.3 mm	Present
E2S4	Circular	White	Entire	Convex	Glossy	0.2 mm	Absent
E2S5	Circular	White	Entire	Convex	Glossy	0.2 mm	Present
E2M1	Circular	White	Entire	Convex	Glossy	0.4 mm	Absent
E2M2	Circular	White	Entire	Convex	Glossy	0.2 mm	Present
E2M3	Circular	White	Entire	Convex	Glossy	0.2 mm	Present
E2M4	Circular	White	Entire	Convex	Glossy	0.3 mm	Absent
E2M5	Circular	White	Entire	Convex	Glossy	0.3 mm	Absent
E2C1	Circular	White	Entire	Convex	Glossy	0.3 mm	Absent
E2C2	Circular	White	Entire	Convex	Glossy	0.2 mm	Present
E2C3	Circular	White	Entire	Convex	Glossy	0.2 mm	Present
E2C4	Circular	White	Entire	Convex	Glossy	0.2 mm	Absent
E2C5	Circular	White	Entire	Convex	Glossy	0.2 mm	Absent
I1S1	Circular	White	Entire	Convex	Glossy	0.3 mm	Present
I1S2	Circular	White	Entire	Convex	Glossy	0.2 mm	Absent
I1S3	Circular	White	Entire	Convex	Glossy	0.2 mm	Present
I1S4	Circular	White	Entire	Convex	Glossy	0.3 mm	Present
I1S5	Circular	White	Entire	Convex	Non-glossy	0.2 mm	Absent
I1M1	Circular	White	Entire	Convex	Glossy	0.4 mm	Absent
I1M2	Circular	White	Entire	Convex	Glossy	0.2 mm	Present
I1M3	Circular	White	Entire	Convex	Glossy	0.2 mm	Present
I1M4	Circular	White	Entire	Convex	Glossy	0.3 mm	Absent
I1M5	Circular	White	Entire	Convex	Non-glossy	0.3 mm	Absent
I1C1	Circular	White	Entire	Convex	Glossy	0.2 mm	Absent
I1C2	Circular	White	Entire	Convex	Glossy	0.2 mm	Present
I1C3	Circular	White	Entire	Convex	Glossy	0.2 mm	Absent
I1C4	Circular	White	Entire	Convex	Glossy	0.2 mm	Present
I1C5	Circular	White	Entire	Convex	Non-glossy	0.2 mm	Absent
I2S1	Circular	White	Entire	Convex	Glossy	0.2 mm	Present
I2S2	Circular	White	Entire	Convex	Glossy	0.3 mm	Present
I2S3	Circular	White	Entire	Convex	Non-glossy	0.2 mm	Absent
I2S4	Circular	White	Entire	Convex	Glossy	0.3 mm	Absent
I2S5	Circular	White	Entire	Convex	Glossy	0.2 mm	Present
I2M1	Circular	White	Entire	Convex	Glossy	0.2 mm	Present
I2M2	Circular	White	Entire	Convex	Glossy	0.3 mm	Absent
I2M3	Circular	White	Entire	Convex	Glossy	0.2 mm	Present
I2M4	Circular	White	Entire	Convex	Non-glossy	0.2 mm	Absent
I2M5	Circular	White	Entire	Convex	Glossy	0.4 mm	Present
I2C1	Circular	White	Entire	Convex	Glossy	0.3 mm	Present
I2C2	Circular	White	Entire	Convex	Glossy	0.3 mm	Present
I2C3	Circular	White	Entire	Convex	Glossy	0.2 mm	Absent
I2C4	Circular	White	Entire	Convex	Glossy	0.2 mm	Absent
I2C5	Circular	White	Entire	Convex	Glossy	0.2 mm	Present

Source of data: Microbiology and Animal Health Laboratory, Faculty of Animal Science, Hasanuddin University, Indonesia, 2026

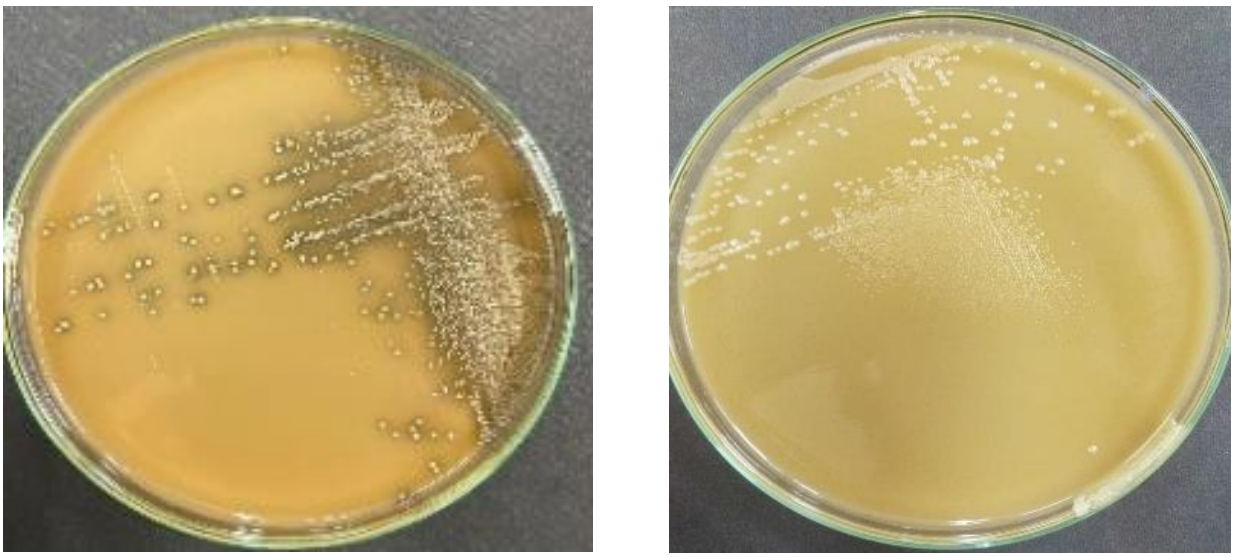
The majority of isolates exhibited colony characteristics consistent with those of LAB, namely a circular shape, white color, entire margin, convex/flat elevation, and glossy surface, with diameters of 0.1-0.4 mm (Table 1; Badis et al., 2022). However, selection analyses demonstrated that not all growing colonies formed a transparent zone. Isolates such

as E1S4, E1M2, E1C2, and I1M1 did not form clear zones, indicating that they were non-acidogenic microbes that merely tolerated the MRSA medium.

The distribution of suspected transparent zone-forming LAB exhibited a pattern correlated with management practices and ecological compartments in duck eggs. On extensive farms (E1 and E2), acidogenic isolates were randomly distributed across all egg compartments. This finding aligned with that of Wierup (2017), who reported that unconfined poultry environments facilitate intensive interactions between chickens and a diverse environmental microflora in rice-field or marsh ecosystems. On intensive farms (I1 and I2), transparent zone-forming isolates were more consistently identified in the outer compartments (eggshell and membrane). This pattern was consistent with the findings of Stepien-Pysniak et al. (2021), suggesting that regulated sanitation and biosecure feeding management limited arbitrary microbial infiltration while promoting the proliferation of specific surface-associated LAB groups.

The characteristics of each egg compartment serve as a primary differentiator. Consistent with the findings of Saputra and Rahayu (2013), who isolated acidogenic LAB from salted duck eggs, the present results indicated that the shell (S) and egg membrane (M) harbored significant acidogenic strains, such as E1S1, E1S5, E2M2, I1S4, and I2M1. This finding confirmed that the outer compartments functioned as a critical biological defense barrier. Organic acids produced by LAB on these surfaces lowered the local pH, thereby inhibiting the colonization of fecal- or soil-borne pathogenic bacteria prior to shell penetration (Stepien-Pysniak et al., 2021).

The successful isolation of bacterial colonies from the egg contents of E1C1, E2C2, E2C3, I1C2, I1C4, and I2C5 samples was particularly notable given the interior's strong antimicrobial environment. Albumen contains lysozyme, ovo transferrin, and avidin (Guyot et al., 2016) and maintains a highly alkaline pH of 9.0 to 9.6 (Wellman-Labadie et al., 2010). Microbes primarily penetrate via transovarian transmission during egg formation or horizontally through eggshell pores compromised by environmental moisture (Baron et al., 2016). A comparative visual representation of colony morphology between acidogenic and non-acidogenic isolates is illustrated in Figure 1.



(a) (b)

Figure 1. Colony morphology of isolates derived from fresh duck eggs in Sidrap Regency, South Sulawesi, Indonesia on De Man, Rogosa, and Sharpe agar supplemented with 1% $CaCO_3$. **a:** Sample E1S1 showing a wide transparent zone, **b:** Sample E1S4 showing no transparent zone.

Microscopic characteristics

Isolates demonstrating positive acidogenic activity underwent further characterization through Gram staining, endospore staining, acid-fast staining, and catalase testing to confirm their classification as LAB and to exclude non-LAB microorganisms or yeasts capable of dissolving $CaCO_3$ (Table 2; Badis et al., 2022). The cellular characteristics predominantly exhibited Gram-positive (+) cocci arranged in chains or pairs, as well as bacillus forms (Table 2). Isolates exhibiting Gram-negative (−) or catalase-positive (+) traits, including E1S5, E1M3, and E2M3, were discarded as environmental contaminants that merely tolerated the acidic medium. This rigorous elimination approach was consistent with the findings of Papadimitriou et al. (2015), who emphasized that catalase positivity and Gram-negativity are indicative of non-LAB taxa. Consequently, isolates E1S1, E1S3, E1M1, E2S2, E2M2, I1M2, and I2M5 were selected for subsequent analyses. Lactic acid bacteria status was confirmed by negative results for endospore staining, acid-fast staining, and catalase tests. In contrast to common egg-contaminating spore-formers such as *Bacillus* species (Liang et

al., 2018; Kurnia et al., 2020), the present isolated indigenous LAB were non-spore-forming. Furthermore, the catalase-negative property reflects an aerotolerant anaerobic metabolic pathway, a fundamental functional hallmark of true LAB.

Table 2. Microscopic characteristics, staining reactions, and biochemical results of lactic acid bacteria isolates derived from fresh duck eggs in Sidrap Regency, South Sulawesi, Indonesia

Isolate code	Gram	Endospore	Acid-fast	Cell shape	Cell arrangement	Catalase
E1S1	(+)	(−)	(−)	Coccus	Chain	(−)
E1S2	(+)	(−)	(−)	Coccus	Chain	(+)
E1S3	(+)	(−)	(−)	Coccus	Paired	(−)
E1S5	(−)	(−)	(−)	Coccus	Single	(+)
E1M1	(+)	(−)	(−)	Bacillus	Single	(−)
E1M3	(−)	(−)	(−)	Coccus	Single	(+)
E1M5	(−)	(−)	(−)	Coccus	Chain	(+)
E2C1	(−)	(−)	(−)	Coccus	Chain	(+)
E2S2	(+)	(−)	(−)	Bacillus	Single	(−)
E2S3	(+)	(−)	(−)	Bacillus	Chain	(+)
E2S5	(+)	(−)	(−)	Coccus	Chain	(+)
E2M2	(+)	(−)	(−)	Coccus	Paired	(−)
E2M3	(−)	(−)	(−)	Coccus	Chain	(+)
E2C2	(−)	(−)	(−)	Coccus	Single	(+)
E2C3	(−)	(−)	(−)	Coccus	Single	(+)
I1S1	(−)	(−)	(−)	Coccus	Chain	(+)
I1S3	(+)	(−)	(−)	Coccus	Single	(+)
I1S4	(+)	(−)	(−)	Coccus	Chain	(+)
I1M2	(+)	(−)	(−)	Coccus	Single	(−)
I1M3	(+)	(−)	(−)	Coccus	Single	(+)
I1C2	(+)	(−)	(−)	Coccus	Chain	(+)
I1C4	(+)	(−)	(−)	Bacillus	Single	(+)
I2S1	(+)	(−)	(−)	Coccus	Chain	(+)
I2S2	(+)	(−)	(−)	Coccus	Chain	(+)
I2S5	(+)	(−)	(−)	Bacillus	Single	(+)
I2M1	(+)	(−)	(−)	Coccus	Single	(+)
I2M2	(+)	(−)	(−)	Coccus	Single	(+)
I2M5	(+)	(−)	(−)	Coccus	Paired	(−)
I2C1	(+)	(−)	(−)	Coccus	Chain	(+)
I2C2	(−)	(−)	(−)	Coccus	Chain	(+)
I2C5	(−)	(−)	(−)	Coccus	Chain	(+)

Source of data: Microbiology and Animal Health Laboratory, Faculty of Animal Science, Hasanuddin University, Indonesia, 2026. E1: Extensive farm 1, E2: Extensive farm 2, I1: Intensive farm 1, I2: Intensive farm 2, S: Eggshell, M: Egg membrane, C: Egg contents, (+): Positive reaction, (−): Negative reaction

Biochemical characteristics and carbohydrate fermentation profiles

The metabolic capacity of the seven selected LAB isolates was evaluated through sugar fermentation analyses (Table 3). All seven selected isolates consistently fermented glucose, exhibiting a homofermentative pattern, as evidenced by a color change from red to yellow, with no CO_2 production (Table 3).

From a physiological perspective, this metabolic pattern confirmed that the isolates effectively convert sugars into lactic acid predominantly via the Embden-Meyerhof-Parnas (EMP) pathway, without generating gaseous byproducts that could potentially compromise or modify the texture of food products (Murtari et al., 2020; Badis et al., 2022). Isolates E2S2, E2M2, and I1M2 fermented glucose, lactose, fructose, and mannitol, while E1S1, E1S3, E1M1, and I2M5 fermented only glucose. The observed differences in sugar fermentation confirmed the diversity of indigenous LAB species, each with distinct competitive advantages in adapting to the fluctuating nutritional environment of the duck egg matrix (Saputra and Rahayu, 2013).

Table 3. Carbohydrate fermentation profiles and metabolic patterns of selected lactic acid bacteria isolates derived from fresh duck eggs in Sidrap Regency, South Sulawesi, Indonesia

Isolate code	Glucose	Lactose	Fructose	Mannitol	Fermentation pattern
E1S1	(+)	(−)	(−)	(−)	Homofermentative
E1S3	(+)	(−)	(−)	(−)	Homofermentative
E1M1	(+)	(−)	(−)	(−)	Homofermentative
E2S2	(+)	(+)	(+)	(+)	Homofermentative
E2M2	(+)	(+)	(+)	(+)	Homofermentative
I1M2	(+)	(+)	(+)	(+)	Homofermentative
I2M5	(+)	(−)	(−)	(−)	Homofermentative

Source of data: Microbiology Laboratory, Faculty of Medicine, Hasanuddin University, Indonesia, 2026. (+): Able to ferment, medium color changed to yellow, (−): Unable to ferment, E1: Extensive farm 1, E2: Extensive farm 2, I1: Intensive farm 1, I2: Intensive farm 2, S: Eggshell, M: Egg membrane

Genus identification of lactic acid bacteria

Cell morphology, metabolic profiles, and other phenotypic data were compiled and compared with Bergey's Manual of Determinative Bacteriology to indicate tentative taxonomic classifications of the selected isolates (Table 4).

Indigenous LAB isolates were classified into two genera, including *Lactobacillus* (E1M1 and E2S2) and *Pediococcus* (E1S1, E1S3, E2M2, I1M2, and I2M5). The prevalence of *Lactobacillus* and *Pediococcus* in fresh duck eggs aligned with the findings of Saputra and Rahayu (2013), thereby confirming that these two genera constituted stable and dominant indigenous microflora in avian egg environments. Furthermore, the capacity of E2S2, E2M2, and I1M2 isolates to ferment mannitol corresponded with the well-established taxonomic characteristics of *Pediococcus pentosaceus* or *Pediococcus acidilactici* (Garvie, 1986).

The presence of *Lactobacillus* and *Pediococcus* extending into the eggshell, membrane, and albumen was associated with physical penetration through shell pores that ranged from 10 to 65 µm in diameter. These pores are significantly larger than LAB cells, which measure between 0.5 and 1.2 µm for *Pediococcus* and 1 to 10 µm for *Lactobacillus*. The present study demonstrates that *Lactobacillus* strains could readily colonize the proteinaceous egg membrane. This finding was consistent with Saputra and Rahayu (2013), who emphasized the strong affinity of *Lactobacillus* cell walls for adhering to keratin fiber networks.

Pediococcus and *Lactobacillus* possess cell wall modifications, including high teichoic acid content and exocellular polysaccharides (Papadimitriou et al., 2015). In accordance with Wang et al. (2021), peptidoglycan modifications in *Lactobacillus* and *Pediococcus* enhance cell wall resistance against lysozyme-mediated degradation compared to other Gram-positive bacteria. Furthermore, their homofermentative EMP pathway facilitates rapid accumulation of lactic acid without the production of gases (Siren et al., 2023), thereby confirming that fresh duck eggs served as a natural reservoir for resilient biopreservative LAB strains.

Table 4. Genus identification of indigenous lactic acid bacteria isolates derived from fresh duck eggs in Sidrap Regency, South Sulawesi, Indonesia

Isolate code	Cell shape	Gram	Catalase	Fermentation pattern	Tentative genus
E1S1	Coccus	(+)	(−)	Homofermentative	*Pediococcus*
E1S3	Coccus	(+)	(−)	Homofermentative	*Pediococcus*
E1M1	Bacillus	(+)	(−)	Homofermentative	*Lactobacillus*
E2S2	Bacillus	(+)	(−)	Homofermentative	*Lactobacillus*
E2M2	Coccus	(+)	(−)	Homofermentative	*Pediococcus*
I1M2	Coccus	(+)	(−)	Homofermentative	*Pediococcus*
I2M5	Coccus	(+)	(−)	Homofermentative	*Pediococcus*

Source of data: Microbiology and Animal Health Laboratory, Faculty of Animal Science, Hasanuddin University, Indonesia, 2026. E1: Extensive farm 1, E2: Extensive farm 2, I1: Intensive farm 1, I2: Intensive farm 2, S: Eggshell, M: Egg membrane

Antimicrobial activity against pathogenic bacteria

Antimicrobial activity testing evaluated the ability of CFS derived from LAB isolates to inhibit the growth of *E. coli* and *S. aureus*. Results comparing inhibition zone diameters at original and neutral pH are presented in Table 5.

Table 5. Inhibition zone diameters (mm) of lactic acid bacteria isolates derived from fresh duck eggs in Sidrap Regency, South Sulawesi, Indonesia against *Escherichia coli* and *Staphylococcus aureus*

Isolate code	*Escherichia coli* (Original pH)	*Escherichia coli* (Neutral pH)	*Staphylococcus aureus* (Original pH)	*Staphylococcus aureus* (Neutral pH)
E1S1	16.0	15.5	16.0	13.0
E1S3	27.5	25.5	17.0	13.0
E1M1	21.5	15.0	19.0	13.0
E2S2	16.5	15.0	15.0	13.0
E2M2	17.0	14.5	15.5	13.0
I1M2	21.0	17.0	16.5	13.0
I2M5	19.0	14.0	15.0	13.0

Source of data: Microbiology and Animal Health Laboratory, Faculty of Animal Science, Hasanuddin University, Indonesia, 2026. The value of 13.0 mm was interpreted as the well diameter, indicating the absence of an inhibition zone against the indicator bacteria.

All seven LAB isolates demonstrated strong antagonistic activity under original acidic pH conditions (Table 5). Isolate E1S3 produced the highest inhibition zone against *E. coli* (27.5 mm), driven primarily by organic acid accumulation. Under original acidic conditions, all seven LAB isolates exhibited strong antagonistic activity against both pathogens (Table 5). This strong inhibition at the original pH was consistent with findings of Septian et al. (2020) and Badis et al. (2022), demonstrating that organic acid accumulation, primarily lactic acid, served as the primary mechanism for LAB strains to suppress pathogens. Isolate E1S3 produced the largest zone against *E. coli* (27.5 mm), as visualized in Figure 2a.

Upon pH neutralization, inhibitory activity against *S. aureus* completely disappeared across all isolates, as indicated by a 13.0 mm well diameter. This finding contrasted with some broad-spectrum bacteriocin studies, but aligned with the findings of Septian et al. (2020), suggesting that the anti-*S. aureus* activity observed in these specific isolates was exclusively dependent on acidity. Conversely, under neutral pH conditions, significant inhibition against Gram-negative *E. coli* was observed in several isolates, notably E1S3 (25.5 mm) and I1M2 (17.0 mm), as depicted in Figure 2. This residual inhibition at neutral pH was consistent with the findings of Septian et al. (2020), indicating that non-acid antimicrobial metabolites such as BLIS maintain activity regardless of pH. The different sensitivity of *E. coli* and *S. aureus* at neutral pH implied that the BLIS or antimicrobial peptides produced have target-specific actions against the outer membranes of Gram-negative bacteria.

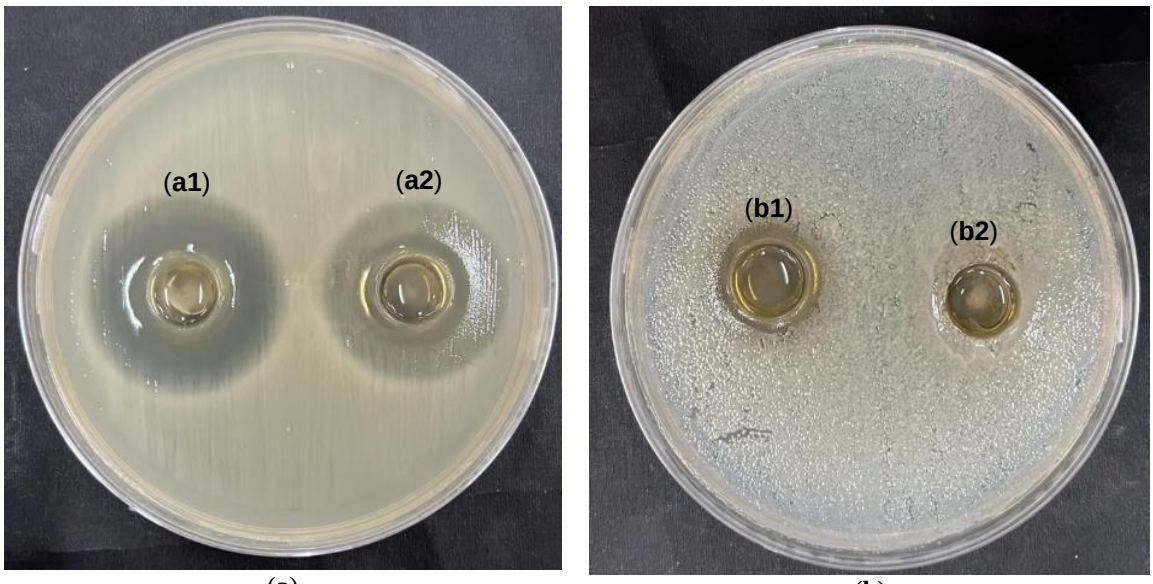


Figure 2. Visualization of antimicrobial activity of cell-free supernatants from lactic acid bacteria isolates. **a**: Isolate E1S3 in comparison to *E. coli*., **b**: Isolate E1S1 in comparison to *S. aureus*, **a1, b1**: Original pH, **a2, b2**: Neutral pH

CONCLUSION

The present study successfully isolated seven indigenous LAB isolates obtained from different compartments of fresh duck eggs in Sidrap Regency, South Sulawesi, Indonesia, each exhibiting strong acidogenic capabilities. These isolates

consisted of five extensive farms, as well as eggshell and egg membrane samples, and two isolates obtained from intensive farms, specifically from the egg membrane. All isolates were Gram-positive, catalase-negative, non-spore-forming, and homofermentative. Tentative phenotypic identification classified the isolates into the genera *Lactobacillus* and *Pediococcus*. At the original pH, all isolates demonstrated strong antimicrobial activity against *E. coli* and *S. aureus*; however, at neutral pH, inhibition persisted only against *E. coli*, thereby confirming BLIS production. Further purification and characterization are needed to verify the nature and spectrum of the putative BLIS produced by these isolates. Conversely, inhibition against *S. aureus* was exclusively attributable to organic acids. The current findings indicated that fresh duck eggs are a promising source of functional LAB for natural biopreservation applications. A limitation of the present study was that identification was based solely on phenotypic characteristics; molecular identification (*16S rRNA* gene sequencing) is recommended in future studies to confirm species-level classification and genetic diversity of the isolates.

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Authors' contributions

Muhammad Irham contributed to data collection, laboratory investigations, and manuscript preparation. Wahniyathi Hatta contributed to study design, manuscript drafting, and manuscript revision. Fatma Maruddin contributed as principal investigator, study design, data interpretation, and final manuscript approval. All authors have read and approved the final edition of the manuscript.

Availability of data and materials

Samples and data are available upon reasonable request from the corresponding author.

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 checked by all the authors. The authors used Gemini (Google) to improve the grammar and clarity of the text. After that, the authors revised the AI-edited text, reviewed it, and took full responsibility for using AI. The authors conducted all intellectual investigations, data collection, analysis, and scientific conclusions without using AI tools. No AI tools were used for data generation or scientific interpretation. All authors are solely responsible for the accuracy, originality, and integrity of the final content before submission, during the revisions, and final publication of the article.

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Prevalence, Risk Factors, and Clinicopathological Characterization of Ocular and Renal Complications of Diabetes Mellitus in Dogs

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ABSTRACT

Canine diabetes mellitus (DM) is an endocrinopathy in dogs, which can lead to diabetic complications in the patient's eyes and kidneys. The present study aimed to describe the prevalence, risk factors, hematologic and serum biochemical findings, and incidence of ocular and renal complications of DM in dogs at veterinary clinics in Ho Chi Minh City, Vietnam. Dogs brought to veterinary clinics with clinical signs of polyuria, polydipsia, polyphagia, and weight loss were examined for DM, including hematology, serum biochemical, and urine analyses, from January to December 2025. Dogs confirmed with DM had hyperglycemia after at least 8 hours of fasting and concomitant glycosuria. The incidence of ocular and renal complications of diabetic dogs was recorded by using slit-lamp and urine parameters (elevated serum creatinine, blood urea nitrogen, microalbuminuria, and urine protein-to-creatinine ratio), respectively. Twenty canine DM cases were identified, accounting for 1.43% of the total dogs brought to veterinary clinics during the study. The mean age of diabetic dogs was 6.35 ± 3.65 years. The highest prevalence of DM was in the 3-8 years old group, the overweight group, and the male-neutered group among male dogs; however, female-entire dogs had the highest overall prevalence (3.36%). Diabetic dogs demonstrated elevated mean corpuscular volume, mean corpuscular hemoglobin, alanine aminotransferase, aspartate transaminase, alkaline phosphatase, creatinine, blood urea nitrogen, and cholesterol, and decreased Lymphocytes, red blood cells, hemoglobin, and hematocrit–in hematology and serum biochemical analyses. Urinalysis results of diabetic dogs demonstrated an increase in creatinine, microalbuminuria, and proteinuria, which could be used to diagnose diabetic renal complications in dogs. Cataract formation was the only ocular complication recorded in the present study, which was recorded in 5 diabetic dogs, and all diagnosed dogs had cataracts in both eyes. Renal failure was observed in 5 diabetic dogs, including 4 dogs with stage 4 renal failure and 1 dog with stage 3 renal failure. No dogs had both ocular and renal complications at the same time. In the current study, dogs that were older, overweight, or male-neutered had higher DM prevalence, with cataracts and stage 4 renal failure being the most common diabetic complications.

Keywords: Diabetes mellitus, Dog, Hematology, Ocular complication, Renal complication

INTRODUCTION

Canine diabetes mellitus (DM) is a common endocrine disease in dogs, which is characterized by persistent hyperglycemia resulting from insulin deficiency in production, action, or both (Ettinger et al., 2017). Depending on the mechanism causing hyperglycemia, canine DM is divided into insulin-dependent and non-insulin-dependent (Vaitaitis et al., 2024). Insulin-dependent diabetes (type I DM) is characterized by the destruction of pancreatic beta cells by immune-mediated mechanisms, usually leading to insulin deficiency (Ettinger et al., 2017; Vaitaitis et al., 2024). Most diabetic dogs are thought to have insulin-dependent DM (Feldman et al., 2015). In previous studies in North America and Europe, the prevalence of canine DM was approximately 0.32% to 1.33% of total dogs brought to veterinary clinics (Guptill et al., 2003; Fracassi et al., 2004; Mattin et al., 2014). Risk factors associated with canine DM were breed, sex, neuter status, age, and body condition (Mattin et al., 2014). Breeds at high risk for DM included Bichon Frise, Australian Terrier, Siberian Husky, Standard Schnauzer, Samoyed, Poodle, and Yorkshire Terrier (Guptill et al., 2003; Fracassi et al., 2004). These breeds had genetic factors that increased the risk of developing DM (Catchpole et al., 2013). Gender was reported to be one of the risk factors for DM in previous studies (Fall et al., 2007). Furthermore, neuter status was reported to be a risk factor for DM in female dogs, as progesterone-induced diabetes occurs during pregnancy (Fall et al., 2007). Previous studies showed that age was one of the risk factors for DM, as DM was most commonly diagnosed in dogs above 10 years old (Guptill et al., 2003; Fall et al., 2007). Overweight was also associated with DM in dogs (Mattin et al., 2014).

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New management strategies and therapies for canine DM might alter the outcome of diabetic patients and increase their lifespan (Mattin et al., 2014). As a result, ocular and renal complications of DM in dogs have also become more common (Miller and Brines, 2018; Priyanka et al., 2018). Cataract formation is one of the most common diabetic complications in dogs (Miller and Brines, 2018). Diabetic cataract, due to the accumulation of sorbitol and galactitol in the lens, is irreversible, which can eventually lead to blindness (Cantero et al., 2023). Diabetic nephropathy is a chronic complication that takes years to progress to renal failure, which (Bloom and Rand, 2013). Consequently, diabetic renal complications were rarely reported in dogs with DM. However, there have been only a few studies on the prevalence of canine DM, as well as ocular and renal complications of diabetes in dogs in Vietnam. Therefore, the present study aimed to investigate the prevalence and risk factors of DM in dogs and the incidence of ocular and renal complications in diabetic dogs in Ho Chi Minh City, Vietnam.

MATERIALS AND METHODS

Ethical approval

Ethics approval was provided by the Animal Ethics Committee, Nong Lam University, Ho Chi Minh City, Vietnam.

Hematology, serum biochemistry, and urine analyses

A total of 1,390 dogs were examined for DM from January to December 2025 at two veterinary clinics in Ho Chi Minh City, Vietnam. Dogs with clinical signs of canine DM, such as polyuria, polydipsia, polyphagia, and weight loss, were examined for DM by veterinarians. A total of 20 blood samples, with 2 mL of blood for each sample, were collected from the cephalic vein and analyzed by an automated biochemistry analyzer (Pointcare V3, MNChip, China) for serum biochemistry analysis and an automated hematology analyzer (DH36Vet, Dymind, China) for hematology analysis. Approximately 5-10 mL of urine was collected and analyzed by urine test strips (Vet11AC, DFI, Korea) and a urine reader (UriDoctor Vet, DFI, Korea). Dogs with at least 8 hours of fasting before blood sampling, confirmed with DM, had persistent fasting hyperglycemia (blood glucose above 6.6 mmol/L) and concomitant glycosuria (Ettinger et al., 2017). Dogs with fasting status less than 8 hours at the time of blood collection were excluded from the study. The recorded data included breed group (domestic, foreign), sex (male, female), neuter status (neutered, entire), age categories (1-3 years, 3-8 years, over 8 years), and body condition, which was determined using the Body Condition Score by the World Small Animal Veterinary Association (WSAVA, 2025).

Ocular and renal complications

Dogs previously diagnosed with DM or newly diagnosed with DM in the study were screened for cataract formation. A slit lamp (PSL One Portable Slit Lamp, Keeler, USA) was used by veterinarians to confirm and determine the stages of cataracts (Cantero et al., 2023). Cataracts were divided into five stages, including initial, immature, mature, hypermature, and intumescent. Diabetic dogs were also tested for renal complications. Diabetic dogs confirmed with renal complications had elevated serum creatinine (> 176.8 µmol/L), blood urea nitrogen (> 10.4 mmol/L), microalbuminuria (or proteinuria), and elevated urine protein-to-creatinine ratio (> 0.5; Bloom and Rand, 2013). The severity of kidney disease was determined by International Renal Interest Society (IRIS) references (IRIS, 2023).

Statistical analysis

Data were collected and analyzed with Minitab statistical software (Minitab 17, Minitab LLC, USA), with statistical significance determined by a p-value less than 0.05. Multiple regression was used to assess the risk factors for diabetes in dogs by breed, sex, and neuter status. Chi-square and Fisher's tests were used to compare the prevalence of diabetes across age groups, body condition, and neuter status within the same sex.

RESULTS AND DISCUSSION

Prevalence of diabetes mellitus

In a total of 1,390 dogs brought to veterinary clinics in this study, there were 20 dogs diagnosed with DM, which accounted for 1.43% of the total cases. The prevalence of DM in dogs was influenced by sample size, population of each dog breed, geographical location, duration of the study, and the care habits of the owners. Previous studies showed that the prevalence of DM in dogs was from 0.34% to 1.33% of total dogs brought to veterinary hospitals (Guptill et al., 2003; Fracassi et al., 2004; Mattin et al., 2014). Diabetes mellitus in dogs can be discovered incidentally when the patients are brought to the veterinary clinics due to other diseases. In the study by Mattin et al. (2014), approximately 47% of DM dogs were incidentally diagnosed with diabetes during veterinary hospital visits for other diseases.

The prevalence of DM in a domestic dog breed (Phu Quoc Ridgeback) and foreign dog breeds (one French Bull, one Golden Retriever, two Labrador Retrievers, two Pugs, two Alaskas, three Poodles) was 1.37% (9/658) and 1.50% (11/732), respectively (Table 1). The results of the current study showed that dog breed did not significantly increase the risk of DM in dogs ($p > 0.05$). However, according to previous studies, dog breed was one of the risk factors for canine diabetes (Catchpole et al., 2013; Feldman et al., 2015). The breeds with a high possibility of DM were Samoyed, Poodle, and Cairn Terrier (Ettinger et al., 2017). Furthermore, Irish and English Setters were proven to have a 2-4 times higher risk of developing diabetes compared to mixed breeds (Fracassi et al., 2004). Differences in the prevalence of diabetes in dog breeds are also influenced by the breed's geographical distribution (Fracassi et al., 2004). Previous studies on diabetes-related genes in dogs have been conducted on certain breeds (Catchpole et al., 2013; Short et al., 2014). However, the number of experimental samples in diabetes-related genetic studies was still relatively low, and the genes selected for veterinary studies were primarily based on human studies (Denyer et al., 2021).

Table 1. Breed, sex, and neuter status of dogs with diabetes mellitus presented to veterinary clinics in Ho Chi Minh City, Vietnam, from January to December 2025

Parameters	Surveyed dogs	Diabetic dogs	Prevalence	P-value
Breed				
Domestic	658	9	1.37[a]	0.833
Foreign	732	11	1.50[a]	
Sex				
Male	774	8	1.03	0.155
Female	616	12	1.95	
Neuter status				
Neutered	588	14	2.38[a]	< 0.05
Entire	802	6	0.75[b]	

[a,b] Mean different superscript letters indicate a significant difference among groups in the same column ($p < 0.05$)

The prevalence of DM in female dogs recorded at the clinic during the survey period was 1.95% (12/616), and in male dogs it was 1.03% (8/774; Table 1). The prevalence of diabetes in neutered dogs was 2.38% (14/588), higher than the prevalence of diabetes in entire dogs, which was 0.77% (6/802; $p < 0.05$). In the current study, the result showed that the prevalence of DM in male dogs (1.05%) was lower than in female dogs (1.95%), but statistical analysis showed no significant difference. The study by Mattin et al. (2014) also showed that sex was not a risk factor for DM in dogs. However, a previous study by Fall et al. (2007) indicated that female dogs were more susceptible to DM than male dogs. In male dogs of the present study, the prevalence of diabetic neutered dogs was 2.32%, which was higher than diabetic male-entire dogs (0.21%; Table 2; $p < 0.05$). Sex hormones are thought to play a significant role in the risk of diabetes in dogs; for instance, male sex hormones help reduce the risk, while female sex hormones increase the risk of DM (Feldman et al., 2015). Hormonal changes in female dogs during pregnancy were considered to be the main cause of increased DM risk in female dogs, while male sex hormones were thought to be the reason why male dogs were less obese than female dogs, which resulted in a lower incidence of DM in male dogs (Mattin et al., 2014). The trend of spaying female dogs to avoid unwanted pregnancies has changed over time, which might also influence the sex-associated incidence of diabetes in dogs (Fall et al., 2007). Therefore, neuter status may affect the prevalence of DM in dogs.

The mean age of diabetic dogs was 6.35 ± 3.65 years. Dogs aged 3-8 years had the highest percentage of DM (1.66%), followed by the above 8 years old group (1.66%), and the 1-3 years old group (0.29%; Table 2; $p > 0.05$). Geriatric dogs are more likely to develop diseases such as DM. In a study by Mattin et al. (2014), dogs in the 8-11 years old group accounted for about 40% of diabetic dogs, followed by the 11-13 years old group (27.3%), 3-8 years old group (19.6%), and the over 13 years old group (12.4%). Another study by Guptill et al. (2003) also indicated that dogs aged 8-15 years had the highest percentage of diabetic dogs, which was approximately 75% of diabetic dogs. The mean age of diabetic dogs in the current study was relatively low (about 6 years old), which indicated a lack of awareness of nutrition and canine DM control in the area.

The overweight group had a diabetic prevalence of 2.60% (13/500), higher than the under ideal (1/412) and ideal (6/478) groups ($p < 0.05$). All female dogs were confirmed not to be pregnant at the time of survey. Neutered male dogs had a prevalence of diabetes of 2.32%, higher than entire male dogs (0.21%; $p < 0.05$). The incidence of diabetes in neutered female dogs was 1.28%, and the incidence of diabetes in entire female dogs was 3.36%. Dogs with DM can have either ideal, under ideal, or over ideal body condition (Ettinger et al., 2017). Untreated DM in dogs over a long

period may lead to weight loss; however, severe loss of muscle mass is rare unless there is another underlying disease, such as exocrine pancreatic insufficiency (Feldman et al., 2015). However, obesity is considered one of the risk factors of DM in dogs (Feldman et al., 2015). A study by Mattin et al. (2014) indicated that overweight dogs were shown to have a higher risk of developing the disease compared to non-overweight dogs. Approximately 15% of diabetic dogs were obese, according to a study by Thao et al. (2015). Dogs diagnosed with diabetes are usually given regular check-ups, and their body weight can be appropriately controlled.

Table 2. Age, body condition, and sex-neuter status of dogs with diabetes mellitus presented to the veterinary clinics in Ho Chi Minh City, Vietnam from January to December 2025

Parameters	Surveyed dogs	Diabetic dogs	Prevalence	P-value
Age group				
1-3 years old	350	1	0.29[a]	
3-8 years old	678	13	1.92[b]	0.105
Above 8 years old	362	6	1.66[b]	
Body conditions				
Under ideal	412	1	0.24[a]	
Ideal	478	6	1.26[ab]	0.011
Overweight	500	13	2.60[b]	
Sex-neuter status				
Male-neuter	302	7	2.32[a]	0.007
Male-entire	472	1	0.21[b]	
Female-neuter	286	7	2.45	0.147
Female-entire	330	5	1.52	

[a,b] Mean different superscript letters indicate a significant difference among groups in the same column ($p < 0.05$)

Hematology, serum biochemistry, and urine results

In the current study, most diabetic dogs had normal hematology results within the reference range. The average results for mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH) were higher than the reference range, while lymphocyte percentage (Lym%) was below the reference range (Table 3). There were 15 diabetic dogs with elevated MCV, and 13 dogs with elevated MCH. The percentage of diabetic dogs with decreased red blood cells (RBC), hematocrit (HCT), and hemoglobin (HGB) was 30%, 40%, and 40%, respectively. Hematological abnormalities could affect other tissues and organs, which could lead to complications such as cataracts and kidney disease in diabetic dogs (Mansour et al., 2023). However, there are not many studies on the relationship between hematological abnormalities and DM in dogs. In the present study, diabetic dogs had Lym, RBC, HGB, HCT, MCV, and MCHC outside the reference ranges reported by Vaden (2009). High blood glucose levels lead to the non-enzymatic binding of glucose to red blood cell protein (HbA1c), causing red blood cells to bind more tightly to oxygen, making it difficult to release oxygen to tissues and resulting in localized tissue hypoxia (Mansour et al., 2023). In cases of inflammation or pancreatitis, neutrophil counts could be elevated (Peter, 2017). A study conducted by Slead et al. (2022) on hematological analysis and blood smears in diabetic dogs showed that RBC, HGB, and HCT counts were lower, and WBC counts in diabetic dogs were higher than the reference range. Furthermore, diabetic ketoacidosis is often found in diabetic dogs with an overproduction of codocytes (Slead et al., 2022).

In the present study, the average values of aspartate aminotransferase (AST), alkaline phosphatase (ALP), creatinine (Crea), blood urea nitrogen (BUN), and cholesterol (CHOL) were higher than the reference range (Table 3). The results showed that approximately 25% of diabetic dogs might have liver diseases, with elevated AST and ALP. In the present study, there were 5 diabetic dogs that showed elevated Crea and BUN, suggesting that diabetes in dogs ~~may~~ be associated with urinary diseases. Elevated CHOL was also observed in 5 dogs, which accounted for 25% of the total number of diabetic cases. Other serum biochemistry parameters were recorded within the reference range in most diabetic dogs. In the study, a proportion of diabetic dogs showed abnormalities in liver and kidney functions. In diabetic dogs, hypovolemia could occur, which leads to hepatocellular damage and elevated serum AST, ALP, and ALT in the serum biochemistry analysis panel (Babazadeh et al., 2022). An increase in serum creatinine and BUN could indicate kidney disease or diabetic renal complications (O'Brien, 2010). Dogs with DM often exhibit hyperketonemia, which eventually could lead to ketoacidosis (Slead et al., 2022). Diabetic ketoacidosis occurs when the body increases ketone production to compensate for inefficient glucose utilization in the blood (Feldman et al., 2015). A previous study demonstrated the potential of HbA1c and fructosamine in canine DM diagnosis (Baldo et al., 2020). However, elevated serum fructosamine levels within the reference range can still occur in dogs with newly diagnosed diabetes shortly before veterinary clinic admission (Ettinger et al., 2017; Norris and Schermerhorn, 2022).

In the present study, there were 7 dogs recorded with hyperketoneuria, and 11 dogs also showed hyperalbuminuria (Table 4). An elevated protein/creatinine ratio was also observed in 5 dogs. Urinalysis is one of the tests that should be performed in cases of diabetic dogs (Ettinger et al., 2017). A study by Mazzi et al. (2008) showed that approximately 25% of diabetic dogs had albuminuria, which could also be an indicator for detecting diabetic kidney damage. In the same study, the percentage of dogs with elevated protein/creatinine and albumin/creatinine levels was 71%. Glucosuria may increase the risk of urinary tract infections (Ettinger et al., 2017). Therefore, bacterial culture and isolation in urine can be performed to assess the presence of urinary tract infections.

Table 3. Hematology and serum biochemistry analysis of diabetic dogs presented to veterinary clinics in Ho Chi Minh City, Vietnam from January to December 2025

Parameter	Unit	Reference range*	Average result	Below reference range	Within reference range	Above reference range
Hematology analysis						
WBC	10^3/µL	6-17	15.61	10%	75%	15%
Lym	10^3/µL	1-5	1.93	15%	80%	5%
Lym percentage	%	24-26	14.57	65%	25%	10%
RBC	10^6/µL	5.4-7.8	6.22	30%	65%	5%
HGB	g/dL	13-20	13.72	40%	60%	-
HCT	%	40-60	40.09	40%	60%	-
MCV	fL	39-52	64.56	-	25%	75%
MCH	pg	13-21	22.01	-	35%	65%
MCHC	g/dL	30-38	34.16	-	95%	5%
PLT	10^3/µL	170-575	337	15%	70%	15%
Serum biochemistry analysis						
ALB	g/L	22-44	34.72	-	100%	-
TP	g/L	57-89	70.94	5%	90%	5%
GLOB	g/L	23-52	38.23	-	90%	10%
A/G			1.5			
TBIL	µmol/L	0-15	7.39	-	100%	-
ALT	U/L	5-130	108.52	-	95%	5%
AST	U/L	0-48	56.33	-	75%	25%
ALP	U/L	14-111	178.34	-	75%	25%
Crea	µmol/L	53-177	254.98	10%	65%	25%
BUN	mmol/L	2.9-10.4	12.00	-	75%	25%
BUN/Crea			19.02			
GLU	mmol/L	3.89-6.66	10.27	-	-	100%
CHOL	mmol/L	1.68-5.81	6.07	-	75%	25%

WBC: White blood cells, Lym: Lymphocytes, RBC: Red blood cells, HGB: Hemoglobin, HCT: Hematocrit, MCV: Mean corpuscular volume, MCH: Mean corpuscular hemoglobin, MCHC: Mean corpuscular hemoglobin concentration, PLT: Platelets, ALB: Albumin, TP: Total protein, GLOB: Globulin, A/G: Albumin/globulin ratio, TBIL: Total bilirubin, ALT: Alanine aminotransferase, AST: Aspartate transaminase, ALP: Alkaline phosphatase, Crea: Creatinine, BUN: Blood urea nitrogen, GLU: Blood glucose, CHOL: Cholesterol. The source of the reference ranges was Vaden (2009).

Ocular and renal complications

In the current study, the incidence of cataract complications in diabetic dogs was 25% (5/20 cases; Table 5). However, cataract was the only ocular complication recorded in diabetic dogs. All cases of diabetic dogs with cataracts in the study were observed in both eyes. There was one case of immature, one case of mature, and three cases of hypermature cataract. There was one case of incipient and intimescent cataracts recorded. The duration of DM and the time of cataract onset were not recorded in any of the cataract cases. Cataract formation is one of the most common eye complications in diabetic dogs (Miller and Brines, 2018). A study by Beam et al. (1999) showed that 14% of dogs diagnosed with DM already had cataract formation. Over 65% of diabetic dogs had cataracts, and approximately 80% of diabetic dogs developed cataracts after about 16 months (Beam et al., 1999). The incidence of cataract in diabetic dogs in the current study was lower than in a previous study, which might be due to the low mean age of diabetic dogs. In a study of cataract formation in dogs by Donzel et al. (2017), the average age of diabetic dogs with cataracts was about 10 years old. While it was hypothesized that glycemic control prolongs the time to cataract development, a recent study by

Arad et al. (2025) indicated that dogs with hyperglycemia and increased glycemic variability had diabetic cataracts develop rapidly.

Table 4. Urinalysis of diabetic dogs presented to the veterinary clinics in Ho Chi Minh City, Vietnam from January to December 2025

Parameter	Unit	Reference range*	Below reference range	Within reference range	Above reference range
GLU	mg/dL	Negative	-	-	100%
Bilirubin		Negative	-	80%	20%
Ketone	mg/dL	Negative	-	65%	35%
USG		1.015-1.025	-	15%	85%
RBC	cells/hpf	< 2-5	-	100%	-
pH		6-7.5	25%	75%	-
Protein	mg/dL	Negative	-	55%	45%
Nitrite		Negative	-	90%	10%
WBC	cells/hpf	< 2-5	-	80%	20%
Microalbumin	mg/L	0-1	-	45%	55%
Creatinine	mg/dL	0-0.5	-	25%	75%
P/C		0-0.5	-	75%	25%

USG: Urine specific gravity, Ketone: Urine ketone, Creatinine: Urine creatinine, P/C: Urine protein/creatinine ratio, RBC: Urine red blood cells, WBC: Urine white blood cells, hpf: High-power field. Source: Vaden (2009)

Table 5. Incidence of cataracts in diabetic dogs presented to veterinary clinics in Ho Chi Minh City, Vietnam from January to December 2025

Stage of cataracts	Number of cases	Percentage
Incipient cataract	-	-
Immature cataract	1	5
Mature cataract	1	5
Hypermature cataract	3	15
Intumescent cataract	-	-
Total	5	25

The incidence of renal complications in dogs was 25% of the total number of diabetic dogs (5/20 cases). Based on the IRIS classification of chronic kidney disease stages in dogs, there were 4 cases classified as stage 4 chronic kidney disease, and 1 case as stage 3. None of the dogs with renal complications also developed cataracts during the study period. Urinalysis for microalbuminuria is a method to detect renal complications due to diabetes. Microalbuminuria occurs in diabetic dogs, and the albumin/creatinine ratio in urine increases before the urine protein and creatinine ratio in urine increases (Mazzi et al., 2008). According to a study by Mazzi et al. (2008), over 50% of diabetic dogs have an elevated albumin/creatinine ratio in their urine.

CONCLUSION

Cataract formation was the most common diabetic ocular disease in dogs. Most diabetic dogs with renal complications had stage 4 chronic kidney disease. However, the associations between diabetic complications and DM in dogs still need more investigation. More studies are needed to investigate the associations between microalbuminuria and renal complications in diabetic dogs. The present study had certain limitations, as only 20 diabetic dogs were recorded, which may not reflect actual clinical effects, despite the statistical difference observed between the groups compared. Additionally, the study did not record or analyze data on dogs' dietary intake and daily exercise, which could influence the risk of developing DM. More studies are needed to investigate the associations between microalbuminuria and renal complications in diabetic dogs.

DECLARATIONS

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Authors' contributions

Thuong Thi Nguyen and Khanh Nguyen Dinh carried out conceptualization, methodology, formal analysis, original draft preparation, review, and editing. Tan Nhat Nguyen participated in formal analysis, original draft preparation, review, and editing of the study. Linh Ngoc Thuy Bui and Tuyen Thi Kim Le participated in original draft preparation. All authors have read and approved the final edition of the manuscript before publication in the present journal.

Availability of data and materials

The authors of the present study confirm that all data supporting the findings of the present study are available upon reasonable request from the corresponding author.

Competing interests

The authors declare no conflict of interest.

Ethical considerations

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Granulocyte Histomorphometry, Granulocyte Counts, and Platelet Profiles Associated with Age and Sex in Sumbawa Horses

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ABSTRACT

The Sumbawa horse (*Equus caballus*) is an indigenous Indonesian horse breed traditionally maintained under an extensive grazing system, characterized by unrestricted grazing and exposure to natural climatic conditions. This study aimed to evaluate granulocyte histomorphometry, granulocyte counts, and platelet profiles in clinically healthy indigenous Sumbawa horses in association with age and sex, providing baseline hematological data for this indigenous horse breed. A total of 80 clinically healthy indigenous Sumbawa horses meeting the predefined inclusion criteria were selected using purposive sampling and enrolled in this cross-sectional study. Peripheral blood smears were used for the histomorphometry evaluation of neutrophils, eosinophils, and basophils, whereas granulocyte counts, platelet counts, and platelet indices, including mean platelet volume (MPV), platelet distribution width (PDW-CV), and plateletcrit (PCT), were analyzed with an automated hematology analyzer. Sex had no significant effect on granulocyte histomorphometry or granulocyte counts. However, female horses exhibited significantly higher platelet counts and PCT values, whereas MPV was significantly higher in males. Adult horses exhibited significantly larger neutrophils and basophils, together with higher neutrophil and eosinophil counts. In contrast, platelet counts and platelet indices did not differ significantly between age groups. These findings indicated that granulocyte structural and quantitative characteristics are more responsive to age-related physiological adaptation, whereas platelet variation is predominantly associated with sex.

Keywords: Granulocyte count, Granulocyte histomorphometry, Platelet indices, Sumbawa horse, Sex

INTRODUCTION

The Sumbawa horse (*Equus caballus*) is one of Indonesia's indigenous equine genetic resources and plays an important socio-economic, cultural, and livestock role in West Nusa Tenggara Province (Sukri et al., 2022). In addition to its genetic importance, the breed is widely used as a working pony that supports transportation and rural livelihoods in the region (Drought et al., 2023) and has recently attracted scientific attention for its immunological and hematological characteristics (Rate et al., 2025). Unlike most horse populations for which hematological reference intervals have been established under intensive management systems in temperate regions, Sumbawa horses are predominantly maintained under the traditional *lar* grazing system, an extensive husbandry practice characterized by year-round communal grazing. They also serve as working ponies that are integral to local transportation and rural livelihoods in West Nusa Tenggara (Drought et al., 2023). The traditional *lar* grazing system provides a unique ecological context that distinguishes Sumbawa horses from horses managed under intensive production systems (Sukri et al., 2022; Rate et al., 2025). Under this extensive management system, horses graze freely throughout the year and are continuously exposed to seasonal fluctuations in forage availability, climatic variation, environmental microorganisms, and naturally occurring antigens (Martinson et al., 2017; Drought et al., 2023). The traditional *lar* grazing system and continuous exposure to natural climatic conditions may contribute to physiological adaptation and variation in hematological characteristics that should be considered when interpreting hematological findings in indigenous horses. Consequently, hematological reference intervals established for horses maintained under intensive management conditions may not accurately reflect the physiological status of indigenous horses adapted to tropical grazing environments (Pratama et al., 2025). Establishing population-specific hematological reference data is therefore essential for accurate clinical interpretation and for advancing the understanding of hematological variation under extensive tropical grazing systems.

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Routine hematological examination provides valuable quantitative information regarding circulating blood cells; however, numerical cell counts alone provide only limited insight into cellular functional status (Brooks et al., 2022). Peripheral blood smear evaluation and quantitative histomorphometry analysis allow assessment of cellular dimensions, nuclear architecture, and morphological characteristics that reflect granulocyte maturation, activation, and physiological adaptation (Brooks et al., 2022; Pratama et al., 2025). Morphological alterations may occur before detectable changes in leukocyte numbers, providing early evidence of cellular responses to environmental and physiological stimuli. Therefore, integrating quantitative hematology with cellular histomorphometry provides a more comprehensive characterization of hematological features associated with innate immune function than either approach alone (Walton and Lawson, 2021). Granulocytes, including neutrophils, eosinophils, and basophils, are the principal effector cells of innate immunity and undergo coordinated structural and functional changes in response to infection, inflammation, and tissue injury (Marshall et al., 2018; Arnold and Munitz, 2024).

Accumulating evidence demonstrates that granulocytes and platelets interact closely during inflammatory and innate immune responses. Beyond their classical role in hemostasis, platelets actively regulate leukocyte recruitment, inflammatory amplification, endothelial activation, and immunothrombosis through continuous communication with granulocytes and vascular cells (Scherlinger et al., 2023). In horses, platelet activation has likewise been associated with systemic inflammatory responses and innate immune regulation (Ehrmann et al., 2021; Walton and Lawson, 2021). Platelet profiles in horses can be more comprehensively characterized by combining platelet count (PLT) with platelet indices, including mean platelet volume (MPV), platelet distribution width (PDW-CV), and plateletcrit (PCT) (Gasparyan et al., 2011; Budak et al., 2016). Simultaneous evaluation of granulocyte histomorphometry, granulocyte counts, platelet count (PLT), and platelet indices provides a comprehensive characterization of hematological profiles in horses by integrating complementary cellular and platelet-related parameters (Satué et al., 2020; Brooks et al., 2022). Moreover, physiological variables such as age and sex influence hematopoiesis, immune maturation, hormonal regulation, and platelet production, making them essential considerations when establishing clinically meaningful hematological reference intervals (Koupenova et al., 2015; Klein and Flanagan, 2016; Miller et al., 2021).

Despite these recognized biological interactions, studies simultaneously evaluating both granulocyte morphology and platelet characteristics in indigenous horses remain unavailable. Previous investigations in Sumbawa horses have independently described hematological profiles, granulocyte counts, histological characteristics of granulocytes, and morphometric features of agranulocytic leukocytes and erythrocytes (Candrianisa et al., 2024; Pratama et al., 2025). However, these studies evaluated hematological profiles, granulocyte counts, granulocyte histological characteristics, and leukocyte morphometric features separately, leaving unresolved how granulocyte morphology relates to granulocyte abundance and platelet profiles in clinically healthy Sumbawa horses. Consequently, the relationships among granulocyte histomorphometry, granulocyte counts, and platelet profiles under extensive tropical grazing conditions remain poorly understood. Addressing this knowledge gap is important because coordinated evaluation of structural and quantitative hematological biomarkers may provide biological information that cannot be obtained from isolated measurements alone.

The present study is among the first to simultaneously evaluate granulocyte histomorphometry, granulocyte counts, platelet count (PLT), and platelet indices in clinically healthy indigenous Sumbawa horses while considering physiological variation associated with age and sex. This integrated approach enables comprehensive characterization of structural and quantitative hematological features within a single analytical framework. Therefore, this study aimed to evaluate age- and sex-related variation in granulocyte histomorphometry, granulocyte counts, PLT, and platelet indices in clinically healthy Sumbawa horses maintained under the traditional *lar* grazing system.

MATERIALS AND METHODS

Ethical approval

This study was approved by the Research Ethics Committee of the Faculty of Veterinary Medicine, Udayana University, Indonesia (Approval No. B/211/UN.14.2.9/PT.01.04/2024). Written informed consent was obtained from the owners of all privately owned Sumbawa horses before sample collection. Although the horses roamed freely on communal grazing land under the traditional *lar* grazing system, each horse had an identifiable owner. All procedures involving animals were conducted in accordance with institutional guidelines for animal welfare and research ethics.

Study design

A cross-sectional observational study was conducted between June and August 2025 to evaluate the associations of age and sex with granulocyte histomorphometry, granulocyte counts, and platelet profiles in clinically healthy Sumbawa horses.

Study area and animals

The study was conducted in Sumbawa regency, West Nusa Tenggara Province, Indonesia (Figure 1). Horses were sampled from Sumbawa Regency between June and August 2025 during the dry season, when ambient temperatures typically ranged from approximately 22°C at night to 31°C during the daytime. The horses were maintained under the traditional *lar* grazing system, in which they grazed freely on communal rangelands during the day and were confined to simple shelters at night. The grazing areas were dominated by Napier grass (*Pennisetum purpureum*), Setaria grass (*Setaria sphacelata*), signal grass (*Brachiaria decumbens*), and native savanna grasses, including *Digitaria* spp., *Chloris* spp., and *Panicum maximum*. The horses primarily consumed these natural forages and received cut forage and rice bran once daily as dietary supplements. All horses were managed under the same feeding regimen regardless of age or sex (Pratama et al., 2025). The study included 80 clinically healthy Sumbawa horses. Eligible animals were selected using purposive sampling and subsequently categorized by sex (male or female) and age (young or adult), generating four study groups of 20 horses each.

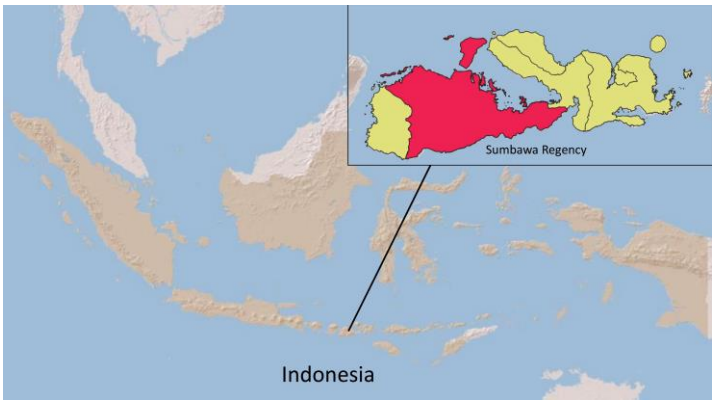


Figure 1. Geographic location of the study area in Sumbawa Regency, Indonesia. The map was prepared by the authors using GADM version 4.1 administrative boundaries (GADM, 2025) and the Esri Shaded Relief basemap.

Animal selection and veterinary clinical examination

Prior to enrolment, all horses underwent a comprehensive clinical examination performed by licensed veterinarians to confirm their health status. The clinical examination consisted of body condition scoring, measurement of rectal temperature, heart rate, respiratory rate, capillary refill time, mucous membrane color, and hydration status, together with evaluation of the respiratory, gastrointestinal, musculoskeletal, and other body systems. Body condition was assessed using the Henneke Body Condition Scoring system (Henneke et al., 1983), and only horses with normal body condition scores were eligible for inclusion.

All physiological variables measured during the clinical examination fell within the normal reference ranges for healthy horses (Hall et al., 2018; Walton and Lawson, 2021), with rectal temperatures ranging from 37.2 to 38.3°C, heart rates from 28 to 48 beats/min, respiratory rates from 8 to 14 breaths/min, and capillary refill times of less than two seconds. The inclusion criteria comprised clinically healthy Sumbawa horses maintained under the traditional *lar* grazing system that exhibited normal physical examination findings and physiological parameters. Horses presenting poor body condition, abnormal vital signs, evidence of systemic disease, gastrointestinal or respiratory disorders, or other clinically significant abnormalities were excluded from the study. All clinical examinations and blood sample collections were performed by licensed veterinarians following standardized procedures to ensure consistency in animal selection and sample collection. According to owner interviews and available health records, none of the horses had previously received any vaccination or anthelmintic treatment.

Faecal examination

To further verify the health status of the animals, gastrointestinal parasite burden was assessed by fecal egg count analysis using the McMaster technique (Nielsen, 2022; Lejeune et al., 2023). In accordance with current recommendations for defining clinically healthy equine populations in hematological investigations (Nielsen, 2021), only horses with negative fecal egg counts were considered eligible for inclusion. Horses with positive fecal egg count results indicative of gastrointestinal parasitism were excluded from further analysis.

Blood collection and sample preparation

Whole blood samples were aseptically obtained from the jugular vein by licensed veterinarians using sterile Venoject® needles and collected directly into EDTA-coated vacuum tubes. Approximately 8 mL of whole blood was

obtained from each horse between 08:00 and 11:00 h to minimize potential diurnal variation in hematological parameters. Immediately after collection, blood tubes were gently inverted 8-10 times to ensure complete mixing with the anticoagulant. Fresh blood samples were immediately used to prepare peripheral blood smears, whereas the remaining EDTA-anticoagulated blood was transported to the laboratory in a portable cooler maintained at 4 ± 1°C and processed within two hours after collection to minimize cellular deterioration and hemolysis (Arnold et al., 2019). The blood smears were air-dried, fixed in absolute methanol for three minutes, and subsequently stained using the Modified Diff-Quik staining kit (MDT IndoReagen®, Indonesia; Catalog No. 710623-MD) according to the manufacturer's instructions and standard veterinary hematology procedures (Brooks et al., 2022).

Histomorphometry analysis

Granulocyte histomorphometry was examined using an Olympus CX33 light microscope (Olympus Corporation, Tokyo, Japan) under 400× and 1000× magnification. Granulocytes were identified according to their characteristic nuclear morphology, cytoplasmic appearance, and specific cytoplasmic granules (Brooks et al., 2022). Morphometric analysis included neutrophils, eosinophils, and basophils. Only well-preserved cells exhibiting intact cellular morphology, distinct nuclear configuration, clearly defined cytoplasmic boundaries, and absence of staining artifacts were included in the analysis. Cells showing overlapping structures, indistinct cellular boundaries, poor staining quality, or smear preparation artifacts (e.g., cell distortion, folding, or mechanical damage) were excluded from the analysis in accordance with standard veterinary hematology procedures (Brooks et al., 2022).

Because eosinophils and basophils are naturally present at low frequencies in the peripheral blood of clinically healthy horses, these cell types were not consistently observed in every blood smear (Brooks et al., 2022; DeNotta and McFarlane, 2023). Therefore, morphometric analysis of eosinophils and basophils was performed only when well-preserved cells fulfilling the predefined morphological criteria were available. Digital morphometric measurements were obtained using EPview software (version 4.0; Olympus Corporation, Tokyo, Japan). The evaluated variables included cell area (μm²), cell perimeter (μm), and cell diameter (μm). All histomorphometric measurements were performed by a trained laboratory technologist following a standardized measurement protocol to ensure consistency throughout the study. Blood smear preparation, staining, and morphometric measurements were performed according to standardized laboratory protocols to minimize analytical variation (Stirn and Freeman, 2022).

Haematological analysis

Whole blood specimens were processed using an automated hematology analyzer (VetScan HM5; Abaxis, Union City, California, USA). The hematological variables evaluated included neutrophil, eosinophil, and basophil counts, as well as platelet count (PLT), mean platelet volume (MPV), platelet distribution width coefficient of variation (PDW-CV), and plateletcrit (PCT). The analyzer was calibrated and subjected to routine internal quality-control procedures, including background verification and analysis of commercial quality-control materials, according to the manufacturer's instructions before sample analysis to ensure analytical accuracy and measurement reliability. Routine internal quality-control procedures were maintained throughout the analytical process to ensure consistency and reliability of hematological measurements (Stirn and Freeman, 2022).

Statistical analysis

Descriptive statistics were calculated for all variables and are presented as mean ± standard deviation (SD). Data normality was assessed using the Shapiro–Wilk test, and homogeneity of variance was evaluated using Levene's test prior to comparative analysis. Comparisons between age groups (young versus adult) and sex groups (male versus female) were performed using the independent-samples t-test. All histomorphometry and hematological variables were analyzed by the independent-samples t-test. All statistical analyses were performed using IBM SPSS Statistics version 29.0 (IBM Corp., Armonk, NY, USA). Differences in all histomorphometry and hematological variables between age and sex groups were considered statistically significant at $p < 0.05$.

RESULTS

Microscopic examination of granulocytes from Sumbawa horses reared under the traditional *lar* grazing system demonstrated characteristic morphological features for each granulocyte type across different age (young and adult) and sex (male and female) groups. Neutrophils (Figure 2) were round in shape and contained nuclei segmented into three to five lobes of varying sizes. Their cytoplasm appeared pale pink with fine granules. Eosinophils (Figure 3) were also round, with segmented nuclei and large pink-orange granules. The cytoplasm contained numerous large, densely packed eosinophilic (globular) granules. Basophils (Figure 4) exhibited a round cell shape with abundant dark blue to purple metachromatic cytoplasmic granules. Their nuclei were indistinct, poorly segmented, and often appeared irregular. Morphological observations indicated that these structural characteristics were consistently observed in both young and adult horses, regardless of sex.

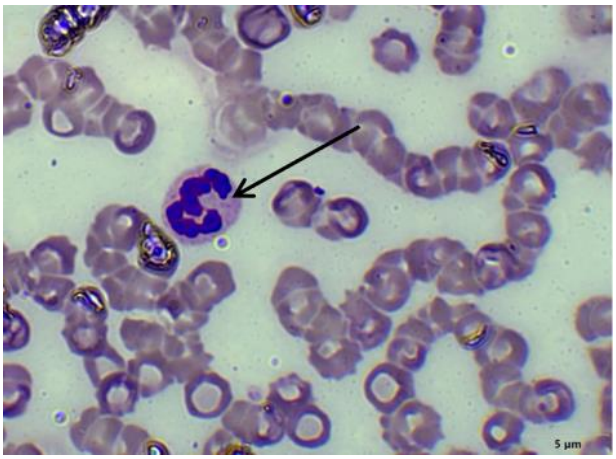


Figure 2. Representative neutrophil in a peripheral blood smear of a Sumbawa horse (arrow), Indonesia.

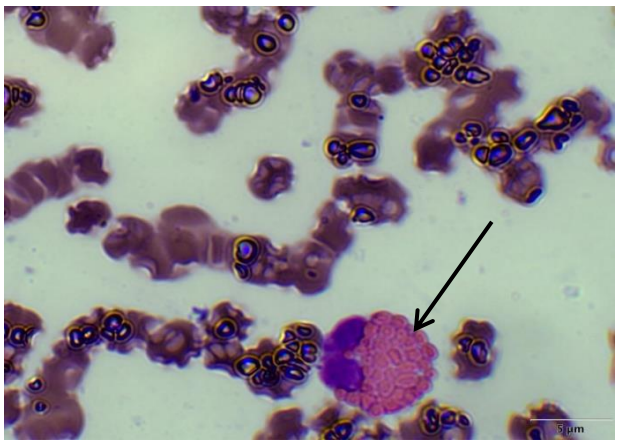


Figure 3. Representative eosinophil in a peripheral blood smear of a Sumbawa horse (arrow), Indonesia.

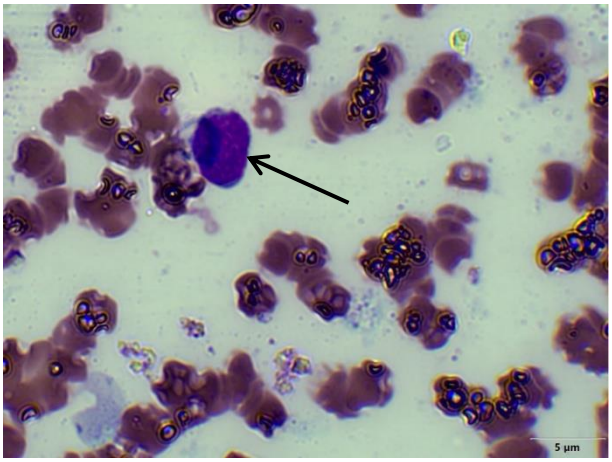


Figure 4. Representative basophil in a peripheral blood smear of a Sumbawa horse (arrow), Indonesia.

Histomorphometry of granulocytes based on sex

No significant sex-related differences were observed in the area, perimeter, or diameter of neutrophils, eosinophils, or basophils (Table 1; $p > 0.05$). Although male horses tended to exhibit slightly larger neutrophil dimensions than females, these differences were not statistically significant. Likewise, eosinophil and basophil morphometric parameters remained comparable between sexes.

Histomorphometry of granulocytes based on age

Age significantly influenced the histomorphometry characteristics of neutrophils and basophils (Table 2). Adult horses exhibited significantly greater neutrophil area, perimeter, and diameter than young horses ($p < 0.05$). Basophils demonstrated the same age-related trend, with adult horses exhibiting significantly larger area, perimeter, and diameter than young horses ($p < 0.05$). In contrast, eosinophil morphometric characteristics did not differ significantly between age groups ($p > 0.05$).

Granulocyte counts and platelet profiles based on sex

Neutrophil, eosinophil, and basophil counts did not differ significantly between male and female horses (Table 3; $p > 0.05$). In contrast, platelet-related variables showed significant sex-associated variation. Female horses exhibited significantly higher PLT and PCT, whereas male horses exhibited significantly higher MPV. PDW-CV did not differ significantly between sexes ($p > 0.05$).

Granulocyte counts and platelet profiles based on age

Adult horses exhibited significantly higher neutrophil and eosinophil counts than young horses (Table 4; both $p < 0.05$), whereas basophil counts did not differ significantly between age groups ($p > 0.05$). None of the platelet-related variables, including PLT, MPV, PDW-CV, and PCT, differed significantly between young and adult horses ($p > 0.05$).

Table 1. Histomorphometry characteristics of granulocytic leukocytes (neutrophils, eosinophils, and basophils) in clinically healthy Sumbawa horses according to sex

Indicator		Male (Mean ± SD)	Female (Mean ± SD)
Neutrophil	Area (μm^2)	23.02 ± 8.53	22.92 ± 9.65
	Perimeter (μm)	16.50 ± 3.06	16.34 ± 3.50
	Diameter (μm)	5.24 ± 0.97	5.20 ± 1.11
Eosinophil	Area (μm^2)	38.13 ± 15.64	39.27 ± 14.60
	Perimeter (μm)	21.43 ± 4.49	21.81 ± 4.25
	Diameter (μm)	6.82 ± 1.43	6.95 ± 1.34
Basophil	Area (μm^2)	21.61 ± 9.85	23.13 ± 9.95
	Perimeter (μm)	16.11 ± 3.44	16.66 ± 3.67
	Diameter (μm)	5.13 ± 1.09	5.30 ± 1.17

Table 2. Histomorphometry characteristics of granulocytic leukocytes (neutrophils, eosinophils, and basophils) in clinically healthy Sumbawa horses according to age

Indicator		Young (Mean ± SD)	Adult (Mean ± SD)
Neutrophil	Area (μm^2)	20.01 ± 7.53 [a]	25.93 ± 9.57 [b]
	Perimeter (μm)	15.60 ± 2.87 [a]	17.75 ± 3.33 [b]
	Diameter (μm)	4.97 ± 0.91 [a]	5.65 ± 1.06 [b]
Eosinophil	Area (μm^2)	37.54 ± 13.88 [a]	39.86 ± 16.23 [a]
	Perimeter (μm)	21.34 ± 4.10 [a]	21.90 ± 4.62 [a]
	Diameter (μm)	6.80 ± 1.29 [a]	6.97 ± 1.47 [a]
Basophil	Area (μm^2)	18.65 ± 7.12 [a]	26.08 ± 10.89 [b]
	Perimeter (μm)	15.01 ± 2.98 [a]	17.76 ± 3.57 [b]
	Diameter (μm)	4.78 ± 0.95 [a]	5.65 ± 1.14 [b]

[a,b] Different superscript letters within the same row indicate significant differences ($p < 0.05$).

Table 3. Granulocyte counts and platelet profiles in clinically healthy Sumbawa horses according to sex

Indicator	Male (Mean ± SD)	Female (Mean ± SD)
NEU (10^9/L)	4.38 ± 1.70 [a]	4.81 ± 1.84 [a]
EOS (10^9/L)	0.30 ± 0.17 [a]	0.26 ± 0.19 [a]
BAS (10^9/L)	0.03 ± 0.03 [a]	0.03 ± 0.03 [a]
PLT (10^9/L)	203.33 ± 71.88 [a]	301.45 ± 104.43 [b]
MPV (fL)	7.77 ± 0.61 [a]	7.50 ± 0.42 [b]
PDW-CV (%)	29.98 ± 2.27 [a]	29.55 ± 1.66 [a]
PCT (%)	0.15 ± 0.05 [a]	0.22 ± 0.08 [b]

[a,b] Different superscript letters within the same row indicate significant differences ($p < 0.05$). NEU: Neutrophil count, EOS: Eosinophil count, BAS: Basophil count, PLT: Platelet count, MPV: Mean platelet volume, PDW-CV: Platelet distribution width, PCT: Plateletcrit.

Table 4. Granulocyte counts and platelet profiles in clinically healthy Sumbawa horses according to age

Indicator	Young (Mean ± SD)	Adult (Mean ± SD)
NEU (10^9/L)	3.97 ± 1.46 [a]	5.21 ± 1.85 [b]
EOS (10^9/L)	0.24 ± 0.18 [a]	0.33 ± 0.14 [b]
BAS (10^9/L)	0.03 ± 0.03 [a]	0.03 ± 0.04 [a]
PLT (10^9/L)	243.30 ± 97.15 [a]	261.48 ± 106.88 [a]
MPV (fL)	7.70 ± 0.48 [a]	7.57 ± 0.58 [a]
PDW-CV (%)	29.72 ± 1.95 [a]	29.81 ± 2.06 [a]
PCT (%)	0.18 ± 0.07 [a]	0.19 ± 0.08 [a]

[a,b] Different superscript letters within the same row indicate significant differences ($p < 0.05$). NEU: Neutrophil count, EOS: Eosinophil count, BAS: Basophil count, PLT: Platelet count, MPV: Mean platelet volume; PDW-CV: Platelet distribution width, PCT: Plateletcrit.

DISCUSSION

This study simultaneously evaluated granulocyte histomorphometry, granulocyte counts, and platelet profiles in clinically healthy Sumbawa horses maintained under the traditional *lar* grazing system while assessing the influence of age and sex. Overall, age exerted a greater influence on granulocyte characteristics than sex, as reflected by the significantly larger neutrophil and basophil dimensions and higher neutrophil and eosinophil counts in adult horses. In contrast, granulocyte morphology and differential counts were largely comparable between males and females, whereas sex-related variation was mainly confined to platelet indices. These findings suggest that aging predominantly influences granulocyte structure and abundance, whereas platelet biology exhibits greater sexual dimorphism. By integrating granulocyte morphometry with granulocyte counts and platelet profiles, this study extends previous investigations in Sumbawa horses and complements existing literature on hematological variation among different horse breeds. Similar age- and sex-related variations in hematological characteristics have been reported in Spanish Purebred horses (Satué et al., 2020). Likewise, studies involving Arabian, Thoroughbred, and local horses demonstrated that hematological profiles are influenced by breed, age, and sex, although the affected parameters differ among breeds (Sawesi et al., 2023). Consistent with these findings, Yavuz et al. (2025) also reported significant age- and sex-related variation in hematological parameters in Arabian and Thoroughbred racehorses. In line with these observations, the present study demonstrated that age and sex also influence granulocyte morphometry, granulocyte counts, and platelet profiles in Sumbawa horses, further emphasizing the importance of establishing breed-specific reference values. These findings complement previous evidence of age- and sex-related variation in immunoglobulin M (IgM) concentrations in Sumbawa horses maintained under the traditional *lar* grazing system (Rate et al., 2025) and extend existing knowledge by providing baseline reference data on granulocyte morphometry, hematological, and platelet parameters for interpreting innate immune status and hematological adaptation in Sumbawa horses maintained under extensive tropical grazing conditions (Candrianisa et al., 2024; Pratama et al., 2025).

Effects of sex on granulocyte histomorphometry

Granulocyte histomorphometry characteristics were comparable between male and female Sumbawa horses, indicating that sex exerts minimal influence on granulocyte morphology under normal physiological conditions. Similar neutrophil, eosinophil, and basophil area, perimeter, and diameter suggest that granulocyte maturation appears to be largely independent of physiological differences in endocrine status. Comparable findings have been reported in healthy Spanish Purebred and Sumbawa horses, where sex has little influence on leukocyte-related hematological characteristics (Satué et al., 2020; Candrianisa et al., 2024; Pratama et al., 2025).

The absence of morphometric differences is biologically plausible because mature granulocytes complete their differentiation before entering the circulation, and their morphology generally remains stable unless altered by inflammation, toxic change, or accelerated granulopoiesis. Consequently, physiological differences between males and females are more likely to influence granulocyte function than cellular structure (Walton and Lawson, 2021; Brooks et al., 2022). Likewise, the similar eosinophil and basophil morphology observed in clinically healthy, parasite-free horses indicates minimal granulocyte activation under normal physiological conditions (Brosnahan, 2020).

These findings suggest that sex-specific reference intervals for granulocyte histomorphometry measurements are unlikely to be required in healthy Sumbawa horses. Despite significant sex-related differences in platelet parameters, granulocyte morphology remained stable, indicating that leukocyte structural characteristics are more tightly conserved than platelet-related variables. The preservation of granulocyte morphology despite sex-related differences in platelet parameters further supports the complementary role of granulocyte histomorphometry in hematological evaluation.

Effects of age on granulocyte histomorphometry

Unlike sex, age significantly influenced the morphology of selected granulocyte populations ($p < 0.05$). Adult Sumbawa horses exhibited significantly greater neutrophil and basophil area, perimeter, and diameter than young horses, whereas eosinophil morphometric characteristics remained unchanged. These findings indicate that age-related structural adaptation is cell-specific and reflects differential responses of granulocyte populations during immune maturation. Similar age-associated variation in equine hematological characteristics has been reported previously, emphasizing age as an important determinant of immune development (Satué et al., 2020; Candrianisa et al., 2024).

The larger neutrophil dimensions observed in adult horses are consistent with progressive maturation of innate immune function. As the predominant circulating phagocytes, neutrophils are continuously exposed to environmental microorganisms and naturally occurring antigens, particularly under extensive grazing conditions. Likewise, basophils exhibited comparable age-related enlargement despite representing only a minor leukocyte population, suggesting that structural adaptation during immune maturation extends beyond the predominant granulocyte population. Because these

morphometric changes occurred without corresponding increases in basophil counts, cellular morphology may respond to age independently of circulating cell abundance (Walton and Lawson, 2021; DeNotta and McFarlane, 2023).

In contrast, eosinophil morphology remained stable despite the slightly higher eosinophil counts observed in adult horses. Because eosinophil differentiation is primarily regulated by antigenic stimulation associated with parasitic infection and allergic responses rather than chronological age, similar cellular dimensions were expected in the absence of major stimulatory factors. The exclusion of horses with detectable gastrointestinal parasitism further supports the interpretation that eosinophil morphology remained under normal physiological regulation rather than environmental or pathological influence (Brosnahan, 2020).

Overall, these findings demonstrated that age influences granulocyte morphology more profoundly than sex and that individual granulocyte populations respond differently to immune maturation. The selective enlargement of neutrophils and basophils, together with the unchanged eosinophil morphology, extends previous descriptions of leukocyte morphology in Sumbawa horses and provides baseline reference information for interpreting age-related granulocyte development in indigenous horses maintained under tropical grazing conditions.

Effects of sex on granulocyte counts and platelet profiles

Unlike platelet-related variables, circulating granulocyte counts were comparable between male and female Sumbawa horses, indicating that sex has little influence on granulopoiesis or peripheral granulocyte homeostasis under normal physiological conditions. Similar neutrophil, eosinophil, and basophil counts have been reported in healthy horses, suggesting that granulocyte populations are maintained through tightly regulated hematopoietic mechanisms rather than sex alone (Satué et al., 2020; Candrianisa et al., 2024). Because all horses were clinically healthy, maintained under identical grazing conditions, and free of detectable gastrointestinal parasitism, environmental factors capable of altering granulocyte populations were minimized, supporting the interpretation that the comparable granulocyte counts reflected normal physiological regulation rather than differences in innate immune competence (Walton and Lawson, 2021; Brooks et al., 2022).

In contrast, platelet indices exhibited clear sexual dimorphism. Female horses had significantly higher PLT and PCT values, whereas males exhibited significantly greater MPV, consistent with physiological variation in thrombopoiesis and platelet turnover rather than pathological alteration (Koupenova et al., 2015; Gaina et al., 2020; Satué et al., 2020). PDW-CV remained comparable between sexes, and all platelet-related variables were within physiological reference ranges, indicating normal biological variation. Overall, these findings show that sex influences platelet profiles more readily than granulocyte populations, with granulocyte morphology and abundance remaining stable despite measurable differences in platelet production and circulating platelet mass.

Effects of age on granulocyte counts and platelet profiles

Age exerted a greater influence on circulating granulocyte populations than on platelet-related variables. Adult Sumbawa horses exhibited significantly higher neutrophil and eosinophil counts than young horses, whereas basophil counts and all platelet indices remained comparable between age groups. Combined with the enlarged neutrophil and basophil dimensions observed in adult horses, these findings support the interpretation that aging is associated with both structural and quantitative granulocyte characteristics, whereas platelet-related variables appear to remain relatively stable. Together, these structural and quantitative changes are consistent with progressive maturation of innate immune function in adult horses. As the principal phagocytic cells of innate immunity, neutrophils continuously respond to environmental stimuli throughout life, whereas eosinophils are influenced by long-term exposure to environmental antigens despite the absence of detectable gastrointestinal parasitism in the present study. These findings support the concept that immune maturation continues beyond early growth and is accompanied by selective changes in circulating granulocyte populations (Gaina et al., 2020; Miller et al., 2021; Satué et al., 2020; DeNotta and McFarlane, 2023). Consistent with this interpretation, Rate et al. (2025) reported age-related variation in immunoglobulin M (IgM) concentrations in Sumbawa horses maintained under the traditional *lar* grazing system, suggesting that both cellular and humoral immune components undergo age-related adaptation in this indigenous horse breed.

In contrast, basophil counts remained unchanged despite the significant enlargement of basophil dimensions observed in adult horses, suggesting that structural adaptation may occur independently of changes in circulating cell numbers. This discrepancy highlights the complementary value of combining histomorphometry and hematological analyses, as subtle cellular adaptations may not be detected through differential leukocyte counts alone (Walton and Lawson, 2021).

Unlike granulocyte populations, PLT, MPV, PDW-CV, and PCT remained stable across age groups, indicating that thrombopoiesis and platelet turnover are maintained within relatively narrow physiological limits throughout adulthood (Satué et al., 2017; Gaina et al., 2020). Overall, these findings demonstrated that age-related hematological adaptation in

Sumbawa horses is characterized primarily by coordinated changes in granulocyte morphology and abundance, whereas platelet physiology remains comparatively stable. These observations establish baseline reference data for interpreting age-related hematological variation in indigenous horses maintained under tropical grazing conditions.

Influences of the traditional lar grazing system

The traditional *lar* grazing system provides an important ecological context for interpreting the hematological characteristics of Sumbawa horses. Unlike intensively managed horses, Sumbawa horses graze freely on communal rangelands throughout the year and are continuously exposed to seasonal climatic variation, diverse forage resources, environmental microorganisms, and naturally occurring environmental antigens associated with pasture-based management (Martinson et al., 2017; Drought et al., 2023). Similar effects of environmental and management conditions have been reported in other horse populations. Francesca Aragona et al. (2023) demonstrated that housing systems and seasonal variation significantly influenced platelet counts and most leukocyte populations, whereas Deniz et al. (2024) reported significant effects of climatic variation on leukocyte populations, including neutrophils, lymphocytes, and monocytes, as well as platelet counts. Likewise, Sawesi et al. (2023) showed that management practices, including exercise and dietary supplementation, affected several hematological indices, while Ono et al. (2021) reported seasonal variations in hematological profiles in indigenous Noma horses. Collectively, these findings support the interpretation that continuous exposure to the traditional *lar* grazing system may contribute to physiological adaptation and variation in the hematological characteristics of Sumbawa horses and should be considered when establishing reference intervals for clinically healthy indigenous populations.

The predominance of age-related over sex-related variation supports the view that long-term environmental adaptation contributes more substantially to granulocyte adaptation than normal physiological differences between males and females. Adult horses exhibited enlarged neutrophils and basophils together with higher neutrophil and eosinophil counts, whereas granulocyte morphology and abundance remained largely comparable between sexes, with sex-related differences primarily confined to platelet parameters. Moreover, structural enlargement of granulocytes was not consistently accompanied by corresponding changes in circulating cell numbers, indicating that cellular morphology and routine hematological variables provide complementary information for identifying subtle physiological adaptations (Stokol, 2020).

CONCLUSION

This study simultaneously evaluated granulocyte histomorphometry, granulocyte counts, and platelet profiles in clinically healthy Sumbawa horses maintained under the traditional *lar* grazing system. Sex had no significant effect on granulocyte histomorphometry or granulocyte counts, whereas age significantly influenced neutrophil and basophil histomorphometry as well as neutrophil and eosinophil counts. In contrast, platelet profiles were primarily influenced by sex rather than age. These findings establish baseline hematological reference data for clinically healthy Sumbawa horses and highlight the importance of considering age and the traditional *lar* grazing system when interpreting hematological characteristics in indigenous horses. They also provide a valuable reference for clinical pathology and future comparative studies of hematological adaptation under tropical grazing conditions. Future studies should include multiple indigenous horse populations from different geographical regions and integrate functional immunological and molecular biomarkers, such as cytokines and oxidative stress indicators, to further elucidate hematological adaptation under diverse environmental and management conditions.

DECLARATIONS

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Authors' contributions

I Putu Gde Surya Nagara Ardiana conceptualized the study, designed the experiments, and conducted data collection and analysis. Ni Ketut Suwiti, Nyoman Sadra Dharmawan, Ida Bagus Komang Ardana, I Nyoman Sulabda, Ni Luh Eka Setiasih, and I Nengah Kerta Besung contributed to blood sample preparation, laboratory management, statistical analyses, and drafting of the manuscript. All authors have read and approved the final edition of the manuscript.

Availability of data and materials

All data supporting the findings of this study are available from the corresponding authors upon a reasonable request.

Competing interests

The authors declare that there is no conflict of interest regarding the publication of this study.

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. No AI tools were used to conduct this study and prepare the manuscript. The authors accept full responsibility for the originality of the study before submission and throughout the entire peer-review process of publication.

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Serological Investigation of Antibodies Induced by Smooth and Rough *Brucella* Lipopolysaccharide in Sheep

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ABSTRACT

Different *Brucella* species, characterized by smooth and rough cell walls, are recognized as etiological agents of ovine brucellosis. While serological studies in adult and older sheep are common, those on the serological status of young animals are limited. The present study aimed to investigate brucellosis in sheep caused by smooth- and rough-walled *Brucella* species at two slaughterhouses in the Marmara Region of Turkey using serological methods. Three different analyses, including the rapid slide test, the tube agglutination test, and the indirect enzyme-linked immunosorbent assay (iELISA), each included smooth- and rough-structured *Brucella* antigens, resulting in a total of six separate tests. Blood sera were randomly obtained from 150 male sheep, approximately 1 year old and weighing about 70 kilograms, from the Merino, Kıvırcık, Lacauné, and Tahirova breeds. These sheep were brought to slaughterhouses from several provinces, including 25 from Tekirdağ, 45 from İstanbul, 43 from Kırklareli, 17 from Çanakkale, and 20 from Kocaeli. Using the rough iELISA, 15 positive cases (10%) were identified, of which eight were concurrently positive with the smooth iELISA. The low positivity rate and negative results from tube agglutination and rapid slide tests decreased the probability that the sheep had active brucellosis. Positive results for the same sample in both S-iELISA and R-iELISA tests may be linked to smooth LPSs, while positivity only in R-iELISA could be attributed to rough LPSs. Although no *Brucella ovis* or *Brucella melitensis* were found in the animals examined in the present study, the positive ELISA results suggested that brucellosis could have important epidemiological implications. Using two iELISA methods with smooth and rough LPS antigens concurrently enhanced the diagnostic sensitivity of indirect serological diagnosis of brucellosis.

Keywords: Brucellosis, Sheep, Slaughterhouse, Rough lipopolysaccharide, Smooth lipopolysaccharide

INTRODUCTION

Brucellosis is a general term referring to the disease caused by bacteria of the genus *Brucella* (*B.*). These *Brucella* species exhibit host specificity; nonetheless, they may infect animals other than their specific hosts. Sheep serve as specific hosts for both *B. melitensis* and *B. ovis* (Alton et al., 1988; WOAH, 2025a). *Brucella melitensis* is the most common species in sheep and the predominant agent of ovine brucellosis (WOAH, 2025a). *Brucella melitensis* infections have long been recognized and remain widespread in Turkey. In Turkey, different control strategies have been implemented to manage brucellosis, specifically *B. melitensis* infections, since the 1980s (Çakır and Yıldırım, 2018). According to the latest national studies, the individual prevalence of ovine and caprine brucellosis in Turkey was estimated at 2.10%, while herd prevalence was 12.34% (Bebek, 2019). In similar epidemiological studies, animals under one year of age were typically excluded from risk groups for statistical reasons. This exclusion was due to the higher likelihood of false positives in young animals, which could be attributable to maternal antibodies or vaccination. While this approach might be epidemiologically justified, it can limit the serological assessment of young animals. Brucellosis is frequently correlated with abortions and reproductive disorders in female animals. In males, infection leads to considerable productivity and economic losses, including epididymitis, orchitis, testicular atrophy, reduced sperm quality, and infertility. The presence of testicular abnormalities in male animals serves as a significant diagnostic indicator of brucellosis. In young animals, subclinical or latent infections may occur, thereby facilitating the persistence and transmission of brucellosis within herds (WOAH, 2025a;b). Monitoring young male animals for brucellosis can provide valuable epidemiological data for herd management.

Numerous studies have addressed the diagnosis of *B. ovis* infections in sheep (Praud et al., 2012; Costa et al., 2016; Branscom et al., 2018). *Brucella ovis* has been reported in multiple countries, and in regions where disease prevalence

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posed a significant threat, effective control strategies have been implemented (Galluzzo et al., 2021; WOAH, 2025b). In Turkey, there is no dedicated control program for managing *B. ovis* infections in sheep (Keskin et al., 2022). Apart from a single bacterial isolation from a ram imported from Syria (Erdenliğ Gürbilek et al., 2018), there have been no comprehensive studies on the prevalence or incidence of *B. ovis* infections across all provinces in Turkey. However, numerous serological studies have indicated *B. ovis* seropositivity (Uçan and Aras, 2007; Tel et al., 2016; Keskin et al., 2022), indicating a strong likelihood of *B. ovis* presence in Turkey.

Brucella are classified as smooth or rough strains based on their Gram-negative cell wall structure. The lipopolysaccharide (LPS) component of the cell wall is the primary determinant of this classification. Smooth *Brucella* strains have an S-LPS molecule on their surface, in which the O-polysaccharide (O-PS) is a key determinant of the cell wall structure. Naturally rough strains, including *B. ovis*, *B. canis*, *B. abortus* RB51, and *B. melitensis* B115, do not include this O-PS molecule. Thus, the presence or absence of O-PS distinguishes smooth strains from rough strains (Nielsen et al., 2004). Field isolates from diseased animals typically are smooth *Brucella* strains, although there have been occasional reports of stable rough field strains (Yang et al., 2020). Some rough strains originate from smooth ones that are compelled to grow under unfavorable conditions, such as stagnant liquid media. Consequently, isolation and preservation conditions significantly influence the bacterial cell wall structure of these isolates (Alton et al., 1988).

The gold-standard test for diagnosing brucellosis is bacterial isolation and identification, although these methods are expensive and time-consuming (Saytekin and Ak, 2018). Additionally, bacterial isolation carries a high risk of contamination, endangering personnel and the environment. Therefore, it is essential to carefully assess and establish the laboratory biosecurity level before handling *Brucella* bacteria (WOAH, 2025a). Many molecular diagnostic methods require direct DNA extraction from bacteria, increasing the risk of bacterial contamination throughout the process. These methods are beneficial because they provide direct diagnosis for brucellosis (Saytekin and Ak, 2018). In contrast to bacterial isolations, serological analyses pose a lower risk of contamination and are more cost-effective and less labor-intensive. However, indirect serological diagnosis has lower specificity because cross-reactions can occur among closely related bacterial species. Therefore, selecting appropriate antigens for serological analyses is important (WOAH, 2025 a;b). Furthermore, Alton et al. (1988) noted that structural differences among *Brucella* strains necessitate selecting suitable serological test antigens, either smooth or rough, depending on the specific strain targeted. Advancements in technology have led to more precise and sensitive serological diagnostic tests. The World Organization for Animal Health (WOAH) noted different assays and tests for diagnosing infections caused by the smooth (*B. melitensis*) and rough (*B. ovis*) *Brucella* species (WOAH, 2025a;b). These methods included several types of enzyme-linked immunosorbent assay (ELISA), a highly sensitive and reliable analytical method for detecting brucellosis, with variants depending on the target antigen and diagnostic goal (Keskin et al., 2022). The present study aimed to assess the presence of antibodies targeting both smooth and rough LPS phenotypes of *Brucella* species using several serological techniques.

MATERIALS AND METHODS

Ethical approval

This study received approval from the İstanbul University-Cerrahpaşa Faculty of Veterinary Science Research Ethics Committee under approval number 2021-44, dated October 26, 2021.

Samples collection and location

Sample size calculation was conducted with a 95% confidence level, an 8% margin of error, and an anticipated prevalence of 50%. Between March 2022 and February 2023, a total of 150 serum samples were randomly collected from male Merino, Tahirova, Lacauné, and Kıvırcık sheep aged between 6 months and 1 year, with an average weight of 70 kg. These sheep were brought to two slaughterhouses located in Istanbul from several provinces, including 25 from Tekirdağ, 45 from İstanbul, 43 from Kırklareli, 17 from Çanakkale, and 20 from Kocaeli, Turkey (Figure 1). During the study period, slaughterhouses were visited every two months, and blood serum was collected from 15 animals during each visit.

The number of animals sampled from each province, their breeds, and the proportional distribution of the samples are presented in Table 1. All blood samples were drawn from the jugular vein using sterile, non-anticoagulant vacuum tubes in accordance with aseptic and antiseptic procedures. Following an overnight stand period, the samples were centrifuged to separate the sera. The collected sera were then transferred into sterile Eppendorf tubes, appropriately numbered, and stored at 2-8°C until further testing. All processes were carried out in the Microbiology laboratory of the Veterinary Faculty of İstanbul University-Cerrahpaşa, Turkey.

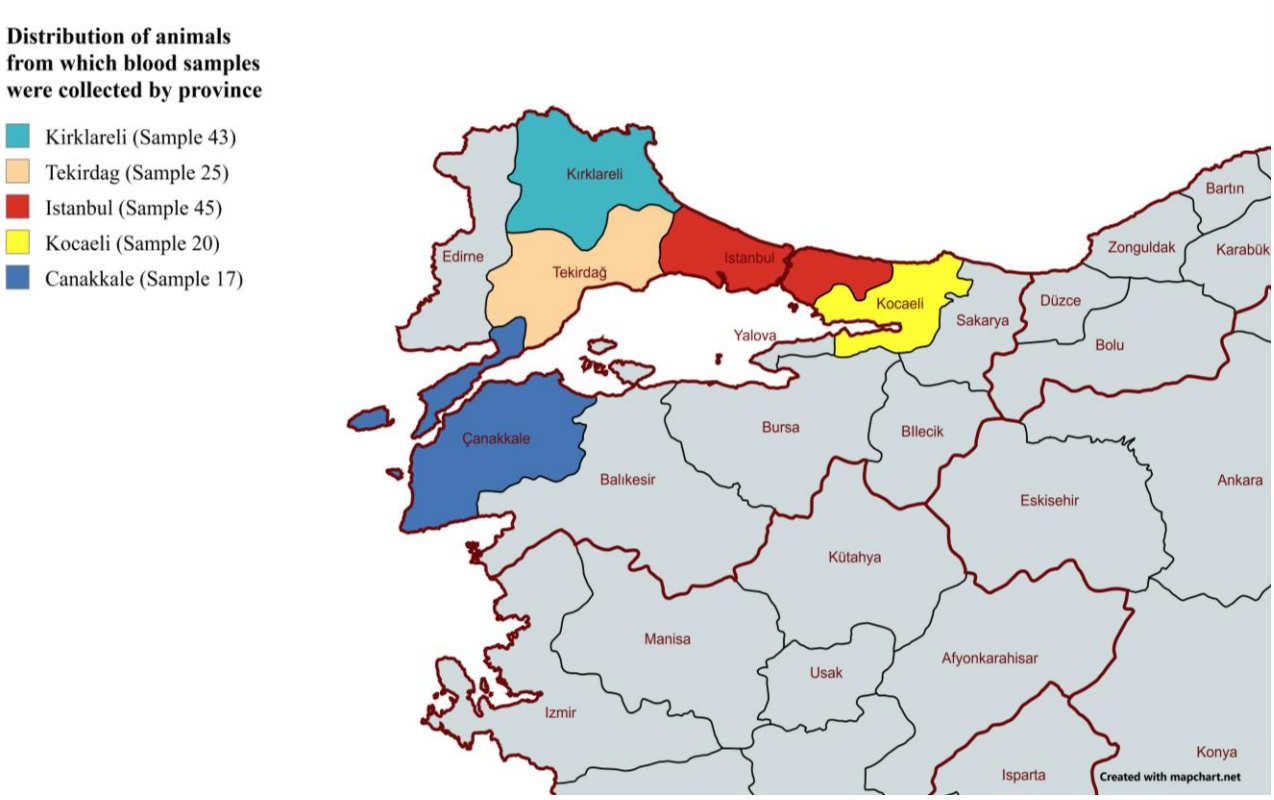


Figure 1. Distribution of sheep blood samples by province in Turkey. Source: Mapchart.net

Table 1. Distribution of sheep blood samples by breed and province, collected from slaughterhouses in Turkey between March 2022 and February 2023

Province	Numbers of serum samples	Sheep breeds and numbers	Percentage
Çanakkale	17	Kıvırcık (17)	11.33
İstanbul	45	Kıvırcık Cross (32) Merino (4) Merino Cross (1) Lacaune (5) Tahirova (3)	30
Kocaeli	20	Merino Cross (20)	13.33
Kırklareli	43	Kıvırcık (30) Kıvırcık Cross (13)	28.67
Tekirdağ	25	Merino (7) Merino Cross (18)	16.67
Total	150		100

Clinical examination

The medical histories and general physical examinations of 150 animals were carried out in the slaughterhouses. Animal owners were asked if the source flocks of these sheep had a history of Brucellosis diagnosis, if any abortion cases had been reported, and whether any animals demonstrated signs of lameness and orchitis. General physical examinations of animals were performed, and body temperatures were measured. The presence of inflammatory signs, such as color change, heat, pain, and swelling in joints or the testis, was evaluated visually and by palpation.

Serological analysis

A total of 150 serum samples were examined for the serological diagnosis of Brucella species with either smooth or rough cell wall structures, utilizing six different tests. The initial analyses involved rapid slide agglutination tests prepared with antigens derived from standard *Brucella* strains possessing smooth and rough cell wall structures, specifically the S-Rose Bengal plate test (S-RBPT) and R-Rose Bengal plate test (R-RBPT). Subsequently, tube agglutination tests were performed; the S-tube agglutination test (S-TAT) and the R-modified 2-mercaptoethanol tube

agglutination test (R-2ME-TAT). Finally, indirect ELISA (iELISA) was performed to detect smooth (S-iELISA) and rough (R-iELISA) *Brucella* strains (Alton et al., 1988; WOAH, 2025a). Among these methods, S-RBPT and S-TAT were commercially acquired. The remaining assays were prepared in-house, adhering to established protocols documented in the studies.

Reference strains and sera

Reference strains and control sera were obtained from two sources. The *B. canis* M [-] strain and positive and negative control sera for the S-RBPT, R-RBPT, S-TAT, and R-2ME-TAT assays were provided by the *Brucella* reference diagnostic laboratory at the Pendik Veterinary Control Institute Directorate, Turkey. Additionally, the Pendik Veterinary Control Institute Directorate, Turkey, supplied the commercial S-RBPT and S-TAT kits (lot numbers RBPT/02/2022 and TAT/02/2022), prepared using the smooth *B. abortus* S99 strain. The R-RBPT and R-2ME-TAT kits were prepared in-house using the *B. canis* M [-] strain, following the method of Alton et al. (1988). Positive and negative control sera for the S-iELISA and R-iELISA with *B. melitensis* B115 were sourced from the Laboratories of the Department of Microbiology, Faculty of Veterinary Medicine, Harran University, Şanlıurfa, Turkey. Additional positive control sera for the R-iELISA were sourced from rams previously diagnosed with *B. ovis* (Erdenliğ Gürbilek et al., 2018). Both the S-RBPT and R-RBPT employed stained antigens and were performed by mixing 50 µL of serum with the antigen on a clean glass slide, with agglutination evaluated within four minutes (Alton et al., 1988; WOAH, 2025a).

The tube agglutination tests, S-TAT and R-2ME-TAT, which were prepared with unstained antigens, were performed in glass tubes. Ten serial dilutions of each suspected serum were prepared in clean glass tubes for each test. Equal volumes (0.5 mL) of the respective test antigen were then added, and after incubation at 37°C for 24 hours, the results were evaluated (Alton et al., 1988; WOAH, 2025a).

For the S-iELISA, ready-to-use S-LPS with a smooth structure was obtained from the United Kingdom Animal and Plant Health Agency (APHA) and used to coat microplates (NUNC, 269620, Denmark). For the R-iELISA, the rough-form antigen was prepared from the *B. melitensis* B115 standard strain by isolating the R-LPS layer using a modified Tri-Reagent method, as described by Yi and Hackett (2000). Colonies of *B. melitensis* B115 grown on tryptic soy agar (Merck, Germany) under microaerobic conditions were collected from the agar surface using sterile phosphate-buffered saline (PBS), inactivated by heating in a water bath at 80°C for 30 minutes. The resulting killed bacterial suspension was subsequently centrifuged at 3500 g at 4°C to remove the supernatant (Yi and Hackett, 2000). The sediment of the killed bacterial pellet was collected. For each gram of bacterial pellet, 2 mL of Tri-Reagent was added. The mixture was incubated at room temperature for 10-15 minutes to ensure complete homogenization. Subsequently, 300 µL of chloroform per gram of bacterial pellet was added to induce phase separation. The suspension was vortexed, incubated for an additional 10 minutes, and centrifuged at 12000 g for 10 minutes to separate the supernatant from the organic phase. The supernatant was transferred to a new 1.5 mL centrifuge tube. In the organic phase, 100 µL of distilled water was added, followed by centrifugation at 12000 g for 10 minutes. The resulting supernatant was combined with the previous aqueous extract, and the crude LPS solution was stored at –20°C for subsequent use as the solid-phase antigen in ELISA (Yi and Hackett, 2000).

Before conducting these analyses, different concentrations of the smooth and rough antigens were prepared and tested through reciprocal titrations (checkerboard analysis) to identify the optimal antigen concentration. Antigen was diluted in carbonate-bicarbonate coating buffer (pH 9.6) to the specified concentration and dispensed into 96-well plates at 100 µL per well. Plates were incubated overnight at 4°C to facilitate antigen coating. After a single wash with PBS-Tween (phosphate-buffered saline containing 0.5% Tween 20), positive and negative control sera, as well as test sera from sheep, were diluted 1:100 and added to the wells as the primary antibody at 100 µL per well. Following another wash, HRPO-conjugated recombinant Protein A/G (Pierce 32490) was diluted 1:20000 in PBS-Tween and added to the wells. Following the additional washing steps, 100 µL of chromogenic substrate, comprising 2 µg of ortho-phenylenediamine in 0.1 M citrate buffer at pH 5.5 with 0.03 % H_2O_2, was added to each well. Plates were incubated at room temperature for 15 minutes. The reaction was terminated by adding 100 µL of 4 NH_2SO_4 to each well. Absorbance was measured at 490 nm using an automated ELISA reader (VERSAmax, USA). The ELISA cut-off values were defined as the mean optical density (OD) of negative sera plus three standard deviations (SD). To determine these values, each reference-positive serum was tested ten times via ELISA, each negative serum five times, and each brucellosis-positive serum ten times. The mean and standard deviation of the results were subsequently calculated.

Statistical analysis

Percentages were calculated using descriptive statistics in Microsoft Excel for Microsoft 365 (Microsoft Corporation, version 2024).

RESULTS AND DISCUSSION

According to the history and clinical findings, there were no previous diagnoses of brucellosis in the herds, and no cases of abortion, lameness, or orchitis were observed. The history indicated no signs of disease in the sheep herds. Additionally, clinical examinations of the sampled sheep exhibited no signs of illness or infection.

The use of R-iELISA on serum samples from sheep in Istanbul, Kocaeli, and Çanakkale yielded 15 positive results (10%), of which eight were confirmed positive by S-iELISA. Consequently, eight sera demonstrated positivity in both R-iELISA and S-iELISA assays. All positive findings marginally exceeded the established cut-off thresholds, indicating borderline results. All results from the slide and tube agglutination analyses were negative. Since eight serum samples tested positive on both S-iELISA and R-iELISA, these positive results were associated with smooth cell structures. Conversely, the positive results exclusive to R-iELISA were associated with rough cell structures. All breeds yielded positive results, yet this positivity was limited to sheep from just three provinces in Turkey. The serological findings are summarized in Table 2.

Table 2. Serum samples testing for ELISA, categorized by province of origin, sheep breed, collection period, and associated test results in Turkey

No	Sheep Breed	Province	Date	R-iELISA	S-iELISA	S-RBPT	R-RBPT	S-TAT	R-2ME-TAT
1	Merino	İstanbul	March 2022	P	P	N	N	N	N
2	Merino	İstanbul	March 2022	P	P	N	N	N	N
3	Lacaune	İstanbul	May 2022	P	P	N	N	N	N
4	Lacaune	İstanbul	May 2022	P	P	N	N	N	N
5	Merino	İstanbul	May 2022	P	P	N	N	N	N
6	Kıvırcık Cross	İstanbul	November 2022	P	N	N	N	N	N
7	Merino Cross	Kocaeli	July 2022	P	N	N	N	N	N
8	Merino Cross	Kocaeli	July 2022	P	N	N	N	N	N
9	Merino Cross	Kocaeli	July 2022	P	N	N	N	N	N
10	Merino Cross	Kocaeli	January 2023	P	N	N	N	N	N
11	Merino Cross	Kocaeli	January 2023	P	N	N	N	N	N
12	Kıvırcık	Çanakkale	March 2022	P	N	N	N	N	N
13	Kıvırcık	Çanakkale	March 2022	P	P	N	N	N	N
14	Kıvırcık	Çanakkale	March 2022	P	P	N	N	N	N
15	Kıvırcık	Çanakkale	January 2023	P	P	N	N	N	N

P: Positive, N: Negative, iELISA: İndirect enzyme-linked immunosorbent assay, RBPT: Rose Bengal plate test, TAT: Tube agglutination test

Brucella melitensis is the primary causative agent of small ruminant brucellosis, which can lead to significant economic losses in sheep and goat farming (WOAH, 2025a). Because it is also zoonotic, it can infect humans and is recognized as the dominant etiologic agent in human brucellosis (Çelik et al., 2023). *Brucella ovis* infects only rams, causing ovine epididymitis, and affects ewes, leading to infertility and culling in sheep flocks (WOAH, 2025b). *Brucella melitensis* and *B. ovis* are etiological agents that cause economic losses worldwide (WOAH, 2025a;b). The present study randomly sampled rams from different provinces and flocks, thereby improving diagnostic evaluation. Notably, Galluzzo et al. (2021) observed seropositivity predominantly in males among both rams and ewes in a *B. ovis*-positive flock, as detected by R-iELISA and the complement fixation test (CFT). The present results were consistent with the findings of Galluzzo et al. (2021), implying that male animals were more likely to yield positive serological findings.

The CFT, double agar gel immunodiffusion (AGID) test, and iELISA are among the most efficient and widely used methods for the serological diagnosis of *B. ovis* (WOAH, 2025b). Standard diagnostic techniques for *B. ovis* have been implemented in numerous countries. Although there is no international standardization, several independent validation studies have demonstrated that iELISA has higher sensitivity compared to the CFT or AGID tests (Nielsen et al., 2004; Praud et al., 2012; WOAH, 2025b). All serological assays used in the present study were among those recommended by the WOAH for detecting antibodies against smooth and rough *Brucella* strains. The iELISA was considered to be a more sensitive technique than other methods and practical for processing large numbers of samples (WOAH, 2025a;b). To detect antibodies produced against smooth *Brucella* strains, standard smooth *Brucella* strains can be used as antigens to coat iELISA plates. Alternatively, only the S-LPS or O-PS layers from the cell walls of these strains can be extracted and used as antigens (WOAH, 2025a). In a large-scale comparative study, Legesse et al. (2023) evaluated 2,317 serum

samples using an iELISA with S-LPS antigen alongside RBPT and CFT, reporting that the iELISA exhibited the highest specificity and sensitivity for diagnosing *B. abortus*. Consistent with these findings and the recommendations of WOAH (2025a), the present study used the standard smooth strain *B. abortus* S99 as the antigen in all tests designed to detect smooth antibodies, whereas the S-LPS layer derived from the same strain was specifically used as the antigen in the iELISA.

Several studies have assessed the effectiveness of ELISA-based methods in diagnosing *B. ovis* infection serologically (Chiarenza et al., 2018; Savić et al., 2018; Elderbrook et al., 2019). Pradu et al. (2012) examined 4,599 ram sera using a commercial iELISA kit and CFT and reported that iELISA exhibited high sensitivity with acceptable specificity, indicating that iELISA could be reliably employed in large-scale epidemiological studies. Similarly, Costa et al. (2016) compared several serological assays for *B. ovis* antibody detection and found ELISA to be the most sensitive. In a broader survey, Elderbrook et al. (2020) tested 2,276 sheep serum samples using two different ELISA techniques, detecting seropositivity rates of 0.88% and 1.10%, respectively, supporting the use of ELISA for population-level screening. More recently, Keskin et al. (2022) developed iELISA assays using four distinct antigenic agents of *B. ovis* and tested them on 84 sheep serum samples. Keskin et al. (2022) found that the results across these assays had no notable differences, indicating that different antigen preparations may produce similar diagnostic results. Consistent with previous studies, the current results demonstrated that the iELISA achieved the highest diagnostic sensitivity among the serological tests used, highlighting its importance as a main screening method for brucellosis in sheep populations.

The WOAH reported that iELISAs targeting S-LPS or O-PS as antigens were highly sensitive in detecting anti-*Brucella* antibodies in cattle, small ruminants, and pigs. It was noted that iELISAs using S-LPS may accidentally detect antibodies against the rough *B. abortus* RB51 vaccine due to shared antigenic structures. Furthermore, the problem of false positivity, especially in pigs, can be mitigated but not entirely eliminated by using iELISAs prepared with extracts from rough *Brucella* strains. Most false positives were reported to result from cross-reactions with the O-PS portion of the S-LPS molecule. Regarding false positivity, the brucellin skin test remains the most specific diagnostic method. Nonetheless, its use was restricted to countries that did not perform vaccination (WOAH, 2025a). The most commonly used method for preparing antigens from the *B. ovis* REO 1982 strain for the serological diagnosis of *B. ovis* infection is the hot saline extract, which obviated the need for carbon dioxide (WOAH, 2025b). The high-water solubility and abundance of cell-surface epitopes explained the excellent performance of *B. ovis* antigens in serological assays.

In areas with *B. melitensis* presence or where sheep were vaccinated with *B. melitensis* Rev.1, the accuracy of *B. ovis* diagnosis was assessed using the results of serological tests for smooth *Brucella* strains (WOAH, 2025b). In the present study as well, sheep sera were tested and evaluated together using different assays with smooth and rough antigens. The positive results observed in S- and R-iELISAs, which were marginally near the cutoff thresholds during the present study, indicated that these sheep may have been exposed, whether recently or historically, via environmental sources or colostrum. However, the probability that the detected antibodies are of maternal origin is minimal, considering the brief half-life of maternal antibodies (Watson, 1992). Several studies have reported that false-positive reactions in serological *Brucella* tests may result from cross-reactions or non-specific agglutination with other Gram-negative bacteria, including certain species of *Yersinia*, *Bordetella*, *Pseudomonas*, *Francisella*, and *Salmonella* (CFSPH, 2025). Additionally, there may be cross-reactions with other bacteria that possess similar R-LPS and S-LPS structures, such as *Dichelobacter nodosus*, *Arcanobacterium pyogenes*, *Corynebacterium ovis* (WOAH, 2025b), and *Yersinia enterocolitica* O:9 (WOAH, 2025a). Hurvell (1972) found serological cross-reactions between antibodies against *Yersinia enterocolitica* O:9 and four *Brucella* species, including *B. abortus*, *B. melitensis*, *B. suis*, and *B. neotomae*, using immunodiffusion and immunoelectrophoresis assays on agar media. All these strains demonstrated similar tendencies to cross-react. However, rough *Brucella* strains (*B. canis* and *B. ovis*) did not exhibit detectable cross-reactions with *Yersinia enterocolitica* O:9. Víctor et al. (2021), comparing crude *B. canis* RM6/66 antigen (CA-iELISA) with the immunodominant GroEL protein (GroEL-iELISA) for canine brucellosis, reported that while both assays demonstrated cross-reactions with *Leptospira interrogans*, the GroEL-iELISA additionally cross-reacted with *Salmonella* spp. In a more extensive evaluation, Yao et al. (2022) assessed iELISAs targeting *Brucella* outer membrane proteins (Omp31, BP26, Omp25) and a multi-epitope fusion protein, using 34 *Brucella*-positive and 62 *Brucella*-negative canine sera. They found that BP26 and Omp31 exhibited high sensitivity but cross-reacted with *Vibrio parahaemolyticus* and *Listeria monocytogenes*, whereas Omp25 demonstrated poor sensitivity and discriminatory ability. The multi-epitope fusion protein, despite exhibiting mild cross-reactions with *Escherichia coli* H7, *Salmonella*, and *Vibrio parahaemolyticus*, exhibited the best overall performance and was identified as the best antigen for worldwide serodiagnosis of canine brucellosis. These findings underscored the importance of selecting appropriate antigens to optimize serological diagnosis. Nielsen et al. (2004) compared serological assays for detecting antibodies against different bacteria and reported that cross-reactions among *E. coli* O157:H7, *Yersinia enterocolitica* O:9, and *B. abortus* could occur depending on the test used. Consequently, extensive cross-reactivity was observed in iELISA,

whereas reactivity was much lower in the fluorescent polarization assay (FPA) and competitive ELISA (cELISA). In the present study, it was considered that all eight positive results obtained by both iELISAs were likely due to exposure to smooth-structured bacterial agents, either *Brucella* or other microorganisms with similar structures. This result was due to S-LPS containing O-PS and shared antigenic structures with R-LPS. In contrast, the eight samples that were positive only on R-iELISA were generally considered indicative of *B. ovis* infection, with a lower likelihood of cross-reactivity with other rough-structured bacteria that share similar epitopes.

Consistent with the present study, which used heterologous antigens from *B. canis* and *B. melitensis* B115, numerous studies have supported the use of heterologous rough *Brucella* strains as test antigens for the serological diagnosis of *Brucella* with rough cell walls. Perletta et al. (2023) demonstrated that serological tests utilizing *B. ovis* antigens are effective for diagnosing *B. canis* in dogs. Additionally, Escobar et al. (2010) found that rough strains of *B. canis*, *B. ovis*, and *B. abortus* RB51 were suitable for diagnosing *B. canis*. Due to shared antigenic structures between *B. ovis* and *B. canis*, ELISAs developed with *B. ovis* antigens can detect *B. canis* infection (López et al., 2006). They noted that *B. canis* antigens could serve as a complementary tool to official tests for diagnosing *B. ovis* in sheep flocks.

CONCLUSION

The detection of iELISA-positive results near the cut-off threshold, along with the age of sheep (≤ 1 year), indicated a low likelihood of active *Brucella* infection with either smooth or rough strains. However, the detection of low-titer anti-*Brucella* antibodies did not entirely exclude prior exposure to *Brucella* agents or previous infections in these flocks, and the epidemiological importance of this result should be carefully evaluated. The combined use of serological analyses encompassing both smooth and rough cell wall structures was an effective approach, significantly enhancing diagnostic accuracy. Using multiple assays together significantly enhances the accuracy of interpreting results. For the serological diagnosis of brucellosis in sheep, it is strongly recommended to utilize antigens derived from both the smooth and rough cell wall structures. Active surveillance in sheep flocks is essential for identifying and preventing the spread of brucellosis, and future studies should focus on diagnosing brucellosis caused by *B. melitensis* and *B. ovis*. Furthermore, additional studies should be conducted across different regions of Turkey to yield more reliable results.

DECLARATIONS

Authors' contributions

Ahmet Murat Saytekin, Hüsamettin Avcı, Ayfer Güllü Yücetepe, Enikö Kiraly-Avcı, Kemal Metiner, and Sevil Erdenliğ Gürbilek have contributed to the investigation, methodology, formal analysis, writing, and reviewing the manuscript. Ramazan Gül and Ayda Nur Aksoy have contributed to the investigation, writing, and editing of the manuscript. All authors have read and approved the final edition of the manuscript.

Availability of data and materials

All data supporting the findings of this study may be requested upon reasonable justification from corresponding author.

Competing interests

The authors declared no competing interests.

Ethical considerations

All authors confirmed that this study is an original article and has not been published before, nor is it under consideration elsewhere. No AI tools were used for preparing and writing the present study. The authors accept the responsibility for the originality of the study in the entire process of publication.

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The Implementation of Biosecurity Measures in Poultry Farms in Haut-Bassins Region, Burkina Faso

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ABSTRACT

Poultry farming is an important socio-economic sector in Burkina Faso. However, disease outbreaks can substantially impact livestock production, so appropriate preventive measures should be implemented. The present study aimed to assess the implementation of biosecurity measures in poultry farms in the Haut-Bassins Region, Burkina Faso. The present cross-sectional study included 79 poultry farms and used a validated questionnaire. Questions were asked of each poultry farmer through interviews, and farms were observed to verify the implementation of certain biosecurity measures, including the presence and quality of foot baths, the presence of stagnant water, the orientation of the buildings, and the presence of gates. The current results indicated that the majority of farms were managed by men (86.1%), and 45.6% of farmers had more than 10 years' experience. Regarding farm capacity, 68.4% could house up to 10,000 poultry, and 64.6% of the reported farms had facilities that could accommodate more than 1,000 poultry. Over 59.5% of farms were located more than 300 meters away from the main road; however, 64.6% were located less than 500 meters from the nearest poultry farm. More than 97.5% of farms were enclosed by fences, and 87.3% of these fences were made from waterproof materials. On farms, 60.8% had functional footbaths, 11.4% produced poultry feed, and 92.4% were not protected against wild birds entering their airspaces. Most farms (97.5%) regularly cleaned and disinfected materials and equipment, but only 38% had suitable waste disposal practices, and 22.8% kept water waste outside the farm. The present study reported that 56.9% of farms implemented a satisfactory level of biosecurity. Farmers who attended poultry farming training were more likely to apply biosecurity practices on their farms. The present study delineated the application of biosecurity measures on farms and emphasized the importance of raising awareness about biosecurity among poultry farmers in the Haut-Bassins Region of Burkina Faso.

Keywords: Biosecurity, Implementation, Poultry farm, Survey, Questionnaire

INTRODUCTION

In Burkina Faso, poultry farming is an important socio-economic sector and represents the primary source of animal protein (Dione et al., 2025). Poultry production is a significant source of income for rural and urban populations throughout all regions of Burkina Faso and plays an important role in ensuring food security (MRA/DGSV, 2006). The livestock sub-sector contributed between 18.3% and 19.5% to the gross domestic product, ranking second after cotton (MRA, 2011). Although livestock production contributes less to the country's economy than gold and cotton production, it still plays an important socio-economic role (FAO, 2018). In Burkina Faso, poultry meat accounts for around 16.5% of total meat production, with estimated per capita consumption of about 8 kg of poultry meat and 1 kg of eggs per year (FAO, 2018). In 2021, national chicken production was estimated at more than 50,413,102 heads (INSD, 2021). Among the different regions of Burkina Faso, the Hauts-Bassins region ranks third in poultry production, with around 6,115,920 poultry heads in 2020 (INSD, 2021). Burkina Faso has two primary livestock production systems; an active traditional and semi-traditional sector, which accounts for 90% of the livestock and is mainly operated by small farmers in rural and semi-urban areas, and a modern sector centered around the country's main cities (MRAH, 2019). However, the development of poultry production is closely linked to proper control of poultry diseases (Adesola et al., 2025). In Burkina Faso, many poultry diseases have been reported, including infectious bursal disease, Newcastle disease, avian coccidiosis, avian pox, and highly pathogenic avian Influenza (HPAI; FAO, 2008), which led to significant mortality and economic losses on farms. Therefore, effective implementation of biosecurity measures can help reduce the risk of pathogens entering farms (Van Limbergen et al., 2018). To date, few studies have examined biosecurity in Burkina Faso.

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Despite the importance of poultry farming in the Hauts-Bassins region, no study has assessed the implementation of biosecurity measures in poultry farms.

The present study aimed to assess the implementation of biosecurity protocols in poultry farms and identify factors associated with biosecurity levels in urban and peri-urban poultry farms in Bobo-Dioulasso, Burkina Faso.

MATERIAL AND METHODS

Ethical approval

Ethical approval was obtained from the Research Ethics Committee of the University Joseph KI ZERBO (agreement reference CE-UJKZ/2020-03). In addition, before interviews, farmers were informed of the study's context and purpose, emphasizing that participation was voluntary and that collected data would remain confidential. Therefore, only poultry farmers who provided consent to participate were included in the study.

Study area and period

The current investigation was conducted from August to November 2022 in the Haut-Bassins region, Burkina Faso, in the communes of Bama, Péni and Bobo-Dioulasso (Figure 1). Bobo-Dioulasso is the region's capital, located in Southwestern Burkina Faso, 365 km from the capital, Ouagadougou, and 125 km from the Mali border. It covers an area of 1,102 km^2 and has one urban commune, 12 rural communes, and 205 villages (INSD, 2021). It is limited to the North by the province of Banwa, to the South by the province of Comoé, and to the East and Southwest by the provinces of Kénédougou and Bougouriba, respectively. The region ranks third in poultry production in Burkina Faso, after the Boucle du Mouhoun and Centre-Ouest regions. The poultry population of the region was estimated at more than 6 million heads in 2020 (INSD, 2021). Poultry production was mainly characterized by traditional and semi-modern systems for local and mixed poultry species. Modern systems are predominantly located near urban areas and focus on egg and broiler production.

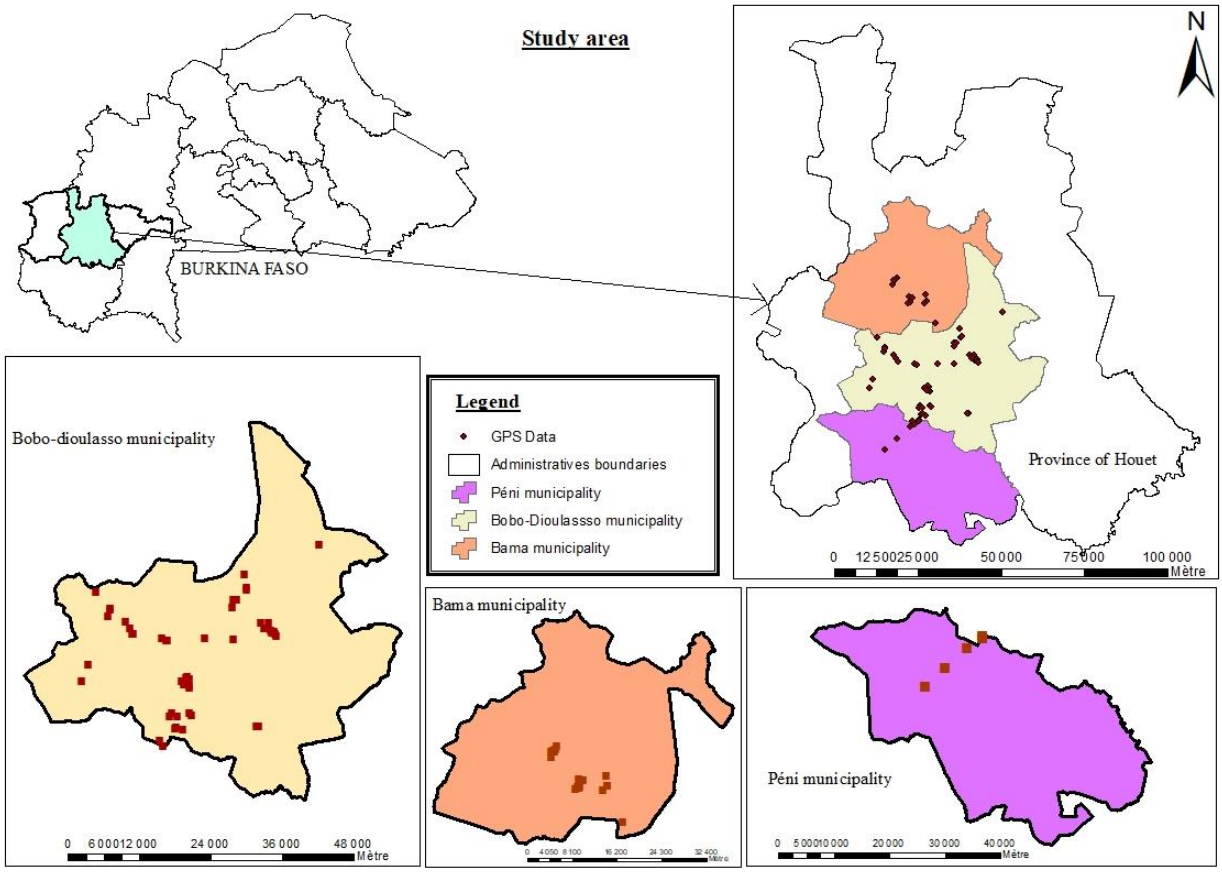


Figure 1. The study site is located in the Haut-Bassins region, Burkina Faso

Sampling and data collection

Poultry farms were selected using snowball sampling within a 30 km radius of Bobo-Dioulasso because of security concerns and limited road connectivity. A total of 79 poultry farms participated in the survey. The questionnaire was

developed from the questionnaire used by Ismael et al. (2021) and validated through a preliminary survey of 10 poultry farmers in the region. The pre-survey assessed participants' understanding of the questions and the interview duration. After the pre-survey, the authors revised the questionnaire based on field feedback and confirmed its validity. A total of 79 farmers were interviewed face-to-face in either their local language or French, based on their understanding, using validated questionnaires (Moore or Dioula). Respondents included farm managers and, when farm managers were absent, senior on-site staff. In addition to the interviews, infrastructure and documents were observed, and distances and building dimensions were measured with a tape measure. The questionnaire was divided into three sections focused on socio-economic characteristics of farm owners, general characteristics of farms, such as ownership of the premises, the type of farming, the number of buildings, the capacity of the farm, and the presence of other species, and conceptual, structural, and operational biosecurity indicators, including location of the farm and orientation of buildings, material used for buildings, feeding and watering equipment, as well as access rules and management of the farms.

Statistical analyses

Data were collected using KoBoCollect and exported to a Microsoft Office 2016 Excel spreadsheet. The data collected were processed and coded. Interview responses were scored according to their accuracy. All biosecurity assessment questions were designed to be answered yes when the assessed parameter was present and no when it was absent. Moreover, when the answer had a positive effect on biosecurity, a score of 1 was assigned; otherwise, the score was 0. Overall, a poultry farmer could attain the highest possible score of 74 by applying all biosecurity indicators assessed in the present study. Farms that achieved a total score of at least 50% (37/74) were considered to be applying a satisfactory level of biosecurity, and those with a total score below 50% were considered to be implementing an unsatisfactory level of biosecurity (Ismael et al., 2021).

Statistical analyses were carried out using R software version 3.5.2. The chi-square test was used to evaluate the association between a satisfactory level of biosecurity and different explanatory variables, such as farm capacity, age of the farm, level of owner education, and location of the farm. For all statistical analyses, the significance level was set at p-value less than 0.05.

RESULTS

Socio-economic characteristics of poultry farmers

Data on the characteristics of poultry farm owners in the study area were reported in Table 1. Of the 79 farmers interviewed, 68 (86.1%) were men, 56 (70.9%) were aged 30 to 50, and 44 (55.7%) held higher education degrees. A total of 36 (45.6%) farm owners practiced full-time poultry farming, while 50 (63.3%) farmers had completed at least one biosecurity training course. In the present study, 61 (77.2%) farmers had 10 years or less of poultry farming experience.

Table 1. Characteristics of poultry farm owners in the Hauts-Bassins region, Burkina Faso, 2022

Variables	Frequency number (%)
Gender	
Woman	11 (13.9)
Man	68 (86.1)
Age class	
Less than 30 years old	10 (12.7)
Between 30 and 50 years old	56 (70.9)
More than 50	13 (16.5)
Level of study	
Primary	5 (6.3)
Secondary	17 (21.5)
Higher education	44 (55.7)
No formal education	13 (16.5)
Main activities	
Full-time poultry farmer	36 (45.6)
Non-agricultural entrepreneur	22 (27.8)
Official	18 (22.8)
Retirement	3 (2.8)
Experience of poultry farmers	
10 years or less	61 (77.2)
More than 10 years	18 (22.8)
Biosecurity training	
Yes	50 (63.3)
No	29 (36.7)

Characteristics of poultry farms

Overall, 75 (94.9%) participants owned their farms, 22 (27.8%) had medium-sized buildings (500-1000 poultry), and 68.4% of farms had multiple buildings with a total production capacity between 1000 and 10,000 poultry. Three main poultry species were identified on farms, including laying hens (83.5%), broiler chickens (24.0%), and local chickens (20.2%). The characteristics of the farms surveyed were presented in Table 2.

Conceptual biosecurity measures

Among the farms, 47 (59.5%) were located more than 300 m away from the main road. However, 64.6% of farms were constructed within 500 meters of the nearest farm (Table 3). The present results demonstrated that 73.4% of farms had buildings less than 20 m from staff living facilities, while only 31.6% of farms were located more than 200 m from the nearest dwellings. Most farms (82.3%) had no stagnant water nearby, 97.5% had mesh fences, and 87.3% were built using impermeable material. In the present study, 81% of buildings were oriented perpendicular to the prevailing winds. Additionally, 69.6% of the farms reported the presence of carnivores and several bird species, including ducks, geese, and guinea fowls.

Structural biosecurity measures

The present study reported that none of the farms had a hatchery; only 5.1% had a functional tire bath, 11.4% produced their own feed, 60.8% had a functional footbath, and 45.6% had complete fencing. Moreover, 48.1% of farms had a gate at the entrance, and among those farms, 73.7% did not keep their gates closed. Among surveyed farms, 26.6% prohibited vehicle access, 94.9% had specific materials and equipment for each poultry building, and 86.1% had a process to control the quality of water used for drinking and cleaning. The current findings reported that 24.1% of farms applied measures to control wild animals and rodents. In 84.4% of farms, wild animals have access to litter. Table 4 reported data on the application of structural biosecurity measures.

Operational biosecurity measures

The present study reported that 31.6% of farms had staff uniforms and 41.8% had staff work shoes. The current results indicated that 2.5% of farms had implemented proper signage, 38.0% had established adapted dumping sites, 22.8% used a high-pressure sprayer during cleaning, 22.8% discharged wastewater outside the farm, 20.3% prohibited staff from contacting other farms, and 82.4% ensured the isolation of sick animals. Among farms, 73.4% contacted a veterinarian or an animal health specialist when sick animals were reported on their farms, and 97.5% of farms were vaccinated against Newcastle disease. Table 5 reported detailed data on operational biosecurity indicators.

Factors associated with a satisfactory level of biosecurity on poultry farms

The present results demonstrated that 56.9% of farms had a satisfactory level of biosecurity. An adequate level of biosecurity was significantly correlated with poultry training ($p < 0.05$), with 72.3% of poultry farmers trained to implement a satisfactory level of biosecurity. Biosecurity level was not significantly associated with farmer experience, farm capacity, farm age, number of buildings, owner gender, owner age, study level, or study location ($p > 0.05$).

Table 2. Characteristics of poultry farms in the Hauts-Bassins region, Burkina Faso, 2022

Variables/modalities	Frequency number (%)
Farm ownership status	
Rental	4 (5.1)
Property	75 (94.9)
Building capacity	
Small scale (< 500)	6 (7.6)
Medium scale (500-1000)	22 (27.8)
Large scale (> 1000)	51 (64.6)
Farm capacity	
Small scale (< 1000)	14 (17.7)
Medium scale (1000-10000)	54 (68.4)
Large scale (> 10000)	11 (13.9)
Farm type *	
Laying hens	66 (83.5)
Broiler chicken	19 (24.0)
Local chicken	16 (20.2)

*In some farms, laying hens, broiler chickens, and local chickens were found.

Table 3. Conceptual biosecurity indicators in poultry farms in the Hauts-Bassins region, Burkina Faso, 2022

Conceptual biosecurity indicators	Frequency number (%)
Distance to main road	
0-50 meters	10 (12.7)
50-100 meters	7 (8.9)
100-300 meters	15 (19.0)
> 300 meters	47 (59.5)
Distance to neighboring farms	
< 500 meters	51 (64.6)
> 500 meters	28 (35.4)
Distance between buildings and dwellings on the farm	
0-20 meters	58 (73.4)
20-100 meters	13 (16.5)
> 100 meters	8 (10.1)
Distance between the farm and the nearest townhouse	
0-20 meters	11 (13.9)
20-200 meters	43 (54.4)
> 200 meters	25 (31.6)
Presence of stagnant water near the farm	
Yes	14 (17.7)
No	65 (82.3)
Building's orientation	
Perpendicular to the prevailing winds	64 (81.0)
Parallel to the prevailing winds	15 (19.0)
Construction of buildings using waterproof material	
Yes	69 (87.3)
No	10 (12.7)

Table 4. Structural biosecurity indicators in poultry farms in the Hauts-Bassins region, Burkina Faso, 2022

Structural biosecurity indicators	Frequency number (%)
Presence of a complete fence	36 (45.6)
Presence of a hatchery	0 (0)
Presence of a gate at the entrance	38 (48.1)
Presence of a gate always closed	28 (73.7)
Presence of functional footbaths	48 (60.8)
Vehicle access prohibited on the farm	21 (26.6)
Existence of a single vehicle entry point	36 (45.6)
Presence of tire bath/spray at the gate	4 (5.1)
Registration of visitors at the entrance to the farm	8 (10.1)
Feed production on the farm	9 (11.4)
Material and equipment specific to each building	75 (94.9)
Farm-specific vehicles	36 (45.6)
Prohibition of access to pets	32 (40.5)
Existence of a permanent rodent control mechanism	19 (24.1)
Existence of a wild animal control mechanism	19 (24.1)
Access of wild birds to buildings	73 (92.4)
Access of wild birds to used litter	67 (84.8)
Access of wild birds to used food	6 (7.6)
Inaccessibility of food to other farm animals	79 (100.0)
Staff informed of the emergence of an illness	62 (78.5)
Control of drinking water	68 (86.1)
Control of cleaning water	68 (86.1)

Table 5. Frequency and percentage of operational biosecurity indicators in the Hauts-Bassins region, Burkina Faso, 2022

Operational biosecurity indicators	Frequency number (%)
Presence of a poultry house per building	74 (93.7)
Use of work attire	25 (31.6)
Use of work shoes	33 (41.8)
Using a work mask	5 (6.3)
Use of work cap	0 (0.0)
Regular washing of work clothing	28 (35.4)
Prohibition of access for non-professional visitors to the building	48 (60.8)
Existence of special clothes for visitors	7 (8.9)
Existence of special shoes for visitors	12 (15.2)
Presence of signage on the farm	2 (2.5)
Poultry raised in an identical age group	74 (93.7)
Applying care starting with the youngest poultry	74 (93.7)
Application of the All-in and All-out system on the farm	73 (92.4)
Regular cleaning and disinfection	77 (97.5)
Disposal of wastewater outside the farm	18 (22.8)
Building cleaning using high-pressure spray	18 (22.8)
Presence of a suitable storage location for waste	30 (38.0)
Prohibiting access of drivers to poultry building	5 (6.3)
Banning staff contact with other poultry farms	16 (20.3)
Proper management of dead birds	61 (77.2)
Storage of soiled litter in a suitable place	54 (68.4)
Appropriate treatment of new litter before using it on the farm	67 (84.8)
Application of sanitary downtime	73 (92.4)
Contact ban with neighboring farms	11 (13.9)
Absence of other species on the farm	44 (55.7)
Absence of pet birds on the farm	40 (50.6)
Isolation of sick animals	65(82.3)
Veterinarian intervention in case of illness	58 (73.4)
Regular serological monitoring of major poultry diseases on the farm	3 (3.8)
Practice of vaccination against Newcastle disease	77 (97.5)
Practice of vaccination against infectious bronchitis	75 (94.9)
Practice of vaccination against infectious bursal disease	76 (96.2)
Practice of vaccination against avian pox	70 (88.6)
Compliance with vaccination instructions	71 (89.9)
Recording of health data	68 (86.1)

Table 6. Factors associated with a satisfactory level of biosecurity in poultry farms in the Hauts-Bassins region, Burkina Faso, 2022

Variables	Number	Frequency number of those with a satisfactory level (%)	p-value
Farm capacity			
Small	14	5 (35.7)	0.068
Medium	54	31 (57.4)	
Large	11	9 (81.8)	
Age of farm			
0-5 years	46	24 (52.2)	0.462
5-10 years	18	10 (55.6)	
> 10 years	14	10 (71.4)	
Poultry training			0.0008
Never	32	11 (34.4)	
Yes	47	34 (72.3)	
Experience in the activity			0.452
10 years and less	61	32 (52.4)	
More than 10 years	18	13 (72.2)	
Number of buildings			
Less than 5	70	39 (55.7)	0.532
More than 5	9	6 (66.7)	
Gender			
Woman	11	7 (63.6)	0.749
Man	68	38 (55.9)	
Age			
< 30 years	10	6 (60.0)	0.251
30-50	56	29 (51.8)	
> 50	13	10 (76.9)	
Study			
Formal education	66	38 (57.6)	0.803
No formal education	13	7 (53.8)	
Location			
Bobo Dioulasso and surrounding areas	25	14 (56.0)	0.906
Far from Bobo-Dioulasso	54	31 (57.4)	

DISCUSSION

The study provided a comprehensive understanding of the biosecurity measures implemented in the Haut-Bassins Region, Burkina Faso. The present findings revealed that farms are mainly managed by men. Some socio-cultural factors, such as patriarchal family management in Burkina Faso, mean men are given the right to be responsible for and manage the family (Gueye, 1998). The average age of poultry farmers was between 30 and 50 years old. Most farmers prioritized poultry farming as their primary activity due to its profitability. Many farmers had experience in poultry farming, while a few had participated in at least one biosecurity training provided by private veterinarians, breeding technicians, or occasionally managers from foreign pharmaceutical companies. Laying hens were the most important breeding animals on farms, consistent with the findings of Tsegaye et al. (2023) in the Arsi and East Showa zones of Ethiopia. In the present study, most buildings had a capacity above 1000, and farm capacity ranged from 1000 to 10,000 individuals. The current findings were lower than those reported by Kouam et al. (2018) in the Western Highlands of Cameroon in Central Africa, where an average flock size of 1181.37 was documented. According to the conceptual biosecurity indicators, the majority of farms were located more than 300 m from the main road. The finding was

considered satisfactory given the elevated risk of pathogen contamination via ambient air, particularly during poultry transport on public roads, including *Mycoplasma* spp. and Newcastle disease virus, which can be transmitted by wind and remain infectious for a long time (Levisohn and Kleven, 2000). Elbers et al. (2022) reported that keeping farms far from main roads is a protective measure that reduces contamination of poultry by windborne pathogens, which is consistent with the present findings. Farms were generally located within 500 meters of each other, which contradicted the recommendation of Gelaude et al. (2014), who stated that farms should be at least 500 meters apart. Most farms were located close to dwellings, between 20 and 200 m, increasing the risk of contamination and hygiene and public health issues for surrounding populations exposed to pathogens from poultry farms. However, Poulsen et al. (2018) recommend greater distances between farms and neighboring homes to reduce contamination risk. Many farms in the present study were not located near stagnant water, which was inconsistent with the study of Kouam et al. (2018), who reported that most farms in Cameroon were located less than 500 m from a watercourse. Because rivers and stagnant water can act as long-term environmental reservoirs that facilitate the spread of infectious bursal disease virus to new farms, keeping farms near watercourses increases the risk of disease transmission (Ahmad et al., 2022).

Therefore, it is imperative to eliminate stagnant water near agricultural areas and establish farms at a considerable distance from watercourses, which also serve as drinking sites for wild birds that may carry important pathogens, including HPAI virus (Fang et al., 2026). In the present study, most buildings were constructed with impermeable materials, and most farms had wire mesh fences. In the present study, buildings were generally well oriented perpendicular to the prevailing winds, which helped reduce humidity and temperature. Supporting the present findings, Oloyo and Ojerinde (2019) reported that this orientation reduced the sidewall exposure to direct sun radiation and limited heat stress in birds. Few farms had a functional tire bath at the entrance, but most had a functional footbath at the entrance of poultry buildings. The limited use of tire baths may be due to poultry farmers' neglect or the cost of installing this equipment, increasing the risk of pathogen entry on farms through vehicle movement from outside (Dorea et al., 2010). Similar observations have been documented in Côte d'Ivoire, where footbaths were reported in only 30.3% of farms (Kadja et al., 2021). Nasr et al. (2018) emphasized the importance of footbath disinfection and designated footwear and clothing on farms prior to entering poultry houses to minimize the risk of pathogen transmission via humans. Rodent and wild-animal control was poorly implemented, as only a few farms safeguard their buildings from domestic and wild birds and animals; therefore, farms may have been exposed to pathogens, as wild birds can carry pathogens, including Newcastle disease virus, *Mycoplasma* spp., *Campylobacter* spp., and *Mycobacterium avium*, which are transmissible to poultry (Chaves Hernández, 2014).

The lack of farm-specific footwear and apparel for both staff and visitors was a notable deficiency. Comparable findings have been reported in Cameroon (Kouam et al., 2018) and Ethiopia (Tsegaye et al., 2023), where there was an absence of designated shoes and clothing for personnel. Poor waste management has been observed in the present study, which was linked to the lack of space for waste storage and treatment. Improper storage of poultry waste, such as manure, dead birds, and litter, can cause serious infections, including those caused by *Salmonella*, *Escherichia coli*, and the HPAI virus (Mozhiarasi and Natarajan, 2022; Rahman et al., 2022). Consistent with the present findings, Ismael et al. (2021) reported that, in Ethiopia, 61.36% of farms lacked a suitable place to dispose of poultry litter and waste. In the present study, only a limited number of farms had signage restricting access to certain areas of their farm, aligning with findings of Ismael et al. (2021) in Ethiopia. Without signage, drivers generally had direct access to buildings, increasing the risk of external pathogen introduction (Tsegaye et al., 2023). In the present study, 45 farms (56.9%) had a satisfactory biosecurity level. Gemeda et al. (2026) reported similar findings, noting that 61.25% of farms in Sebeta town, Ethiopia, achieved a biosecurity score above 50%. However, Ismael et al. (2021) found that only 25% of farms in Bishoftu Town, Ethiopia, implemented adequate biosecurity practices. The satisfactory biosecurity level was notably associated with staff training in poultry farming. In the present study, farmers who attended biosecurity training implemented biosecurity practices at much higher rates than those who had not been trained.

CONCLUSION

The present findings indicated that farms in the Hauts-Bassins region of Burkina Faso implemented different biosecurity practices, but only 56.9% of farms had a satisfactory biosecurity level. The current results indicated the need to train poultry farmers and assess the relationship between biosecurity measures and farm infection. The main limitation of the study was the inaccessibility of certain areas of the region due to insecurity and poor road conditions. Future studies should assess the impact of biosecurity measure implementation on the prevalence of diseases such as Newcastle disease, avian colibacillosis, and avian coccidiosis.

DECLARATIONS

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Authors' contributions

Laibané Dieudonné Dahourou designed the study. Bali Joël Wilfried Bama, Madi Savadogo, and B. Ido Apollinaire Bako conducted the field survey and drafted the statistical analysis. Laibané Dieudonné Dahourou verified the statistical analysis, which was validated by Amadou Traore and Mireille Catherine Kadja Wonou. Laibané Dieudonné Dahourou and Bali Joël Wilfried Bama prepared the draft of the manuscript. All authors contributed to revising the manuscript. All authors read and approved the final edition of the manuscript.

Availability of data and materials

The data from the present study are available upon reasonable request from the corresponding author.

Competing interests

The authors declare no competing interests.

Ethical considerations

All authors thoroughly reviewed the ethical considerations, including issues related to plagiarism, consent for publication, misconduct, data fabrication and/or falsification, duplicate publication and/or submission, and redundancy before submission. No AI tools/applications were used to conduct, write, or prepare any part of the study. The authors confirmed the data's originality before submission and after publication.

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Effects of *Cinnamomum verum* Supplementation on Physicochemical and Sensory Properties of Goat Milk Yogurt

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ABSTRACT

Yogurt made from goat's milk has a pungent odor that persists in the product. Cinnamon is high in cinnamaldehyde, a compound responsible for its sweet, slightly spicy flavor, distinctive aroma, and warming sensation, all of which can enhance the flavor of goat milk yogurt. The present study aimed to evaluate the physicochemical and sensory properties of goat milk yogurt products supplemented with cinnamon. A completely randomized design was employed, with five treatment groups and four replications for each. The control group included goat milk yogurt without cinnamon, and the remaining treatment groups included goat milk yogurt with cinnamon at concentrations of 0.1%, 0.2%, and 0.3%. The physicochemical characteristics, including fat content, moisture content, viscosity, pH, and acidity, as well as sensory attributes (color, aroma, taste, texture, and overall acceptability) were evaluated. The present results indicated that different cinnamon concentrations influenced the fat content, moisture level, and pH of the yogurt, but did not affect its acidity or viscosity. Sensory evaluation of the goat milk yogurt demonstrated that cinnamon addition impacted the yogurt's color, aroma, taste, texture, and overall acceptability. The 0.2% cinnamon increased fat and pH levels, decreased moisture, and did not alter acidity or viscosity. The current findings suggest that 0.1% cinnamon enhanced the aroma of goat milk yogurt without reducing acceptability, whereas higher concentrations of cinnamon may be less favored.

Keywords: Cinnamon, Goat milk, Physicochemical, Sensory, Yogurt

INTRODUCTION

Yogurt is a dairy product produced through the fermentation of milk by lactic acid bacteria (LAB; Ichimura, 2024; Liu and Peng, 2024). Milk used in yogurt production can be derived from different animals, including cows, goats, and buffaloes, each imparting unique physicochemical and sensory qualities to the final product (Andiç et al., 2026; Jankowska et al., 2026). Cow's milk is widely used as the primary substrate in yogurt production; however, milk from other species, such as goats, may serve as an alternative raw material, possessing distinctive physicochemical properties (Wang et al., 2023; Demirhan et al., 2026). Goat milk has been reported to offer several health benefits due to its bioactive compounds, including potential benefits for cardiovascular health, lipid metabolism, and immune function (dos Santos et al., 2023). Goat's milk has several advantages, including a high content of essential minerals such as calcium and phosphorus, as well as vitamins A, E, and B complex. In addition, goat's milk contains bioactive compounds with natural antimicrobial properties, such as lactoferrin-derived peptides and other bioactive peptides (ALKaisy et al., 2023; dos Santos et al., 2023).

Nevertheless, goat milk is frequently associated with a distinctive goat aroma, primarily due to short-chain fatty acids, which can reduce consumer acceptability (Clark and García, 2017). Flavor plays a critical role in the consumer acceptability of dairy products; thus, natural flavoring substances are commonly added to improve the sensory quality of goat milk products (Li et al., 2024). Cinnamon (*Cinnamomum verum*) is a potential natural additive, known for distinctive aromatic compounds such as cinnamaldehyde and eugenol, which contribute to its warm, pleasant flavor. These compounds can reduce unpleasant odors and improve the overall sensory acceptability of dairy products (Alonso et al., 2024).

Cinnamomum verum is widely recognized as a culinary spice employed internationally, including in Indonesian cuisine, where it serves as a flavor-enhancing ingredient across various food products (Rao and Gan, 2014; Spence,

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2024). In addition to its culinary applications, cinnamon is rich in phenolic and volatile bioactive compounds, including cinnamic acid, eugenol, and linalool, which are known for their potent antioxidant properties (Guo et al., 2024; Karimirad et al., 2025). Cinnamon contains essential oil compounds such as cinnamaldehyde and eugenol, which contribute to a sweet, spicy aroma and a warming sensation (Ranasinghe et al., 2013). Flavonoids are widely recognized for their diverse biological activities, including antioxidant, antimicrobial, anti-inflammatory, antifungal, and antidiabetic effects (Shamsudin et al., 2022; Hao et al., 2024). Therefore, *Cinnamomum verum* extract can be used as an alternative food additive to reduce the pungent aroma of goat milk in yogurt production. The present study aimed to investigate the physicochemical and organoleptic characteristics of goat milk yogurt containing *Cinnamomum verum*.

MATERIALS AND METHODS

Ethical approval

The experimental laboratory study was designed to evaluate the physicochemical and sensory qualities of goat milk yogurt with the addition of cinnamon extract. The present study was conducted in accordance with the guidelines of the Department of Animal Husbandry, Faculty of Agriculture, University of Lampung, Indonesia.

Study materials

The materials used in the present study included fresh goat milk collected from CV Telaga Rizky Metro City, Lampung Province, a commercial starter culture containing *Lactobacillus bulgaricus*, *Streptococcus thermophilus*, *Lactobacillus acidophilus*, and *Bifidobacterium* (1×10^{10}CFU.g^{-1}; PT. Diamond Cold Storage, Indonesia), dried cinnamon extract, 0.1 N NaOH solution, distilled water, and a 1% phenolphthalein (PP) indicator. The equipment used in the present study consisted of an autoclave, gas stove, pot, beaker, stirrer, refrigerator, dropper, measuring flask, Erlenmeyer flask, funnel, filter paper, a sieve, a thermometer, a digital scale, a Bunsen burner, tissue, scissors, rubber gloves, tweezers, a stainless steel sieve, sterile glass bottles, a digital pH meter (EZ-9909, China), and a Brookfield viscometer (Brookfield Engineering Laboratories, USA).

Study design

The experiment was arranged in a completely randomized design (CRD) with four treatment levels and five independent replicates per treatment, resulting in a total of 20 experimental units. The control group comprised goat milk yogurt without cinnamon extract (P0), while the experimental groups consisted of cinnamon extract at 0.1% (P1), 0.2% (P2), and 0.3% (P3), expressed as a percentage (v/v) relative to the 250 mL volume of goat milk per unit.

Study procedure

Process of making cinnamon extract

The process of preparing cinnamon extract followed the modified procedure outlined by Widiyanto et al. (2013), which involved reducing the size of the cinnamon pieces by pounding them to facilitate grinding, then grinding them into a fine powder using a blender. The cinnamon powder was filtered using a 40-mesh sieve to obtain fine cinnamon powder (Figure 1a) and macerated using methanol at a ratio of 1:6. The mixture was left to stand for two days and subsequently filtered using Whatman No. 1 filter paper (11 µm pore size) to obtain the extract (Figure 1b) by evaporation using a rotary evaporator at a constant temperature of 50°C (Figure 1c). The process was continued until all methanol had evaporated, resulting in the formation of cinnamon oleoresin, a concentrated extract containing both volatile and non-volatile bioactive compounds (Jayaprakasha et al., 2007; Ranasinghe et al., 2013). Subsequently, the filtrate was left to harden and then oven-dried at 40°C for approximately 24 hours, after which it was ground in a mortar (Figure 1d).

Making yogurt

The equipment for making yogurt was sterilized. Cinnamon extract was added to 250 mL of goat milk according to the treatment concentrations, followed by pasteurization at 85°C for 15 seconds with continuous stirring (Hanum et al., 2021). The mixture was subsequently cooled to a temperature of 43-45°C (Wanniatie et al., 2022) and inoculated with a 10% LAB starter culture, followed by thorough mixing (Kirana et al., 2025). Fermentation was carried out at room temperature for 48 hours (Kirana et al., 2025). After fermentation, the yogurt was stored under refrigeration for seven days before analysis. The resulting product was evaluated for fat content, moisture content, pH, total titratable acidity, viscosity, and sensory properties (AOAC, 2005; Tamime and Robinson, 2007; Lawless and Heymann, 2010).



Figure 1. Preparation and extraction of bioactive compounds from cinnamon. **a**: Cinnamon powder filtering process, **b**: Filtration of the yield, **c**: Filtrate evaporation, **d**: Cinnamon extract grinding.

Sample analysis

Fat and moisture contents were analyzed according to the standard methods of the Association of Official Analytical Chemists (AOAC, 2005). Total titratable acidity was determined by titration, following the AOAC method for measuring lactic acid content (AOAC, 2005). The pH was measured using a digital pH meter (EZ-9909, China). A 50 mL sample was transferred into a 100 mL beaker. Before measurement, the pH meter was calibrated using a standard buffer solution at pH 4 and then gently dried with tissue paper. The electrode was then immersed in the sample, and the pH was noted (AOAC, 2005).

Viscosity was measured using a Brookfield viscometer (Brookfield Engineering Laboratories, USA). A spindle was immersed in the yogurt sample, rotated at a constant speed, and the resistance torque was measured. The torque value directly correlated with viscosity and was expressed in centipoise (cP). All measurements were conducted under controlled temperature conditions (Rao, 2013; Brookfield Engineering Laboratories, 2017).

Sensory evaluation was conducted using a hedonic test with 28 panelists (Lawless and Heymann, 2010). Approximately 30 g of yogurt was weighed and placed in cups labeled with random codes. Each panelist received four samples with different treatments and was asked to evaluate the samples using a structured questionnaire based on a 5-point hedonic (Likert-type) scale (Lawless and Heymann, 2010). The following criteria included color (1: Typical white milk to 5: Brownish white), aroma (1: Typical fermented milk to 5: Very cinnamon-like aroma), texture (1: Very thin to 5: Very thick), taste (1: Very little cinnamon flavor to 5: Very strong cinnamon flavor), and overall acceptability (1: Very dislike to 5: Very like).

Statistical analysis

Data on physicochemical properties, including fat content, moisture content, viscosity, pH, and total titratable acidity, were subjected to one-way analysis of variance (ANOVA). When significant effects were detected ($p < 0.05$), mean comparisons were performed using the least significant difference (LSD) test. Sensory evaluation data, collected on a 5-point hedonic scale, were analyzed with the nonparametric Kruskal Wallis test. When significant differences were

observed ($p < 0.05$), pairwise comparisons were conducted with the Mann–Whitney U test, with appropriate adjustment for multiple comparisons. All statistical analyses were performed using SPSS version 21 (IBM Corp., Armonk, NY, USA).

RESULTS AND DISCUSSION

The effects of adding *Cinnamomum verum* at different levels on the physicochemical properties of yogurt are presented in Table 1. The average scores for the addition of *Cinnamomum verum* on the organoleptic quality of cow's milk yogurt are presented in Table 2.

Table 1. Physicochemical qualities of goat milk yogurt containing *Cinnamomum verum*

Cinnamomum verum concentration / Parameter	P0	P1	P2	P3
Fat (%)	12.50 ± 3.70^{b}	13.00 ± 3.60^{b}	17.30 ± 5.40^{c}	7.90 ± 0.50^{a}
Water (%)	81.96 ± 2.23^{b}	81.52 ± 1.74^{b}	78.13 ± 0.77^{a}	85.57 ± 0.91^{c}
pH	4.00 ± 0.02^{b}	4.00 ± 0.01^{b}	4.04 ± 0.01^{c}	3.95 ± 0.01^{a}
Total acid (%)	0.72 ± 0.07	0.76 ± 0.08	0.73 ± 0.07	0.62 ± 0.07
Viscosity (cP)	14670 ± 2166.4	12058 ± 1848.5	12720 ± 1928.2	11283 ± 788.7

P0: Control group containing 0% *Cinnamomum verum* concentration, P1: 0.1% *Cinnamomum verum* solution, P2: 0.2% *Cinnamomum verum* solution, and P3: 0.3% *Cinnamomum verum* solution. ^{abc} Means different superscript letters on the same row indicate significant differences ($p < 0.05$).

Table 2. Average score of goat milk yogurt's organoleptic test containing *Cinnamomum verum*

Cinnamomum verum concentration / Parameter	P0	P1	P2	P3
Color	1.25 ± 0.441^{a}	3.86 ± 0.525^{b}	4.00 ± 0.000^{b}	4.00 ± 0.000^{b}
Aroma	1.14 ± 0.525^{a}	2.75 ± 0.585^{b}	3.71 ± 0.713^{c}	4.11 ± 0.786^{c}
Taste	1.07 ± 0.378^{a}	3.07 ± 0.766^{b}	4.21 ± 0.738^{c}	4.32 ± 0.819^{c}
Texture	3.68 ± 0.670^{b}	3.46 ± 0.693^{b}	2.93 ± 0.766^{a}	3.14 ± 0.891^{a}
Overall acceptability	3.79 ± 0.995^{c}	3.25 ± 0.967^{b}	2.96 ± 1.138^{a}	2.79 ± 1.343^{a}

P0: Control group containing 0% *Cinnamomum verum* concentration, P1: 0.1% *Cinnamomum verum* solution, P2: 0.2% *Cinnamomum verum* solution, and P3: 0.3% *Cinnamomum verum* solution. ^{abc} Means different superscript letters on the same row indicate significant differences ($p < 0.05$).

Fat

The addition of cinnamon in yogurt production had a significant effect on fat content ($p < 0.05$). The control group did not differ significantly from Group P3 ($p > 0.05$). However, there was a significant difference in yogurt fat content between the control group and groups P1 and P2 ($p < 0.05$). Adding 0.2% cinnamon (P2) increased fat content to 17.30% ± 5.40, compared to the control group (12.50% ± 3.70). However, increasing the cinnamon concentration to 0.3% (P3) reduced fat content. This finding was consistent with those of Günes-Bayir and Bilgin (2019), who reported that yogurt containing 0.3-2.5% cinnamon demonstrated a proportional decrease in fat content.

Water

The present results demonstrated that the addition of cinnamon extract significantly affected the moisture content of yogurt ($p < 0.05$). The water content of Group P1 demonstrated no significant difference from the control group ($p > 0.05$), but differed significantly from groups P2 and P3 ($p < 0.05$). Pectin, a polysaccharide in cinnamon, can absorb water and increase water-holding capacity/viscosity (Chandel et al., 2022). The addition of 0.2% cinnamon in Group P2 increased the viscosity (12720 cP ± 1928.2) and retained water in yogurt (78.13% ± 0.77). Meanwhile, increasing the cinnamon concentration to 0.3% increased the water content (85.57% ± 0.91), thereby reducing the viscosity of yogurt (11283 cP ± 788.7). In addition to pectin, polyphenols in cinnamon, including proanthocyanidins and tannins, can bind to milk proteins, such as casein, forming complexes. This interaction does not directly bind water, unlike oligosaccharides; however, the formation of protein-polyphenol complexes modifies the matrix structure, enhancing the system's ability to retain water (Tosif et al., 2021).

pH

The present results indicated that the mean pH of goat milk yogurt with different cinnamon concentrations ranged from 3.95 to 4.04 (Table 1), which was consistent with the range outlined by INS (2009; 3.80-4.50). The addition of

cinnamon to goat milk yogurt significantly affected the yogurt's pH ($p < 0.01$). Group P0 demonstrated no significant difference in pH compared to Group P1 (0.1%), but differed significantly from groups P2 (0.2%) and P3 (0.3%).

The addition of cinnamon affected the pH of yogurt, depending on the concentration used. At a concentration of 0.1%, there was no significant effect on pH. However, increasing the concentration to 0.2% increased the pH, which might be due to the presence of active compounds in cinnamon, such as cinnamaldehyde and eugenol. These compounds exhibit antimicrobial activity and can inhibit LAB growth, thereby reducing acid production (Hyldgaard et al., 2012; Ranasinghe et al., 2023). Conversely, at a concentration of 0.3%, the pH decreased, possibly due to the dominance of cinnamic acid compounds, which disrupted the balance of the fermentation process. Therefore, adding cinnamon at specific concentrations can alter the yogurt's pH, either raising or lowering it depending on the amount used. Hastuti and Rustanti (2014) stated that cinnamon extract contains cinnamaldehyde, an acidic compound, so adding higher concentrations can create a more acidic environment.

Total acid

The present results indicated that the average total acidity of goat milk yogurt with cinnamon inclusion ranged from 0.62% to 0.76% (Table 1). The addition of cinnamon at concentrations ranging from 0.1% to 0.3% did not significantly influence the total acidity of goat milk yogurt ($p > 0.05$). Group P1 had a higher average acidity of 0.76%, while Group P3 had a lower acidity of 0.62%. According to INS (2009), the total acidity level of yogurt ranges from 0.5-2.0%, which indicates that the present values were within the established standard limits.

Cinnamon extract at 10% concentration has been shown to lower the pH of yogurt to around 4.65 (Marnianti et al., 2021), and this decrease in pH was accompanied by an increase in total titratable acidity due to lactic acid production during fermentation (Lindasari, 2013). However, the present result demonstrated a different trend, in which groups P1, P2, and P3 had lower pH values, and the average total acid content was low (Table 1). A decrease in pH does not necessarily correspond to an increase in total titratable acidity, as these two parameters reflect different aspects of acidity. The discrepancy might be due to the antimicrobial activity of cinnamon compounds, which can affect LAB and alter fermentation processes (Lucey, 2002; Choi et al., 2016). This condition can occur because lactic acid produced during yogurt production is a weak acid, resulting in a low pH (Rahmawati, 2021). The titration method used to measure total acid was designed to detect only strong acids, such as lactic acid, and to exclude weak acids or phenolic compounds, such as cinnamic acid, from cinnamon. Therefore, despite the decrease in pH, phenolic and other bioactive compounds derived from cinnamon were not included in the measurements of total titratable acidity. This was because the method primarily quantified organic acids, such as lactic acid, thereby yielding comparatively low total acidity values (Lucey, 2002; Tamime and Robinson, 2007). An increase in total acidity does not necessarily result in a proportional decrease in pH. This phenomenon can be attributed to the buffering capacity of milk proteins and the incomplete dissociation of lactic acid, a weak organic acid, which limits the concentration of free hydrogen ions (H^+) in the solution despite the accumulation of total acids (Lucey, 2002; Tamime and Robinson, 2007). Mursyida and Wati (2021) indicated that a low concentration of H^+ ions suggested that the acid in the product was not completely dissociated, thereby classifying it as a weak acid.

Viscosity

The current results indicated that the average viscosity of goat milk yogurt with different cinnamon concentrations ranged from 11,283 to 14,670 cP (Table 1). The current results demonstrated that different cinnamon concentrations did not significantly affect yogurt viscosity ($p > 0.05$). However, despite the lack of a significant effect, a decrease in viscosity was observed in Group P3. The highest average viscosity was observed in Group P0 at 14670 cP, while the lowest average viscosity was observed in Group P3 at 11283 cP. The viscosity values obtained in the present study (11,283-14,670 cP) were within the typical range reported for yogurt, which generally ranges from approximately 1,000 to over 10,000 cP depending on composition and consistency (Lucey, 2002; Hoxha et al., 2023). Higher doses of cinnamon extract within each treatment group yielded different viscosity values, which tended to decrease. The decrease in viscosity values might be attributed to the antimicrobial activity of cinnamon compounds such as cinnamaldehyde and eugenol, which can inhibit LAB, thereby reducing lactic acid production and affecting protein coagulation (Lucey, 2002; Burt, 2004; Upadhyay et al., 2016). According to Lindasari et al. (2013), the properties of the active compounds in cinnamon can affect the protein structure and gel matrix in yogurt.

The viscosity results of the present study indicated that cinnamon at 0.1-0.3% did not affect viscosity. Nevertheless, the viscosity values had a relatively high range across all treatment groups, indicating that the produced yogurt exhibited excellent consistency and was consistent with typical yogurt characteristics. The current results demonstrate higher values than those reported by Savitry and Setiani (2017), who documented a viscosity range of 1815-9012 cP in liquid skim milk yogurt containing tomato juice. According to Dobozi et al. (2023), increased lactic acid production enhances

casein coagulation by lowering pH, thereby facilitating the formation of a protein gel network that influences yogurt viscosity.

Sensoris

Color

The organoleptic evaluation on a 1-5 scale revealed that the addition of cinnamon extract significantly affected the color of goat milk yogurt ($p < 0.05$). The mean color score for Group P0 (1.25) was described as white, typical of milk, whereas groups P1 (3.86), P2 (4.00), and P3 (4.00) exhibited a brownish-white appearance. Further analysis utilizing the Mann–Whitney test revealed significant differences between Group P0 and all treatments containing cinnamon ($p < 0.05$). Conversely, no significant differences were observed among P1, P2, and P3 ($p > 0.05$). This finding suggested that adding 0.1% cinnamon extract was sufficient to enhance the yogurt's visual color intensity. Cinnamon contains phenolic compounds, such as tannins, which can contribute to browning reactions, resulting in a brownish-white color when added to goat milk yogurt (Rizki et al., 2024). Phenolic compounds, such as cinnamaldehyde in cinnamon, can interact with milk proteins through the Maillard reaction, a reaction between sugars and proteins that can improve the sensory quality of fermented products (Mehanna et al., 2014). Color changes during yogurt processing are more probably caused by ingredient additions and physicochemical interactions rather than Maillard reactions, which typically occur at higher temperatures (Yu et al., 2020). Figure 2 illustrates the color variations in goat milk yogurt after adding cinnamon extract.



Figure 2. Yogurt color after addition of *Cinnamomum verum* based on treatment levels. **a**: Control group, **b**: 0.1% *Cinnamomum verum*, **c**: 0.2% *Cinnamomum verum*, **d**: 0.3% *Cinnamomum verum*

Aroma

The organoleptic evaluation on a 1-5 scale indicated that the addition of cinnamon extract significantly influenced the aroma of the goat milk yogurt ($p < 0.05$). The average aroma score increased with the addition of cinnamon extract, with Group P0 (1.14), P1 (2.75), P2 (3.71), and P3 (4.11). The Mann-Whitney test indicated significant differences between Group P0 and all cinnamon treatments ($p < 0.05$), as well as between P1, P2, and P3 ($p < 0.05$). However, there was no significant difference between P2 and P3 ($p > 0.05$). Increasing the cinnamon concentration to 0.2% and 0.3% (P2 and P3) significantly intensified the cinnamon aroma in the yogurt

The organoleptic test results indicated that adding cinnamon extract to goat milk yogurt enhanced its floral aroma without compromising the typical sour flavor of fermented products. This effect was attributed to the bioactive compounds in cinnamon, such as linalool, eugenol, and cinnamaldehyde, which have floral, spicy, and warm aromatic characteristics (Pratiwi and Subarnas, 2020). Furthermore, the distinct aroma of cinnamon can enhance the overall warm and spicy aroma profile of yogurt (Boycheva et al., 2011; Honestin et al., 2021).

Texture

The organoleptic quality evaluation, conducted using a 1-5 scale, indicated that the addition of cinnamon extract significantly affected the texture of goat milk yogurt ($p < 0.05$). The highest mean texture scores were observed in groups P0 (3.68) and P1 (3.46), both characterized by a thick consistency, whereas the lowest scores were recorded in groups P2 (2.93) and P3 (3.14), which exhibited a slightly thick texture. The Mann-Whitney test indicated a significant

difference between groups P0 and P1 with P2 and P3 ($p < 0.05$). However, there was no significant difference between groups P2 and P3 ($p > 0.05$). Increasing the concentration of cinnamon extract decreased panelists' texture ratings of the yogurt.

The decrease in viscosity of goat milk yogurt in groups P2 and P3 likely results from interactions between cinnamon polysaccharides and milk proteins, which interfere with the formation of a stable gel structure (Hege et al., 2020). Excess polysaccharides can interfere with protein-protein interactions in the gel structure, leading to a weaker structure and a thinner texture (Li et al., 2023; Leal et al., 2024). Polysaccharides isolated from *Cinnamomum cassia* are mainly composed of glucose and arabinose and have been reported to exhibit significant biological activities (Zhang et al., 2024). Conversely, at lower concentrations, such as 0.1% cinnamon, acceptable texture formation was still supported because the gel was more stable. In addition, the decrease in viscosity was attributed to antimicrobial compounds in cinnamon, such as cinnamaldehyde and eugenol, which could inhibit LAB growth (Kusumawati et al., 2019). Eugenol exhibits antibacterial activity by disrupting bacterial cell membranes, potentially inhibiting LAB and thereby influencing fermentation processes in yogurt (Burt, 2004; Upadhyay et al., 2016).

Taste

The results of organoleptic quality testing using a 1-5 scale demonstrated that adding cinnamon extract significantly affected the taste of goat milk yogurt ($p < 0.05$). The average taste scores for each treatment were 1.07 in Group P0 for neutral taste, 3.07 in Group P1 for slightly cinnamon-flavored, and 4.21 and 4.32 in groups P2 and P3, respectively, for strongly cinnamon-flavored. The Mann-Whitney test demonstrated statistically significant differences between groups P0, P1, P2, and P3 ($p < 0.05$). However, there were no significant differences between P2 and P3 ($p > 0.05$). Cinnamon plays an important role in reducing the rancid taste and excessive acidity in goat milk yogurt. Cinnamon can improve the sensory properties of yogurt by eliminating undesirable flavors, such as sourness and goaty odor, thereby increasing overall acceptability (Lindasari, 2013; Legowo et al., 2024).

As the concentration of cinnamon extract increased, panelists reported a stronger cinnamon flavor. The active compounds, cinnamaldehyde and eugenol, in cinnamon play an important role in creating the sweet, warm flavor (Kusumawati et al., 2019). Adding cinnamon extract balanced the sourness of fermentation with its sweet flavor, resulting in a more layered flavor (Marnianti et al., 2021). However, excessively high concentrations can produce a dominant warm flavor and disrupt the flavor balance (Ramayanti et al., 2018). Therefore, the selection of the concentration is very important for maintaining the organoleptic quality of yogurt.

Overall acceptability

The hedonic test results demonstrated that yogurt without cinnamon extract (P0) was the most preferred yogurt by the panelists, receiving the highest scores for color (4.29), which was typical of milk, aroma (3.79) typical of fermented milk, texture (3.86) described as thick, and taste (2.79) with minimal cinnamon flavor. Group P1 (0.1%) remained acceptable to the panelists, whereas groups P2 (0.2%) and P3 (0.3%) demonstrated a decline in preference.

The decrease in color preference in groups P2 and P3 was due to a shift toward a darker shade caused by the addition of cinnamon. Consistent with the findings of Rohman and Maharani (2020), consumers tend to prefer bright, fresh colors because colors that are closer to the base ingredients, such as milk or fruit additives, are considered more natural and healthier (Gitama, 2020). Furthermore, in terms of aroma, cinnamon was considered too dominant, reducing the expected freshness of yogurt (Dyah and Larasati, 2022). In terms of texture, the interaction between milk proteins and cinnamon polysaccharides can alter the structure of yogurt, thereby reducing its viscosity (Smykov, 2020). Similarly, in terms of taste, the score was relatively low due to the addition of high concentrations of cinnamon, which enhanced the spicy and warm flavors that consumers disliked (Setiawan et al., 2019). Ramayanti et al. (2018) reported that using high concentrations of cinnamon extract can decrease preference. Wijaya and Carolina (2022) suggest that this decline in acceptance may be due to the panelists' age, with those aged 22-25 years generally being less familiar with spicy flavors. Preference for sweet taste was generally higher among adolescents and young adults and was influenced by individual factors, including gender and biological variation (Guarneri et al., 2025).

CONCLUSION

The observed changes in physicochemical properties of goat milk yogurt might be attributed to interactions between bioactive compounds in cinnamon and the milk matrix during fermentation. The increase in fat content and pH at 0.2% of cinnamon suggested a potential modulation of microbial activity and protein-fat interactions. Meanwhile, the reduction in moisture content might be attributable to structural changes in the yogurt gel that enhance water retention within the matrix. Meanwhile, the absence of significant effects on the acidity and viscosity of goat milk yogurt indicated

that the addition of cinnamon did not substantially interfere with LAB activity or gel structure formation. For sensory evaluations, the acceptability of yogurt with 0.1% cinnamon indicated that low concentrations were sufficient to enhance aroma without adversely affecting taste and texture, whereas higher concentrations produced a strong flavor that reduced consumer preference. It is recommended that studies be continued by increasing the storage period to determine the effectiveness of cinnamon in maintaining the physicochemical and sensory characteristics of yogurt.

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Authors' contributions

Veronica Wanniatie contributed to the study design, analysis, and data interpretation, edited the manuscript, revised the final version, and coordinated the authors. Ema Nurlatifah made major contributions to the study design, realization, and supervision of laboratory investigations, data analysis and interpretation, and manuscript editing. Nandini Fitaloka made major contributions to the study design, realization, and supervision of laboratory investigations, data analysis and interpretation, and manuscript editing. Ratna Ermawati made contributions to the study design and revised the final edition of the manuscript. Arif Qisthon and Ali Husni made contributions to the study design, analysis, and data interpretation. All authors have read and approved the final edition of the manuscript to be published.

Availability of data and materials

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

Competing interests

The authors have not declared any conflicts of interest.

Ethical considerations

This article is not submitted anywhere else, and the findings are analyzed and written under the supervision of all authors. All authors wrote the article and checked the last draft of the manuscript for the similarity index. The authors declared that no AI tools were used for writing and preparing the article. The authors accept the full responsibility of originality of the manuscript in the entire publication process.

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Association of Metabolic Profile and Body Condition Score with Productive and Reproductive Parameters in Dairy Cows in a High-altitude Environment

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ABSTRACT

High-altitude environments impact metabolic adaptation during the transition from late gestation to early lactation, thereby affecting milk production, metabolic health, and reproductive recovery. The present study aimed to evaluate the associations between metabolic status and body condition with milk yield and composition, metabolic disorders, and reproductive intervals in Brown Swiss cows grazing in high-altitude areas. A prospective longitudinal observational study was conducted at a single production facility in Peru, located at an altitude of 3,829 meters above sea level. Sixty-one multiparous cows, approximately 5.5 to 7 years old, with 4 to 5 recorded calvings, had an average prepartum body weight of 692.4 ± 123.6 kilograms. These cows were monitored from 4 weeks before calving until 9 weeks postpartum. Metabolic adaptation during the transition period was evaluated prepartum and at postpartum weeks 1, 3, 5, 7, and 9. Levels of β-hydroxybutyrate, glucose, cholesterol, triglycerides, albumin, total protein, ionized calcium, body condition score, estimated body weight, milk yield and composition, and reproductive intervals were measured. Energy-lipid imbalance was determined by measuring β-hydroxybutyrate, glucose, cholesterol, and triglycerides, whereas serum protein levels were assessed by measuring albumin and total protein. Both integrated indicators, energy–lipid imbalance and serum protein status, demonstrated non-linear patterns, peaking at their highest posterior levels around postpartum week 5. Postpartum week had a greater impact on milk production and composition than the animals' metabolic status. The body condition score demonstrated the strongest association with ketosis, where a 1-standard-deviation increase was associated with lower odds of ketosis. Ketosis prevalence was highest at week 5 (19.6%), whereas hypocalcemia persisted as a common condition throughout the follow-up period. Higher serum protein levels were associated with a shorter calving-to-conception interval, although this finding was imprecise. The postpartum period was more strongly associated with milk production, whereas body condition was the most indicative measure of ketosis risk.

Keywords: Body condition score, Dairy cow, Ketosis, Milk yield, Postpartum period, Transition period

INTRODUCTION

The interval from late pregnancy to the start of lactation involves significant physiological and metabolic changes in the dairy cow's production cycle (LeBlanc, 2010). Immediately following calving, lactation markedly increases nutrient demands, while feed intake and metabolic adaptation may be postponed during the initial weeks postpartum (Villar et al., 2024). In the early postpartum period, cows often mobilize fat reserves to balance the temporary mismatch between energy required for lactation and nutrient intake. This mobilization redirects nutrient utilization primarily toward maintenance and milk production, which may reduce resources available for reproduction (Nigussie, 2018). These coordinated metabolic changes can be detected in the bloodstream through levels of glucose, β-hydroxybutyrate, cholesterol, triglycerides, proteins, and minerals. Additionally, changes in body condition score (BCS) and body weight (BW) can serve as indicators of these integrated metabolic adaptations. Excessive or prolonged adjustments can increase the risk of metabolic disorders such as ketosis and may impair productivity and postpartum reproductive recovery (Çolakoğlu et al., 2019; Zhao et al., 2019).

Dairy cows raised at high altitude are exposed to additional environmental challenges, particularly reduced oxygen availability, low ambient temperatures, and fluctuations in forage availability, which may alter nutrient consumption and metabolic adaptation during the postpartum period (Estremadoyro et al., 2024). Reduced oxygen availability in high-altitude environments may affect tissue oxygenation and metabolic efficiency, whereas exposure to low ambient temperatures can increase the energy required for thermoregulation. In addition, seasonal changes in forage availability

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and quality may limit nutrient intake, thereby increasing the metabolic challenge faced by dairy cows during early lactation (Bartl et al., 2009). Consequently, the metabolic patterns observed in lowland or intensive production settings might not accurately reflect how cows in highland environments respond (Kong et al., 2021). Evaluating Brown Swiss dairy cows raised under high-altitude grazing conditions within their local production environment is therefore necessary to differentiate physiological postpartum adaptation from metabolic changes that may adversely affect health, milk production, or reproductive performance.

Metabolic assessment is particularly relevant for evaluating the transition period because alterations in energy, protein, and mineral metabolism can be associated with changes in milk production, postpartum health, and the return of reproductive function during early lactation (LeBlanc, 2010; Caixeta and Omontese, 2021). β-Hydroxybutyrate and glucose are widely used to characterize energy metabolism, while cholesterol and triglycerides offer additional insights into lipid transport and liver metabolic adaptation (Puppel and Kuczyńska, 2016; Spaans et al., 2022). Albumin and total serum protein contribute to assessing protein metabolism, while albumin may also reflect changes in hepatic synthetic activity and the inflammatory response during the transition period (Bertoni and Trevisi, 2013; Spaans et al., 2022). Ionized calcium plays a crucial role around the calving period, as the start of colostrum and milk production sharply increases the mother's calcium requirements (Weaver et al., 2016). In dairy cows, the BCS provides a practical estimate of body energy reserves and their mobilization during the transition from late gestation to early lactation (Roche et al., 2009), supplementing data from circulating metabolic biomarkers (Ghaffari et al., 2023). Nevertheless, the BCS does not fully reflect metabolic adaptation, and analyzing a single biomarker is complicated by factors such as lactation stage, feeding, herd dynamics, and individual variation. Therefore, considering indicators of energy and lipid metabolism, such as β-hydroxybutyrate, glucose, cholesterol, and triglycerides, along with serum proteins, ionized calcium, and BCS, may provide a broader assessment of dairy cows' metabolic status (Bertoni and Trevisi, 2013; Puppel and Kuczyńska, 2016).

Studies on transition dairy cows often assess metabolic biomarkers, body condition, production performance, and reproductive results, typically using separate or outcome-specific analyses (LeBlanc, 2010; Abdelli et al., 2017; Kerwin et al., 2022). Longitudinal studies have documented temporal changes in metabolic and inflammatory indicators, whereas other investigations have focused specifically on the relationships among energy balance, blood metabolites, and fertility (Wathes et al., 2007; Spaans et al., 2022). Previous studies conducted in high-altitude or high-tropical conditions have generally focused on specific sources of variation, including altitude and diet (Bartl et al., 2009), geographic differences among high-altitude production areas (Kong et al., 2021), and parity or seasonal effects during the early postpartum period (Vallejo-Timarán et al., 2020). In contrast, there is a limited number of longitudinal studies that examine metabolic status alongside both productive and reproductive outcomes in the same cows from prepartum to early lactation. Consequently, the specific relationships among the integrated energy–lipid state, serum protein status, calcium concentration, and body condition in relation to milk yield, milk composition, metabolic disorders, and reproductive recovery under high-altitude conditions remain insufficiently defined. Multivariate approaches identify coordinated patterns among circulating metabolites in dairy cows, enabling biomarkers to be assessed within broader metabolic profiles rather than as independent measurements. These approaches help characterize metabolic adaptation and identify patterns linked to different health conditions during the transition period (Hailemariam et al., 2014; Kayano and Kida, 2015). Accordingly, the present study aimed to investigate the associations between metabolic status and BCS and milk production, milk composition, and reproductive performance in dairy Brown Swiss cows grazing at high altitudes.

MATERIALS AND METHODS

Ethical approval

The present study and its field procedures received authorization from the Regional Directorate of Agriculture of Junín via the Agricultural Agency of Peru, under Letter No. 007-GRJ-DRA-AAC-PERÚ-2025. All procedures involving animal handling, clinical assessments, and the collection of biological samples were performed by trained personnel in accordance with animal welfare principles and to minimize stress and discomfort for the animals.

Study area

The study was conducted at a single dairy production unit in Yanacancha District, Chupaca Province, Junín Region, central Peruvian Andes, Peru, at approximately 3,829 m above sea level. The production unit was chosen because it maintained a Brown Swiss herd that met the study's criteria, allowing for repeated monitoring of the same cows from prepartum through early lactation. The study area was a dry, temperate high-Andean climate with temperatures ranging from approximately 3 to 18.5°C, a mean annual precipitation of approximately 726.6 mm, and marked seasonal variation, often experiencing low temperatures and frost (Figure 1; Marmolejo Gutarra and Ruiz Velásquez, 2018).

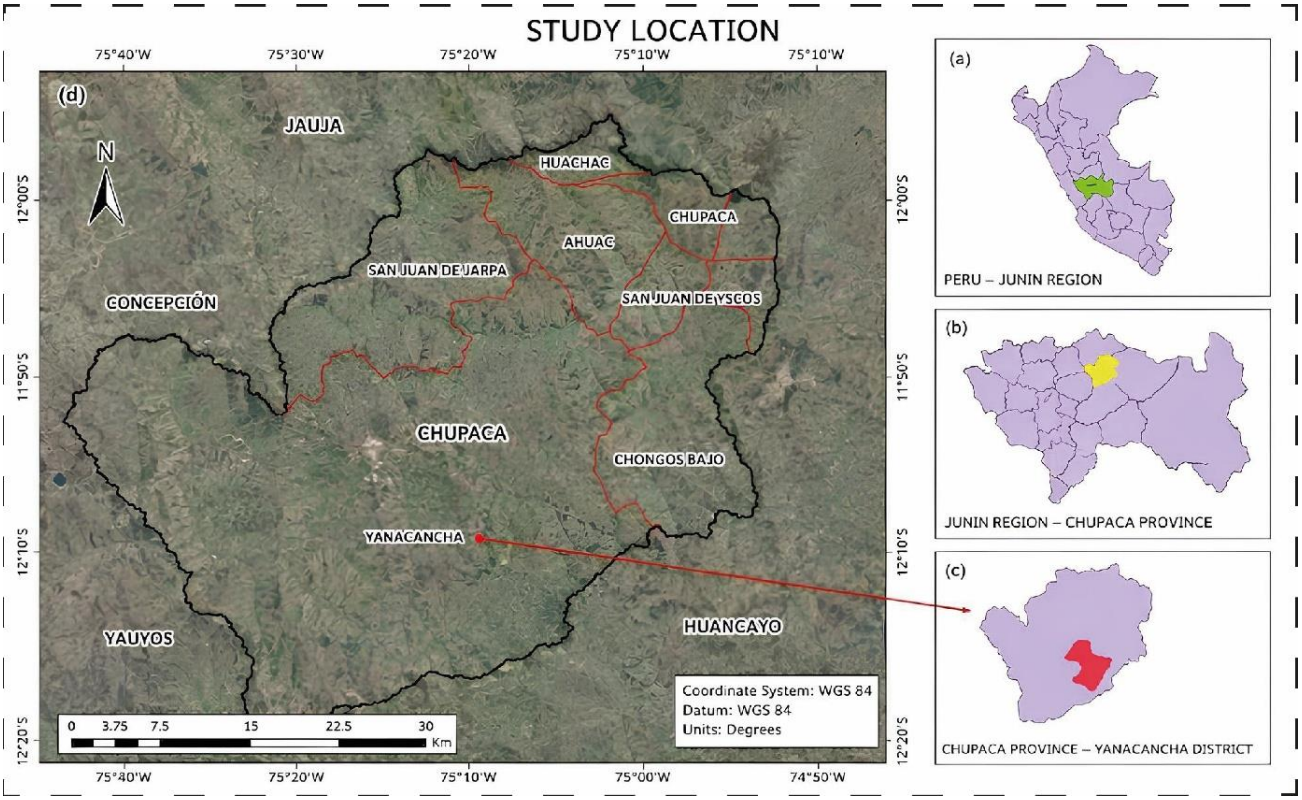


Figure 1. Geographic location of the study area in Yanacancha District, Chupaca Province, Junín Region, Peru

Animals and distribution

The study involved 61 multiparous Brown Swiss cows, aged around 5.5 to 7 years, each with four to five calvings and a full set of permanent teeth, including eight incisors. All 61 cows included in the study were available during the study period and met the preset inclusion criteria for breed, parity, reproductive status, and health; therefore, a formal a priori sample size calculation was not conducted. During the prepartum assessment, the average live weight was 692.44 ± 123.55 kg. Before the study, the cows received the routine antiparasitic treatment used in the herd, consisting of oral administration of Ranide Forte® (Tecnología Química y Comercio S.A., Lima, Peru) at 1 mL/10 kg of body weight, equivalent to 10.0 mg/kg of rafoxanide and 9.0 mg/kg of albendazole, according to the manufacturer's recommendations (Tecnología Química Y Comercio S.A., 2024). The cows were maintained on a grazing-based feeding system, with alfalfa as the primary forage, and no experimental dietary treatment was imposed.

Cows were repeatedly evaluated from late gestation through week 9 postpartum. Six assessment intervals were scheduled at approximately four weeks prior to calving and at postpartum weeks 1, 3, 5, 7, and 9. Blood sampling, body condition scoring, and live-weight estimation were scheduled at all six assessment times. Milk yield was obtained from individual milking records, and milk composition was determined at postpartum weeks 1, 3, 5, 7, and 9 using an ultrasonic milk analyzer BOECO Milk Analyzer LAC-SP (Boeckel + Co. GmbH + Co. KG, Hamburg, Germany).

Blood sampling and biochemical parameters

Blood samples were collected at 06:00 a.m., before morning feeding. Approximately 4.5 mL of blood was obtained from the coccygeal vein using sterile needles and red-top evacuated Vacutainer tubes without anticoagulant. Samples were labeled with the individual cow identification code and sampling period and transported on the same morning to the Animal Health Laboratory of the Faculty of Animal Science, Universidad Nacional del Centro del Perú, where laboratory processing was performed between 10:00 and 11:00 a.m.

Blood samples were left to clot for approximately 30 minutes at room temperature and then centrifuged at 3,000 rpm for 10 minutes. The resulting serum samples were analyzed on the same day. Serum glucose, cholesterol, triglycerides, total protein, albumin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), direct bilirubin, and total bilirubin were determined using a WP21A semi-automated chemistry analyzer (Genrui Biotech Inc., Shenzhen, China) with commercial reagents (QCA, Amposta, Spain), following the recommended procedures for each assay. The reagent reference codes were 998282 for glucose, 995282 for cholesterol, 992330 for triglycerides, 997180 for total protein, 997258 for albumin, 999500 for AST, 999200 for ALT, 990208 for direct bilirubin, and 992714 for total bilirubin.

Serum β-hydroxybutyrate (BHB) concentration was measured in mmol/L using a NovaVET Xpress ketone meter (Nova Biomedical Corporation, Waltham, MA, USA). Ionized calcium (iCa) was determined in mmol/L using a

LAQUAtwin portable calcium ion meter (HORIBA Advanced Techno Co., Ltd., Japan). The BHB and iCa were evaluated as continuous variables and as binary classifiers for metabolic disorders, with ketosis defined as a BHB of 1.4 mmol/L or greater (BHB ≥ 1.4 mmol/L; Denis-Robichaud et al., 2014) and hypocalcemia operationally defined as iCa concentration below 0.86 mmol/L (iCa < 0.86 mmol/L). At each assessment, prevalence was determined by dividing the number of cows meeting the specific criterion by the total number of cows with available measurements for that biomarker (Thrusfield et al., 2018).

Milk sampling and productive measurements

Milk samples were collected during postpartum evaluations. Following udder and teat cleaning, the initial streams of milk were discarded, and approximately 50 mL of milk was collected per cow into an individually identified container. Samples were immediately placed in a cooler with ice packs and transported to the Microbiology Laboratory at the Universidad Nacional del Centro del Perú for physicochemical analysis. Milk fat, lactose, protein, and solids-not-fat (SNF) were measured using a BOECO Milk Analyzer LAC-SP ultrasonic milk analyzer (Boeco, Hamburg, Germany). The instrument was used and calibrated following the manufacturer's guidelines. The milk fat-to-protein ratio was calculated for each observation as the ratio of measured fat to protein percentages, following its conventional use in dairy cow metabolic assessment (Cabezas-Garcia et al., 2021). Whenever the original ratio differed from the ratio calculated directly from the relevant fat and protein measurements, the calculated value was used. Prepartum milk data were not available in the dataset because the cows had not yet calved, so no zero values were imputed. Individual milk yield was recorded for each cow from their production records and linked to their postpartum assessment via animal identification code. The outcomes related to milk, including milk yield, fat, lactose, protein, SNF, and the fat-to-protein ratio, were assessed.

Body condition score and live-weight measurements

The BCS was evaluated on a five-point scale, with higher scores indicating greater body energy reserves. The BCS was treated as a continuous variable in the statistical analyses, and its scores were assigned in 0.25-point increments when appropriate, following the criteria described by Ferguson et al. (1994). The BCS assessment was performed by the same trained veterinarian throughout the study to minimize inter-observer variability (Edmonson et al., 1989).

Body weight (kg) was estimated using a bovine heart-girth weight tape. The tape was placed around the thorax immediately behind the forelimbs, and the corresponding BW estimate was recorded for each cow, following the heart-girth approach described for cattle (Wood et al., 2015).

Reproductive records

Reproductive information was collected from the individual records maintained for each cow, including calving date, first postpartum service, subsequent services, and the service associated with confirmed pregnancy. Pregnancy was confirmed by rectal palpation by a veterinarian and cross-checked with the reproductive record.

The calving-to-first-service interval was calculated as the number of days from calving to the first postpartum service, while the calving-to-conception interval represented the number of days from calving to the service that led to a confirmed pregnancy (Coleman et al., 2009). Accordingly, the conception date was attributed to the recorded service that subsequently resulted in a positive pregnancy diagnosis. These reproductive intervals were utilized as outcome variables within the Bayesian reproductive models.

Statistical analysis

A conservative sensitivity analysis was conducted to assess the statistical power of the available sample. The analysis used a multiple-regression model with four predictors, assuming a two-sided alpha of 0.05, 80% power, and a sample size of 61 cows. Under these assumptions, the smallest detectable overall effect was Cohen's $f^2 = 0.213$, corresponding to approximately 17.6% of explained variance ($R^2 \approx 0.176$), which represents a roughly moderate effect. All analyses were conducted using R version 4.4.1. The data were structured in long format, with repeated measurements nested within each cow. Missing observations were not imputed, and prepartum milk measurements were regarded as structurally unavailable. Continuous predictors in the regression models were standardized by centering each observation on the overall mean and dividing by the corresponding standard deviation (SD; Schielzeth, 2010).

$$z_{ij} = \frac{x_{ij} - \bar{x}}{s_x} \quad \text{(Formula 1)}$$

z_{ij} is the standardized value of predictor x for cow i at observation j, x_{ij} is the original observed value, $\bar{x}$ is the overall mean of the predictor, and s_x is its SD. Thus, continuous predictors were expressed on a common scale with a mean of

zero and a SD of one. This standardization enables interpretation of model coefficients in terms of a one-standard-deviation increase in each predictor.

Energy–lipid imbalance was represented as a latent factor derived from Bayesian confirmatory factor analysis of standardized levels of BHB, glucose, cholesterol, and triglycerides. The measurement model was defined as follows (Merkle and Rosseel, 2018).

$$z_{kij}=\nu_k+\lambda_k F_{EL,ij}+\varepsilon_{kij},\quad \varepsilon_{kij}\sim N(0,\theta_k)\ \#(\text{Formula 2})$$

z_{ijk} is the standardized concentration of metabolic indicator k for cow (i) at assessment (j), k represents BHB, glucose, cholesterol, or triglycerides; ν_k is the intercept of indicator k, λ_k is its factor loading; $F_{EL,ij}$ is the latent energy–lipid imbalance score for cow (i) at assessment (j), ε_{ijk} is the indicator-specific residual error, and θ_k is the corresponding residual variance. For model identification and orientation, the BHB loading was fixed at λ_BHB = 1.

A broader hepatic–protein latent construct was initially assessed but was not retained due to its inability to provide a sufficiently stable factor solution. Therefore, serum protein status was indicated by a composite score derived from standardized measurements of albumin and total serum protein concentrations. For each cow and assessment, this composite was calculated according to the following formula (Merkle and Rosseel, 2018).

$$P^{*}_{ij}=\left(\frac{1}{m_{ij}}\right)\sum_{k\in\{Alb,TP\}} z_{kij}\ \#\ (\text{Formula 3})$$

P^{*}_{ij} is the raw serum protein composite for cow (i) at assessment (j), z_{kij} is the standardized value of albumin (Alb) or total serum protein (TP), and m_{ij} is the number of available protein markers for that observation. When both markers were available, m_{ij} = 2, when only one marker was available, m_{ij} = 1, and when neither marker was available, the composite was treated as missing. The resulting composite was subsequently standardized according to Formula 1 and used as a predictor of serum protein status.

Milk yield and milk-composition outcomes were analyzed using Bayesian mixed-effects models implemented in the brms package (Bürkner, 2017). A Student-t likelihood was used for milk yield, fat, lactose, solids-not-fat, and the fat-to-protein ratio, whereas milk protein was modeled using a Gaussian likelihood.

$$Y_{ij}\sim t_\nu\left(\mu_{ij},\sigma\right)\ \#(\text{Formula 4})\qquad Y_{ij}\sim N\left(\mu_{ij},\sigma\right)\ \#(\text{Formula 5})$$

$$\mu_{ij}=\beta_0+\beta_{week(j)}+\beta_1 F_{EL,ij}+\beta_2 P_{ser,ij}+\beta_3 zCa_{ij}+\beta_4 zBCS_{ij}+b_i\ \#(\text{Formula 6})$$

Y_{ij} is the observed milk outcome for cow (i) at assessment (j), μ_{ij} is its expected value, ν is the degrees-of-freedom parameter of the Student-t distribution, σ is the residual SD, β_0 is the model intercept, β_week(j) represents the fixed effect of postpartum week at assessment (j), β_1 to β_4 are the regression coefficients associated with the established predictors, $F_{EL,ij}$ is the established latent energy-lipid imbalance score, $P_{ser,ij}$ is the established serum protein status, zCa_{ij} is established ionized calcium, $zBCS_{ij}$ is established BCS, and b_i is the cow-specific random intercept, assumed to follow $b_i \sim N(0, \sigma^2_b)$, in which σ^2_b represented the between-cow variance. Posterior means and pairwise contrasts were then estimated for each postpartum week. The overall metabolic-productive analysis framework is summarized in Figure 2.

Ketosis and hypocalcemia were analyzed separately using Bayesian logistic mixed-effects models in brms (Bürkner, 2017). The binary response for each metabolic disorder was calculated using the following formulas.

$$Y_{ij}\sim \text{Bernoulli}(p_{ij})\ \#(\text{Formula 7})\qquad \text{logit}\left(p_{ij}\right)=\beta_0+\beta_{week(j)}+\beta_1 zBCS_{ij}+\beta_2 zBW_{ij}+b_i\ (\text{Formula 8})$$

Y_{ij} represents the binary indicator of metabolic-disorder status in cow (i) at assessment (j), where 1 indicated the presence of a disorder, and 0 indicates its absence. p_{ij} is the probability of the disorder, β_0 is the intercept, $\beta_{week(j)}$ represents the fixed effect of sampling period, β_1 and β_2 are the regression coefficients for standardized body condition score ($zBCS_{ij}$) and standardized body weight (zBW_{ij}), respectively, and b_i is the cow-specific random intercept, with $b_i \sim N(0, \sigma^2_b)$. Regression coefficients were exponentiated and expressed as odds ratios (OR = exp[β]). Values greater than 1 indicated higher odds, while those less than 1 indicated lower odds of the respective disorder.

The calving-to-first-service and calving-to-conception intervals were analyzed using Bayesian lognormal regression models implemented in brms (Bürkner, 2017). For each cow, early-postpartum metabolic predictors were summarized as the mean of available standardized measurements from postpartum weeks 1, 3, and 5.

$$T_i\sim \text{Lognormal}\left(\mu_i,\sigma\right)\ \#(\text{Formula 9})\qquad \mu_i=\beta_0+\beta_1\bar{F}_{EL,i}+\beta_2\bar{P}_{ser,i}+\beta_3\,z\bar{C}a_i+\beta_4\,zB\bar{C}S_i\ \ (\text{Formula 10})$$

T_i is the reproductive interval in days for cow (i), μ_i is the expected value of the natural logarithm of the reproductive interval, σ is the residual SD on the log scale, β_0 is the intercept, β_1 to β_4 are the regression coefficients, $\bar{F}_{EL,i}$ is the mean standardized energy-lipid imbalance score, $\bar{P}_{ser,i}$ is the mean standardized serum protein status, $z\bar{C}a_i$ is the mean standardized ionized calcium concentration; and $zB\bar{C}S_i$ is the mean standardized body condition score across postpartum weeks 1, 3, and 5. Exponentiated coefficients were reported as time ratios (TR = exp[β]); values greater than 1 indicated a longer reproductive interval, whereas values below 1 indicated a shorter interval.

Models were fitted using four Markov chains and model-specific weakly informative priors to provide regularization while allowing the observed data to primarily determine the posterior estimates (Gelman et al., 2008; Bürkner, 2017). Posterior distributions were summarized by their means, 95% credible intervals (CrI), and probability of direction (Pdir), following the guidelines of Makowski et al. (2019).

$$P_{dir} = \max[P, (\beta>0|data)P(\beta<0|data)] \quad \text{(Formula 11)}$$

β is the model coefficient of interest, P(β > 0 | data) is its posterior probability of being positive, and P(β < 0 | data) is its posterior probability of being negative. Therefore, Pdir calculated the posterior probability that the estimated effect aligned with the most probable direction. Convergence was evaluated with the rank-normalized $\hat{R}$ diagnostic, effective sample sizes (ESS) for bulk and tail, and the presence of divergent transitions (Vehtari et al., 2021). Convergence was considered satisfactory when $\hat{R} < 1.01$, both bulk and tail ESS exceeded 400 posterior samples, and no divergent transitions occurred. The ESS referred to the effective number of approximately independent posterior samples generated by the Markov chains and did not represent the number of cows or observations included in the study. Model adequacy was examined using posterior predictive checks (Gabry et al., 2019), while out-of-sample predictive performance was evaluated using Pareto-smoothed importance-sampling leave-one-out cross-validation (PSIS-LOO; Vehtari et al., 2017).

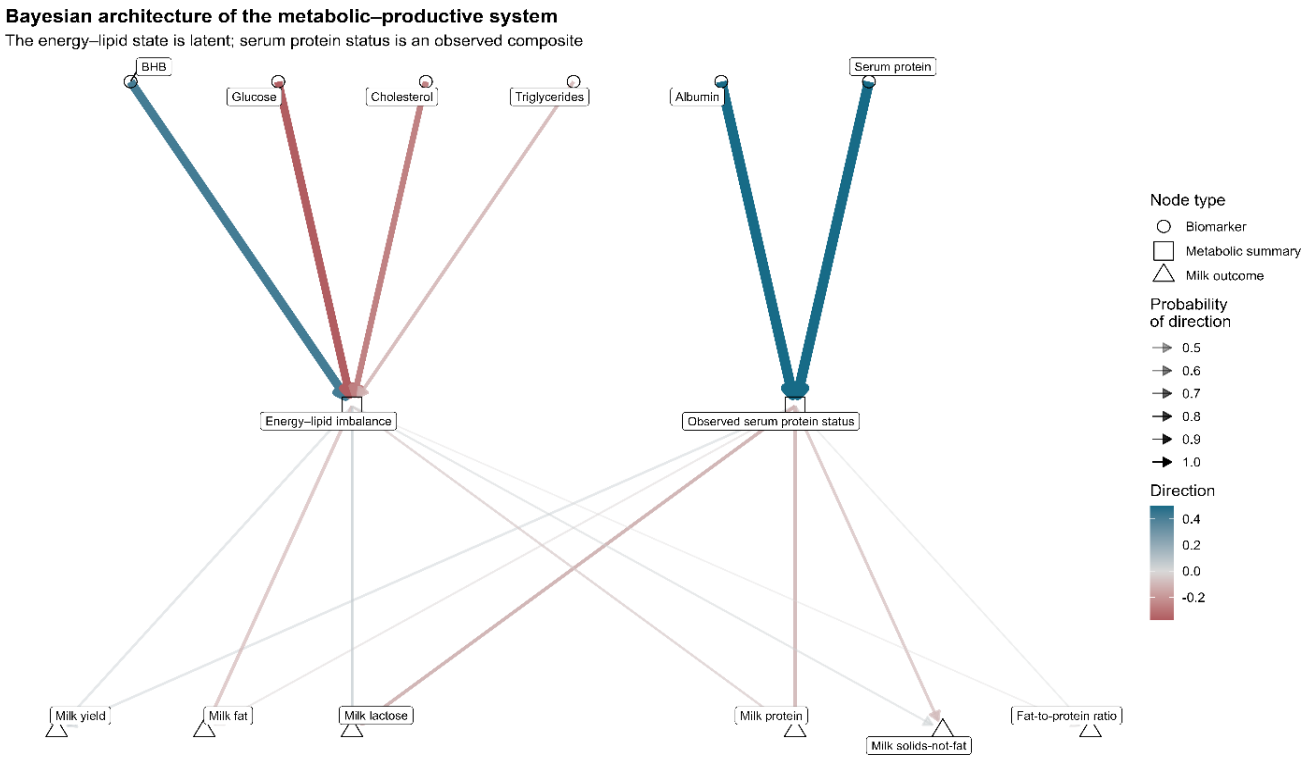


Figure 2. Bayesian architecture of the metabolic-productive system in Brown Swiss dairy cows from approximately 4 weeks prepartum to 9 weeks postpartum under high-altitude grazing conditions in Peru. β-Hydroxybutyrate, glucose, cholesterol, and triglycerides were integrated into a latent energy-lipid imbalance score, whereas albumin and total serum protein were combined into an observed serum protein status index.

RESULTS

Descriptive characteristics and metabolic profile

The main temporal changes in serum biochemical indicators, body measurements, and milk traits are summarized in Table 1. Body condition score had its lowest mean at postpartum week 1 (2.66 ± 0.08) and its highest at the prepartum assessment (3.40 ± 0.20), then increased progressively to 3.19 ± 0.30 at week 9. Body weight was highest prepartum (692.44 ± 123.55 kg), declined to its lowest value at week 5 (618.57 ± 101.76 kg), and subsequently increased to 632.05 ± 100.34 kg by week 9, although it remained below the prepartum mean. Among the metabolic indicators, β-hydroxybutyrate reached its highest concentration at week 5 (1.04 ± 0.57 mmol/L) and its lowest concentration at week 9 (0.83 ± 0.25 mmol/L). Triglycerides decreased from a maximum of 51.85 ± 25.53 mg/dL prepartum to a minimum of 30.39 ± 9.54 mg/dL at week 5, whereas cholesterol increased from its lowest mean prepartum (82.80 ± 22.59 mg/dL) to its highest value at week 9 (104.86 ± 38.39 mg/dL). Total bilirubin declined from 0.59 ± 0.29 mg/dL prepartum to 0.33 ± 0.21 mg/dL at week 9, while AST decreased from 126.46 ± 48.11 U/L prepartum to its lowest mean of 97.38 ± 37.64 U/L at week 7. Serum glucose differed across assessments, with the lowest mean at week 5 (50.55 ± 26.51 mg/dL) and the highest at week 9 (67.95 ± 24.69 mg/dL).

Table 1. Serum biochemical, metabolic, body condition, and milk parameters of Brown Swiss dairy cows across the prepartum and early postpartum periods under high-altitude grazing conditions in Peru

Parameters	PRE	S1	S3	S5	S7	S9
Serum biochemical and metabolic indicators						
Albumin (g/dL)	3.89 ± 0.86	4.27 ± 1.70	4.08 ± 1.86	4.68 ± 2.24	4.31 ± 2.06	3.22 ± 1.69
Direct bilirubin (mg/dL)	0.29 ± 0.24	0.31 ± 0.35	0.18 ± 0.20	0.23 ± 0.26	0.19 ± 0.28	0.34 ± 0.68
β-Hydroxybutyrate (mmol/L)	0.85 ± 0.46	0.88 ± 0.32	0.90 ± 0.32	1.04 ± 0.57	0.93 ± 0.27	0.83 ± 0.25
Total bilirubin (mg/dL)	0.59 ± 0.29	0.45 ± 0.19	0.42 ± 0.22	0.38 ± 0.23	0.34 ± 0.17	0.33 ± 0.21
Ionized calcium (mmol/L)	0.71 ± 0.10	0.78 ± 0.28	0.73 ± 0.24	0.79 ± 0.23	0.77 ± 0.24	0.75 ± 0.21
Cholesterol (mg/dL)	82.80 ± 22.59	89.51 ± 41.25	98.11 ± 46.92	104.28 ± 46.67	93.88 ± 38.88	104.86 ± 38.39
Glucose (mg/dL)	53.71 ± 20.25	51.45 ± 29.10	61.96 ± 28.35	50.55 ± 26.51	55.98 ± 28.55	67.95 ± 24.69
Total serum protein (g/dL)	6.21 ± 3.34	9.61 ± 5.19	8.73 ± 5.31	9.68 ± 5.62	10.11 ± 6.62	7.71 ± 6.01
Aspartate aminotransferase (U/L)	126.46 ± 48.11	118.51 ± 40.04	110.49 ± 44.62	112.85 ± 49.13	97.38 ± 37.64	100.56 ± 35.41
Alanine aminotransferase (U/L)	32.30 ± 6.65	30.03 ± 9.46	33.27 ± 14.26	30.79 ± 11.45	30.15 ± 12.90	29.67 ± 8.85
Triglycerides (mg/dL)	51.85 ± 25.53	39.39 ± 29.91	33.94 ± 19.69	30.39 ± 9.54	32.26 ± 15.72	31.21 ± 10.14
Body measurements						
BCS (1–5)	3.40 ± 0.20	2.66 ± 0.08	2.93 ± 0.17	3.05 ± 0.33	3.09 ± 0.27	3.19 ± 0.30
BW (kg)	692.44 ± 123.55	653.70 ± 103.85	620.04 ± 99.39	618.57 ± 101.76	625.13 ± 104.42	632.05 ± 100.34
Milk production and composition						
Milk yield (L)	NA	15.34 ± 7.52	14.63 ± 6.86	14.63 ± 6.86	11.43 ± 6.59	9.08 ± 6.12
Milk fat (%)	NA	4.33 ± 2.53	4.89 ± 2.09	4.46 ± 2.58	3.90 ± 2.45	4.00 ± 2.03
Milk lactose (%)	NA	4.67 ± 1.19	4.03 ± 0.77	4.00 ± 1.11	3.98 ± 1.41	3.77 ± 1.60
Milk protein (%)	NA	3.15 ± 1.16	2.91 ± 1.30	3.22 ± 1.65	3.83 ± 2.04	3.79 ± 2.21
Milk solids-not-fat (%)	NA	8.30 ± 2.26	7.06 ± 2.00	6.80 ± 2.69	6.64 ± 3.22	6.30 ± 3.18
Fat-to-protein ratio	NA	1.59 ± 1.21	1.95 ± 1.04	1.73 ± 1.49	1.27 ± 1.17	1.35 ± 1.07

BCS: Body condition score, BW: Body weight, PRE: Assessment approximately 4 weeks before calving, S1, S3, S5, S7, and S9 represent assessments at postpartum weeks 1, 3, 5, 7, and 9. Values are presented as mean ± standard deviation. NA indicates that the variable was not assessed during that period. The effective sample size may vary among variables because of missing observations.

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Milk yield was highest at 15.34 ± 7.52 L in week 1 and progressively declined to its lowest mean at 9.08 ± 6.12 L in week 9. Milk fat reached its highest value at week 3 (4.89 ± 2.09%) and its lowest level at week 7 (3.90 ± 2.45%), whereas lactose decreased from 4.67 ± 1.19% at week 1 to 3.77 ± 1.60% at week 9. Milk protein level was 2.91 ± 1.30% at week 3, the lowest value, while its highest level was 3.83 ± 2.04% at week 7. Solids-not-fat decreased from 8.30 ± 2.26% at week 1 to 6.30 ± 3.18% at week 9. The fat-to-protein ratio reached its maximum level at week 3 (1.95 ± 1.04) and its minimum value at week 7 (1.27 ± 1.17).

Reproductive outcomes are summarized separately in Table 2. The calving-to-first-service interval averaged 75.48 ± 37.62 days for 25 cows, whereas the calving-to-conception interval averaged 104.44 ± 40.78 days for 23 cows.

Both integrated metabolic indicators changed non-linearly across the transition period (Figure 3). The energy-lipid imbalance remained close to the prepartum level at postpartum week 1 (S1) and postpartum week 3 (S3), increased at S5, and then decreased to its lowest estimate after S9. Serum protein level demonstrated a similar temporal pattern, increasing after calving, peaking at S5, remaining elevated at S7, and declining by S9. Compared with the prepartum period (PRE), the most evident directional increase was observed at S5 for both energy-lipid imbalance (Pdir = 95.2%) and serum protein status (Pdir = 97.4%). At S9, the posterior distributions were generally lower than the prepartum observations. When both metabolic indicators were considered simultaneously, cows at S5 and S7 tended to exhibit higher values of energy-lipid imbalance and serum protein levels, whereas at S9 energy-lipid imbalance and serum protein levels were lower. However, the distributions across the different assessment periods overlapped significantly, indicating that metabolic responses differed among cows and that the temporal changes did not clearly distinguish all sampling periods.

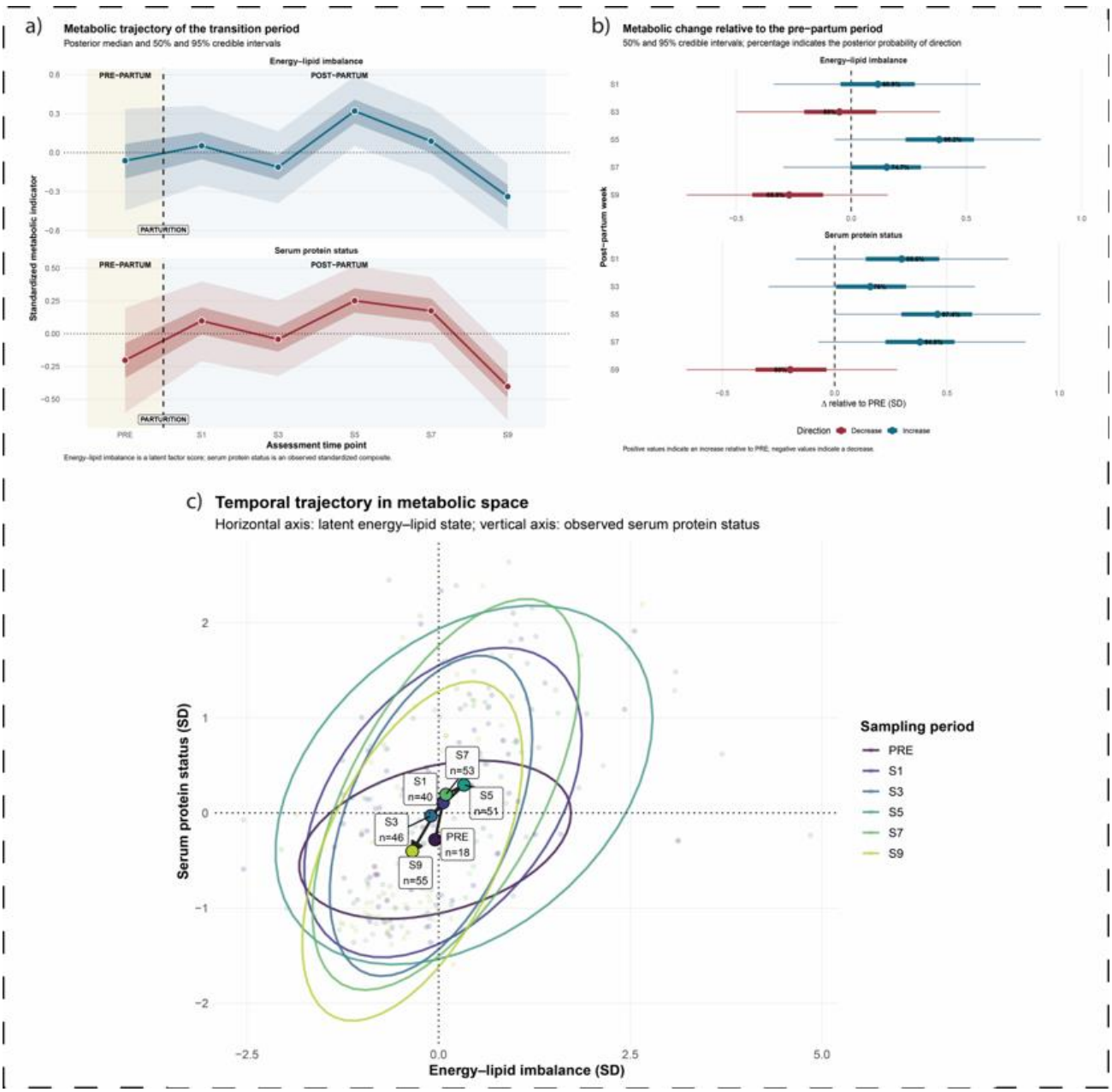


Figure 3. Posterior trajectories and joint distribution of energy–lipid imbalance and serum protein status in Brown Swiss cows in Peru, from prepartum to postpartum week 9. **a:** Temporal trajectories with 50% and 95% credible intervals (CrIs). **b:** Changes relative to the prepartum period (PRE); percentages indicate the posterior probability of direction (Pdir). **c:** Joint distribution of standardized indicators; points show period centroids and sample sizes, and ellipses show within-period dispersion.

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Table 2. Reproductive intervals of Brown Swiss dairy cows during the early postpartum period under high-altitude grazing conditions in Peru

Reproductive outcome	n	Mean ± SD (days)
Calving-to-first-service interval	25	75.48 ± 37.62
Calving-to-conception interval	23	104.44 ± 40.78

The calving-to-first-service interval was calculated from calving to the first recorded postpartum service. The calving-to-conception interval was calculated from calving to the service that resulted in a subsequently confirmed pregnancy. n: Number of cows included in the analysis for each reproductive outcome. Values are presented as mean ± standard deviation (SD)

Associations between metabolic indicators and milk outcomes

Table 3 and Figure 4 summarize the posterior associations between metabolic indicators and milk outcomes. Overall, the estimated effects were modest and uncertain, with all 95% credible intervals including zero. Milk yield demonstrated no clear association with any of the evaluated predictors. The present results indicated that serum protein level was consistently associated with lactose and milk protein, ionized calcium with solids-not-fat, and BCS with milk fat. However, these patterns remained uncertain and were not interpreted as conclusive effects. Associations with the fat-to-protein ratio were generally weak. A visual overview of the direction and relative strength of these posterior associations, emphasizing that the evidence was primarily directional rather than conclusive, is presented in Figure 4.

Postpartum changes in milk production and composition

Milk production and composition differed across postpartum weeks, whereas model-predicted differences associated with changes in the integrated metabolic indicators were comparatively small (Table 4 and Figure 5). Adjusted milk yield increased from 9.97 L at S1 to 11.08 L at S3, remained similar at S5 (11.02 L), and subsequently decreased to 9.96 L at S7 and 8.30 L at S9. Compared with postpartum week 9 (S9), milk yield was higher at S3 by 2.76 L (95% HPD interval of 1.04 to 4.61 L), at S5 by 2.69 L (95% HPD interval of 0.98 to 4.49 L), and at S7 by 1.64 L (95% HPD interval of 0.10 to 3.36 L). These posterior differences indicate the estimated variation in the adjusted mean milk yield between the two postpartum weeks. Milk fat reached its highest adjusted mean at S3 (5.05%) and was higher at S3 than at S7 and S9. Lactose concentration was highest at S1, decreased at S3, then increased again at S5 and S7. Milk protein levels were lowest at S3 and highest at S7, with an estimated increase of 0.33 percentage points between these stages. Solids-not-fat exhibited a similar pattern, whereas the fat-to-protein ratio peaked at S3 and decreased at S7 and S9.

Figure 5 illustrated model-based predictions across three predefined metabolic conditions, including the average level of each metabolic indicator, one SD above the mean (+1 SD), and one SD below the mean (−1 SD). For energy-lipid imbalance, predictions under these three conditions were very similar for milk yield, solids-not-fat, and the fat-to-protein ratio, with only small differences in milk fat, lactose, and protein. Predictions based on serum protein levels demonstrated limited differentiation among the three conditions. The significant overlap of the 95% credible intervals indicated that differences across postpartum weeks are more distinctly identifiable than the predicted changes resulting from a one-standard-deviation increase or decrease in either metabolic indicator.

Ketosis and hypocalcemia

Ketosis remained relatively infrequent throughout the study, with a temporary increase at postpartum week 5 to 19.6%, then declining to 3.6% at week 9 (Table 5). In contrast, hypocalcemia was frequent throughout the study, with prevalence decreasing from 88.9% prepartum to its lowest value of 60.8% at week 5. The percentages indicated the observed frequency of each disorder, while the Bayesian logistic mixed-effects models assessed the adjusted associations with postpartum period and animal-level predictors (Table 6).

Body condition score demonstrated the clearest association with ketosis. A one-standard-deviation increase in BCS was associated with substantially lower odds of ketosis (OR = 0.207; 95% credible interval: 0.103-0.386), corresponding to approximately a 79% reduction in odds. No other ketosis predictor had a 95% credible interval that excluded 1. Regarding hypocalcemia, none of the predictors had a 95% credible interval that excluded 1; however, the posterior estimates indicated lower odds in postpartum weeks 5 and 9 compared to the prepartum period, with Pdir values of 96.6% and 95.1%, respectively. Overall, the strongest model-supported result was the inverse relationship between BCS and ketosis, while the evidence for temporal changes in hypocalcemia was consistent in direction but not conclusive.

Early metabolic status and reproductive recovery

Table 7 and Figure 6 illustrate the relationships between early-postpartum metabolic health and reproductive recovery. None of the evaluated predictors had a 95% credible interval for the time ratio that excluded 1, indicating that the estimated associations remained uncertain. For the calving-to-first-service interval. The posterior estimates generally suggested longer intervals characterized by greater energy-lipid imbalance and increased BCS, while higher serum protein levels were generally linked to shorter intervals. For the calving-to-conception interval, serum protein level demonstrated the strongest directional association, with a time ratio of 0.771, a 95% credible interval of 0.579-1.035, and a Pdir value of 96.3%, indicating a shorter interval as serum protein level increased, although the credible interval included 1. Figure 6 illustrates the combined predictions for energy-lipid imbalance and serum protein status. Regarding reproductive outcomes, longer predicted intervals were predominantly observed with early energy-lipid imbalance and reduced serum protein levels, with the effect most pronounced for the calving-to-conception interval. Conversely, a higher serum protein level combined with a lower energy-lipid imbalance was associated with shorter predicted intervals.

However, the gradual changes across the predictions, along with the credible intervals reported in Table 7, indicated that these associations were uncertain rather than conclusive.

Table 3. Posterior associations of metabolic indicators and body condition score with milk yield and composition in Brown Swiss dairy cows from postpartum weeks 1 to 9 under high-altitude grazing conditions in Yanacancha, Junín, Peru

Milk outcome	Predictor	β	95% CrI	Pdir
Milk yield	Energy–lipid imbalance	0.06	[−0.65, 0.74]	56.9%
	Serum protein status	0.04	[−0.71, 0.70]	57.3%
	Ionized calcium	−0.04	[−0.73, 0.62]	53.5%
	BCS	0.13	[−0.79, 1.07]	61.0%
Milk fat	Energy–lipid imbalance	−0.12	[−0.48, 0.24]	74.4%
	Serum protein status	−0.03	[−0.35, 0.29]	56.9%
	Ionized calcium	−0.16	[−0.48, 0.16]	83.7%
	BCS	0.23	[−0.13, 0.62]	88.8%
Milk lactose	Energy–lipid imbalance	0.07	[−0.11, 0.24]	77.3%
	Serum protein status	−0.12	[−0.27, 0.03]	94.6%
	Ionized calcium	−0.04	[−0.18, 0.11]	69.9%
	BCS	−0.08	[−0.26, 0.09]	82.1%
Milk protein	Energy–lipid imbalance	−0.05	[−0.20, 0.10]	75.1%
	Serum protein status	−0.10	[−0.24, 0.03]	92.6%
	Ionized calcium	−0.07	[−0.21, 0.06]	86.2%
	BCS	−0.11	[−0.26, 0.05]	91.4%
Milk solids-not-fat	Energy–lipid imbalance	0.04	[−0.25, 0.32]	59.8%
	Serum protein status	−0.09	[−0.33, 0.14]	78.3%
	Ionized calcium	−0.18	[−0.42, 0.06]	93.4%
	BCS	−0.15	[−0.43, 0.13]	85.4%
Fat-to-protein ratio	Energy–lipid imbalance	−0.00	[−0.19, 0.18]	51.8%
	Serum protein status	0.01	[−0.16, 0.18]	54.3%
	Ionized calcium	0.02	[−0.14, 0.18]	60.8%
	BCS	0.06	[−0.15, 0.27]	72.3%

BCS: Body condition score, β: Posterior mean, CrI = Credible interval, P_{dir}: Posterior probability of the estimated direction. All continuous predictors were standardized; therefore, β represents the expected change in the outcome associated with a one-standard-deviation increase in the predictor.

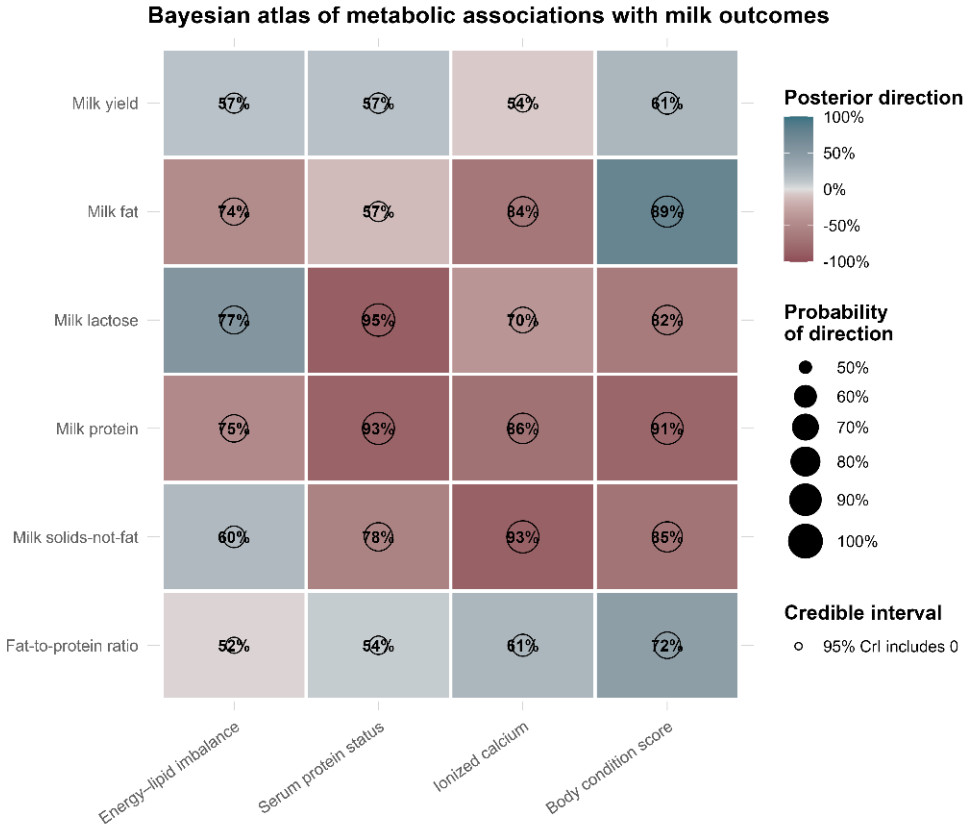


Figure 4. Bayesian atlas of posterior associations between metabolic indicators and milk production traits in Brown Swiss dairy cows from postpartum weeks 1 to 9 under high-altitude grazing conditions in Peru. Cell color indicates the direction and relative magnitude of the posterior association, with blue representing positive effects and red representing negative effects. Circle size and the percentage shown within each cell represent the posterior probability of direction (P_{dir}). Open circles indicate that the 95% credible interval included zero.

Table 4. Adjusted posterior estimates of milk production and composition from postpartum weeks 1 to 9 in Brown Swiss dairy cows under high-altitude grazing conditions in Yanacancha, Junín, Peru

Panel A. Adjusted posterior marginal means

Outcome	S1	S3	S5	S7	S9
Milk yield (L/day)	9.97 [6.27, 13.66]	11.08 [7.72, 14.37]	11.02 [7.86, 14.47]	9.96 [6.54, 13.06]	8.30 [4.95, 11.60]
Milk fat (%)	4.73 [3.97, 5.55]	5.05 [4.37, 5.73]	4.61 [3.95, 5.25]	3.99 [3.39, 4.65]	3.95 [3.25, 4.55]
Milk lactose (%)	4.17 [3.71, 4.60]	3.60 [3.20, 4.00]	3.86 [3.46, 4.25]	4.03 [3.65, 4.40]	3.76 [3.36, 4.15]
Milk protein (%)	3.85 [3.29, 4.41]	3.56 [2.98, 4.11]	3.70 [3.15, 4.27]	3.89 [3.36, 4.45]	3.77 [3.22, 4.34]
Milk solids-not-fat (%)	6.70 [5.72, 7.76]	6.02 [4.99, 6.91]	6.26 [5.32, 7.19]	6.58 [5.66, 7.49]	6.40 [5.46, 7.30]
Fat-to-protein ratio	1.59 [1.17, 2.03]	1.98 [1.59, 2.32]	1.71 [1.36, 2.08]	1.31 [1.00, 1.63]	1.38 [1.05, 1.71]

Panel B. Pairwise posterior contrasts with 95% HPD intervals excluding zero

Outcome	Comparison	Posterior difference [95% HPD]	Interpretation
Milk yield (L/day)	S3 vs. S9	2.76 [1.04, 4.61]	Higher at S3
Milk yield (L/day)	S5 vs. S9	2.69 [0.98, 4.49]	Higher at S5
Milk yield (L/day)	S7 vs. S9	1.64 [0.10, 3.36]	Higher at S7
Milk fat (%)	S3 vs. S7	1.05 [0.17, 1.85]	Higher at S3
Milk fat (%)	S3 vs. S9	1.11 [0.26, 1.94]	Higher at S3
Milk lactose (%)	S1 vs. S3	0.57 [0.19, 0.97]	Higher at S1
Milk lactose (%)	S3 vs. S7	−0.43 [−0.78, −0.07]	Higher at S7
Milk protein (%)	S3 vs. S7	−0.33 [−0.66, −0.01]	Higher at S7
Milk solids-not-fat (%)	S3 vs. S7	−0.56 [−1.13, −0.01]	Higher at S7
Fat-to-protein ratio	S3 vs. S7	0.66 [0.22, 1.09]	Higher at S3
Fat-to-protein ratio	S3 vs. S9	0.59 [0.14, 1.05]	Higher at S3

S1, S3, S5, S7, and S9: Postpartum weeks 1, 3, 5, 7, and 9. HPD: Highest posterior density. Values in Panel A are adjusted posterior marginal means (95% HPD interval). In Panel B, values are posterior differences (95% HPD interval). Positive values indicate a higher mean in the first week, and negative values indicate a higher mean in the second week. Only contrasts whose 95% HPD intervals exclude zero are shown.

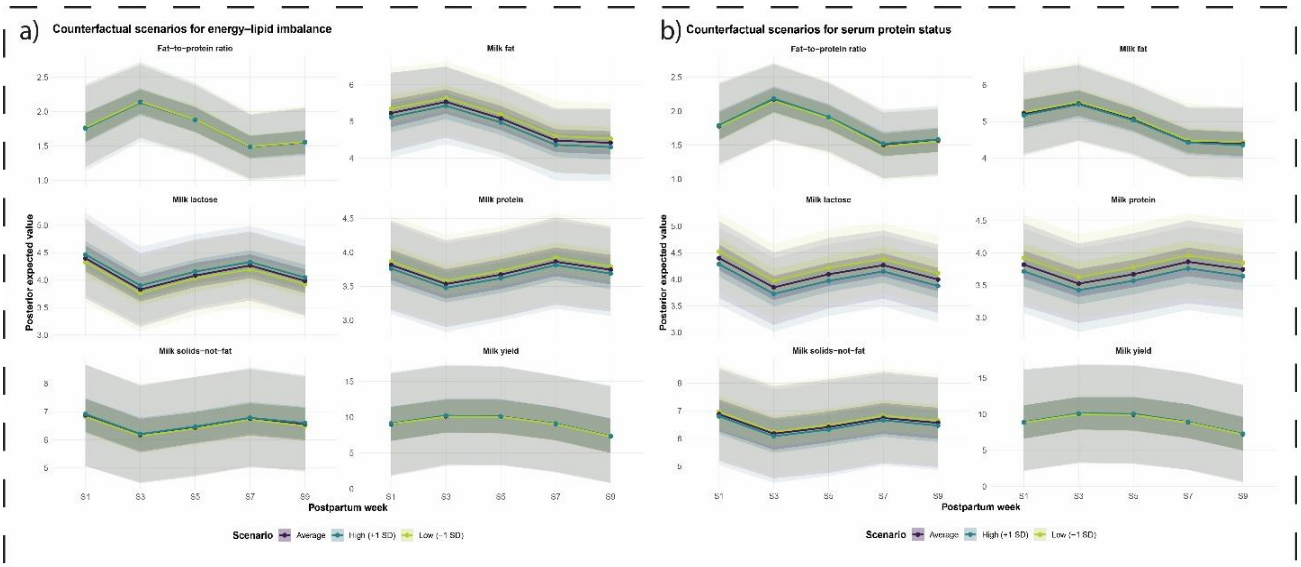


Figure 5. Posterior counterfactual trajectories of milk yield and composition according to energy–lipid imbalance and serum protein status in Brown Swiss dairy cows from postpartum weeks 1 to 9 under high-altitude grazing conditions in Yanacancha, Junín, Peru. **a:** Posterior expected trajectories of milk yield, milk fat, lactose, protein, solids-not-fat, and the fat-to-protein ratio under average, high $(+1\ \mathrm{SD})$, and low $(-1\ \mathrm{SD})$energy–lipid imbalance scenarios. **b:** Corresponding posterior trajectories under average, high $(+1\ \mathrm{SD})$, and low $(-1\ \mathrm{SD})$serum protein status scenarios. Points represent posterior expected values and shaded bands represent 95% credible intervals. Predictions were obtained for postpartum weeks 1, 3, 5, 7, and 9, with the remaining covariates held constant.

Table 5. Prevalence of ketosis and hypocalcemia across sampling periods in Brown Swiss dairy cows under high-altitude grazing conditions in Yanacancha, Junín, Peru

Sampling period	Ketosis, n/N (%)	Hypocalcemia, n/N (%)
Prepartum	1/18 (5.6)	16/18 (88.9)
Postpartum week 1	4/40 (10.0)	31/40 (77.5)
Postpartum week 3	4/46 (8.7)	31/46 (67.4)
Postpartum week 5	10/51 (19.6)	31/51 (60.8)
Postpartum week 7	3/53 (5.7)	37/53 (69.8)
Postpartum week 9	2/55 (3.6)	35/55 (63.6)

Values are presented as n/N (%), n: The number of cows classified as cases, N: The total number of cows evaluated at each sampling period. Ketosis was defined as a serum β-hydroxybutyrate concentration ≥ 1.4 mmol/L, and hypocalcemia as an ionized calcium concentration < 0.86 mmol/L. Prevalence was calculated using only cows with a valid measurement for the corresponding biomarker.

Table 6. Bayesian logistic mixed-model associations with ketosis and hypocalcemia from prepartum to postpartum week 9 in Brown Swiss dairy cows under high-altitude grazing conditions in Yanacancha, Junín, Peru

Outcome	Predictor	β (95% CrI)	OR	Pdir (%)
Ketosis	Week 1 vs. prepartum	−0.992 (−2.249, 0.213)	0.371	94.5
	Week 3 vs. prepartum	−0.219 (−1.480, 1.007)	0.803	63.6
	Week 5 vs. prepartum	0.987 (−0.152, 2.137)	2.683	95.5
	Week 7 vs. prepartum	−0.260 (−1.558, 0.991)	0.771	65.6
	Week 9 vs. prepartum	−0.341 (−1.745, 1.019)	0.711	68.9
	BCS , per 1 SD	−1.575 (−2.272, −0.951)	0.207	100.0
	Body weight, per 1 SD	0.263 (−0.389, 0.862)	1.301	79.7
Hypocalcemia	Week 1 vs. prepartum	0.086 (−1.047, 1.229)	1.090	56.2
	Week 3 vs. prepartum	−0.497 (−1.474, 0.477)	0.608	83.8
	Week 5 vs. prepartum	−0.901 (−1.868, 0.053)	0.406	96.6
	Week 7 vs. prepartum	−0.428 (−1.349, 0.518)	0.652	81.6
	Week 9 vs. prepartum	−0.796 (−1.733, 0.142)	0.451	95.1
	BCS , per 1 SD	−0.018 (−0.407, 0.373)	0.982	54.4
	Body weight, per 1 SD	0.212 (−0.266, 0.711)	1.236	80.9

β values are posterior mean regression coefficients on the log-odds scale, with the numbers in parentheses representing the lower and upper limits of the 95% credible interval (CrI). OR: Posterior mean odds ratio, calculated as exp(β), Pdir: Posterior probability of the estimated direction, SD: Standard deviation. Week effects were estimated relative to the prepartum period. Body condition score and body weight were standardized, and models included a cow-specific random intercept. Bold values indicate that the 95% CrI for β excluded zero.

Table 7. Bayesian lognormal regression of early-postpartum metabolic status (weeks 1, 3, and 5) and reproductive intervals in Brown Swiss dairy cows under high-altitude grazing conditions in Yanacancha, Junín, Peru

Outcome	Predictor	β (95% CrI)	Time ratio	Pdir (%)
IP–PC	Energy–lipid imbalance	0.121 (−0.227, 0.471)	1.129	76.3
	Serum protein status	−0.175 (−0.553, 0.209)	0.839	82.5
	Ionized calcium	0.034 (−0.414, 0.481)	1.035	56.2
	BCS	0.175 (−0.146, 0.491)	1.191	86.7
IP–C	Energy–lipid imbalance	0.068 (−0.195, 0.331)	1.070	69.7
	Serum protein status	−0.260 (−0.547, 0.034)	0.771	96.3
	Ionized calcium	−0.030 (−0.373, 0.308)	0.970	57.2
	BCS	0.126 (−0.120, 0.368)	1.134	84.7

BCS: Body condition score, β values are posterior mean regression coefficients, and the numbers in parentheses represent the lower and upper limits of the 95% credible interval (CrI); for example, 0.121 (−0.227, 0.471) indicates a posterior mean β of 0.121 with a 95% CrI from −0.227 to 0.471. Time ratios were obtained as exp(β) and are presented as posterior means; values > 1 indicate longer reproductive intervals and values < 1 indicate shorter intervals. Pdir: Posterior probability of the estimated direction; IP–PC: Calving-to-first-service interval; IP–C: Calving-to-conception interval. All continuous predictors were standardized.

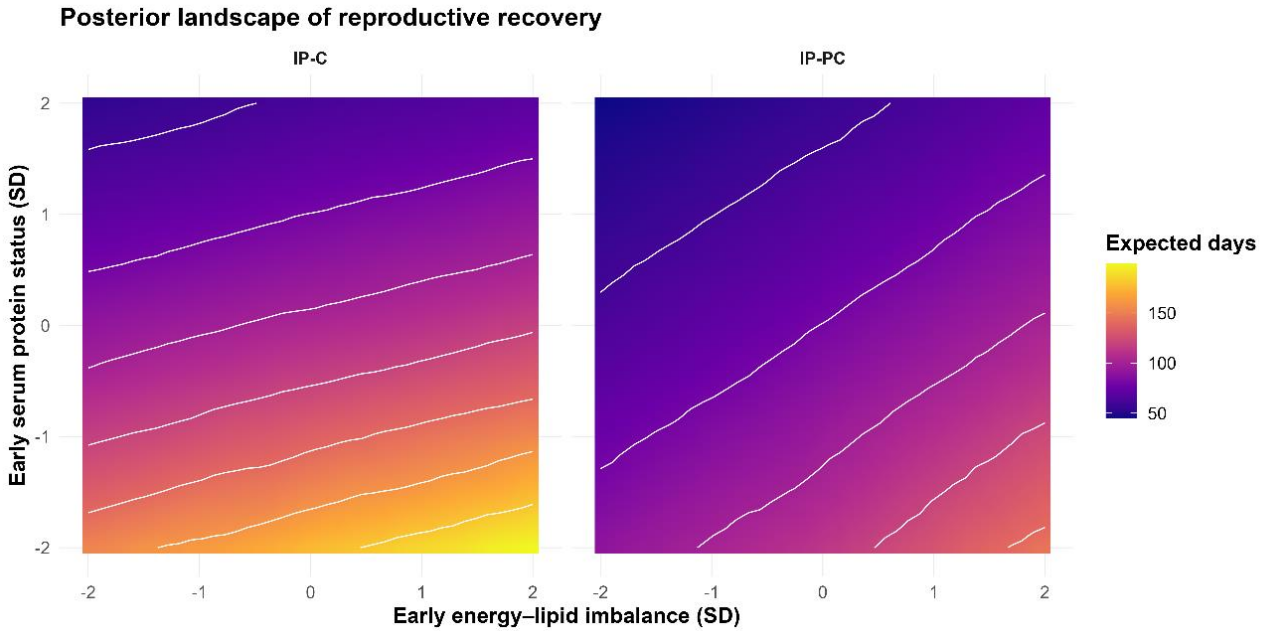


Figure 6. Posterior landscape of reproductive recovery according to early-postpartum energy–lipid imbalance and serum protein status in Brown Swiss dairy cows from postpartum weeks 1 to 5 under high-altitude grazing conditions in Yanacancha, Junín, Peru. IP–C = calving-to-conception interval; IP–PC = calving-to-first-service interval.

DISCUSSION

Energy-lipid imbalance and serum protein levels reached their highest posterior values around postpartum week 5 and subsequently declined, whereas BCS and BW decreased after calving. These patterns were consistent with the substantial metabolic adjustments that occur during early lactation (LeBlanc, 2010), when nutrient requirements for milk synthesis increase more rapidly than feed intake and metabolic capacity (Bertoni and Trevisi, 2013; Caixeta and Omontese, 2021; Spaans et al., 2022). The increase in energy-lipid imbalance around week 5 may reflect a greater reliance on endogenous energy reserves at the initiation of lactation. When nutrient intake does not fully meet energy requirements, adipose tissue mobilization increases the supply of fatty acids to the liver, alters circulating glucose and lipid metabolites, and may promote ketone body production (Roche et al., 2009; LeBlanc, 2010; Zhao et al., 2019). The observed concurrent decline in BCS and BW in the present study supported this finding. However, the energy-lipid indicator, comprising β-hydroxybutyrate, glucose, cholesterol, and triglycerides, reflected a broader metabolic pattern rather than a direct measure of negative energy balance. Similar multimetabolite methods have been employed to analyze metabolic adaptation and disease-related profiles in transition dairy cows (Hailemariam et al., 2014; Kayano and Kida, 2015). The considerable overlap in sampling periods indicated that the extent of metabolic adaptation differed among cows. Under high-altitude grazing conditions, this variability may be affected by factors such as environmental temperature, forage availability, diet, and hypoxic exposure (Bartl et al., 2009; Kong et al., 2021).

The postpartum week had a more pronounced influence on milk production and composition than the metabolic conditions. Milk yield remained comparatively higher during the middle of the observation period before declining at week 9, whereas fat, lactose, protein, solids-not-fat, and the fat-to-protein ratio demonstrated different temporal patterns. These changes were expected, as nutrient distribution, mammary demand, dry matter intake, and body reserve mobilization vary considerably during early lactation (Roche et al., 2009; LeBlanc, 2010; Caixeta and Omontese, 2021). Predictions at the mean and ±1 SD for energy-lipid imbalance and serum protein level overlapped extensively. This finding suggested that differences in these integrated indicators, within the observed range, had a smaller effect on milk-trait variability than the postpartum period. This finding did not suggest that metabolism was unimportant for lactation; instead, metabolic effects on milk production occur through different interconnected pathways and can be influenced by diet, physiological stage, individual capacity, and environmental factors (Bertoni and Trevisi, 2013; Puppel and Kuczyńska, 2016; Spaans et al., 2022). Studies conducted in high-altitude and high-Andean environments similarly demonstrated that factors such as diet, breed, season, and production environment can influence milk yield and metabolic characteristics (Bartl et al., 2009; Estremadoyro et al., 2024).

The correlation between higher serum protein levels and decreased lactose and milk protein should be interpreted with caution. The serum protein indicator used in the present study combined albumin and total serum protein, thereby

representing the systemic biochemical status of proteins rather than directly indicating the supply of metabolizable protein or amino acids to the mammary gland. Albumin and total protein levels can change due to hepatic synthesis, inflammatory responses, hydration status, and physiological stages (Bertoni and Trevisi, 2013; Puppel and Kuczyńska, 2016; Spaans et al., 2022). Therefore, the observed changes might reflect relationships among systemic metabolic adaptation, lactation stage, and milk production, rather than a direct inhibitory impact of circulating proteins. The negative association between ionized calcium and solids-not-fat had a credible interval of zero. The present findings indicated that individual biomarkers need to be interpreted within a wider metabolic framework, rather than evaluated only by the sign of an estimated coefficient (Hailemariam et al., 2014; Caixeta and Omontese, 2021).

The strongest animal-level link observed in the present study was the inverse relationship between BCS and ketosis. Within the range observed in these cows, higher BCS was associated with substantially lower odds of ketosis, possibly because lower scores during early lactation reflect greater prior mobilization of adipose reserves, thereby increasing fatty acid delivery to the liver and favoring ketogenesis (Roche et al., 2009; Çolakoğlu et al., 2019; Zhao et al., 2019; Ghaffari et al., 2023). Cows with a high BCS may have reduced feed intake and increased postpartum lipid mobilization. However, early lactation and the extent of body condition loss are distinct indicators of changes in energy reserves (Roche et al., 2009; Çolakoğlu et al., 2019; Ghaffari et al., 2023). The peak in energy-lipid imbalance at week 5, coinciding with the highest observed prevalence of ketosis, further supported the link between increased lipid mobilization and ketogenesis during this period (LeBlanc, 2010; Caixeta and Omontese, 2021).

In contrast, a high number of cows were classified as hypocalcemic throughout the study, which could not be explained solely by the conventional periparturient decline in calcium. Calcium demand increases abruptly around calving due to colostrum and milk synthesis; therefore, disturbances in calcium homeostasis are most commonly associated with the immediate periparturient period (Weaver et al., 2016; Caixeta and Omontese, 2021). The persistence of low ionized calcium levels in later postpartum assessments indicated that additional factors, such as mineral intake, individual calcium regulation, analytical methods, or the suitability of the threshold, could be contributing. Ionized and total calcium are related but not diagnostically suitable, and their relationship may change in early lactation (Weaver et al., 2016; Cohrs et al., 2023). Therefore, the high prevalence observed in the present study should be interpreted as a deficiency of ionized calcium, not as clinical milk fever.

The associations between early postpartum metabolic state and reproductive recovery were less strong and less precise. A greater energy-lipid imbalance was associated with longer reproductive intervals, while higher serum protein levels generally reduced the duration, particularly in the calving-to-conception interval. Prolonged negative energy balance impairs insulin/IGF signaling, follicular development, steroidogenesis, and resumption of ovarian activity (Roche et al., 2009; Nigussie, 2018; Çolakoğlu et al., 2019). Conversely, a more favorable overall metabolic condition might facilitate faster recovery of reproductive function (Wathes et al., 2007). Nevertheless, serum protein levels should not be viewed as a direct indicator of dietary protein sufficiency. However, since all 95% credible intervals for the reproductive time ratios include 1, these associations should be considered suggestive rather than conclusive. Additionally, their uncertainty might be due to the fewer cows with full reproductive results.

CONCLUSION

Brown Swiss cows raised in high-altitude grazing areas exhibited a heterogeneous, non-linear metabolic transition from the prepartum period through early lactation, with the greatest energy-lipid imbalance occurring approximately during the postpartum week 5. Changes in milk yield and composition were more closely linked to postpartum stage than to variations in energy-lipid imbalance and serum protein status. Body condition score demonstrated the strongest association with ketosis, with higher BCS associated with lower odds of ketosis. Associations between hypocalcemia and reproductive intervals were less precise. The current findings were limited by the observational design, evaluation within a single high-altitude production setting, a limited number of cows with complete reproductive data, and the lack of a low-altitude comparison group. Future studies should include larger populations from multiple high-altitude herds, verify ionized calcium reference thresholds specifically for cows living at high altitudes and explore whether integrating repeated metabolic assessments with BCS improves early detection of cows at higher metabolic risk.

DECLARATIONS

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Authors' contributions

Lucy Remigio-Lorenzo was responsible for funding acquisition, project administration, field sample collection, laboratory analyses, and the preparation of the original draft. Fernando Arauco Villar contributed to the study design, methodology, funding acquisition, project administration, review, and editing. Noemí Mayorga Sánchez performed the laboratory analyses and provided resources. Yakelin Mauricio-Ramos performed the laboratory analyses. Ide Unchupaico-Payano reviewed and critically revised the manuscript. Jordan Ninahuanca performed the statistical analyses and drafted the manuscript. All authors reviewed and approved the final edition of the manuscript.

Availability of data and materials

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

Competing interests

The authors declare no conflict of interest.

Ethical considerations

Generative artificial intelligence was used only to support English-language editing, improve grammatical accuracy, and enhance sentence clarity. Artificial intelligence tools were not used to generate, modify, or analyze the study data, nor to make scientific decisions or independently interpret the results. All AI-assisted content was critically reviewed, verified, and revised by the authors. The authors accept full responsibility for the accuracy, originality, integrity, and scientific content of the manuscript, both before submission and after acceptance.

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Effects of Adding *Aquilaria malaccensis* Leaf Extract on Motility and Post-Thaw Semen Quality Using Computer-Assisted Sperm Analysis in Saanen Goats

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ABSTRACT

The quality of frozen semen from Saanen goats plays a vital role in the success of artificial insemination. The present study aimed to evaluate the effects of different semen diluents on post-thaw semen quality and sperm kinematic parameters using computer-assisted sperm analysis (CASA). Fresh semen samples were obtained from two 3-4-year-old Saanen goats, weighing 64 kilograms and 92 kilograms, with an average fresh motility of 80%. Semen quality was assessed under a microscope, focusing on motility, viability, abnormalities, concentration, and total motile spermatozoa. In the current laboratory experiment, five treatment groups were used with 10 replicates. The first group served as the negative control and consisted of tris-egg yolk without *Aquilaria malaccensis* leaf extract, the second group contained tris-egg yolk with 0.10 mg of *Aquilaria malaccensis* leaf extract, the third group consisted of tris-egg yolk with 0.15 mg of *Aquilaria malaccensis* leaf extract, the fourth group contained tris-egg yolk with 0.20 mg of *Aquilaria malaccensis* leaf extract, and the fifth group received the AndroMed® (positive control). The present results indicated that the use of different diluents had a significant effect on post-thawing semen quality, including motility, progressive motility, average path distance, curved line distance, straight line distance, average path velocity, straight line velocity, curvilinear velocity, amplitude of lateral head, beatcross frequency, and wobble. However, there was no significant difference in post-thawing semen quality in terms of individual motility, viability, abnormalities, concentration, total motile spermatozoa, straightness, and linearity. The use of tris-egg yolk diluents supplemented with *Aquilaria malaccensis* leaf extract at a concentration of 0.10 mg and AndroMed® indicated that the highest results among all treatments were demonstrated by the highest post-thaw semen quality.

Keywords: Antioxidant, *Aquilaria malaccensis*, Sperm kinematic, Saanen goat

INTRODUCTION

The agricultural and livestock sectors in Indonesia have exhibited significant development and are considered promising contributors to future economic growth. Livestock husbandry, particularly the production of small ruminants such as goats, constitutes a vital component of the agricultural sector. Recent statistics from the Central Statistics Agency of Indonesia reported that the goat population in Indonesia was approximately 15.7 million head (Directorate General of Livestock and Animal Health Services, 2024). Saanen goats are among the most important dairy goat breeds due to their high milk production capacity and adaptability to diverse environmental conditions (Zeljić Stojiljković et al., 2025). Previous studies reported that Saanen goats produced an average of 2.55 kg of milk per day, a yield that was higher than that of most dairy goat breeds (Ferro et al., 2017). Therefore, Saanen goats were extensively used as a genetic foundation for dairy goat improvement programs worldwide (Upadhyay et al., 2024). Genetic quality improvement in livestock can be achieved by increasing livestock productivity through the application of artificial insemination (AI) technology (Mansour, 2025).

Artificial insemination improves reproductive performance and pregnancy outcomes while reducing the need to maintain breeding males, thereby increasing production efficiency (Ayantoye et al., 2025). The success of AI is largely dependent on the quality of frozen-thawed semen. Luo et al. (2019) reported that outcomes are influenced by post-thaw semen quality, the timing of insemination, and the effectiveness of estrus synchronization protocols. Sharafi et al. (2022) reported that the cryopreservation of goat semen is associated with several physiological challenges due to the high sensitivity of spermatozoa to cold shock. During cooling, freezing, and thawing, sperm cells may experience membrane

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damage, reduced motility, osmotic stress, and elevated reactive oxygen species (ROS) levels, which can ultimately impair semen quality. The ROS-induced oxidative stress is a major factor contributing to sperm damage during cryopreservation. High ROS levels can lead to DNA fragmentation, lipid peroxidation, acrosomal damage, and mitochondrial dysfunction, all of which reduce sperm viability and fertilization capacity (Wang et al., 2025). Therefore, the quality of semen after thawing is often lower than that of fresh semen, underscoring the need to develop appropriate diluents that protect spermatozoa from oxidative stress. Zhang et al. (2022) further indicated that excessive ROS disrupts plasma membrane integrity, acrosome integrity, sperm motility, and fertilization function.

The effectiveness of a semen diluent depends on its ability to provide metabolic substrates, maintain physicochemical stability, protect sperm membrane integrity, and minimize cryoinjury during freezing and thawing (Bustani and Baiee et al., 2021). Liang et al. (2023) reported that goat semen cryopreserved with Optidyl® (egg yolk-based) and Andromed® (soybean lecithin-based) diluents exhibited significantly higher post-thaw sperm motility than semen preserved in skim milk-based diluents. Moreover, semen frozen with skim milk diluent demonstrated lower membrane integrity, acrosome integrity, and mitochondrial activity after thawing. Different types of diluents frequently used in semen cryopreservation include tris-egg yolk and skim milk. In addition to conventional diluents, commercial formulations such as AndroMed® and Ovixcell® have been developed and are widely used for the cryopreservation of Saanen goat semen due to their standardized composition and protective effects on sperm quality (Nisfimawardah et al., 2023). Adding 2% of the natural antioxidant black cincau leaf extract to skim milk yolk diluent resulted in a 50.40% increase in motility compared to 43.42% without the addition (Wahjuningsih et al., 2021). The integration of natural antioxidants offers a viable alternative, as it is more environmentally sustainable compared to antioxidants derived from egg yolk. Tris-egg yolk diluent contains natural antioxidant compounds derived from egg yolk, including low-density lipoproteins (LDL), phospholipids, and α-tocopherol, which contribute to membrane protection and reduce oxidative damage during cryopreservation (Mahiddine and Kim, 2021).

Aquilaria malaccensis leaf extract (ALE) contains bioactive compounds such as flavonoids, phenolics, and terpenoids that act as antioxidants and anti-inflammatory agents (Batubara et al., 2020). The antioxidant potential of ALE can improve membrane integrity, prevent lipid peroxidation, and maintain sperm motility during freezing (Ismail et al., 2019). The application of ALE in goat semen cryopreservation remains limited. Furthermore, comprehensive studies have compared the effects of different diluents on frozen-thawed semen quality, evaluated using both conventional microscopic assessment and computer-assisted sperm analysis (CASA; Anand and Yadav, 2016). The CASA is widely recognized as a reliable tool for the objective evaluation of sperm motility and kinematic characteristics, providing more precise and reproducible measurements than conventional microscopic assessments (Shah et al., 2023). The present study aimed to assess the potential of ALE as a natural antioxidant and to identify the optimal diluent formulation for improving the cryopreservation and post-thaw quality of Saanen goat semen.

MATERIALS AND METHODS

Ethical approval

All study procedures were carried out in accordance with Standard Operating Procedures (SOPs) at the Artificial Insemination Center (AIC) in Lembang, Bandung, West Java, Indonesia, in accordance with ISO/IEC 17025-2008 and were supervised by a veterinarian. Ethical guidelines and research procedures were provided by AIC and approved by the Ethics Committee of the Faculty of Veterinary Medicine, Brawijaya University, Malang, Indonesia, with number No. 77-KEP-FKHUB-2026.

Study design

This study utilized two male Saanen goats aged 3 to 4 years, located at the AIC in Lembang, Bandung, West Java, Indonesia. The goats were allowed to graze in the morning on forage primarily composed of Indigofera zollingeriana and *Calliandra calothyrsus*. Each goat received a daily diet comprising 2 kg of forage, 2.5 kg of leguminous feed, and 1 kg of concentrate. The concentrate used at AIC Lembang was reported to have contained 16–18% crude protein (CP) and 60–75% total digestible nutrients (TDN). The goats weighed 64 kg and 92 kg, respectively. Semen collection was performed twice a week for five weeks using an artificial vagina (IMV, France). Initially, semen was collected using false mounting to enhance libido and facilitate collection within the goat enclosure.

Extract preparation

Aquilaria malaccensis leaves were collected from an agarwood plantation located in Lamandau Regency, Central Kalimantan, Indonesia. The preparation of ALE was initiated by drying the leaves in an oven at 55°C for 72 hours. The dried leaves were subsequently macerated in 70% ethanol at a 1:10 (w/v) ratio (100 g leaf powder in 1000 mL solvent)

for 24 hours. The mixture was then filtered to separate the filtrate from the residue, and the maceration extract was collected and stored. The remaining residue was further extracted using microwave-assisted extraction (MAE). The resulting extract was centrifuged at 3500 rpm for four minutes, and the supernatant was collected. Subsequently, the supernatant was filtered through Whatman No. 1 filter paper, and the ethanol was removed on a rotary vacuum evaporator at 60°C for 45 minutes, yielding a concentrated extract. Finally, the crude extract was stored in dark glass bottles and kept under refrigerated conditions until further use.

Diluent preparation

The present study used two types of diluents, namely tris-egg yolk (AIC, Lembang, Indonesia) with the addition of ALE at 0 mg (T0), 0.10 mg (T1), 0.15 mg (T2), 0.20 mg (T3), and the patented AndroMed® diluent (minitube, Germany; T4). The tris-based diluent consisted of a buffer solution containing 2.42 g of tris hydroxymethyl aminomethane (Merck, Germany) supplemented with antibiotics, 1.28 g of citric acid (Merck, Germany), 2.16 g of fructose (Merck, Germany), 0.1 g of penicillin (Meiji, Japan), 0.1 g of streptomycin (Meiji, Japan), and 100 ml of water for injection. The buffer was stirred with a magnetic stirrer for 4 hours, then 74 mL of the homogenized buffer was removed. This formed the basis of the Andromed thinner buffer, which was then mixed with 20 mL of egg yolk and 6 mL of glycerol (Merck, Germany) to create the tris egg yolk diluent. Homogenization was performed for 30 minutes, followed by centrifugation at 4000 rpm for 30 minutes to separate the solution from the precipitate. The resulting solution was then stored in a refrigerator at 4-5℃ (AIC, 2026). The AndroMed® diluent contained buffer, citric acid, glycerol, fructose, aquabidest, phospholipids, spectinomycin, 15 mg of lincomycin, 5 mg of tylocin, and 25 g of gentamicin.

The process of preparing semen diluent was carried out by placing tris-egg yolk diluents, AndroMed®, and ALE in a water bath at 37℃. There were five treatment groups. The first group received a tris-egg yolk diluent without ALE (T0), the second group was treated with a tris-egg yolk diluent containing 0.10 mg/100 mL of ALE (T1), the third group comprised a tris-egg yolk diluent with 0.15 mg/100 mL of ALE (T2), the fourth group was administered a tris-egg yolk diluent with 0.20 mg/100 mL of ALE (T3), and the fifth group solely received AndroMed® (T4). Following supplementation, each diluent was vortex-mixed for three minutes and maintained in a water bath to preserve temperature stability. Treatment T4 was prepared by diluting the commercial diluent with distilled water at a 1:4 ratio (20 mL of AndroMed® to 80 mL of distilled water), yielding a final volume of 100 mL without ALE supplementation (Nisfimawardah et al., 2023). Semen dilution was performed in a single step by adding fresh semen to the five dilution treatments, using Formula 1.

$$\text{Volume total} = \frac{\text{fresh semen (mL)} \times \text{concentration} \times 10^6}{50 \times 10^6} \times 0.25 \text{ (mL)} \qquad \text{(Formula 1)}$$

Semen dilution at the AIC in Lembang, Indonesia, was performed in a one-step procedure, in which the calculated volume of semen was mixed directly with the total volume of diluent at one time. To ensure the quality of semen during cold storage, semen mixed with a diluent at a temperature of 37°C was placed within a water jacket filled with water maintained at 37°C and subsequently stored in a refrigerator set to a temperature of 4-5°C for a duration of 30 minutes. The semen mixture is then removed from the water jacket and kept at 4-5°C for an additional three hours. The pre-freezing motility analysis should meet a minimum threshold of 55%, in accordance with the Indonesian National Standard (INS, 2014). When motility fell at or below 55%, the freezing procedure was not initiated.

Semen collection

Semen was collected twice weekly in the morning using a goat artificial vagina (IMV, France) containing 500 mL of warm water maintained at 50°C. The artificial vagina was pumped and coated with lubricating gel to prevent injury when the semen was collected. The volume of Saanen goat semen obtained was 1-2 mL. Once the semen had been collected, the samples were sent to the laboratory at AIC in Lembang, Indonesia, which specialized in semen quality testing. Then, the semen samples were placed in a container designed to protect them from environmental exposure, such as light.

Semen freezing procedures

The cryopreservation procedure consisted of three evaluation stages, including fresh semen, chilled semen prior to freezing, and frozen-thawed semen. During the fresh semen stage, semen quality was assessed based on motility (≥ 70%), viability, and sperm abnormalities using a microscope (Olympus BX-53, Japan) at 400x magnification. Then, equilibration was carried out in a cool top for three hours, and a pre-freezing analysis was performed to assess semen quality, including motility ≥ 55%, viability, and abnormalities, using a light microscope (Olympus BX-53, Japan) at 400x magnification. Semen samples meeting the quality requirements were subsequently packaged and sealed into 0.25-

mL straws in accordance with the designated treatment groups. The filled straws were subsequently subjected to a 7-minute pre-freezing step at approximately −136°C, followed by immersion in liquid nitrogen (LN_2) at −196°C and storage. Each stage of evaluating fresh semen, liquid semen (before freezing), and frozen semen (after freezing) was compared using CASA (AndroVision®, Germany).

Evaluation of semen quality

Spermatozoa motility

Sperm motility was assessed by preparing a 20 µL semen sample on an object glass and covering it with a coverslip. A light microscope at 400x magnification was used to observe the semen sample. Sperm motility was assessed at three stages following the INS (2014) guidelines, immediately after collection (fresh semen, ≥70%), prior to cryopreservation (liquid semen, ≥ 55%), and after thawing (frozen semen, ≥ 40%). Motility assessment was performed using five fields of view, according to Formula 2 (Susilawati, 2011).

$$\text{Motility (\%)} = \frac{\text{Motility 1 + Motility 2 + Motility 3 + Motility 4 + Motility 5}}{5} \times 100\% \quad \text{(Formula 2)}$$

Spermatozoa viability

Viability or sperm survival was determined by preparing a semen smear from 10 µL, adding 20 µl of eosin-nigrosin dye, mixing thoroughly, and spreading the mixture thinly and slowly for evaluation. Sperm viability was assessed using a light microscope at 400× magnification. Spermatozoa that absorbed the stain were classified as non-viable, whereas unstained spermatozoa were considered viable, which indicated live cells (Zou et al., 2021). The evaluation was performed by counting at least 200 live and dead spermatozoa, as described by Formula 3 (Susilawati, 2011).

$$\text{Viability (\%)} = \frac{\text{Viability}}{\text{Viability + nonviability}} \times 100\% \quad \text{(Formula 3)}$$

Spermatozoa abnormality

Sperm abnormalities were assessed by preparing a semen smear of 10 µl, adding 20 µl of eosin-nigrosin stain, mixing thoroughly, and then spreading the mixture thinly and slowly for evaluation. The evaluation of abnormalities was performed using a microscope (Olympus BX-53, Japan) set at 400x magnification. The abnormalities assessed include the shape of the head (too large, small, or flat) and the middle or tail (broken, curved, or abnormal). The evaluation was performed by counting at least 200 live and dead spermatozoa using Formula 4 (Susilawati, 2011).

$$\text{Abnormality (\%)} = \frac{\text{Sperm Abnormal}}{\text{Sperm abnormal + sperm normal}} \times 100\% \quad \text{(Formula 4)}$$

Spermatozoa concentration

Sperm concentration was measured as the number of sperm per unit volume to determine the dilution requirements for semen. The concentration was determined using an SDM 6 spectrophotometer (Minitube, Germany) in accordance with the semen freezing protocol established by the AIC, Lembang, Indonesia, which incorporated the addition of fresh semen, 0.9% NaCl, and aquabidest. The findings were subsequently presented on the device in units of millions per milliliter.

Total motile spermatozoa

Total motile spermatozoa (TMS) were the total number of motile spermatozoa calculated by multiplying motility by concentration. At the time of post-thawing, TSM was calculated as the product of motility and concentration (Zou et al., 2021).

Statistical analysis

The present study used a randomized block design (RBD) with five treatment groups and 10 replicates. The data obtained in this study were analyzed using RStudio version 4.4.3 and one-way ANOVA with Duncan's Multiple Range Test. The significance level was established at a p-value less than 5% ($p < 0.05$) for all statistical analyses.

RESULTS AND DISCUSSION

Fresh Saanen goat semen

Fresh semen was an indicator of semen quality before freezing. Fresh semen was obtained immediately after semen collection and placed in a sample test tube in the laboratory. In accordance with INS (2014), fresh semen exhibiting motility ≥ 70% was deemed eligible for the dilution stage. The current results on the quality of fresh Saanen goat semen are presented in Table 1.

The fresh semen exhibited a volume of 2.5 ± 0.53 mL, a milky-white color, a pH of 6.5, a precise odor, and a thick consistency. These macroscopic characteristics indicated normal semen quality and were consistent with previous findings reported by Cardenas-Padilla et al. (2024), who observed fresh semen motility of more than 70% accompanied by high semen consistency. Microscopic evaluation revealed a mass motility score of +++ (vigorous wave motion), along with individual motility of 80.00 ± 10.54%, viability of 75.50 ± 0.74%, abnormalities of 4.46 ± 0.31%, sperm concentration of 3578 ± 223.32 × 10^6 spermatozoa/mL, and TMS of 2850 ± 302.59 × 10^6 spermatozoa/mL. These findings indicated that the ejaculates met the minimum quality requirements for further processing, as individual motility exceeded the recommended threshold of 70%. The current findings were consistent with those of Dapawole and Sirappa (2021), who indicated that fresh semen used in dilution should be at least 70% in goats.

Table 1. Fresh semen quality of Saanen goats

Parameter	**Macroscopic finding** (Mean + SD)
Volume (ml)	2.5 ± 0.53
Color	Milky white
pH	6.5
odor	Typical
Consistency	Thick
Parameter	**Microscopic finding** (Mean + SD)
Mass motility	+++
Individual motility (%)	80 ± 10.54
Viability (%)	75.50 ± 0.74
Abnormality (%)	4.46 ± 0.31
Concentration (million/ml)	3578 ± 223.32
Total motile spermatozoa (million/ml)	2850 ± 302.59

Mass motility was assessed based on the intensity of wave motion. +: Weak wave motion samples, ++: Moderate wave motion samples, and +++: Vigorous wave motion samples

Evaluation of Saanen goats' frozen semen

The supplementation of ALE in the tris-egg yolk diluent was intended to enhance the antioxidant capacity of the medium and provide additional protection to spermatozoa during the cryopreservation process. This finding was consistent with previous studies showing improved sperm preservation during cryopreservation. The present study focused on the effect of AML supplementation on post-thaw semen quality parameters. The beneficial effects of ALE might be attributed to its high content of bioactive compounds, including flavonoids, phenolic compounds, tannins, and terpenoids, which exhibit strong antioxidant activity. These compounds act as free radical scavengers, thereby reducing ROS accumulation and minimizing oxidative stress during the cryopreservation process (Eissa et al., 2022). AndroMed® was used as the commercial diluent due to its soy lecithin content and being free of animal-derived ingredients such as egg yolk; therefore, it protected the spermatozoa during cold storage (Bustani and Baiee, 2021). The current results on Saanen goats' semen quality were presented in Table 2.

There was a significant difference in pre-freezing sperm motility across the groups ($p < 0.05$; Table 2). Motility was assessed using a microscope across five fields of view as a percentage. Treatment T1 demonstrated higher motility after freezing than T0 (42.00 ± 10.33%), T3 (39.00 ± 13.08%), and T4 (43.50 ± 11.80%). The current results demonstrated that Treatment T1, supplemented with 0.10 mg of ALE, exhibited superior post-thaw motility compared with T0 (42.00 ± 10.33%), T3 (39.00 ± 13.08%), and T4 (43.50 ± 11.80%). These findings indicated that adding 0.10 mg of ALE provided optimal protection during cryopreservation, thereby preserving plasma membrane integrity and maintaining the functional activity of the sperm flagellar apparatus. Studies evaluating the supplementation of *Aquilaria malaccensis* extract in a tris-egg yolk extender were limited; therefore, the present findings were discussed in comparison with previous studies that employed extracts from other plant sources. Similar results were reported by Wahjuningsih et al. (2019), who reported post-thawing motility of 63% by adding 3% moringa leaves' extract. The differences in motility among treatments were due to the bioactive content of ALE, including flavonoids and phenolics, which can protect the sperm against oxidative stress induced by ROS (Ismail et al., 2019). Kaltsas (2023) indicated that mitochondrial integrity and ATP production decreased due to ROS, thereby impairing motility. This finding was consistent with the current results, which demonstrated that 0.20 mg of ALE was mildly toxic when the antioxidant concentration exceeded normal limits. The current result was consistent with those of Isnaini et al. (2019), who reported that the addition of 6% mangosteen peel extract antioxidants resulted in only 24.5% motility. This suggested that excessive antioxidant addition may induce toxic effects on spermatozoa.

Table 2. Evaluation of Saanen goats' frozen semen using a light microscope (400 × magnification)

Parameter	**Fresh semen** (Mean + SD)	**Before freezing** (Mean + SD)					**After freezing** (Mean + SD)				
		T0	T1	T2	T3	T4	T0	T1	T2	T3	T4
Motility (%)	80 ± 10.54	60.00 ± 6.67^{ab}	63.00 ± 7.89^{a}	58.00 ± 5.37^{bc}	56.50 ± 5.80^{c}	63.50 ± 4.74^{a}	42.00 ± 10.33	49.00 ± 9.66	40.00 ± 16.83	39.00 ± 13.08	43.50 ± 11.80
Viability (%)	75.50 ± 0.74	62.60 ± 5.53^{b}	67.45 ± 6.43^{a}	64.20 ± 5.41^{ab}	66.19 ± 4.62^{ab}	68.30 ± 6.09^{a}	54.31 ± 9.49	57.74 ± 9.24	55.51 ± 10.91	56.94 ± 9.99	51.65 ± 8.79
Abnormality (%)	4.46 ± 0.31	6.35 ± 1.02	5.55 ± 0.73	5.85 ± 0.95	6.41 ± 0.96	5.87 ± 1.14	6.96 ± 1.29	6.42 ± 0.83	6.82 ± 0.94	7.56 ± 0.59	6.47 ± 0.95
Concentration (million/straw)	3578 ± 223.32	61.14 ± 1.95	63.68 ± 3.12	63.24 ± 3.82	64.14 ± 2.54	64.65 ± 4.11	60.63 ± 2.47	63.12 ± 3.34	62.64 ± 3.86	63.57 ± 2.56	64.08 ± 4.16
TMS (million/straw)	2850 ± 302.59	36.75 ± 4.8	40.13 ± 5.6	36.69 ± 4.2	36.30 ± 4.5	41.09 ± 4.4	25.46 ± 6.48	31.04 ± 6.90	25.17 ± 11.20	24.78 ± 8.40	27.73 ± 7.39

^{ab}Different supperscript letters in the same row indicate a significant difference ($p < 0.05$). TSM: Total motile spermatozoa, ALE: *Aquilaria malaccensis* leaf extract, T0: Tris-egg yolk treatment group, T1: Tris-egg yolk + ALE at 0.10 mg, T2: Tris-egg yolk + ALE at 0.15 mg, T3: Tris-egg yolk + ALE at 0.20 mg, T4: AndroMed®

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Viability is the survival rate of spermatozoa assessed using semen preparation with eosin-nigrosin staining (Abedin et al., 2023). The use of different diluent formulations resulted in significant differences in sperm viability prior to freezing ($p < 0.05$); however, post-thaw viability did not differ significantly among treatments ($p > 0.05$). Despite the lack of statistical significance after thawing, treatments supplemented with ALE (T1, T2, and T3) consistently exhibited higher viability than the control treatment (T0). The current results indicated that the antioxidant compounds present in ALE may have provided protective effects against cryodamage by maintaining sperm plasma membrane stability throughout the freezing and thawing procedures. The current results indicated that spermatozoa cell membranes were susceptible to lipid peroxidation because ruminants have a high content of unsaturated fatty acids in their cell membranes. Saratsi et al. (2024) indicated that spermatozoa viability decreased with storage time, while the addition of cryoprotectants to the diluent increased the viability of spermatozoa stored in liquid nitrogen.

Sperm abnormalities were evaluated by examining morphological defects in spermatozoa, including abnormalities of the head, midpiece, and tail regions. These defects might be attributable to inherent developmental disorders or to physical damage sustained during cryopreservation (Humann-Ziehank, 2026). Sperm abnormality rates did not differ among the diluent treatments either before freezing or after thawing ($p > 0.05$). Nevertheless, numerical variations were observed, with Treatment T3 exhibiting the highest percentage of abnormalities before freezing (6.41 ± 0.96%). Following thawing, the highest rate of abnormalities was observed in T0 (6.96 ± 1.29%), whereas T1 demonstrated the lowest rate of abnormalities (6.42 ± 0.83%). The current findings indicated that ALE supplementation did not adversely affect sperm morphology and may have helped maintain structural integrity during cryopreservation. The increase in sperm abnormalities observed after cryopreservation may have been attributed to structural damage induced during the freezing and thawing processes (Zang et al., 2025). Cryoinjury was reported to affect sperm membranes, acrosomes, mitochondria, and tail structures, leading to morphological defects and reduced sperm quality. The addition of 1% and 2% soybean lecithin to tris egg yolk diluents was reported to enhance membrane protection during cryopreservation, thereby minimizing sperm morphological damage and improving post-thaw semen quality (Suwor et al., 2025). The abnormality values observed in the present study remained below 5%, indicating that the tris-egg yolk diluent supplemented with ALE, as well as the AndroMed® diluent, did not induce substantial structural damage to spermatozoa and remained within the acceptable range for semen cryopreservation. Representative images of sperm viability and morphological abnormalities are illustrated in Figure 1.

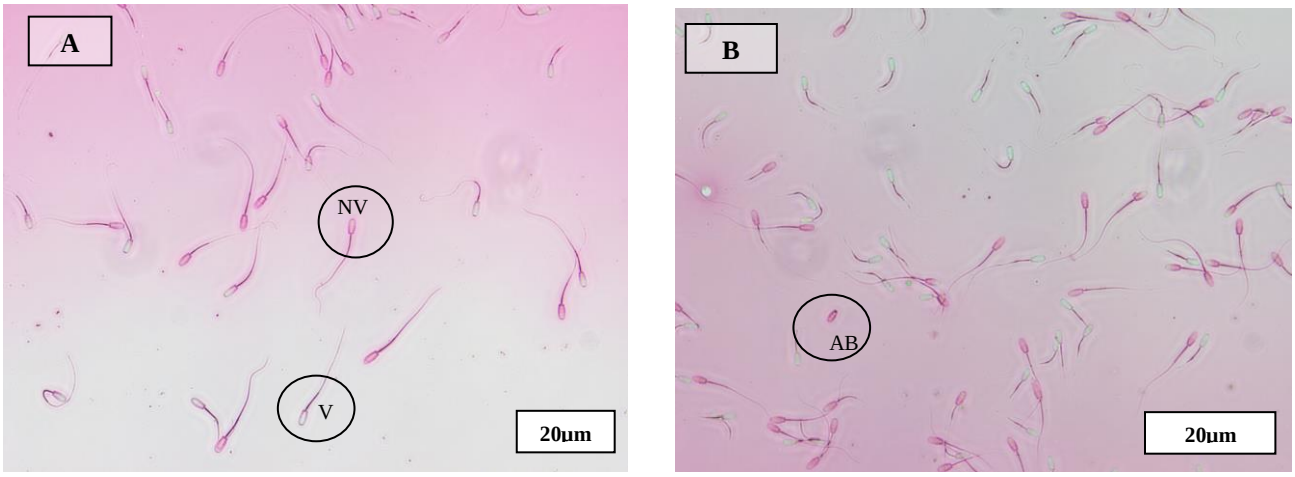


Figure 1. Spermatozoa viability and abnormalities post-thawing using a light microscope. **A**: Viability preparation, **NV**: Non-viable, **V**: Viable (head does not absorb color), **B:** Abnormality preparation, **AB**: Abnormal spermatozoa (the sperm head was detached from the tail). Eosin–nigrosin staining, 400x magnification.

Sperm concentration was measured before freezing and after thawing. No significant differences in sperm concentration were observed among the diluent treatments at either evaluation stage ($p > 0.05$). The lowest post-thawing concentration was observed in T0, which was 60.63 ± 2.47 million/straw, and the highest post-thawing concentration was observed in Treatment T4, which was 64.08 ± 4.16 million/straw. The concentration across treatment groups was stable across diluents, ranging from 60 to 64 million/straw. This finding indicated that supplementation with ALE did not affect the number of spermatozoa but helped maintain their functional quality during cryopreservation. Similar results were reported by Hassan et al. (2021), who found that adding natural antioxidants to semen diluents improved post-thaw sperm motility and viability without affecting sperm concentration. Likewise, El-Seadawy et al. (2022) reported that antioxidant supplementation primarily protected sperm membranes against oxidative damage and enhanced sperm function, whereas sperm concentration remained unchanged. Therefore, the beneficial effects of ALE were likely associated with its ability to preserve sperm physiological integrity rather than alter sperm cell numbers. Sperm concentration was influenced by individual male factors, such as age, genetic background, reproductive status, and

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testicular size, as well as by semen collection frequency. Similarly, Kumar et al. (2025) found that genetic factors, breed, boar age, and physiological conditions affect fresh semen concentration and sperm survival during freezing.

The TMS was a combined parameter that integrated sperm concentration and motility (Novita et al., 2021). The TMS was considered essential for evaluating semen quality prior to AI, as it provided a comprehensive estimate of the number of motile spermatozoa available for fertilization. The current results indicated that the use of a diluent did not significantly affect TMS before freezing and post-thawing ($p > 0.05$). The highest post-thaw TMS was observed in Treatment T1 (31.04 ± 6.90 million/straw), whereas Treatment T3 demonstrated the lowest value (24.78 ± 8.40 million/straw). These findings indicated that adding 0.10 mg of ALE to the tris-egg yolk diluent improved sperm survival during cryopreservation. The phospholipid content of egg yolk and the antioxidant properties of ALE suppressed oxidative damage. These findings are consistent with those of Isnaini et al. (2019), who found that freezing semen using 2% mangosteen peel extract resulted in a motility value of 56.5%. Additionally, Tethool et al. (2022) indicated that egg yolk protected spermatozoa motility.

Evaluation of motility

The evaluation of frozen semen, before and after freezing, can be conducted either visually with a microscope or systematically using CASA to assess different motility parameters of spermatozoa (Fernandez-Novo et al., 2021). The inclusion of CASA reduced potential bias in semen evaluation by objectively quantifying sperm motility and motion characteristics through computerized analysis. This approach minimized the influence of observer subjectivity, fatigue, and differences in technical expertise, resulting in more accurate and reproducible assessments compared with conventional visual examination. Additionally, sperm motility parameters are crucial indicators of fertility, helping to identify males with lower reproductive potential and to select genetically superior sires for breeding programs (Hackerova et al., 2025). A total of 14 parameters were used in CASA, including motile, progressive, distance average path (DAP), distance curved line (DCL), distance straight line (DSL), velocity average path (VAP), velocity straight line (VSL), velocity curved line (VCL), straightness (STR), linearity (LIN), amplitude of lateral head (ALH), beat cross frequency (BCF), wobble (WOB), and head activity (HAC; Blagojević et al., 2024). The results of sperm motility using CASA before and after freezing stages are indicated in Tables 3 and 4.

Table 3. Evaluation of sperm motility before freezing using computer-assisted sperm analysis in Saanen goat semen

Before freezing / Kinematic parameters	T0	T1	T2	T3	T4
Motile (%)	75.42 ± 8.72	79.50 ± 8.99	79.36 ± 8.81	77.39 ± 11.06	75.03 ± 3.81
Progressive (%)	71.27 ± 10.74	76.34 ± 10.72	76.04 ± 9.67	73.08 ± 13.47	69.27 ± 6.03
DAP (µm)	17.05 ± 5.93	15.56 ± 3.46	16.11 ± 4.60	17.74 ± 7.08	14.54 ± 6.03
DCL (µm)	35.50 ± 12.50^{a}	33.90 ± 8.27^{ab}	34.90 ± 10.47^{ab}	37.51 ± 15.06^{a}	30.72 ± 11.40^{b}
DSL (µm)	12.95 ± 4.85	11.07 ± 2.63	11.76 ± 3.60	13.35 ± 5.92	11.58 ± 5.80
VAP (µm/sec)	52.76 ± 15.27	57.58 ± 12.72	56.06 ± 11.42	55.49 ± 15.08	55.22 ± 9.59
VSL (µm/sec)	41.70 ± 12.43	44.04 ± 9.31	43.38 ± 8.58	43.55 ± 12.50	45.84 ± 9.90
VCL (µm/sec)	105.85 ± 31.90	119.18 ± 29.62	115.86 ± 26.38	107.49 ± 39.55	112.75 ± 21.06
STR (%)	0.79 ± 0.02^{ab}	0.77 ± 0.01^{b}	0.77 ± 0.02^{b}	0.78 ± 0.02^{b}	0.82 ± 0.04^{a}
LIN (%)	0.40 ± 0.03^{b}	0.37 ± 0.02^{b}	0.38 ± 0.02^{b}	0.39 ± 0.01^{ab}	0.41 ± 0.04^{a}
ALH (µm)	0.90 ± 0.37^{b}	1.38 ± 0.30^{a}	1.37 ± 0.31^{a}	1.37 ± 0.40^{a}	1.21 ± 0.22^{b}
BCF (Hz)	9.01 ± 2.00	11.47 ± 1.07	11.69 ± 1.46	11.96 ± 2.28	11.60 ± 2.88
WOB (%)	0.50 ± 0.02	0.49 ± 0.02	0.49 ± 0.02	0.50 ± 0.02	0.49 ± 0.04
HAC (rad)	0.29 ± 0.08	0.32 ± 0.07	0.32 ± 0.07	0.31 ± 0.09	0.30 ± 0.05

^{a,b} Mean different superscript letters that vary in each row indicate significant differences at $p < 0.05$. T0: Tris-egg yolk, T1: Tris-egg yolk + 0.10 mg of ALE, T2: Tris-egg yolk + 0.15 mg of ALE, T3: Tris-egg yolk + 0.20 mg of ALE, T4: AndroMed®. DAP: Distance average path, DCL: Distance curved line, DSL: Distance straight line, VAP: Velocity average path, VSL: Velocity straight line, VCL: Velocity curved line, STR: Straightness, LIN: Linearity, ALH: Amplitude of lateral head, BCF: Beat cross frequency, WOB: Wobble, HAC: Head activity.

Table 4. Evaluation of sperm motility after freezing using computer-assisted sperm analysis in Saanen goat semen

After freezing / Kinematic parameters	T0	T1	T2	T3	T4
Motile (%)	66.11 ± 18.34[a]	68.01 ± 17.63[a]	63.81 ± 19.89[a]	57.65 ± 22.48[a]	45.15 ± 15.72[b]
Progresif (%)	61.74 ± 20.71[a]	62.79 ± 20.76[a]	58.41 ± 22.56[a]	53.25 ± 24.54[a]	40.56 ± 16.19[b]
DAP (µm)	15.44 ± 4.94[a]	16.06 ± 6.21[a]	14.39 ± 6.56[a]	15.10 ± 7.05[a]	11.10 ± 4.87[b]
DCL (µm)	30.66 ± 9.76[a]	30.86 ±10.99[a]	29.32 ± 11.81[a]	30.13 ± 13.66[a]	23.77 ± 10.31[b]
DSL (µm)	12.50 ± 4.52[a]	13.21 ± 5.91[a]	11.56 ± 6.08[ab]	12.47 ± 6.53[a]	9.25 ± 4.50[b]
VAP (µm/sec)	44.45 ± 16.23[a]	46.41 ± 17.90[a]	42.45 ± 18.18[ab]	40.46 ± 18.84[ab]	34.08 ± 13.94[b]
VSL (µm/sec)	36.89 ± 13.95[a]	39.03 ± 16.33[a]	35.06 ± 16.28[ab]	34.02 ± 16.69[ab]	29.20 ± 12.36[b]
VCL (µm/sec)	87.42 ± 35.17[a]	88.38 ± 35.02[a]	84.67 ± 35.32[a]	79.50 ± 37.88[ab]	70.73 ± 30.49[b]
STR (%)	0.83 ± 0.04	0.83 ± 0.05	0.82 ± 0.04	0.83 ± 0.05	0.85 ± 0.03
LIN (%)	0.43 ± 0.05	0.44 ± 0.07	0.41 ± 0.05	0.42 ± 0.05	0.41 ± 0.02
ALH (µm)	1.03 ± 0.34[a]	1.02 ± 0.34[a]	1.01 ± 0.37[a]	0.95 ± 0.39[ab]	0.78 ± 0.29[b]
BCF (Hz)	12.42 ± 3.24[a]	12.76 ± 3.66[a]	11.75 ± 3.27[a]	11.54 ± 4.20[a]	9.26 ± 3.47[b]
WOB (%)	0.52 ± 0.05[a]	0.53 ± 0.05[a]	0.50 ± 0.03[ab]	0.51 ± 0.03[ab]	0.48 ± 0.02[b]
HAC (rad)	0.23 ± 0.09[a]	0.23 ± 0.09[a]	0.22 ± 0.09[a]	0.21 ± 0.10[ab]	0.18 ± 0.07[b]

[a,b] Mean different superscript letters that vary in each row indicate significant differences at $p < 0.05$. T0: Tris-egg yolk, T1: Tris-egg yolk + 0.10 mg of ALE, T2: Tris-egg yolk + 0.15 mg of ALE, T3: Tris-egg yolk + 0.20 mg of ALE, T4: AndroMed®. DAP: Distance average path, DCL: Distance curved line, DSL: Distance straight line, VAP: Velocity average path, VSL: Velocity straight line, VCL: Velocity curved line, STR: Straightness, LIN: Linearity, ALH: Amplitude of lateral head, BCF: Beat cross frequency, WOB: Wobble, HAC: Head activity.

According to the present results, diluent type did not significantly influence TSM prior to cryopreservation ($p > 0.05$). However, significant differences were observed among treatments after thawing ($p < 0.05$), indicating that the protective effects of the diluents became more pronounced following freeze-thaw stress. Treatment T1 exhibited the highest total motility values both before freezing (79.50 ± 8.99%) and after thawing (68.01 ± 17.63%). A similar pattern was observed for progressive motility, which did not differ among the treatment groups prior to the freezing process ($p > 0.05$) but was significantly influenced by the treatments following thawing ($p < 0.05$). Treatment T1 exhibited the highest progressive motility values both before freezing (76.34 ± 10.72%) and after thawing (62.79 ± 20.76%). Although progressive motility decreased across all treatments following cryopreservation, the extent of the decline differed among different diluent formulations. The lowest post-thaw progressive motility was observed in Treatment T4 (40.56 ± 16.19%), whereas Treatment T1 maintained the highest value. The current findings indicated that supplementation with ALE enhanced the diluent's ability to preserve sperm motility during cryopreservation, thereby reducing the detrimental effects of freeze-thaw stress on sperm function. Treatment T1 consistently exhibited higher total and progressive motility values, suggesting that supplementation with 0.10 mg of ALE enhanced sperm resistance to cryoinjury during freezing and thawing. The improved post-thaw motility observed in the present study indicated a protective effect on the integrity of the sperm plasma membrane and flagellar function. Damage to the flagellar apparatus during cryopreservation may impair flagellar motility and propulsion, thereby decreasing sperm motility after thawing. However, motility and progression remained above 40% in Treatment T1, likely due to flavonoids and phenolics in the diluent acting as potent antioxidants. The present results were in agreement with the findings of Hassan et al. (2021), who reported that *Turraea fischeri* leaf extract enhanced post-thaw sperm quality due to its high flavonoid and phenolic content. These antioxidant compounds have been demonstrated to neutralize excessive ROS produced during equilibration and cryopreservation, thereby safeguarding spermatozoa from oxidative damage. Consequently, the preservation of membrane integrity and mitochondrial function contributed to improved sperm motility following thawing. Meanwhile, the reduction in motility and progression observed in Treatment T4 suggested that patented or practical diluents containing lesitin were not fully effective in mitigating oxidative stress in goat semen, which was highly sensitive to thermal and osmotic pressures (Nisfimawardah et al., 2023). These findings were likely associated with the inherent sensitivity of goat spermatozoa to cryopreservation-induced oxidative stress, as their plasma membranes were rich in polyunsaturated fatty acids, which are particularly susceptible to lipid peroxidation induced by ROS. The results of the CASA motility assessment were presented in Figure 2.

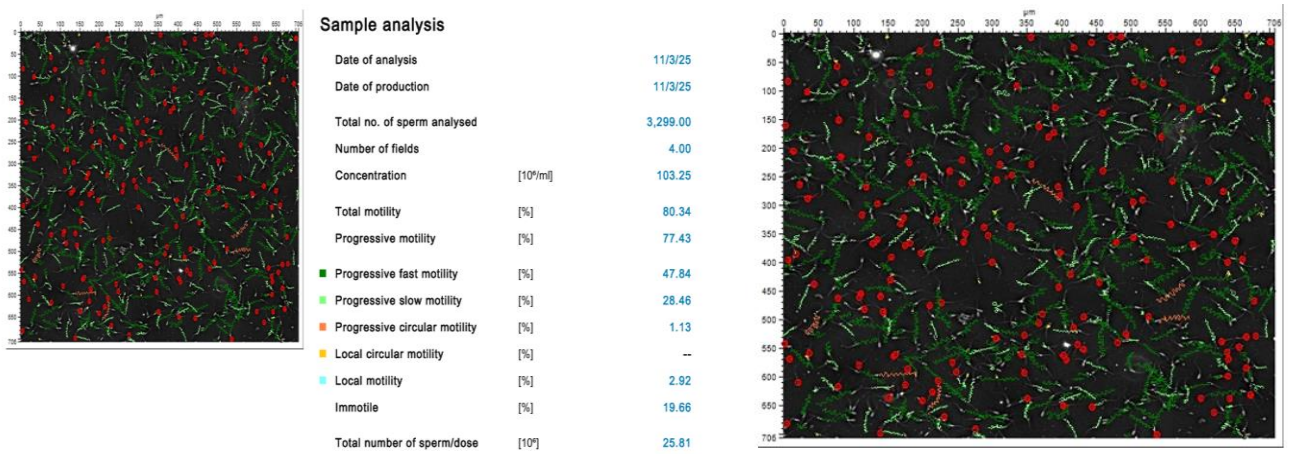


Figure 2. Kinematic parameters using computer-assisted sperm analysis AndroVision® (Minitube, Germany)

The CASA-derived parameters (DAP, DCL, and DSL) and velocity parameters (VAP, VSL, and VCL) exhibited patterns that closely aligned with the motility results. Higher values of these kinematic parameters were generally associated with improved sperm motility, suggesting enhanced preservation of sperm movement efficiency and fertilizing potential following cryopreservation. Treatments T1 and T2 demonstrated higher values for DAP, DCL, and DSL than Treatment T4, indicating that spermatozoa movement patterns were more directed and stable. The spermatozoa exhibited an enhanced capacity to maintain longer, more consistent movement paths, as evidenced by increased DAP, DCL, and DSL values. These parameters indicated that the spermatozoa moved greater distances while maintaining forward progression more efficiently. Additionally, treatments T1 and T2 exhibited higher VAP and VCL values, indicating enhanced preservation of sperm kinematic performance following thawing. These improvements may have reflected improved mitochondrial function and energy metabolism, as sperm motility largely depends on ATP generated by mitochondria in the midpiece. Preservation of mitochondrial structural integrity during cryopreservation likely contributed to the maintenance of sperm velocity and overall motility (Zhang et al., 2025). However, the highest ALE supplementation level (0.20 mg) in Treatment T3 resulted in lower VAP and VSL values than in treatments T1 and T2. However, VAP and VSL values remained higher than those observed in Treatment T4 supplemented with AndroMed®. The current findings indicated that increasing the extract concentration beyond the optimal level did not further enhance sperm kinematic performance and may have reduced the beneficial effects of antioxidant supplementation. A similar trend was reported by Hassan et al. (2021), who observed that moderate concentrations of *Turraea fischeri* leaf extract improved post-thaw sperm quality, whereas excessive supplementation did not provide additional benefits and adversely affected sperm function. These findings indicated that the addition of high doses of ALE can negatively affect enzymatic activity and flagellar motility. The present results were consistent with those of Wahjuningsih et al. (2019), who reported that 6% *Moringa oleifera* leaf extract reduced sperm motility, indicating that high concentrations of antioxidants may exert detrimental effects on sperm physiological function. Adding 0.10 mg of ALE in the present study maintained greater movement and velocity after thawing, indicating that this concentration provided optimal protection against cryopreservation-induced damage while preserving sperm kinematic performance.

The STR, LIN, and WOB parameters, along with head-oscillation metrics such as ALH displacement, BCF, and HAC, were used to describe sperm movement patterns in greater detail. These kinematic indicators provided valuable information regarding movement efficiency, trajectory control, and flagellar function, thereby enabling a more comprehensive assessment of sperm quality following cryopreservation. The highest post-thaw STR and LIN values were recorded in treatments T4 and T1, respectively, indicating that spermatozoa exhibited straighter swimming and greater forward progression after cryopreservation. This finding indicated that 0.10 mg of ALE contributed to the preservation of sperm kinematic function by reducing unnecessary lateral movement and improving swimming efficiency. The present findings were consistent with those of İnanç et al. (2018), who reported that increased sperm LIN was positively correlated with penetration distance and reproductive performance. In the current study, treatments T1 and T2 exhibited the highest post-thaw ALH and BCF values, indicating higher preservation of sperm flagellar function following cryopreservation. Higher ALH and BCF values reflect stronger flagellar movement and optimal tail-beat activity. These are crucial for sustaining sperm hyperactivation, aiding capacitation, and enhancing the spermatozoa's capacity to penetrate the zona pellucida during fertilization. This finding was consistent with the study conducted by Xu et al. (2023), who demonstrated that the capacitation process was influenced by sperm motility and metabolic alterations following thawing. Similar observations were reported by Giaccagli et al. (2021), who found that mitochondrial activity was essential for sperm hyperactivation and fertilizing ability by regulating sperm motility. Furthermore, Tourmente et

al. (2022) found that capacitation involves metabolic changes that boost sperm motility and energy use, highlighting a strong link between energy metabolism, sperm movement, and fertilization ability. In contrast, Treatment T4, which used the AndroMed® diluent, exhibited lower ALH, BCF, WOB, and HAC values after thawing, suggesting impaired head oscillation and flagellar activity resulting from cryopreservation-induced structural damage. The reduction in these kinematic parameters indicated a decline in sperm movement efficiency and functional performance following the freeze-thaw process. Conversely, treatments T1 and T2 maintained relatively stable values for these parameters, indicating that the combination of tris-egg yolk diluent and ALE provided superior protection against cryoinjury. The present findings indicate that the ALE-supplemented diluent more effectively preserved sperm kinematic function and structural integrity than treatments T0 and T4. This observation was consistent with that of Rizal et al. (2025), who reported that the motility of frozen goat semen was 47.5% with AndroMed® diluent alone and increased to 56.25% when 4% moringa leaf extract was added to the diluent. The improved post-thaw motility and kinematic parameters observed in treatments T1 and T2 indicated that the antioxidant capacity of ALE was most effective at appropriate concentrations, thereby enhancing sperm protection during freezing and thawing.

CONCLUSION

Adding 0.10 mg of *Aquilaria malaccensis* leaf extract to a tris-egg yolk diluent enhanced protection against cryopreservation-induced damage, as evidenced by higher post-thaw motility, progressive motility, and improvements in movements, velocity, and head oscillation measures. Therefore, *Aquilaria malaccensis* leaf extract exhibited potential as a natural antioxidant additive to enhance the functional quality of frozen Saanen goat semen and supported artificial insemination programs. The utilization of *Aquilaria malaccensis* has the potential to enhance the efficacy of future artificial insemination programs by enhancing the availability of high-quality frozen semen and promoting the dissemination of superior genetic resources.

DECLARATIONS

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Authors' contributions

Lailatun Nisfimawardah, Firdaus Husein, Monasdir, Ika Fitriana Dyah Ratnasari, Eros Sukmawati, and Sri Wahjuningsih contributed to manuscript writing, data analysis, and study design. Lailatun Nisfimawardah collected data from the field and laboratory. All authors contributed to reviewing the data of the study, performed statistical analysis, and approved the final draft of the manuscript. All authors have read and approved the final edition of the manuscript before publication.

Availability of data and materials

All data generated and analyzed during this study were included in this published article. Additional data supporting the findings of the present study were available from the corresponding author upon reasonable request.

Competing interests

The authors declared no personal or financial interests in this study.

Ethical considerations

The authors declared that this manuscript has been checked by all authors and was originally submitted to the journal for publication. No AI tools were used to conduct or write this study. The authors accept full responsibility for the originality of the study before submission and before publication.

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Assessment of Livestock Owners' Knowledge, Attitude and Practice towards Antimicrobial Usage and Resistance in Amhara Region, Ethiopia

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ABSTRACT

Antibiotic resistance is a major concern for humans and animals, especially in less developed countries such as Ethiopia. Livestock owners often misuse antibiotics, use incorrect doses, and ignore instructions for proper medication use. The present study aimed to evaluate the knowledge, attitudes, and practices regarding antimicrobial use (AMU) and antimicrobial resistance (AMR) in Ethiopia. A cross-sectional study was conducted to assess livestock owners' knowledge, attitudes, and practices (KAP) regarding antibiotic use, antibiotic residues, and antibiotic resistance in Ethiopia. Structured interviews were conducted with eligible participants to gather insights on their perceptions and self-reported practices related to AMU and AMR. A survey involving 240 livestock owners assessed their backgrounds, knowledge, attitudes, and practices. Among the livestock owners, 84.6% were men, 36.2% had only completed primary education, and 88.8% were aware of AMR and AMU. Antimicrobial use and AMR were not comprehended by 58.8% of respondents. A significant number of respondents mistakenly believed that antibiotics were effective against viral infections. Additionally, 76.7% of respondents reported consuming animal-derived products at home from animals treated with over-the-counter medications without observing the recommended withdrawal periods, even though 75% of respondents were aware of the antibiotic withdrawal periods for ill animals. In the present study, 47.9% of respondents reported giving antibiotics to their animals in the past month, primarily for illness prevention or treatment. There was a significant association across KAP scores and gender, education, income, and farming experience. Although livestock owners have adequate knowledge and positive attitudes toward antibiotic use, many still engage in practices that may contribute to AMR.

Keywords: Antimicrobial use, Antimicrobial resistance, Drug residue, Livestock owner, Veterinary

INTRODUCTION

In developing countries such as Ethiopia, the increasing demand for animal- derived food products has led to a substantial rise in the use of antimicrobials in food animal production. Global antimicrobial consumption in livestock was projected to increase by approximately 67% between 2010 and 2030 (Van Boeckel et al., 2015; Ibrahim et al., 2020).

Ethiopia possesses the largest livestock population in Africa, with an estimated 59 million cattle, 30.7 million sheep, 30.2 million goats, 56.87 million poultry, and 11 million equine (CSA, 2017). Despite this extensive livestock resource, the sector's productivity remains below its potential (FAO, 2019). Several factors contribute to low livestock productivity, including traditional husbandry practices, poor genetic potential, inadequate nutrition, and a high prevalence of infectious diseases (Belay et al., 2012; CSA, 2017). Antimicrobial use (AMU) plays a critical role in animal health management and is widely used for therapeutic purposes, disease prevention such as prophylaxis and metaphylaxis, and, in some countries, growth promotion (Clement et al., 2019). However, inappropriate and excessive use of antimicrobials has become a major concern for animal and public health. In Ethiopia, the regulation and supervision of veterinary AMU are still quite limited, and livestock owners often have easy access to veterinary medicines without adequate professional guidance. Such practices may contribute to the emergence and spread of antimicrobial resistance (AMR) among animals and humans (Clement et al., 2019).

Antimicrobial resistance is a growing global health threat that reduces the effectiveness of antimicrobial medicines, resulting in treatment failure, increased morbidity and mortality, and higher economic losses in livestock production (Murray et al., 2022). Resistant pathogens can spread between animals and humans through direct contact, contaminated environments, and consumption of animal-derived food products such as meat and milk (Van Boeckel et al., 2019). Pathogens, including *Salmonella* spp. and *Escherichia coli*, have been identified as important zoonotic organisms capable of transmitting resistant strains to humans, posing significant public health risks (Ngangom et al., 2019; Almansour et al., 2023).

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Several studies conducted in Ethiopia have reported the presence of antimicrobial residues in foods of animal origin and inappropriate AMU practices among livestock owners (Gebeyehu et al., 2021; Gemeda et al., 2021). The livestock sector, which plays a vital role in income generation and livelihoods, is increasingly threatened by the misuse of commonly used veterinary medicines.

Assessing livestock owners' knowledge, attitudes, and practices (KAP) regarding AMU and AMR is essential for developing effective intervention strategies (Gemeda et al., 2020). These assessments provide essential insights for developing awareness campaigns, educational programs, and policies aimed at promoting the responsible use of antimicrobials and preventing AMR. In urban and peri-urban areas such as Debre Markos, Ethiopia, where veterinary services and livestock activities are prevalent, assessing livestock owners' KAP can play a key role in enhancing antimicrobial stewardship and protecting public health (Yizengaw et al., 2026).

Although KAP is a key determinant of AMU practices, limited information is available regarding these factors among livestock owners in Debre Markos, Ethiopia. Livestock owners often purchase veterinary medicines without prescriptions, administer antimicrobials without professional consultation, and fail to observe recommended withdrawal periods (Gemeda et al., 2020). Furthermore, weak regulatory enforcement and unrestricted access to veterinary medicines may facilitate inappropriate AMU and accelerate the development of AMR (Gebeyehu et al., 2021).

The lack of baseline data on AMU and AMR in the study area represents a significant evidence gap and should be considered essential for developing effective behavioral and policy strategies. Therefore, the present study aimed to assess livestock owners' KAP regarding AMU and AMR practices in the Amhara Region, Ethiopia.

MATERIALS AND METHODS

Ethical approval

The present study was approved by the Ethics Committee of the East Gojjam Zone Livestock Development Office, Ethiopia (No. 0054EHLDF/2025) on December 18, 2025. All participants received a thorough explanation of the study's goal, and prior to the interview, informed verbal consent was acquired. Participants received assurances that the data they provided would be kept private and used exclusively for the present study. Respondents were free to leave the study at any time without facing any repercussions, as participation was entirely voluntary.

Study location

The study was conducted in Debre Markos city, located in East Gojjam Zone of the Amhara National Regional State, Ethiopia, from September to November 2025 (Figure 1). Debre Markos serves as the administrative center of East Gojjam Zone and is situated in Northwestern Ethiopia, approximately 300 km from Addis Ababa and 265 km from Bahir Dar, the regional capital. Geographically, the city is located at 10º 21′ North latitude and 37º 43′ East longitude.

East Gojjam Zone comprises 18 districts and 7 urban administrations. The area is characterized by diverse agro-ecological conditions, with approximately 60% of the land classified as highland and 40% as midland. The elevations range from 2,800 to 4,100 meters above sea level, and the annual rainfall ranges between 900 and 1,200 mm (CSA, 2017).

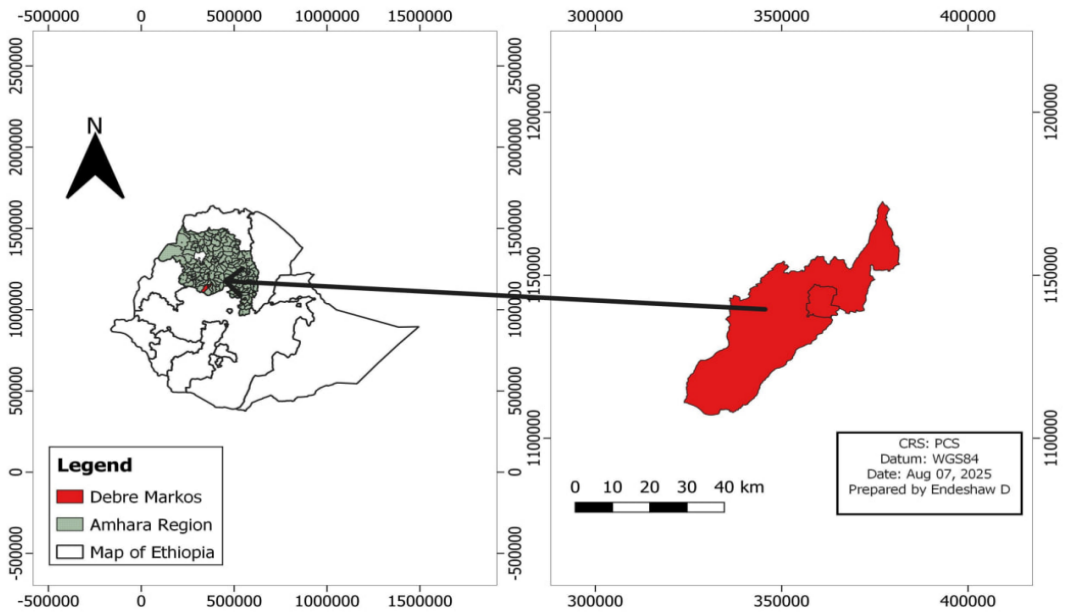


Figure 1. The study area, Debre Markos City, Ethiopia. Source: Generated using ArcGIS software version 3.1 (Designed by authors)

The zone has an estimated 342,000 households (CSA, 2017) and is predominantly characterized by a mixed crop-livestock production system. The major livestock species raised in the area include cattle, sheep, goats, equines, and poultry. The population in and around Debre Markos City is highly dependent on livestock production and veterinary services for their livelihoods, making the area a suitable and strategic location for conducting this study (CSA, 2017).
The study included 240 livestock owners living in Debre Markos town who were actively involved in livestock production. The study included livestock owners raising cattle, sheep, goats, poultry, and equine. The inclusion criteria were: being a livestock owner actively involved in livestock production and management, aged 18 years and above, and their willingness to participate in the study. Exclusion criteria included individuals who were unwilling to participate, did not own livestock, were aged less than 18 years old, or were unable to provide reliable information during the interview.

Study design and period

A cross-sectional study was conducted to assess KAP of livestock owners on AMU, AMR, and antimicrobial residues. The study was conducted from September to November 2025.

Sample size and sampling method

A multistage sampling method was used. First, kebeles were selected randomly, followed by systematic random selection of livestock owners.

The sample size for this field study was determined using a single population-based formula for cross-sectional surveys to assess KAP (Lwanga and Lemeshow, 1991). Because no prior study had documented the percentage of livestock owners with sufficient KAP regarding AMU and AMR in the study area, a 95% confidence level (Z = 1.96), a5%margin of error(e = 0.05), and an expected proportion of 50% (p = 0.5) were assumed.

$n = Z^2p(1-p)/e^2 = (1.96^2 \times 0.5 \times 0.5)/(0.05^2)$

The initial sample size was 384 participants. A finite population correction was applied because the total number of livestock-owning households in the chosen kebeles of Debre Markos town in Ethiopia was 500, which was less than 10,000. The corrected sample size, nf = n/[1 + (n/N)], where nf was the final sample size following correction, n was the original sample size (384), and N was the overall population size (500), was used to calculate the corrected sample size. The total number of participants was 218, calculated as follows.

nf = 384/[1 + (384/500)] = 384/1.768 = 218 participants

After adding a 10% contingency for potential non-response and missing questionnaires (218 + [218 × 0.10] = 240), the final sample size for the study was 240 livestock owners (Lwanga and Lemeshow, 1991). The additional 10% was added only during sample size determination to account for possible nonresponse and incomplete questionnaires. The additional 10% was not applied during data analysis and therefore did not introduce bias, resulting in a total sample size of 240 respondents.

Data collection methods

A standardized semi-structured questionnaire was developed and used to assess the livestock farmers' KAP. The questionnaire was initially developed in English and translated into the Amharic language for the interview process to ensure clarity and effective communication with the participants. To ensure the cooperation of livestock owners and to obtain reliable data, the objectives of the study were clearly explained before the start of each interview.

Study variables

The dependent variable of the study was the level of KAP related to AMU and AMR among livestock owners. The independent variables include different demographic and related factors such as age, gender, educational background, occupation, income level, access to veterinary services, and exposure to training on AMU and AMR.

Data analysis

Data collected through interviews were entered into Microsoft Office Excel 2010 for data cleaning and coding, and then exported to SPSS version 20 for statistical analysis. Descriptive statistics were employed to summarize the data, with frequencies and percentages calculated for categorical variables including respondents' socio-demographic characteristics and their KAP regarding AMU and AMR. The current results were presented in tables to facilitate the clear interpretation of the findings.

The association between respondents' socio-demographic characteristics and their KAP levels was assessed using Chi-square ($\chi 2$). A p-value of less than 0.05 was considered statistically significant ($p < 0.05$).

RESULTS

Socio-demographic characteristics of the respondents

According to the socio-demographic analysis, most respondents were male (84.6%). Regarding educational status, 36.2% of the participants had completed primary school. The majority of respondents (88.3%) were married. In terms of income, 47.5% reported a monthly income of less than 5,000 Ethiopian birr. Regarding farming experience, 30.4% had worked in the livestock sector for five or more years (Table 1).

Table 1. Socio-demographic characteristics of participants in Debre Markos, Ethiopia in 2025

Household variables	Categories	Frequency	Percentage
Sex of respondent	Male	203	84.6
	Female	37	15.4
Age	≤ 30 years	60	25
	31-40 years	113	47
	41-50 years	47	19.5
	≥ 51 years	20	8.3
Education level	Not able to read and write	68	28.3
	Primary school	87	36.2
	High school	38	15.8
	College/University	47	19.6
Marital status	Single	28	11.7
	Married	212	88.3
	Divorced/Widowed	0	0
Monthly income in Ethiopian birr	< 5000	114	47.5
	5000-15,000	96	40
	> 15,000	30	12.5
Years of farming experience (years)	< 5	114	47.5
	5-10 years	73	30.4
	11-15 years	31	12.9
	≥ 16 years	10	4.2

Knowledge of antimicrobial use, resistance, and residue

The study findings indicated that livestock owners' understanding of medication residues, AMU, and AMR differed. While the majority of respondents (80.8%) were aware that antibiotics are used to treat bacterial illnesses, 58.7% mistakenly believed that antibiotics were effective against viral infections. Although 51.7% of respondents were aware that resistant bacteria can spread from animals to people, over half of the respondents (60%) knew that the misuse or overuse of antibiotics might contribute to AMR. Only 31.7% of respondents were aware that AMR can result from inappropriate AMU, which includes early treatment termination, incorrect dosage, failure to adhere to the recommended treatment duration, and the use of inappropriate medicines. Furthermore, 92.5% of respondents acknowledged the possible health hazards connected to antibiotic residues in food products of animal origin, and 75% of respondents were aware of the withdrawal time after antimicrobial treatment. Fewer respondents mentioned overcrowding and poor hygiene (19.2%) and inadequate feeding (27.9%) as potential causes of treatment failure. Only 64.6% of respondents identified AMR as a contributing factor in treatment failure. Additionally, 68.3% of respondents linked treatment failure to insufficient diagnostic testing before treatment, 67.1% pointed to poor-quality medicines, and 65.4% identified the inappropriate choice of antimicrobial agents as potential causes. Overall, livestock owners recognized antibiotic use as the primary factor but had limited awareness of indirect contributors, such as poor hygiene and inadequate diet, that contribute to treatment failure (Table 2).

As can be seen in Figure 2**,** the respondents reported that antimicrobial treatment failure may be caused by a number of factors. Antimicrobial resistance was reported by 64.6% of the respondents, while 68.3% mentioned inadequate diagnostic testing prior to treatment. In addition, 67.1% linked treatment failure to poor-quality medications, and 65.4% to inappropriate AMU. Overcrowding or poor sanitary practices (80.8%) and poor feed quality (72.1%) were identified as significant contributing factors. Approximately 95.8% of respondents reported self-prescription and unsupervised AMU by livestock owners as the most frequently reported cause of treatment failure.

Table 2. Knowledge about antimicrobial usage and resistance in Debre Markos, Ethiopia in 2025

Knowledge question	Categories respondents	Number	Percentage
Do you know that antibiotics are used to treat bacterial infections?	Yes	194	80.8
	No	46	19.2
Do you know that antibiotics do not work against viral infections?	Yes	141	58.8
	No	99	41.3
Do you know that misuse of antibiotics can cause resistance in animals?	Yes	144	60
	No	96	40
Can resistant bacteria spread from animals to humans?	Yes	124	51.7
	No	116	48.3
Do you know that incomplete antibiotic treatment can lead to resistance?	Yes	76	31.7
	No	164	68.3
Are you aware of the drug withdrawal period after antibiotic use?	Yes	180	75
	No	60	25
Do you know that antibiotic residues in animal products can affect human health?	Yes	222	92.5
	No	18	7.5
Do you know that AMR is a global public health problem?	Yes	107	44.6
	No	133	55.4

AMR: Antimicrobial resistance

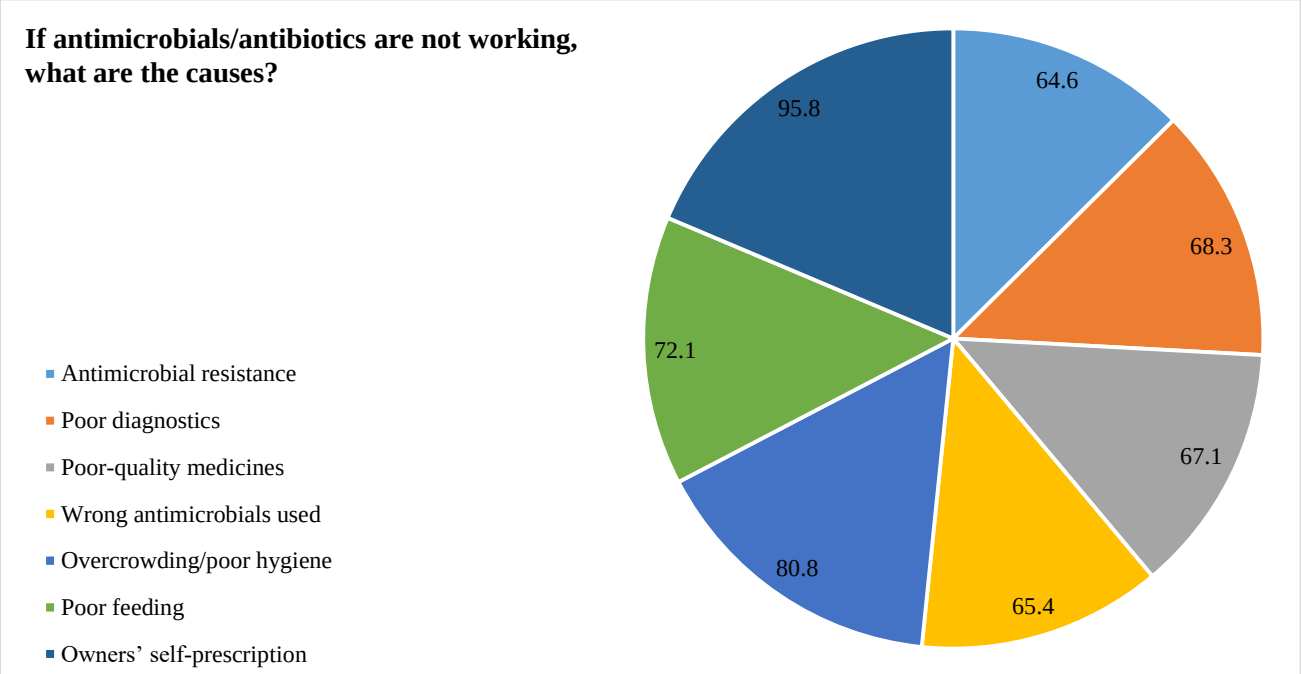


Figure 2. Perceived causes of antimicrobial treatment failure among livestock owners in Debre Markos, Ethiopia, in 2025

Attitudes toward antimicrobial use, resistance, and residue

Regarding the usage of antibiotics in animals, most responses were in agreement. Almost 88% of respondents highlighted the importance of performing diagnostic analyses prior to treatment. Nearly all respondents (99.9%) stated that antibiotics should only be administered under a veterinarian's prescription. A statistically significant proportion of respondents (87.4%) agreed that medicine misuse can lead to AMR ($p < 0.05$). Additionally, 80% of respondents mentioned that performing adequate farming practices such as biosecurity, vaccination, and hygiene helps reduce the demand for antibiotics. However, there was a lack of clarity regarding who was responsible for managing AMR; 75% of respondents believed that farmers should be responsible for managing AMR, while the rest of the respondents were either neutral or disagreed. Most respondents (92.2%) demonstrated a high level of awareness about Antimicrobial Resistance (AMR) and prudent (responsible) use of antibiotics in livestock farming. (Table 3).

Table 3. Attitude about antimicrobial usage and resistance in Debre Markos, Ethiopia in 2025

Characteristics	Categories respondents	Response		Chi-square tests	
		Number	Percentage	Value	Df
Antibiotics should only be used when prescribed by a veterinarian	Strongly agree	191	79.5	22.115	2
	Agree	49	20.4		
Laboratory testing before antibiotic use is important	Strongly agree	153	63.75	18.150	1
	Agree	87	36.25		
Antibiotic misuse in animals contributes to AMR	Strongly agree	182	75.8	262.022	8
	Agree	28	11.6		
	Strongly disagree	30	15.8		
Regulations on antimicrobial usage are necessary to prevent resistance	Strongly agree	123	51.25	95.282	10
	Agree	39	16.25		
	Neutral	28	11.66		
	Disagree	19	7.9		
	Strongly disagree	31	12.9		
AMR is a serious threat to animal health	Strongly agree	125	52.08	95.588	6
	Agree	78	32.5		
	Neutral	37	15.41		
AMR is a serious threat to public health.	Strongly agree	123	51.25	209.766	10
	Agree	47	19.58		
	Neutral	30	12.5		
	Disagree	29	12.08		
	Strongly disagree	11	4.58		
Proper farm biosecurity and vaccination can reduce antibiotic use.	Strongly agree	173	72.08	107.386	8
	Agree	9	3.75		
	Neutral	19	7.91		
	Disagree	10	4.1		
	Strongly disagree	29	12.08		
Farmers should be trained regularly about prudent antibiotic use	Strongly agree	140	58.3	183.74	8
	Agree	40	16.6		
	Disagree	20	8.3		
	Strongly disagree	40	16.6		

AMR: Antimicrobial resistance, DF: Degree of freedom

Practices toward antimicrobial use, resistance, and residue

A total of 47.9% of farmers reported using antimicrobials during the previous month, primarily for treatment (20.4%) or prevention (27.5%), while 52.1% did not use any antimicrobials. The majority of respondents (94.6%) used medications prescribed by experts and relied on veterinary advice. Triclabendazole (also known as Fasinex) and albendazole were antiparasitic medications, and tetracycline and penicillin were the most frequently reported antibiotics used by farmers. Half of the participants reported using antimicrobials only once a month, indicating that these agents were primarily employed infrequently. Medicines were primarily obtained from veterinary clinics (52.9%) and pharmacies (39.2%).

Additionally, incomplete treatment practices were observed; approximately 87.1% of respondents did not complete the full prescribed duration, primarily due to increasing prescription costs, and 12.1% of respondents quit treatment early. The majority of expired prescriptions (44.2%) were appropriately disposed of; however, numerous respondents were unable to verify the expiry date of the medications. Regarding withdrawal periods, the majority of respondents (76.7%) continued to use products from treated animals, while others reported using products at home or selling or providing milk or meat from treated animals to dogs, calves, or households (Table 4).

Table 4. Community response to each practice regarding antimicrobial usage and resistance in Debre Markos, Ethiopia in 2025.

Characteristics	Categories respondents	Response		Chi-square test	
		Number	Percentage	Value	Df
Have you ever prescribed antimicrobials for your animals in the last month?	Yes	115	47.90%	9.893	2
	No	125	52.10%		
If yes, why did you use antimicrobials?	To treat sick animals	49	20.40%	54.357	4
	As a preventive measure (Prophylaxis/Metaphylaxis)	66	27,5%		
If applicable, how did you choose the medication?	Based on recommendations from animal health professionals	203	84.60%	75.996	4
	Self-selection	28	11.70%		
	Recommendations from neighbors				
Who administers antimicrobials?	Veterinarians or animal health professionals	213	88.80%	106/479	4
	The animal owner (without a veterinarian)	18	7.50%		
Which antimicrobials are commonly used on your farm?	Albendazole	18	7.50%	242.943	12
	Tetracycline	49	20.40%		
	Penicillin	20	8.30%		
	Fasinex	28	11.70%		
	Don't know	45	18.70%		
How often did you use antimicrobials in the last month?	Once	116	48.30%	73.631	8
	2 to 5 times	96	40%		
	More than 5 times	19	7.90%		
	6 times	9	3.80%		
What are the sources of antimicrobials you use?	Veterinary pharmacy/drug shops	94	39.20%	111.366	4
	Veterinary clinic	127	52.90%		
	Both	19	7.90%		
Do you ever stop treatment before completing the prescribed course?	Yes	29	12.10%	14.899	2
	No	211	87.90%		
What are the main reasons for stopping antimicrobial treatment early?	Medication is not working/animal died	9	3.80%		
	Animal has shown improvement	10	4.20%		
	Saving for future use (stockpiling)	10	4.20%		
Do you give the full amount and complete course of medicine as written on the label?	Yes	209	87.10%	24.994	2
	No	31	12.90%		
What are the main reasons for not completing the prescribed medicine courses?	Not enough money	21	8.80%	24.994	4
	Thought it was enough	10	4.20%		
If the medicine has passed its expiry date, what do you do?	Do not use it	59	24.60%	78.374	4
	Throw it away	106	44.20%		
	Did not check the expiry date	75	31.20%		
What do you do with meat, eggs, or milk from animals that are being treated or in the withdrawal period?	Feed it to calves, dogs, or pets	184	76.70%	167.433	8
	Sell it to neighbors or contractors	38	15.80%		
	Use it at home	18	7.50%		

DF: Degree of freedom

Socio-Demographic determinants

The respondents' knowledge level was significantly correlated with several sociodemographic characteristics, including age, gender, monthly income, number of animals owned, education level, and marital status. ($p < 0.05$; Table

5). Attitudes exhibited a significant correlation solely with marital status, whereas knowledge level was strongly linked to sociodemographic variables (Table 6; $p < 0.05$).

Additionally, the study discovered a significant correlation between livestock owners' antimicrobial activities and several sociodemographic factors, including gender, marital status, and educational background ($p < 0.05$). Furthermore, a significant correlation was observed between proper practices and monthly income, with farmers earning more than 5,000 birr more likely to practice proper practices than those earning less than 5,000 birr ($p < 0.05$). Additionally, 84.21% of respondents over 40 years old demonstrated high-level proper habits ($p < 0.05$). On the other hand, AMU practice was not significantly impacted by the number of animals kept or farming experience (Table 7).

Table 5. Association of knowledge with socio-demographic characteristics in Debre Markos, Ethiopia in 2025

Socio-demographic characteristics	Categories Respondents	Low (%)	Moderate (%)	High (%)	P-value
Gender	Female	75.68	24.32	0	0.046
	Male	24.14	43.35	32.51	
Marital status	Married	36.32	36.79	32.14	0.027
	Single	0	67.86	57.45	
Educational level	College/university	0	42.55	57.45	0.003
	No formal education	55.88	29.41	14.71	
	Primary	44.83	33.33	21.84	
	Secondary	0	73.68	26.32	
Monthly income	5000 birrs	50.88	25.44	23.68	0.033
	5000-15000	19.79	50	30.21	
	> 15000	0	66.67	33.33	
Number of animals owned	20-49	0	50	25.45	
	< 20	35	39.55	100	0.047
Years of experiences	5-10 years	0	0	100	
	11-15 years	64.52	35.48	0	0.089
	> 16 years	10.47	55.81	33.72	
Age group	20-30	45.71	45.71	8.57	0.002
	31-40	31.86	39.82	28.32	
	> 40	15.79	35.09	49.12	

Table 6. Association of attitude with socio-demographic characteristics in Debre Markos, Ethiopia in 2025

Socio-demographic characteristics	Categories Respondents	Low (%)	Moderate (%)	High (%)	P-value
Gender	Female	75.68	0	24.32	0.865
	Male	77.34	0	22.66	
Marital status	Married	78.3	0	21.7	0.002
	Single	67.86	0	32.14	
Educational level	College/University	61.7	0	38.3	0.004
	No formal education	100	0	0	
	Primary	100	0	0	
	Secondary	70.83	0	29.17	
Monthly income	5000birr	100	0		0.054
	5000-15000	75	0	25	
	> 15000	100	0		
Number of animals owned	20-49	63.01	0	37	0.006
	< 20	75	0	25	0.058
Years of experiences	5-10 years	63.01	0	36.99	
	11-15 years	100	0		
Age group	31-40	91.15	0	8.85	0.701
	> 40	50.88	0	49.12	

Table 7. Association of practice with socio-demographic characteristics in Debre Markos, Ethiopia in 2025

Socio-demographic characteristics	Categories	Low (%)	Moderate (%)	High (%)	P-value
Gender	Female	24.32	24.32	51.35	0.044
	Male	15.27	23.15	61.58	
Marital status	Married	18.87	22.17	58.86	0.019
	Single	0	32.14	67.86	
Educational level	Primary	0	22.99	77.01	0.024
	No formal education	30.88	26.47	42.65	
Monthly income	5000birr	16.67	24.56	58.77	
	> 15000	33.33	0	66.67	0.037
Number of animals owned	20-49	50	0	50	
	< 20	13.64	25.45	60.91	
Years of experiences	5-10 years	35.48	0	100	0.076
	11-15 years	35.48	32.26	32.26	
	> 16 years	0	20.93	79.07	
Age group	20-30	24.29	20	55.71	0.002
	31-40	20.35	29.2	50.44	
	> 40	0	15.79	84.21	0.045

DISCUSSION

The observed discrepancy between livestock owners' knowledge and their AMU practices in the present study was consistent with a previous study conducted in Ethiopia, including Gemeda et al. (2020) who found that 72.3% of pastoralists did not follow the full treatment course, and Tufa et al. (2023), who reported that 94% of farmers had poor knowledge, but 80% still showed good behavior scores. Specifically, livestock owners frequently misused antimicrobials and demonstrated poor adherence to appropriate antimicrobial administration guidelines, despite having awareness of AMU and AMR practices.

Notably, the present study revealed a notable gap between livestock owners' knowledge and their practical application concerning antimicrobials. Although most participants were aware that antimicrobials are used to treat animal illnesses, their actual practices conflicted with this understanding. Key issues included non-prescribed administration, incomplete treatment courses, and non-adherence to recommended dosages or timelines. Such misuse accelerated AMR in livestock, ultimately threatening public health through potential foodborne transmission and environmental contamination pathways.

Additionally, the present study revealed a low level of awareness among livestock owners regarding the development and consequences of AMR, aligning closely with the findings of Gebeyehu et al. (2021). Similarly, Beyene et al. (2015) observed that the use of veterinary medicines in Ethiopia is frequently irrational, further evidence of widespread antimicrobial misuse. Notably, while 75% of respondents in the current study claimed to be aware of medicines withdrawal periods, they still engaged in high-risk behaviors, such as feeding calves or companion animals with products sourced from treated livestock. Such practices increased antimicrobial exposure, promoting the development of resistant bacteria. This result aligned with the findings of Gemeda et al. (2021), who reported higher levels of antimicrobial residues in animal products due to non-adherence to withdrawal periods and improper disposal methods.

The current results further indicated that many livestock owners failed to recognize how suboptimal management practices contributed to treatment failure. Notably, a large proportion of respondents disagreed that overcrowding, poor hygiene, and inadequate feeding practices contributed to disease occurrence and therapeutic failure. These findings suggested that livestock owners relied heavily on antimicrobials as a safety framework, while overlooking important animal husbandry and biosecurity practices. As McKernan et al. (2021) emphasized, robust biosecurity practices and proper animal management are indispensable factors of responsible AMU practices. Similarly, reports from the CSA (2017) indicated that traditional livestock production systems often facilitated disease transmission, underscoring the urgent need to strengthen husbandry practices alongside veterinary antimicrobial stewardship efforts.

Despite generally positive attitudes toward rational medicines administration, several inappropriate practices were identified. For instance, most participants agreed that antimicrobials should only be administered under veterinary prescription, reflecting strong confidence in professional veterinary advice and aligning with Food and Agriculture Organization (FAO) recommendations. However, this positive attitude did not consistently translate into practice, as self-prescription and early discontinuation of therapy were still documented. Similar behavioral gaps were noted by Tufa et al. (2023), who observed that treatments were often cut short due to perceived animal recovery, financial constraints, or a desire to save remaining medication for future use. Mckernan et al. (2021) and Tang et al. (2017) highlighted that non-compliance can lead to the spread of antimicrobial-resistant microorganisms, emphasizing how vital it is to follow prescribed treatments properly.

The present study demonstrated that most antimicrobials were sourced from private veterinary clinics and private pharmacies. However, insufficient regulatory oversight on veterinary medicine distribution, along with the ongoing availability of these medicines without a prescription, greatly contributed to their inappropriate use. Similar concerns regarding unrestricted or uncontrolled access to critical antimicrobials have been widely documented in the literature. Furthermore, the present study demonstrated a remarkable association between KAP outcomes and factors such as educational level, livestock-derived income, and years of herd management experience. These findings highlighted that interventions aimed at improving antimicrobial use should consider the distinct socioeconomic and educational backgrounds of livestock owners. Implementing focused awareness campaigns, customized farmer training sessions, and strengthened veterinary extension services can be essential for encouraging responsible AMU practices and reducing the broader risks of AMR (Gemeda et al., 2020).

The current findings reinforced the global concerns raised by WHO (2019) and FAO, WOAH, and WHO (2020) regarding the growing threat of AMR. Livestock-dependent communities continue to play a pivotal role in the emergence and dissemination of AMR, underscoring an urgent need for coordinated, multi-sectorial efforts. These initiatives should specifically aim to improve antimicrobial stewardship, reinforce national regulatory frameworks, and boost public awareness campaigns focused on responsible use of pharmaceuticals.

CONCLUSION

The present study revealed a substantial gap between livestock owners' knowledge and their actual AMU practice in Debre Markose City, Ethiopia. Many respondents demonstrated adequate knowledge of appropriate AMU practice and the risks associated with AMR. Inappropriate practices, including self-medication, early discontinuation of treatment, use of remaining antimicrobials, purchasing medicines without a veterinary prescription, and failure to observe withdrawal periods, remained common challenges among farmers and livestock owners. The lack of awareness regarding the importance of hygiene and nutrition for disease prevention indicated a significant shortfall in livestock management practices among farmers. Addressing these challenges requires strengthening veterinary extension services through farmer training and community awareness programs, enforcing regulations on antimicrobial sale and use, and improving animal husbandry and biosecurity practices. Further laboratory-based and longitudinal studies are recommended to monitor antimicrobial residue and resistance patterns in livestock and animal-derived food products across the Amhara region in Ethiopia.

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Authors' contributions

Getahun Simeneh conducted data collection, data analysis, and manuscript writing. Yibeltal Simeneh contributed supervision, data interpretation, and manuscript revision. All authors reviewed and approved the final edition of the manuscript.

Availability of data and materials

All data generated during the study are included in the present published article.

Competing interests

The authors declare no conflicts of interest.

Ethical considerations

All authors confirmed that this study is an original article and has not been published before, nor is it under consideration elsewhere. No AI tools were used for preparing the current study. The authors accept full responsibility for the originality of the study before submission and before publication.

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Hematological and Biochemical Dysregulation During Acute *Babesia ovis* Infection in Sheep

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ABSTRACT

Babesiosis, caused by *Babesia ovis*, is a significant tick-borne disease affecting small ruminants, characterized by hemolytic anemia, liver dysfunction, and metabolic imbalances. The current study aimed to investigate the clinical, hematological, and biochemical alterations linked to naturally occurring ovine babesiosis in the Shirvan-Salyan region of Azerbaijan. A total of 21 Garadolag-breed lambs, comprising 11 females and 10 males, all under 1 year old and weighing 15-18 kg, were purposively sampled from three farms. The present study included animals exhibiting clinical signs such as hyperthermia and anemia, as well as healthy animals from the same flocks for comparison. A total of 21 Garadolag sheep were examined, consisting of eight with severe clinical signs of babesiosis and thirteen that appeared healthy. Because clinical signs were observed in the flock, all animals underwent a thorough examination. Babesiosis was confirmed by microscopic examination of Giemsa-stained blood smears, and hematological as well as serum biochemical parameters were determined using automated analyzers. The infected sheep exhibited significant reductions in red blood cell count, hemoglobin concentration, hematocrit, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration, alongside leukocytosis, eosinophilia, and monocytosis, indicating severe hemolytic anemia. Blood smear examination revealed anisocytosis and poikilocytosis, indicating erythrocyte destruction and a regenerative response. Serum biochemical evaluation revealed elevated levels of bilirubin, blood urea nitrogen, creatinine, total cholesterol, and triglycerides, as well as increased levels of alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, and lactate dehydrogenase. Conversely, the levels of total protein, albumin, and globulin decreased, indicating compromised liver synthetic function and changes in protein metabolism. The present study demonstrated that acute *Babesia ovis* infection in Garadolag lambs was characterized by distinct clinical signs, including primarily hyperthermia, icterus, and significant parasitemia, alongside specific systemic disturbances.

Keywords: *Babesia ovis*, Biochemical parameter, Hemolytic anemia, Liver dysfunction, Metabolic disorder

INTRODUCTION

Babesia ovis (*B. ovis*), a protozoan hemoparasite belonging to the family Babesiidae and order Piroplasmida, is among the most widely distributed tick-borne pathogens in mammals (following *Trypanosoma* spp.) and the causative agent of ovine babesiosis, an economically important disease affecting sheep in the Mediterranean Basin, the Middle East, North Africa, and Central and Western Asia (Homer et al., 2000; Uilenberg, 2006). The disease is characterized by fever, hemolytic anemia, jaundice, hemoglobinuria, lethargy, and hepatic and renal dysfunction, resulting in reduced productivity, increased treatment costs, and mortality in severely affected animals (Schnittger et al., 2012).

The primary vectors of *B.ovis* are ticks of the genus *Rhipicephalus* (specifically *R.bursa* and *R.turanicus*), with *R.bursa* uniquely confirming transovarial transmission and playing a key role in parasite transmission (Bonnet et al., 2021; Martínez-García et al., 2021; Firat et al., 2024). Although other tick species are also evaluated as potential vectors, infection typically begins when infected ticks inoculate sporozoites into the host's bloodstream during blood feeding (Jalovecka et al., 2019).

Previous studies conducted in Azerbaijan indicated that pathological examinations of small ruminants that died from babesiosis often detect concurrent parasitic co-infections, including dicroceliosis, echinococcosis, and gastrointestinal and respiratory strongyloidiasis (Azizova, 2022). Recent studies indicated that babesiosis is a globally emerging infectious disease, with certain causative species spreading as their tick vectors expand their geographic range (Bajer et al., 2022). In 2015, the World Health Organization (WHO) recognized vector-borne diseases as a major public health issue (WHO, 2014). Although human babesiosis is relatively rare compared with other vector-borne diseases, its

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incidence has been increasing worldwide in recent decades (Kumar et al., 2021). A global meta-analysis including 22 countries and 69 studies reported that human babesiosis increased between 2006 and 2021, indicating a high public health concern (Karshima et al., 2022). The emergence of zoonotic babesiosis in Asia is of particular concern and requires the implementation of appropriate control measures (Islam et al., 2022).

Microscopic analysis of Giemsa-stained blood smears is a vital, affordable, and rapid diagnostic method for field studies, even though molecular methods such as polymerase chain reaction (PCR) provide higher sensitivity. Furthermore, PCR can simultaneously assess parasitemia and erythrocyte morphology, underscoring its value for routine veterinary and epidemiological surveillance in resource-limited areas (Nyifi and Bilbonga, 2023; Sumbria et al., 2024).

Hematological and serum biochemical parameters are crucial for diagnosing ovine babesiosis (Esmaeilnejad et al., 2012). Similarly, serum biochemical markers, including elevated alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), bilirubin, blood urea nitrogen (BUN), and creatinine, along with oxidative stress markers, indicate hepatic and renal dysfunction. These markers are valuable for assessing disease severity and prognosis in babesiosis (Nevidnyk-Pravda and Ushakova, 2025). A recent study on Lohi sheep in Pakistan confirmed highly significant erythron alterations, along with marked changes in BUN, creatinine, and total bilirubin, in animals infected with *B. ovis* compared with uninfected controls (Sajid et al., 2023). Studies on bovine babesiosis confirmed the multiorgan impact of *Babesia* infection (Alqhtani et al., 2026). Epidemiological studies in Türkiye have consistently shown that ovine babesiosis is prevalent among sheep flocks (Dumanli et al., 1997; Aktaş et al., 2001; Emre et al., 2001; Çiçek et al., 2004; Ceylan and Sevinc, 2020).

Shirvan-Salyan region in Azerbaijan features a semi-arid climate, vast lowland pastures, and traditional sheep farming, creating favorable conditions for the maintenance of *Rhipicephalus* ticks and the spread of tick-borne diseases. By integrating multi-organ pathology with hematological and metabolic profiling in indigenous breeds, the present study addressed an important regional research gap and established the first reference framework to enhance the management and control of ovine babesiosis in Azerbaijan. Therefore, the present study aimed to investigate the clinical, hematological, and biochemical alterations associated with naturally occurring ovine babesiosis in local sheep within this endemic region.

MATERIALS AND METHODS

Ethical approval

The present study was carried out with the consent of the farm owners and in compliance with established animal welfare standards. Furthermore, the protocol was approved by the Ethics Committee of the Azerbaijan Veterinary Scientific Research Institute (Ministry of Agriculture of the Republic of Azerbaijan).

Study area and animal selection

This clinical and epidemiological study was conducted in the Shirvan-Salyan region of Azerbaijan. A total of 21 Garadolag-breed lambs, comprising 11 females and 10 males, all under 1 year old and weighing 15-18 kg, were purposively sampled from three farms. The present study included animals exhibiting clinical signs such as hyperthermia and anemia, as well as healthy animals from the same flocks for comparison. All animals were maintained under traditional pastoral conditions, including open grazing and communal watering. A clinical examination of the animals was conducted to assess their health status. Each sheep underwent a thorough physical examination prior to inclusion in the study, including assessment of body condition score, measurement of vital signs (heart rate, respiratory rate, rectal temperature), and examination of mucous membranes. These assessments ensured the absence of clinical abnormalities and infectious diseases. The study population was divided into two distinct groups. The infected group consisted of eight sheep (6 females and 2 males) that exhibited clinical signs of acute babesiosis, including hyperthermia, icterus, and lethargy, which were subsequently confirmed by microscopic examination. The control group comprised 13 sheep (5 females, 8 males) that were clinically healthy and tested negative for hemoparasites. The adequacy of the sample size was assessed using a power analysis in G*Power to ensure statistical reliability according to Formula 1 (Faul et al., 2007).

$1 - \beta = 0.80, \alpha = 0.05$ (Formula 1)

In this study, statistical significance was determined using a significance level of $\alpha = 0.05$, which represents the probability of making a Type I error—that is, incorrectly rejecting a true null hypothesis (a false positive). A Type II error, denoted by β, represents the probability of failing to reject a false null hypothesis (a false negative). The expression $1 - \beta = 0.80$ indicates that the statistical power of the test is 80%, meaning there is an 80% probability of correctly detecting a true effect or difference when one actually exists.

Sample collection and microscopic examination

The diagnosis of ovine babesiosis was based on clinical findings and microscopic examination of Giemsa-stained thin blood smears (Suleiman and Al-lahaibi, 2024). In clinical screening, sheep exhibiting one or more of the following signs- including a rectal temperature exceeding 40.5°C, pale or icteric mucous membranes, lethargy, anemia, and generalized weakness were suspected of babesiosis. Blood samples of approximately 5 mL were obtained from all animals via jugular venipuncture. The samples were collected into EDTA-K3 tubes for hematological examination and into plain tubes for serum biochemistry analysis. For parasitological verification, thin blood smears were prepared from capillary blood obtained from the ear vein of each individual. These smears were air-dried, fixed in 100% methanol for 5 minutes, and subsequently stained with a 10% Giemsa solution (pH 7.2) for 30 minutes. Preparations were examined at 1000× magnification using a Bell light microscope (Bell Technologies, Italy). *Babesia ovis* identification was based on its characteristic paired, obtuse-angled pyriform morphology, following established diagnostic criteria (Uilenberg, 2006; OIE, 2018). Clinically healthy sheep with negative blood smears for *Babesia ovis* served as the control group. Furthermore, ticks were collected from the ears, perineum, and axillary regions of infected animals and identified as *R. bursa* using established taxonomic features (Walker et al., 2003).

Hematological profiling

Hematological analysis was performed within 4 hours post-collection using a calibrated Mindray BC-3000 Plus automated analyzer (Mindray Bio-Medical Electronics Co., Shenzhen, China). Measured parameters included white blood cell (WBC), red blood cell (RBC), hemoglobin (Hb), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC). Differential leukocyte counts were performed automatically and confirmed by manual microscopic examination of stained smears, following established veterinary diagnostic protocols (Meyer and Harvey, 2004; Weiss and Wardrop, 2010).

Biochemical assays

Serum was separated by centrifugation at 3000×g for 10 minutes at room temperature and stored at −20°C until analysis. Serum biochemical profiles were measured using a Mindray BS-120 Vet automated clinical chemistry analyzer (Shenzhen, China). Diagnostic kits manufactured by BioSystems S.A. (Barcelona, Spain) were used for all analyses, strictly following the manufacturer's analytical protocols. The biochemical assessment measured total protein using the Total Protein kit (BioSystems, Barcelona, Spain) and albumin using the Albumin kit (BioSystems, Barcelona, Spain), with globulin calculated as the difference between total protein and albumin. Total and direct bilirubin concentrations were measured directly using BioSystems bilirubin assay kits, whereas indirect bilirubin was calculated as the difference between total and direct bilirubin. Renal function, lipid metabolism, and hepatic enzymes were assessed by measuring BUN, creatinine, total cholesterol, triglycerides, ALT, AST, ALP, and lactate dehydrogenase (LDH) concentrations using commercial enzymatic colorimetric kits from BioSystems S.A. (Barcelona, Spain), strictly following the manufacturer's analytical protocols.

Statistical analysis

Statistical analysis was performed using SPSS (version 13.0, Chicago, IL, USA). Data were presented as mean ± standard deviation (SD). The Shapiro-Wilk test was applied to assess normality. The independent Student's t-test was used to compare the infected and control groups, while Pearson's correlation coefficient (r) was used to assess relationships between hematological variables and liver/renal biomarkers. Results were considered statistically significant at p-values less than 0.05.

RESULTS

Clinical parameters, including heart rate, respiratory rate, and rectal temperature, were obtained from eight sheep naturally infected with *B. ovis* and compared with those of 13 clinically healthy sheep in the control group. The normal physiological ranges for these parameters were interpreted according to established veterinary reference values (Radostits et al., 2007; Constable et al., 2017). There was a significant disparity in clinical parameters between infected and healthy sheep. Animals with babesiosis exhibited elevated heart rates, respiratory rates, and body temperatures; clinically apparent jaundice was also observed (Table 1). All clinical parameters were significantly higher in the infected sheep than in the healthy controls ($p < 0.05$).

Microscopic examination of Giemsa-stained peripheral blood smears obtained from sheep exhibiting clinical suspicion of babesiosis demonstrated the presence of ring-shaped *B. ovis* parasites, approximately 1.2-2.2 µm in size, as well as paired pyriform *B. ovis* parasites, approximately 1.3-2.6 µm in size, within erythrocytes (Figure 1).

Hematological and biochemical profiles were compared between sheep infected with *B. ovis* and healthy ones (Table 2). Compared with the control group, sheep naturally infected with *B. ovis* demonstrated significantly higher total

leukocyte (WBC) and eosinophil counts ($p < 0.05$). In contrast, RBC, HCT, Hb, PCV, and MCH counts were significantly reduced in sheep infected with *B. ovis* compared to the healthy sheep ($p < 0.05$). No significant differences were observed in lymphocyte, monocyte, MCV, or MCHC counts between the groups ($p > 0.05$). A significant decrease in the neutrophil percentage was observed in the infected sheep compared with the control group ($p < 0.05$). In addition, microscopic examination of peripheral blood smears from sick animals revealed morphological changes in erythrocytes, including anisocytosis and poikilocytosis (Figure 2).

Serum biochemical parameters for all sheep were evaluated (Table 3). Significant increases in total, direct, and indirect bilirubin, BUN, creatinine, ALT, AST, ALP, and LDH were observed in naturally infected sheep ($p < 0.05$), indicating hemolysis with concurrent hepatic and renal dysfunction. In contrast, total protein, globulin, cholesterol, and triglyceride did not differ significantly between infected and healthy sheep ($p > 0.05$). Serum albumin concentration was significantly lower in infected sheep (3.53 ± 0.24 g/dL) than in healthy controls (4.33 ± 0.11 g/dL; $p < 0.05$).

Table 1. Clinical parameters of naturally *Babesia ovis*-infected sheep and healthy control sheep in the Shirvan-Salyan region of Azerbaijan during June 2023

Parameters	Healthy sheep	Infected sheep	p-value
Heartbeat /minuts	78.57 ± 12.49	116.82 ± 7.39	< 0.05
Respiratory rate /minuts	26.94 ± 5.46	58.25 ± 2.43	< 0.05
Body temperature /°C	39.33 ± 0.16	40.89 ± 0.92	< 0.05

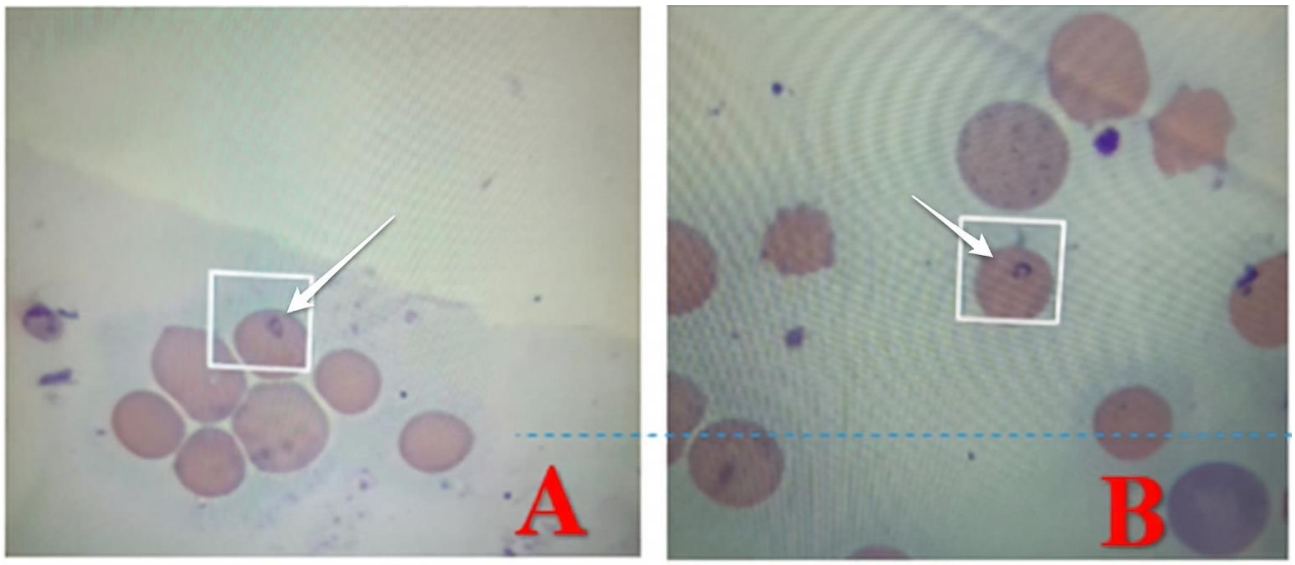


Figure 1. *Babesia ovis* in Garadolag lamb erythrocytes. **A:** Typical acute-stage paired pear-shaped merozoites from Garadolag lambs in the Shirvan-Salyan region of Azerbaijan (Giemsa stain, 1000× magnification); **B:** Microphotograph of *Babesia ovis* ring-shaped forms within erythrocytes. The small, round trophozoites, and some showing chromatin dots (Giemsa staining, 1000× magnification).

Table 2. Hematological parameters of naturally *Babesia ovis*-infected sheep and healthy sheep in the Shirvan-Salyan region of Azerbaijan, June 2023

Parameters	Healthy sheep	Infected sheep	p-value
WBC (10^9/µL)	8.92 ± 3.14	12.31 ± 2.88	< 0.05
Lymphocytes (%)	65.75 ± 2.13	62.40 ± 2.65	>0.05
Monocytes (%)	2.20 ± 0.02	2.60 ± 0.10	>0.05
Neutrophils (%)	30.42 ± 1.25	24.19 ± 1.34	< 0.05
Eosinophils (%)	5.40 ± 0.23	8.30 ± 0.12	< 0.05
RBC (10^6/µL)	6.73 ± 0.84	3.42 ± 0.99	< 0.05
MCV (fL)	37.40 ± 1.65	39.74 ± 0.86	>0.05
HCT (%)	26.40 ± 4.51	20.30 ± 5.74	< 0.05
Hb (g/dL)	11.70 ± 1.13	6.98 ± 0.85	< 0.05
PCV (%)	31.40 ± 2.34	22.73 ± 1.77	< 0.05
MCHC (g/dL)	32.50 ± 6.30	28.20 ± 5.40	> 0.05
MCH (pg)	13.90 ± 0.44	10.40 ± 0.21	< 0.05

WBC: White blood cells, RBC: Red blood cells, MCV: Mean corpuscular volume, HCT: Hematocrit, Hb: Hemoglobin, MCH: Mean corpuscular hemoglobin, MCHC: Mean corpuscular hemoglobin concentration. Data are expressed as mean ± SD. Statistical significance was defined as $p < 0.05$, with $p > 0.05$ denoting lack of statistical significance.

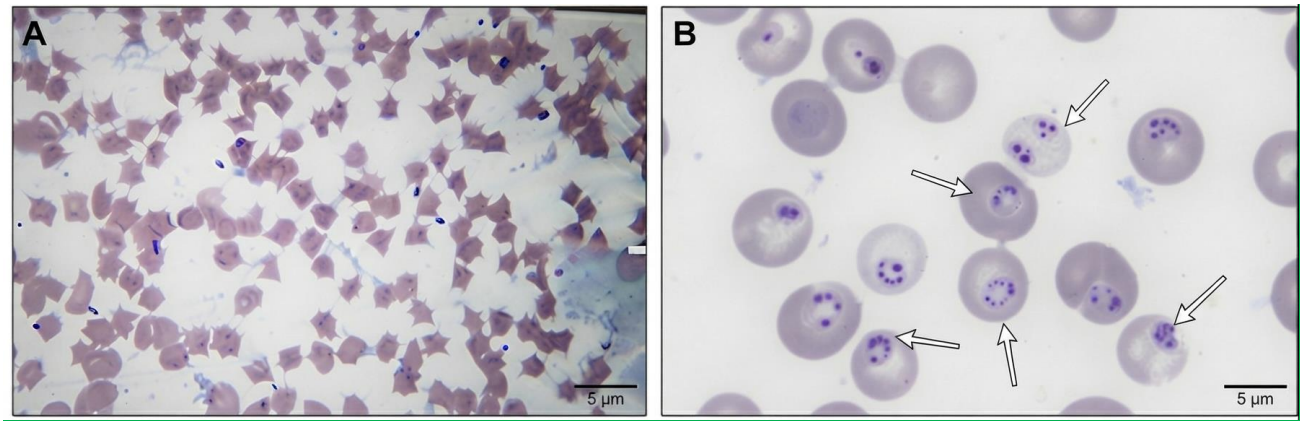


Figure 2. Anisocytosis and poikilocytosis in erythrocytes of a Garadolag lamb infected with *Babesia ovis* from the Shirvan-Salyan region of Azerbaijan (Giemsa stain, 1000× magnification).

Table 3. Biochemical parameters of naturally *Babesia ovis*-infected sheep and healthy control sheep in the Shirvan-Salyan region of Azerbaijan, June 2023

Parameters	Healthy sheep	Sick sheep	p-value
Total protein (g/d)	6.33 ± 0.82	5.87 ± 0.77	> 0.05
Total bilirubin (mg/dL)	0.42 ± 0.23	1.58 ± 0.34	< 0.05
Direct bilirubin (mg/dL)	0.25 ± 0.14	0.44 ± 0.17	< 0.05
Indirect bilirubin (mg/dL)	0.13 ± 0.15	1.29 ± 0.14	< 0.05
Albumin (g/dL)	4.33 ± 0.11	3.53 ± 0.24	< 0.05
Globulin (g/dL)	2.75 ± 0.66	1.64 ± 0.31	> 0.05
Creatinine (mg/dL)	1.41 ± 0.32	1.82 ± 0.43	< 0.05
BUN (mg/dL)	24.73 ± 1.67	57.93 ± 4.22	< 0.05
Cholesterol (mg/dL)	4.81 ± 1.19	5.52 ± 1.08	>0.05
Triglyceride (mg/dL)	5.72 ± 1.32	5.98 ± 1.24	> 0.05
ALT (U/L)	28.84 ± 3.29	34.49 ± 1.45	< 0.05
AST (U/L)	144.28 ± 11.76	196.82 ± 18.84	< 0.05
ALP (U/L)	118.3 ± 5.53	286.8 ± 8.37	< 0.05
LDH (U/L)	353.4 ± 21.32	469.7 ± 28.86	< 0.05

BUN: Blood urea nitrogen, ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, ALP: Alkaline phosphatase, LDH: Lactate dehydrogenase. Data are expressed as mean ± SD. Statistical significance is indicated by $p < 0.05$, non-statistical significance by $p > 0.05$.

DISCUSSION

Babesia ovis infection caused notable hematological changes in the affected sheep. Infected animals exhibited notably lower RBC, Hb, and HCT levels than healthy controls, while their MCV was markedly higher. These observations were aligned with previous findings on small ruminants in Khorasan province, Iran, and Eastern Turkey (Ceylan, 2020; Razmi, 2022; Ulucesme et al., 2023). Similar hematological alterations, including reductions in RBC count and Hb concentration, have been reported in horses infected with *Theileria equi* (formerly *Babesia equi*) and in dogs infected with *Babesia gibsoni* (Karasová et al., 2022; Salinas-Estrella et al., 2022).

The mean rectal temperature of the healthy control sheep (39.33 ± 0.16°C) was within the normal physiological range reported for sheep (38.3-39.9°C). The rectal temperature in healthy lambs, though within normal limits, aligns with the slightly elevated basal metabolic rate typical of young animals compared to adult sheep (Radostits et al., 2007; Constable et al., 2017). The relatively high value could be due to environmental factors during sampling, as animals were observed in the semi-arid Shirvan-Salyan region during the warm season. Furthermore, handling and restraint prior to blood collection may have caused temporary stress, resulting in a slight elevation in rectal temperature (Merck and Co., Inc., 2024). The hematological findings in the present study were consistent with previous reports regarding erythrocyte-related parameters in *B. ovis*-infected animals. The notable reductions in RBC count, Hb, and HCT levels confirmed the anemic characteristic of ovine babesiosis, aligning with findings reported in naturally and experimentally infected sheep (Esmaeilnejad et al., 2012; Sajid et al., 2023). In contrast, leukocyte responses, particularly neutrophil alterations, were not consistent across studies. While some investigations have reported increased neutrophil counts during *B. ovis* infection, the present study demonstrated a remarkable reduction in neutrophil percentage, indicating that the leukocyte response may vary according to infection stage, parasite burden, host-related factors, and the timing of sample collection (Esmaeilnejad et al., 2012; Haq et al., 2021; Korelidou et al., 2026). Differences in study design, such as using naturally versus experimentally infected animals, and in infection duration and severity may also lead to disparities in leukocyte

profiles. Therefore, the consistent decrease in erythrocyte-related parameters across studies indicated that anemia is a relatively stable hematological characteristic of *B. ovis* infection. In contrast, changes in leukocyte and neutrophil levels are more variable and might indicate the host's dynamic response to the infection.

The anemia associated with *B. ovis* infection is primarily attributed to intravascular and extravascular hemolysis, as well as to immune-mediated erythrocyte destruction triggered by antibodies against erythrocyte membrane antigens (Zahidullah et al., 2023). The detection of anisocytosis and poikilocytosis in blood smears was consistent with regenerative hemolytic anemia and indicated increased erythrocyte production. The increase in MCV may result from the release of larger immature erythrocytes (reticulocytes) from the bone marrow during recovery from anemia. Meanwhile, the decrease in MCHC level was associated with the lower Hb concentration of these immature cells (Abdalla et al., 2020; Sajid et al., 2023). Therefore, these findings indicated that anemia in *B. ovis* infection results from both intravascular and extravascular hemolysis, accompanied by immune-mediated erythrocyte destruction and increased erythrophagocytosis by macrophages (Tufani et al., 2017; Djokic et al., 2021). These hematological changes reflected the pathophysiological response to *B. ovis* infection.

The observed biochemical alterations indicate hepatic dysfunction, impaired renal function, and systemic metabolic disturbances associated with *B. ovis* infection. Similar increases in serum bilirubin, BUN, creatinine, and hepatic enzyme activities, with decreases in serum protein concentrations, have been reported in naturally and experimentally infected sheep with *B. ovis* (Esmaeilnejad et al., 2012; Sevinc et al., 2013). The biochemical profile observed in the present study indicated that *B. ovis* infection was associated with systemic metabolic alterations involving renal function, lipid metabolism, and serum protein homeostasis. The notable increases in serum creatinine, cholesterol, and triglyceride concentrations were consistent with the findings of Bozan et al. (2024) in sheep with babesiosis, supporting the occurrence of renal and metabolic disturbances during infection. Elevated creatinine and BUN levels may indicate impaired kidney function caused by hemoglobin-related kidney injury, decreased renal blood flow due to anemia, or dehydration. Similarly, the increases in cholesterol and triglycerides may indicate altered lipid metabolism and reduced lipid clearance in response to hepatic dysfunction and systemic inflammation (Sivajothi et al., 2022). Alterations in serum protein concentrations supported the systemic inflammatory and metabolic effects of babesiosis. Some studies have reported elevated total protein and globulin concentrations; these differences among studies may be attributable to disease stage, parasite load, and the intensity of the host's immune response (Adewumi et al., 2022; Salinas-Estrella et al., 2022). In contrast, the reduction in albumin concentration observed in the present study was consistent with the negative acute-phase response during babesiosis and may be associated with impaired hepatic protein synthesis and reduced feed intake during the acute stage of infection (Kuleš et al., 2021). Therefore, the current findings indicated that the biochemical response to *B. ovis* infection was not limited to hemolysis but also encompassed renal dysfunction, hepatic and lipid metabolic disturbances, systemic inflammation, and nutritional alterations.

Mortality was observed in lambs aged 5 to 6 months, accompanied by severe anorexia, indicating that young animals are especially vulnerable to the systemic effects of *B. ovis* infection. Similar pathological mechanisms have been documented in naturally and experimentally infected sheep. *Babesia ovis* infection results in more severe clinical signs and a less favorable prognosis in lambs than in adult sheep (Sevinc et al., 2013). The hematological and biochemical changes observed in the present study were consistent with the findings of Esmaeilnejad et al. (2012), who reported that progressive hemolysis led to hepatic dysfunction, renal impairment, and metabolic disturbances in ovine babesiosis.

CONCLUSION

Babesia ovis is a significant etiological agent of ovine babesiosis in Azerbaijan. The current study demonstrated that sheep naturally infected with this pathogen exhibited notable hematological and biochemical changes, including anemia, elevated bilirubin levels, increased BUN and creatinine, elevated hepatic enzyme activities, elevated lipid levels, and decreased serum protein concentrations and erythrocyte indices. The current findings indicated hemolysis, hepatic dysfunction, altered renal function, and systemic metabolic disturbances associated with *B. ovis* infection. The combined evaluation of clinical signs, hematological and serum biochemical parameters improved the diagnosis and clinical assessment of ovine babesiosis and provided baseline data for disease monitoring in endemic areas of Azerbaijan. Therefore more study should be conducted in different regions of Azerbaijan to evaluated *Babesia ovis* hematological and biochemical dysregulation.

DECLARATIONS

Authors' contributions

Aygun Azizova contributed to the design, conceptualization, collection of samples, sample analysis, and manuscript preparation. Ugur Uslu participated in the statistical analysis and drafting of the manuscript. Gular Mammadova was

involved in sample collection, sample analysis, and manuscript writing. Elnura Aslanova contributed to the design and manuscript preparation. Yusif Yusifli participated in sample collection. All authors have reviewed and approved the final edition of the manuscript.

Availability of data and materials

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

Competing interests

The authors declare no conflicts of interest regarding the publication of this manuscript. There are no financial, personal, or professional relationships with individuals or organizations that could have inappropriately influenced or biased the findings presented in this work.

Ethical considerations

The authors declared that this manuscript is original, has not been published previously, and is not under consideration for publication elsewhere. All authors have read and approved the final version of the manuscript and agree to its submission. The use of artificial intelligence (AI)-assisted tools (Grammarly) was limited to language editing and readability improvements. All authors critically reviewed and revised the AI-edited language edition of the manuscript and the final manuscript, and take full responsibility for their accuracy, originality, and integrity before submission and accepted manuscript before publication.

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Morphological and Molecular Investigations of *Sarcocystis* spp. in *Camelus dromedaries* in Kuwait

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ABSTRACT

Sarcocystosis is a disease caused by *Sarcocystis* parasites, which are widely distributed among mammals, birds, and reptiles and may cause myositis and encephalitis. The present study aimed to investigate the prevalence, morphological and histopathological characteristics, and molecular identification of *Sarcocystis* infection in dromedary camels (*Camelus dromedarius*) in Kuwait using pepsin digestion, histopathological examination, and molecular characterization based on the 18S rRNA gene. A total of 1,350 tissue samples from muscles, diaphragm, tongue, heart, and esophagus were collected from 270 camels slaughtered in Kuwait. The camels comprised 214 males and 56 females and were categorized into three age groups, including younger than 8 months (n = 99), between 8 months and 3 years old (n = 113), and older than 3 years old (n = 58). Microscopic examination revealed *Sarcocystis* infection in 194 of 270 camels (71.85%). Infection was not significantly associated with sex, whereas age and season were significantly associated with *Sarcocystis* prevalence. *Sarcocystis* prevalence was 63.3% in muscle, 63.0% in diaphragm, 62.2% in tongue, 62.0% in heart, and 56.3% in esophagus. Histopathological examination revealed three cyst morphologies, including spindle-shaped, ellipsoidal, and irregularly elongated. Pepsin digestion revealed three distinct bradyzoite morphologies, including banana-shaped, sausage-shaped, and round forms. Five tissue samples were selected for molecular analysis using 18S rRNA sequencing. The BLASTn analysis demonstrated the highest similarity to *Sarcocystis* spp. isolated from *Camelus dromedarius* in Iran (GenBank accession no. GU074011), with 69% query coverage and a sequence identity of 98.4-98.67%. The present study reported a high prevalence of *Sarcocystis* infection (71.85%) among dromedary camels in Kuwait, with infection rates significantly influenced by age and season. Three distinct cyst and bradyzoite morphologies, along with molecular data showing high sequence similarity to Iranian dromedary isolates, strongly suggested that *Sarcocystis* infections are widespread and diverse among camel populations in Kuwait.

Keywords: *Camelus dromedarius*, Molecular analysis, *Sarcocystis* spp., Season

INTRODUCTION

Sarcocystosis is a zoonotic parasitic disease caused by intracellular coccidian protozoa of the genus *Sarcocystis*, which belongs to the phylum Apicomplexa (Latif and Muslim, 2016; Muslim and Chin Heo, 2022). Members of the genus *Sarcocystis* are found worldwide and infect a broad range of intermediate and definitive hosts, such as mammals, birds, and reptiles (Latif and Muslim, 2016; Muslim and Chin Heo, 2022). Infections with *Sarcocystis* spp. are particularly common in dromedary camels (*Camelus dromedarius* [*C. dromedarius*]) and have been reported in several camel-producing countries. High infection rates have been documented in Saudi Arabia (Fatani et al., 1996), Iraq (Latif et al., 1999), Iran (Valinezhad et al., 2008; Motamedi et al., 2011), Egypt, and other endemic regions (Gareh et al., 2020). Valinezhad et al. (2008) identified *Sarcocystis* infection in 83.6% of 250 camels examined in Eastern Iran, illustrating the high prevalence of the parasite in camel populations.

Sarcocystis infection in camels was first documented in the early twentieth century, when Mason (1910) described the cysts, their morphology, and cyst-wall dimensions. Subsequent studies identified multiple *Sarcocystis* species in camels. Nonetheless, the classification of these parasites remained unclear because some species lacked detailed morphological characterization or had no type specimens. Dubey et al. (2015) conducted a comprehensive review of the available literature and provided information on the sarcocysts of *Sarcocystis cameli* (*S. cameli*) and *Sarcocystis ippeni* (*S. ippeni*) from *C. dromedarius*, utilizing light microscopy and transmission electron microscopy. In addition, Dubey et al. (2015) clarified key morphological features of *S. cameli* and *S. ippeni* and considered *S. camelicanis*, *S. camelocanis*, and *S. miescheri* as invalid species.

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Camels become infected with *Sarcocystis* by consuming food or water contaminated with sporulated oocysts or sporocysts from the definitive hosts. After ingestion, the parasite undergoes developmental stages in the intermediate host and ultimately forms sarcocysts in the muscles (Valinezhad et al., 2008). While many infections are asymptomatic or subclinical, severe diseases can arise. *Sarcocystis* infection can induce inflammatory responses, tissue damage, necrosis, and fibrosis, particularly when cysts degenerate (Valinezhad et al., 2008).

Sarcocystis infection significantly impacts camel production and meat quality. Macroscopic sarcocysts negatively impact the visual appeal and acceptability of meat, potentially leading to carcass or tissue condemnation. Although microscopic sarcocysts may remain undetected during routine meat inspection, their high prevalence can lead to economic losses and affect animal health and productivity. In animals exhibiting clinical signs, reported manifestations include fever, anemia, anorexia, muscle weakness, decreased productivity, abortion, and, in severe cases, mortality (Valinezhad et al., 2008; Metwally et al., 2020).

Diagnosing *Sarcocystis* infection in camels can be difficult, especially when there are no visible macroscopic cysts or clinical symptoms. Several diagnostic methods are employed, including muscle compression or squash techniques, pepsin and trypsin digestion, histopathological analysis, and molecular assays. Each method has distinct diagnostic capabilities, and the use of morphological, histopathological, and molecular techniques can improve the detection and characterization of *Sarcocystis* infections (Saeed et al., 2018a).

Although *Sarcocystis* infection has been extensively documented in camels in several countries, information on its prevalence, tissue distribution, morphological characteristics, and molecular identity in dromedary camels in Kuwait remains limited. Given the economic importance of camels in Kuwait as sources of meat and milk, it is essential to determine the epidemiological status of *Sarcocystis* infection. Therefore, the present study aimed to determine the prevalence and tissue distribution of *Sarcocystis* infection in camels slaughtered in Kuwait and to characterize the infection using pepsin digestion, histopathological examination, and molecular analysis.

MATERIALS AND METHODS

Ethical approval

This study was conducted in accordance with the regulations established by the Public Authority of Agriculture Affairs and Fish Resources (PAAFR), Rabia, Kuwait, for research activities, and received approval from the technical committee of the Animal Resources sector.

Study design and samples

A cross-sectional study was conducted over one year, from January to December 2020. Given the climatic conditions in Kuwait, the year has been divided into two distinct seasons, including a dry season from April to November, and a wet season from December to March (Halwagy et al., 1974). The study population comprised one-humped camels (*Camelus dromedarius*) of both sexes and different age groups, reared under an extensive husbandry system that allowed free grazing. Information on the specific diets and husbandry practices of individual camels was unavailable because the camels originated from different owners and farms. A total of 270 camels were slaughtered at two slaughterhouses in Kuwait, including Al-Jahra Slaughterhouse in Northern Kuwait (Al-Jahra Governorate) and Al-Dhahar Slaughterhouse in Southern Kuwait (Mubarak Al-Kabeer Governorate). The sample size in the present study was determined based on previous studies by Gareh et al. (2020) and Metwally et al. (2020). Camels were selected from animals that were presented for slaughter during the study period.

Information on sex, age, body condition, and breed was recorded at the time of sampling. Age was determined through data obtained from owners and through dentition assessment. In accordance with the classification outlined by Bello et al. (2013), the camels were categorized into three groups, including less than 8 months, between 8 months and 3 years, and over 3 years.

Microscopic examination

Pepsin digestion technique

In the present study, over a period of one year and through bi-weekly visits to two slaughterhouses, approximately 20 grams of tissue samples from five organs, namely the diaphragm, esophagus muscles, skeletal muscles, tongue, and heart, were collected, minced, and subsequently incubated in 100 mL of digestion solution at 40°C for 30 minutes. The digestion solution contained pepsin (1.3 g), HCl (3.5 mL), and NaCl (2.5 g) dissolved in 500 mL of distilled water (Dubey et al., 1989). Following digestion, samples were centrifuged at 3500 × g for 3 minutes, stained with Giemsa, and examined under a light microscope (Hamidinejat et al., 2013).

Histopathological examination

Collected samples, each measuring 2×1×1 cm^3, were fixed in 10% formalin for histological preparation (Bancroft and Gamble, 2007). Paraffin embedding was performed, followed by sectioning of 5-µm-thick slices and hematoxylin and eosin (H&E) staining. Cysts were measured using an ocular micrometer under a microscope and photographed with the AX10 microscope (Zeiss, Germany) connected to the high-resolution camera (HRC; FLIR, UK). All measurements were recorded in micrometers.

Molecular analysis

Eight positive samples, prepared using pepsin digestion, were chosen for the molecular analysis. The sediments were preserved in 80% alcohol until use and were transported by airplane to Iran. All molecular techniques were performed at the Parasitology Department, Medical Sciences Faculty, Tarbiat Modares University, Tehran, Iran. DNA from eight samples was extracted using a DNA purification kit (Yekta Tajhiz Azma, YTA Genomic DNA Extraction Mini kit for Tissue, Tehran, Iran) according to the manufacturer's instructions and stored at -20°C for molecular analysis. Polymerase chain reaction (PCR) was performed to identify *Sarcocystis* species by amplifying a partial sequence of the small subunit ribosomal RNA (18S rRNA) gene from *Sarcocystis* in camels. For amplification of *Sarcocystis* species 18S rRNA, primers SarcoF 5'-CGCAAATTACCCAATCCTGA-3' and SarcoR 5'-ATTTCTCATAAGGTGCAGGAG-3' were used (Moréet al., 2011; Bräunig et al., 2016). The 20 µL PCR mixture included 1 µL of template DNA, 10 µL of Taq DNA Polymerase 2x Master Mix RED (Ampliqon, 2 mM $MgCl_2$), and 1 µM of each primer. The PCR process consisted of 35 cycles (95°C for 30 s, 57°C for 30 s, and 72°C for 1 min), starting with an initial denaturation at 95°C for 5 minutes and ending with a final extension at 72°C for 10 minutes.

Phylogenetic analysis

For sequence analysis, five isolates were selected from 8 positive tissues, including isolates 1 and 5 from skeletal muscle, isolates 2 and 3 from the tongue, and isolate 4 from the diaphragm. These five positive samples were sequenced on the ABI3730XL sequencer (Japan). The sequences were edited and aligned using Clustal W and compared with reference sequences from GenBank (NCBI). For *Sarcocystis* species, the phylogenetic tree was constructed using the maximum-likelihood algorithm in the Molecular Evolutionary Genetics Analysis (MEGA) software (version 7.0; USA). Sequences of representative *Sarcocystis* species infecting camels were included from NCBI GenBank. *Neospora caninum* (LC897915) was used as an outgroup owing to its classification within the family *Sarcocystidae*, despite belonging to a different genus (Table 1). The sequences from the five samples were deposited in GenBank (NCBI) with accession numbers PQ120680- PQ120684.

Table 1. Coccidian species employed in phylogenetic analysis, along with their corresponding GenBank accession numbers

Accession number	Parasite species	Host	Country
PQ120680	*Sarcocystis* sp. Isolate 1	*Camelus dromedarius*	Kuwait
PQ120681	*Sarcocystis* sp. Isolate 2	*Camelus dromedarius*	Kuwait
PQ120682	*Sarcocystis* sp. Isolate 3	*Camelus dromedarius*	Kuwait
PQ120683	*Sarcocystis* sp. Isolate 4	*Camelus dromedarius*	Kuwait
PQ120684	*Sarcocystis* sp. Isolate 5	*Camelus dromedarius*	Kuwait
GU074011	*Sarcocystis* sp.	*Camelus dromedarius*	Iran
OP959654	*Sarcocystis cameli*	*Camelus dromedarius*	India
OM462705	*Sarcocystis cameli*	*Camelus dromedarius*	Not shown
OM462704	*Sarcocystis cameli*	*Camelus dromedarius*	India
KU527109	*Sarcocystis masoni*	*Lama guanicoe*	Argentina
KU527110	*Sarcocystis masoni*	*Lama glama*	Argentina
KU527111	*Sarcocystis masoni*	*Lama glama*	Argentina
KU527113	*Sarcocystis masoni*	*Vicugna pacos*	Argentina
LC171832	*Sarcocystis fayeri*	*Equus caballus*	Canada
MH025628	*Sarcocystis bertrami*	*Equus caballus*	China
KT901104	*Sarcocystis sinensis*	*Bubalus bubalis*	Egypt
KF954731	*Sarcocystis hominis*	Cattle	Germany
KY019056	*Sarcocystis silva*	*Capreolus capreolus*	Italy
KR186117	*Sarcocystis fusiformis*	*Bubalus bubalis*	Egypt
KJ778019	*Sarcocystis cafferi*	*Syncerus caffer*	South Africa
KU247910	*Sarcocystis buffalonis*	*Bubalus bubalis*	Egypt
KC209741	*Sarcocystis hirsuta*	*Bos taurus*	Argentina
L76473	*Sarcocystis moulei*	Experimentally infected goats	Germany
KC209733	*Sarcocystis gigantea*	*Ovis aries*	Norway
KF831275	*Sarcocystis alceslatrans*	*Alces alces*	Canada
AF017123	*Sarcocystis aucheniae*	Not specified	Australia
JX110662	*Sarcocystis aucheniae*	*Lama glama*	Argentina
JX110663	*Sarcocystis aucheniae*	*Lama glama*	Argentina
KU527114	*Sarcocystis aucheniae*	*Lama guanacoe*	Argentina
KU527116	*Sarcocystis aucheniae*	*Lama glama*	Argentina
KU527118	*Sarcocystis aucheniae*	*Lama guanicoe*	Argentina
KF383267	*Sarcocystis aucheniae*	Not specified	Argentina
KF383268	*Sarcocystis aucheniae*	Not specified	Argentina
LC897915	*Neospora caninum* (outgroup)	*Columba livia domestica*	Not shown

Statistical analysis

Data were analyzed and compared statistically using IBM SPSS software (version 23). Associations between parasitism and different parameters were assessed using the Pearson chi-square test. A p-value less than 0.05 was considered significant.

RESULTS

No macroscopic *Sarcocystis* spp. were found in any of the examined camels. However, microscopic *Sarcocystis* spp. were detected in 194 of 270 camels, yielding an overall prevalence of 71.85% using the pepsin digestion method. The rate of infection was not significantly associated with gender ($p > 0.05$), although males had a higher infection rate (74.3%) than females (62.5%; Table 2). In contrast, age and season significantly influenced the prevalence of *Sarcocystis* infection ($p < 0.05$). The highest infection rates were observed among camels aged between 8 months and 3 years and during the wet season, compared to other age groups and the dry season, respectively (Tables 2 and 3). Thus, camels aged > 8 months–3 years had a significantly higher prevalence than both other age groups. The groups 0–8 months and > 3 years did not differ significantly. A total of 1,350 tissue samples, including skeletal muscles, diaphragm, tongue, heart, and esophagus, from 270 camels were analyzed for *Sarcocystis* spp. infection using the pepsin digestion method. The prevalence rates were 63.3% in skeletal muscles, 63.0% in the diaphragm, 62.2% in the tongue, 62.0% in the heart, and 56.3% in the esophagus.

Table 2. Prevalence of *Sarcocystis* species according to gender and age groups in Kuwait

Category		Number of negative (%)	Number of positive (%)	Total number (%)	P-value
Gender	Male	55 (25.7)	159 (74.3)	214 (79.3)	p > 0.05
	Female	21 (37.5)	35 (62.5)	56 (20.7)	
Age	0-8 months	40 (40.4)	59 (59.6)	99 (36.6)	p < 0.05
	> 8 months-3 years	16 (14.2)	97 (85.8)	113 (42)	
	> 3 years	20 (34.5)	38 (65.5)	58 (21.4)	
Season	Dry	70 (37.6)	116 (62.4)	186 (68.8)	p < 0.05
	Wet	6 (7.2)	78 (92.8)	84 (31.2)	
	Total	76 (28%)	194 (72%)	270	

Table 3. Prevalence of *Sarcocystis* species according to age groups in Kuwait using post-hoc pairwise comparisons with Bonferroni correction

Pairwise comparison	Positive prevalence	Raw P-value	Bonferroni-adjusted P	Interpretation
0-8 months versus > 8 months-3 years	59.6% versus 85.8%	< 0.001	< 0.001	Significant
0-8 months versus > 3 years	59.6% versus 65.5%	0.461	1.000	Not significant
> 8 months-3 years versus > 3 years	85.8% versus 65.5%	0.002	0.006	Significant

Morphological results

Cysts

Three morphological types of cysts were observed in the examined tissues, including spindle, ellipsoid, and irregularly elongated. The length of the elongated shape was 2.75 × 1.2 µm in esophagus samples and 286.9 × 78.2 µm in muscle samples, with 144.8 ± 200.9 µm length and 39.7 ± 54.45 µm width. The measured ellipsoid shape was 113.4 × 86.4 µm in muscle samples and 38.8 × 31 µm in tongue samples. The spindle shape was observed only in a single diaphragm sample measuring 387.4 × 148.8 µm. The cyst wall was thin in elongated and spindle-shaped cysts, whereas it was thick in ellipsoid-shaped cysts (Figure 1).

Bradyzoites

Three morphological forms of bradyzoites were observed. The first form included banana-shaped bodies, measuring 15.5-29.6 µm in length (20.62 ± 5.84 µm) and 3.5-18.5 µm in width (10.46 ± 7.0 µm). Each bradyzoite had a pointed, arrow-like tip at one end and a rounded tip at the other end, producing a lancet-like shape (Figure 2A). The second form was sausage-shaped, measuring 9-26 µm in length (17.64 ± 6.87 µm) and 7.2-13.2 µm in width (9.86 ± 2.3 µm; Figure 2B). The third form of bradyzoites was spherical, with a diameter ranging from 7.8 to 16.5 µm (12.34 ± 3.7 µm; Figures 2A and 2B).

Molecular results

The PCR assay yielded a specific amplicon of approximately 700 bp. Clear bands were observed in six samples, while the remaining two samples exhibited very weak bands (Figure 3). DNA fragments of approximately 655 bp were successfully obtained from all five isolates, and their sequences were subsequently deposited in GenBank under accession numbers PQ120680-PQ120684. Isolates 1 and 5 had identical nucleotide compositions, including 194 adenine (A), 113 cytosine (C), 153 guanine (G), and 195 thymine (T). Isolates 2 and 3 exhibited identical sequences with compositions of 194 A, 114 C, 152 G, and 195 T. Isolate 4 demonstrated minor variations in nucleotide composition, with 193 A, 114 C, 152 G, and 196 T. The five isolates exhibited a pairwise sequence identity of 99.69%. Phylogenetic

analysis of the 18S rRNA sequences revealed that all five *Sarcocystis* isolates obtained from camels in Kuwait clustered within the same clade as *Sarcocystis* sp. isolated from *Camelus dromedarius* in Iran (GenBank accession number GU074011; Figure 4). The BLASTn analysis of the 18S rRNA sequences against the GenBank database revealed that the *Sarcocystis* sp. from *Camelus dromedarius* in Iran (GU074011) had the highest similarity to the Kuwaiti isolates, with 69% query coverage and 98.4-98.67% sequence identity. In contrast, the sequences of *Sarcocystis* sp. from *Camelus dromedarius* in India (OP959654, OM462704, and OM462705) exhibited 68% query coverage and 95.11% sequence identity with the isolates of the present study.

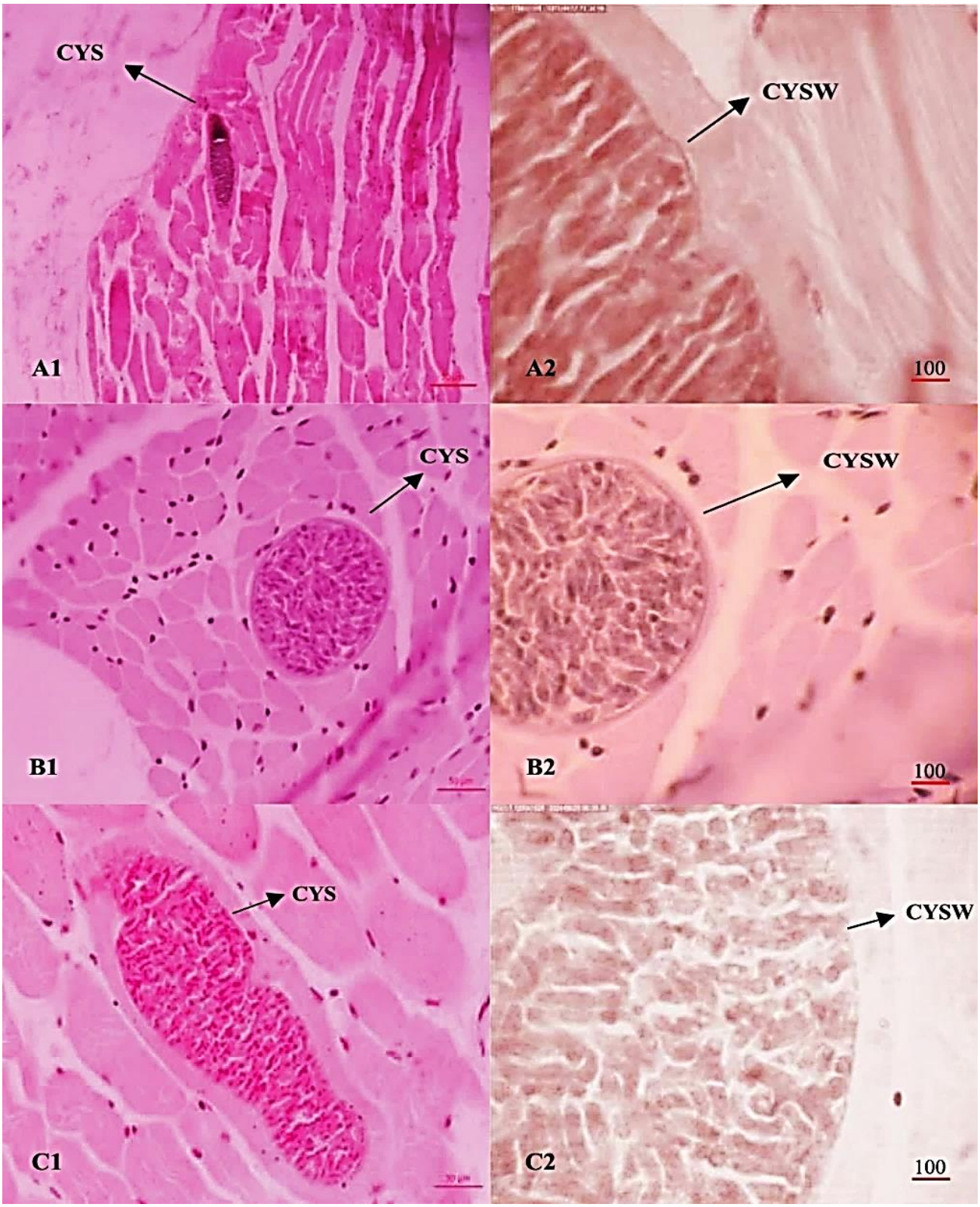


Figure 1. Muscle sections of *Camelus dromedarius* infected with *Sarcocystis* species showing different cyst types and their cyst walls. **A1:** Spindle-shaped cyst from the muscle tissue, **A2:** The cyst wall of A1. **B1:** An ellipsoid cyst from the tongue tissue, **B2:** The cyst wall of B1, **C1**: Elongated, irregular-shaped cyst from the diaphragm, **C2**: The cyst wall of C1, CYS: Cyst. CYSW: Cyst wall (100x magnification).

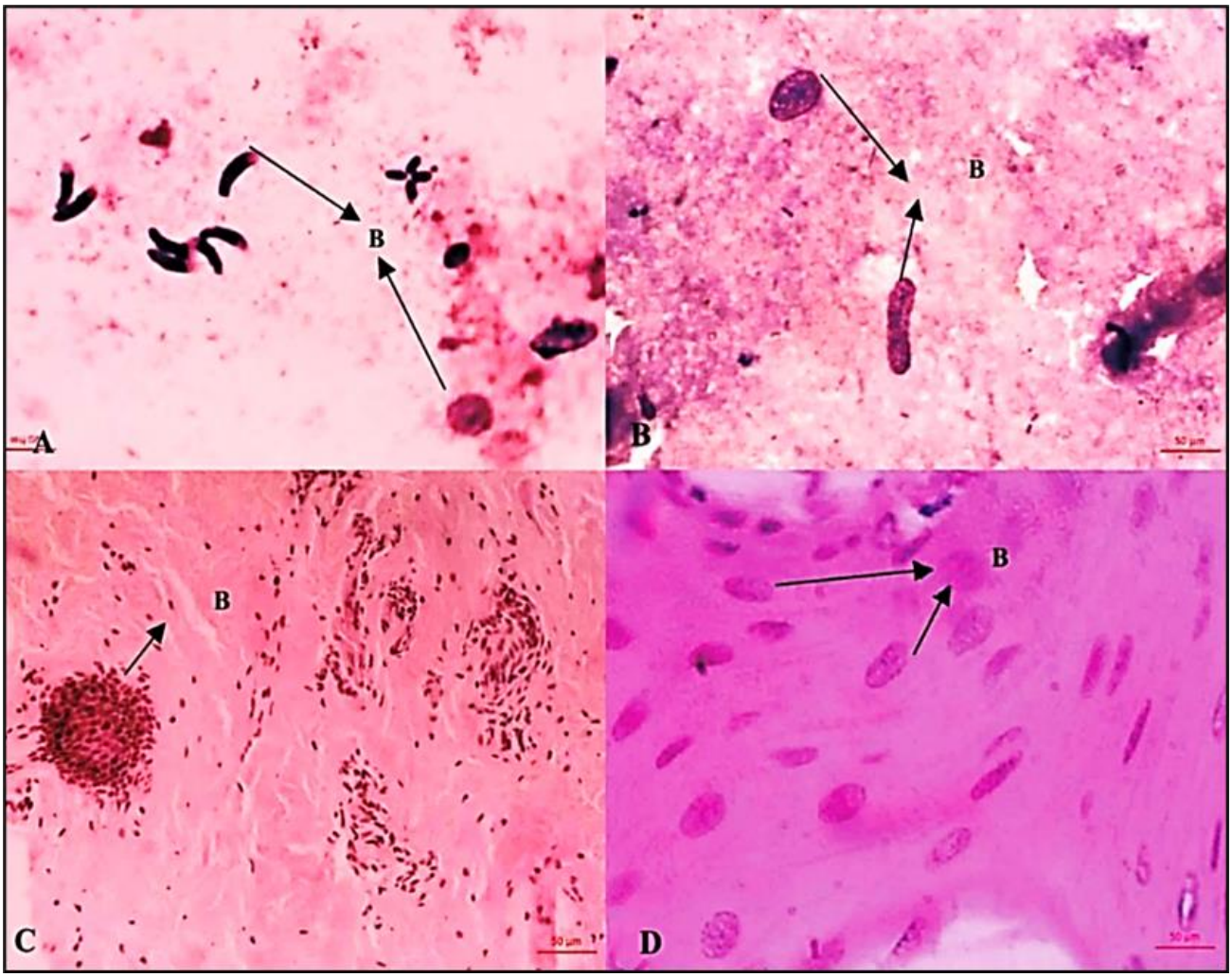


Figure 2. Pepsin digestion method used on muscles from Camelus dromedarius raised in Kuwait, with different types of bradyzoites. **A:** Banana and round-shaped bradyzoites, **B:** Round and sausage-shaped bradyzoites, **C** and **D:** Intensive distribution of bradyzoites, B: Bradyzoite.

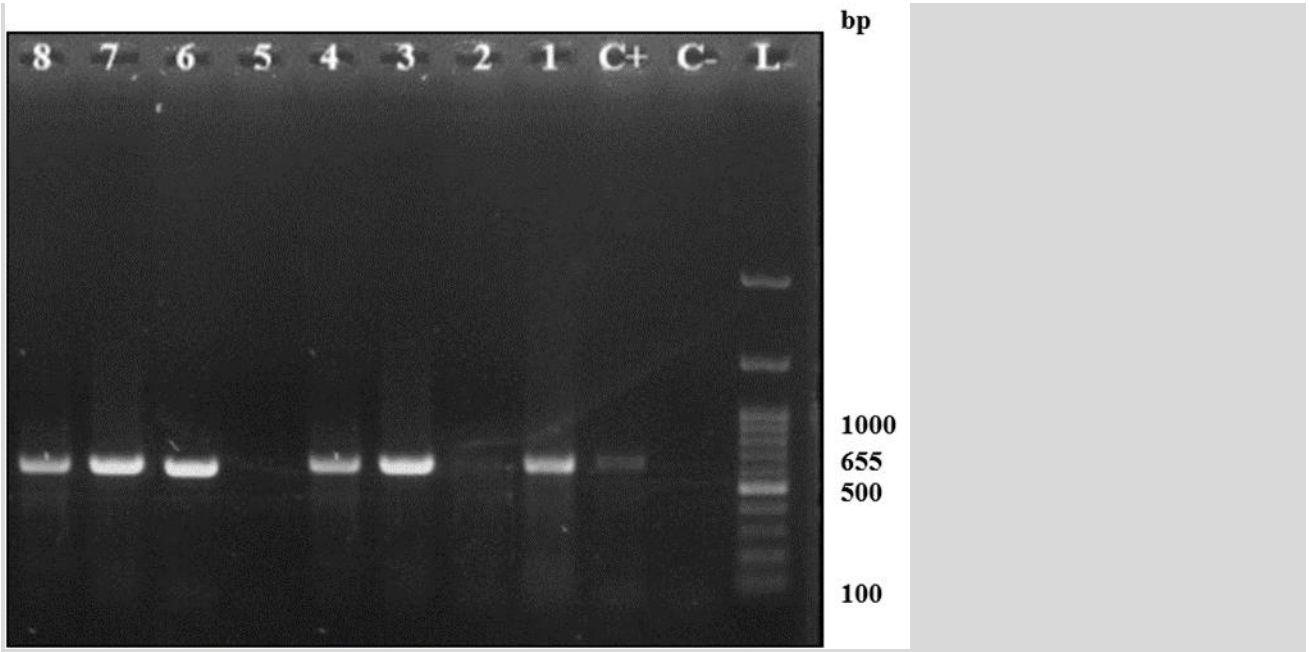


Figure 3. Agarose gel electrophoresis (1.5%) of 18S rRNA PCR products (700 bp) from *Sarcocystis*-positive tissues of *Camelus dromedarius*. Lanes 1 and 7: *Sarcocystis* DNA extracted from muscles, Lane 2: *Sarcocystis* DNA extracted from heart, Lanes 3 and 4: *Sarcocystis* DNA extracted from tongue, Lane 5: *Sarcocystis* DNA extracted from esophagus, Lane 6: *Sarcocystis* DNA extracted from diaphragm, Lane 8: *Sarcocystis* DNA extracted from esophagus of *Camelus dromedarius*, C+: Positive control, C: Negative control, L: Ladder 1000bp.

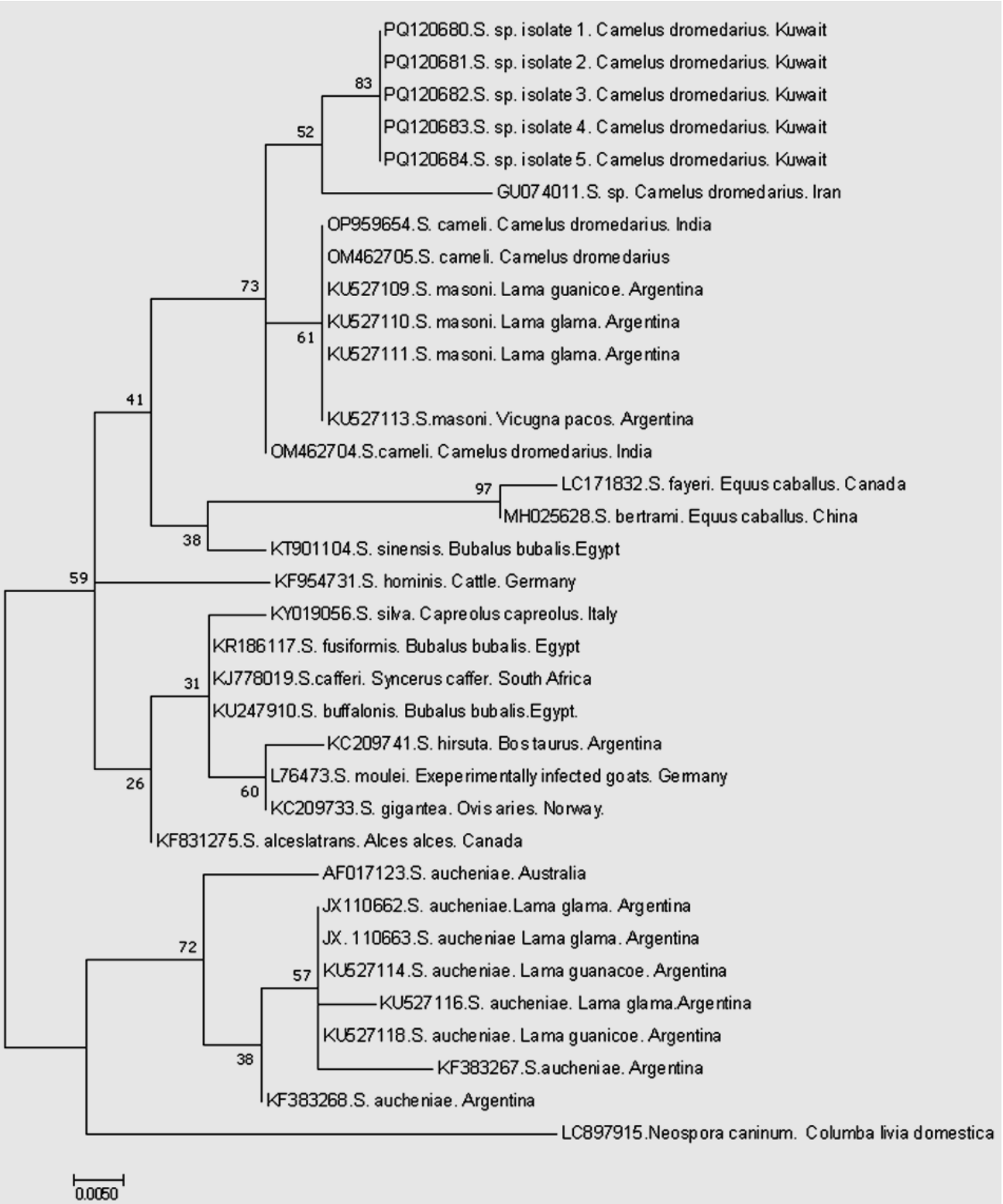


Figure 4. Maximum likelihood algorithm of the phylogenetic relationship between the obtained sequences (isolates 1-5) of *Sarcocystis* species and other sequences from GenBank (NCBI) based on 18S rRNA.

DISCUSSION

The present study is one of the initial investigations about the prevalence of *Sarcocystis* infection among camels in Kuwait. No macroscopic cysts were observed during the gross examination of the camel carcasses, in contrast to the findings reported previously in Iraq by Dubey et al. (2017).

In the present study, 194 of the 270 camels examined were positive for *Sarcocystis* infection by pepsin digestion, yielding a relatively high prevalence of 71.85%. This finding was consistent with the prevalence reported by Gareh et al. (2020). Additionally, high prevalence rates have been reported in previous studies using different diagnostic techniques,

including light microscopy, transmission electron microscopy, and histopathological examination (Valinezhad et al., 2008; Rabie et al., 2021). Enzymatic digestion methods, such as pepsin and trypsin, are reported to be more sensitive than muscle squash preparations or histological methods. This increased sensitivity might be due to their ability to examine larger tissue samples and effectively release bradyzoites from sarcocysts (Saeed et al., 2018a).

The prevalence of infection was higher in males (74.3%) than in females (62.5%), consistent with previous reports from Egypt (Gareh et al., 2020; Rabie et al., 2021). The higher number of infected male camels can be due to the higher number of males slaughtered and included in the study. The increased demand for tender camel meat could lead to more slaughtering of younger animals, particularly those aged between 8 months and 3 years. In the current study, camels aged between 8 months and 3 years demonstrated the highest infection rates (85.8%), indicating a notable association between age and *Sarcocystis* infection. In contrast, Shekarforoush et al. (2006) reported a higher prevalence among older animals, possibly due to longer exposure to infective oocysts or sporocysts.

Season was substantially associated with *Sarcocystis* infection, with a higher prevalence in the wet season (92.8%) than in the dry season (62.4%). This variation could result from differences in the number and traits of animals slaughtered across the two seasons. In Kuwait, the wet season is shorter than the dry season, which could influence the availability of animals for slaughter and affect the distribution of the examined samples.

Among the tissues examined, the esophagus had the lowest *Sarcocystis* infection rates, whereas the other tissues had relatively similar rates. Histopathological examination was useful for evaluating the size and shape of the sarcocysts. Saeed et al. (2018b) indicated that microscopic *Sarcocystis* cysts may differ in shape depending on the tissue, appearing as round, spherical, elongated, ellipsoid, spindle-shaped, or spiral forms. However, cyst shape alone is insufficient for species identification. Rabie et al. (2021) identified *S. cameli* and *S. ippeni*, exhibiting similar thin-walled cyst morphology. Factors including cyst age, location within the body, and tissue processing methods can affect the morphological features of sarcocysts. *Sarcocystis cameli* and *S. ippeni* can be differentiated based on the shape of their villar protrusions, which are finger-like at one end and conical at the other (Saeed et al., 2018a). Therefore, the morphology of the cyst wall is an important criterion for identifying and classifying *Sarcocystis* species. Dubey et al. (2015) stated that several species reported in camels, namely *S. camelicanis*, *S. camelocanis*, and *S. miescheri*, are invalid, with *S. camelicanis* considered similar to *S. cameli*.

Although *sarcocystosis* is generally considered asymptomatic or subclinical in camels, microscopic infection may be associated with pathological alterations. These pathological changes include pyrexia, anorexia, anemia, peripheral eosinophilia, hyperproteinemia, hyperglobulinemia, and hemorrhagic lesions affecting several organs, including the omentum, mesenteric lymph nodes, urinary bladder, myocardium, brain, lungs, and skeletal muscles. In the United States, eosinophilic myositis associated with natural *Sarcocystis* infection was reported in an alpaca that exhibited clinical signs including recumbency, hyperthermia, reduced responsiveness to external stimuli, dyspnea, marked muscle tremors, decreased milk production, and abortion. Furthermore, experimental infection of young alpacas with sporocysts resulted in reduced body weight gain and hematocrit levels compared to uninfected controls, along with a high mortality rate (Saeed et al., 2018 a,b). These observations indicated that microscopic *Sarcocystis* infection could have pathological implications under specific conditions.

It has been suggested that consuming raw or undercooked *Sarcocystis*-infected camelid meat may cause gastroenteritis due to exposure to sarcocyst-derived toxins, although no confirmed human cases have been reported. Experimental studies indicated that boiling, grilling, or frying infected meat inactivated the toxin, as assessed in rabbits inoculated with sarcocyst homogenates. In contrast, while charqui preparation prevented parasite transmission to dogs, it did not completely eliminate the toxic effects of sarcocysts in rabbits. Additionally, data on the impact of sarcocystosis on productivity parameters in Old World camels are scarce (Wieser et al., 2024).

Previous molecular investigations have used mitochondrial cytochrome c oxidase subunit I (*cox1*) sequences to detect *Sarcocystis* in Saudi Arabia (Metwally et al., 2020), Iraq (Aggar et al., 2023), and India (Narnaware et al., 2024). The 18S rRNA gene has been utilized to molecularly characterize *Sarcocystis* isolates from camels in Iran (Motamedi et al., 2011), Saudi Arabia (Omer et al., 2017), Egypt (Rabie et al., 2021), and India (Narnaware et al., 2024). In a recent study, Wieser et al. (2024) used 18S rRNA gene sequences to construct a phylogenetic tree that included *Sarcocystis* species from New World and Old World camelids. Wieser et al. (2024) found that *S. masoni* and *S. cameli* grouped within the same clade, clearly separated from *S. aucheniae*, which aligns with the present findings, as the Kuwaiti isolates clustered with *S. masoni*, whereas *S. aucheniae* appeared more distantly related. These results indicated a strong genetic connection between the isolates identified in the present study and those reported by Wieser et al. (2024) for *S. cameli* and *S. masoni*.

The 18S rRNA fragment obtained in the present study was 655 bp, compared with fragments of 539 bp and 610 bp reported in studies from India and Iran, respectively. Sequence identities between the Kuwaiti isolates and those from Iran ranged from 98.4% to 98.67%, indicating a strong genetic connection. The transportation and importation of camels

across countries can facilitate the spread of genetically related *Sarcocystis* isolates. However, the current data did not identify the exact source of infection.

The discrepancies in prevalence, histopathological findings, and molecular characteristics between the present study and those from other countries (Fukuyo et al., 2002; Valinezhad et al., 2008; Metwally et al., 2020) could be explained by several factors, including differing diagnostic techniques, geographical settings, tissue selection, age of animals, sample sizes, herd management, sanitation, and the occurrence of definitive hosts, especially dogs (Saeed et al., 2018a). These factors are important to consider when comparing the epidemiology and molecular characteristics of *Sarcocystis* infection across different camel populations.

CONCLUSION

A high prevalence of microscopic *Sarcocystis* infection in camels was detected by pepsin digestion, whereas no macroscopic cysts were observed during gross examination. Histopathological analysis characterized sarcocyst morphology. Molecular analysis of the 18S rRNA gene revealed that the Kuwaiti isolates were genetically closely related to previously reported *Sarcocystis* isolates, especially those in Iran. Since the 18S rRNA gene is relatively conserved among closely related *Sarcocystis* species, relying on this marker alone may not yield reliable species-level identification. Further studies incorporating additional variable molecular markers, such as cox1, alongside comprehensive morphological and ultrastructural characterization, are required to attain more precise identification of *Sarcocystis* species infecting camels in Kuwait.

DECLARATIONS

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Author's contributions

Ameen Ashour, Hoda Taha, and WessamYoussef conceived, supervised, and designed the study. Hoda Taha wrote and drafted the manuscript. Adawia Henedi and Abdolhossein Dalimi conducted the investigation and performed the formal analysis. All authors reviewed and approved the final edition of the manuscript.

Availability of data and materials

The data supporting the findings of this study are accessible from the first and corresponding authors upon reasonable request. The nucleotide sequences of the 18S rRNA obtained have been deposited in GenBank under accession numbers (PQ120680-PQ120684).

Competing interests

The authors declared no potential conflicts of interest with respect to the study, authorship, and/or publication of this article.

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 affirmed that no artificial intelligence tools were used in the writing and preparation of the manuscript. The authors accept full responsibility for the originality of the data both prior to submission and throughout the peer review process.

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Physicochemical and Microbiological Characteristics of Processed Goat Milk with Lontar Fruit Using Different Types and Levels of Sweeteners

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ABSTRACT

Fresh goat milk is perishable and has a distinctive flavor that may reduce consumer acceptance. Incorporating lontar fruit and sweeteners such as honey and stevia may improve its physicochemical properties. The present study aimed to investigate the effects of different sweetener types and concentrations on the characteristics of goat milk supplemented with lontar fruit puree. A completely randomized design was employed, comprising seven treatments, including a control group with 5% sucrose, honey at three concentrations (2%, 4%, 6%), and stevia at three concentrations (0.05%, 0.1%, 0.15%), each with four replicates. Antioxidant activity, antibacterial activity, degree of acidity (pH), viscosity, and color values, including brightness (L*), redness (a*), and yellowness (b*), were evaluated. Honey-containing treatments demonstrated higher viscosity and lower pH than stevia-containing treatments, while the highest antioxidant activity was observed in the 0.15% stevia treatment (33.80%). No microbiological inhibition was observed against *Escherichia coli* and *Staphylococcus aureus*. The inclusion of honey in goat milk supplemented with lontar fruit puree resulted in higher viscosity than treatments containing stevia. Formulations with milk and honey had a lower pH than those with stevia. The 0.05% stevia treatment had the highest L* value (brightest color) and a negative a* value (a greener hue), while honey increased the intensity of red and yellow coloration with increasing concentration. The 0.15% stevia treatment demonstrated the highest antioxidant activity and favorable physicochemical characteristics.

Keywords: Goat milk, Lontar fruit, Microbiological characteristic, Physicochemical characteristic, Sweetener

INTRODUCTION

Goat milk is a nutrient-rich substance comprising proteins, fats, minerals, and other nutritional constituents that enhance its nutritional value (Nayik et al., 2021). Additionally, goat milk contains short- and medium-chain fatty acids, including caproic, caprylic, and capric acids, which contribute to its distinctive flavor and are readily digested and absorbed (Nayik et al., 2022). Furthermore, goat milk contains different bioactive components associated with potential health benefits (Verruck et al., 2019). However, fresh goat milk is highly perishable and prone to spoilage, and its characteristic flavor can limit consumer acceptance. Fresh goat milk is highly perishable and can spoil rapidly due to bacterial growth, which may compromise its quality during storage (Razali et al., 2021). Therefore, appropriate heat treatment is necessary to reduce microbial contamination and maintain the microbiological quality and safety of goat milk during storage (Prasantha and Wimalasiri, 2019).

Against this background, the development of dairy products combining appropriate heat treatment with natural ingredients may provide an approach to improving product quality, stability, and functional properties. Incorporating natural ingredients such as fruit purees, juices, and plant extracts not only improves sensory qualities but also increases levels of bioactive compounds, including polyphenols, flavonoids, and carotenoids, which may offer health benefits (Prestes et al., 2022).

One of the indigenous natural ingredients with potential for the development of processed dairy products is the lontar fruit (*Borassus flabellifer* L.; Rahman et al., 2023). Lontar fruit is known to contain several bioactive compounds, including flavonoids, saponins, tannins, and phenolic compounds, with potential antioxidant and antibacterial activities. In addition, lontar fruit contains protein, fat, and micronutrients such as calcium and phosphorus (Mary and Jasmin, 2022). These compounds help prevent food spoilage by inhibiting oxidation in fats and proteins (Rahman et al., 2023). Konay et al. (2019) reported that ethanol extract of lontar fruit exhibited antibacterial activity against the growth of *Staphylococcus aureus* (*S. aureus*). Rahman et al. (2023) demonstrated that the addition of lontar fruit to goat milk products can increase antioxidant activity and affect the physical characteristics of the product.

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Excessive use of sucrose is currently a concern due to its association with increased risk of obesity, diabetes, and other cardiometabolic diseases (Malik and Hu, 2022). The increasing public awareness regarding healthy lifestyles is motivating consumers to choose food products that incorporate natural sweeteners, such as honey and stevia. These natural sweeteners may provide potential health benefits beyond their primary role as sweeteners (Schiatti-Sisó et al., 2023). Dias (2023) stated that increasing consumer awareness of the negative impacts of excessive sugar consumption has increased the demand for healthier natural sweeteners.

Honey contains high levels of simple sugars, particularly fructose (35.6-41.8 g/100 g) and glucose (25.4-28.1 g/100 g), as well as several minor components, including vitamins, minerals, flavonoids, phenolic compounds, and enzymes, such as diastase, invertase, glucose oxidase, and catalase (Alaerjani et al., 2022; Al-Kafaween et al., 2023). These bioactive components, particularly phenolic compounds, flavonoids, and enzymes, contribute to the antioxidant and antibacterial properties of honey. Almasaudi (2021) stated that the antibacterial activity of honey comes from its high sugar content, polyphenols, hydrogen peroxide, and bee defensin-1. In addition, honey has a distinctive aroma and flavor that may enhance the product's sensory characteristics. Its elevated sugar and total solids content are correlated with its viscosity and may impact the viscosity of the resulting dairy beverage (Evahelda et al., 2017).

Stevia is a natural, low-calorie sweetener that is approximately 150 to 450 times sweeter than sucrose (Peteliuk et al., 2021). Stevia comprises steviol glycosides, mainly stevioside and rebaudioside A, which exhibit adequate stability at elevated temperatures and across a wide pH range, thereby supporting their use in food products subjected to high-temperature processing (Satar-AlDavdi, 2022). In addition to providing sweetness, stevia contains bioactive compounds such as flavonoids, phenolics, tannins, and ascorbic acid, which have potential antioxidant and antibacterial properties (Ahmad et al., 2020; Chakma et al., 2023). Furthermore, stevia is considered safer for diabetics and consumers on low-sugar diets because it is non-caloric (Papaefthimiou et al., 2023). Therefore, the present study aimed to examine the physicochemical and microbiological characteristics of processed goat milk supplemented with lontar fruit, with honey and stevia added as natural sweeteners, as alternatives to sucrose.

MATERIALS AND METHODS

Ethical approval

The present study did not involve human participants or animals; therefore, ethical approval was not required. Additionally, the final product was not consumed by any living humans, animals, or other organisms. The study was conducted in accordance with the guidelines established by Hasanuddin University, Indonesia.

Experimental design

The main ingredients used in this study were goat milk obtained from Al-Fatih Farm, Gowa, Indonesia, fresh lontar fruit (*Borassus flabellifer* L.) purchased from a local market in Makassar, Indonesia, Uray® commercial honey (PT Rejeki Tujuh Alam, Indonesia); Stetes® commercial stevia (PT Ananta Hira Boga Pratama, Indonesia), and commercial granulated sucrose purchased from a local market in Makassar, Indonesia. The present study employed a Completely Randomized Design (CRD) with seven treatments and four replicates.

Product preparation and treatment groups

Fresh goat milk served as the base at 100%, with lontar fruit puree incorporated at 10% (w/v). The treatment groups comprised natural sweeteners at different concentrations, expressed as a percentage of the total volume (w/v). The control group (C) received 5% commercial granulated sucrose purchased from a local market in Makassar, Indonesia. For the stevia treatments, the sweetener was added at concentrations of 0.05% (S0.05), 0.1% (S0.1), and 0.15% (S0.15). For the honey treatments, honey was added at 2% (H2), 4% (H4), and 6% (H6). After adding the sweeteners, each combination was autoclaved (Tomy SX-500, Japan) at 105°C for five minutes. The heated products were then cooled to room temperature over 30 minutes.

Preparation of lontar fruit stock

The mature lontar fruit was utilized. The inner skin layer (mesocarp) was initially peeled, and the flesh (endosperm) was blended until smooth. Subsequently, lontar fruit puree was incorporated at a concentration of 10% (v/v), based on the volume of goat milk (Rahman et al., 2023).

Antioxidant activity

A volume of 3.9 mL of 75 µM 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution prepared in methanol was combined with 100 µL of the methanol extract. The mixture was incubated for 30 minutes, after which its absorbance was recorded

at 517 nm. The percentage of antioxidant activity was determined using the DPPH assay according to Formula 1 (Hafeez et al., 2025).

$$Free\ radical\ scavenging\ activity(\%) = \frac{\text{Absorbance of reagent blank} - \text{Absorbance of sample}}{\text{Absorbance of reagent blank}} x\ 100\% \quad \text{(Formula 1)}$$

Antibacterial activity

The antibacterial activity was assessed through the well diffusion assay against *Escherichia coli* (*E. coli*) and *S. aureus*. Samples were placed in wells prepared on nutrient agar and incubated at 37°C for 24-48 hours. The diameter of the inhibition zone was measured five times for each well, and the mean value was used for calculation according to Formula 2 (Maruddin et al., 2019).

Antibacterial activity = Inhibition zone area - Well area (Formula 2)

Zone area = π.r^2 (r = radius of inhibition zone)

Well area = π.r^2 (r = radius of ring)

where π (constant) was 3.14, the ring diameter was 0.83 mm, and r (radius) was calculated as 1/2 × the diameter.

Degree of acidity (pH)

Prior to measuring the samples, the pH meter was calibrated using standard buffer solutions at pH 7.00 and pH 4.01. After calibration, the pH of each sample was measured using the calibrated pH meter (Liu et al., 2024).

Viscosity

An Ostwald viscometer (Pyrex®, USA) was used to determine viscosity based on the flow-time method (Thakur and Wadhwa, 2017; Formula 3). The flow time of the sample between the two calibrated marks of the U-shaped capillary tube was recorded. The same procedure was performed using water as the reference liquid (Thakur and Wadhwa, 2017). $\eta = \eta_0 \times \frac{d \cdot t}{d0 \cdot t0}$ (Formula 3)

where η was the viscosity of the sample (cP), η0 was the viscosity of water (cP), d was the density of the sample (g/mL), d_0 is the density of water (g/mL), t was the flow time of the sample (seconds), and t_0 was the flow time of water (seconds).

Color

Color was measured using the CIE Lab color space. In this system, the L* value represented lightness and ranged from 0 (black) to 100 (white), while the a* and b* values ranged from −120 to +120. Positive a* indicated redness and negative a* indicated greenness, whereas positive b* indicated yellowness and negative b* indicated blueness. Hue angle and chroma were calculated from these values. Measurements were performed using a colorimeter (TES-135A, TES Electrical Electronic Corp., Taiwan; Minz and Saini, 2021).

Data analysis

Data obtained in the present study were analyzed using analysis of variance (ANOVA) under a completely randomized design (CRD) to evaluate the effects of different types and levels of natural sweeteners on the observed parameters. When significant differences were identified, Duncan's multiple range test was employed at a significance level of p value less than 0.05 ($p < 0.05$). All statistical analyses were conducted using SPSS, version 25.

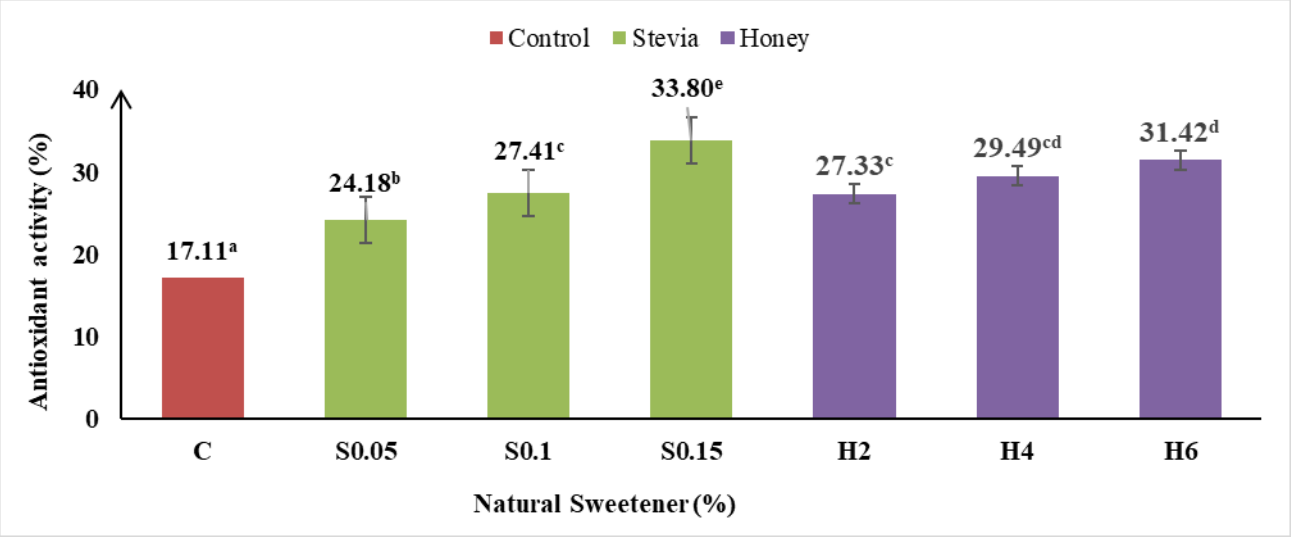
RESULTS

Antioxidant activity

The types and levels of natural sweeteners significantly affected the antioxidant activity of the product ($p < 0.05$; Graph 1). Compared with the control treatment, the addition of honey and stevia generally resulted in higher free-radical inhibition, with inhibition values ranging from 17.11% to 33.80%. The lowest antioxidant activity was observed in the control group (17.11%), whereas the highest inhibition value was recorded in the S0.15 treatment group at 33.80%.

Antibacterial activity

The antibacterial activity results demonstrated that all treatments, whether using honey or stevia at different concentrations, did not exhibit any inhibitory effect against *E. coli* and *S. aureus*. The absence of antibacterial activity was indicated by the absence of an inhibition zone (clear zone) around the sample area on the growth medium. Honey and stevia did not function as antibacterial agents in the goat milk supplemented with lontar fruit puree. As shown in Figure 1, honey and stevia did not function as antibacterial agents in the goat milk supplemented with lontar fruit puree.



Graph 1. Antioxidant activity of goat milk products supplemented with lontar fruit and different concentrations of stevia and honey as natural sweeteners. [a,b,c] Mean different superscript letters indicate a significant difference ($p < 0.05$). C: Control (5% sucrose), S: Stevia, H: Honey.

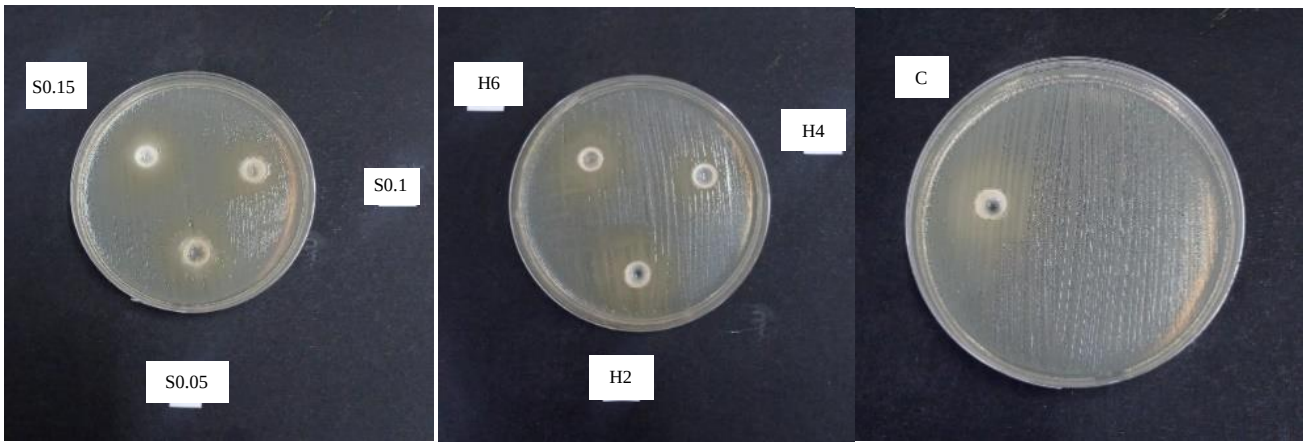


Figure 1. Antibacterial activity of goat milk products supplemented with lontar fruit and different concentrations of stevia and honey as natural sweeteners. C: Control (5% sucrose), S0.05: Stevia 0.05%, S0.1: Stevia 0.1%, S0.15: Stevia 0.15%, H2: Honey 2%, H4: Honey 4%, H6: Honey 6%.

Degree of acidity (pH)

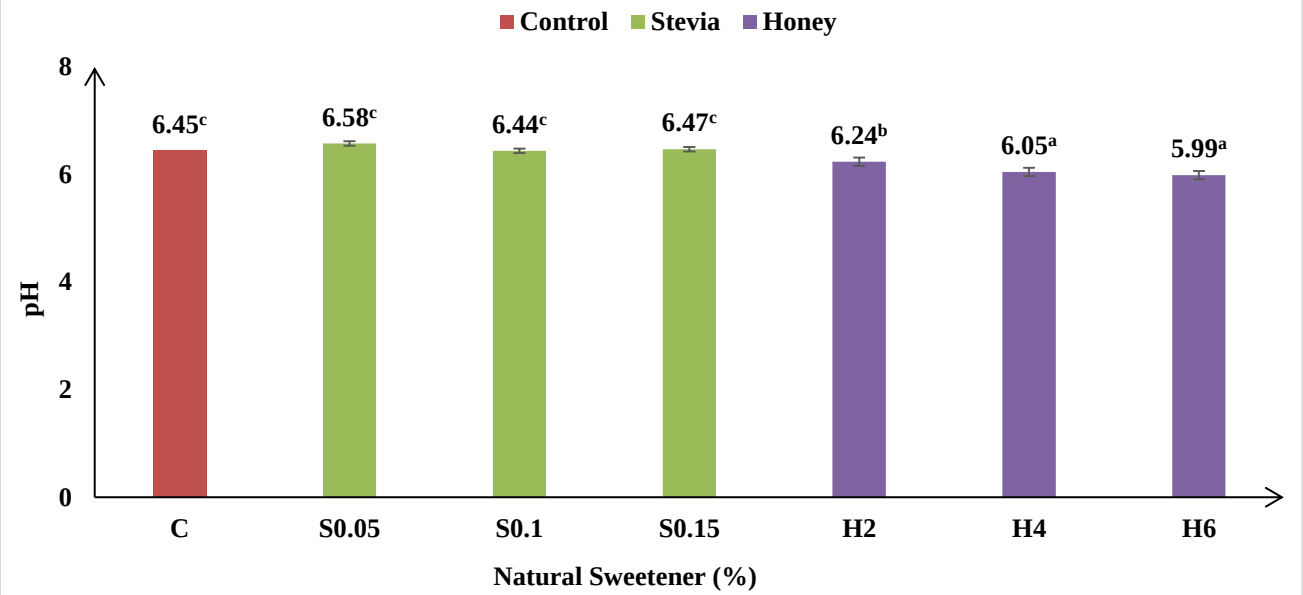
Different concentrations of stevia and honey significantly affected the pH of goat milk supplemented with lontar fruit puree ($p < 0.05$; Graph 2). The present results indicated that the average treatment values ranged from 5.99 to 6.58, with the highest value observed in the S0.05 treatment group (6.58) and the lowest pH in the H6 treatment group (5.99). The pH values for the control and all stevia treatment groups were within the normal range for fresh goat milk, which is 6.4-6.7 (Benyahia et al., 2025).

Viscosity

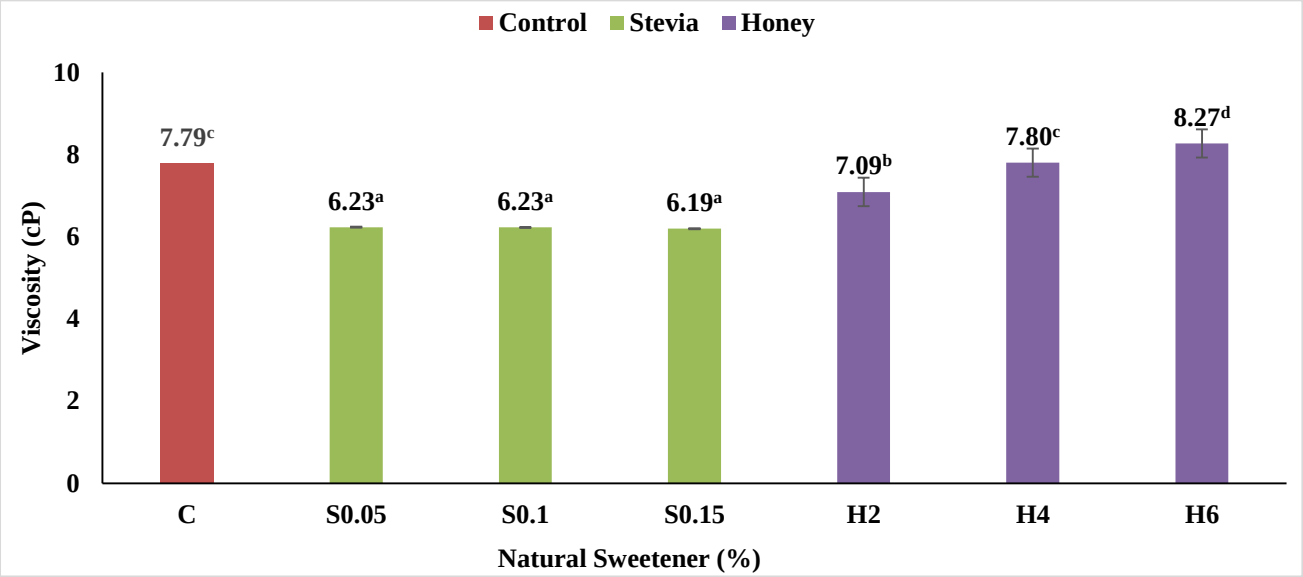
Different concentrations of stevia and honey significantly affected the viscosity of goat milk supplemented with lontar fruit puree ($p < 0.05$; Graph 3). Viscosity values ranged from 6.19 cP to 8.27 cP. The highest viscosity was found in the H6 treatment group at 8.27 cP, while the lowest viscosity was observed in the S0.15 treatment group at 6.19 cP.

Color

The current results indicated that the type and concentration of natural sweeteners significantly affected the color values of goat milk supplemented with lontar fruit puree ($p < 0.05$; Table 1). Adding honey reduced the L* value (brightness) as its concentration increased. Similarly, higher stevia levels decreased the L* value. The a* value (redness/greenness) for all treatments was negative, indicating a tendency toward green color. Stevia reduced the a* value (greener) with increasing its concentration, whereas honey increased the a* value (more red) with increasing the honey concentration. The b* value (yellowness/blueness) was positive across all treatments, indicating a yellowish color, with stevia producing lower b* values (3.65-4.15) than honey, which yielded higher b* values (7.71-9.89).



Graph 2. The acidity level (pH) of goat milk products supplemented with lontar fruit and different concentrations of stevia and honey as natural sweeteners. [a,b,c] Mean different superscript letters indicate a significant difference ($p < 0.05$). C: Control (5% sucrose), S: Stevia, H: Honey.



Graph 3. Viscosity of goat milk products supplemented with lontar fruit and different concentrations of stevia and honey as natural sweeteners. [a,b,c] Mean different superscript letters indicate a significant difference ($p < 0.05$). C: Control (5% sucrose), S: Stevia, H: Honey.

Table 1. Color values (L*a*b) of goat milk supplemented with lontar fruit and different concentrations of stevia and honey as natural sweeteners

Treatment groups	L* (Lightness)	*a (Redness/Greenness)	b* (Yellowness/Blueness)
C	77.75 ± 0.93^{bc}	-5.31 ± 0.58^{a}	3.90 ± 0.25^{a}
S0.05	81.30 ± 1.34^{e}	-5.49 ± 0.84^{a}	3.65 ± 0.73^{a}
S0.1	80.70 ± 0.54^{e}	-5.85 ± 0.13^{a}	3.85 ± 0.87^{a}
S0.15	80.05 ± 0.86^{e}	-6.05 ± 1.22^{a}	4.15 ± 1.87^{a}
H2	78.92 ± 0,29^{cd}	-3.65 ± 0,55^{b}	7.71 ± 0,28^{b}
H4	76.29 ± 1.59^{b}	-3.23 ± 0.50^{bc}	8.49 ± 1.10^{bc}
H6	74.54 ± 1.48^{a}	-2.44 ± 0.07^{c}	9.89 ± 0.55^{c}

[a,b,c] Mean different superscript letters within each column indicate significant differences ($p < 0.05$). C: Control group, S: Stevia, H: Honey

DISCUSSION

Antioxidant activity

Stevia exhibited superior antioxidant activity compared to the honey treatments and the control group containing 5% commercial granulated sucrose. The superior antioxidant activity of stevia was demonstrated by the DPPH free-radical inhibition value, which reached 33.80% in the 0.15% stevia treatment group, compared with 31.42% in the 6% honey treatment and 17.11% in the control group. The high antioxidant activity observed in the stevia treatment might be due to bioactive compounds present in *Stevia rebaudiana* leaves. Papaefthimiou et al. (2023) and Ibrahim et al. (2024) reported that phenolic compounds, flavonoids, and vitamin C (ascorbic acid) in stevia act as natural antioxidants. The use of stevia as a natural sweetener has been reported in different food and beverage products, including jelly candy, functional okra-ginger beverages, and green tea kombucha (Amelia et al., 2019; Apriyanto et al., 2020; Rukman and Haerussana, 2023). Stevia exhibited higher free-radical-scavenging activity than honey, as indicated by the DPPH assay results (Rukman and Haerussana, 2023). This difference in antioxidant activity among natural sweeteners can be attributed to the presence of steviol glycosides and phenolic compounds in stevia, which may enhance its antioxidant properties (Eliwa and Ahmed, 2025). Peasura and Sinchaipanit (2022) reported that stevia at low concentrations increased antioxidant activity in guava nectar. The addition of 0.05% stevia resulted in the highest free radical-scavenging activity, as measured by the DPPH assay, compared with the other guava nectar formulations. The higher antioxidant activity was attributed to the phenolic and other bioactive compounds present in stevia, which can act as natural antioxidants (Peasura and Sinchaipanit, 2022). Furthermore, the antioxidant activity tended to increase proportionally with increasing honey concentration. The inhibition value increased from 27.33% in 2% honey to 31.42% in 6% honey. This increase demonstrated that honey has potential as a natural antioxidant source in food products. Septiana et al. (2021) indicated that the primary compounds responsible for this protective activity in honey are phenolic and flavonoid compounds. Phenolic and flavonoid compounds are the main compounds acting as antioxidants. These compounds serve as natural antioxidants by donating hydrogen atoms or electrons to unstable molecules (Zeb, 2020). The phenolic content of honey is positively associated with its antioxidant activity, with higher phenolic content corresponding to greater free-radical scavenging activity as determined by the DPPH assay (Abdul, 2021).

Antibacterial activity

The absence of an inhibition zone observed in the honey group could be attributed to the dilution of honey within the milk. This dilution may have reduced the concentration and efficacy of honey antimicrobial activity, thereby weakening its inhibitory activity against the tested bacteria (Luca et al., 2024). Honey contains natural antibacterial components, including the enzyme glucose oxidase, which plays an important role in generating hydrogen peroxide, a key antimicrobial agent in honey. The heating process could diminish the antimicrobial efficacy of honey by impacting the activity of glucose oxidase, the production of hydrogen peroxide, and the content of defensin-1, thereby compromising its antibacterial effects (Bucekova et al., 2018; Mat Ramlan et al., 2021). The reduced antimicrobial activity observed in the present study may be attributable to the availability of nutrients in the milk, including protein, lactose, and fat, which can support the growth of *E. coli* and *S. aureus*. The observed reduction in antibacterial activity in the present study was consistent with the findings of Rababah et al. (2024), who reported that heating can affect the physicochemical properties and biological activity of honey. Although stevia exhibited greater thermal stability than honey, the incorporation of stevia in the current antibacterial assay did not produce an inhibition zone. Stevia contains bioactive compounds such as flavonoids, alkaloids, and tannins, which may compromise the integrity of microbial cell membranes (Singh et al., 2024a). However, the stevia concentrations used in the present study (0.05%, 0.1%, 0.15%) may not have reached the minimum inhibitory concentration required to effectively suppress bacterial growth. The absence of an inhibition zone indicated that, although steviol glycosides are heat-stable and can remain stable within the applied thermal treatment range (Singh et al., 2024b), their concentration was inadequate to effectively suppress bacterial metabolism in milk media. Ahmed et al. (2025) reported that the antimicrobial activity of *Stevia rebaudiana* leaf extract was concentration-dependent, with higher concentrations of bioactive compounds producing stronger inhibitory effects against bacteria. The concentration-dependent antimicrobial activity reported by Ahmed et al. (2025) is relevant to the present study, as the relatively low stevia concentrations used (0.05%, 0.1%, and 0.15%) may have resulted in an insufficient concentration of bioactive compounds to produce a detectable inhibition zone.

Degree of acidity

Stevia supplementation resulted in relatively stable pH values, ranging from 6.44 to 6.58. The pH values of the stevia treatments did not differ from that of the control group (pH 6.45), indicating that the addition of stevia did not notably alter the pH of the final product. The stability of stevia pH values could be attributed to the low concentration of

stevia used as a non-nutritive sweetener, while its major compounds, stevioside and rebaudioside, remained stable across different pH levels and temperatures. The high stability of steviol glycosides across different pH levels and temperature conditions has been documented by Chughtai et al. (2025), suggesting that stevia is unlikely to notably alter the chemical properties of food products. The high thermal stability of steviol glycosides prevents heat reactions from generating organic acid byproducts; therefore, the inclusion of stevia in goat milk supplemented with lontar fruit maintained the milk pH as stable and within the normal range of fresh milk. In contrast to stevia, honey supplementation exhibited a decline in pH value with increasing concentration, from 6.24 at 2% to 6.05 at 4%, then to 5.99 at 6% honey inclusion level. Honey has a naturally acidic pH, typically ranging from 3.2 to 4.5, and its incorporation can increase the final product's acidity (Gadge et al., 2024; Inaudi et al., 2025). The acidic nature of honey is consistent with the present results, as the increasing honey concentration progressively decreased the pH of the product from 6.24 to 5.99. The greater amount of acidic honey added to the product likely contributed to the increased acidity and consequently the lower pH observed at higher honey concentrations. The reduction in pH value might be due to the natural organic acid content of honey, particularly gluconic acid, formed by the oxidation of glucose by the enzyme glucose oxidase. Goat's milk exhibits strong resistance to pH changes due to its protein, particularly casein, phosphate, and mineral components, all of which remarkably enhance its buffering capacity (Zakaria et al., 2020). Despite exposure to high-temperature heating, the pH of the product remained relatively stable, which could be attributed to the high buffering capacity of goat's milk.

Viscosity

The higher viscosity observed in the honey treatment groups might be attributed to the high content of simple sugars, particularly fructose and glucose, which are important contributors to honey's rheological properties (Oroian et al., 2014; Faustino and Pinheiro, 2021). Fructose and glucose are the predominant sugars in honey and contribute substantially to its total soluble solids content. Higher sugar concentrations typically increase viscosity, as elevated dissolved sugar levels can impede molecular motion and reduce fluid flow. Faustino and Pinheiro (2021) reported that the rheological properties of honey are strongly influenced by its glucose and fructose contents. The high concentrations of these sugars can increase the viscosity of honey by increasing the concentration of dissolved solids and restricting the mobility of water molecules, thereby reducing fluid flow. This mechanism is consistent with the present study, in which increasing honey concentration resulted in a progressive increase in viscosity from 7.09 cP at 2% honey to 7.80 cP at 4% and 8.27 cP at 6% honey. Similarly, Anwar et al. (2021) reported that increasing honey concentration in kefir increased viscosity, attributed to honey's high carbohydrate content and its ability to bind water. A similar pattern was observed in the present study, where increasing honey concentration resulted in a progressive increase in viscosity from 7.09 cP at 2% honey to 7.80 cP at 4% and 8.27 cP at 6% honey. Greater water binding by carbohydrates can reduce the mobility of water molecules, thereby increasing product viscosity. Stevia is a non-nutritive sweetener characterized by a very high level of sweetness and is therefore used at very low concentrations in the present study. Consequently, the incorporation of stevia did not impact the product's viscosity as much as honey or table sugar, which have high sugar content. Astuti et al. (2021) indicated that stevia, particularly the extract from *Stevia rebaudiana* leaves, did not contain high concentrations of simple sugars such as glucose, sucrose, maltose, or fructose, and therefore did not affect the total dissolved solids of the product. The low contribution of simple sugars from stevia is consistent with the limited change in viscosity observed in the present study, where viscosity remained nearly unchanged at 6.23, 6.23, and 6.19 cP with the addition of 0.05%, 0.10%, and 0.15% stevia, respectively. The low concentration of stevia and its limited contribution to dissolved solids may therefore account for its minimal effect on product viscosity.

L* Color

The addition of honey tended to decrease the L* value as its concentration increased. The L* values decreased from 78.92 at 2% honey to 76.29 at 4% honey and 74.54 at 6% honey, indicating a gradual reduction in lightness. The decrease in L* value may be attributed to the natural yellow-to-brown color of honey and the formation of brown pigments through Maillard reactions, which could contribute to darker product coloration during heating (Starowicz et al., 2021). The observed decrease in L* value was consistent with the findings of Anwar et al. (2025), who reported that increasing honey concentration tended to decrease L*. In addition, Mercan and Akın (2017) reported that adding honey increased total phenolic content and reduced L* values, resulting in darker yogurt coloration, which is consistent with the present findings. Conversely, the addition of stevia resulted in higher L* values than the addition of honey and the 5% commercial granulated sucrose, indicating a lighter product color. The lighter color might be due to the absence of reducing sugars in steviol glycosides, which limited the potential for non-enzymatic browning reactions during heating. Consequently, stevia was less likely to induce browning through Maillard reactions or caramelization of sugars than sweeteners containing glucose and fructose. The current findings were consistent with those of Sulistyawati et al. (2024),

who reported that stevia had a lower potential to participate in Maillard reactions than sucrose-based sweeteners. The lower potential of stevia to participate in Maillard reactions may have contributed to the higher L* values observed in the stevia treatments in the present study by limiting brown pigment formation during heating. Similarly, Shahu et al. (2023) discovered that stevia leaf extract in water notably reduced the formation of Maillard reaction products, supporting the lighter color observed in the stevia treatments compared with the honey and sucrose treatments in the present study. However, as the stevia concentration increased, the L* value decreased. This finding was consistent with that of Rungraeng et al. (2022), who reported that the lightness value decreased with increasing levels of steviol bioside (the main component of stevia). The decrease in L* with increasing stevia concentration was further supported by the findings of Buniowska et al. (2020) and Roy et al. (2025), who indicated that the coloration of stevia extract was derived from natural pigments, including tannins and phenolic compounds, and that increasing the concentration of the extract could reduce clarity and increase turbidity, consequently leading to a reduction in the lightness value (L*).

a* Color

All treatments exhibited negative a* values, indicating that the product color was predominantly within the green spectrum. The stevia treatment groups exhibited lower (more negative) a* values than the other treatment groups, indicating a greater intensity of greenish color in the product. The change to a greener hue might result from natural compounds in stevia extract, such as chlorophyll, polyphenols, and flavonoids, which can contribute to the product's greenish appearance. The current findings are consistent with Maharika et al. (2024), who reported that increasing the concentration of stevia leaves resulted in progressively darker green coloration. This color alteration might be attributed to non-sugar compounds in stevia leaf extract, particularly chlorophyll, the primary contributor to the green color, and flavonoids, which may influence the resulting greenish-to-brownish coloration. Conversely, in the honey treatment, the a* value increased from -3.65 and -3.23 to -2.44, indicating that the product color became less green and shifted toward red as the honey concentration increased. This color shift toward red could be attributed to the natural pigments in honey and to the formation of reddish-brown compounds from the Maillard reaction during heating. The increase in the a* value was consistent with the findings of Anwar et al. (2025), who investigated probiotic yogurt beverages produced from different milk sources with varying honey concentrations and reported an increase in redness (a*) with increasing honey concentration.

b* Color

The b* values increased with higher stevia concentrations; however, the b* values in stevia treatment groups remained comparatively low compared to other treatment groups, resulting in a less yellow product color. Maharika et al. (2024) reported that the substitution of stevia leaf powder in aloe vera dodol increased the b* value, which might be related to the presence of β-carotene and carotenoids in stevia leaves that contribute to red, orange, and yellow coloration. A similar increase in b* value was observed in the present study as the stevia concentration increased, although the b* values remained relatively low compared with the honey and control treatments. The increase in b* value may be associated with the contribution of pigments present in stevia leaves, including β-carotene and carotenoids. In addition, quercetin 3-β-rutinoside, a flavonoid glycoside found in stevia, may contribute to the yellow coloration. The relatively low b* values observed in the stevia treatment groups, compared with those in the honey and control treatment groups, may be attributed to the predominance of chlorophyll pigments in the stevia extract. Chlorophyll pigments impart a green coloration, which may diminish the true intensity of yellow. Phenolic and flavonoid compounds present in stevia may also influence light absorption, resulting in less pronounced color intensity. Habsari et al. (2026) reported that the predominance of chlorophyll can mask yellow coloration, thereby leading to lower b* values. The relatively low b* values observed in the stevia treatments in the present study are consistent with the reported effect of chlorophyll, as the green pigment may have masked the yellow coloration. Furthermore, Indiarto et al. (2024) reported that phenolic and flavonoid compounds can influence light absorption, thereby reducing color brightness and producing a darker or more subdued appearance. The presence of these compounds in stevia may also have contributed to the relatively low b* values observed in the present study. The honey treatment groups exhibited higher b* values than the other treatment groups. The b* value increased from 7.71 to 8.49 and 9.89 as the honey concentration increased, indicating a progressively more yellow product color. The increase in b* value may be attributed to natural pigments in honey, including carotenoids, as well as the formation of yellow to brown compounds through non-enzymatic browning reactions, such as the Maillard reaction, during heating. The observed color changes in the present study were consistent with the findings of Brudzynski (2023), who stated that the concentration of pigmented compounds in honey, such as flavonoids, quinones, carotenoids, and Maillard reaction products, plays an important role in the formation of honey color, which can range from yellow, orange, and red to brown. Additionally, chemical reactions involving phenolic

compounds may contribute to color changes, particularly through the oxidation of polyphenols to semiquinones and quinones, which can produce yellow-to-brown coloration.

CONCLUSION

The present study demonstrated that incorporating natural sweeteners, particularly stevia at 0.15%, significantly enhanced the functional properties of goat milk supplemented with lontar fruit by increasing the antioxidant activity and maintaining favorable physicochemical characteristics, including pH. Although neither honey nor stevia exhibited antibacterial effects in the present study, stevia at 0.15% demonstrated potential as a natural sweetener to increase the antioxidant activity of goat milk supplemented with lontar fruit puree. Further studies should investigate a wider range of stevia concentrations and processing conditions and evaluate the physicochemical, antioxidant, microbiological, and sensory stability of goat milk supplemented with lontar fruit puree during storage.

DECLARATIONS

Acknowledgments

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Authors' contributions

Hilma Hamimah contributed to the methodology, data collection, data analysis, data interpretation, and manuscript preparation. Fatma Maruddin was responsible for study design, data interpretation, and manuscript review and editing. Ambo Ako contributed to manuscript review and editing. All authors have read and approved the final edition of the manuscript.

Availability of data and materials

All data obtained and analyzed in this study are included in the manuscript. No supplementary materials are available for this article. Further data may be requested from the corresponding author upon reasonable request.

Competing interests

The authors declare no conflicts of interest.

Ethical considerations

The authors confirm that the present study is original, has not been submitted elsewhere, and has been examined for misconduct and plagiarism prior to submission to the present journal. The authors accept full responsibility for the originality and integrity of this study before and after submission and publication. No artificial intelligence tools were used in conducting the study, including research design, data collection, laboratory analyses, or data analysis.

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Prevalence and Antimicrobial Resistance of *Streptococcus dysgalactiae* in Commercial Broiler Farms in Barishal, Bangladesh

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ABSTRACT

Streptococcus dysgalactiae (*S. dysgalactiae*) is a significant pathogen affecting veterinary and public health. However, data on its presence and antimicrobial resistance in poultry production in Bangladesh are scarce. The present study aimed to investigate the prevalence, identification methods, and antibiotic susceptibility profiles of *S. dysgalactiae* in poultry, water, and feed samples collected from poultry farms in the coastal regions of Barishal, Bangladesh. A total of 64 samples were randomly collected, including poultry samples (heart, intestine, feces) and farm-associated sources (water and feed). These samples were analyzed using conventional phenotypic methods, including culture characteristics, hemolytic activity on blood agar, Gram staining, and catalase testing. Antimicrobial susceptibility was determined by the Kirby-Bauer disk diffusion method. Molecular confirmation by polymerase chain reaction (PCR) was not performed. *S. dysgalactiae* was found in 26 (40.62%) samples, with a significantly higher occurrence in poultry samples (52.63%) compared to environmental samples (23.08%). Among poultry samples, intestinal specimens exhibited the highest isolation frequency. All isolates were resistant to ampicillin and penicillin, while moderate resistance was observed to amoxicillin, azithromycin, streptomycin, doxycycline, and oxytetracycline. Comparatively lower resistance rates were detected for ciprofloxacin, levofloxacin, and gentamicin. Multidrug resistance was identified among isolates collected from poultry and environmental samples. The current findings demonstrated the presence of multidrug-resistant *S. dysgalactiae* in poultry production systems in coastal Barishal and provided baseline epidemiological data for Bangladesh. The study highlighted the need for prudent antimicrobial use, stronger farm biosecurity, and continuous surveillance.

Keywords: Antibiotic resistance, Broiler chicken, Poultry, Prevalence, *Streptococcus dysgalactiae*

INTRODUCTION

Avian streptococcosis is an important bacterial disease of poultry caused by different *Streptococcus* species, including *Streptococcus dysgalactiae* (*S. dysgalactiae*; McDougall et al., 2014; Watanabe et al., 2016). Streptococci are Gram-positive, catalase-negative, chain-forming cocci that commonly inhabit the skin and mucosal surfaces of animals as opportunistic pathogens (De Vos et al., 2009). Under favorable conditions, *S. dysgalactiae* can cause septicemia and a range of systemic infections affecting the cardiovascular, reproductive, musculoskeletal, and ocular systems, leading to significant morbidity and mortality in poultry (Watanabe et al., 2016; Idalan et al., 2026). Of the more than 60 recognized Streptococcus species, only a few, such as *S. dysgalactiae*, have been identified in poultry, underscoring its growing significance in veterinary settings (McDougall et al., 2014; Watanabe et al., 2016).

S. dysgalactiae is traditionally classified into two subspecies, including *S. dysgalactiae* subsp. *dysgalactiae* and *S. dysgalactiae* subsp. *equisimilis*. *The subspecies dysgalactiae* typically demonstrates alpha-hemolysis or is non-hemolytic on blood agar and is frequently associated with Lancefield group C (Vandamme et al., 1996). In contrast, *S. dysgalactiae* subsp. *equisimilis* typically forms β-hemolytic colonies and is most often categorized within Lancefield groups C and G (Vieira et al., 1998). *S. dysgalactiae* subsp. *equisimilis* has been identified as a pathogen affecting humans and different domestic mammals. However, recent reports have documented its isolation from poultry with septicemia, endocarditis, and ocular infections, thereby indicating that poultry may also constitute a susceptible host (McDougall et al., 2014; Watanabe et al., 2016; Idalan et al., 2026). The increasing detection of *S. dysgalactiae* in poultry and farms underscores its growing significance in veterinary medicine and highlights the need to understand its epidemiology, pathogenic potential, and antimicrobial resistance in poultry production systems (Watanabe et al., 2016; Glambek et al., 2024). The infections can have significant economic impacts, especially in developing countries where poultry farming is vital for food security and rural livelihoods (FAO, 2019).

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The pathogenicity of *S. dysgalactiae* is associated with different virulence factors, including adhesins, hemolysins, and immune-evasion mechanisms that facilitate colonization and persistence within the host (Fulde and Valentin-Weigand, 2013; Walker et al., 2014). In addition, antimicrobial-resistant strains have increasingly been reported worldwide (Glajzner et al., 2023; López de Egea et al., 2023). Mobile genetic elements, such as plasmids, transposons, and integrative conjugative elements, contribute to the dissemination of antimicrobial resistance genes among streptococci and other bacteria. The pattern raises concerns about potential treatment failures and resistance distribution in animal production systems (Glajzner et al., 2023; López de Egea et al., 2023) and underscores the importance of integrated surveillance from a One Health perspective (Glambek et al., 2024).

Poultry production systems can also serve as reservoirs for antimicrobial-resistant bacteria. Continuous or improper antibiotic use in poultry farming can exert selective pressure, promoting the emergence and persistence of resistant bacterial populations (WOAH, 2023). Farm environments, such as drinking water, feed, litter, and equipment, can support bacterial survival and transmission among chickens, contributing to the maintenance of antimicrobial resistance in poultry systems and potentially increasing the risk of resistant bacteria spreading to other populations via environmental routes (Hossain et al., 2020; Glambek et al., 2024).

In Bangladesh, the irrational use of antimicrobials in food-producing animals significantly contributed to antimicrobial resistance, underscoring the need for antimicrobial stewardship and coordinated One Health interventions (Islam et al., 2026). Previous studies have primarily focused on antimicrobial-resistant bacteria such as *Escherichia coli*, *Salmonella* spp., and *Staphylococcus* spp. found in different environmental settings (Hossain et al., 2020). Different factors in the coastal region of Barishal, Bangladesh, including elevated humidity, seasonal flooding, saline intrusion associated with climate change, and intensive poultry production with different biosecurity practices, may collectively facilitate bacterial persistence (Islam et al., 2026). However, information specifically describing the occurrence and antimicrobial resistance of *S. dysgalactiae* in poultry and associated farm environments remains limited, particularly in the coastal region of Barishal, Bangladesh. Consequently, the epidemiological distribution and antimicrobial susceptibility characteristics of *S. dysgalactiae* in poultry production systems remain poorly understood. Therefore, monitoring bacterial occurrence and antimicrobial resistance in this region is important for supporting disease control strategies and antimicrobial stewardship (WOAH, 2023; Glambek et al., 2024). The present study aimed to investigate the prevalence and antimicrobial resistance patterns of *S. dysgalactiae* isolated from poultry tissue and farm environmental samples in coastal Barishal, Bangladesh.

MATERIALS AND METHODS

Ethical approval

The study used samples from broiler chickens collected during routine farm management and slaughter processes. No live animals were specifically handled for the experiment. Sample collection was carried out with farm owners' permission, following proper biosafety and animal welfare protocols.

Study location

A cross-sectional study was carried out from July 2024 to June 2025 in selected commercial broiler chicken farms within specific areas of Barishal, Bangladesh (Figure 1).

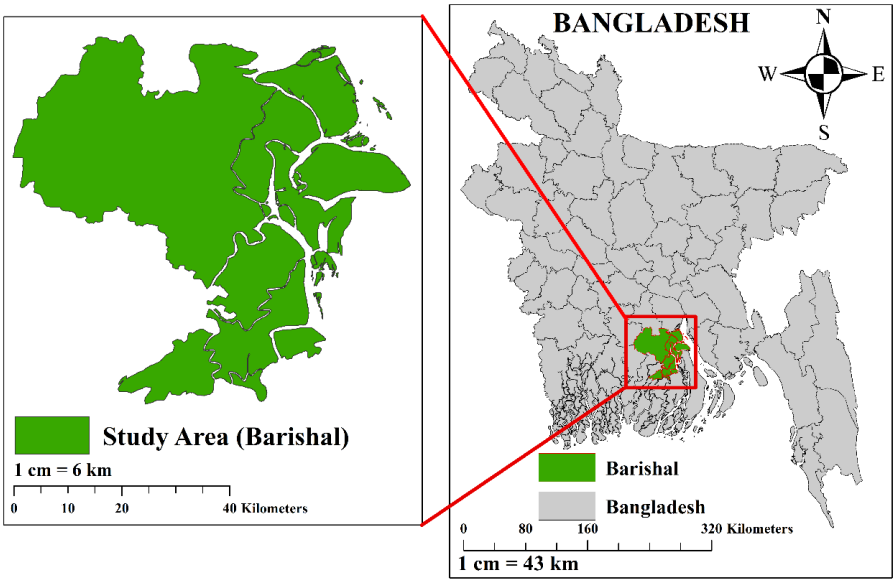


Figure 1. Location of the selected commercial broiler chicken farms in specific areas of Barishal, Bangladesh. The figure was designed using ArcGIS Software version 8

Sample collection

A total of 64 samples were collected from 10 commercial broiler chicken farms using a simple random sampling approach, consisting of 38 poultry-derived samples from heart (n = 8), intestine (n = 18), and feces (n = 12), and 26 farm-environment samples, including 14 samples collected from drinking water and 12 from poultry feed. All poultry flocks were fed commercially formulated, balanced broiler feeds obtained from established local commercial feed suppliers, according to the recommended nutritional requirements of the respective broiler breeds and growth stages.

Certified veterinarians conducted routine clinical examinations of the broiler chicken flocks at all participating farms to assess flock health and identify any clinical signs suggestive of infectious diseases. Samples collected from farms were included in the present study because they presented possible sources of bacterial exposure and transmission within the poultry production system. The samples were used to examine the presence and antimicrobial resistance patterns of *S. dysgalactiae*. To prevent selection bias, samples were collected from randomly selected poultry farms during the sampling period. As a result, not every chicken contributed all three sample types, yielding 38 independent poultry samples rather than matched tissue samples from the same chicken. Heart and intestinal samples were aseptically collected immediately after slaughter using sterile scissors and forceps. Approximately 5-10 g of intestinal tissue and heart tissue were placed separately into sterile sample containers. Fresh fecal samples (approximately 5 g) were collected directly from poultry house floors immediately after defecation using sterile spatulas. For farm-associated sampling, approximately 100 mL of drinking water was aseptically collected into sterile, screw-capped bottles from the drinking system of each poultry house. Feed samples (approximately 50 g) were collected individually from feeders using sterile gloves and transferred into sterile polyethylene bags. Each sample was analyzed individually; no pooled samples were included. Immediately after collection, all samples were transported to the laboratory under refrigerated conditions (4-8˚C) and processed within 6 hours of collection. Isolation and presumptive identification of *S. dysgalactiae***.** Due to financial constraints, molecular confirmation was not performed; bacterial identification was carried out using traditional phenotypic methods, including colony morphology, hemolytic features, Gram staining, and catalase testing.

Isolation and identification of *Streptococcus dysgalactiae*

Approximately 1 g of tissue or fecal sample was homogenized in 9 mL of sterile physiological saline. Water samples were mixed thoroughly before processing, while feed samples were homogenized in sterile saline at a 1:10 dilution. Sterile cotton swabs soaked in the prepared suspensions were streaked onto 5% sheep blood agar (HiMedia, India) and incubated aerobically at 37˚C for 24-48 hours. Presumptive streptococcal colonies were selected based on α- and β-hemolysis, colony morphology, Gram staining (Gram-positive cocci arranged in chains), and a catalase-negative reaction (Quinn et al., 2011; Markey et al., 2013). Selected isolates were inoculated onto Mannitol salt agar (MSA; HiMedia, India) to assess growth in high-salt conditions and to exclude salt-tolerant Staphylococcus species. The MSA results were used only as supportive observations and were not considered a definitive criterion for identifying *S. dysgalactiae* (Vieira et al., 1998; Quinn et al., 2011).

Antibiogram profiling of *Streptococcus dysgalactiae*

Antimicrobial susceptibility testing was performed using the Kirby-Bauer disk diffusion method according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI VET01S)**.** Fresh bacterial colonies were inoculated into sterile nutrient broth and incubated overnight. The suspension was uniformly spread onto Mueller-Hinton agar supplemented with 5% sheep blood using sterile cotton swabs. Plates were incubated at 35 ± 2˚C for 18-24 hours. The diameter of inhibition zones was measured in millimeters using a digital caliper and interpreted according to CLSI guidelines (CLSI, 2023). The commercially available discs (Himedia, India) were used, including ampicillin (10 µg), penicillin G (10 units), amoxicillin (25 µg), vancomycin (30 µg), oxytetracycline (30 µg), azithromycin (15 µg), doxycycline (30 µg), gentamycin (10 µg), streptomycin (10 µg), ciprofloxacin (5 µg), and levofloxacin (5 µg). For antimicrobials without an established resistance breakpoint for *Streptococcus* spp., resistance was interpreted using breakpoints for either another antimicrobial in the same drug class or another pathogen group (Zhang et al., 2018).

Statistical analysis

Data were entered into Microsoft Excel and analyzed using IBM SPSS Statistics version 26.0 (IBM Corp., USA). The prevalence of presumptive *S. dysgalactiae* was calculated as the percentage of positive samples among the total samples examined. Categorical variables were expressed as frequencies and percentages. Differences in prevalence between poultry and farm-associated samples were evaluated using the Chi-square test or Fisher's exact test, depending on the expected cell frequencies. Differences among poultry sample types (heart, intestine, and feces) and farm-associated sample types (water and feed) were similarly assessed using Fisher's exact test where appropriate. All statistical tests were two-tailed, and p-values less than 0.05 were considered statistically significant.

RESULTS

Streptococcus dysgalactiae isolation and identification

A total of 64 poultry and farm-associated samples were examined for the presence of presumptive *S. dysgalactiae*. Identification of *S. dysgalactiae* was based on conventional phenotypic characteristics, including colony morphology, hemolytic activity on blood agar, Gram staining, and catalase testing. The presumptive isolates demonstrated α-hemolytic colonies on 5% sheep blood agar, no or minimal growth on MSA, Gram-positive cocci arranged in chains, and catalase-negative reactions (Figures 2 and 3). Based on these phenotypic characteristics, the isolates were identified as *S. dysgalactiae.*



Figure 2. *Streptococcus dysgalactiae* isolated from broiler chicken fecal samples from July 2024 to June 2025 in Barishal, Bangladesh. **A:** Growth of *Streptococcus dysgalactiae* on 5% sheep blood agar with α-hemolysis, **B:** Poor or no growth on Mannitol salt agar without mannitol fermentation, consistent with *Streptococcus dysgalactiae* growth, **C:** Growth with mannitol fermentation characteristic of *Staphylococcus* spp., indicated by a yellow color change on Mannitol salt agar.

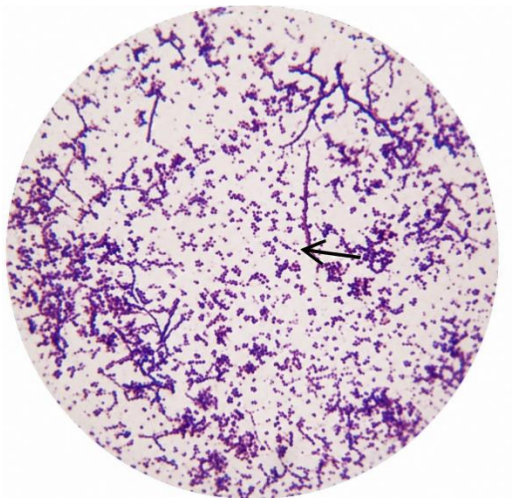


Figure 3. Gram staining characteristics of *Streptococcus dysgalactiae* showing Gram-positive cocci arranged in chains

Prevalence of *Streptococcus dysgalactiae* across different sample sources

Among the 64 samples examined, 26 were positive for *S. dysgalactiae*, resulting in an overall prevalence of 40.62%. The prevalence of *S. dysgalactiae* differed between poultry and farm-associated samples (Table 1). Among poultry-derived samples, 20 of 38 samples (52.63%) tested positive, while 6 of 26 farm-associated samples (23.08%) were positive for *S. dysgalactiae*. The difference between poultry and farm-associated samples was statistically significant ($p < 0.05$), indicating that poultry samples were more frequently positive than farm-associated samples during the present study.

Prevalence of *Streptococcus dysgalactiae* among poultry sample types

The prevalence of *S. dysgalactiae* among different poultry sample types is presented in Table 2. Isolates of *S. dysgalactiae* were identified in 4 out of 8 heart samples (50.00%), 10 out of 18 intestinal samples (55.55%), and 6 out of 12 fecal samples (50.00%). No statistically significant differences were observed among the three poultry sample types ($p > 0.05$), suggesting similar prevalence across the sampled tissues. Because different sample types were collected from

randomly selected poultry rather than the same individuals, the current findings confirmed the presence of *S. dysgalactiae* in multiple poultry samples but did not indicate systemic colonization or dissemination within individual chickens.

Prevalence of *Streptococcus dysgalactiae* in farm-associated samples

The prevalence of *S. dysgalactiae* among farm-associated samples is presented in Table 3. *S. dysgalactiae* was found in 4 out of 14 water samples (28.57%) and 2 out of 12 feed samples (16.66%). While the prevalence was greater in water samples, this difference was not statistically significant ($p > 0.05$).

Table 1. Prevalence of *Streptococcus dysgalactiae* among poultry-derived and farm-associated samples collected from July 2024 to June 2025 in Barishal, Bangladesh

Sample source	Sample type	Total samples (n)	Positive samples	Prevalence (%)	95% CI (%)	Statistical comparison
Heart, intestine, and feces	Poultry	38	20	52.63	36.50 - 68.30	p = 0.018*
Water and feed	Farm-associated samples	26	6	23.08	9.00 - 43.00	

n: Number of samples, *: Significantly lower than poultry

Table 2. Prevalence of *Streptococcus dysgalactiae* among different poultry tissue samples

Sample type	Total samples (n)	Positive samples	Prevalence (%)	95% CI (%)	Statistical comparison
Heart	8	4	50.00	15.70 - 84.30	
Intestine	18	10	55.55	30.80 - 78.50	p = 0.943*
Feces	12	6	50.00	21.10 - 78.90	

n: Number of samples, *: P-value versus Heart/Feces

Table 3. Prevalence of *Streptococcus dysgalactiae* among farm-associated samples from July 2024 to June 2025 in Barishal, Bangladesh

Farm-associated source	Total samples (n)	Positive samples	Prevalence (%)	95% CI (%)	Statistical comparison
Water	14	4	28.57	8.40 - 58.10	p = 0.473*
Feed	12	2	16.66	2.10 - 48.40	

n: Number of samples, *: P-value versus Water

Antimicrobial resistance patterns of *Streptococcus dysgalactiae*

The antimicrobial resistance profiles of *S. dysgalactiae* isolates recovered from poultry and farm-associated samples are presented in Table 4. Resistance to β-lactam antibiotics was widespread, with all isolates resistant to ampicillin and penicillin. Different resistance rates were observed for amoxicillin, azithromycin, doxycycline, oxytetracycline, and streptomycin, whereas resistance to ciprofloxacin, levofloxacin, and gentamicin remained relatively low. Although resistance percentages differed among sample types, no statistically significant differences were detected between poultry and farm-associated isolates for any of the antimicrobial agents tested ($p > 0.05$).

Poultry isolates multidrug resistance patterns

Among poultry-derived *S. dysgalactiae* isolates, antimicrobial resistance patterns differed by sample type. All heart isolates (100%) were resistant to ampicillin, penicillin, and amoxicillin, whereas no resistance was detected to ciprofloxacin, levofloxacin, or gentamicin. Resistance to azithromycin, doxycycline, oxytetracycline, vancomycin, and streptomycin was observed in 50% of heart isolates. Similarly, all intestinal isolates (100%) were resistant to ampicillin and penicillin. Resistance was also observed to azithromycin (60%), doxycycline (60%), amoxicillin (60%), and vancomycin (40%), while lower resistance rates were detected for ciprofloxacin (20%), levofloxacin (20%), gentamicin (20%), and streptomycin (20%). Among fecal isolates, resistance was highest to ampicillin and penicillin (100%), followed by amoxicillin (83.3%). Resistance to azithromycin, doxycycline, oxytetracycline, vancomycin, and streptomycin ranged from 50.0% to 66.7%, whereas resistance to ciprofloxacin was comparatively low (16.7%). No gentamicin resistance was detected among heart isolates, while intestinal (20.0%) and fecal (33.3%) isolates exhibited low resistance rates. Although resistance percentages differed numerically among heart, intestinal, and fecal isolates, antimicrobial resistance patterns did not differ significantly among poultry sample types ($p > 0.05$). This result indicated a generally similar distribution of resistance patterns across the different tissues sampled.

Among the farm-associated *S. dysgalactiae* isolates, all isolates collected from water samples (100%) were resistant to ampicillin and penicillin, with high resistance observed to amoxicillin (75.00%) and streptomycin (75.00%). Resistance to azithromycin, doxycycline, oxytetracycline, vancomycin, gentamicin, ciprofloxacin, and levofloxacin ranged from 25.00% to 50.00%. Similarly, all feed isolates (100%) were resistant to ampicillin, penicillin, and amoxicillin. No resistance was detected to ciprofloxacin or levofloxacin, whereas 50.0% of feed sample isolates were resistant to azithromycin, doxycycline, oxytetracycline, vancomycin, gentamicin, and streptomycin.

Table 4. Antibiotic resistance patterns of *Streptococcus dysgalactiae* isolated from poultry tissues and farm-associated samples from July 2024 to June 2025 in Barishal, Bangladesh

Source	Sample type	Antibiotic resistance against *Streptococcus dysgalactiae* (%)										
		CIP	AZM	DOX	OXY	VAN	LEV	AMP	CN	P	S	AMX
Poultry	Heart	0	50	50	50	50	0	100	0	100	50	100
	Intestine	20	60	60	40	40	20	100	20	100	20	60
	Feces	16.7	50	50	33.3	16.7	33.3	100	33.3	100	66.7	83.3
Farm-associated samples	Water	25	25	50	50	25	25	100	25	100	75	75
	Feed	0	50	50	50	50	0	100	0	100	50	100

AMX: Amoxicillin, AMP: Ampicillin, AZM: Azithromycin, CIP: Ciprofloxacin, DOX: Doxycycline, CN: Gentamicin, LEV: Levofloxacin, P: Penicillin, OXY: Oxytetracycline, S: Streptomycin, VAN: Vancomycin

Poultry and farm-associated multidrug resistance patterns

Streptococcus dysgalactiae isolates derived from poultry and farm-associated samples demonstrated resistance to multiple antimicrobial agents. Resistance was particularly frequent against β-lactam antimicrobials, while different resistance patterns were observed to macrolides, tetracyclines, aminoglycosides, glycopeptides, and fluoroquinolones**.** Among isolates derived from heart samples, multidrug resistance (MDR) profiles exhibited resistance to multiple antibiotic classes, including β-lactams (ampicillin, penicillin, amoxicillin), macrolides (azithromycin), tetracyclines (doxycycline, oxytetracycline), glycopeptides (vancomycin), and aminoglycosides (streptomycin). Isolates from intestinal samples demonstrated broader MDR profiles, with resistance to β-lactams, macrolides, tetracyclines, glycopeptides, and aminoglycosides. Additionally, some isolates were resistant to fluoroquinolones (ciprofloxacin and levofloxacin) and gentamicin. Similarly, isolates derived from fecal samples were resistant to β-lactams, macrolides, tetracyclines, glycopeptides, and aminoglycosides, whereas resistance to fluoroquinolones and gentamicin was less frequent. Furthermore, isolates from farm-associated samples exhibited MDR patterns. Water-derived isolates demonstrated concurrent resistance to β-lactams, tetracyclines, macrolides, glycopeptides, and aminoglycosides. Feed-derived isolates were resistant to β-lactams, macrolides, tetracyclines, glycopeptides, and aminoglycosides; however, no resistance to fluoroquinolones was observed.

DISCUSSION

Detection of presumptive *Streptococcus dysgalactiae* in poultry-derived and farm-associated samples indicates its possible occurrence in commercial broiler chicken production systems in coastal Barishal, Bangladesh. The higher detection rate in poultry-derived samples (52.63%) than in farm-associated samples (23.08%) suggests a greater association with poultry-derived materials, while detection in drinking water and feed indicates possible persistence or dissemination within farm systems. However, as the isolates were identified presumptively based on phenotypic characteristics without molecular confirmation, the findings should be interpreted cautiously and do not establish definitive species-level occurrence or reservoir status. Previous studies have reported *Streptococcus* spp. in livestock production systems in Bangladesh (Hassan et al., 2023), including molecularly confirmed *S. dysgalactiae* from bovine mastitis in southern Bangladesh (Farzana et al., 2023). *S. dysgalactiae* has also been molecularly characterized in bovine mastitis in China (Xu et al., 2021) and in slaughtered pigs in India, where antimicrobial resistance was reported (Patel et al., 2024). Differences in host, sample type, geographical setting, and diagnostic methods limit direct comparison among these studies.

The high level of antimicrobial resistance observed in the present study indicated that antimicrobial-resistant *S. dysgalactiae* was circulating within commercial poultry production systems in Barishal, Bangladesh. Similar resistance patterns of *S. dysgalactiae* have been identified in *isolates* collected from food-producing animals in China (Zhang et al., 2018). The widespread and frequently inappropriate use of antimicrobial agents in livestock production is a well-recognized major contributor to antimicrobial resistance. The extensive use of antimicrobials can create selective pressure that promotes the emergence and persistence of resistant bacteria (WHO, 2019). No statistically notable differences in antimicrobial resistance were found between poultry and farm-associated isolates, suggesting that similar resistance profiles circulated within the farm ecosystem, likely due to shared antimicrobial exposure and bacterial exchange between chickens and their environment. Although the present study did not investigate transmission routes of *S. dysgalactiae*, it highlighted the necessity of careful antimicrobial use, enhanced farm biosecurity measures, and comprehensive One Health surveillance to prevent the development and dissemination of antimicrobial-resistant *S. dysgalactiae* within poultry production systems (Glambek et al., 2024; Hossain et al., 2020).

Poultry-derived and environmental samples were MDR, indicating that resistant *S. dysgalactiae* strains were present within poultry production systems. Similar findings have been reported in food-producing animals regarding livestock-associated streptococci, highlighting the growing challenge of antimicrobial resistance (FAO, 2019).

CONCLUSION

The present study demonstrated the occurrence of *S. dysgalactiae* in poultry and environmental samples collected from commercial broiler chicken farms in the coastal region of Barishal, Bangladesh. Isolates from poultry samples exhibited a significantly higher *S. dysgalactiae* prevalence than farm-associated samples. Antimicrobial susceptibility results demonstrated high resistance to β-lactam antibiotics, especially ampicillin and penicillin, and revealed MDR patterns among the isolates derived from poultry- and farm-associated samples. Although bacterial identification was based solely on conventional phenotypic methods without molecular confirmation, the present study provided valuable baseline information on the occurrence and antimicrobial resistance patterns of *S. dysgalactiae* in coastal poultry farms in Barishal, Bangladesh. In addition, the relatively small sample size and descriptive analysis might have limited the generalizability of the current findings. Future investigations incorporating molecular identification, antimicrobial resistance gene characterization, and larger sample sizes are recommended to comprehensively understand the epidemiology and public health significance of *S. dysgalactiae* in Bangladesh.

DECLARATIONS

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Authors' contributions

Farzana Islam Rume conceptualized, supervised the study, and revised the final draft. Md. Yeasin Arafat and Arobi Jahan Disha conducted the experiments and documented the results. Md. Yeasin Arafat, Wahedul Karim Ansari, Md. Kamruzzaman Akimul, and Nasrin Akter Sumona performed the formal analysis, drafted the manuscript, generated figures, and created the maps. Farzana Islam Rume provided critical reviews and contributed to the final edition of the manuscript. All authors read and approved the final edition of the manuscript.

Availability of data and materials

All data generated and analyzed during the present study are included in this published article. No supplementary materials are associated with this manuscript. Requests for additional information or data related to this study should be directed to the corresponding author.

Competing interests

The authors have no competing interests to declare.

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. During the

preparation of this manuscript, the authors utilized ChatGPT (version 4.0) for linguistic improvements. The authors carefully reviewed and revised the AI-edited language content to ensure accuracy, completeness, and adherence to the journal's requirements, and take full responsibility for the final content of the published article. The authors assume full responsibility for the originality of the data prior to the submission of the article, throughout the peer review process, and subsequent to acceptance.

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Three-Dimensional Imaging and Plastination in Veterinary Anatomy Education

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ABSTRACT

Veterinary anatomy plays a crucial role in veterinary education. However, cadaveric dissection encounters significant challenges such as high costs, ethical concerns, and limited accessibility, particularly in developing countries. The present study aimed to develop supplementary, cost-effective educational tools using smartphone-based three-dimensional (3D) imaging and a modified Elnady plastination technique applied to ox and camel organs to enhance gross anatomy teaching. Digital photogrammetry was employed to create 3D models of the axis, skull, femur, lumbar vertebrae, and soft-tissue specimens of the head, including the nasal and oral cavities of an adult bull, utilizing a smartphone camera. Subsequently, the 3D models were processed and refined using the KIRI Engine 3D Scanner application. Bull specimens were obtained from the Anatomy Laboratory at the College of Veterinary Medicine, King Faisal University, Al-Ahsa, Saudi Arabia, from the existing collection. Meanwhile, anatomical specimens from five adult male camels (5 hearts, 10 kidneys, 5 penises, and 5 testes) and six adult bulls (6 testes) were plastinated using a modified version of the Elnady protocol to create long-lasting teaching tools. The 3D imaging produced precise, high-resolution, and interactive models of the ox skeleton, which clearly exhibited anatomical structures and spatial relationships with adjacent bones. The ox skeletal and soft-tissue 3D models demonstrated excellent detail, allowing clear visualization of anatomical structures. Plastinated specimens of the adult male camel preserved their anatomical integrity, exhibited minimal shrinkage, had adequate structural clarity, remained dry, odorless, and suitable for repeated handling. Among the ox specimens, shrinkage was observed in the testicular parenchyma following plastination. Using 3D imaging alongside plastination enhanced visual and tactile learning. The current findings indicated that smartphone-based 3D photogrammetry and the modified Elnady plastination technique offered a practical, scalable approach to modernizing anatomy teaching, especially in limited-resource settings.

Keywords: Anatomy, Education, Ox, Plastination, Veterinary, 3D imaging

INTRODUCTION

Veterinary anatomy is essential in veterinary education, forming the fundamental knowledge needed for clinical and diagnostic practices (Gummery et al., 2024). Anatomical knowledge and practical skills provide students with a strong academic foundation essential for advanced studies and clinical practice, including radiology, pathology, and surgery (Older, 2004; Sugand et al., 2010). Anatomy is widely considered one of the most challenging subjects due to the complexity of anatomical structures and the vast volume of information that students are required to master, despite its significant role in veterinary education (Dickson et al., 2022; Inpanbutr et al., 2024). Traditional teaching methods are often uninspiring, which can reduce student motivation and hinder knowledge retention (Warren and Donnon, 2013; Armitage-Chan et al., 2018). Cadaver-based instruction poses challenges, including health risks from formalin exposure, high costs, and ethical concerns (Ye et al., 2020).

In recent years, the global effort to reduce animal use in educational settings, coupled with financial constraints, infrastructural limitations, and reduced instructional time, has substantially affected veterinary anatomy education (King, 2004; Dickson et al., 2022). Collectively, these factors may result in deficiencies in anatomical knowledge and diminished student confidence. Consequently, there is a growing necessity to revise and modernize veterinary anatomy education, particularly in developing countries and other low- and middle-income countries such as Sudan, where resource constraints require cost-effective and sustainable teaching strategies (Sugand et al., 2010; Ozkadif and Eken, 2012; Chang Chan et al., 2024).

Advances in educational technologies have reshaped anatomy teaching, with growing emphasis on methods that reduce reliance on cadavers while enhancing learning outcomes (Guevar, 2020). Although cadaver dissection is valuable, educators are increasingly integrating innovative tools such as artificial intelligence (AI; Martín-Alguacil et al., 2025),

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digital imaging (Alcobaça et al., 2025), virtual platforms, three-dimensional (3D) visualization and modeling, 3D printing (Choudhary and Sarkar, 2025; Doğan and Kaçmaz, 2025), and plastinated specimens (Bernal et al., 2022). These teaching methods promoted active learning, enhanced understanding of complex anatomical connections, and addressed challenges in veterinary anatomy education related to specimen availability, safety, and ethics (Choudhary, 2026).

Imaging techniques, which broadly refer to methods that generate visual representations of internal body structures, play a crucial role in modern anatomy education (Hussain et al., 2022). However, students frequently encounter difficulties when transforming 2D images into 3D anatomical models (Preece et al., 2013; Wainman et al., 2018). The incorporation of 3D models into anatomical education effectively enhances spatial cognition and promotes understanding of complex anatomical relationships, especially for students with limited spatial visualization skills (Höffler, 2010; Schirone et al., 2024).

Plastination is now widely used in veterinary education as either an alternative or supplementary resource to traditional cadaveric specimens (Riederer, 2014). Plastinated specimens are widely accepted and have demonstrated educational benefits, particularly at the College of Veterinary Medicine, University of Murcia, Spain (Latorre et al., 2007). The plastination technique comprises four main stages, including fixation, dehydration, forced impregnation, and curing (von Hagens et al., 1987). Throughout these procedures, water and lipids within tissues are replaced with polymers such as silicone, polyester, or epoxy (Henry et al., 2019). The plastinated specimens provide sufficient contrast and structural clarity for educational purposes (Bernal et al., 2022). The Elnady technique is a recent advancement in plastination, offering a simpler, more cost-effective method for producing long-lasting specimens with minimal shrinkage and superior morphological preservation (Elnady et al., 2015; Elnady, 2016; 2019). Despite the growing adoption of low-cost 3D imaging and simplified plastination methods in anatomy education, their application remains limited within veterinary anatomy, especially in resource-limited settings. Few studies have integrated 3D imaging and simplified plastination approaches as supplementary educational tools. Therefore, the present study aimed to develop smartphone-based 3D imaging to generate 3D models of ox skeletal specimens, and a modified Elnady plastination technique to produce plastinated camel and ox specimens as low-cost educational resources for veterinary gross anatomy.

MATERIALS AND METHODS

Ethical approval

All experimental procedures were carried out in strict accordance with institutional and national regulations concerning the ethical use of animals in research. The present procedures received approval from the Animal Ethics Committee of King Faisal University (Ref. No. KFU-2026-ETHICS4248), Saudi Arabia. Biological samples were sourced from animals processed for commercial slaughter at an authorized abattoir in Al-Ahsa, Saudi Arabia.

Sampling

The present study was conducted in the dissecting room of the Department of Anatomy at the College of Veterinary Medicine, King Faisal University, Al-Ahsa, Saudi Arabia, over 16 weeks. Four skeletal specimens from an adult ox, consisting of the axis, skull, femur, and a lumbar vertebra, were selected from the Department of Anatomy teaching collection at King Faisal University, Saudi Arabia, for 3D imaging. The sample size was consistent with previous specimen-based plastination studies using a limited number of animal specimens (Medina et al., 2025). An expert veterinary anatomist with a PhD in Veterinary Anatomy selected these specimens based on their completeness, structural integrity, and the absence of visible damage or deformity, thereby ensuring precise 3D reconstruction. Camel bones were not included because the study focused on demonstrating the feasibility of photogrammetry rather than on species comparison.

Soft-tissue specimens were collected from animals slaughtered at a local abattoir in Al-Ahsa, Eastern Province, Saudi Arabia. Samples included one heart, two kidneys, one penis, and one testis from each of five healthy adult male dromedary camels (*Camelus dromedarius*), totaling 25 camel organ specimens. Additionally, one testis was collected from each of six healthy adult bulls, resulting in six bull testicular specimens. Bull testes were included to demonstrate the preservation technique in another domestic species, not for interspecies comparison. Soft tissues intended for the plastination process were obtained from 11 animals, comprising five camels and six bulls, all healthy and free of gross pathological lesions.

Preparation of 3D anatomical images

Digital photogrammetry was employed to produce original 3D anatomical models of gross specimens. Each specimen was documented utilizing a smartphone, equipped with a 48-megapixel Fusion main camera (Apple Inc.,

Cupertino, CA, USA). Images were systematically captured under consistent diffuse lighting conditions as the specimen was rotated incrementally at approximately 10-15° intervals until a comprehensive 360° image set was acquired. This process yielded approximately 60 to 130 overlapping photographs per specimen, depending on the specimen's size and complexity.

The photographs were processed using the KIRI Engine 3D Scanner (Version V4.2.8, KIRI Innovations, Hong Kong, China), with high-quality reconstruction settings, automatic image alignment, and quad-mesh generation with Physically Based Rendering (PBR) materials. The resulting models were subsequently refined in Blender (Version 5.3.0, Blender Foundation, Amsterdam, The Netherlands) by removing background artifacts, smoothing the mesh as needed, and optimizing the geometry while preserving the original anatomical morphology. Anatomical labels were subsequently added using Sketchfab (Epic Games Inc., New York, NY, USA) and the Emb3D Viewer application (Theia Interactive, USA) to produce interactive educational 3D models. The finalized 3D models were incorporated into gross anatomy lectures and practical laboratory sessions following previously described digital anatomy teaching workflows (Silva et al., 2026).

Preparation of plastinated specimens

The main steps of the modified Elnady plastination technique used in the present study are summarized in Table 1. After collection of the specimens from the slaughterhouse, the soft-tissue specimens (heart, kidney, penis, and testes) were immediately washed under running tap water for 10-15 minutes to remove residual blood, dirt, and tissue debris before fixation (Bancroft and Gamble, 2008). The specimens were then blotted dry with absorbent paper and air-dried at room temperature (approximately 25°C) for 15-30 minutes to remove surface moisture before fixation, following a modified protocol adapted from Bancroft and Gamble (2008).

Table 1. Modified Elnady technique steps for preparation of adult ox and camel plastinated specimens

Stage	Description	Procedure	Duration	Conditions	Notes
1	Collection and washing	Specimens collected post-slaughter and washed under running tap water	10-15 minutes	RT	Removal of blood and debris
2	Initial drying	Air-dried on absorbent paper	15-30 minutes	RT	Surface moisture removal
3	Fixation	Immersion in 10% buffered formalin (≥ 10x specimen volume)	2-3 weeks	RT, sealed containers	Formalin was changed twice; specimens were kept submerged using a cotton cloth
4	Post-fixation drying	Air-dried on absorbent paper	Overnight	RT	Preparation for dehydration
5	Dehydration (80% acetone)	Immersion in 80% acetone (at least 10x specimen volume)	1-2 weeks	RT	Acetone was tested every 2-3 days
6	Dehydration (90% acetone)	Immersion in 90% acetone	1-2 weeks	RT	Concentration maintained
7	Dehydration (100% acetone)	Immersion in 100% acetone	2-3 weeks	RT	Completed when water content was <1%
8	Post-dehydration drying	Air-dried on absorbent paper	Overnight	RT	Before impregnation
9	Impregnation	Immersion in glycerin at a 5:1 glycerin-to-specimen volume ratio in a sealed container	5-8 weeks	RT	Duration depends on specimen size
10	Draining	Specimens drained	3-14 days	RT	Excess glycerin removal
11	Curing (cornstarch method)	Stored in cotton sacks inside buckets covered with cornstarch (no direct contact)	1-3 weeks	RT	Until glycerin exudation stops
12	Final cleaning and storage	Brushing/air compressor cleaning	—	RT	Stored at room temperature

The reference is adopted from Bancroft and Gamble (2008) and Elnady (2016)**.** RT: Room temperature

Fixation

The specimens were immersed in 10% neutral buffered formalin in sealed plastic containers, with a fixative volume at least 10 times the specimen volume to ensure adequate fixation (Bancroft and Gamble, 2008). Cotton cloths kept the specimens fully submerged. Fixation time was adjusted based on specimen size, with smaller specimens fixed for 2 weeks and larger specimens for 3 weeks to ensure adequate penetration of fixative into deeper tissues. The formalin

solution was replaced on days 7 and 14 to maintain fixation quality. After fixation, the specimens were removed from formalin and air-dried overnight on absorbent paper at room temperature before dehydration (Bancroft and Gamble, 2008).

Dehydration

Dehydration was performed at room temperature (25°C) following a protocol adapted from Elnady (2016), with modifications to acetone concentration and exposure duration based on specimen type. Gradual acetone exchanges were employed to remove water and lipids, with specimens sequentially immersed in baths of 80%, 90%, and 100% acetone (Honeywell, Charlotte, NC, USA). Smaller specimens remained at each concentration for one week, whereas larger specimens required two weeks per step. The final dehydration step in 100% acetone lasted 2-3 weeks, depending on the specimen size, using about ten times the specimen volume of acetone. The purity of acetone was monitored every 2-3 days using a dual-scale hydrometer (DURAC® 50325 Specific Gravity/Baumé, SP Bel-Art, Wayne, NJ, USA), with the acetone solution replaced whenever its concentration fell below the target level. Dehydration was considered complete once the acetone concentration exceeded 99%, indicating that residual water was below 1%, as confirmed by hydrometer measurements. After dehydration, the specimens were extracted from the acetone and air-dried overnight at room temperature (25°C).

Impregnation

Dehydrated specimens were immersed in glycerin (Sigma-Aldrich, St. Louis, MO, USA) in sealed containers at a volume ratio of approximately 5:1 (glycerin to specimen). The impregnation period ranged from 5 to 8 weeks, depending on specimen size and tissue density, following the modified Elnady plastination technique (Elnady, 2016). Glycerin impregnation was considered complete when the specimens exhibited uniform pliability throughout the tissue, and no further visible changes in tissue consistency were observed during routine inspection. Vacuum impregnation was not performed because the modified Elnady plastination technique relied on prolonged passive glycerin infiltration at atmospheric pressure instead of forced polymer impregnation under vacuum (Elnady et al., 2016).

Curing

After impregnation, specimens were removed and allowed to drain at room temperature (25°C) for 3-14 days. Excess glycerin was gently removed using absorbent paper. Specimens were then placed in cotton bags and suspended within sealed plastic containers filled with cornstarch powder (Jeddah, Saudi Arabia), ensuring no direct contact between the specimens and the powder. Following the revised Elnady plastination protocol, cornstarch was used as a desiccant to absorb excess glycerin migrating from the specimens, as documented by Elnady (2016). Subsequent to the curing process, any remaining cornstarch particles were thoroughly eliminated using soft brushes prior to storage at ambient temperature (25°C). The plastinated specimens have preserved their integrity and have been used for teaching without any visible deterioration for over 12 months after preparation.

Data analysis

The 3D models and plastinated specimens were descriptively evaluated for anatomical accuracy, structural integrity, and preservation quality. The current findings were recorded through photographs, so no statistical analysis was conducted.

RESULTS

Smartphone-based 3D imaging

The smartphone-based 3D imaging technique yielded precise, detailed 3D models of bone specimens from an adult ox (Figures 1 and 2). The 3D model of an axial vertebra of the ox accurately depicted the vertebral body, dorsal spinous process, ventral crest, cranial and caudal articular processes, transverse processes, vertebral foramen, and alar foramina. Surface morphology and bony landmarks were clearly visualized from multiple viewing angles, enabling a detailed examination of the anatomical features (Figures 1A and 1B). The color-enhanced 3D skull models from an adult ox enabled distinct differentiation of the individual cranial and facial bones. The boundaries between adjacent bones, including the frontal, parietal, temporal, maxillary, nasal, lacrimal, zygomatic, and occipital bones, as well as major foramina and external bony landmarks, were readily distinguished, facilitating understanding of their anatomical layout and spatial relationships (Figures 1C and 1D). The femur models derived from an adult ox demonstrated the proximal and distal extremities, incorporating the head, trochanters, trochanteric fossa, shaft, condyles, and trochlea, all of which were distinctly preserved in the reconstructed model (Figures 1E and 1F). The lumbar vertebrae of an adult ox were meticulously replicated, demonstrating the sequential arrangement of the vertebrae while maintaining the normal orientation, intervertebral spacing, vertebral bodies, transverse processes, spinous processes, and articular processes. This

precise reproduction facilitated a clear visualization of their 3D anatomical relationships (Figures 1G and 1H). The 3D imaging method reproduced soft-tissue specimens from the head of the adult ox (Figure 2). The 3D models preserved the morphology and major anatomical features of the nasolabial region and hard palate, including the muzzle and nostrils, and surrounding soft tissues, whereas the nasal cavity model displayed the nasal septum, nasal conchae, and meatuses. The hard palate model exhibited the palatine raphe and transverse palatine ridges with adequate anatomical detail suitable for interactive visualization and educational demonstration.

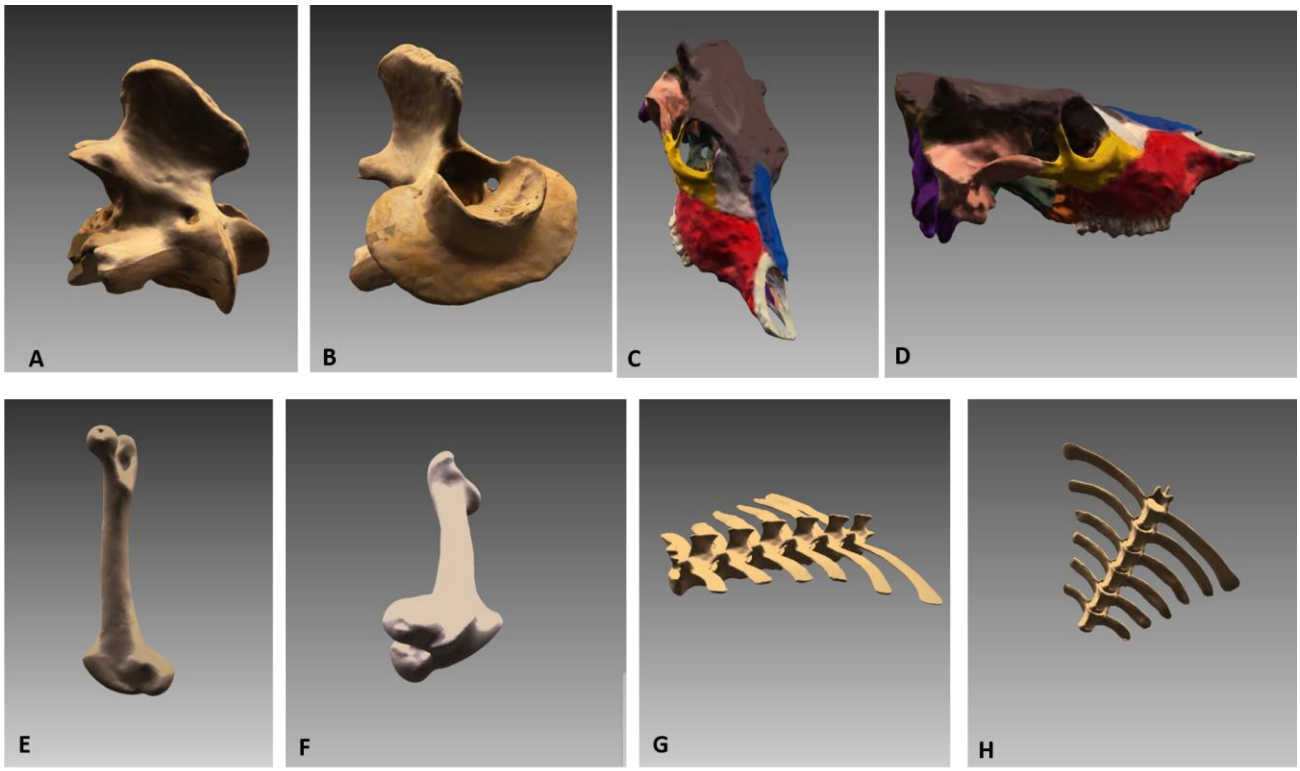


Figure 1. Smartphone-based 3D reconstructed skeletal models of an adult ox. **A** and **B:** Axis vertebra showing preserved morphology from different views, **C** and **D:** Skull models, including color-enhanced segmentation illustrating cranial bone differentiation, **E** and **F:** The femur demonstrates clear proximal and distal anatomical landmarks, **G:** Lumbar vertebrae showing sequential alignment, **H:** Associated rib structures illustrating spatial relationships

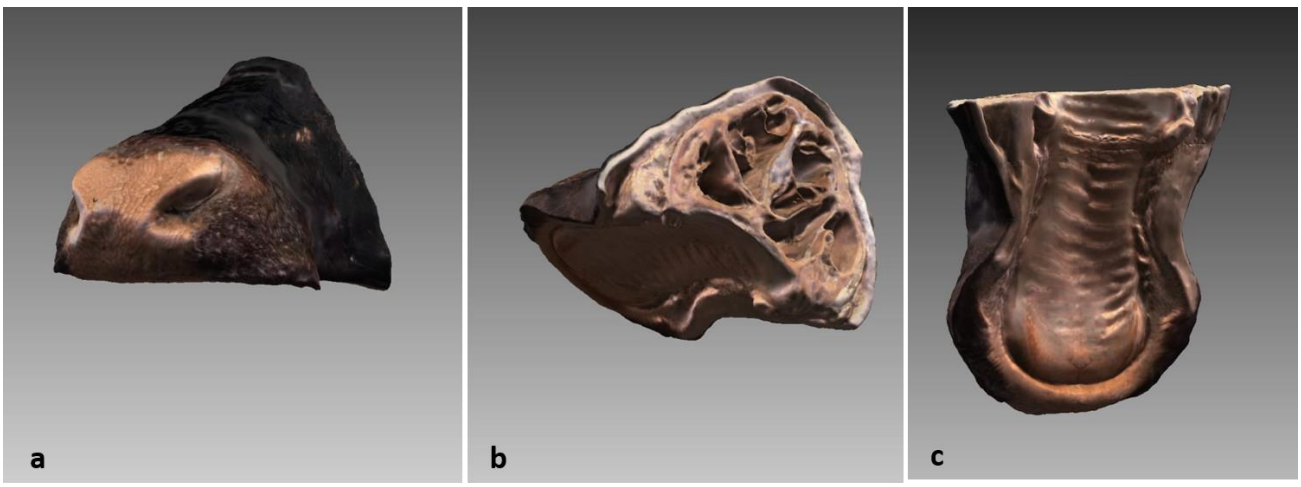


Figure 2. Smartphone-based 3D reconstructed soft tissue models of the head of an adult ox. **a**: External view showing the muzzle and nasolabial region, **b:** Sagittal section of the head showing the nasal cavity and associated internal structures, **c:** Internal view of the oral cavity showing the hard palate and palatine ridges

Plastinated specimens

The modified Elnady plastination method produced durable anatomical specimens with well-preserved morphology (Figure 3). The plastinated heart from an adult male dromedary camel maintained its shape and key features, including a well-defined apex and base, ventricular and coronary grooves, and visible openings of major blood vessels (Figure 3A).

The left kidney from an adult male camel demonstrated preserved structure with a typical bean shape, smooth external surface, distinct poles, a well-preserved hilum, and an outer capsule (Figure 3B). The testicular specimen from an adult male camel retained surface features such as the tunica albuginea, a smooth texture, epididymal attachment, and spermatic cord (Figure 3C). The penis of an adult male camel retained its natural curvature and structure, with the shaft, glans, and connective tissue clearly visible after plastination (Figure 3D).

Shrinkage artifact

Visual examination demonstrated that some tissue shrinkage was observed in plastinated testicular samples from an adult ox (Figure 4). The reduction in size was most noticeable in the testicular parenchyma, whereas the tunica albuginea remained intact, and the overall external morphology of the testes was preserved in ox plastinated models. Conversely, no remarkable shrinkage was observed in the plastinated heart, kidney, or penis of adult male dromedary camels, which retained their gross anatomical morphology following plastination (Figure 3).

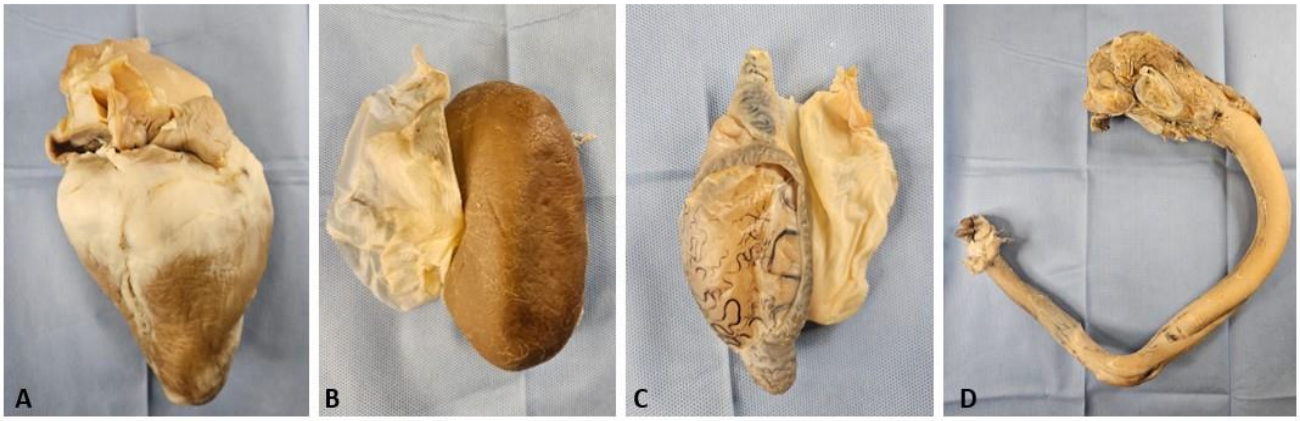


Figure 3. Plastinated anatomical specimens from an adult male dromedary camel prepared with the modified Elnady technique. **A**: Heart with preserved external morphology and anatomical contours, **B**: Left kidney with maintained shape and smooth surface texture, **C**: Testis showing the preserved tunica albuginea, smooth external surface, epididymal attachment, and spermatic cord, **D**: Penis demonstrating the retained natural curvature and preserved shaft, glans, and surrounding connective tissue.

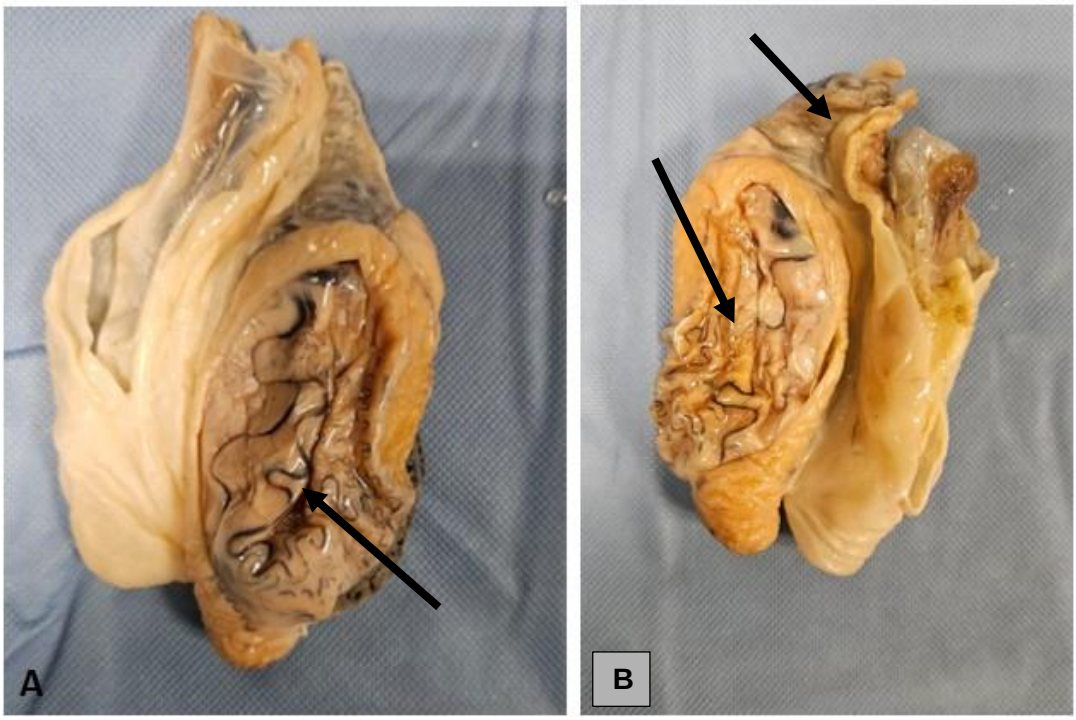


Figure 4. Plastinated testicular specimens from an adult ox showing different degrees of tissue shrinkage and surface wrinkling following the modified Elnady plastination technique. **A** and **B** represent two different plastinated testicular specimens illustrating variation in the extent of shrinkage. The arrows indicate areas of tissue shrinkage and surface wrinkling.

DISCUSSION

The present study demonstrated that smartphone-based photogrammetry successfully generated high-resolution interactive 3D models of both skeletal and soft-tissue specimens of an adult ox. Similar findings were reported by Silva et al. (2026), who used the KIRI Engine to reconstruct equine bones for veterinary anatomy education and found that the resulting models accurately preserved major anatomical landmarks and served as an accessible educational resource. Similarly, Morichon et al. (2024) demonstrated that smartphone photogrammetry can produce anatomically realistic 3D models suitable for anatomy education without requiring specialized imaging equipment. Consequently, these findings indicated that smartphone-based photogrammetry is a reliable and cost-effective method for generating interactive anatomical models for veterinary education.

In addition to ensuring anatomical accuracy, the reconstructed 3D models enabled rotation, magnification, and examination from multiple perspectives, thereby enhancing the visualization of complex spatial relationships among anatomical structures. Preece et al. (2013) and Wainman et al. (2018) documented that physical 3D models improved anatomical learning compared with computer-based counterparts. These findings partially supported the current results, affirming the educational importance of 3D visualization and emphasizing the benefits of interactive 3D anatomical models. Moreover, smartphone-based photogrammetry has demonstrated that detailed anatomical models can be produced without expensive imaging systems or specialized infrastructure, thereby making the approach especially appropriate for veterinary institutions with limited financial and technical resources (Chang Chan et al., 2024). The findings of Chang Chan et al. (2024) are consistent with the present results, demonstrating clear and detailed 3D anatomical models using smartphone-based photogrammetry. As a result, these accessible digital models have the potential to enhance spatial visualization and supplement traditional anatomy teaching, especially for students with lower spatial visualization abilities as reported by Höffler (2010). However, Schirone et al. (2024) found no notable advantage despite students' preference for 3D models.

The modified Elnady plastination technique resulted in durable, dry, and well-preserved anatomical specimens. The current results aligned with the findings of Elnady et al. (2015), Elnady (2016), and Bernal et al. (2022), who demonstrated that glycerin-based plastination produced durable teaching specimens without requiring vacuum impregnation. Additionally, Latorre et al. (2007) observed excellent preservation of gross morphology in veterinary specimens prepared using conventional plastination techniques, which is consistent with the current findings. In the present study, the modified Elnady technique achieved similar anatomical preservation through a simpler, more cost-effective process, highlighting its suitability for resource-limited veterinary institutions, as previously demonstrated in Egypt (Elnady, 2016). While the gross morphology of the plastinated specimens was largely maintained, shrinkage was observed in the testicular parenchyma, whereas the heart, kidney, and penis exhibited no notable deformation. Elnady (2016) similarly reported that tissue shrinkage during plastination depends on tissue composition, specimen size, and dehydration conditions, with soft parenchymatous tissues being more susceptible than dense fibrous tissues. The shrinkage observed in the bull testicular parenchyma in the present study was likely attributable to both acetone-induced dehydration and lipid extraction, consistent with the mechanism described by von Hagens et al. (1987). During dehydration, acetone removes water from tissues and dissolves lipids, reducing tissue volume and causing shrinkage. Despite the reduction in testicular parenchyma, the tunica albuginea and external anatomical landmarks remained intact, preserving the overall morphology of the specimens. These findings suggested that the modified Elnady plastination technique offered a practical balance among anatomical preservation, specimen durability, affordability, and procedural simplicity, supporting its applications in routine veterinary anatomy teaching.

CONCLUSION

The present study demonstrated that smartphone-based photogrammetry and the modified Elnady plastination technique were complementary, low-cost tools for veterinary gross anatomy education. These techniques provided interactive digital models and durable anatomical specimens that support practical instruction while reducing reliance on costly equipment and traditional preservation methods, especially for veterinary institutions with limited financial and technical resources. Using digital models for visual learning alongside plastinated specimens for tactile learning provides a multimodal educational approach that addresses different learning preferences. The present study was limited by the small sample size and the absence of student-based evaluation of the educational effectiveness of 3D imaging and plastination techniques. Future studies should investigate the educational impact of these techniques through comprehensive student-based assessments, including anatomical knowledge, understanding of the 3D relationships among anatomical structures, student engagement, and satisfaction.

DECLARATIONS

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Authors' contributions

Ayoub Ben Hadj contributed to the study's conceptualization, methodology, and investigation. Abdelrahman Mohamed Ali Elseory contributed to the investigation, provided funding, and helped review and edit the manuscript. Mohammed Sami Aleid contributed to the conceptualization, methodology, and investigation. Abdulla Al-Ramadan contributed to the conceptualization, methodology, and investigation. Mortada Mahgoub Osman Elhassan contributed to the review and editing of the manuscript. Lemiaa Eissa contributed to the conceptualization and editing of the manuscript. Abdelhay Mohamed Ali contributed to the review and editing of the manuscript. Thnaian Ali Al-Thnaian contributed to the review and editing of the manuscript and supervised the study. All authors read and approved the final edition of the manuscript.

Availability of data and materials

All data generated and analyzed during this study are included in this manuscript. No supplementary data files were generated. Requests for additional information related to this study should be directed to the corresponding author upon reasonable request.

Competing interests

The authors declare no conflicts of interest.

Ethical considerations

The authors confirmed that this manuscript is original, has not been published previously, is not under consideration for publication elsewhere, and contains no redundant publication, plagiarism, data fabrication, data falsification, or other forms of scientific misconduct. The authors employed the Superhuman AI Writing Assistant (Superhuman Platform Inc., USA, Version 2024.6) to enhance the manuscript's grammar and linguistic quality. The authors meticulously reviewed, edited, and authenticated all AI-generated suggestions and take full responsibility for the manuscript's accuracy, integrity, and final content. All authors have approved the originality of the manuscript before submission and during the peer review process, and accept full responsibility for its content.

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Effects of *Acetobacter* species Isolated from Palm Vinegar on TNF-α and Blood Profile in Rats

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ABSTRACT

Palm vinegar (*Arenga pinnata*), a traditional fermented product, contains acetic acid bacteria, particularly *Acetobacter* sp., which may serve as a natural immunomodulatory agent. The current study aimed to evaluate the effects of *Acetobacter* sp. isolated from palm vinegar on the immune response of white rats. A total of 24 male rats, aged 6-8 weeks and weighing 150-200 g, were randomly assigned to four groups of six rats each. The study included a control group that received normal saline and three treatment groups that received McFarland at doses of 0.5 (1.5×10^8 CFU/mL), 2 (6.0×10^8 CFU/mL), and 4 (1.2×10^9 CFU/mL) orally over 21 days. *Acetobacter* sp. was administered once a day for 14 days, and effects were monitored through blood samples collected weekly. Serum samples were collected during the first and second weeks of observation to assess tumor necrosis factor-alpha (TNFα) levels by enzyme-linked immunosorbent assay (ELISA) and for hematological examination. The results showed no significant difference in TNF-α levels between the first and second weeks. In the first week, mean TNF-α decreased in a dose-dependent manner, with the lowest value in the McFarland 4 group. During the second week of observation, measured parameters fluctuated across the treatment groups. Levels in the control group and McFarland 2 increased, while the McFarland 0.5 and 4 groups continued to decrease. Red blood cell (RBC) and WBC analysis showed no significant differences between observation times and between treatment groups. Descriptively, in the first week, the control group had the highest score and McFarland's 0.5 the lowest, but the values at doses 2 and 4 remained within the normal physiological range. In the second week, most groups showed an upward trend, except McFarland 0.5, which remained low. It can be concluded that *Acetobacter* sp. isolated from palm vinegar is effective as an immunomodulatory agent through a decrease in TNF-α levels. The stability of RBC and WBC parameters within normal limits confirms the safety of these bacteria, making them functional probiotic candidates that do not trigger systemic inflammation or blood balance disorders.

Keywords: *Acetobacter*, Palm vinegar, Probiotic, Tumor necrosis factor-alpha

INTRODUCTION

Indonesia is a biodiversity-rich region with a wide variety of plants that provide significant benefits for human life. One such plant is the sugar palm (*Arenga pinnata*), which belongs to the *Arecaceae* family (Martini et al., 2012). Local communities have long used the *Arenga pinnata* plant in daily life, particularly as a food source (Nuryanti et al., 2015). Palm sap is a sweet, clear liquid with a distinctive aroma obtained from the inflorescence of palm trees such as *Arenga pinnata* (Kurniawan et al., 2025). Fresh sap contains about 80-85% water, 15% sucrose, 7.61% glucose, 4.17% fructose, 1.5% minerals, and 0.8% vitamins, making it a suitable substrate for fermentative microorganisms (Saputro et al., 2019). Dominant microorganisms involved in sap fermentation include acetic acid bacteria, particularly *Acetobacter* sp. (Rastina et al., 2026). One product of palm sap fermentation is palm vinegar (Igbinadolor, 2009).

Palm vinegar is a natural preservative that is relatively safe for consumption and readily available (Masyitah et al., 2017). It is produced by naturally fermenting fresh palm sap in closed containers. The fermentation process begins with activity, particularly *Saccharomyces cereviceae*, followed by oxidative fermentation (aerobic respiration) by acetic acid bacteria such as *Acetobacter* sp. This bacterium has been reported to possess probiotic potential (Ilahi et al., 2025). *Acetobacter* is a Gram-negative bacterium with ellipsoidal to rod-shaped (bacillary) morphology, which may appear as single cells, pairs, or chains (Guerrero, 2001). It exhibits aerobic metabolism and plays a key role in fermentation by oxidizing ethanol produced by *Saccharomyces cereviceae* and other carbohydrates into acetic acid (Artnarong et al., 2016).

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Several studies show that acetic acid bacteria have probiotic potential because they can inhibit pathogenic bacteria, modulate gut microbiota, and enhance host immunity. Furthermore, bioactive compounds in palm vinegar, such as polyphenols and flavonoids, support the immunomodulatory role of acetic acid bacteria by contributing to antioxidant activity and antibody production (Dwisandi et al., 2021). In addition, *Acetobacter* sp. produces antimicrobial metabolites that can inhibit the growth of pathogenic bacteria in the gastrointestinal tract (Rastina et al., 2026), while also supporting gut microbiota balance and digestive processes through its enzymatic activity (Wei et al., 2017)

The effects of *Acetobacter* sp. administration as a probiotic candidate on systemic health can be evaluated through tumor necrosis factor-alpha (TNFα) levels and hematological parameters, as blood serves as an important indicator of immunological and metabolic status (Fikri et al., 2025). Oral probiotic administration has been reported to influence hematological parameters, including erythrocyte and leukocyte counts and hemoglobin levels, which are associated with oxygen transport, immune response, and tissue repair (Quinn et al., 2016). Imbalances in hematological parameters may reflect infection, inflammation, or metabolic disturbances (Ayodeji and Babafemi, 2020). Palm vinegar has long been used in culinary applications in Acehnese dishes (Erfiza et al., 2018). However, its potential as a natural probiotic source remains underexplored. Consequently, the present study aimed to determine the efficacy of *Acetobacter* sp. from palm vinegar as a natural probiotic by examining its impact on immune regulation and the maintenance of systemic equilibrium in rats.

MATERIALS AND METHODS

Ethical approval

All experimental procedures involving animals were conducted in strict accordance with the ethical guidelines established by the Veterinary Ethics Committee, Faculty of Veterinary Medicine, Syiah Kuala University, Indonesia (Approval No. 456/KEPH/XII/2025).

Study period and location

The study was conducted from October to November 2025 at the Faculty of Veterinary Medicine, Universitas Syiah Kuala, Banda Aceh, Indonesia.

Preparation of *Acetobacter* species

The procedure for identifying *Acetobacter* sp. bacteria isolated from palm vinegar (Arenga pinnata) began by taking 1 mL of vinegar and transferring it into a test tube. Next, the Pepton Yeast Glucose Broth (PYGB) medium (USA) was added to the tube and homogenized. The mixed suspension was incubated at 28°C for 24-48 hours. After incubation, 1 mL of culture was transferred to a Petri dish, then PYG agar medium was added and homogenized. After the agar hardened, the Petri dish was incubated upside down at 28°C for 24-48 hours. Bacterial colonies were then identified by Gram staining and a series of biochemical tests, including Indole, Methyl Red, Voges-Proskauer, and Citrate tests. In addition, fermentation tests were carried out on various types of sugar such as maltose, sucrose, lactose, glucose, and mannitol. All fermentation tests were carried out by incubation for 24 hours at a temperature of 37°C (Ilahi et al., 2025).

Study design

A total of 24 male white rats, aged 6-8 weeks and weighing 150-200 g, were purchased from the Integrated Service Unit of Experimental Animals, Faculty of Veterinary Medicine, USK, Aceh, Indonesia, and maintained under standard laboratory conditions. The rats were acclimatized in their cages for one week before the study began. The rats were maintained in clean plastic cages with a stainless-steel top grill that provided proper aeration and a 12-hour light/dark cycle (Agustiningsih, 2025). The cages were cleaned weekly to maintain a clean environment. The white rats received pelleted feed (Rat Bio 1, Indonesia) and unrestricted access to water (Pritchett and Corning, 2004).

Administration of *Acetobacter* species

A total of 24 white rats were divided into 4 groups; treatment group one received standard feed and 5 mL distilled water and served as the test control. This treatment used McFarland's standard method to determine the bacterial concentration. Treatment group tow included of *Acetobacter* sp. bacteria with a McFarland dose of 0.5 (1.5×10^8 CFU/mL) as a low dose, then the Treatment group three was given standard feed and *Acetobacter* sp. bacteria with a dose of McFarland 2 (6.0×10^8 CFU/mL) and the treatment group four was given standard feed and *Acetobacter* sp. with a dose of McFarland 4 (1.2×10^9 CFU/mL as a high dose (Ahmadi-Soleimani et al., 2026). The experimental procedure involved daily oral administration of *Acetobacter* sp. via orogastric intubation (1 mL per rat) for 21 days. Blood and serum samples were collected from each group at weekly intervals to evaluate TNF-α levels and comprehensive hematological profiles.

Sample collection

Following a seven-day acclimatization period (week 1), blood samples were collected on day 14 (week 2) and day 21 (week 3). The male white rats were placed under mild anesthesia (ophthalmic drops, Indonesia) during the procedure. Approximately 1-3 mL of blood was collected from each rat via the retro-orbital sinus. The samples were then distributed into micro tubes containing ethylenediaminetetraacetic acid (EDTA) for hematological assessment and into plain tubes (without anticoagulants) to measure TNF-α levels via Enzyme-linked immunosorbent assay (ELISA).

Tumor necrosis factor-alpha

Tumor necrosis factor-alpha levels were measured using serum samples obtained from blood collected via the retro-orbital sinus under anesthesia using a combination of ketamine100 mg/kg and xylazine 10 mg/kg, respectively (Rastina et al., 2026). The collected blood was placed in tubes without anticoagulants to allow serum separation, then centrifuged at 3000 rpm for 15 minutes. The resulting supernatant (serum) was then collected and analyzed for TNF-α levels using a Rat TNF-α ELISA kit (Bioassay Technology Laboratory, Cat. No. E0764Ra, China; Diehl et al., 2001; Parasuraman et al., 2010).

Hematological analysis

Hematological analysis was performed using an automated hematology analyzer (Licare CC-3200Vet, China). Blood samples were collected on day 14 (week 2) and day 21 (week 3) into EDTA vacutainer tubes via the retro-orbital sinus under mild anesthesia (ophthalmic drops, Indonesia), with approximately 50 µL of EDTA-treated blood obtained from each animal, following the manufacturer's instructions. Analyzed parameters included red blood cell (RBC) count, hemoglobin (Hb) concentration, hematocrit (HCT) value, and white blood cell (WBC) count (Lippi et al., 2005).

Statistical analysis

Comparisons between treatment groups and observation time points for all measured parameters were performed using Two-Way Repeated-Measures Analysis of Variance (ANOVA). Duncan's multiple range test was applied as a post hoc analysis using SPSS® version 25. Statistical significance was set at a p-value less than 0.05.

RESULTS

Tumor necrosis factor-alpha

The results showed no significant difference in TNF-α levels between the first and second weeks of observation ($p > 0.05$; Table 1). However, descriptively, mean TNF-α levels showed a decreasing trend in the first week with increasing doses of *Acetobacter* sp., with the McFarland 4 group exhibiting the lowest mean value among all groups ($p > 0.05$). In the second week, TNF-α levels exhibited fluctuations across treatment groups. The control and McFarland 2 groups showed a non-significant increase in mean TNF-α levels compared with the first week ($p > 0.05$). In contrast, the McFarland 0.5 and McFarland 4 groups showed a non-significant decrease ($p > 0.05$). The standard deviations recorded during the second week reflect high inter-individual variation in biological responses within each group. TNF-α levels differed significantly among treatment doses ($p < 0.05$). The results demonstrated that the McFarland 4 group formed a distinct subset, significantly different from the control and McFarland 2 groups ($p < 0.05$). Meanwhile, the McFarland 0.5 group formed a subset, showing no significant difference compared with the control, McFarland 2, or McFarland 4 groups ($p > 0.05$).

Table 1. Tumor necrosis factor-alpha levels were measured in male rats aged 6–8 weeks during the first and second weeks after administration of *Acetobacter* sp.

Parameters		Treatment groups (Mean ± SD)			
	Time interval (week)	**Control**	**McFarland 0.5**	**McFarland 2**	**McFarland 4**
Tumor Necrosis factor-alpha pg/mL)	First week	31.82 ± 19.19	28.05 ± 18.22	25.11 ± 13.40	17.93 ± 15.88
	Second week	32.67 ± 27.83	5.80 ± 6.27	37.85 ± 32.91	5.23 ± 9.74
	Mean ± SE	32.24 ± 5.39^{b}	16.93 ± 5.39^{ab}	31.48 ± 5.39^{b}	11.58 ± 5.39^{a}

^{a,b,c,d} Means within a row with different superscripts differ significantly ($p < 0.05$). SD: Standard deviation, SE: Standard error

Red blood cell profile

Red blood cell analysis showed no significant differences between the first and second weeks of observation in rats administered *Acetobacter* sp. ($p > 0.05$; Table 2). During the first week, the control group had higher RBC values than the treatment groups, with the lowest value in the McFarland 0.5 group; however, these differences were not significant ($p > 0.05$). Concurrently, the McFarland 2 and McFarland 4 groups showed fluctuating RBC counts that remained within the established physiological reference range for white rats (5.6-7.89 × 10^6/µL), with no statistically significant variation when compared to the control group ($p > 0.05$). In the second week, RBC values were observed in the control, McFarland 2, and McFarland 4 groups, whereas the McFarland 0.5 group remained at relatively lower values. Although descriptive fluctuations occurred, all RBC counts remained within the normal physiological range of 5.6-7.89 × 10^6/µL, and results showed no significant differences across the treatment groups ($p > 0.05$). However, RBC levels did not differ significantly across treatment groups ($p > 0.05$). The results indicated that all groups were classified within the same subset, suggesting no statistically significant variation in RBC values among groups ($p > 0.05$).

Hemoglobin (Hb) results in Table 2 remained stable throughout the study, as statistical analysis showed no significant effect of observation time between the first and second weeks ($p > 0.05$). Descriptively, the control group had a higher mean Hb level compared to all treatment groups, while the McFarland 0.5 group recorded the lowest value. The McFarland 2 and McFarland 4 groups showed relatively comparable Hb levels. Although numerical variations were observed, Hb levels did not differ significantly among the treatment groups ($p > 0.05$).

In the second week, Hb levels increased in the McFarland 2 and McFarland 4 groups, while the McFarland 0.5 group showed lower Hb values than the other groups. The results revealed no significant differences in Hb levels among treatment groups ($p > 0.05$).

The haematocrit (HCT) results in Table 2 for rats administered *Acetobacter* sp. showed no significant effect of observation time between the first and second weeks ($p > 0.05$). Descriptively, during the first week, the control group tended to have higher HCT values than the treatment groups, with the lowest value in the McFarland 0.5 group; however, statistical analysis showed no significant differences among groups ($p > 0.05$) across all treatment doses. The McFarland 2 and McFarland 4 groups showed fluctuating hematocrit (HCT) levels within the established normal physiological range for white rats (36.0-46.0%) and no statistically significant differences compared with the control group ($p > 0.05$). In the second week, HCT values exhibited an increasing trend in the control, McFarland 2, and McFarland 4 groups, while the McFarland 0.5 group continued to show relatively lower values; however, statistical analysis confirmed that these variations were not significant ($p > 0.05$), as all experimental groups were categorized into a single homogeneous subset, confirming the absence of statistically significant differences. However, statistical analysis indicated no significant differences among treatment groups ($p > 0.05$).

Table 2. Red blood cell parameters were measured in male rats aged 6-8 weeks during the first and second weeks after administration of *Acetobacter* sp.

Parameters		**Treatment groups (mean ± SD)**			
Red blood cells	**Time interval (mean ± SD)**	**Control**	**McFarland 0.5**	**McFarland 2**	**McFarland 4**
(erythrocytes) (10^6/µL)	First week	31.82 ± 19.19	28.05 ± 18.22	25.11 ± 13.40	17.93 ± 15.88
	Second week	32.67 ± 27.83	5.80 ± 6.27	37.85 ± 32.91	5.23 ± 9.74
	Mean ± SE	32.24 ± 5.39^{b}	16.93 ± 5.39^{ab}	31.48 ± 5.39^{b}	11.58 ± 5.39^{a}
Hemoglobin (g/dL)	First week	4.34 ± 0.91	2.88 ± 3.58	2.99 ± 2.72	2.99 ± 2.77
	Second week	4.88 ± 2.18	2.16 ± 1.03	5.42 ± 1.05	6.05 ± 2.21
	Mean ± SE	4.61 ± 0.95^{a}	2.52 ± 0.95^{a}	4.20 ± 0.95^{a}	4.52 ± 0.95^{a}
Hematocrit (%)	First week	28.2 ± 7.10	17.7 ± 20.6	18.1 ± 15.5	19.5 ± 19.1
	Second week	29.8 ± 12.5	13.8 ± 5.95	27.7 ± 16.5	37.7 ± 13.9
	Mean ± SE	29.0 ± 5.85^{a}	15.7 ± 5.85^{a}	22.9 ± 5.85^{a}	28.6 ± 5.85^{a}

^{a,b,c,d} Means within a row-with different superscripts differ significantly ($p < 0.05$). SD: Standard deviation, SE: Standard error

White blood cell profile

White blood cell counts showed no significant differences between the first and second weeks of observation in rats administered *Acetobacter* sp. ($p > 0.05$; Table 3). In the first week of observation, WBC counts were highest in the control group and lowest in the McFarland 0.5 group, with significant variations observed across all treatment doses

($p > 0.05$). Although the McFarland 2 and 4 groups showed variable values, their counts remained within the established physiological limits for rats (2.9-15.3 $\times 10^3/\mu L$).

In the second week, WBC values tended to decrease in the control group. In contrast, during the second week, the treatment groups showed more uniform leukocyte profiles, particularly in the McFarland 2 group, whose values closely mirrored the control group. Across treatment groups, WBC counts differed significantly ($p < 0.05$).

The analysis of lymphocyte counts showed no significant effect of observation time between the first and second weeks ($p > 0.05$; Table 3). Descriptively, during the first week, the highest lymphocyte counts were observed in the control group, while the lowest values were found in the McFarland 0.5 group, followed by the McFarland 2 and McFarland 4 groups. In the second week, lymphocyte levels in the control group tended to decrease, whereas the McFarland 2 and McFarland 0.5 groups showed increases ($p > 0.05$). The results indicated significant differences in lymphocyte counts ($p < 0.05$). Monocyte analysis showed no significant differences between observation times ($p > 0.05$; Table 3). Descriptively, during the first week, the highest monocyte values were observed in the control group, while the lowest values were found in the McFarland 0.5 group, followed by the McFarland 2 and McFarland 4 groups. In the second week, monocyte values decreased in the control group but remained relatively stable in the treatment groups. The results indicated significant differences among treatment groups ($p < 0.05$).

Granulocyte analysis showed in Table 3 that observation time had no significant effect between the first and second weeks ($p > 0.05$). Descriptively, during the first week, the highest granulocyte values were observed in the control group, while the lowest values were found in the McFarland 0.5 group, followed by the McFarland 2 and McFarland 4 groups. In the second week, granulocyte levels decreased in the control group, while treatment groups remained relatively stable, with a slight increase in the McFarland 0.5 group. Despite these descriptive variations, the results revealed no significant differences among treatment groups ($p > 0.05$).

The results showed no significant differences in eosinophil percentages between the first and second weeks of observation ($p > 0.05$; Table 3). Descriptively, eosinophil values were relatively uniform across all groups during the first week. Meanwhile, the McFarland 2 and McFarland 4 groups exhibited variable RBC values that remained within the normal physiological range ($p > 0.05$).

Table 3. White blood cell parameters were measured in male rats aged 6-8 weeks during the first and second weeks after administration of *Acetobacter* sp.

Parameters		**Treatment groups (mean ± SD)**			
Red blood cell	**Time interval (mean ± SD)**	**Control**	**McFarland 0.5**	**McFarland 2**	**McFarland 4**
Erythrocytes ($10^6/\mu L$)	First week	31.82 ± 19.19	28.05 ± 18.22	25.11 ± 13.40	17.93 ± 15.88
	Second week	32.67 ± 27.83	5.80 ± 6.27	37.85 ± 32.91	5.23 ± 9.74
	Mean ± SE	32.24 ± 5.39^b	16.93 ± 5.39^{ab}	31.48 ± 5.39^b	11.58 ± 5.39^a
Hemoglobin (g/dL)	First week	4.34 ± 0.91	2.88 ± 3.58	2.99 ± 2.72	2.99 ± 2.77
	Second week	4.88 ± 2.18	2.16 ± 1.03	5.42 ± 1.05	6.05 ± 2.21
	Mean ± SE	4.61 ± 0.95^a	2.52 ± 0.95^a	4.20 ± 0.95^a	4.52 ± 0.95^a
Hematocrit (%)	First week	28.2 ± 7.10	17.7 ± 20.6	18.1 ± 15.5	19.5 ± 19.1
	Second week	29.8 ± 12.5	13.8 ± 5.95	27.7 ± 16.5	37.7 ± 13.9
	Mean ± SE	29.0 ± 5.85^a	15.7 ± 5.85^a	22.9 ± 5.85^a	28.6 ± 5.85^a
White blood cell	**Time interval (mean ± SD)**	**Control**	**McFarland 0.5**	**McFarland 2**	**McFarland 4**
Leukocyte ($10^3/\mu L$)	First week	20.2 ± 3.74	1.67 ± 1.58	9.63± 8.16	7.56± 9.26
	Second week	9.10 ± 6.36	8.23 ± 3.83	9.70 ± 2.35	4.86 ± 1.78
	Mean ± SE	14.6 ± 2.19^b	4.95 ± 2.19^a	9.66 ± 2.19^{ab}	6.21 ± 2.19^a
Lymphocyte (cells/L)	First week	7.40 ± 1.11	0.76 ± 0.55	3.10 ± 1.37	3.16 ± 3.85
	Second week	4.70 ± 3.40	2.76 ± 1,19	4.26 ± 2.15	2.60 ± 0.08
	Mean ± SE	6.05 ± 0.90^b	1.76 ± 0.90^a	3.68 ± 0.90^{ab}	2.88 ± 0.90^a
Monocyte ($10^3/\mu L$)	First week	0.83 ± 0.32	0.33 ± 0.05	0.50 ± 0.60	0.23 ± 0.32
	Second week	0.30 ± 0.17	0.33 ± 0.15	0.30 ± 0.17	0.16 ± 0.05
	Mean ± SE	0.56 ± 0.10b	0.18 ± 0.10a	0,.0 ± 0.10ab	0.20 ± 0.10a
Granulocyte (cells/L)	First weeks	11,9 ± 2.90	0.86 ± 0.98	6.03 ± 6.22	4.16 ± 5.09
	Second weeks	4.10 ± 2.78	5.13 ± 2.51	5.13 ± 1.65	2.10 ± 0.90
	Mean ± SE	8.03 ± 1.50a	3.00 ± 1.50a	5.58 ± 1.50a	3.13 ± 1.50a
Eosinophil (%)	First weeks	2.73 ± 0.11	3.20 ± 0.40	2.80 ± 0.26	2.83 ± 0.05
	Second weeks	3.03 ± 0.11	2.63 ± 0.05	2.83 ± 0.55	3.10 ± 0.10

	Mean ± SE	2.88 ± 0.13a	2.91 ± 0,13a	2.81 ± 0.13a	2.96 ± 0.13a

[a,b,c,d] Means within a row with different superscripts differ significantly ($p < 0.05$). SE: Standard error.

DISCUSSION

Immunomodulatory effects on tumor necrosis factor-α level

Acetobacter sp. isolated from palm vinegar demonstrated a significant dose-dependent reduction in serum TNF-α levels, particularly at the McFarland 4 dose. As a pro-inflammatory cytokine, TNF-α is a critical biomarker for immune activation; its suppression suggests that the probiotic candidate effectively regulates systemic inflammation (Abbas et al., 2025). The immunomodulatory effect likely involves interactions between bacterial structural components such as peptidoglycan and exopolysaccharides with pattern recognition receptors, which modulate the NF-κB signaling pathway to prevent excessive inflammatory responses (Kim et al., 2023).

The fluctuations observed in the second week across different doses may reflect trained immunity or immune priming, where prior microbial exposure dynamically enhances innate cytokine responses to eventually reach a stable, regulated homeostatic state (Netea et al., 2020).

Stability of erythrocyte indices

Analysis of RBC counts, hemoglobin, and hematocrit revealed no statistically significant differences across observation periods or treatment groups. The observed numerical variations, particularly the slight dip in the first week, can be explained by a single unified mechanism: the initial physiological adaptation to Gram-negative bacterial exposure.

Lipopolysaccharides (LPS) in *Acetobacter* sp. can trigger a transient innate immune response, increasing TNF-α levels, which in turn stimulates hepatic hepcidin synthesis (Ganz and Nemeth, 2015). Elevated hepcidin restricts iron availability for erythropoiesis by inhibiting ferroprotein-mediated iron export, a process characteristic of mild "anemia of inflammation" (Weiss et al., 2019). However, stabilization and an upward trend in RBC, Hb, and HCT levels by the second week, especially in higher-dose groups, suggest that the host adapted successfully to the treatment (Markowiak and Śliżewska, 2017). *Acetobacter* sp. administration does not induce hemolysis or hematotoxicity and may eventually support erythrocyte stability by improving nutrient absorption through gut microbiota modulation (Wen et al., 2023).

Dynamics of leukocyte profiles and immune priming

Some WBC parameters, including lymphocytes and monocytes, varied significantly between groups; however, all values remained strictly within the normal physiological range for rats (Kurniawan et al., 2020). The initial decrease in circulating leukocytes and lymphocytes during the first week likely represents "leukocyte redistribution," where immune cells migrate from peripheral circulation to gut-associated lymphoid tissues (GALT) upon first encountering the probiotic (Trinh et al., 2023). The subsequent stabilization in the second week indicates an adaptive immune adjustment rather than pathological suppression (Tari et al., 2018).

Furthermore, stable granulocytes and eosinophils across all groups confirmed the safety of the bacterial administration. The slight descriptive increases in granulocytes at higher doses may reflect innate immune priming, where bacterial metabolites such as acetate interact with receptors such as FFAR2/FFAR3 to enhance the readiness of the nonspecific immune system without triggering acute systemic inflammation (Mousa et al., 2023). The absence of eosinophilia or sustained leukocytosis further supports that *Acetobacter* sp. is a safe, non-allergenic immunomodulatory agent that maintains immune homeostasis (Balta et al., 2021).

CONCLUSION

Administration of *Acetobacter* sp. from palm vinegar in rats significantly reduced TNF-α levels, with the most pronounced effect observed at the McFarland 4 dose. Red blood cell parameters remained stable and within physiological ranges, indicating no adverse effects on erythrocyte homeostasis. White blood cell parameters fluctuated significantly in the 0.5 and 4 McFarland treatment groups compared with the control group; however, these values remained within normal limits and did not indicate systemic inflammation. These findings suggest that *Acetobacter* sp. is safe for use and has potential as a probiotic candidate that can modulate immune responses without disrupting hematological balance. Future studies should identify the specific molecular mechanisms and signaling pathways, such as NF-κB regulation and gut microbiota interaction, to better understand the precise mode of action by which *Acetobacter* exhibits its immunomodulatory potential. Additionally, further studies are warranted to evaluate the

therapeutic efficacy of *Acetobacter* sp. in specific disease models, such as chronic inflammatory or infectious conditions, to confirm its clinical potential beyond healthy physiological states.

DECLARATIONS

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Authors' contributions

Rastina was responsible for the overall design and execution of the study, data collection, and analysis. Writing the manuscript and ensured all necessary revisions were made by all authors. Darmawi assisted in the conceptualization of the study and contributed to the experimental design. Additionally, Safika helped with data analysis and interpretation. Zinatul Hayati contributed to the literature review and interpretation of results, and Winda Yulia contributed to final 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 the present study should be directed to the corresponding author.

Competing interests

The authors declare no conflicts of interest.

Ethical considerations

The authors confirm that the present study is original, has not been previously submitted for publication or under review elsewhere, and adheres to strict ethical standards regarding data integrity and the avoidance of plagiarism. The authors explicitly confirm that they take full responsibility for all use of artificial intelligence in the preparation of the present study, and they did not use any AI tools for conducting the study or preparing this manuscript. The authors affirm sole responsibility for the accuracy, originality, and integrity of the final content before submission, during the revisions, and throughout the final publication of the article.

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Effects of Dietary *Cordyceps militaris* on Sperm Quality and Reproductive Performance in Rabbits

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ABSTRACT

Cordyceps militaris is a medicinal mushroom rich in bioactive compounds and has been investigated for its potential antioxidant properties. The present study aimed to evaluate the effects of dietary supplementation with *Cordyceps militaris* extract on semen quality and reproductive performance in male rabbits. A total of 15 adult male rabbits, with an average body weight of 3.56 ± 0.34 kg, were randomly assigned to five treatments receiving 0 (control), 20, 40, 80 and 160 mg of *Cordyceps militaris* extract daily for 14 weeks. Semen was collected at weeks 6, 8, 10, 12, and 14, with an average volume of 0.4 ± 0.1 mL per collection, and evaluated for sperm viability, motility, acrosome and membrane integrity, DNA fragmentation, and antioxidant capacity. A total of 15 adult female rabbits, with an average body weight of 3.37 ± 0.26 kg, were used for mating and were managed under the same experimental conditions. After eight weeks of supplementation, treated males were naturally mated with female rabbits to evaluate fertility and litter performance. Compared with the control group, *Cordyceps militaris* supplementation improved sperm motility, viability, membrane integrity, acrosome integrity, and antioxidant capacity, while reducing DNA fragmentation. The 40 mg/day treatment showed the most favourable overall effects on semen quality across the evaluation period. At week 8, rabbits receiving 40 mg/day showed higher sperm motility (74.34%), viability (90.85%), acrosome integrity (99.92%), membrane integrity (39.79%), and seminal antioxidant capacity (35.04%), together with lower DNA fragmentation (35.76%), compared with the control group. The 40 mg/day group showed a significantly higher pregnancy rate at first mating than the control group, reaching 100%. Rabbits that received 40 mg/day achieved a 100% first-mating pregnancy rate, while litter traits and offspring growth did not differ significantly among treatments. Overall, dietary supplementation with *Cordyceps militaris* improved semen quality and reproductive performance in male rabbits, with 40 mg/day providing the most favourable overall response among the tested supplementation levels.

Keywords: Antioxidant capacity, Dietary supplementation, DNA fragmentation, Sperm, Reproductive performance

INTRODUCTION

Rabbit farming is an important component of small-scale farming systems in many countries due to the nutritional value of rabbit meat, which is characterized by a high protein content and relatively low levels of fat and cholesterol (Gašperlin et al., 2006). Furthermore, rabbit farming is recognized as a potentially sustainable livestock production system due to their efficient feed utilization and high reproductive productivity (Siddiqui et al., 2024). Improving reproductive performance, particularly male fertility, is a key factor contributing to productivity and sustainability in rabbit farming systems (Fadl et al., 2024). Semen quality in breeding males is of particular importance, as reproductive efficiency directly influences conception rates, litter performance, and overall productivity in rabbit production (Brun et al., 2016). Nevertheless, male reproductive performance is susceptible to nutritional and environmental conditions, particularly oxidative stress (Agarwal et al., 2014). An imbalance between ROS production and antioxidant defenses can adversely affect sperm membrane function, motility, and DNA integrity, thereby compromising fertility (Agarwal et al., 2014; Sies, 2017).

Natural plant-based feed additives with antioxidant properties have received increasing attention as potential alternatives to synthetic antioxidant vitamins in livestock nutrition (Manuelian et al., 2021). Bioactive compounds derived from medicinal plants, herbs, and other natural sources have been studied for their potential to enhance animal health and reproductive performance through regulating oxidative status (Manuelian et al., 2021). Dietary antioxidant supplementation with vitamin C, vitamin E, and selenium has been reported to improve semen quality and reproductive performance in male rabbits, potentially through enhanced antioxidant status and attenuation of oxidative damage (Yousef et al., 2003; Fadl et al., 2024).

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Cordyceps militaris (*C. militaris*) is a medicinal fungus containing a variety of bioactive compounds, including cordycepin, adenosine, polysaccharides, and phenolic compounds (Jędrejko et al., 2021; Wang et al., 2022). Previous studies have reported antioxidant, anti-inflammatory, and immunomodulatory activities of *C. militaris* and its bioactive components, particularly polysaccharides and cordycepin (Miao et al., 2022; Phull et al., 2022). Recent studies in livestock have shown that supplementation with *C. militaris* or its spent mushroom substrate can enhance antioxidant capacity and reduce oxidative stress markers in pigs (Boontiam et al., 2020). In addition, dietary *C. militaris* supplementation has been reported to improve intestinal barrier function and modulate gut microbiota in pigs (Zheng et al., 2022). Furthermore, supplementation with fermented products from *C. militaris* has been reported to enhance reproductive development and support testicular function in male rats, suggesting a potential beneficial role in male reproduction (Lin et al., 2022). Thus, the findings suggest that the bioactive components of *C. militaris* may contribute to improved reproductive performance through their antioxidant and cytoprotective effects.

Although the biological properties of *C. militaris* have been investigated in several species, information regarding its effects on semen quality and reproductive performance in rabbits remains limited. Therefore, the present study aimed to evaluate the effects of different dietary supplementation levels of *C. militaris* extract on semen quality, antioxidant capacity, and reproductive performance in rabbits.

MATERIALS AND METHODS

Ethical approval

All experimental procedures were approved by the Can Tho University Animal Ethics Committee (Approval No. CTU-AEC24036), Can Tho City, Vietnam.

Animals

Rabbits were housed individually in elevated cages under natural environmental conditions. Throughout the experimental period, all rabbits were maintained under the same feeding conditions and were provided with fresh elephant grass (*Pennisetum purpureum*), fresh clean vegetables, and commercial pelleted rabbit feed (De Heus 5640, De Heus Vietnam, Vietnam). Fresh drinking water was provided *ad libitum*. The same types of feed were provided to all experimental animals throughout the study to maintain consistent nutritional conditions among experimental groups. Animals were monitored daily throughout the experiment.

Cordyceps militaris extract

Cordyceps militaris extract used in the current study was provided by the Institute of Pharmaceutical and Health Food, Thu Dau Mot University, Ho Chi Minh City, Vietnam (Nguyen et al., 2024). The extract was administered orally at doses of 0, 20, 40, 80, and 160 mg/day per rabbit according to the experimental treatments. The extract quality and composition were analyzed and certified in accordance with ISO/IEC 17025:2017 standards. The cordycepin content in the extract was determined to be 0.15% (w/w).

Study design

The study was conducted using 15 adult male rabbits (3.56 ± 0.34 kg) and 15 adult female rabbits (3.37 ± 0.26 kg) aged 9–12 months at the experimental farm of Can Tho University, Vietnam. A total of 15 adult male rabbits were randomly allocated to five dietary treatments (three rabbits in each treatment), receiving 0 (control), 20, 40, 80, and 160 mg of *C. militaris* extract daily for 14 weeks. The *C. militaris* extract was mixed into pelleted feed (De Heus 5640, De Heus Vietnam, Vietnam) with the daily diet throughout the experimental period. Semen was obtained by artificial vagina collection once per week. Assessments were performed at weeks 6, 8, 10, 12, and 14 to characterize sperm quality, including motility, viability, plasma membrane, and acrosomal integrity. Antioxidant status and sperm DNA fragmentation were also examined to further evaluate sperm functional and cellular integrity. After eight weeks of supplementation, treated males were naturally mated with female rabbits to evaluate reproductive performance. Pregnancy rate, gestation length, litter size, and offspring growth were recorded.

Assessment of sperm quality

Motility

Sperm motility was observed under an Eclipse Si microscope (Nikon, Japan) at 40× magnification. The percentage of total motile sperm was determined by the proportion of total motile sperm to the total number of sperm counted (Tran et al., 2023).

Viability

Sperm viability was assessed using the Eosin–Nigrosin vital staining method and examined under a light microscope (Nikon, Japan; Figure 1A). Sperm viability was determined following the method of Tran et al. (2023). Dead sperm cells were identified by their purple staining, while viable cells remained unstained. Viability was expressed as the proportion of live spermatozoa among the total number of sperm cells evaluated.

Membrane integrity

Sperm plasma membrane integrity was assessed using the hypo-osmotic swelling test (HOST) following the procedure described by Tran et al. (2025). Briefly, semen (20 µL) was diluted with 80 µL of hypo-osmotic solution and maintained at 37°C for 30 min. Following incubation, an aliquot of the suspension was transferred onto a glass slide and examined microscopically (400× magnification) to determine sperm membrane integrity. Spermatozoa showing coiled or swollen tails were considered to have intact plasma membranes, whereas spermatozoa without tail swelling were considered membrane-damaged (Figure 1B). At least 200 spermatozoa were evaluated per sample.

Acrosome integrity

Acrosome integrity was assessed using the Giemsa staining method described by Nguyen et al. (2025). Spermatozoa were fixed on glass slides and air-dried at room temperature for 10 minutes. The samples were then immersed in 96% ethanol for 15 seconds for fixation, followed by air-drying at room temperature (25ºC) for an additional five minutes. Subsequently, the slides were stained with 10% Giemsa solution for 10 minutes. After staining, the slides were gently rinsed with water, air-dried at room temperature, and examined under the microscope (Figure 1C). Spermatozoa with intact acrosomes exhibited purple staining at the head, whereas spermatozoa with damaged or incomplete acrosomes did not.

Antioxidant capacity

Seminal antioxidant activity was determined by measuring the ABTS radical-scavenging capacity based on the method of Nenadis et al. (2004), with slight modifications. Fresh semen (10 µL) was added to 990 µL of $ABTS^+$ working solution and allowed to react for 6 minutes at room temperature (28-30°C). The absorbance of the reaction mixture was recorded at 734 nm using a UV–Vis spectrophotometer (Thermo Fisher Scientific, USA). Each sample was analyzed in three independent measurements, and antioxidant activity was reported as the percentage of $ABTS^+$ radical scavenging.

DNA fragmentation

DNA fragmentation was assessed using the Sperm Chromatin Dispersion (SCD) method. Sperm samples were stained with Giemsa and observed under the microscope (Figure 1D). Spermatozoa with intact DNA displayed a halo around the nucleus, whereas those with fragmented DNA did not, as described by Absalan et al. (2012).

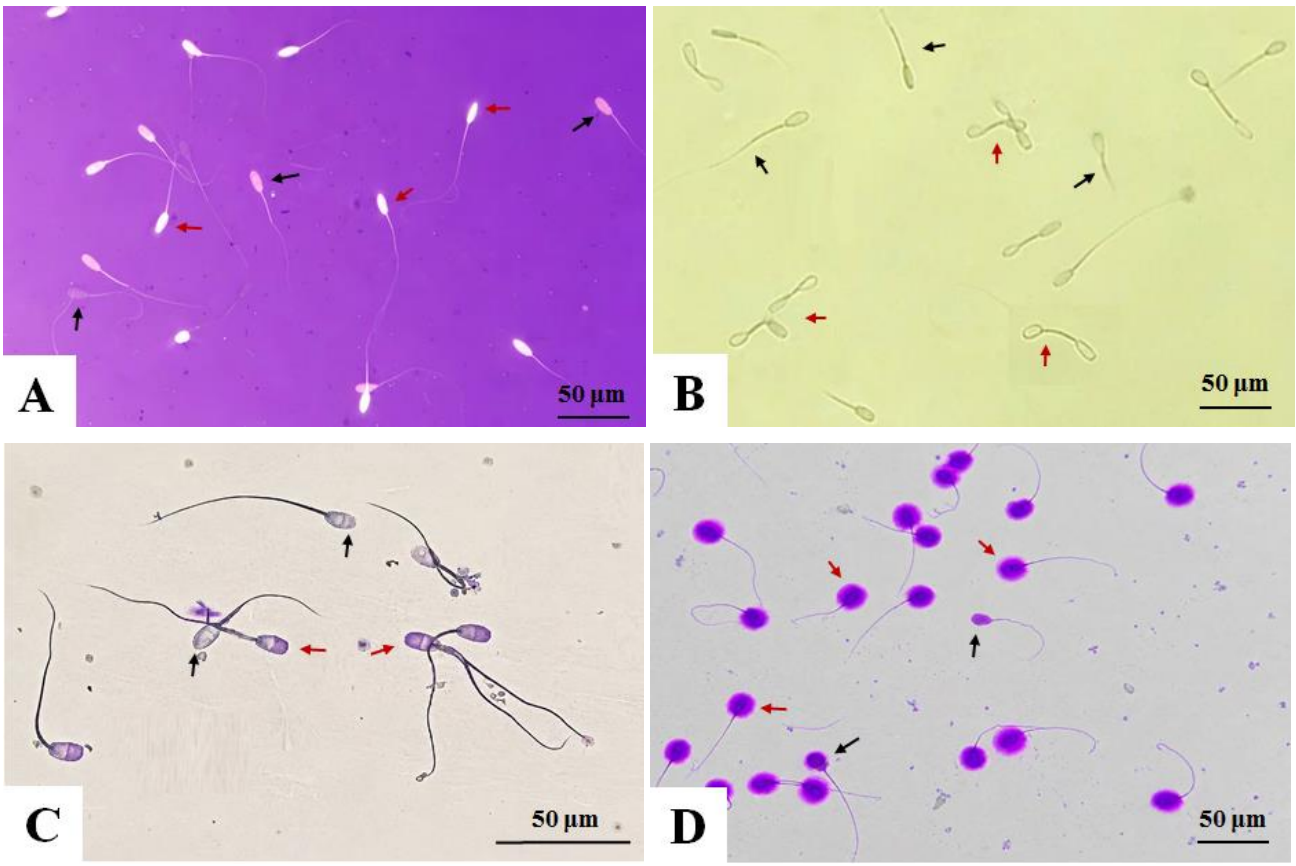


Figure 1. Representative photomicrographs of rabbit sperm used for semen quality evaluation. **A**: Viable spermatozoa (red arrow) and dead spermatozoa (black arrow), **B**: Spermatozoa with intact plasma membranes (red arrow) and damaged membranes (black arrow), **C**: Spermatozoa with intact acrosomes (red arrow) and damaged acrosomes (black arrow), **D**: Spermatozoa with non-fragmented DNA (red arrow) and fragmented DNA (black arrow).

Assessment of *in vivo* reproductive performance of female rabbits

The *in vivo* reproductive performance of female rabbits was evaluated based on pregnancy rate, gestation length, litter size, and offspring body weight. Mating was conducted in up to three consecutive rounds, and pregnancy was diagnosed 10 days after mating. Non-pregnant or pseudo-pregnant females were remated after a resting period of at least one month. Gestation length was calculated as the interval from mating to parturition and typically ranged from 28 to 32 days. Litter size was determined by counting the number of live-born kits at birth, while dead kits were recorded separately. Neonatal body weight was measured using an electronic balance before suckling, and total litter weight as well as average kit weight were calculated at birth and again at eye opening (10-12 days of age; Morshedy et al., 2022).

Statistical analysis

Data were analyzed using Minitab 16 software. Semen quality parameters measured at different sampling times were analyzed using repeated measures analysis of variance (ANOVA), with nutritional supplementation level as the inter-subject factor and sampling time as the intra-subject factor. When significant differences were detected, the mean values of the treatment groups were compared using Tukey's post hoc test. Reproductive performance and offspring growth parameters were analyzed using one-way ANOVA, with nutritional supplementation level as the primary factor, followed by Tukey's post hoc test. Normality of residuals and homogeneity of variance were assessed before statistical analysis. Data are presented as mean ± standard deviation (SD), and differences were considered statistically significant at p-value less than 0.05.

RESULTS

Semen quality

Semen quality parameters of male rabbits supplemented with different levels of *C. militaris* extract are presented in Table 1. Dietary supplementation affected several semen quality traits during the experimental period. In general, supplemented groups showed higher sperm motility, viability, membrane integrity, acrosome integrity, and antioxidant capacity, together with lower DNA fragmentation than the control group during weeks 6-12 ($p < 0.05$). Among the treatment groups, the 40 mg/day group generally produced the most favorable response across the measured parameters ($p < 0.05$). Subsequently, from week 14 onwards, several semen quality parameters declined significantly.

Overall sperm motility

Differences in sperm motility among dietary treatments became evident during the later stages of the experiment (Table 1). Between weeks 6 and 12, rabbits receiving *C. militaris* supplementation consistently maintained higher motility than those in the control group, with significant differences observed among all supplemented treatments ($p < 0.05$). Among the supplemented treatments, the 40 mg/day group generally showed the highest motility values, reaching 74.34% at week 8 and remaining above 70% until week 12 ($p < 0.05$). In comparison, sperm motility in the control group remained relatively stable at approximately 54-55% during the same period. Although supplementation levels of 80 and 160 mg/day also improved sperm motility compared with the control treatment, their values were not significantly higher than those observed in the 40 mg/day group ($p > 0.05$). Sperm motility changed significantly throughout the experimental period across all treatment groups from week 6 to week 14. Sperm motility increased from week 6 to week 8 and remained relatively stable through week 12, followed by a decline at week 14 in all treatment groups. Compared with the control group, sperm motility was significantly higher in the supplemented groups during weeks 6-12 ($p < 0.05$), whereas no significant difference was observed at week 14 ($p > 0.05$).

Sperm viability

Dietary supplementation with *C. militaris* increased sperm viability compared with the control group during weeks 6-12 (Table 1; $p < 0.05$). By week 14, no significant differences in sperm viability were detected among any of the treatment groups, including the control group ($p > 0.05$). The maximum viability was recorded at week 8 in rabbits receiving 40 mg/day. No significant differences in sperm viability were observed among the supplemented groups, suggesting that increasing the supplementation level beyond 20 mg/day did not provide an additional beneficial effect on sperm viability.

Membrane integrity

Supplementation with *C. militaris* extract improved sperm membrane integrity compared to the control group during weeks 6-12 ($p < 0.05$). Specifically, at week 8, the 40 mg group achieved the highest value (39.79%) compared to the control group (30.00%), and maintained high levels at week 10 (39.27% with 29.97%) and week 12 (38.53% with

29.61%). Doses of 20, 80, and 160 mg also showed higher values than the control group (approximately 36.4-37.7%), but there were no significant differences between these doses ($p > 0.05$). Overall, the 40 mg dose provided the best and most stable results across all time points. By week 14, sperm membrane integrity had decreased across all groups, with values ranging from approximately 28.39% to 30.01%. No significant differences were detected among the groups at this time point ($p > 0.05$).

Table 1. Effects of *Cordyceps militaris* extract supplementation on sperm quality parameters in 9-month-old male rabbits over 14 weeks

Parameter (%)	Dose (mg)	Week 6	Week 8	Week 10	Week 12	Week 14
Overall sperm motility	0	$55.08^{b} \pm 1.31$	$55.12^{c} \pm 1.19$	$55.06^{c} \pm 1.33$	$54.41^{c} \pm 1.22$	$54.19^{ab} \pm 1.35$
	20	$68.80^{a} \pm 1.23$	$70.05^{b} \pm 1.25$	$69.14^{b} \pm 1.23$	$67.83^{b} \pm 1.21$	$52.83^{b} \pm 0.94$
	40	$70.24^{a} \pm 1.15$	$74.34^{a} \pm 1.22$	$73.38^{a} \pm 1.20$	$71.99^{a} \pm 1.18$	$56.06^{a} \pm 0.92$
	80	$69.82^{a} \pm 1.50$	$71.08^{ab} \pm 1.53$	$70.16^{ab} \pm 1.51$	$68.83^{ab} \pm 1.44$	$53.60^{ab} \pm 1.15$
	160	$69.07^{a} \pm 0.53$	$69.97^{b} \pm 0.57$	$69.06^{b} \pm 0.49$	$67.76^{b} \pm 0.52$	$52.77^{b} \pm 0.40$
Membrane integrity	0	$29.97^{b} \pm 1.55$	$30.00^{b} \pm 1.67$	$29.97^{b} \pm 1.61$	$29.61^{b} \pm 1.59$	$29.49^{a} \pm 1.21$
	20	$37.01^{a} \pm 0.73$	$37.68^{a} \pm 0.93$	$37.19^{a} \pm 0.98$	$36.48^{a} \pm 0.97$	$28.41^{a} \pm 0.75$
	40	$37.59^{a} \pm 1.00$	$39.79^{a} \pm 1.05$	$39.27^{a} \pm 1.04$	$38.53^{a} \pm 1.02$	$30.01^{a} \pm 0.79$
	80	$36.12^{a} \pm 0.53$	$36.94^{a} \pm 1.14$	$37.44^{a} \pm 0.87$	$37.02^{a} \pm 0.81$	$27.82^{a} \pm 0.68$
	160	$36.97^{a} \pm 0.98$	$37.64^{a} \pm 1.07$	$37.15^{a} \pm 0.66$	$36.45^{a} \pm 0.76$	$28.39^{a} \pm 0.73$
Viability	0	$64.15^{b} \pm 2.31$	$64.21^{b} \pm 2.27$	$64.14^{b} \pm 2.12$	$63.37^{b} \pm 2.28$	$63.12^{a} \pm 2.35$
	20	$85.43^{a} \pm 2.89$	$86.98^{a} \pm 2.95$	$85.85^{a} \pm 2.83$	$84.23^{a} \pm 2.81$	$65.60^{a} \pm 2.20$
	40	$85.84^{a} \pm 4.54$	$90.85^{a} \pm 2.80$	$89.67^{a} \pm 2.74$	$87.98^{a} \pm 2.60$	$68.52^{a} \pm 2.62$
	80	$84.27^{a} \pm 2.14$	$87.70^{a} \pm 2.64$	$85.12^{a} \pm 2.67$	$84.59^{a} \pm 2.77$	$65.78^{a} \pm 2.32$
	160	$85.35^{a} \pm 2.62$	$86.89^{a} \pm 2.87$	$85.76^{a} \pm 2.45$	$84.14^{a} \pm 2.64$	$65.53^{a} \pm 2.29$
Acrosome integrity	0	$85.50^{b} \pm 3.08$	$85.57^{c} \pm 3.09$	$85.48^{c} \pm 3.01$	$84.46^{c} \pm 3.14$	$84.12^{a} \pm 3.03$
	20	$92.93^{a} \pm 1.39$	$94.61^{b} \pm 1.44$	$93.38^{b} \pm 1.40$	$91.62^{b} \pm 1.35$	$71.35^{b} \pm 1.06$
	40	$94.41^{a} \pm 1.41$	$99.92^{a} \pm 1.49$	$98.62^{a} \pm 1.38$	$96.76^{a} \pm 1.45$	$75.36^{b} \pm 1.13$
	80	$92.93^{a} \pm 1.33$	$94.61^{b} \pm 1.53$	$93.38^{b} \pm 1.46$	$91.62^{b} \pm 1.47$	$71.35^{b} \pm 1.10$
	160	$92.84^{a} \pm 1.25$	$94.52^{b} \pm 1.21$	$93.29^{b} \pm 1.33$	$91.53^{b} \pm 1.26$	$71.28^{b} \pm 1.23$
Antioxidant capacity	0	$22.60^{b} \pm 0.77$	$22.62^{b} \pm 0.83$	$22.60^{b} \pm 0.82$	$22.33^{b} \pm 0.76$	$22.24^{b} \pm 0.80$
	20	$33.01^{a} \pm 1.19$	$33.61^{a} \pm 1.22$	$33.18^{a} \pm 1.28$	$32.55^{a} \pm 1.12$	$25.35^{a} \pm 0.95$
	40	$33.54^{a} \pm 1.21$	$35.50^{a} \pm 1.18$	$35.04^{a} \pm 1.16$	$34.37^{a} \pm 1.24$	$26.77^{a} \pm 0.97$
	80	$33.01^{a} \pm 1.11$	$33.61^{a} \pm 1.27$	$33.18^{a} \pm 1.30$	$32.55^{a} \pm 1.37$	$25.35^{a} \pm 0.88$
	160	$32.98^{a} \pm 1.07$	$33.58^{a} \pm 1.11$	$33.14^{a} \pm 1.25$	$32.52^{a} \pm 1.33$	$25.32^{a} \pm 0.91$
DNA fragmentation	0	$45.38^{a} \pm 1.08$	$45.42^{a} \pm 1.12$	$45.37^{a} \pm 1.23$	$44.83^{a} \pm 1.09$	$44.65^{a} \pm 1.16$
	20	$38.24^{b} \pm 1.38$	$37.48^{bc} \pm 1.35$	$37.86^{bc} \pm 1.37$	$38.28^{bc} \pm 1.38$	$38.78^{bc} \pm 1.40$
	40	$37.62^{b} \pm 1.36$	$35.76^{c} \pm 1.29$	$35.98^{c} \pm 1.30$	$36.23^{c} \pm 1.31$	$36.55^{c} \pm 1.32$
	80	$38.24^{b} \pm 1.31$	$37.48^{bc} \pm 1.24$	$38.01^{bc} \pm 1.28$	$38.58^{bc} \pm 1.44$	$39.14^{bc} \pm 1.41$
	160	$39.06^{b} \pm 1.41$	$39.84^{b} \pm 1.44$	$40.56^{b} \pm 1.46$	$41.34^{ab} \pm 1.49$	$42.02^{ab} \pm 1.53$

[a,b,c,d] Means within a column of each parameter with different superscript letters differ significantly ($p < 0.05$)

Acrosome integrity

The results showed that supplementation with *C. militaris* increased acrosome integrity compared to the control group at all time points ($p < 0.05$). Specifically, the 40 mg group achieved the highest level at week 8 (99.92%) compared to 85.57% in the control group, and maintained high levels at week 10 (98.62%) and week 12 (96.76%; $p < 0.05$). The 20, 80, and 160 mg/day supplementation groups also exhibited significantly higher values than the control group, ranging from approximately 91% to 94% ($p < 0.05$). By week 14, acrosome integrity values decreased to approximately 71.28-75.36%; however, they remained statistically significant compared to the control group ($p < 0.05$).

Antioxidant capacity

Antioxidant capacity in semen samples was significantly higher in supplemented groups than in the control group throughout the experimental period (Table 1; $p < 0.05$). The control group-maintained values of approximately 22%

during weeks 6-14, whereas supplemented groups showed values ranging from approximately 32% to 35% during weeks 6-12. The 40 mg/day treatment showed the highest antioxidant capacity, particularly at weeks 8 and 10. No significant differences in antioxidant capacity were observed among the supplemented groups ($p > 0.05$). At week 14, antioxidant capacity decreased compared with week 12 across all supplemented groups.

DNA fragmentation index

A significant reduction in sperm DNA fragmentation was observed in rabbits receiving *C. militaris* supplementation compared with the control group (Table 1; $p < 0.05$). The difference in DNA fragmentation index was evident from weeks 6 to 12, during which all supplemented groups exhibited significantly lower DNA fragmentation levels than the control group ($p < 0.05$). During weeks 8-12, the 40 mg/day group showed the lowest numerical sperm DNA fragmentation among the supplemented groups, although the differences among supplementation levels were not statistically significant ($p > 0.05$). Sperm DNA fragmentation in the 160 mg/day group increased progressively over the experimental period and was no longer significantly different from the control group by week 14 ($p > 0.05$).

***In vivo* fertility and offspring**

Pregnancy rate

Pregnancy rate following mating differed among treatments during the first mating period (Table 2). Female rabbits mated with males receiving *C. militaris* supplementation generally showed significantly higher pregnancy rates than those mated with control males ($p < 0.05$). The control group recorded the lowest first-mating pregnancy rate (33.33%), whereas the 40 mg/day treatment achieved 100% pregnancy. Intermediate values (66.67%) were observed in the 20, 80, and 160 mg/day treatments. Following the second mating, pregnancy rate reached 100% in all remaining groups, and no significant differences among treatments were detected ($p > 0.05$).

Table 2. Pregnancy rates of female rabbits after mating with males supplemented with *Cordyceps militaris*

Treatment group	First mating (%)	Second mating (%)
0 mg	33.33[c]	100
20 mg	66.67[b]	100
40 mg	100.00[a]	Not applicable (all females pregnant after first mating)
80 mg	66.67[b]	100
160 mg	66.67[b]	100

[a,b,c,d] Means within a column with different superscript letters differ significantly ($p < 0.05$)

Reproductive and offspring parameters

Table 3 summarizes the reproductive performance and early growth characteristics of the offspring. No significant differences were detected among the treatment groups for gestation length, litter size, litter birth weight, litter weight at two weeks, individual pup birth weight, or pup weight at two weeks ($p > 0.05$). Gestation length remained relatively stable across treatments, ranging from 30.1 to 31.3 days.

Although the differences were not statistically significant, numerical variations were observed among the supplemented groups in several reproductive and offspring growth parameters, including litter size, litter weight, and pup body weight ($p > 0.05$). Litter size increased from 3.67 kits in the control group to 6.67 kits in the 160 mg/day treatment. Similarly, supplemented groups showed a tendency toward higher litter birth weight and litter weight at two weeks of age, particularly at the higher supplementation levels. However, the increase in total litter weight was not consistently associated with higher individual pup weight. Pup weight at birth and at two weeks remained relatively similar among the control, 20 and 40 mg/day treatments, whereas lower values were observed in the 80 mg/day group.

Table 3. Reproductive performance and offspring development parameters in female rabbits mated with males treated with different doses of *Cordyceps militaris*

Parameter	Treatment				
	0 mg	20 mg	40 mg	80 mg	160 mg
Gestation length (days)	30.7 ± 0.6	30.7 ± 0.6	30.7 ± 0.6	31.3 ± 0.6	30.0 ± 1.0
Litter size (kits)	3.7 ± 0.6	4.0 ± 1.0	5.0 ± 2.6	6.0 ± 1.0	6.7 ± 2.5
Litter birth weight (g)	186 ± 43	207 ± 61	227 ± 101	260 ± 78	323 ± 121
Litter weight at 2 weeks (g)	367 ± 96	409 ± 121	474 ± 176	473 ± 128	590 ± 219
Pup weight at birth (g)	50.5 ± 5.9	51.3 ± 2.5	46.5 ± 5.6	42.9 ± 8.2	48.4 ± 5.4
Pup weight at 2 weeks (g)	100.2 ± 18.5	101.6 ± 4.9	99.7 ± 17.8	77.9 ± 10.9	88.6 ± 1.0

There were no significant differences between the treatments in reproductive performance and offspring development parameters ($p > 0.05$)

DISCUSSION

The improvement in semen quality observed following *C. militaris* supplementation is consistent with previous evidence that dietary antioxidants can enhance reproductive function in male rabbits. Vitamin E and selenium, for example, have been reported to improve sperm quality, antioxidant status, and fertility in rabbit bucks. Fadl et al. (2024) found that supplementation with vitamin E and selenium significantly improved fresh and post-thaw semen characteristics and increased conception and kindling rates in sub-fertile rabbits. Similarly, dietary organic selenium at 0.3 mg/kg dry matter improved sperm functional characteristics, total antioxidant capacity, DNA integrity, and fertility in rabbit bucks exposed to natural heat stress (Hosny et al., 2020). These findings are in agreement with the present results, in which *C. militaris*-supplemented rabbits showed improved sperm motility, viability, membrane and acrosome integrity, antioxidant capacity, and reduced DNA fragmentation compared with the control group during weeks 6–12.

Other natural antioxidant sources, including vitamin E and propolis, have also demonstrated beneficial effects on rabbit semen quality (Hashem et al., 2013). Dietary supplementation with vitamin E or propolis at 150 mg/kg diet for 10 consecutive weeks improved semen quality and oxidative status in rabbit bucks during the hot season (Hashem et al., 2013). In another study, dietary supplementation with vitamin E at 200 mg/kg diet, combined with vitamin C at 0.5 g/L drinking water, was provided for 120 days. The combination of these vitamins at supranutritional levels inhibited oxidative processes and improved the characteristics of fresh and stored rabbit semen (Castellini et al., 2003). More recently, *Origanum vulgare* essential oil, applied *in vitro* at a 10^{-2} dilution, was reported to improve sperm motility in rabbits and increase the proportions of sperm subpopulations characterized by high velocity and progressivity (Zarhouti et al., 2024). Taken together, these findings support the view that enhancement of antioxidant protection is a common mechanism through which nutritional and phytogenic additives may help preserve sperm function. The present study provides further evidence that *C. militaris* supplementation was associated with improvements in multiple indicators of sperm quality and a higher first-mating pregnancy rate.

However, the magnitude and nature of the response vary depending on the antioxidant interventions and experimental conditions (Fadl et al., 2024). Previous studies have demonstrated the beneficial effects of common antioxidants in rabbits, particularly under conditions inducing oxidative stress. For example, Hosny et al. (2020) reported that dietary supplementation with organic selenium at a dosage of 0.3 mg/kg dry matter for 12 consecutive weeks improved sperm functional parameters, plasma membrane and DNA integrity, antioxidant status, and reproductive performance in male rabbits exposed to natural heat stress. Similarly, a recent study on male rabbits with impaired fertility showed that combined supplementation with vitamin E (100 or 200 IU/head) and selenium (0.1 or 0.2 mg/kg body weight), respectively, once weekly for 8 consecutive weeks improved fresh and post-thaw semen quality and increased conception and kidding rates, with the higher supplementation level producing the most favorable responses (Fadl et al., 2024). The results reinforce the protective role of antioxidant supplementation in maintaining male reproductive function under conditions of oxidative stress or fertility impairment (Hosny et al., 2020; Fadl et al., 2024). In contrast, the current study was conducted on healthy male rabbits under standard experimental conditions, which may have resulted in a less pronounced response to nutrient supplementation. Furthermore, differences in supplementation strategies, dosages, and the physiological status of the animals among the cited studies make direct comparisons of efficacy difficult. In the present study, supplementation with 40 mg/day of *C. militaris* yielded positive responses (in terms of data) regarding certain semen parameters, although the differences between supplementation levels were not always statistically significant. Therefore, the current results should be viewed as evidence of the potential beneficial effects of *C. militaris* supplementation on semen quality, rather than as conclusive proof that this additive outperforms traditional antioxidants such as vitamin E or selenium.

The beneficial effects observed in the present study may be related to the bioactive compounds present in *C. militaris*, including cordycepin, adenosine, and polysaccharides. Among these constituents, *C. militaris* polysaccharides have been reported to possess antioxidant activity, while cordycepin and adenosine have also been identified as important bioactive constituents of this fungus (Jiang et al., 2011; Zhang et al., 2019; Gu et al., 2022). The favorable changes in sperm function observed in the present study may be linked to the biological effects of cordycepin. In experimental rats, cordycepin supplementation was associated with enhanced antioxidant defense and attenuation of age-related testicular dysfunction (Kopalli et al., 2019). Previous studies indicated that oral administration of cordycepin isolated from *C. militaris* at 5, 10, and 20 mg/kg body weight/day for six months attenuated testicular inflammation in naturally aged rats, with the effects associated with modulation of the NF-κB/MAPK signaling pathway (Kopalli et al., 2022). Therefore, the increased antioxidant capacity and reduced DNA fragmentation observed in supplemented rabbits in the present study may be associated with the protective effects of *C. militaris*-derived bioactive compounds against oxidative damage in sperm cells (Jiang et al., 2011; Zhang et al., 2019; Gu et al., 2022). Previous studies have also reported beneficial effects of *C. militaris* on antioxidant status and male reproductive function in several animal species.

In male Sprague–Dawley rats, dietary supplementation with *C. militaris* mycelium at 1% or 5% for 6 weeks increased epididymal sperm count and sperm motility, with the effects becoming evident after 5 weeks of supplementation (Chang et al., 2008). Similarly, in juvenile male mice, dietary administration of different *C. militaris* fermentation products at 5% of the diet for 28 days increased sperm concentration and the proportion of motile sperm in several treatment groups, while some fermentation products also promoted testicular development and testosterone synthesis (Lin et al., 2022). These findings were broadly consistent with the present study in male rabbits, in which dietary supplementation with *C. militaris* extract was associated with improvements in sperm motility, viability, plasma membrane and acrosome integrity, antioxidant capacity, and DNA integrity. However, the present study differs from previous studies in both animal species and supplementation strategy. Whereas Chang et al. (2008) and Lin et al. (2022) evaluated *C. militaris* mycelium or fermentation products in rats and mice, respectively, the present study evaluated a standardized *C. militaris* extract in adult male rabbits at doses of 20, 40, 80, and 160 mg/day over a longer 14-week experimental period. In addition, the present study assessed a broader range of sperm functional parameters and reproductive outcomes, including pregnancy rate following natural mating. These differences suggest that the reproductive effects of *C. militaris* may depend on the animal species, form of supplementation, dosage, and duration of administration.

The higher first-mating pregnancy rate observed in the present study is consistent with previous evidence that improving semen quality through nutritional supplementation can enhance reproductive performance in male rabbits. Fadl et al. (2024) reported that repeated intramuscular administration of vitamin E (100 or 200 IU/head) combined with selenium (0.1 or 0.2 mg/kg body weight, respectively) once weekly for 8 consecutive weeks improved sperm characteristics and increased conception and kindling rates in sub-fertile rabbit bucks. Similarly, Hosny et al. (2020) found that dietary supplementation with organic selenium at 0.3 mg/kg dry matter for 12 consecutive weeks (84 days) improved semen quality, DNA integrity, antioxidant status, and fertility in rabbit bucks exposed to natural heat stress. The reproductive response observed following *C. militaris* supplementation may therefore be associated with the improvement in sperm functional characteristics and antioxidant status observed in the present study. In contrast, the absence of significant effects on litter size and early offspring growth differs from the fertility-related improvements reported in some antioxidant supplementation studies. Such differences may be related to variation in animal physiological status, supplementation strategy, experimental duration, and sample size among studies (Hosny et al., 2020; Fadl et al., 2024).

CONCLUSION

The findings of the current study indicated that dietary *C. militaris* supplementation, particularly at 40 mg/day, has the potential to improve semen quality and reproductive performance in rabbit bucks. The relatively low sample size and limited physiological measurements represent important limitations of the present study. Further studies should determine the optimal supplementation dose, duration and clarify the underlying physiological mechanisms.

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Authors' contributions

Bui Le Khanh Ngoc, Cao Quoc Huy, and Le Hoang Kim Lan contributed to the conception, design, and methodology. Le Hoang Phuong Anh, Nguyen Luong Hao, and Nguyen Lam Khanh Duy have participated in the investigation, acquisition, analysis, and interpretation of data. Nguyen Thi Lien Thuong has contributed to methodology. Nguyen Trong Hong Phuc and Tran Thi Thanh Khuong conducted the funding acquisition, writing-review, editing, supervision, and project administration. 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 supporting the findings of the present study are available within the manuscript upon reasonable request from the corresponding author.

Ethical considerations

All authors confirmed that the present study is an original article, has not been published previously, and is not under consideration for publication elsewhere. ChatGPT (OpenAI) was used as an artificial intelligence (AI) assistance tool during the preparation and revision of the manuscript, mainly for language editing, grammar checking, and

improving the clarity and readability of the text. No AI tools were used to generate, analyze, or interpret the experimental data. All AI-assisted revisions were carefully reviewed and approved by the authors, who take full responsibility for the accuracy, originality, scientific content, and integrity of the final manuscript.

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Competing interests
The authors declare no conflict of interest.

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Comprehensive Preclinical Evaluation of a Taste-Masked Praziquantel Formulation as a Feline Anthelmintic

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ABSTRACT

Helminthiasis is common in domestic cats, compromising their welfare and posing a zoonotic risk. Praziquantel is a highly effective agent against cestodes; however, its bitter taste may cause hypersalivation and resistance to oral administration. The present randomized, controlled trial aimed to evaluate the pharmaceutical qualities, anthelmintic efficacy, tolerability, and systemic exposure to praziquantel (Anthel Cat), formulated with taste-masking and solubilizing excipients, compared with a commercially available product. A total of 30 domestic short-haired cats (*Felis catus*), aged 1-3 years and weighing 3.5 ± 1.1 kilograms (kg), comprising 20 males and 10 females, all of which had naturally acquired gastrointestinal helminth infection confirmed by fecal examination, were randomly allocated into three groups of ten. The control group received distilled water, the commercial group received a commercially available product, and the Anthel group received Anthel Cat tablets. Both active treatments contained 20 mg of praziquantel and 230 mg of pyrantel pamoate per tablet and were administered at the recommended dose of 1 tablet per 4 kg of body weight, as a single oral dose. Cats were monitored for 14 days. Active ingredient content, disintegration time, fecal egg counts determined by flotation and sedimentation, hematology, serum biochemistry, taste acceptance, and plasma praziquantel concentration were assessed. Anthel Cat active ingredient contents were 107.88% of pyrantel pamoate and 99.17% of praziquantel, both within pharmacopoeial limits, with a mean disintegration time of 7 minutes 22 seconds. By day 14, fecal egg counts in both treated groups declined to near zero by sedimentation, whereas flotation did not demonstrate a difference among groups. Hematological and serum biochemical parameters remained within reference ranges and did not differ among groups, which indicated no hepatic or renal toxicity. Peak plasma praziquantel levels occurred 1 hour after dosing in both treated groups, and Anthel Cat did not differ from the commercial product. For taste acceptance, Anthel Cat produced no measurable saliva volume. Salivation duration was elevated in both treated groups. The result demonstrated that Anthel Cat has the same quality as the commercial product in pharmaceutical quality, anthelmintic efficacy, tolerability, and systemic exposure. However, limiting the salivary aversion response, particularly saliva volume.

Keywords: Anthelmintic, Cat, Efficacy, Praziquantel, Pyrantel pamoate

INTRODUCTION

Gastrointestinal helminth infections are prevalent among domestic cats, with reported prevalence rates that differ considerably across geographic regions, environmental conditions, and husbandry practices (Symeonidou et al., 2025 *Toxocara cati* is consistently the most common endoparasite of owned cats, with a prevalence of 19.7% in a multicenter European survey of 1,519 cats (Beugnet et al., 2014). In Indonesia, a retrospective study of 171 pet cats in Bogor detected *Toxocara* spp. in 38.0% and Dipylidium caninum in 4.7% of the animals (Ridwan et al., 2023). Several feline helminths are zoonotic; therefore, effective parasite control is crucial for animal welfare and public health (FAO/OIE/WHO, 2021). Praziquantel is widely used as a cestocidal agent in felines, and its anthelmintic efficacy has been linked to the activation of transient receptor potential channels in parasites. This activation leads to calcium influx, prolonged muscle contractions, and damage to the tegument, potentially increasing exposure of parasitic antigens to the host's immune system (Park et al., 2021; Waechtler et al., 2023).

Despite the efficacy of praziquantel, its significant bitter taste can complicate oral administration (Kuevi et al., 2023). Interaction of the medicine with lingual bitter-taste receptors can trigger hypersalivation, foaming, and dose rejection (Waechtler et al., 2023). Incomplete or unsuccessful dose administration may reduce medicine exposure and compromise treatment efficacy (Waechtler et al., 2023). Administering medication orally can be especially challenging in cats, a species in which owners often struggle to administer medication reliably (Papich, 2020).

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Several pharmaceutical strategies have been investigated to reduce the bitterness of praziquantel (Kuevi et al., 2023). Microencapsulation of praziquantel in polymer matrices, such as the gastro-soluble polymethacrylate, limits drug release in saliva and hence its contact with taste receptors, while allowing rapid dissolution in gastric and intestinal fluids (Münster et al., 2017; Machado et al., 2018). Forming complexes with cyclodextrins or maltodextrin can also decrease the amount of unbound praziquantel dissolved in saliva (Münster et al., 2017). Studies using electronic tongue and rodent taste-aversion methodologies confirm that taste masking effectively reduces the bitterness of praziquantel liquids (Münster et al., 2017; Kuevi et al., 2023). Nevertheless, excipients used for taste masking may influence medicine release and absorption (D'Abbrunzo et al., 2024). Praziquantel is a compound with low water solubility and high permeability, where dissolution could limit its systemic absorption (Waechtler et al., 2023; D'Abbrunzo et al., 2024). Coating materials and complexing agents designed to reduce oral exposure to medicines can delay or alter drug release, potentially affecting overall systemic exposure and therapeutic effectiveness (Ahmad et al., 2021; D'Abbrunzo et al., 2024). Conversely, appropriately selected solubilizing excipients may improve medicine dissolution (D'Abbrunzo et al., 2024). To properly assess a taste-masking formulation, its impact on pharmaceutical quality and *in vivo* therapeutic performance should be evaluated concurrently in the target species (Gupta et al., 2021).

Taste-masking excipients are not completely inert in drug delivery (Waechtler et al., 2023). Coating layers and complexing agents, which prevent the medicine from interacting with lingual receptors, also slow the release of taste-masking excipients. For compounds where absorption is limited by dissolution rather than permeability, this delayed release could reduce systemic exposure and potentially decrease efficacy of the active compounds (Waechtler et al., 2023; D'Abbrunzo et al., 2024). Therefore, the efficacy of a taste-masked formulation should be tested in the target species before replacing an existing product to ensure it performs comparably. Anthel Cat is an Indonesian-approved tablet for cats, containing 20 mg of praziquantel and 230 mg of pyrantel pamoate. Anthel Cat uses a new milk-flavor masking to reduce the bitterness of the active ingredients. The use of Praziquantel combined with Pyrantel pamoate is common in feline veterinary treatments, but it lacks extra taste-masking and solubilizing excipients (Papich, 2020). The present study aimed to assess whether the taste-masked formulation of Anthel Cat was equivalent to the commercial product in terms of quality, palatability, efficacy, tolerability, and praziquantel exposure in naturally infected cats.

MATERIALS AND METHODS

Ethical approval

The present study was conducted in accordance with institutional guidelines for the care and use of animals in research. Ethical approval was obtained from the Animal Ethics Committee of the School of Veterinary Medicine and Biomedical Sciences, IPB University, Bogor, Indonesia, prior to the commencement of all experimental procedures (Approval No. 290A/KEH/SKE/I/2025).

Animals and housing

A total of 30 domestic short-haired cats (*Felis catus*) of local origin were obtained from iRATco Laboratory Services in Bogor, Indonesia. Animals were aged 1 to 3 years, weighed 3.5 to 4 kg, and comprised 20 males and 10 females. All cats underwent a 7-day acclimatization period before treatment. The present study was conducted as a controlled comparative trial focusing on efficacy and tolerability, rather than detecting a specific effect size. The number of cats was minimized in accordance with the 3Rs principles and in accordance with institutional approval for animal use. A group of 10 cats exceeded the minimum requirement of six adequately infected animals per group, as recommended by the World Association for the Advancement of Veterinary Parasitology for controlled anthelmintic efficacy studies (Beugnet et al., 2022).

Cats were housed individually in cages that met laboratory animal welfare standards and had free access to commercial feed and water. Eligibility was determined through clinical examination and quantitative fecal analysis conducted on two consecutive days prior to treatment. Cats qualified when their average fecal egg count reached at least 80 eggs per gram. Cats with no detectable helminth eggs and those treated with any anthelmintic in the past three months were excluded. Selected cats were allocated to three groups of 10 using simple randomization. Allocation was not stratified by age, sex, or body weight, and since all animals were domestic short-haired cats, breed was homogeneous by the inclusion criterion. Cats in the control group received distilled water orally as a vehicle, cats in the second group received the commercially available antiparasitic product, and cats in the third group received the Anthel Cat tablets. The trade name of the commercial product was withheld for commercial reasons; consequently, the product was identified throughout by its composition, strength, and regulatory status. Both antiparasitic products contained 20 mg of praziquantel and 230 mg of pyrantel pamoate per tablet, with a similar dosage of one tablet per 4 kg of body weight, equivalent to 5 mg/kg of praziquantel and 57.5 mg/kg of pyrantel pamoate. Regarding the dosing procedure, each tablet

was dissolved in physiological saline (0.9% NaCl) at a 1:1 (w/v) ratio and administered orally via syringe to ensure the complete delivery of the intended dose to each cat. Any immediate signs of taste aversion, such as hypersalivation, head-shaking, or spitting, were observed during daily clinical observations. All treatments were administered as a single dose at the start of the study, and the animals were monitored for 14 days.

Test material

Anthel Cat (PT Agroveta Husada Dharma, a subsidiary of PT Kalbe Farma Tbk in East Jakarta, Indonesia) is a solid oral tablet containing 20 mg of praziquantel and 230 mg of pyrantel pamoate. Anthel Cat is officially registered with the Indonesian Ministry of Agriculture under registration number D.22037309 PKM and is indicated for the treatment of gastrointestinal nematode and cestode infections in feline patients. The tablet was divided into sections, and its distinguishing feature relative to existing combination products was the addition of a milk flavoring to reduce bitterness. The commercial film-coated tablet contained 20 mg of praziquantel and 230 mg of pyrantel pamoate, approved for use in cats in Indonesia and prescribed for *Toxocara cati*, *Ancylostoma tubaeforme*, *Dipylidium caninum*, and *Taenia taeniaeformis*. Since the two products were identical in active ingredients, potency, and dosing schedule, and differed only in taste-masking excipients, any differences in performance were attributed to formulation rather than dosage.

Taste acceptance assessment

Both antiparasitic products were administered as solutions. Taste acceptance was objectively assessed by monitoring the salivary aversion response during dosing in a subset of five cats per group. Immediately after each syringe-administered dose, salivation duration was recorded with a stopwatch, and the saliva produced was collected by allowing it to drip into a one mL microtube; its volume was then measured with a micropipette. All recordings were performed by a single observer who was blinded to the treatment groups. The duration of salivation and saliva volume were subsequently compared among the control, commercial, and Anthel Cat groups.

Active ingredient content and disintegration time analyses

The amounts of pyrantel pamoate and praziquantel were measured using a validated in-house reversed-phase high-performance liquid chromatography (HPLC) method, which was developed based on established protocols for their simultaneous analysis in solid dosage forms (Tatar et al., 2015; Rajesh and James, 2019) and validated for accuracy, precision, and reproducibility according to the International Council for Harmonization Q2(R2) guideline. Standards and sample solutions were prepared using gravimetric methods on a calibrated analytical balance, then homogenized and filtered through a 0.45 µm nylon membrane filter. Separation was performed on a Phenomenex Luna C18 column (250 × 4.6 mm, 5 µm) with water and acetonitrile (20:80, v/v) as the mobile phase, delivered isocratically at one mL/minute, with an injection volume of 20 µL and ultraviolet detection at 220 nm. Analyses were carried out on a Shimadzu Prominence liquid chromatograph (Shimadzu Corporation, Japan) comprising an LC-20AT pump, SIL-20A autosampler, SPD-20A ultraviolet-visible detector, and CTO-20A column oven. Peak areas for each analyte were quantified against a standard calibration curve, and results were assessed against the acceptance limits for tablet content stated in the Indonesian Pharmacopeia (Ministry of Health of the Republic of Indonesia, 2020).

Disintegration time was determined using an ED-2L disintegration test apparatus (Electrolab, India) in 700 mL of demineralized water maintained at 37 ± 2°C, following the tablet disintegration analysis described in the Indonesian Pharmacopeia (Ministry of Health of the Republic of Indonesia, 2020). Disintegration was considered complete when there was no solid residue on the mesh, and all particles had dispersed into the medium. Three replicates were performed for each product.

Efficacy and safety analysis

Fecal samples were collected on days 0, 7, and 14. Egg counts were determined in parallel by flotation and sedimentation and expressed as the mean number of helminth eggs per microscopic field, counted across 5 fields per sample at 400x magnification. For flotation, 1 g of feces was homogenized in saturated sugar-salt solution, transferred to a 15 mL tube until a convex meniscus formed, and covered with a coverslip for 10 minutes before microscopic examination. For sedimentation, one g of feces was homogenized in sodium chloride solution, strained, centrifuged at 3,000 rpm for 10 minutes, and the resuspended sediment was examined under a light microscope (ZEISS, Germany). The primary efficacy endpoint was the percentage reduction in egg count on day 14 relative to day 0, calculated using Formula 1.

$$\text{Primary efficacy endpoint} = ([\text{day 0 count} - \text{day 14 count}] / \text{day 0 count}) \times 100 \quad \text{(Formula 1)}$$

Complete clearance was characterized by the absence of detectable eggs in all examined fields. Endpoint definitions followed the World Association for the Advancement of Veterinary Parasitology guidelines for the evaluation of

anthelmintic efficacy in dogs and cats (Beugnet et al., 2022). Clinical observations concerning activity, appetite, response to stimuli, salivation, and overall condition were documented daily throughout the 14-day period. Blood samples were obtained from the brachial vein on days 0, 7, and 14. A total volume of 1.5 mL was divided between an EDTA tube for hematological analysis, including erythrocyte count, leukocyte count, hemoglobin levels, hematocrit, and differential leukocyte count, while the remaining volume was collected into a plain tube for serum biochemistry analysis. Hematological analyses were performed by a BC-5000 Vet Auto Hematology Analyzer (China). Serum glutamic pyruvic transaminase (SGPT) and serum glutamic oxaloacetic transaminase (SGOT) were determined by the optimized IFCC method, blood urea nitrogen (BUN) by the kinetic ultraviolet glutamate dehydrogenase method, and creatinine by an enzymatic method, using Glory Diagnostics reagent kits (Linear Chemicals S.L.U., Spain) according to the manufacturer's instructions, on an RT-6100 microplate reader (Rayto Life and Analytical Sciences, China)

Bioavailability measurement

Plasma praziquantel levels were measured primarily to qualitatively confirm systemic absorption of the active ingredient, rather than to detail its pharmacokinetics; therefore, the sampling schedule was designed accordingly. Plasma praziquantel was measured by competitive enzyme-linked immunosorbent assay (ELISA) with the ECALBIO praziquantel ELISA kit (Cat. No. PZQ-ELISA-96; Wuhan Ecalbio, China), following the provided assay protocol. The blood sampling timetable and the approach to describing plasma praziquantel disposition were based on the published characterization of oral praziquantel pharmacokinetics in cats (Xie et al., 2023). Blood was collected at 0, 1, 2, 4, 8, 10, and 12 hours after dosing on day zero. These samples were centrifuged at 4,000 rpm for five minutes. From each sample, 50 µL of supernatant was diluted with 950 µL of redissolving solution before analysis. Blood samples and standards were measured in duplicate, and absorbance was read at 450 nm using an RT-6100 microplate reader (Rayto Life and Analytical Sciences, China). Concentrations were measured using the established curve as a reference.

Statistical analysis

Baseline comparability of age, sex, body weight, and fecal egg count on the first day among the three groups was assessed prior to the primary analysis. Data were presented as mean ± standard deviation. Distributions were assessed using the Shapiro–Wilk test, and homogeneity of variance was assessed using Levene's test. Fecal egg counts, hematological, and serum biochemical parameters were analyzed using repeated-measures ANOVA, considering group as the between-subject factor and day as the within-subject factor. For the comparisons between groups on days 7 and 14, day 0 values were used as a covariate in an analysis of covariance. No prior sample-size or power calculation was conducted. Statistical significance was set at p less than 0.05. Analyses were performed in R version 4.6.1 (R Core Team, 2026).

RESULTS AND DISCUSSION

The antiparasitic treatment groups exhibited a higher salivary response than the control group; however, the taste-masked formulation demonstrated greater tolerability, as reflected in the volumetric measurements (Table 1). Salivation duration was significantly longer in the commercial product (10.60 ± 1.67 seconds; $p < 0.05$) and Anthel Cat (7.80 ± 2.68 seconds; $p < 0.05$) than in the control group. The commercial product significantly increased saliva volume (0.46 ± 0.11 mL; $p < 0.05$), while Anthel Cat did not cause a significant change (0.00 ± 0.00 mL), which did not differ significantly from the control group ($p > 0.05$). Anthel Cat did not produce a measurable volume of saliva, whereas the commercial product produced a measurable volume of saliva. This contrast indicated that the taste-masking strategy successfully limited the salivary aversion response.

The pyrantel pamoate content was 107.88% in Anthel Cat and 108.49% in the commercial product, while praziquantel was 99.17% and 97.18%, respectively (Table 2). Both products were within the Indonesian Pharmacopeia acceptance range of 90.0-110.0% and the United States Pharmacopeia acceptance range. Since both products contained 20 mg of praziquantel per tablet, the actual doses were 19.83 mg for Anthel Cat and 19.44 mg for the commercial product, representing about a 2% difference. Since both products delivered equivalent praziquantel doses, any difference in systemic exposure could be attributed to formulations rather than dosages (Papich, 2020). Content uniformity within pharmacopoeial limits indicated a homogeneous distribution of active substances during blending and compression, demonstrating effective control of granulation, binder addition, and lubrication steps (Cutolo et al., 2022). Maintaining consistent active ingredient levels is clinically crucial, as subtherapeutic exposure may lead to anthelmintic resistance (Gupta et al., 2021). The slight variation in praziquantel content between the two products was due to differences in excipient composition or the medicine's physicochemical properties during granulation and drying. Interactions between active ingredients and excipients can influence the stability of poorly soluble compounds, especially those susceptible to

polymorphism (Papich, 2020). The mean disintegration time was 7 minutes and 22 seconds for Anthel Cat, compared to 5 minutes and 42 seconds for the commercial product (Table 2). Disintegration time was influenced by excipient composition, tablet hardness, particle size, and porosity. It is important to note that a longer disintegration time did not necessarily correlate with reduced absorption, as praziquantel absorption is primarily limited by dissolution rather than membrane permeability. Furthermore, exceptionally rapid disintegration may lead to the release of the medicine at a rate faster than its dissolution rate (Waechtler et al., 2023; D'Abbrunzo et al., 2024). Consequently, the disintegration profile of Anthel Cat was consistent with the active ingredient's effective release.

Table 1. Salivation duration and saliva volume in domestic short-haired cats

Parameter	Control	Commercial	Anthel Cat
Salivation duration (seconds)	0.00 ± 0.00^{a}	10.60 ± 1.67^{b}	7.80 ± 2.68^{b}
Saliva volume (mL)	0.00 ± 0.00^{a}	0.46 ± 0.11^{b}	0.00 ± 0.00^{a}

[a,b] Means within a row with different superscript letters differ significantly ($p < 0.05$).

Table 2. Active ingredient content and disintegration time of Anthel Cat and the commercial product

Product	Content		Disintegration time		
	Pyrantel pamoate	Prazyquantel	1	2	3
Anthel Cat	107.88%	99.17%	0:07:15	0:07:22	0:07:30
Commercial	108.49%	97.18%	0:05:15	0:05:54	0:05:58

Anthelmintic efficacy

Using sedimentation, the fecal egg counts in both treatment groups decreased significantly by day 7 and nearly reached zero by day 14, whereas the Fecal egg counts in the untreated control group remained elevated throughout the study period (Figure 1; $p < 0.05$). Through flotation, counts in the treated groups declined over the same interval; however, the differences among the groups were not statistically significant ($p > 0.05$). The flotation findings were not significant across the treatment groups compared to the control groups. The difference between the two techniques was probably due to their unique egg-recovery properties rather than any biological impact. Sedimentation tended to recover eggs with higher specific gravity more effectively, whereas the sugar-salt flotation technique used in the present study did not float all helminth eggs with equal efficiency (Beugnet et al., 2022). The reduction in fecal egg excretion to near-zero levels within two weeks indicated substantial clearance of gastrointestinal helminths and was considered an established marker of effective anthelmintic therapy. Based on sedimentation results, both Anthel Cat and the commercial product demonstrated comparable reductions in fecal egg counts. These findings are consistent with the high efficacy reported for praziquantel-based combinations against feline gastrointestinal helminths (Gupta et al., 2021; Tielemans et al., 2021).

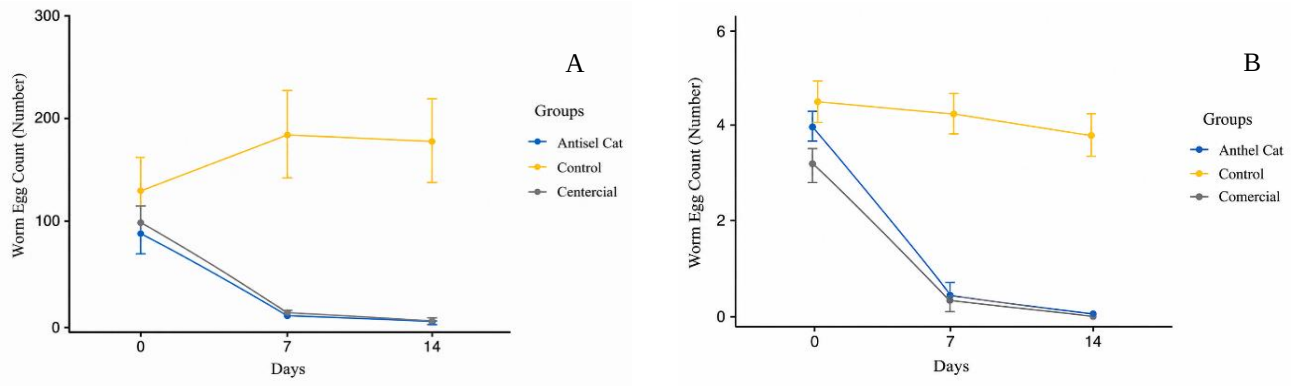


Figure 1. Fecal egg counts in domestic short-haired cats after receiving a single oral dose of distilled water (control), a commercial anthelmintic, and Anthel Cat on days 0, 7, and 14. **A**: Counts determined by the flotation method, **B**: Counts determined by the sedimentation method, Values are mean ± standard deviation

Tolerability

No clinical sign of toxicity was recorded in any group during the 14-day observation period. Hematological parameters did not differ significantly among the control, commercial, and Anthel Cat groups at any time point (Table 3; $p > 0.05$), and all values remained within published commercial ranges for the domestic cat (Brooks et al., 2022). Leukocyte counts declined gradually in both treated groups over the observation period, while the remaining hematological parameters were within commercial limits.

Table 3. Hematological parameters of domestic short-haired cats after receiving a single oral dose of distilled water (control), a commercial anthelmintic, and Anthel Cat on days 0, 7, and 14

Groups / Parameters	Control			Commercial			Anthel Cat		
	Days								
	0	7	14	0	7	14	0	7	14
WBC (10^9/L)	20.62 ± 11.3^{ns}	19.3 ± 9.4^{ns}	19.35 ± 8.0^{ns}	18.49 ± 7.7^{ns}	15.86 ± 5.9^{ns}	14.39 ± 6.0^{ns}	23.0 ± 5.0^{ns}	18.52 ± 5.1^{ns}	15.63 ± 4.9^{ns}
Neu (10^9/L)	12.8 ± 8.3^{ns}	12.4 ± 7.5^{ns}	12.19 ± 5.9^{ns}	12.7 ± 6.4^{ns}	11.2 ± 5.2^{ns}	9.6 ± 4.6^{ns}	8.35 ± 5.2^{ns}	7.57 ± 4.3^{ns}	6.61 ± 3.9^{ns}
Lym (10^9/L)	5.2 ± 2.9^{ns}	4.3 ± 2.4^{ns}	4.38 ± 1.9^{ns}	3.5 ± 1.1^{ns}	3.02 ± 1.2^{ns}	3.56 ± 1.6^{ns}	10.65 ± 4.5^{ns}	8.2 ± 2.9^{ns}	7.67 ± 3.0^{ns}
Mon (10^9/L)	1.16 ± 1.8^{ns}	0.91 ± 1.0^{ns}	0.98 ± 0.9^{ns}	0.88 ± 1.2^{ns}	0.53 ± 0.4^{ns}	0.54 ± 0.3^{ns}	0.93 ± 0.3^{ns}	0.86 ± 0.4^{ns}	0.89 ± 0.3^{ns}
Eos (10^9/L)	1.46 ± 1.0^{ns}	1.69 ± 0.8^{ns}	1.81 ± 0.9^{ns}	1.41 ± 0.4^{ns}	1.1 ± 0.5^{ns}	0.7 ± 0.3^{ns}	3.06 ± 0.4^{ns}	1.9 ± 0.7^{ns}	0.47 ± 0.2^{ns}
Bas (10^9/L)	0.0 ± 0.0^{ns}	0.0 ± 0.0^{ns}	0.0 ± 0.0^{ns}	0.0 ± 0.0^{ns}	0.0 ± 0.0^{ns}	0.0 ± 0.0^{ns}	0.01 ± 0.0^{ns}	0.0 ± 0.0^{ns}	0.0 ± 0.0^{ns}
RBC (10^{12}/L)	5.43 ± 2.1^{ns}	6.77 ± 1.2^{ns}	5.23 ± 2.5^{ns}	6.12 ± 1.4^{ns}	6.75 ± 2.1^{ns}	4.13 ± 1.2^{ns}	8.18 ± 1.7^{ns}	6.95 ± 2.0^{ns}	7.01 ± 1.8^{ns}
HGB (g/L)	92.7 ± 31.5^{ns}	105.9 ± 18.3^{ns}	84.2 ± 39.0^{ns}	101.5 ± 21.6^{ns}	100.2 ± 2.1^{ns}	70.0 ± 18.0^{ns}	140.1 ± 30.9^{ns}	114.9 ± 9.6 ^{ns}	127.7 ± 30.5^{ns}

ns: Non-significant, meaning there was no statistically significant difference in each parameter ($p > 0.05$). WBC: White blood cells, Neu: Neutrophils, Lym: Lymphocytes, Mon: Monocytes, Eos: Eosinophils, Bas: Basophils, RBC: Red blood cells, HGB: Hemoglobin

Table 4. Serum biochemical parameters of domestic short-haired cats after receiving a single oral dose of distilled water (control), a commercial anthelmintic, and Anthel Cat on days 0, 7, and 14

Groups / Parameters	Control			Commercial			Anthel Cat		
	Days								
	0	7	14	0	7	14	0	7	14
SGPT	30.12 ± 11.27^{ns}	37.48 ± 9.11^{ns}	36.51 ± 18.67^{ns}	34.16 ± 7.83^{ns}	43.04 ± 12.77^{ns}	61.96 ± 31.13^{ns}	39.17 ± 18.62^{ns}	32.33 ± 8.91^{ns}	32.33 ± 8.91^{ns}
SGOT	22.52 ± 16.97^{ns}	22.73 ± 17.91^{ns}	35.47 ± 35.74^{ns}	19.29 ± 17.13^{ns}	20.84 ± 19.4^{ns}	30.05 ± 14.77^{ns}	19.93 ± 13.26^{ns}	21.01 ± 12.72^{ns}	21.01 ± 12.72^{ns}
BUN	18.04 ± 2.43^{ns}	19.11 ± 3.22^{ns}	20.8 ± 3.38^{ns}	18.58 ± 3.06^{ns}	21.6 ± 3.54^{ns}	18.78 ± 4.67^{ns}	17.83 ± 3.27^{ns}	18.72 ± 5.23^{ns}	16.5 ± 4.24^{ns}
Creatinine	2.71 ± 0.96^{ns}	2.49 ± 1.08^{ns}	2.61 ± 1.01^{ns}	3.07 ± 1.25^{ns}	3.01 ± 1.25^{ns}	2.61 ± 0.79^{ns}	2.76 ± 0.87^{ns}	2.41 ± 0.82^{ns}	2.4 ± 0.61^{ns}

ns: Non-significant, meaning there was no statistically significant difference in each parameter ($p > 0.05$). SGPT: Serum glutamic pyruvic transaminase, SGOT: Serum glutamic oxaloacetic transaminase, BUN: Blood urea nitrogen

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The observed trends aligned with reduced immune stimulation following parasite clearance. However, the differences were not statistically significant. Eosinophil counts demonstrated the greatest alterations, decreasing in both treated groups from day 0 to day 14, with a slight increase in the control group. Considering the role of eosinophils in the antiparasitic response, the observed pattern was consistent with successful parasite clearance; however, the change was not statistically significant ($p > 0.05$). Erythrocyte and hemoglobin levels remained stable across all groups.

Serum biochemical parameters did not differ significantly among groups at any time point (Table 4; $p > 0.05$). The SGPT, SGOT, BUN, and creatinine levels remained within the published reference ranges for domestic shorthair cats (Reynolds et al., 2008). Furthermore, no progressive elevation was observed in any group, indicating that neither product caused detectable hepatocellular injury nor impairment of glomerular filtration throughout the study period. The absence of clinicopathological changes aligns with the established safety profile of praziquantel in cats. Target animal safety investigations involving praziquantel-based formulations have documented no significant changes in BUN, creatinine, SGPT, and SGOT levels following single or repeated administration of praziquantel at doses up to 5 times the recommended label dose. Additionally, histopathological examinations revealed no hepatic or renal lesions (Gupta et al., 2021; Tielemans et al., 2021). The considerable safety margin was attributable to the mechanism of action, given that the transient receptor potential channels targeted by praziquantel are specific to parasites and lack mammalian counterparts (Park et al., 2021).

Systemic praziquantel exposure

In both treated groups, plasma praziquantel concentrations increased rapidly following administration compared with the control group, reaching their maximum at approximately one hour, then gradually declining and remaining detectable at 12 hours (Figure 2). The peak observed in the Anthel Cat group surpassed 200 ng/mL.

The plasma concentration-time profile exhibited rapid gastrointestinal absorption followed by first-order elimination, consistent with the pharmacokinetic characteristics reported for praziquantel in felines following oral administration (Xie et al., 2023). Peak concentration was regarded as the pharmacologically important metric because the cestocidal efficacy of praziquantel depends on reaching a sufficient level at the parasite's tegument rather than on maintaining prolonged low-level exposure (Waechtler et al., 2023). Praziquantel is a poorly soluble, highly permeable compound where absorption depends mainly on dissolution rather than membrane permeability. Therefore, any excipient that slows down release might decrease systemic exposure (D'Abbrunzo et al., 2024). Overall, the taste-masked formulation demonstrated a plasma concentration time profile similar to the commercial product, indicating that its taste-masking excipients did not significantly hinder absorption or affect therapeutic levels.

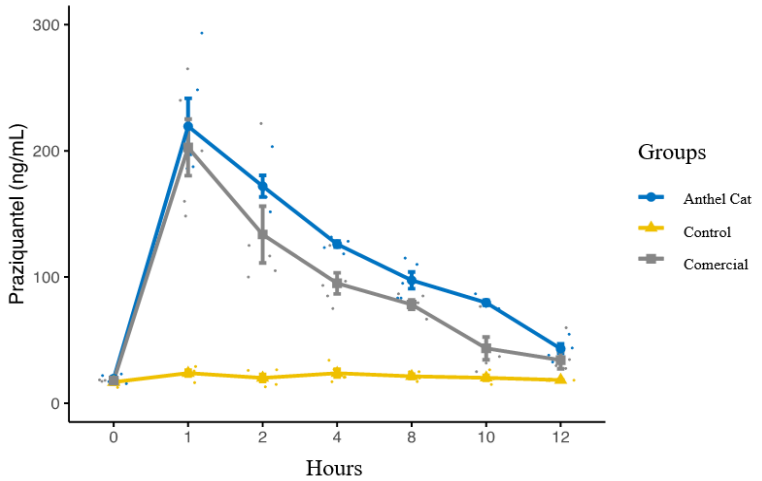


Figure 2. Plasma praziquantel concentration in domestic short-haired cats at 0, 1, 2, 4, 8, 10, and 12 hours after a single oral dose of distilled water (control), a commercial anthelmintic, and Anthel Cat. Values are mean ± standard deviation, n: 10 per group

CONCLUSION

Anthel Cat complied with pharmacopoeial specifications for active ingredient content and disintegration time. In naturally infected domestic cats, a single oral dose resulted in fecal egg counts decreasing to near zero by day 14, as evidenced by sedimentation examination, a result comparable to that of the commercial product. However, the flotation data did not reveal a significant difference among the groups. Hematological and serum biochemical parameters did not differ among groups over 14 days, indicating no detectable hepatotoxicity or nephrotoxicity. Plasma praziquantel

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concentration peaked one hour after administration at a level at least comparable to that of the commercial product, indicating that the taste-masking excipients did not significantly delay absorption. The addition of taste-masking excipients did not compromise the formulation's pharmaceutical quality, anthelmintic efficacy, tolerability, or systemic exposure. The current findings were limited by the modest sample size, the absence of quantitative palatability and pharmacokinetic measurements, and the identification of helminths only to genus level. Future studies ought to incorporate an adequately powered efficacy trial alongside species-level parasite identification, a formal assessment of palatability, and chromatographic measurement of plasma praziquantel accompanied by comprehensive pharmacokinetic characterization.

DECLARATIONS

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Authors' contributions

Mawar Subangkit conceived and designed the study, supervised its execution, and critically revised the manuscript. Elpita Tarigan conducted the laboratory analyses and contributed to data interpretation. Yusa Irarang performed the parasitological examinations and contributed to data analysis. Sindi Farhana carried out the animal procedures and sample collection. Hamdika Yendri Putra performed the statistical analysis and drafted the manuscript. Sabhina Rona Djuwita provided the test materials and contributed to the interpretation of formulation data. All authors have read and approved the final edition of the manuscript prior to publication in the present journal.

Availability of data and materials

The datasets generated and analyzed during the present study are available from the corresponding author on reasonable request.

Competing interests

The authors declare no competing interests in relation to the processing of this manuscript.

Ethical considerations

All authors have reviewed and addressed ethical considerations, including plagiarism, consent for publication, misconduct, data fabrication and falsification, double publication or submission, and redundancy. AI-assisted tools (Claude version Opus 4.8) were employed solely for linguistic editing. The entirety of the data, analyses, and scientific content is authored by the present authors themselves. The authors accepted full responsibility for any artificial intelligence tools utilized in the preparation of this manuscript and confirmed that they bear sole responsibility for the accuracy, originality, and integrity of the content throughout submission, revision, and final publication.

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Antibiotic Use in Veterinary Surgery: Perioperative Practices and Associated Factors in Kenya

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ABSTRACT

Antibiotics are extensively used in animals undergoing surgery to prevent surgical site infections (SSIs) and improve surgery outcomes. The current study aimed to evaluate perioperative practices among Kenyan veterinarians and determine their correlation with perioperative antibiotic administration. The present study employed a cross-sectional survey design, in which a structured questionnaire was distributed to veterinarians in Kenya who were registered with the Kenya Veterinary Board. Detailed information on respondents' demographics, types of surgical procedures, perioperative practices, antibiotic usage patterns, and the incidence of SSI was collected. Eighty-three veterinarians participated in the study from January 1, 2023, to June 30, 2023, comprising 48 (57.8%) males and 35 (42.2%) females. The average number of surgeries performed monthly was 8.7, primarily elective and reconstructive procedures. Hand scrubbing before surgery was widely practiced (86.7%); However, only 42.2% adhered to recommended techniques, which include using a scrub solution followed by 70% ethyl alcohol disinfection. The primary sterilization methods were chemical immersion (49.4%) and autoclaving (37.5%). The majority of respondents (85.5%) considered antibiotic use optimal, primarily based on their personal experience (74.7%). Antibiotics were predominantly administered postoperatively (67.5%) and were highly likely to be used in contaminated, dirty, and emergency surgeries. Penicillins and cephalosporins were the most commonly used antibiotic classes perioperatively. Antibiotic usage was notably correlated with the surgeon's educational background, routine hand scrubbing, and adherence to ideal scrubbing protocols. The present study highlighted the existing perioperative antibiotic practices and identified main risk factors for antimicrobial misuse among veterinarians in Kenya.

Keywords: Antibiotic use, Infection, Antimicrobial resistance, Surgery, Veterinarian

INTRODUCTION

Antimicrobial resistance (AMR) is a growing global health challenge affecting both humans and animals, identified as one of the top ten threats to public health worldwide (CDC, 2020; WHO, 2020). Given the widespread and often indiscriminate use of antibiotics, antimicrobial resistance may emerge as the next global pandemic unless immediate and effective measures are implemented (Eshrati et al., 2025). A recent World Bank model predicted that by 2050, drug-resistant infections may lead to substantial economic losses and a sharp rise in mortality (World Bank, 2017). In veterinary medicine, antibiotics are commonly used to treat bacterial infections and prevent complications such as surgical site infections (SSIs); however, the misuse or overuse of antibiotics can accelerate AMR (Weese et al., 2015; Cavalli et al., 2025). A recent study found that antibiotics are administered to animals predominantly for metaphylaxis or growth promotion, thereby directly contributing to the emergence of resistant pathogens such as *Salmonella* and *Campylobacter* species (Vasantha et al., 2025). The non-therapeutic application of antibiotics leads to antibiotic-resistant pathogens, thereby compromising critical treatments for animal health and productivity and posing a risk to human health (Adesokan et al., 2022).

In small animal surgery, antibiotics are routinely administered prophylactically to reduce the risk of SSIs (Cavalli et al., 2025). Despite the advantages of antibiotics, there is a growing concern about the lack of standardized guidelines, particularly in regions such as Africa, where the high prevalence of multidrug-resistant pathogens and limited diagnostic resources necessitate effective stewardship (Adekanye et al., 2020; Odoi et al., 2021). Numerous high-income countries have developed and/or implemented evidence-based veterinary perioperative antibiotic protocols, including those from the World Small Animal Veterinary Association (WSAVA) and the International Society for Companion Animal Infectious Diseases (ISCAID), to promote antimicrobial stewardship (Weese et al., 2019; Frey et al., 2022). These guidelines are established based on species-specific responses, appropriate dosing based on pharmacokinetic-pharmacodynamic (PK/PD) targets, and emphasize the critical importance of administering within one hour prior to

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incision (Pelligand et al., 2024). In countries such as Kenya, where there are no locally validated frameworks, veterinary practitioners often depend on outdated texts or directly replicate protocols from disparate geographic regions. These practices might not properly address local antimicrobial resistance patterns, accessible drug formularies, or endemic pathogens (Odoi et al., 2021; Ogwuche et al., 2021).

Although guidelines exist, perioperative antibiotics are frequently misused or overused in veterinary medicine because of misinterpretation or incomplete risk assessment. Prophylactic antibiotic use should be based on the type of surgery and patient-specific factors (Sørensen et al., 2024). Concerns about prolonged surgical durations, breaches in aseptic technique, or inadequate case preparation are frequently addressed with excessively prolonged antibiotic protocols rather than by addressing the underlying technical or environmental issues (Grubb et al., 2020; Garcia Stickney et al., 2022). Antibiotics should not replace surgical management. It is more effective to control factors such as surgical duration, intraoperative contamination, and post-operative care by improving surgical practices and protocols (Weese et al., 2015). The misuse ultimately contributes to antimicrobial resistance, increasing the threat to both animal and public health (Weese et al., 2015; Odoi et al., 2021). The aim of the present study was to assess current perioperative antibiotic use practices among veterinary surgeons in Kenya and to identify key factors that influence antibiotic use.

MATERIALS AND METHODS

Ethical approval

Ethical approval was obtained from the Faculty of Biosafety, Animal Use and Ethics Committee, University of Nairobi, Nairobi, Kenya (FVM BAUEC/2023/630).

Study design

The present study was a cross-sectional study carried out between January 2023 and June 2023. The sample size was calculated using Formula 1 for estimating a single proportion in a cross-sectional study (Stevenson, 2021).

$$N = \frac{Z^2.p.(1-p)}{d^2} \quad \text{(Formula 1)}$$

Where n is the initial required sample size, Z is the standard normal deviate corresponding to the desired confidence level, assuming a 95% confidence level, a margin of error of d = 5%, and an expected proportion of p = 50%. The calculation yielded an initial sample size of 384. Since the total number of registered veterinary clinics in Kenya was approximately 100, a finite population correction was employed, leading to a minimum required sample size of approximately 80 clinics. All eligible clinics were selected to maximize participation and enhance representativeness (Stevenson, 2021).

Veterinary clinics and surgeons' selection

Sampling was conducted at the clinic level rather than the individual veterinarian level because perioperative antibiotic practices were influenced by clinic-specific protocols, available resources, and institutional norms. Furthermore, with only around 100 registered veterinary clinics in Kenya, selecting clinics at the individual level was more feasible and enabled coverage of a wider range of geographic areas and practice types. When multiple veterinarians were present at a clinic, clinic management selected one respondent directly involved in perioperative decision-making to avoid clustering effects and overrepresentation of individual clinics.

To achieve targeted sampling, a list of all registered and active small animal veterinary practices in Kenya was obtained from the Kenya Veterinary Board. Formal invitations to participate in the study were sent to the head veterinarian at each clinic. A total of 112 veterinary clinics across Kenya were invited to participate in the study. The aim of the survey was to purposively interview at least one veterinarian at each veterinary clinic in Kenya. Questionnaires were distributed through Google Forms to veterinarians who provided consent to participate in the study. The Google Forms remained accessible for six months, with bi-weekly reminders sent to all veterinarians who had not yet completed and returned the questionnaires.

Data collection

Data on knowledge and practices toward antibiotic use in the perioperative period were collected using an established questionnaire. The questionnaire was distributed to a licensed veterinarian employed at a recognized veterinary clinic in Kenya. The questionnaire was divided into four sections and included both closed-ended and a few open-ended questions. Section 1 included questions to collect respondents' demographics, such as gender, qualification level, years of practice, and monthly surgical procedures. Section 2 focused on perioperative surgical practices, including scrubbing methods, sterilization of surgical instruments, and aseptic preparation. Section 3 was the pivotal part of the

questionnaire and aimed at collecting data on antibiotic use across different surgical procedures, preferred antibiotic groups, timing and duration of administration, knowledge of and relevance to perioperative prophylactic antibiotics, and sources of information on antibiotic use in surgical patients. Lastly, Section 4 focused on knowledge of postoperative surgical site infections. The questionnaire was pretested, and its questions were refined before distribution to veterinarians. Additionally, the questionnaires were serialized to identify respondents, particularly those who preferred to remain anonymous.

Data analysis

Data were initially entered into Microsoft Excel 2010, coded, and imported into Stata 15.1 software for statistical analyses. For categorical variables, proportions were calculated; for continuous variables, the range, standard deviation, mean, mode, and median were computed.

To identify factors associated with perioperative antibiotic use, a multilevel mixed-effects logistic regression analysis was used. Antibiotic use was considered ideal when the veterinarian selected optimal use, and suboptimal when the veterinarian selected either underuse or overuse. Ideal perioperative antibiotic use was coded as 0, while suboptimal use was coded as 1. Univariable regression analysis was initially performed to assess the associations between all predictor variables and antibiotic use. All variables with $p \leq 0.3$ were then included in the multivariable logistic regression. Initially, correlations between predictor variables were evaluated using pairwise correlations. The final model was then fitted manually using backward stepwise elimination, retaining predictor variables with P-values of 0.05 or less. Plausible biological interactions between significant explanatory variables in the final model were analyzed. The overall performance of the model was evaluated using the area under the receiver operating characteristic curve (AUC).

Final multivariate logistic regression model checking was conducted to assess whether the model fit the data. The goodness-of-fit test was performed with Pearson chi-square ($P \geq 0.05$) and was considered acceptable; when the fit was not acceptable, the covariate patterns were restricted to 10, and the Hosmer-Lemeshow chi-square test was conducted. Overall predictive ability was assessed by evaluating the model's sensitivity and specificity, and a receiver operating characteristic curve was generated. Multicollinearity was assessed using the variance inflation factor, with values equal to or less than 10 (≤ 10) considered acceptable.

RESULTS

The demographic of the respondents

The questionnaire was distributed to 112 veterinarians; however, only 83 veterinarians provided consent and completed the questionnaire, resulting in an overall response rate of 74.1% (Table 1). Of the 83 respondents, 48 (57.8%) were male, and 35 (42.2%) were female. Regarding education, 80.7% (67/83) of respondents had only a Bachelor of Veterinary Medicine degree, while the remaining respondents (16/83) had attained postgraduate training at the Master's or Doctoral level. Most respondents had 2-5 years of clinical practice experience. Additionally, 50.6% (42/83) of the veterinarians were involved in mixed-animal practice, while 41% (34/83) and 8.4% (7/83) were exclusively in small- and large-animal practices, respectively.

Table 1. Demographic profile of the veterinarians who participated in the study between January and June 2023, Kenya

Demographic	Category	Number of respondents (n = 83)	Percentage of the respondents (%)
Gender	Male	48	57.8
	Female	35	42.2
Level of study	Undergraduate	67	80.7
	Postgraduate	16	19.3
Country of training	Kenya	81	97.6
	Outside Kenya	2	2.4
Duration in practice	< one year	18	21.7
	2-5 years	41	49.4
	6-9 years	16	19.3
	> 10 years	8	9.6
Type of practice	Mixed practice	42	50.6
	Small animal practice	34	41.0
	Large animal practice	7	8.4

Surgical practices among the respondents

Type and frequency of surgical procedures

The median number of surgeries per practice in a month was 5, with a range from 1 to 100 and an IQR of 7. When the respondents were asked to indicate the types of surgeries, they had frequently performed in the last six months, elective procedures such as spays and castrations were the most commonly mentioned, with 43 out of 83 respondents citing these surgeries. The elective surgeries were followed by reconstructive surgeries (42/83), orthopedic surgeries (5/83), and abdominal and emergency surgeries (2/83), while ocular and neurological surgeries were each reported by one respondent (Figure 1).

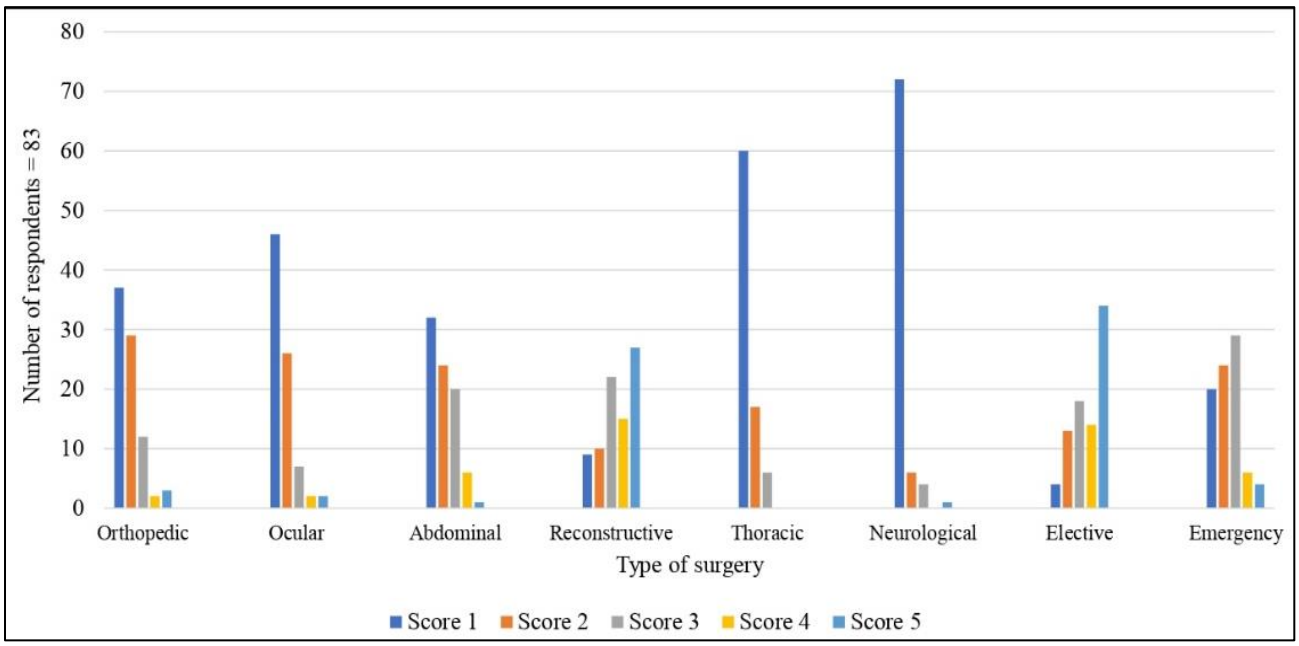


Figure 1. Different types of surgical procedures performed by veterinarians in Kenya between January and July 2023. Score 1: Very low, Score 2: Low, Score 3: Moderate, Score 4: High, Score 5: Very high

Anesthesia and analgesia practices

Most respondents reported using alpha-2 adrenoceptor agonists (90.4%; n = 75) and cyclohexamines (67.5%; n = 56) as common anesthetic agents during surgery. In contrast, benzodiazepines and opioids were rarely used, with only 10.8% (n = 9) and 7.2% (n = 6) of respondents, respectively. Regarding pain management, 55.4% (46/83) of respondents always administered analgesics to surgical patients, 37.3% (31/83) administered them based on the type of surgery, and 7.2% (6/83) did not administer them perioperatively. The most commonly used analgesics were non-steroidal anti-inflammatory drugs (65.1%), followed by corticosteroids (19.3%), drug combinations (7.2%), opioids (6.0%), and local analgesics (2.4%). Regarding the preferred timing for analgesic administration, 51.8% preferred giving analgesics before and after surgery, 34.9% preferred administration postoperatively, and 13.3% preoperatively only.

Preparation for aseptic surgery

The majority of respondents (86.7%) scrubbed their hands prior to a surgical procedure, with 41.0% scrubbing meticulously using a scrub solution, followed by disinfecting their hands with 70% ethyl alcohol. Furthermore, 48% of respondents consistently wore a head covering, 48% utilized a face mask, and 71% wore sterile surgical gloves during surgical procedures. The additional prevalent preoperative surgical practices are summarized in Table 2. Overall, 95.2% (n = 79) of respondents consistently sanitized the surgical site prior to aseptic procedures. The majority of respondents meticulously scrubbed the operative field with a scrub solution and then disinfected the site with 70% ethyl alcohol. Furthermore, 77.1% (n = 64) of respondents were involved in draping the surgical site, and 97.6% (n = 81) utilized sterilized surgical instruments. The predominant sterilization method for surgical instruments was chemical immersion (49.4%), followed by autoclaving (37.4%) and boiling (13.3%; Table 2). Regarding surgical infrastructure, 96.4% (80/83) of the practices had a room dedicated solely to patient preparation, while only 65% had a room dedicated solely to surgery. Surgical theatres were mainly disinfected after every surgery, as stated by 39.8% of the respondents (Table 2).

Table 2. Perioperative practices among veterinary clinicians in Kenya between January and June 2023

Perioperative practice	Category of the perioperative practice	Number of respondents (n = 83)	Percentage of the respondents (%)
Hand scrubbing	Do not scrub hands	11	13.3
	Always scrubs hands	72	86.7
Hand scrubbing technique	Disinfect my hands with alcohol	2	2.4
	Always scrub thoroughly using a scrub solution like chlorhexidine	18	21.7
	Scrub thoroughly using the scrub solution and disinfect the hands with alcohol	35	42.2
	Wash my hands using soap/hand wash solutions and disinfect my hands with alcohol	28	33.7
Use of head cover	Do not adorn a head cover	48	57.8
	Always adorn a head cover	35	42.2
Use of face mask	Do not wear a face mask	35	42.2
	Always wear a face mask	48	57.8
Use of sterile gloves	Do not wear sterile gloves	12	14.5
	Always wear sterile gloves	71	85.5
Scrubbing of surgical site	Do not scrub the surgical site	4	4.8
	Always scrub the surgical site	79	95.2
Draping of surgical Sites	Do not drape surgical site	19	22.9
	Always drape surgical site	64	77.1
Use of sterile surgical equipment	Do not use sterilized instruments	2	2.4
	Always use sterilized instruments	81	97.6
Sterilization method	Autoclave	31	37.4
	Boiling	11	13.2
	Chemical sterilization	41	49.4
Clinic has a dedicated surgical theatre	Yes	54	65.1
	No	29	34.9
Disinfecting surgical heaters	Never	10	12
	Twice per year	3	3.6
	Every 3 months	4	4.8
	Monthly	7	7
	Weekly	11	13.3
	Daily	15	18.1
	After every surgery	33	39.8

Antibiotic use perioperatively

The majority of veterinarians (85.5%) rated their perioperative antibiotic use as optimal, and only 6% (n = 5) reported overuse. Regarding the factors influencing perioperative antibiotic use and selection, 74.7% (n = 62) of respondents indicated that their prior knowledge and experience played important roles, while 37.3% (n = 31) used culture and sensitivity results (Table 3). Antibiotics were administered primarily postoperatively (67.5%), with the primary goals of prophylaxis and therapy, as reported by 66.3% (n = 55) of respondents. Furthermore, 59.1% (n = 49) of veterinarians either agreed or strongly agreed that administering an antibiotic was unnecessary when meticulous perioperative asepsis was carried out (Figure 2).

A general trend was observed in the frequency of antibiotic use across different surgical procedures. The utilization of antibiotics was more prevalent in contaminated surgical procedures compared to clean surgeries. In clean surgeries, the most frequently preferred classes of antibiotics were penicillin and tetracycline, which were cited by 68.7% and 12% of respondents, respectively. In clean-contaminated surgeries, penicillin emerged as the most commonly mentioned class, accounting for 69.9% of responses, while sulfonamides, tetracycline, and cephalosporins were each noted by 6%. Among contaminated surgeries, cephalosporins were the most commonly used antibiotic class (Table 4).

The frequency of antibiotic use during specific surgical procedures indicated that the highest usage was observed in emergency surgeries at 49.2% (n = 41), followed by reconstructive surgeries at 43.2% (n = 36), abdominal surgeries at 38.4% (n = 32), thoracic surgeries at 36% (n = 30), and orthopedic surgeries at 33.6% (n = 28; Figure 3).

Penicillin was the most preferred class of antibiotics in elective, emergency, and reconstructive surgical procedures, as indicated by 74.7% (n = 62), 59.0% (n = 49), and 57.5% (n = 46) of the respondents, respectively. In ocular surgeries, respondents predominantly favored penicillin at 32.1% (n = 26), followed by aminoglycosides at 21% (n = 17), and antibiotic combinations at 16.0% (n = 13). Furthermore, penicillin, sulphonamide, and antibiotic combinations were the preferred antibiotics for application in abdominal surgeries. Moreover, 38% (n = 30) of the veterinarians preferred penicillin for orthopedic procedures, whereas 22.8% (n = 18) preferred cephalosporins. Antibiotic combinations and penicillin were the most commonly used medications in thoracic and neurological surgeries. The details regarding the preferred antibiotics for particular surgical procedures are presented in Table 5.

Table 3. Antibiotic prescription, factors influencing antibiotic use, and intended purposes of perioperative antibiotic administration among veterinarians in Kenya between January and June 2023

Variable	Category of the variable	Number of respondents (n = 83)	Percentage of the respondents (%)
Prescription rate	Optimal	71	85.5
	Under	3	3.6
	Over	5	6.0
	Not sure	4	4.8
Factors influencing the choice of antibiotic	Bacterial culture and susceptibility	31	37.3
	Peer-reviewed scientific literature	14	16.9
	Textbooks	23	27.7
	Online sources	11	13.3
	Recommendation	24	28.9
	Prior knowledge and experience	62	74.7
Purpose of antibiotic therapy	Prophylactic	19	22.9
	Therapeutic	8	9.6
	Both prophylactic and therapeutic	55	66.3
	Don't give antibiotics perioperatively	1	1.2
Timing of antibiotic administration	Pre-operative	4	4.8
	Post-operative	56	67.5
	Intraoperative	3	3.6
	Preoperatively and postoperatively	19	22.9
	Do not give antibiotics perioperatively	1	1.2

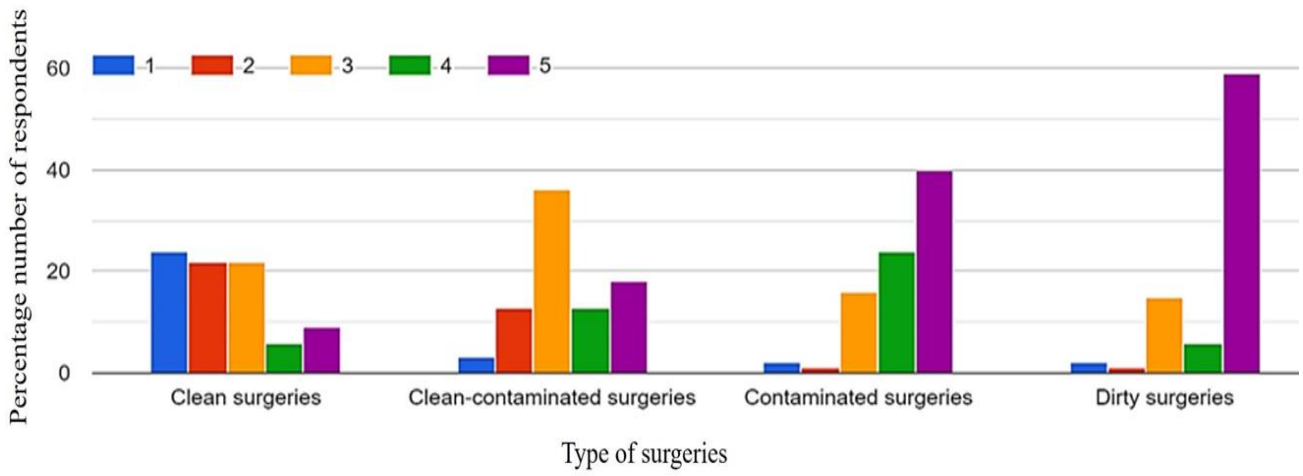


Figure 2. Different types of surgical procedures grouped by surgical sterility in Kenya between January and July 2023. Score 1: Very low, Score 2: Low, Score 3: Moderate, Score 4: High, Score 5: Very high

Table 4. Antibiotics used in elective, emergency, ocular, abdominal, reconstructive, orthopedic, thoracic, and neurologic veterinary surgeries in Kenya between January and June 2023

Surgery	Preferred antibiotics	Number of respondents (n = 83)	Percentage of the respondents (%)
Elective	Penicillin	62	74.7
	Drug combinations	5	6
	Tetracycline	5	6
	Others	11	13.3
Emergency	Penicillin	49	59
	Drug combination	11	13.3
	Tetracycline	7	8.4
	Cephalosporin	6	7.2
	Others	10	12.1
Ocular	Penicillin	26	32.1
	Aminoglycosides	17	21
	Drug combinations	13	16
	Lincosamides	4	4.9
	Others	23	26
Abdominal	Penicillin	24	29.6
	Sulphonamides	17	21
	Drug combinations	17	21
	Tetracycline	7	8.6
	Cephalosporin	5	6.2
	Others	13	13.6
Reconstructive	Penicillin	46	57.5
	Drug combinations	10	12.5
	Tetracycline	6	7.5
	Cephalosporin	5	6.2
	Sulphonamide	3	3.7
	Lincosamides	3	3.7
	Other drugs	10	12.1
Orthopaedic	Penicillin	30	38
	Cephalosporin	18	22.8
	Drug combination	14	17.7
	Fluoroquinolone	4	5.1
	Other drugs	17	16.4
Thoracic	Penicillin	28	35.4
	Drug combination	16	20.3
	Aminoglycoside	9	11.4
	Tetracycline	6	7.6
	Cephalosporin	5	6.3
	Other drugs	19	19
Neurological	Drug combinations	20	25.6
	Penicillin	19	24.4
	Cephalosporin	8	10.3
	Aminoglycoside	5	6.4
	Tetracycline	4	5.1
	Fluoroquinolone	4	5.1
	Others	25	27.7

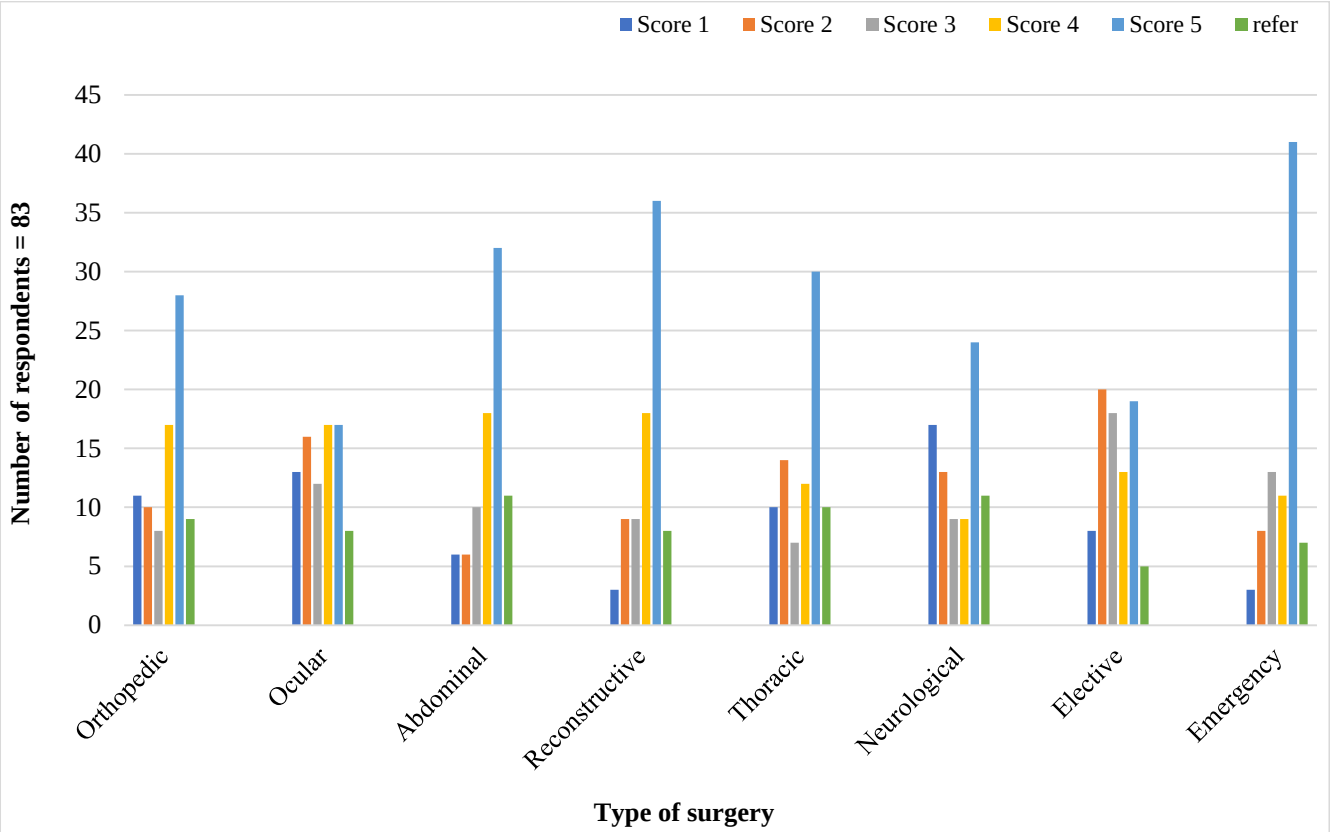


Figure 3. Antibiotic use in different small animal surgical procedures in Kenya between January and July 2023. High antibiotic use was observed in reconstructive, orthopedic, abdominal, thoracic, neurological procedures, elective, and emergency surgeries. Score 1: Very low, Score 2: Low, Score 3: Moderate, Score 4: High, Score 5: Very high

Table 5. Univariable predictors of antibiotic use among veterinary surgical patients in Kenya between January and July 2023

Predictor variable	P-value
Surgeons' level of education	0.192
The country where the veterinary degree was acquired	0.202
Perform reconstructive surgery often	0.073
Anesthesia where alpha-2 agonists are used	0.068
Surgeons always scrub their hands	0.005
Surgeons always wear gloves	0.272
Use of the ideal hand scrubbing technique	0.010
Method of instrument sterilization	0.185
Antibiotic use based on culture and sensitivity	0.127
Antibiotic use based on online literature	0.207
Antibiotics based on recommendations	0.298
Antibiotic use based on prior knowledge	0.168
Surgical site infections in emergency surgery	0.112
Surgical site infections in ocular surgery	0.218
Surgical site infections in abdominal surgery	0.028
Surgical site infections in reconstructive surgery	0.033
Surgical site infections in other surgeries	0.141
Use of the ideal surgical site scrubbing technique	0.010
Chemical sterilization of the instrument	0.067
Autoclave surgical instrument	0.127

Univariable associations $p \leq 0.3$ of predictor variables with antibiotic use in veterinary surgical patients in Kenya

Surgical site infection

Occurrence of surgical site infections

The majority of respondents (85.5%, 71/83) reported a low frequency of postoperative SSI (score <2), whereas only a small proportion (4%, 3/83) assigned a high score (4). However, the incidence of SSI was significantly higher in reconstructive and orthopedic surgeries (scores 4-5) than in ocular and elective procedures (scores 1-2; Figure 4).

The most frequently selected antibiotic class for SSI treatment was penicillin (42.2%, 35/83), with 22.9% of respondents choosing two or more antibiotics and 15.7% (13/83) selecting cephalosporins (Figure 5). Furthermore, 68% of respondents either agree or strongly agree that the use of prophylactic antibiotics can significantly reduce SSI (Figure 6).

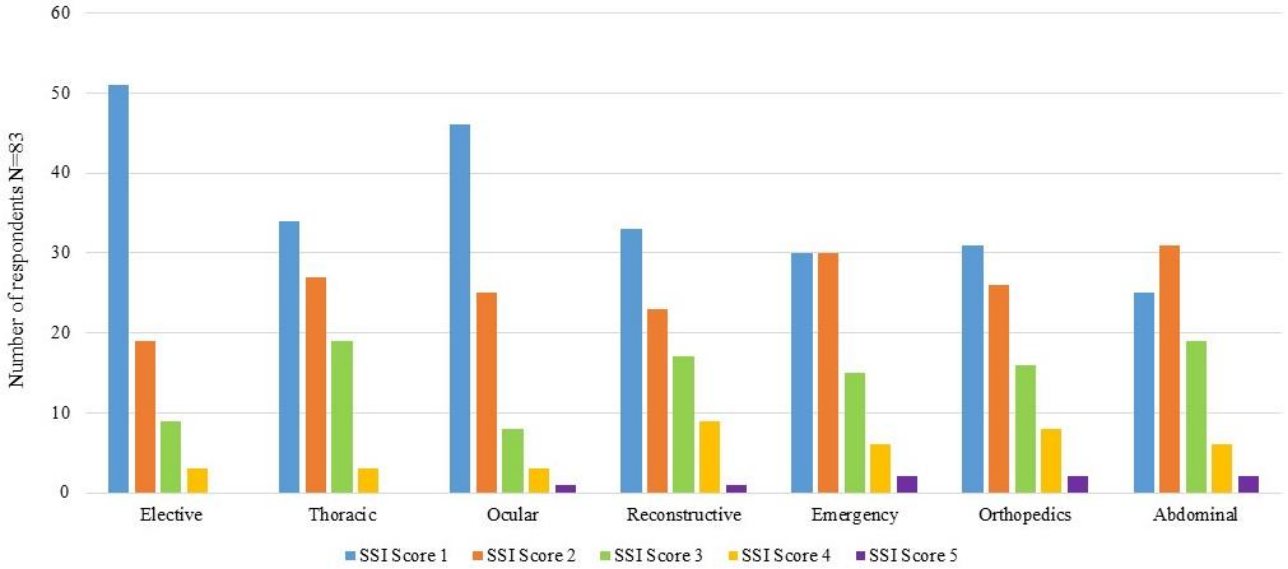


Figure 4. Surgical site infection rates in different small animal procedures in Kenya between January and July 2023. SSI score 1: Very low occurrence, SSI score 2: Low occurrence, SSI score 3: Moderate occurrence, SSI score 4: High occurrence, SSI score 5: Very high occurrence

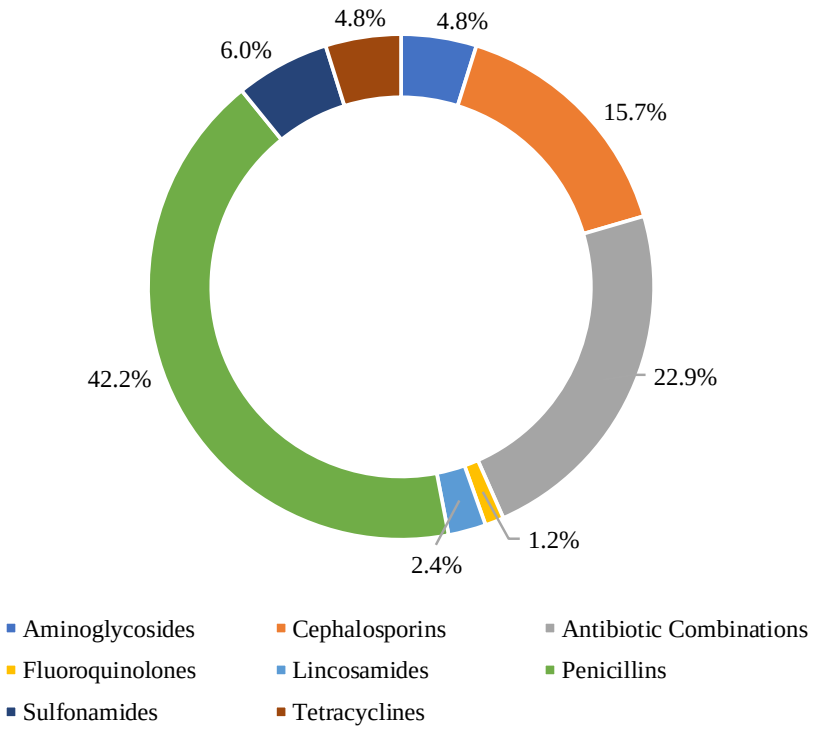


Figure 5. Preferred choice of antibiotics to treat surgical site infections in small animal surgeries in Kenya between January and July 2023.

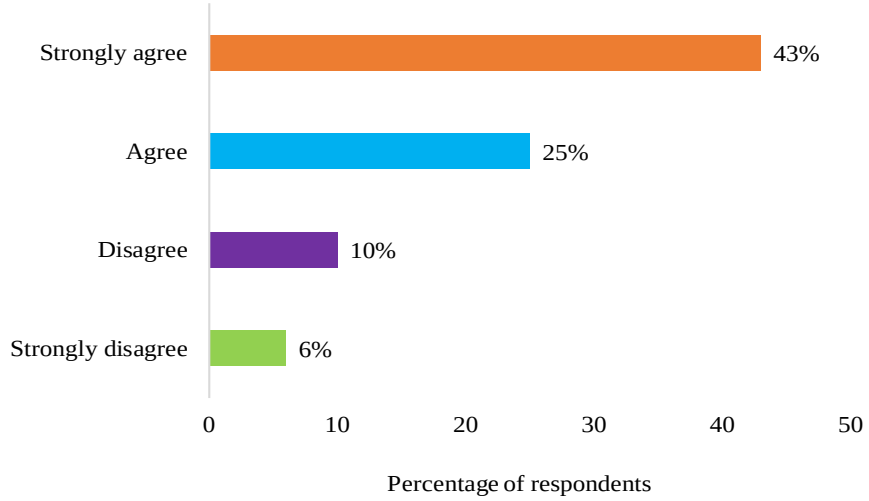


Figure 6. Perceptions of 83 veterinarians who participated in this survey regarding the role of prophylactic antibiotics in minimizing surgical site infections in small-animal surgeries in Kenya between January and July 2023.

Factors associated with antibiotic use in veterinary surgical patients

The final antibiotic-use model identified three variables significantly associated with antibiotic use in veterinary surgical procedures ($p < 0.05$). These variables were surgeons' level of education, whether surgeons always scrub their hands, and the use of optimal surgical site scrubbing techniques (applying a scrub solution followed by disinfecting the site with 70% ethyl alcohol; Table 6). The likelihood of antibiotic use in veterinary surgical cases was decreased by 10% when surgeons consistently performed hand scrubbing prior to surgery, and by 8% when the surgical site was prepared using an optimal sterilizing technique. Surgeons with a Bachelor of Veterinary Medicine degree exhibited a times higher likelihood of administering antibiotics to surgical patients compared to those with postgraduate qualifications.

Table 6. Final multilevel mixed-effects logistic regression model of antibiotic use in veterinary surgical procedures in Kenya between January and July 2023

Variable	Category	OR	95% CI	P-value
Educational level of surgeons	Bachelor of veterinary medicine	10.01	1.39 - 72.12	0.022
Surgeons always scrub their hands	Yes	0.10	0.17 - 0.58	0.01
Use of ideal surgical site scrubbing technique	Yes	0.08	0.01 - 0.50	0.008

OR: Odds ratio, CI: Confidence interval

DISCUSSION

The current study is among the first to report antimicrobial use practices during the perioperative period among veterinarians in Kenya. Most respondents in the present study were young veterinarians primarily engaged in mixed- and small-animal practices. The veterinarians have completed a curriculum that highlighted antimicrobial resistance and antimicrobial stewardship, incorporating these essential subjects into their clinical practice. Furthermore, due to their limited clinical experience, young veterinarians might be more likely to overuse antimicrobials during the perioperative period as a preventive strategy against SSI. Since the majority of respondents were veterinarians working in clinical settings, their perioperative medication decisions were likely influenced by individual clinical judgment and the operational practices of their respective hospitals or clinics.

The present study revealed that veterinarians primarily performed elective and reconstructive surgeries, and antibiotic administration during the perioperative period was often determined by the nature of the surgical procedure. Elective surgeries are categorized as clean procedures that generally do not require antibiotic administration (Jessen et al., 2019), particularly when aseptic protocols and meticulous surgical techniques, including surgical scrubbing, gowning, and the use of sterilized tools and materials, are followed (Nelson, 2011; Frey et al., 2022). These protocols and evidence were consistent with the present findings, as very few veterinarians reported administering antibiotics during elective and ocular surgeries, which were predominantly clean procedures. Notably, a study by Vasseur et al.

(1985) found no difference in infection rates between dogs subjected to clean surgical procedures who received prophylactic ampicillin and those who received a placebo, supporting the present results. Supporting the present results, Välkki et al. (2020) and Sørensen et al. (2024) indicated that antibiotic prophylaxis did not markedly reduce infection rates in clean, uncomplicated soft-tissue surgeries compared to strict aseptic technique alone

Numerous veterinarians assessed their antibiotic prescription practices as optimal, likely due to the comparatively low incidence of SSI, which was predominantly attributable to rigorous perioperative aseptic protocols. In the present study, the likelihood of antibiotic use in surgical procedures diminished by 10% when surgeons consistently performed hand scrubbing prior to surgery, and by 8% when the surgical site was prepared using an optimal technique. Furthermore, the majority of veterinarians agreed that antibiotics were unnecessary when meticulous perioperative asepsis was maintained. The present findings highlighted the vital role of aseptic preparation in reducing antibiotic use in veterinary patients, as supported by Nelson (2011) and Jessen et al. (2019). Rocktäschel et al. (2020) demonstrated that pre-surgical hand scrubbing notably reduced bacterial counts on the hands in a veterinary setting. Furthermore, proper hand and site preparation can effectively decrease the presence of *Staphylococcus pseudintermedius*, the most frequently isolated organism from canine SSIs, thereby lowering the risk of contamination (Nelson, 2011; Crosse, 2022; Singleton, 2024).

In the current study, veterinarians reported administering antibiotics primarily for prophylaxis rather than therapy. This empirical method for perioperative selection, based on existing knowledge and experience, was anticipated in Kenya. In surgical procedures, antibiotics are primarily used to prevent infections, as the likelihood of infection during surgery is generally low (Cavalli et al., 2025). Therefore, surgeons often cannot obtain samples for culture and sensitivity testing, except in cases of confirmed SSIs (Weese et al., 2015). Previous studies indicated that antibiotic prophylaxis was particularly beneficial in high-risk procedures, such as neurosurgery, where infections could be catastrophic, or orthopedic surgery involving implants and substantial tissue trauma (Nelson, 2011; Välkki et al., 2022; Gulaydin et al., 2024). In these surgeries, antibiotics, particularly cefuroxime or amoxicillin-clavulanic acid, were administered 30-60 minutes prior to the initial incision during orthopedic procedures in both animals and humans, with redosing every 90 minutes intraoperatively until completion of the surgery; after that, no further antibiotic use was warranted (Weese et al., 2015; Sørensen et al., 2024). These findings were inconsistent with the present results, as the majority of veterinarians administered antibiotics postoperatively, whereas only a minority adhered to prophylaxis protocols prior to or during surgery.

In the present study, the decision to use perioperative antibiotics was predominantly influenced by the extent of surgical contamination, with veterinarians reporting higher use in contaminated and dirty procedures than in clean and clean-contaminated surgeries. In clean surgeries, such as elective procedures without entry into contaminated viscera, antibiotic prophylaxis was generally not required. However, Mangram et al. (1999) and Weese et al. (2015) suggested that prophylaxis may be necessary for cases involving longer operative times, implant placement, or immunocompromised patients. This suggestion aligns with the current findings, where administering penicillin prophylactically in orthopedic surgeries notably decreased the rate of surgical site infections by 10%. Clean-contaminated surgeries, involving controlled entry into the gastrointestinal, respiratory, or urogenital tracts, require prophylactic agents such as cefazolin or amoxicillin-clavulanic acid (Boothe and Boothe, 2015). These antibiotics target common skin bacteria, particularly *Staphylococcus pseudintermedius*, which is a frequent cause of surgical site infections (Weese and Van Duijkeren, 2010; Nelson, 2011). Contaminated surgeries, such as those involving fresh traumatic wounds or procedures with gross spillage of gastrointestinal contents, require broader antibiotic coverage, typically including metronidazole for anaerobic activity (Boothe and Boothe, 2015; Weese et al., 2015). The duration of antibiotic administration is typically restricted to 24 hours postoperatively (Eugster et al., 2004; Frey et al., 2022). In contaminated surgeries with existing infections, such as abscess drainage, septic peritonitis, bite wounds, or septic pyothorax, antibiotics function primarily as a treatment rather than a preventive measure (Boothe and Boothe, 2015). In contaminated surgeries, immediate, culture-guided antibiotic treatment targeting pathogens such as anaerobes and *Pasteurella* spp. is necessary, with therapy frequently extending over several weeks (Boothe and Boothe, 2015; Ribeiro et al., 2024). The present study revealed widespread postoperative use of penicillins and cephalosporins across different surgical procedures, often without appropriate indications, such as culture and sensitivity results or evidence of gross contamination or dirty wounds. Previous studies have demonstrated that prolonged postoperative antibiotic administration was not only ineffective for infection control but also contributed to the development of antimicrobial resistance (Nelson, 2011; Cavalli et al., 2025). Effective infection prevention during surgery relies on strict aseptic techniques and the application of fundamental surgical principles such as debridement, lavage, and drainage (Jessen et al., 2019).

CONCLUSION

The present study demonstrated that adherence to perioperative practices that promote aseptic surgery can effectively reduce reliance on antibiotic use in Kenya. The majority of respondents (85.5%) considered their perioperative antibiotic use optimal, with only a small number of veterinarians rating it suboptimal. An important limitation of the present study was the uneven distribution of the outcome variable used in the regression analysis. The current findings may be influenced by decision-making and social desirability biases, as veterinarians might have reported practices they perceived as appropriate rather than their actual practices. Future longitudinal or interventional studies should be conducted to enhance understanding of how perioperative practices affect antibiotic use in Kenyan veterinary practice.

DECLARATIONS

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Authors' contributions

Moffat Njuguna conceptualized the study, collected and analyzed data, and prepared the first draft of the manuscript. Willy Mwangi conceptualized and designed the study, prepared the data collection tools, analyzed data, and revised and proofread the final manuscript. John Mande participated in data interpretation as well as preparation, proofreading, and approval of the final manuscript. All authors have read and approved the final edition of the manuscript before publication in the present journal.

Availability of data and materials

Inquiries regarding the questionnaire and data can be directed to the corresponding author.

Competing interests

The authors declare no conflict of interest.

Ethical considerations

The authors have 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 AI tools were used for data generation, analysis, or interpretation. The authors are solely responsible for the accuracy, originality, and integrity of the final content before submission, during the revisions, and final publication of the article.

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Influences of Genetic, Feed, and Technological Factors on the Productivity and Reproductive Capacity in Farm Animals

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ABSTRACT

Productive and reproductive performance in farm animals is shaped by the combined effects of genetic potential, nutritional conditions, and technological management, although the relative importance of these factors differs among species. The present study aimed to identify the main genetic, nutritional, and technological factors influencing the productive and reproductive performance of farm animals and to assess their relative contributions and interactions. A 12-month controlled comparative study included 480 clinically healthy animals, including 120 cattle, 160 pigs, and 200 laying hens of the selected breeds. Within each species and breed, eligible animals were grouped according to age, sex, physiological status, and body condition score. Animals with similar values for these variables were then matched and non-randomly allocated to control and experimental groups to minimize pre-intervention differences between the groups. In cattle, the assessed parameters included milk yield, milk fat and protein content, liveweight gain, pregnancy rate after first insemination, calf birth weight, and calf survival to weaning. In pigs, average daily gain, slaughter yield, intramuscular fat content, litter size, piglet birth weight, and pre-weaning survival were evaluated. In laying hens, egg production, egg weight, the proportion of eggs with shell or shape defects, hatchability, and chick survival were assessed. Measurements were obtained from July 2024 to June 2025 from production records, electronic weighing, infrared milk analysis, ultrasonography, Soxhlet extraction, and automated egg analysis. In cattle, genetic factors, particularly breed and breeding improvement, had the greatest influence in increasing milk yield by 5.8-9.3% and milk protein content by 0.08-0.12 percentage points. In pigs, nutritional factors made the greatest contribution, including optimization of the dietary protein-to-energy ratio, which increased average daily gain by 7.0%, litter size by 4.5%, and intramuscular fat content by 16.7%. In laying hens, combined nutritional and genetic factors increased egg production by 3.9-6.0% and reduced the proportion of non-standard eggs by 31.1%. Technological optimization of microclimate, stocking density, reproductive management, and stress reduction stabilized performance and increased piglet survival by 1.5-2.8%. The findings support species-specific integration of breeding, nutritional, and management strategies.

Keywords: Breeding, Microclimatic condition, Optimization of feeding, Product quality, Reproduction indicator

INTRODUCTION

Improving the productive and reproductive efficiency of farm animals is essential for maintaining the economic sustainability of livestock production and meeting the growing demand for animal-derived food products. These outcomes are determined by the combined effects of genetic potential, nutritional management, and production technologies, yet the relative contribution and interaction of these factors remain insufficiently understood across different animal species (Tsvihun et al., 2025). The intensification of livestock production requires the integrated application of selective breeding, nutritionally balanced feeding strategies, advanced housing and management technologies, and biotechnological methods. Rising demand for livestock products coincides with increasing pressure on natural and economic resources, while climate-related changes further undermine the stability of production systems (Szőllősi et al., 2021; Nurgaliyev et al., 2024).

Kramarenko et al. (2023) examined the factors associated with an increased risk of stillbirth in Ukrainian Meat breed sows. Their findings demonstrated that deviations from recommended indoor microclimatic parameters and non-compliance with prescribed housing and management practices were associated with higher reproductive losses. They noted that optimizing temperature, humidity, and ventilation in sow housing is a key measure for reducing reproductive losses during and immediately after farrowing. Kramarenko et al. (2025) examined the effect of lactation duration on dairy animal productivity and found that even minor deviations from the optimal lactation period were associated with fluctuations in annual milk yield and, consequently, production profitability. The authors substantiated the feasibility of systematic monitoring and planning of lactation duration as a factor in improving the overall efficiency of the herd.

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Dayoub et al. (2024) reviewed the application of smart agriculture technologies in animal husbandry, including sensor-based monitoring systems, automated feeding equipment, environmental control technologies, and artificial intelligence tools for data analysis and management decision-making. It was noted that sensor-based animal monitoring, automated feeding systems, and artificial intelligence combined with data analytics can improve the accuracy of management decisions and reduce farm operating costs associated with labour, feeding, routine monitoring, and resource use (Sychov et al., 2024). A complex of agronomic and breeding methods for increasing the nutritional value of forage crops is considered in the study of Chand et al. (2022). The authors emphasized that improving the protein and mineral balance of feed was associated with higher animal productivity and better physiological condition. Mueller and Van Eenennaam (2022) examined the combined use of genomic selection, assisted reproductive technologies, and gene editing to accelerate genetic improvement in cattle. According to the authors, combining these methods can speed up genetic improvement in cattle and help breeders introduce desirable productive and adaptive traits more quickly. A review by Akintan et al. (2024) highlighted the latest approaches to the development of diets that combine environmental sustainability and high nutritional value. Innovative technologies for processing and selecting feed ingredients can improve nutrient digestibility, optimize the balance of protein, energy, and minerals, and reduce feed losses (Nurgul et al., 2025). As a result, animals use nutrients more efficiently, productivity increases, and the environmental burden of livestock production is reduced through lower feed consumption, nutrient excretion, and waste generation.

Menchaca (2021) reviewed the prospects of gene editing in cattle, pigs, sheep, and goats, particularly its potential to improve productivity and product quality, increase resistance to infectious diseases and environmental stress, and enhance animal welfare by reducing the need for invasive husbandry procedures. Gene editing is regarded as an important component of future strategies for strengthening food security. Melak et al. (2024) analyzed the use of machine-learning and computer-vision systems, sensor-based monitoring tools, robots, and drones for managing animal health, behavior, feeding, and welfare on livestock farms. Automated forecasting and monitoring systems enable farm managers to detect changes in animal health, behavior, and feeding patterns at an early stage, allowing timely intervention before these changes lead to reduced productivity. Davis and White (2020) showed that improving reproductive efficiency in cattle can reduce the number of breeding cows required to produce a given quantity of beef, thereby lowering resource use and environmental costs, while reproductive technologies can accelerate genetic improvement in fertility, productivity, and adaptation. The integration of selective breeding, reproductive biotechnologies, and effective reproductive management can improve fertility, accelerate genetic progress, and increase both the quantity and quality of livestock products. Taken together, these studies indicate that the current study increasingly supports the integration of genetic improvement, reproductive biotechnologies, precision feeding, and advanced management systems to enhance animal productivity and reproductive performance while maintaining animal welfare and production efficiency.

Under these conditions, there was a need for a systematic study of the factors that determine the level of productivity and reproductive capacity of different types of farm animals and to establish relationships between them to optimize production parameters. The study novelty lies in the comparative cross-species evaluation of these factors within a single analytical framework, which allowed the leading factor and the most effective combinations to be identified for each animal species. Therefore, the present study aimed to assess the effects of genetic, nutritional, and technological factors on the productive and reproductive performance of cattle, pigs, and laying hens.

MATERIALS AND METHODS

Ethical approval

All experimental procedures were performed in accordance with Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes (European Parliament and Council of the European Union, 2010).

Study location and timing

The 12-month study was conducted from July 2024 to June 2025 under commercial production conditions at the Agro-Oven (2025) agricultural enterprise in Mahdalynivka, Dnipropetrovsk Oblast, Ukraine, which operated cattle, pig, and poultry production units (Figure 1).

Study design

A prospective, non-randomized controlled field study was conducted at a single agricultural enterprise selected purposively because it maintained cattle, pigs, and laying hens under commercial production conditions, thereby allowing genetic, nutritional, and technological factors to be assessed within the same production setting. Animals were

recruited from the available farm population using purposive non-probability sampling. Only clinically healthy animals that met the predefined criteria for breed, age, sex, physiological status, and body condition were eligible for inclusion.

The sample size was determined pragmatically by the number of eligible animals available during the study period and the need to obtain equal representation of the selected breeds within each animal category (Lakens, 2022). The final sample comprised 480 animals, including 120 cattle, including 30 Holstein, 30 Ukrainian Black-and-White Dairy, 30 Aberdeen Angus, and 30 Limousine cattle; 160 pigs, including 80 Large White and 80 Landrace pigs; and 200 laying hens, including 100 Hy-Line Brown and 100 Leghorn hens.

The breeds were selected because they were available at the study enterprise, represented distinct dairy, beef, pig, and egg-production types, and had established commercial relevance. Holstein and Ukrainian Black-and-White Dairy cattle are widely represented in high-producing Ukrainian dairy herds, while Hy-Line Brown hens are characterized by high egg production under commercial production systems.



Figure 1. Location of the Agro-Oven agricultural enterprise in Mahdalynivka, Dnipropetrovsk Oblast, Ukraine. Source: Agro-Oven (2025).

Animals and grouping

For cattle and pigs, four groups were formed for each species, including one control group and three experimental groups differing in the genetic, nutritional, or technological factor under evaluation. For laying hens, three groups were formed, including one control group and two experimental groups. Thus, the study comprised 11 groups in total, with animals within each species matched for age, sex, breed, physiological status, and body condition. Animals were eligible for inclusion if they were clinically healthy, belonged to the selected breed and predefined age, sex, and physiological categories, and had a body condition score of 3.0-3.5 on a 5-point scale.

Animals were excluded if, at the time of screening, they had acute or chronic disorders capable of affecting productive or reproductive performance, or if they had received antibiotic treatment within the 30 days preceding enrolment. The experimental conditions were organized according to three categories of factors. Genetic factors included breed and membership in breeding-improved lines; nutritional factors included the dietary protein-to-energy ratio, feeding frequency, and feed supplementation; and technological factors included microclimatic conditions, stocking density, reproductive procedures, and exposure to transport, noise, or heat stress. Animals in the control groups were fed twice daily at fixed times according to the standard feeding schedule used at the farm. The control groups were maintained under the farm's standard housing conditions, with an ambient temperature of 22-24°C and relative humidity of 60-65%. Stocking density was set at 10 cattle per 10 m², 8 pigs per 10 m², and 6 laying hens per 1 m². In the nutritional experimental groups, the diets were adjusted according to animal species; the feed for laying hens contained 16.5% crude protein, and animals were fed three times daily, compared with twice-daily feeding in the control groups. In the technological experimental groups, stocking density and microclimatic conditions were adjusted to a temperature of

18-20°C and relative humidity of 55-60%, while ventilation for pigs was maintained at 0.2 m³/h per animal. For each animal species, the study included a control group maintained under the farm's standard production conditions and separate experimental groups in which one predefined nutritional or technological factor, such as the protein-to-energy ratio, feeding frequency, or stocking density, was modified.

The allocation of animals to the control and experimental groups is described below. Productive and reproductive outcomes were compared between the control group and each experimental group. Three control-versus-treatment comparisons were conducted for cattle and pigs, covering genetic improvement, nutritional optimization, and technological management, whereas two comparisons were conducted for laying hens, covering nutritional and technological interventions. For each animal species, productive and reproductive outcomes were compared between a control group and experimental groups exposed to predefined genetic, nutritional, or technological conditions. The control groups were maintained according to the farm's standard production protocol, which included twice-daily feeding; an ambient temperature of 22-24°C; relative humidity of 60-65%; standard stocking densities of 10 cattle per 10 m², 8 pigs per 10 m², and 6 laying hens per 1 m²; and conventional reproductive management. The experimental groups differed from the control group in one principal factor, including breeding improvement, optimization of the diet and feeding frequency, or modification of microclimatic, stocking-density, and reproductive-management conditions. The exact number of animals allocated to each control and experimental group is presented in Tables 1-4.

Control and breeding-improved groups

The control group comprised animals from standard farm lines that had not been included in targeted selection programmed based on pedigree and productivity records. These animals were maintained under the farm's conventional feeding, housing, and reproductive-management conditions. The breeding-improved group included animals from lines selected over several generations for superior productive and reproductive traits, including milk yield in dairy cattle, live-weight gain in pigs, and egg production in laying hens. Animals in the breeding-improved group belonged to high-performing lines selected on the basis of productive and reproductive records. Optimized diets with a balanced protein-to-energy ratio were provided to the nutritional experimental groups and were evaluated separately from the effect of genetic selection. Female cattle and sows in the breeding-improved groups were artificially inseminated using commercial semen doses obtained from breeding males selected on the basis of documented pedigree, performance records, and estimated breeding values provided by the semen supplier.

Experimental factors and conditions

The influence of three groups of factors was studied, including genetic factors, nutritional factors, and technological factors related to animal housing and management. Two main breeds of cattle were selected for genetic factors, including Holstein and Ukrainian Black-and-white Dairy, meat pigs (Large White and Landrace), and egg-laying hens (Hy-Line Brown and Leghorn). Breeding-improved groups were selected from within each breed based on documented participation in productivity improvement programmed conducted during the preceding 5-7 years. The selection of breeding-improved groups within each breed was based on official breeding records and documented productivity data collected over several generations. Breeding improvement was assessed in terms of milk yield, weight gain in pigs, and egg production in hens. For feed factors, rations were formed in accordance with farm standards and the protein-energy ratio was optimized. For example, for cattle, the diet contained 16% protein per digestible protein and 14.0 MJ/kg of metabolic energy. For pigs and hens, the feed contained 20% protein and 15.0 MJ/kg of energy for pigs, 16.5% protein and 12.0 MJ/kg of energy for hens, respectively (Table 1).

For the technological experimental groups of cattle, pigs, and laying hens, the housing microclimate was maintained at 18-20°C and 55-60% relative humidity, with air velocity not exceeding 0.2 m/s. In the control groups, conditions were standard for the farm, with a temperature of 22-24°C and humidity of 60-65%. For insemination, high-quality sperm from the manufacturer Genus Public Limited Company (GPLC; United Kingdom) was used, which provided a high concentration of spermatozoa. Stress factors included four-hour transportation of cattle and pigs, continuous elevated environmental noise in the pig housing facility, and exposure of laying hens to ambient temperatures above 30°C. Noise exposure was assessed in the pig technological group. Sound pressure levels were measured in dB(A) using a calibrated integrating sound-level meter conforming to IEC 61672-1, with measurements taken at approximately animal-head height at several locations within the housing area. The equivalent continuous A-weighted sound level, LAeq, was recorded during the six-hour observation period. Noise exposure was defined as a continuous sound level of approximately 85 dB(A), whereas the control group remained under the ordinary background-noise conditions of the facility. The selected threshold was based on Council Directive 2008/120/EC (European Union, 2008), according to which continuous noise levels of 85 dB(A) or higher and constant or sudden noise should be avoided in pig housing. Animal responses were evaluated through serum cortisol concentrations and behavioral indicators before and after

exposure.

Table 1. Dietary composition, and feeding management in the control and experimental farm animals

Animal species	Group	Crude protein content	Metabolizable energy	Main feed additives	Feeding frequency	Feeding equipment
Cattle	Control	Farm-standard level (National Academies of Sciences, Engineering, and Medicine, 2021)	Farm-standard level (National Academies of Sciences, Engineering, and Medicine, 2021)	Standard mineral and vitamin supplementation used at the farm (National Academies of Sciences, Engineering, and Medicine, 2021)	Twice daily	Trioliet Solomix feed dispenser
Cattle	Experimental nutritional group	16.0%	14.0 MJ/kg	Calcium phosphate, copper, zinc, and selenium, mineral and vitamin permix	Three times daily	Trioliet Solomix feed dispenser
Pigs	Control	Reduced or farm-standard protein level (National Academies of Sciences, Engineering, and Medicine, 2021)	Farm-standard level (National Academies of Sciences, Engineering, and Medicine, 2021)	Standard supplementation used at the farm (National Academies of Sciences, Engineering, and Medicine, 2021)	Twice daily	Big Dutchman automated feeder
Pigs	Experimental nutritional group	20.0%	15.0 MJ/kg	Lysine 98% manufactured by Adisseo, methionine, calcium phosphate, copper, zinc, and selenium	Three times daily	Big Dutchman automated feeder
Laying hens	Control	14.0%	Farm-standard level (National Academies of Sciences, Engineering, and Medicine, 2021)	Standard vitamin and mineral supplementation used at the farm (National Academies of Sciences, Engineering, and Medicine, 2021)	Twice daily	Big Dutchman automated feeder
Laying hens	Experimental nutritional group	16.5%	12.0 MJ/kg	Poultry Boost™ manufactured by DSM company, methionine, calcium phosphate, copper, zinc, and selenium, vitamins A, D, and E	Three times daily at fixed intervals	Big Dutchman automated feeder

All feed additives were administered according to the manufacturers' recommendations. The control diets followed the farm's standard feeding practices, whereas the experimental diets were formulated by optimizing the protein-to-energy ratio, supplementation, and feeding frequency. The manuscript does not provide the proportions of individual feed ingredients; therefore, the table presents the available nutrient specifications and feeding conditions rather than a complete ingredient formulation.

Productivity and reproductive outcome measures

Milk yield was recorded from individual production records, while milk fat and protein contents were determined using a MilkoScan FT 120 infrared analyzer. Pregnancy rate after first insemination was confirmed by ultrasonography on day 30, calf birth weight was measured using electronic scales, and survival to weaning was determined from individual farm records in accordance with ICAR guidelines. Pregnancy after first insemination was diagnosed by transrectal ultrasonography on day 30 and reconfirmed on day 60 by repeat ultrasonography or rectal palpation. Milk yield and milk composition were recorded monthly throughout the 12-month study, whereas pregnancy status, calf birth weight, and survival to weaning were assessed at the relevant physiological stages. Average daily weight gain, slaughter yield, multiparity, and offspring survival were determined in pigs. Average daily increments were measured monthly using electronic scales. At the end of the study, slaughter yield was calculated from preslaughter live weight and carcass weight, while intramuscular fat content was determined using Soxhlet extraction (Melak et al., 2024). Extraction was performed with n-hexane at 70°C for 4 hours. The measurements were performed at the end of 12 months of the study. Multiparity was determined at the time of farrowing, and offspring survival was determined before weaning. Egg production was measured monthly by counting the number of eggs per laying hen for 30 days. Egg weight was

determined using an EggAnalyzer™ automatic analyser (Orka Food Technology, Israel). Egg hatching was evaluated on day 21 of incubation by counting the number of hatched chicks compared to the number of incubated eggs.

Measurement equipment

Milk fat and protein contents were determined using a MilkoScan FT 120 infrared analyser (FOSS, Denmark). Cattle and pigs were weighed using an AXIS BDU1500 electronic scale (AXIS, Poland). Microclimate parameters were monitored using Testo 635-2 thermohygrometers and Testo 416 anemometers (Testo, Germany). Egg quality was determined by an automatic EggAnalyzer™ analyser (Orka Food Technology, Israel). Trioliet Solomix feed mixers (Trioliet, the Netherlands) were used to mix and distribute cattle rations, while Big Dutchman automatic feeders (Big Dutchman, Germany) were used to dispense feed to pigs and laying hens. The proportion of eggs with visible shell defects, cracks, or abnormal shape was determined by visual inspection in accordance with the State Consumer Standard of Ukraine (2008).

Statistical analysis

Statistical analyses were performed using Statistica 10.0 (StatSoft, USA) and IBM SPSS Statistics 25 (IBM, USA). The student's t-criterion was used to compare the two groups, and the univariate or multivariate analysis of variance (ANOVA) was used for three or more groups. For a multivariate ANOVA, interactions between major factors such as genetics × feed, genetics × technology, feed × technology, and between all three factors (genetics × feed × technology) were analyzed. This allowed identification of the influence of each factor separately, in addition to their combined interaction on the productivity and reproductive indicators of animals. For qualitative indicators, the chi-square ($\chi 2$) test was used. The statistical significance level was taken as $p < 0.05$, and the confidence intervals were calculated with $\alpha=0.05$. Quantitative data were presented as mean ± standard deviation (SD), together with absolute and percentage changes between groups.

RESULTS AND DISCUSSION

The analysis revealed statistically significant differences in productive performance among cattle, pigs, and laying hens according to breed and breeding-improvement status ($p < 0.05$). In cattle, breeding improvement increased milk yield from 5,800 to 8,200 kg per cow per year and milk protein content from 3.1% to 3.5% ($p < 0.05$). In meat cattle, average daily weight gain increased from 1.05 to 1.25 kg/day ($p < 0.05$). In pigs, breeding improvement increased average daily liveweight gain from 730 to 820 g and multiparity from 10.8 to 12.5 piglets per farrowing ($p < 0.05$). In laying hens, breeding improvement increased annual egg production from 305 to 314 eggs per hen and egg weight from 60.5 to 61.4 g ($p < 0.05$). The findings were consistent with Yang et al. (2023), who identified significant genetic associations among key reproductive traits in pigs.

Reproductive performance differed according to genotype, including pregnancy rate and calf survival in cattle, litter size and piglet survival in pigs, and hatchability and chick survival in laying hens. The average birth weight of calves ranged from 34.5 to 38.0 kg ($p < 0.05$), and the survival rate of young animals before weaning was 96-98% ($p < 0.05$). The findings were consistent with Carvalho et al. (2023), who reported genetic associations between semen-quality traits and reproductive performance in cows. The results similarly demonstrated an association between reproductive traits and productive performance in cattle.

Large White and Landrace sows had litter size ranging from 10.8 to 12.5 piglets per farrowing, with the difference between genotypes reaching 15.7% ($p < 0.01$). The survival rate of newborn piglets ranged from 88% to 94% ($p < 0.05$), while their average birth weight ranged from 1.25 to 1.40 kg ($p < 0.05$). These findings were consistent with Alam et al. (2021), who reported breed-related variation in productive and reproductive traits in pigs. In the present study, Large White and Landrace pigs differed significantly in litter size ($p < 0.01$), piglet survival, and birth weight ($p < 0.05$).

In Hy-Line Brown and Leghorn hens, egg hatchability ranged from 80% to 86% and differed significantly between the two breeds ($p < 0.05$). Chick survival to 8 weeks of age ranged from 94% to 97%, with a significant difference between the breeds ($p < 0.05$). The duration of the productive period ranged from 68 to 74 weeks ($p < 0.05$). These differences in egg hatchability and chick survival support the conclusion of Chebo et al. (2022) that targeted breeding strategies can improve reproductive performance in laying hens. Consistent with Chebo et al. (2022), the results indicated that systematic selection and performance evaluation improved reproductive traits, particularly egg hatchability and chick survival. Epigenetic mechanisms were not examined in the present study; therefore, the observed differences in reproductive performance cannot be attributed to DNA methylation or histone modifications. Their potential role should be investigated in future molecular studies, as suggested by Wang and Ibeagha-Awemu (2021).

Across cattle, pigs, and laying hens, breeding-improved groups showed significantly better productive and reproductive performance than the corresponding control groups ($p < 0.05$). In dairy cattle, breeding improvement increased milk yield from 5,800 to 8,200 kg per cow per year, representing an increase of 2,400 kg ($p < 0.05$), while milk protein content increased from 3.1% to 3.5% ($p < 0.05$). In meat cattle, average daily weight gain increased from 1.05 to 1.25 kg/day in the breeding-improved groups ($p < 0.05$). In pigs, breeding improvement increased average daily liveweight gain from 730 to 820 g, multiparity from 10.8 to 12.5 piglets per farrowing, and survival rate of young animals from 88% to 94% ($p < 0.05$). In laying hens, breeding improvement increased annual egg production from 305 to 314 eggs per hen, egg weight from 60.5 to 61.4 g, and output from 83.2% to 85.3% ($p < 0.05$). These results were consistent with Djermanovic et al. (2021), who emphasised the importance of genetic factors and breeding strategies in improving reproductive performance. The effects of breeding improvement and their statistical significance are summarised in Table 2.

Table 2. Effects of breeding improvement on productive and reproductive performance of farm animals

Animal species	Breed	Parameters (units)	Control	Breeding-improved group	p-value
Cattle	Dairy	Milk yield (kg/year)	5800 ± 65.6	8200 ± 85.4	p < 0.05
Cattle	Dairy	Protein content in milk (%)	3.1 ± 0.05	3.5±0.04	p < 0.05
Cattle	Meat	Weight gain (kg/day)	1.05 ± 0.02	1.25 ± 0.03	p < 0.05
Pigs	Meat	ADLWG (g)	730 ± 10.5	820 ± 12.3	p < 0.05
Pigs	Meat	Multiparity (head/farrowing)	10.8 ± 0.3	12.5 ± 0.2	p < 0.05
Pigs	Meat	Survival rate of young animals (%)	88 ± 1.2	94 ± 1.1	p < 0.05
Hens	Laying hen	Egg production (egg/year)	305 ± 5.3	314 ± 6.1	p < 0.05
Hens	Laying hen	Egg weight (g)	60.5 ± 0.3	61.4 ± 0.2	p < 0.05
Hens	Laying hen	Output (%)	83.2 ± 1.1	85.3 ± 1.2	p < 0.05

ADLWG: Average daily live weight gain, kg: Kilogram, g: Gram, p: Probability value

Breeding improvement significantly enhanced productive and reproductive performance in cattle, pigs, and laying hens ($p < 0.05$). The most pronounced numerical improvements were observed in dairy cattle milk yield and pig reproductive performance, whereas improvements in laying hens were smaller but remained statistically significant.

The dietary protein-to-energy ratio significantly affected both quantitative and qualitative productivity indicators ($p < 0.05$). Dairy cows receiving the optimal ratio of digestible protein to metabolizable energy (1:5.5) produced 8,050 kg of milk annually, compared with 7,460 kg in cows fed the lower-protein diet ($p < 0.05$). The fat content of milk at the optimal protein level was 4.05%, which exceeded the control by 0.12 percentage points ($p < 0.05$). Pigs fed the balanced diet had an average daily weight gain of 840 g, compared with 785 g in pigs receiving the lower-protein diet ($p < 0.01$). Intramuscular fat content was 2.8% in pigs receiving the optimised diet, compared with 2.4% in the control group ($p < 0.05$). In laying hens, a diet containing 16.5% protein increased annual egg production to 318 eggs per hen, compared with 306 eggs in hens receiving 14% protein ($p < 0.05$). These findings support Cheng et al. (2022), who emphasised that balanced diets help maintain livestock productivity under changing climatic conditions. Consistent with Cheng et al. (2022), the present results showed that optimising the dietary protein-to-energy balance improved milk yield, live-weight gain, and product quality. The findings were consistent with Walmsley et al. (2016), who emphasised the importance of adequate protein and energy supply for cattle productivity. In the present study, the balanced diet increased milk yield and improved milk composition, particularly fat and protein contents.

The feeding schedule, including feeding frequency and consistency of feed delivery times, significantly affected productive and reproductive performance. Dairy cows fed three times daily at evenly spaced intervals produced 8,100 kg of milk per year, compared with 7,750 kg in cows fed twice daily ($p < 0.05$). The pregnancy rate after first insemination was 67.4% in cows maintained on a consistent feeding schedule, compared with 63.2% in the control group ($p < 0.05$). In pigs, feeding three times daily increased average daily weight gain by 5.6% and pre-weaning survival by 2.3% compared with feeding twice daily (both $p < 0.05$). In laying hens, consistent feed delivery times reduced egg-weight variability, with the coefficient of variation declining from 4.2% to 2.9%, and increased hatchability from 84.1% to

85.9% (both $p < 0.05$). These findings were consistent with Muro et al. (2023), who showed that feeding frequency and regularity contributed to maintaining optimal body condition and reproductive performance in sows. They also agreed with Theil et al. (2023), who emphasised that modern sow-feeding systems should ensure a regular nutrient supply. Overall, a consistent feeding schedule improved both productive and reproductive performance.

Dietary interventions, including optimisation of the protein-to-energy ratio, adjustment of feeding frequency, and targeted supplementation with feed additives and premixes, significantly improved productive and reproductive indicators across the studied species (Jonova et al., 2018). Optimisation of the dietary protein-to-energy ratio increased milk yield in dairy cows from 7,460 to 8,050 kg/year and milk protein content from 3.93% to 4.05% ($p < 0.05$). In pigs, a balanced protein-to-energy ratio increased average daily growth from 785 to 840 g ($p < 0.01$) and intramuscular fat content from 2.4% to 2.8% ($p < 0.05$). In laying hens, a diet containing 16.5% protein increased egg production from 306 to 318 eggs/year and egg weight from 60.8 to 62.1 g ($p < 0.05$). Targeted supplementation also improved specific reproductive and quality indicators: a mineral and vitamin complex increased milk protein content in dairy cows from 3.94% to 4.03%, an amino acid premix increased multiparity in pigs from 11.2 to 11.7 piglets per farrowing, and supplementation with vitamins A, D, E, and trace elements reduced the proportion of non-standard eggs from 4.5% to 3.1% ($p < 0.05$). These findings were consistent with Zhang et al. (2021), who reported beneficial effects of biologically active feed components on poultry productivity and health, and with Du et al. (2020), who emphasised the role of balanced nutrition in maintaining reproductive performance in laying hens. The effects of the investigated nutritional factors are summarised in Table 3.

Table 3. Comparative indicators of productivity and reproduction under different feeding conditions of farm animals

Animal species	Feeding conditions	Indicator (units)	Control	Experimental group	P-value
Cattle	Optimal B/E ratio	Milk yield (kg/year)	7,460 ± 85.2	8,050 ± 90.1	$p < 0.05$
Cattle	Optimal B/E ratio	Protein content in milk (%)	3.93 ± 0.03	4.05 ± 0.02	$p < 0.05$
Pigs	Optimal B/E ratio	Average daily growth (g)	785 ± 10.5	840 ± 12.2	$p < 0.01$
Pigs	Optimal B/E ratio	Intramuscular fat content (%)	2.4 ± 0.1	2.8 ± 0.2	$p < 0.05$
Hens	Optimal protein 16.5%	Egg production (egg/year)	306 ± 5.6	318 ± 6.2	$p < 0.05$
Hens	Optimal protein 16.5%	Egg weight (g)	60.8 ± 0.3	62.1 ± 0.2	$p < 0.05$
Cattle	3-time feeding	Percentage of fertilization (%)	63.2 ± 1.0	67.4 ± 1.2	$p < 0.05$
Pigs	3-time feeding	Piglet survival rate (%)	90.1 ± 1.3	92.2 ± 1.1	$p < 0.05$
Hens	Stable feeding time	Output (%)	84.1 ± 0.8	85.9 ± 0.7	$p < 0.05$
Cattle	Mineral and vitamin complex	Protein content in milk (%)	3.94 ± 0.02	4.03 ± 0.02	$p < 0.05$
Pigs	Premix with amino acids	Multiparity (head/farrowing)	11.2 ± 0.3	11.7 ± 0.4	$p < 0.05$
Hens	Vitamins A, D, E + trace elements	Percentage of non-standard eggs (%)	4.5 ± 0.2	3.1 ± 0.2	$p < 0.05$

SD: Standard deviation, B/E ratio: Protein-to-energy ratio, kg: Kilogram, g: Gram, p: Probability value.

Optimizing the dietary protein-to-energy ratio, maintaining consistent feeding schedules, and applying targeted feed supplementation significantly improved productive and reproductive indicators across cattle, pigs, and laying hens ($p < 0.05$). The observed effects varied depending on the animal species and the nutritional intervention applied, with improvements detected in growth performance, product quality, reproductive indicators, and egg quality parameters.

Microclimatic conditions and stocking density significantly affected productive and reproductive performance across the studied animal species ($p < 0.05$). Dairy cows maintained at 18-20°C and 55-60% relative humidity produced 8,120 kg of milk annually, compared with 7,720 kg in cows exposed to 24-26°C and 75-80% relative humidity ($p < 0.05$). Pregnancy rate after first insemination was 68.1% under the predefined microclimatic conditions and 63.9% under the less favourable conditions ($p < 0.05$). In pigs, a ventilation rate of 0.15-0.20 m^3/min per animal and a temperature of 18-20°C increased average daily weight gain to 845 g, which was 6.3% higher than in pigs maintained at a ventilation rate of 0.08-0.10 m^3/min per animal and a temperature of 24-25°C ($p < 0.05$). Litter size also increased by 0.4 piglets per litter ($p < 0.05$). In laying hens, a stocking density of 6-7 hens/m^2 resulted in annual egg production of 320 eggs per hen, compared with 312 eggs at 9-10 hens/m^2 ($p < 0.05$). Hatchability increased from 83.5% to 85.7% ($p < 0.05$). These findings were consistent with Racewicz et al. (2021), who emphasised the importance of appropriate housing conditions

for maintaining pig health and productivity, and with Zhong et al. (2022), who reported that improvements in housing and management conditions enhanced productivity in pig production. Overall, the predefined microclimatic conditions and lower stocking density improved productive and reproductive performance in cattle, pigs, and laying hens.

The insemination method significantly affected pregnancy rate and offspring survival. In the experimental group subjected to oestrus synchronisation followed by artificial insemination, the pregnancy rate after first insemination was 69.4%, which was 5.1 percentage points higher than in the control group inseminated without prior synchronisation ($p < 0.05$). In pigs, artificial insemination with diluted semen containing a high sperm concentration increased litter size by 0.5 piglets per litter and pre-weaning piglet survival by 2.0% (both $p < 0.05$). In laying hens, incubation with automatic egg turning increased hatchability by 2.4% and reduced the proportion of weak chicks from 5.8% to 4.1% (both $p < 0.05$). These findings were consistent with Gordon (2017), who reported that oestrus synchronisation and the use of high-quality semen improve pregnancy outcomes and offspring viability. Similarly, the application of these reproductive technologies in the present study enhanced reproductive performance. Elliott et al. (2013) similarly noted that the adoption of reproductive technologies in cattle production depends on whether improvements in pregnancy rate and calf output outweigh the associated costs. The results of this study confirmed the economic feasibility of introducing modern insemination technologies due to the improvement of productive and reproductive indicators.

Exposure to technological stressors affected productive and reproductive performance across the studied animal species. In cattle, transport stress reduced annual milk yield from 8,050 to 7,630 kg per cow ($p < 0.05$). In pigs, exposure to noise stress decreased average daily growth from 820 to 780 g ($p < 0.05$). In laying hens, temperature stress above 30°C reduced annual egg production from 315 to 296 eggs per hen ($p < 0.05$). In contrast, optimisation of technological conditions improved performance indicators: maintaining an optimal microclimate increased milk yield in cattle from 7,720 to 8,120 kg/year and improved fertilisation rate from 63.9% to 68.1% ($p < 0.05$); optimal ventilation increased average daily growth in pigs from 795 to 845 g and multiparity from 11.3 to 11.7 piglets per farrowing ($p < 0.05$); and optimal stocking density increased egg production in laying hens from 312 to 320 eggs/year and output from 83.5% to 85.7% ($p < 0.05$). Reproductive technologies, including cycle synchronisation, concentrated sperm insemination, and automatic egg turning, also improved reproductive indicators ($p < 0.05$). These findings were consistent with Szőllősi et al. (2021), who reported that stress-reducing technologies improved poultry productivity and reduced non-standard product rates. They also supported Birhanu et al. (2021), who emphasised the importance of reducing environmental stress to maintain production efficiency. The effects of the investigated technological factors are summarised in Table 4.

Table 4. Comparative productive and reproductive performance under different technological conditions

Animal species	Technological factor	Indicator (Units)	Control	Experimental group	p-value
Cattle	Optimal microclimate	Milk yield (kg/year)	7,720 ± 85.5	8,120 ± 90.2	$p < 0.05$
Cattle	Optimal microclimate	Percentage of fertilisation (%)	63.9 ± 1.1	68.1 ± 1.2	$p < 0.05$
Pigs	Optimal ventilation	Average daily growth (g)	795 ± 10.4	845 ± 12.5	$p < 0.05$
Pigs	Optimal ventilation	Multiparity (head/farrowin)	11.3 ± 0.3	11.7 ± 0.2	$p < 0.05$
Hens	Optimal density	Egg production (pcs/year)	312 ± 5.1	320 ± 5.3	$p < 0.05$
Hens	Optimal density	Output (%)	83.5 ± 0.9	85.7 ± 1.0	$p < 0.05$
Cattle	Cycle synchronisation	Percentage of fertilisation (%)	64.3 ± 1.0	69.4 ± 1.1	$p < 0.05$
Pigs	Insemination with concentrated sperm	Multiparity (head/farrowing)	11.4 ± 0.2	11.9 ± 0.3	$p < 0.05$
Hens	Automatic egg turning	Output (%)	84.2 ± 0.8	86.2 ± 0.9	$p < 0.05$
Cattle	Transport stress	Milk yield (kg/year)	8,050 ± 87.7	7,630± 62.4	$p < 0.05$
Pigs	Noise stress	Average daily growth (g)	820 ± 9.7	780 ± 11.3	$p < 0.05$
Hens	Temperature stress > 30°C	Egg production (egg/year)	315 ± 4.3	296 ± 5.1	$p < 0.05$

SD: Standard deviation, kg: Kilogram, g: Gram, pcs: Pieces, °C: Degrees Celsius, p: Probability value

Technological management practices, including optimisation of microclimatic conditions, ventilation, stocking density, and reproductive technologies, significantly affected productive and reproductive indicators across cattle, pigs, and laying hens ($p < 0.05$). Conversely, exposure to transport, noise, and heat stress was associated with reduced performance parameters, demonstrating the importance of appropriate environmental and management conditions in animal production systems.

Comparison of the three investigated factor groups showed that genetic, nutritional, and housing and management factors significantly affected productive and reproductive performance ($p < 0.05$), although their relative contribution differed among animal species. In dairy cattle, genetic factors, particularly breed and assignment to breeding-improved groups, had the strongest association with milk yield and milk composition. These factors increased milk yield by 5.8-9.3% ($p < 0.05$) and milk protein content by 0.08–0.12 percentage points ($p < 0.01$). Nutritional factors, particularly optimisation of the dietary protein-to-energy ratio, further increased milk yield by 7.9% and improved milk composition, particularly fat and protein contents ($p < 0.05$). Housing and management factors, particularly maintaining the prescribed temperature, relative humidity, and ventilation conditions, stabilised performance and reduced seasonal variation, increasing milk yield by 5.2% and pregnancy rate after first insemination by 6.6% ($p < 0.05$).

In pigs, nutritional factors, particularly a balanced dietary protein-to-energy ratio and adequate amino acid supply, had the strongest effect (Shahini et al., 2023; Balanchuk and Umanets, 2025). A balanced diet increased average daily weight gain by 7.0% ($p < 0.01$), litter size by 4.5% ($p < 0.05$), and intramuscular fat content by 16.7% ($p < 0.05$). Genetic factors, particularly assignment to breeding-improved groups, increased average daily weight gain by 5.1-7.4% ($p < 0.05$) and litter size by 0.4-0.6 piglets per litter ($p < 0.01$). In pigs, technological factors, especially optimal ventilation and temperature control, provided an additional increase in average daily increments of 6.3% ($p < 0.05$) and multiplicity of 3.5% ($p < 0.05$), in addition to reducing the negative impact of stress on reproduction rates. In laying hens, nutritional factors made the largest contribution to improved performance. An optimal dietary protein level increased egg production by 3.9%, while vitamin–mineral supplementation increased it by 3.4% and reduced the proportion of eggs with shell defects, cracks, or abnormal shape by 31.1% (all $p < 0.05$). Genetic improvement increased annual egg production by 6-10 eggs per hen and average egg weight by 0.7-1.1 g ($p < 0.05$). Housing and management factors, particularly appropriate stocking density and consistent feed delivery times, reduced the coefficient of variation in egg weight from 4.2% to 2.9% and increased hatchability by 2.1-2.6% ($p < 0.05$). Overall, genetic factors had the strongest effect in cattle, nutritional factors in pigs, and a combination of nutritional and genetic factors in laying hens. The improvements were achieved when genetic, nutritional, and housing and management factors were applied together rather than separately.

CONCLUSION

The study demonstrated that genetic, nutritional, and housing and management factors significantly influenced productive and reproductive performance, although their relative importance differed among species. In cattle, genetic improvement increased milk yield by 5.8-9.3% and milk protein content by 0.08-0.12 percentage points. In pigs, a balanced protein-to-energy ratio increased average daily weight gain by 7.0% and intramuscular fat content by 16.7%. In laying hens, nutritional optimization increased egg production by 3.9-6.0%, while genetic improvement increased egg weight by 0.7-1.1 g. Maintaining appropriate microclimatic conditions and reducing stress further improved milk yield, pregnancy rate, and piglet survival. Overall, the greatest improvements were achieved through the combined application of genetic, nutritional, and management interventions. The findings support breed-focused improvement in dairy cattle, precisely balanced diets in pigs, and combined genetic selection and vitamin–mineral supplementation in laying hens. The study was limited by its 12-month duration and the relative uniformity of the experimental groups. Further studies should examine long-term and epigenetic effects, develop predictive productivity models, and evaluate the economic feasibility of the proposed interventions.

DECLARATIONS

Authors' contributions

Serhii Luhovyi contributed to the study design, data analysis, and manuscript preparation. Tetiana Pidpala conducted the data collection and interpretation of the results. Halyna Danylchuk contributed to the literature review and methodological framework. Liudmyla Onyshchenko was responsible for editing and final approval of the manuscript. All authors read and approved the final edition of the manuscript.

Availability of data and materials

The authors confirmed that the data supporting the findings of this study are available in the article from corresponding author.

Competing interests

The authors declare no conflict of interest.

Ethical considerations

The authors declared that the manuscript is original, has not been published elsewhere, and confirmed that the article has been checked for plagiarism, misconduct, and fabrication before submission. The authors confirmed that no AI tools were used for writing and preparing the article. All authors accept full responsibility for the originality of the manuscript before submission and before publication.

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Safety Evaluation of Inactivated Polyvalent Vaccine Against Bluetongue Virus Serotypes 10, 11, and 17 in Pregnant Ewes

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ABSTRACT

Bluetongue is a noncontagious, vector-borne Orbivirus-induced disease of domestic and wild ruminants. Both live and inactivated monovalent vaccines are used to control bluetongue disease worldwide. The objective of this study was to evaluate the safety and post-vaccination seroconversion of an inactivated polyvalent bluetongue virus (BTV) vaccine containing serotypes 10, 11 and 17 in pregnant ewes. The vaccine was evaluated by vaccinating 66 pregnant ewes (33 for each study group). The ewes enrolled for this study represented 24 dairy, 30 meat, and 12 crossbreds and were aged 1-9 years. The average body weight of the animals ranged from 73 to 82 kg. Pregnant ewes representing all three trimesters of pregnancy were used in this study. Each animal was vaccinated with a 2.0 ml dose of the vaccine subcutaneously. All ewes were revaccinated similarly after one month. Local injection site reactions after both vaccinations, systemic and other adverse reactions, effects on pregnancy, fetal development, delivery of the lambs, and post-vaccination virus-neutralizing (VN) antibody titers were monitored after each vaccination. Mild to moderate injection site reactions such as swelling or lump formation were seen in 29 injection sites (22.0%). All injection site reactions were resolved completely within 2 – 7 weeks without further complications and treatment. Mild to moderate post-vaccination systemic reactions such as anorexia/lethargy/laminitis were seen only in two ewes, which lasted for four days. Bluetongue vaccination in the pregnant ewes did not affect the pregnancy, fetal development, and delivery of the lambs. There were no premature abortions or any form of teratogenic deformities in any of the lambs delivered. The vaccine in the pregnant ewes induced post-vaccination VN antibody titers against all three serotypes of BTV. Lambs born to the vaccinated ewes also showed a protective level of VN antibody titers against all three serotypes of the virus following passive transfer of maternal antibodies. The levels of VN antibody titers seen in the ewes and lambs were considered sufficient to provide protection against field virus challenge. The results of the current study indicated that inactivated polyvalent bluetongue virus vaccine had no adverse effects on pregnancy and lamb delivery.

Keywords: Bluetongue, Inactivated vaccine, Maternal antibody, Pregnant ewe, Safety, Virus-neutralizing antibody

INTRODUCTION

Bluetongue is a noncontagious, Orbivirus induced, insect-transmitted disease of domestic and wild ruminants (MacLachlan et al., 2009; McVey and MacLachlan, 2015). Biological vectors such as the hematophagous biting midges or gnats of the genus *Culicoides* transmit the virus from animal to animal (Tabachnick, 2004; 2010; MacLachlan, 2011; MacLachlan and Mayo, 2013a; Sarma et al., 2026). The disease has been recognized in the United States since the late 1940s. Since then, at least 15 serotypes of the virus have been isolated and identified from different parts of the USA (Johnson, 2007; MacLachlan, 2011; MacLachlan et al., 2013b).

The only vaccine currently available in the United States for use in sheep is a modified live virus vaccine against bluetongue virus (BTV) serotype 10 (McVey and MacLachlan, 2015). No vaccine is presently available for immunization of livestock against the other serotypes of the virus (McVey and MacLachlan, 2015). Although the modified live virus vaccines can induce a protective immune response in vaccinated animals against BTV infection, there are some disadvantages, including reduced milk production in lactating ewes, abortion, early embryonic death, and teratogenesis when used in pregnant animals (McVey and MacLachlan, 2015). Additionally, the live virus vaccines cause a milder form of the disease with viraemia (Van der Sluijs et al., 2013). Biological vectors such as midges or gnats could spread the virus with reversion to virulence and genetic reassortment (Savini et al., 2008).

Conversely, inactivated vaccines are generally safe and induce complete and long-lasting immunity in vaccinated animals (Savini et al., 2008; Sarma et al., 2026). However, to be effective, inactivated vaccines need to be manufactured using higher doses of the antigens, appropriate adjuvants, and generally require a booster dose (Wilson-Welder et al., 2009). The safety of the subcutaneously administered monovalent inactivated BTV vaccines has been reported earlier

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from vaccination studies in sheep, goats, and other animals (Hammers et al., 2009a; Zientara et al., 2010; McVey and MacLachlan, 2015). These studies revealed that the monovalent inactivated vaccines were safe and caused no systemic reactions, including fever, weight loss, loss of appetite, and reproductive anomalies in the vaccinated animals. Except for some vaccine-induced transient local injection site reactions of variable severity (mild to moderate), no safety issues were observed in any of these studies with the monovalent inactivated vaccines (Hammers et al., 2009 a,b; Sarma et al., 2026).

In the United States, inactivated BTV vaccines are not yet commercially available. Autogenous vaccines are produced to meet customers' demand when needed. Due to the lack of published information on the safety of using a polyvalent inactivated vaccine containing BTV serotypes 10, 11, and 17 in pregnant ewes, this study was undertaken to evaluate a polyvalent vaccine for its suitability for use in pregnant animals as well as to determine the potential of maternal antibody transfer to the newborns to provide early protection against the disease.

MATERIALS AND METHODS

Ethical approval

The present study was conducted under field conditions in a commercial sheep ranch. The project was approved by the institutional R and D committee (Reference number: HPRD-RR24) of Hygieia Biological Laboratories (USA).

Study location and animals

This study consisted of two consecutive trials (Trial 1 and Trial 2) conducted at the same geographical location (Burton sheep ranch, Sheridan, California). In Sheridan, the average summer temperatures range from daytime highs of about 32.8°C to 35.6°C, and the average winter temperatures range from 12.8°C to 14.4°C. The rainy season starts in October and lasts until March/Apil. This location is 32 to 34 meters above sea level, and its latitude is approximately 38.98°N. The ranch is a commercial sheep ranch, consisting of both dairy and meat breeds. A total of 66 randomly selected pregnant ewes (33 for each trial), including both pure dairy breeds (East Friesian and Lacaune), meat breeds (Tunis and Suffolk), and crossbreds, were used to evaluate the safety of the inactivated BTV vaccine. The animals' age ranged from 1 to 9 years (Table 1). The average weight of the animals ranged from 73 to 82 kg. Pregnant ewes used for the study encompassed all three trimesters of pregnancy. The gestational status of each ewe was confirmed by ultrasound (SonoSite Edge II ultrasound system, USA) using either the 8-3 MHz transducer or the 5-2 MHz transducer. The ewes were housed in dry lot pens of 20-40 head per pen. Each pen measured approximately 23 × 61 meters. All animals had daily access to seasonal pasture consisting of native grasses. All animals had access to barns within pens for protection from environmental elements such as rain and sun. Pens had shared fence lines. Ewes were fed balanced rations as required by each stage of gestation. The dietary schedule consisted of a standard protocol adopted by the ranch owner and operator. Water and trace minerals (Wind and Rain sheep minerals, catalog number 3005607-106, Purina, USA) were provided *ad libitum*. All animals appeared healthy at the time of study enrolment when examined by a veterinarian.

Table 1. Pregnant ewes enroled in the bluetongue virus vaccine safety study

Trial number	Number of pregnant Ewes vaccinated	Age at vaccination			Breed type			Previous BTV vaccination history*	Previous BTV field virus exposure	Environmental conditions at time of vaccinations
		1-3 (years)	4- 6 (years)	7-9 (years)	Purebred dairy (East Friesian and Lacaune)	Purebred meat (Tunis, Suffolk)	Crossbreds			
1	33	3	18	12	3	27	3	Yes	Yes	Late Spring to Summer (35°C-37.8°C)
2	33	17	16	0	21	3	9	Yes	Yes	Winter (18.3°C-21.1°C)
Total	66	20	34	12	24	30	12	-	-	-

*: For animals ≥ 6 years old, BTV: Bluetongue virus

Vaccine

Experimental serials of BTV inactivated vaccine (Serial # BTV001, BTV002 and BTV003) manufactured by Hygieia Biological Laboratories, Woodland, California 95776, USA were used for vaccination and revaccination of the pregnant ewes. Since BTV serotypes 10, 11, and 17 are the most prevalent serotypes in the USA, the vaccines used in

this study were manufactured using inactivated viruses of these three serotypes. The vaccine was adjuvanted with a water-in-oil emulsion. Binary ethyleneimine was used as an inactivating agent for the virus, and the emulsion was prepared by mixing the inactivated antigens with mineral oil (Univar Solutions, Sacramento, California 95838, USA). The vaccines (Ref. # PC1075.10) contained the same quantity of inactivated antigens and adjuvant per dose and were manufactured following the identical vaccine manufacturing protocol (PO1075.10). Prior to use, the vaccines were tested for sterility, purity, laboratory animal safety, and virus inactivation following the applicable guidelines as stated in the Code of Federal Regulations, Title 9, Sections 113.200 and 113.303. The vaccines passed all required tests and were stored refrigerated at 2-7ºC until used.

Method of vaccination

The vaccination was done in two consecutive trials to verify the repeatability of the results as well as to find out the effect of environmental conditions (Summer vs Winter) on the performance of the vaccine. Prior to vaccination, the bottles containing the vaccine were shaken vigorously for uniform mixing. Due to the slightly thicker viscosity of the mineral oil-adjuvanted vaccine, as well as to get rid of the bubbles created by the mixing process, the vaccine bottles after thorough mixing were allowed to sit at room temperature for about an hour to make the process of vaccine administration easier and more accurate. Consistent with industry practice, injection sites were not clipped or sanitized prior to vaccination but were marked with paint to enable subsequent evaluation of injection site reactions. For initial vaccination, each pregnant ewe received 2.0 ml of the vaccine subcutaneously in the middle of the left side of the neck. An identical dose was administered one month later subcutaneously on the right side of the neck. Separate needles were used for vaccinating each animal. A one-month interval between the first and second vaccination was chosen in order to allow sufficient time to heal the injection site reactions that may have developed following administration of the first dose of the vaccine.

Vaccination trial 1

Trial 1 consisted of 33 ewes, including 27 meat breed ewes, three dairy ewes, and three crossbreed ewes. With the exception of one animal, the ewes in both trials were in their second or third trimester of pregnancy when vaccines were administered. All pregnant ewes were selected for the study based on availability. Pregnant ewes in trial 1 were vaccinated with the experimental vaccines from late spring to summer during exceptionally high and uncomfortable environmental temperatures (35°C-37.8°C). In this trial, all pregnant ewes received several additional treatments simultaneously with the first vaccination with the experimental bluetongue vaccine. Ewes were dewormed with an Ivermectin drench, treated for external parasites with a permethrin topical pour-on, vaccinated against Clostridial diseases utilizing Covexin 8 (Merck Animal Health, catalog 067696, Nebraska 68103 USA), and injected with selenium (Intervet/Merck Animal Health, New Jersey 07065 USA). To reduce stress for gestating ewes from frequent handling during extremely inclement weather, the vaccinated ewes were not bled pre- or post-vaccination for serological assessment of BTV antibodies in trial 1.

Vaccination Trial 2

Trial 2 consisted of 33 vaccinated pregnant ewes, including three meat breed ewes, 21 dairy ewes, and nine crossbred ewes. Unlike trial 1, the pregnant ewes of trial 2 were vaccinated with the BTV vaccine during mid-winter, with more comfortable environmental temperatures (18.3°C-21.1°C). Moreover, in trial 2, fewer interventions coincided with the BTV vaccinations. The animals were handled only for the BTV vaccinations, collection of pre-and post-vaccination blood samples for assessment of virus-neutralizing antibodies against BTV serotypes 10, 11 and 17, ultrasound examinations for pregnancy, evaluation of the injection site reactions and prenatal administration of Covexin 8, *E. coli*-J-5 (Hygeia Biological Laboratories, Woodland, California USA), vitamins A/D and selenium (one month prior to parturition) following manufacturers' directions.

Injection site reactions

The injection sites of the vaccinated ewes after first and second vaccinations were palpated weekly through eight weeks post-vaccination for development of firm or fluctuant lumps or masses, abscess formation, ulceration, and healing. The post-vaccination observations for injection site reactions continued until eight weeks after administering the second dose of the vaccine. For palpating the injection sites and to measure the size of the lumps as well as for ultrasound examinations, the animals were handled using chutes and headgate. The size of the masses was measured roughly using digital palpation, where one finger width was considered approximately equal to one-half inch. Based on size measurement, the lumps or masses at the injection sites were identified as mild reaction (< 1.0 to 1.5 inches in diameter), moderate reaction (1.6 to 2.0 inches in diameter), and severe reaction (> 2.0 inches in diameter).

Post-vaccination observation

All vaccinated ewes were observed daily for the development of any post-vaccination adverse or systemic reactions such as anaphylaxis, anorexia, lethargy, laminitis, abortion, and mortality for up to 30 days following each of the two doses administered subcutaneously. Based on the duration of disappearance of the post-vaccination systemic reactions, the observations were recorded as mild (lasting < 4 days) and moderate to severe (lasting > 4 days). In trials 1 and 2, ewe and lamb mortality was examined by a veterinarian, and samples were sent to the California Animal Health and Food Safety (CAHFS) laboratory, Davis, California, for complete necropsy.

Pregnancy and lamb delivery

Pregnant ewes were monitored daily during the entirety of gestation until the cessation of lambing season. Ewes were observed for premature abortions, stillbirths, mastitis, uterine and/or vaginal prolapses, and other health issues by a veterinarian. Ewes were also observed at the time of delivery to assess normal delivery and/or dystocia. Assisted deliveries were performed whenever necessary. Ewes were treated with antibiotics (Oxytetracycline 300 mg/ml, 4.5 ml per 45 kg) following assisted invasive deliveries and the treatment followed the attending veterinarian's standard practice. Data collected during lambing season included the number of lambs born to each ewe, any weak, stillborn lambs, or lambs with teratogenic deformity. Aborted lambs and dead lambs born with or without assisted deliveries were categorized as dead on arrival (DOA). The lambs that died prior to weaning were transported directly to CAHFS for necropsy. Lambs were identified with USDA scrapie tags, paint branded with their mother's flock tag number, and injected with selenium (subcutaneous BoSe injectable 2.19 mg sodium selenite per mL, equivalent of 1 mg selenium; used 1 mL per 18 kg body weight) within the first three days postpartum. No other additives or medications were used in the newborns.

Antibody screening of pre- and post-vaccination serum samples

At least 5.0 ml of blood was collected by jugular venipuncture from all vaccinated ewes in trial 2 immediately prior to first vaccination and one month following administration of the first and second doses of the vaccine. The pre-vaccination blood collection was done to screen the animals for residual antibodies to BTV acquired either through previous vaccination, active infection due to field exposure to the virus, or passive transfer through colostrum from the antibody-positive dams. The post-vaccination bleeding was done to test the ewe sera for BTV serotype-specific virus-neutralizing (VN) antibody titer. The pre-and-post-vaccination serum samples collected from the pregnant ewes in trial 2 were screened for VN antibodies against BTV serotypes 10, 11, and 17 using a virus neutralization assay. The virus neutralization assay was performed following the previously described procedure (Sarma et al., 2026).

Briefly, using a 96-well plate, the serum samples were serially diluted 2-fold in serum free cell maintenance medium containing Dulbecco's modified Eagle's medium (DMEM, Ref. # 10566-016, Source: Gibco, Grand Island, New York 14072, USA) with 100 IU/ml penicillin and 30 µg/ml streptomycin (Pen Strep, Ref. # 15140-122, Source: Gibco, Grand Island, New York 14072, USA). For each dilution, 50 µl of the diluted serum was mixed with 50 µl of cell maintenance medium containing 100 to 200 $TCID_{50}$ of the BTV serotype 10, 11, or 17. The plate containing the serum-virus mixtures was incubated at 37°C for one hour. Following incubation, the serum-virus mixtures from the plate were transferred to the corresponding wells of a flat-bottomed 96-well cell culture plate with near-confluent monolayers of Vero cells and incubated for three days at 37ºC in a humidified CO_2 incubator. Appropriate cell controls without virus and virus controls without BTV antibody-positive serum were included in each assay for accurate interpretation of the test results. The same procedure was followed for screening each test serum sample separately against each of the three serotypes of the virus (BTV-10, BTV-11, and BTV-17) used in the vaccine.

At the end of the incubation period, the cell monolayer in each well of the plate was examined under 100x magnification of an inverted microscope (Nikon Eclipse TS2-S-SM, Nikon Corporation, Japan) to check for development of BTV-induced cytopathic effect (CPE) in the cells. The CPE-positive cells become enlarged, rounded, and eventually die and detach from the surface. The VN antibody titers for each serum sample against BTV serotypes 10, 11, and 17 were calculated from the reciprocal of the highest serum dilution that completely (100%) inhibited CPE development in the Vero cell monolayer (Sarma et al., 2026).

Screening for maternal antibody in the lambs

In trial 2, serum samples collected from the lambs were subsequently screened for maternal antibody status against BTV serotypes 10, 11, and 17 using the previously described virus neutralization assay (Sarma et al., 2026). The lambs were bled for serum collection within 26 days of birth.

Handling of the animals

Handling of the pregnant ewes for vaccination, blood collection, post-vaccination observations for local and systemic reactions, monitoring of the pregnant animals throughout the pregnancy until lambing, and monitoring of the newborns for their health status and blood collection were all done by the trained personnel under the supervision of a veterinarian and following the standard protocols practiced by the ranch owner.

Statistical analysis

The VN antibody titer data of the pregnant ewes were analyzed for statistical significance by using a t-test, and the maternal antibody titer data of the lambs were analyzed by using a correlation coefficient test. Chi-square statistics were applied to analyze the post-vaccination injection site reactions data. All statistical tests were performed with Microsoft Excel 365.

RESULTS

Injection site reactions

The results of injection site examination following the first and second vaccinations are presented in Table 2. Post-vaccination site reactions in the pregnant ewes were observed after the first and second vaccinations in both trials 1 and 2. In trial 1, 10 injection sites had positive site reactions following the first vaccination, and five injection sites had positive site reactions following the second vaccination. A total of 15 injection sites out of 66 (22.7%) manifested positive site reactions in trial 1. In trial 2, eight injection sites revealed positive site reactions following the first vaccination and six injection sites exhibited positive site reactions following the second vaccination. A total of 14 injection sites out of 66 (21.2%) manifested positive site reactions in trial 2. The combination of trials 1 and 2 yielded a total of 29 injection sites out of 132 (22.0%) that were positive for post-vaccination reactions.

Table 2. Post-vaccination injection site reactions after administering the initial and booster doses of the bluetongue virus vaccine

Trial number	Number of pregnant Ewes vaccinated	Number of post-first vaccination injection sites with reactions					Number of post-second vaccination injection sites with reactions					Total number of positive sites	(%) total positive*
		Mild	Moderate	Severe	Abscess**	Total positive	Mild	Moderate	Severe	Abscess**	Total positive		
1	33	7	3	0	0	10	5	0	0	0	5	15***	22.7
2	33	6	2	0	0	8	6	0	0	0	6	14***	21.2
Total	66	13	5	0	0	18	11	0	0	0	11	29[a]	22.0

*: The difference in percentages of total positive injection site reactions between trial 1 and 2 were not statistically significant ($p > 0.05$), **: Abscess at the injection site due to BTV vaccination, Mild reaction < 1.0 to 1.5 inch in diameter, Moderate reaction 1.6 to 2.0 inch in diameter, Severe reaction > 2.0 inch in diameter, Pos: positive, ***: Out of 66 sites from two vaccinations of 33 ewes; [a]: Out of 132 sites from two trials combined.

Breed and age susceptibility

The relationship of development of post-vaccination injection site reactions to the age and breed of the vaccinated animals and time required to resolve the site reactions revealed that the development of post-vaccination injection site reactions was not correlated to either of those factors. Positive site reactions were observed across the board irrespective of the age and breed of the vaccinated ewes. Most of the injection site reactions in both trials 1 and 2 were mild and resolved completely in 2-7 weeks without any further complications or treatment.

Systemic reactions

The results of evaluating post-vaccination adverse/systemic reactions in the pregnant ewes are shown in Table 3. None of the vaccinated ewes of trials 1 and 2 had any anaphylactic reactions following administration of both doses of the vaccine through the subcutaneous route. Only one vaccinated ewe showed mild symptoms, and another ewe developed moderate to severe anorexia/lethargy/laminitis in the first trial. Another ewe from the first trial also died of environmental stress unrelated to vaccinations. None of the ewes in trial 2 developed anorexia/lethargy/laminitis after vaccinations. However, two vaccinated ewes in the second trial died (one due to cardiac contraction band necrosis and the other due to *Pseudomonas* mastitis). All mortalities in these two trials were unrelated to BTV vaccination as determined by CAHFS.

Table 3. Post-vaccination adverse/systemic reactions in the pregnant ewes

Trial number	Number of pregnant ewes vaccinated	Number of pregnant ewes showing post-vaccination adverse/systemic reactions					
		Anaphylaxis	Anorexia / Lethargy / Laminitis		Total *	Mortality	Cause of mortality
			Mild (< 4 days)	Moderate to severe (> 4 days)			
1	33	0	1	1	2	1	Environmental stress (heat shock)
2	33	0	0	0	0	2	Cardiac contraction band necrosis (1 ewe) *Pseudomonas* mastitis (1 ewe)
Total	66	0	1	1	2	3	Mortalities not related to BTV vaccination

BTV: Bluetongue virus, *: Total number of ewes showing reactions.

Effects on pregnancy, fetal development, and lamb delivery

Bluetongue virus vaccination in the pregnant ewes did not negatively affect the pregnancy, fetal development, or parturition of the lambs (Table 4). There were no abortions or any form of teratogenic deformities in any of the lambs delivered. Out of 66 pregnant ewes, 57 ewes delivered 106 normal healthy lambs, and nine ewes delivered 12 mature stillborn lambs due to prolonged obstructive dystocia. Stillborn lambs were part of multiple births and had one or more surviving siblings.

Table 4. Effects of bluetongue virus vaccination on pregnancy, fetal development and delivery of the newborn lambs

Trial number	Number of pregnant ewes vaccinated	Total number of ewes delivering lambs				Premature abortion	Total number of healthy lambs delivered	Total number of stillborn lambs delivered *
		Normal healthy	Mature stillborn due to dystocia	Teratogenic deformities	Abnormal/ weak			
1	33	29	4	0	0	0	43	4
2	33	28	5	0	0	0	63	8
Total	66	57	9	0	0	0	106	12

*: All stillborn were due to prolonged obstructive dystocia and not related to bluetongue virus vaccination

Pre- and post-vaccination virus-neutralizing antibody titers in the pregnant ewes

The pre- and post-vaccination VN antibody titers in the pregnant ewes against BTV serotype 10, 11, and 17 are shown in Table 5. The present results showed that the post-first and second vaccination mean antibody titers of all the serum samples tested from the pregnant ewes were significantly higher than the corresponding pre-vaccination titers against all three serotypes of the virus ($p < 0.05$). The mean pre-vaccination VN antibody titers against BTV serotypes 10, 11, and 17 were 65, 57, and 115 respectively. After the first vaccination, the titers significantly increased to a mean value of 142, 108, and 189 against each of the three serotypes of the virus, respectively ($p < 0.05$). The second vaccination significantly boosted these titers further to a mean value of 258, 148, and 333 against BTV serotypes 10, 11, and 17, respectively ($p < 0.05$). In general, all serum samples assessed showed more than a 2-fold increase in VN antibody titers from pre-vaccination to post-vaccination. Although administering the second dose of the vaccine considerably increased the titers from the pre-vaccination levels, this increase in antibody titer was not statistically significant when post-second vaccination titers were compared to post-first vaccination titers ($p > 0.05$).

Maternal virus-neutralizing antibody status in the lambs

The serum samples from all the lambs tested were found positive for maternally derived antibodies against all three serotypes of the virus (Table 6). The mean titers of all the serum samples tested were 28, 14, and 25 against BTV serotype 10, 11, and 17, respectively. More than 60% of the lamb serum samples tested had maternal antibody titers ≥ 8 against all three virus serotypes.

Table 5. Virus-neutralizing antibody titer against bluetongue virus in the serum samples collected from the pregnant ewes from trial 2

BTV serotype	Number of pre-vaccination serum samples showing VN antibody titer					Number of post-first vaccination serum samples showing VN antibody titer					Number of post-second vaccination serum samples showing VN antibody titer				
	2 - 8	16 - 32	64 -128	256 -1024	Mean titer[a]	5 -10	20 -40	80 -160	320 -1280	Mean titer[b]	5 -10	20 -40	80 -160	320 -1280	Mean titer[c]
10	14	8	7	4	65 (n = 33)	2	17	5	7	142 (n = 31)	1	7	13	8	258 (n = 29)
11	14	13	5	1	57 (n = 33)	1	6	19	4	108 (n = 30)	0	4	21	4	148 (n = 29)
17	11	11	7	4	115 (n = 33)	1	15	8	7	189 (n = 31)	1	2	19	7	333 (n = 29)

[a,b] Post-1st vaccination mean VN antibody titers were significantly higher ($p < 0.05$) than the pre-vaccination mean VN antibody titers, [a,c] Post-2nd vaccination mean VN antibody titers were significantly higher ($p < 0.05$) than the pre-vaccination mean VN antibody titers, [b,c] Post-2nd vaccination mean VN antibody titers were not significantly higher ($p > 0.05$) than the post-1st vaccination mean VN antibody titers. BTV: Bluetongue virus.

Table 6. Maternal virus-neutralizing antibody titer in the serum samples collected from the lambs delivered by the pregnant ewes from Trial 2

BTV serotype	Mean VN antibody titers in the ewes before lambing [b]	Number of lamb serum samples showing VN maternal antibody titer								Total number of lamb serum samples with maternal antibody titer ≥ 8[a]	Mean VN maternal antibody titer in the lambs [c]
		2	4	8	16	32	64	128	256		
10	258	7	7	16	7	8	4	1	2	38 (73.0%)	28 (n = 52)
11	148	6	9	16	12	7	1	0	0	36 (70.6%)	14 (n = 51)
17	333	10	9	8	5	8	6	3	0	30 (61.2%)	25 (n = 49)

[a]: VN antibody titer of ≥ 8 is likely to provide protection against clinical signs of the disease, [b, c]: A correlation coefficient analysis of the data under these two columns indicated a positive correlation between the mean VN antibody titers in the ewes and corresponding maternal antibody titers in the lambs ($r = 0.814$, $p < 0.05$), BTV: Bluetongue virus

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DISCUSSION

Bluetongue in sheep and other ruminants is quite prevalent in the USA and in several other parts of the world (Sarma et al., 2026). Immunization with a safe and efficacious vaccine is one of the preferred methods to control the disease (Savini et al., 2008). Vaccination results in the induction of VN antibodies, which may prevent viraemia and protect the animals against the development of clinical signs of the disease. Prior to this study, Hygieia Biological Laboratories' polyvalent bluetongue virus vaccine using serotypes 10, 11, and 17 was tested for its post-vaccination safety and seroconversion in other ruminants (Sarma et al., 2026). However, it was not tested in pregnant animals.

The post-vaccination injection site reactions in the pregnant ewes were mostly mild, resolving completely within 2 - 7 weeks (Table 2). The reasons for slightly higher injection site reactions in trial 1 could be due to unpleasantly high environmental temperature at the time of initial vaccination and revaccination. Development of mild to moderate tissue reactions resulting in the formation of lumps or masses at the sites of vaccine administration are common sequelae with the use of mineral oil-adjuvanted inactivated vaccines. The mineral oil-adjuvanted vaccines usually form a depot at the site of vaccine administration. The antigen is released slowly from this depot under the skin for a better and prolonged immune response. Therefore, formation of sterile lumps or masses at the vaccine injection site is expected. However, severe tissue reaction leading to formation of large sterile tissue masses at the vaccine injection sites could be due to accidental injection of the vaccine into the underlying musculature.

The vaccine also did not induce any adverse systemic reactions leading to death of the vaccinated animals, premature abortion, anaphylaxis, teratogenic deformities in the lambs, weak lambs, and stillbirths (Table 3). Twelve stillborn lambs delivered by nine of 66 pregnant ewes were due to prolonged obstructive dystocia and were not associated with vaccination. Only two out of 33 pregnant ewes in trial 1 developed mild to moderate anorexia/lethargy/laminitis lasting for about four days. This was most likely due to adverse and uncomfortable environmental conditions and the multitude of simultaneous other treatments given. All vaccinations in trial 1 were done in late spring to summer when the environmental temperature was unusually high (35°C-37.8°C) and uncomfortable. None of the pregnant ewes in trial 2 showed any sign of anorexia/lethargy/laminitis. In trial 2, all vaccinations were done during mid-winter when the weather was very pleasant (environmental temperature was between 18.3°C-21.1°C).

Vaccination of pregnant ewes with this polyvalent inactivated BTV vaccine adjuvanted with mineral oil appeared to be safe as evidenced by the absence of any vaccine-associated adverse systemic reactions and relatively low injection site reactions. Compared to the bluetongue attenuated live virus vaccines which have several disadvantages including its lack of safety in the pregnant animals (Monaco et al., 2004; Veronesi et al., 2009; McVey and MacLachlan, 2015), the inactivated vaccines containing only one serotype of BTV have been reported to have multiple advantages, particularly with regard to their safety and higher serotype specific seroconversion in the vaccinated animals (DiEmidio et al., 2004; McVey and MacLachlan, 2015). However, no published information was yet available on the safety and efficacy of inactivated vaccines containing three or more serotypes of the virus in pregnant sheep or in any other ruminants. This study shows that this polyvalent vaccine was equally safe for use in pregnant ewes.

The results of serological evaluation of the vaccinated pregnant ewes of trial 2 indicated that, besides being reasonably safe, the vaccine was also capable of inducing VN antibody response when administered as a two-dose regimen at a monthly interval. The VN antibody titers increased in most of the ewes following vaccination and revaccination after one month (Table 5), with some increasing by more than fourfold compared to the pre-vaccination titers. In order to avoid frequent handling of the pregnant ewes during adverse environmental conditions (unusually high environmental temperatures), no serological evaluation was done for the pregnant ewes of trial 1.

Previous BTV vaccine evaluation studies in other ruminants indicated an appropriate correlation between VN antibody titer and protection against challenge (Oura et al., 2009; McVey and MacLachlan, 2015; Sarma et al., 2026). An animal with a VN antibody titer of eight or above could be protected against the disease. In this trial, the post-vaccination antibody titers were all above this threshold. The authors of the current study also observed that there was a variation in individual immune response from animal to animal to the virus serotypes used in the vaccine. A few animals did not respond robustly to vaccination, as evidenced by a lack of significant increase in post-vaccination antibody titers compared to their pre-vaccination status. This could be due to individual animal response to the immunogen, concurrent infection with other pathogenic microorganisms, or other immunosuppressive factors associated with the health status of the animal. No consistent interference was observed with respect to the ability of the animals to seroconvert to each serotype of the virus present in this polyvalent vaccine. Moreover, the authors of the current study also observed that induction of injection site reactions did not apparently influence antibody production in the vaccinated animals. The VN antibody titer responses in the study animals that developed injection site tissue reactions were similar to the animals that did not develop injection site tissue reactions.

The presence of VN antibodies in the pre-vaccination serum samples of all pregnant ewes used for trial 2 was likely due to either prior exposure to the field strains of the virus inducing clinical or sub-clinical infection. This could also be

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due to vaccination with either live or inactivated BTV vaccine prior to initiation of this trial. The previous vaccination history, however, did not indicate use of any live virus vaccine in the ewes used in this trial. An experimental inactivated BTV vaccine manufactured by Hygieia Biological Laboratories, Woodland, California 95776, USA was, however, used to vaccinate some of the ewes aged six years or older about five years prior to initiation of this trial. The pre-vaccination VN antibodies in the pregnant ewes of trial 2 could, therefore, be either the residual antibodies from the previous bluetongue virus vaccination with the inactivated vaccine or induced by field virus exposure causing subclinical infection. Long-term persistence of post-vaccination antibodies following administration of inactivated BTV vaccines has been reported in other studies (Oura et al., 2009; McVey and MacLachlan, 2015). This could be an added advantage in using inactivated vaccines since vaccination with the adjuvanted inactivated BTV vaccine in the presence of residual antibodies will likely boost immunity by increasing the antibody titer and thus help protect the animals against field virus infection.

The presence of BTV antibodies in sheep under field conditions has been reported earlier from systematic serological and epidemiological surveys in the USA (Hilke et al., 2019). Most of the samples tested showed the presence of antibodies against multiple serotypes of the virus. However, in most cases, the available low-level residual antibodies were believed to be insufficient to provide adequate protection against virulent field strains of the virus, thus indicating the necessity of vaccination for complete protection.

Results of maternal antibody evaluation in the lambs born out of the vaccinated pregnant ewes (Table 6) indicated the benefits of vaccinating pregnant animals with inactivated BTV vaccine. All serum samples tested from the lambs were seropositive against all three serotypes of the virus. The antibody titers in more than 60% of the serum samples were eight or higher, which may be sufficient to provide protection to the lambs against the virulent field strains of BTV early in their life.

In this study, the efficacy of the vaccine in pregnant ewes was evaluated based on the post-vaccination VN antibody titers. Since this study was conducted in pregnant ewes, it was not possible to conduct a controlled vaccination - challenge experiment to correlate antibody titers with protection. Moreover, the ranch owner was not in favor of allowing Hygieia Biological Laboratories to bring virulent strains of BTV onto the premises with a large population of susceptible sheep. Also, due to similar reasons, no other ewes at the trial location could be kept as unvaccinated controls. However, as discussed before, previous BTV vaccination studies conducted in sheep and other ruminants demonstrated a high correlation between VN antibody titers and protection against challenge (Savini et al., 2008; Oura et al., 2009; Wackerlin et al., 2010). On the basis of the results reported from these earlier studies, it is anticipated that animals with a post-vaccination virus-neutralizing antibody titer of eight or higher may be protected in the case of field exposure to bluetongue virus.

CONCLUSION

The vaccine was found to be reasonably safe for use in the pregnant ewes. Although some animals revealed mild to moderate injection site reactions after administering the vaccine subcutaneously, no abscesses or severe site reactions were observed in any of the vaccinated ewes. All injection site reactions resolved completely within 2-7 weeks without further complications or treatment. Only a few animals developed mild to moderate post-vaccination systemic reactions lasting for about 4 days. Vaccination in the pregnant ewes did not affect the pregnancy, fetal development, and delivery of the lambs. There were no premature abortions or any form of teratogenic deformities in any of the lambs delivered. The vaccine also induced VN antibody titers against all three serotypes of BTV. Lambs born to the vaccinated ewes also showed maternally transferred VN antibody titers against all three serotypes of the virus. The levels of VN antibody titers seen in the ewes and lambs were most likely sufficient to provide protection against field virus challenge. The data from this study demonstrated promise for the safety and efficacy of a polyvalent inactivated BTV vaccine in pregnant ewes and further the benefits of vaccination to provide maternal antibody-mediated protection in the neonatal lambs. A vaccination and challenge study using a large number of animals including both positive and negative controls will help to add further confidence to the current findings.

DECLARATIONS

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Authors' contributions

Girish Sarma came up with the concept of this study, designed the experiment, manufactured the experimental vaccine, evaluated the serum samples for VN antibodies, collected, assembled, analyzed, and interpreted the data, and wrote the manuscript. Tammy Burton assisted in vaccination and revaccination of the ewes, collected the serum samples, observed the ewes daily for reactions throughout pregnancy and lambing, and critically reviewed the manuscript. Laurel Moody conducted the vaccination, sample collection and processing, collection of the data, and critical revision of the article. Dale Wallis supervised the study, helped in vaccination, blood collection, evaluation of the post-vaccination reactions, and critically reviewed the manuscript. All authors approved the final manuscript prior to submission for publication.

Availability of the data and materials

The results presented in this article represent a summarized version of the original data collected during this study. The original data pool is maintained by the Hygieia Biological Laboratories and may be available upon reasonable request from the corresponding author.

Competing interests

The authors declare no conflicts of interest.

Ethical considerations

The authors declare that the present original article has not been submitted simultaneously to another journal for publication and no AI tools were used in the preparation of the manuscript. All data were originally collected and statistically analyzed without using AI tools. The authors accept all responsibility for the original text and the present data before submission and at final stage of publication.

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Isolation of *Candida* spp. from Broiler Chickens' Crop at a Slaughterhouse in Banda Aceh, Indonesia

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ABSTRACT

Candida spp. are opportunistic yeasts commonly found in the gastrointestinal tract of poultry. The present study aimed to determine the distribution and compare the colony counts of presumptively identified *Candida* spp. recovered from the crop of broiler chickens at a poultry slaughterhouse in Banda Aceh, Indonesia. Ten crop samples collected from individual broiler chickens were enriched in peptone water and subsequently cultured on CHROM agar™ Candida. Presumptive identification of *Candida* spp. was based on characteristic colony color and morphology. The results revealed marked differences in colony counts among the three presumptively identified *Candida* species. *Candida albicans* (*C. albicans*) exhibited the highest mean colony count (9.10 ± 0.88 colonies/plate), followed by *C. krusei* (6.60 ± 0.97 colonies/plate) and *C. tropicalis* (6.10 ± 0.99 colonies/plate). Pairwise comparisons revealed that *C. albicans* had significantly higher colony counts than both *C. krusei* and *C. tropicalis*, whereas no significant difference was observed between *C. krusei* and *C. tropicalis*. The present findings indicated that *C. albicans* was the predominant presumptively identified *Candida* species in broiler chicken crops and highlight the need to further investigate their occurrence, epidemiology, and potential public health implications in poultry production and processing systems.

Keywords: Broiler chicken, *Candida albicans*, *Candida krusei*, *Candida tropicalis*, Crop

INTRODUCTION

Broiler chickens (*Gallus gallus domesticus*) represent an important source of animal protein in Indonesia and are commonly raised under traditional or semi-intensive management systems (Dewi et al., 2022). Traditional and semi-intensive production systems increase exposure to environmental microorganisms, including opportunistic fungi that may colonize the gastrointestinal tract of poultry (Domán et al., 2023; Haroun et al., 2026). Although *Candida* spp. are generally commensal microorganisms that constitute part of the normal mycobiota, they can act as opportunistic pathogens under certain conditions, such as physiological stress, immunosuppression, or microbial dysbiosis, potentially resulting in candidiasis (Talapko et al., 2021).

The crop is an important compartment of the upper gastrointestinal tract in chickens and has been documented as a site from which *Candida* spp. can be recovered (Wyatt and Hamilton, 1975; Haroun et al., 2026). Previous studies in broiler chickens demonstrated that *Candida* spp. could be recovered from crop contents, with *C. albicans* representing the predominant species among the isolates examined (Wyatt and Hamilton, 1975).

A previous study in Banda Aceh has also documented *Candida* spp. in chicken crop samples (Erina et al., 2019). Erina et al. (2019) reported the isolation of *Candida* spp. from crop samples collected from chickens at the Peunayong poultry slaughterhouse in Banda Aceh, providing context for the present study's focus on broiler chicken crops at this site.

Despite these previous observations, quantitative information on the distribution of individual *Candida* species in broiler chicken crops at the slaughterhouse level remains limited. In particular, information regarding differences in colony counts among presumptively identified *Candida* species recovered from broiler chicken crop samples in Banda Aceh is scarce, limiting understanding of their occurrence and relevance at this stage. Therefore, the present study aimed to characterize the distribution and compare the colony counts of presumptively identified *Candida* spp. recovered from the crops of broiler chickens at a poultry slaughterhouse in Banda Aceh, Indonesia.

MATERIALS AND METHODS

Study location and timing

The present study was conducted at the Microbiology Laboratory, Faculty of Veterinary Medicine, Syiah Kuala University, Banda Aceh, Indonesia. Sample collection was carried out at a poultry slaughterhouse located in Peunayong Market, Banda Aceh, Indonesia. The study was conducted from December 2018 to January 2019.

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Equipment and materials

The equipment used in this study included Petri dishes (OneMed, Indonesia), test tubes (OneMed, Indonesia), sterile containers, test tube racks, inoculating loops, Bunsen burners, forceps, bent glass rods, plastic pipettes, and a micropipette. The materials consisted of ten crop samples obtained from broiler chickens (*Gallus gallus domesticus*), aquabidest, peptone water (Sigma-Aldrich, Germany), gentamicin (Sigma-Aldrich, USA), and CHROM agar Candida (CAC; Himedia Laboratories, India).

Sample collection

Ten crop samples from ten broiler chickens during slaughter were collected to examine individual chickens. Each crop sample represented one individual chicken. The crops were rinsed once with running water in sterile containers and subsequently rinsed with aquabidest before being transported to the Microbiology Laboratory, Faculty of Veterinary Medicine, Syiah Kuala University, Indonesia, at 37˚C. Sample processing followed previously described methods (Thompson, 1969; Hastiono, 1987), with additional reference to a recent study on the isolation of *Candida* spp. from chicken crops in Banda Aceh (Erina et al., 2019).

Sample preparation and enrichment

Upon arrival at the laboratory, the crop samples were handled aseptically and rinsed three times with aquabidest containing gentamicin (0.1 mL/100 mL) to reduce contamination. The crop samples were aseptically cut into approximately 3-5 mm sections and transferred into sterile containers containing peptone water at a sample-to-medium ratio of 1:9 to enrich Candida spp. Each sample was enriched in peptone water at 37˚C for 24 hours.

Cultivation on CHROM agar Candida

Following enrichment, 0.2 mL of each enriched sample was inoculated onto a CHROM agar Candida plate using a micropipette and incubated at 37˚C for 48 hours to allow colony development. Each enriched crop sample was inoculated onto a single CHROM agar Candida plate, with one plate representing one individual crop sample. No technical replicates or duplicate plating were performed, and no serial dilution was conducted before inoculation.

Presumptive identification of *Candida* spp.

Following incubation, *Candida* colonies were presumptively identified based on their colony color and morphology on CHROM agar Candida. Colonies exhibiting characteristic color patterns were recorded according to the presumptive identification criteria for *C. albicans*, *C. krusei*, and *C. tropicalis* to distinguish the species. Colony counts for each presumptively identified species were recorded as colonies per plate.

Colony counting

After incubation, colonies with the characteristic color and morphology of each presumptively identified species were counted manually to quantify growth on each plate. Because no dilution was performed, colony counts were expressed directly as colonies per plate.

Statistical analysis

Colony counts were summarized as the mean ± standard deviation (SD) and range. Differences among the three presumptively identified *Candida* species were analyzed using the Friedman test. When a significant overall difference was detected, pairwise comparisons were performed using the Wilcoxon signed-rank test with Bonferroni adjustment. All statistical analyses were performed using IBM SPSS Statistics version 25, and statistical significance was set at $p < 0.05$.

RESULTS

Ten crop samples from broiler chickens were examined for *Candida* spp. following enrichment in peptone water and subsequent culture on CHROM agar Candida. Individual colony counts recovered from each crop sample are presented in Table 1. Among the isolates, *Candida albicans* indicated the highest colony counts, ranging from 8 to 10 colonies/plate, with a mean ± SD of 9.10 ± 0.88 colonies/plate, indicating the greatest recovery. *C. krusei* exhibited colony counts ranging from 5 to 8 colonies/plate, with a mean ± SD of 6.60 ± 0.97 colonies/plate. *C. tropicalis* exhibited colony counts ranging from 5 to 8 colonies/plate, with a mean ± SD of 6.10 ± 0.99 colonies/plate, indicating lower recovery. The Friedman test revealed a significant difference in colony counts among the three *Candida* species (χ^2 (2) = 15.44, $p < 0.05$; Kendall's $W = 0.77$). Pairwise comparisons using the Wilcoxon signed-rank test with Bonferroni adjustment indicated that C. albicans had significantly higher colony counts than *C. krusei* and *C. tropicalis* (both $p < 0.05$). No significant difference was observed between *C. krusei* and *C. tropicalis* ($p > 0.05$).

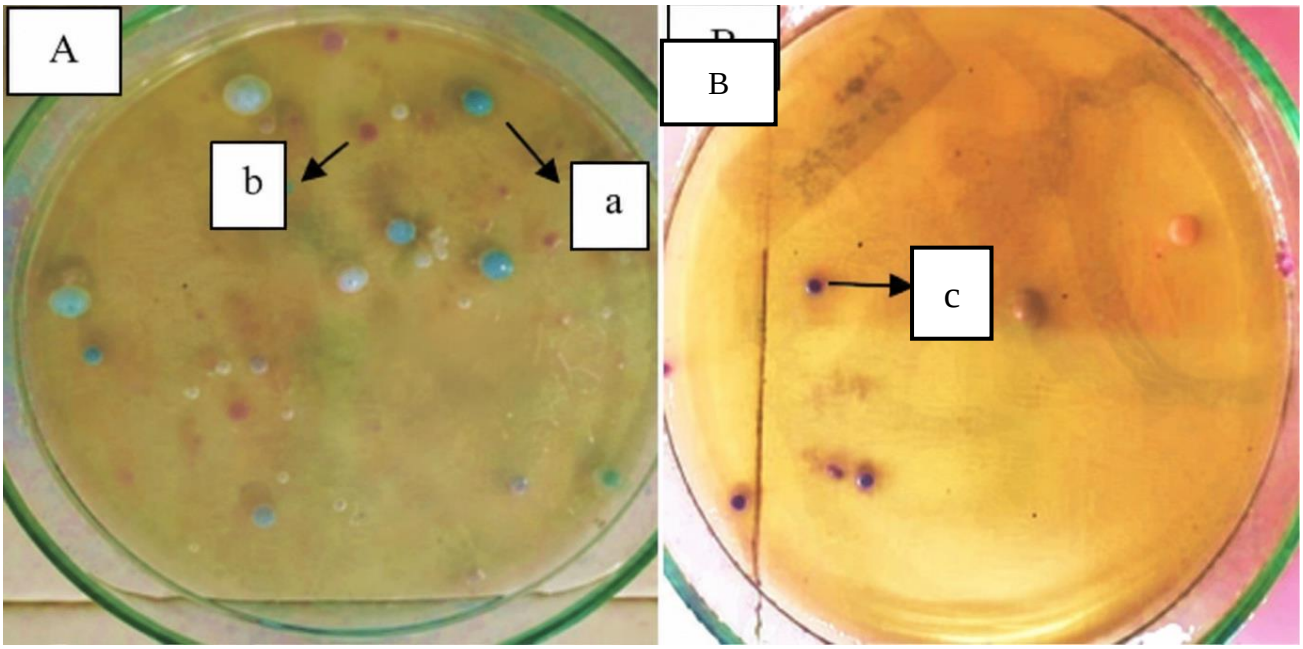


Figure 1. Representative colony morphology of presumptively identified *Candida* spp. recovered from broiler chicken crop samples cultured on CHROM agar™ Candida. Representative culture plates **(A-B)** showing colonies of **a:** *Candida albicans*, **b:** *Candida krusei*, and **c**: *Candida tropicalis*, differentiated based on their characteristic colony color and morphology.

Table 1. Colony counts of presumptive *Candida* spp. isolated from broiler chicken crop samples cultured on CHROM agar™ Candida

Chicken	*Candida albicans*	*Candida krusei*	*Candida tropicalis*
1	9	7	5
2	8	7	6
3	9	5	6
4	10	6	6
5	8	6	7
6	9	7	8
7	10	8	5
8	10	6	7
9	8	6	5
10	10	8	6
Mean ± SD	9.10 ± 0.88	6.60 ± 0.97	6.10 ± 0.99
Median	9.0	6.5	6.0
Range	8-10	5-8	5-8

Values represent the number of colonies recovered per CHROM agar Candida plate (colonies/plate).

DISCUSSION

The present study revealed that *Candida albicans*, *Candida krusei*, and *Candida tropicalis* were presumptively recovered from broiler chicken crops, with *C. albicans* indicating the highest mean colony count (Wyatt and Hamilton, 1975). A previous study by Wyatt and Hamilton (1975) has also demonstrated the recovery of *Candida* spp. from the crops of healthy broiler chickens. The occurrence of *Candida* spp. in broiler chicken crops observed in the present study was therefore consistent with previous evidence of *Candida* recovery from the avian crop (Wyatt and Hamilton, 1975).

The presence of *Candida* spp. in crop samples should not, however, be interpreted as evidence of clinical candidiasis. *Candida* spp. may occur as commensal yeasts in the avian gastrointestinal tract, whereas clinical candidiasis develops when host or gastrointestinal conditions favor fungal overgrowth and tissue invasion. Predisposing conditions described in poultry include immunosuppression, nutritional deficiencies, excessive antimicrobial exposure, poor

husbandry, and crop stasis. Accordingly, the detection of *Candida* spp. in the present study indicates recovery of these yeasts from the crop, not clinical disease or tissue invasion (Talapko et al., 2021).

The higher colony count of *C. albicans* observed in the present study may have been related to its established occurrence in the avian gastrointestinal tract and its frequent association with the crop. Wyatt and Hamilton (1975) similarly identified *C. albicans* as the predominant *Candida* species recovered from broiler chicken crops, accounting for 95% of the isolates in their survey. However, the present study did not investigate virulence factors, strain characteristics, host immune status, or interspecies interactions; therefore, the biological basis for the higher colony count of *C. albicans* remains undefined in the present data.

The present findings are also relevant to previous observations from Banda Aceh, where *Candida* spp. were isolated from crop samples of broiler and indigenous chickens collected at the Peunayong poultry slaughterhouse (Erina et al., 2019). Erina et al. (2019) reported *Candida* spp. in all examined crop samples, providing local evidence of *Candida* in chicken crops in the study area. However, direct comparison between the two studies should be made cautiously because of differences in study design, sampling populations, culture procedures, and reporting criteria.

The statistical analysis further demonstrated that *C. albicans* had significantly higher colony counts than both *C. krusei* and *C. tropicalis*. In contrast, the difference between *C. krusei* and *C. tropicalis* was not significant. The observed pattern indicated that the overall difference among the three species was driven primarily by the higher colony counts of *C. albicans*. Because the present study was designed to characterize colony counts under the specified culture conditions, these statistical differences should not be interpreted as evidence of differences in pathogenicity or colonization capacity.

CONCLUSION

Candida albicans, *Candida krusei*, and *Candida tropicalis* were presumptively recovered from the crops of broiler chickens sampled at a poultry slaughterhouse in Banda Aceh, Indonesia. *C. albicans* indicated the highest colony count and was significantly more abundant than *C. krusei* and *C. tropicalis*, whereas no significant difference was observed between *C. krusei* and *C. tropicalis*. The present findings indicated that *Candida* spp. occurred in broiler chicken crops and were differentially recovered under the culture conditions used in this study. Further investigations involving larger sample sizes, molecular confirmation, and sampling across different stages of poultry production and processing are warranted to better characterize the distribution and potential significance of Candida spp. This study was limited by its small sample size, sampling from a single slaughterhouse, and lack of molecular confirmation. Therefore, further studies involving larger sample sizes and broader sampling are warranted.

DECLARATIONS

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Author’s contributions

Nur Rahmi, Sedrisa Lidya Pertiwi, and Sucia Fadillah contributed to the study's conception and design. Nur Rahmi wrote the first draft of the manuscript. All authors commented on previous versions. Analysis of data was checked by Nur Rahmi, Sedrisa Lidya Pertiwi, and Sucia Fadillah. All authors read and approved the final version of the manuscript.

Availability of data and materials

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

Competing interests

The authors have no conflict of interest.

Ethical considerations

The authors confirm that the present manuscript is original and has not been published or submitted for publication elsewhere. ChatGPT (OpenAI) was used solely for language editing, grammar correction, and sentence clarity. The authors reviewed and revised all AI-assisted text and take full responsibility for the final content. No AI tools were used to generate, analyze, or manipulate the data or results.

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Non-Surgical Contraception in Male Dogs and Cats: Mechanisms and Clinical Perspectives

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ABSTRACT

Surgical sterilization is widely used for population control and reproductive management in dogs and cats; however, its irreversible nature has increased interest in non-surgical contraceptive methods. Hormonal approaches, including progestins, GnRH agonists, and GnRH antagonists, as well as immunocontraceptive and chemical methods, have been investigated for fertility control in male dogs and cats. The present review summarizes the current literature on non-surgical contraceptive methods in male dogs and cats, focusing on their mechanisms of action, effectiveness, reversibility, duration of effect, and reported side effects. Non-surgical approaches include GnRH agonists and antagonists administered systemically or via implants, immunocontraceptive methods, and chemical castration agents administered by intratesticular or intraepididymal injection. GnRH agonists have been reported to provide prolonged and reversible suppression of reproductive function, whereas GnRH antagonists generally have a more rapid onset but a shorter duration of action. Immunocontraceptive methods can suppress testosterone concentrations and spermatogenesis, although their effects may vary among individuals. Chemical castration can impair spermatogenesis and semen quality, with outcomes depending on the chemical agent and administration technique. In conclusion, non-surgical contraceptive methods revealed potential for fertility control in male dogs and cats.

Keywords: Cat, Chemical castration, Dog, Gonadotropin-releasing hormone, Immunocontraception

INTRODUCTION

Contraception is the prevention of reproduction through surgical or medical interventions; non-surgical (pharmacological) methods, including progestins, androgens, and GnRH analogs, act either by directly blocking reproductive hormone receptor-mediated events or by indirectly suppressing gonadotropin secretion through negative feedback (Kutzler and Wood, 2006). Interest in non-surgical methods as alternatives to surgical procedures for population control and reproductive management in domestic cats and dogs has increased, particularly since the 1970s (Rhodes, 2017). Historically, the lack of safe anesthetics, together with limited surgical equipment and instruments, increased surgical risks, making sterilization of dogs and cats uncommon until the latter half of the twentieth century (Brent, 2019). Although opinions on the risks, benefits, and appropriate age for surgical sterilization in dogs vary in the literature, the number of studies on medical contraceptive methods aimed at reversible or permanent fertility suppression has increased significantly in recent years (Tez et al., 2019). Progestins have been investigated as hormonal approaches for fertility control in male dogs (England, 1997). Gonadotropin-releasing hormone (GnRH) analogs, particularly GnRH agonists, have been widely investigated for fertility control in dogs and cats (Lucas, 2014). Different medical contraceptive methods, including GnRH antagonists, immunocontraceptive vaccines, and chemical sterilization approaches, have been developed, and research in this field remains active (Kutzler and Wood, 2006).

The present review aimed to examine non-surgical contraceptive methods in male cats and dogs, including GnRH agonists and antagonists, progestins, immunocontraceptive approaches, and intratesticular and intraepididymal administration of chemical agents, and to compare them in terms of efficacy, duration of effect, reversibility, safety, and clinical applicability.

METHOD

The present narrative review was based on a literature search conducted using Google Scholar and PubMed. Publications from 1997 to 2026 were considered. The search terms included “male dog”, “male cat”, “non-surgical contraception”, “GnRH agonist”, “GnRH antagonist”, “progestin”, “immunocontraception”, “chemical castration”, “intratesticular

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injection", and "intraepididymal injection". Relevant studies on non-surgical contraceptive methods in male dogs and cats were selected and evaluated based on their mechanisms of action, efficacy, duration of effect, reversibility, safety, and clinical applicability. Among non-surgical approaches for suppressing fertility in male cats and dogs, immunocontraception has emerged as an important area of research. A summary of the included studies were presented in Table 1.

MECHANISM OF IMMUNOCONTRACEPTION

Immunocontraception uses the immune response to suppress reproductive functions by inducing the formation of specific antibodies against reproduction-related protein antigens (Levy, 2004). Immunocontraception can, in principle, target different reproductive antigens, including gonadotropin-releasing hormone (GnRH), luteinizing hormone (LH), follicle-stimulating hormone (FSH), zona pellucida proteins, and sperm-associated antigens (Kutzler and Wood, 2006; Rhodes, 2017). However, some of these targets, particularly zona pellucida proteins, are specific to the female reproductive system (Kutzler and Wood, 2006). In male cats and dogs, GnRH-based immunocontraceptive approaches have been more widely investigated (Levy et al., 2004).

Based on the established role of immunological mechanisms in reproductive physiology, GnRH-based vaccines, in particular, have shown efficacy in suppressing gonadal function in males (Levy et al., 2004; Talwar et al., 2009; Munks, 2012). GnRH is a highly conserved decapeptide with a similar structure across mammalian species and is one of the key regulators of the hypothalamic-pituitary-gonadal axis (Padula, 2005). Antibodies produced against GnRH suppress reproductive function by interfering with GnRH activity, resulting in reduced testosterone concentrations and impaired spermatogenesis (Levy et al., 2004). To enhance immunogenicity, GnRH can be conjugated to carrier proteins and combined with adjuvants to induce a stronger immune response (Levy et al., 2004; Munks, 2012).

EXPERIMENTAL AND CLINICAL STUDIES

GnRH vaccines developed for immunocontraception have been reported to reduce testosterone levels and decrease sperm count and motility in male cats and dogs (Levy, 2004). The GonaCon™ (National Wildlife Research Center, Fort Collins, CO, USA) vaccine, developed for free-ranging animal populations, has been reported to produce effects lasting approximately one year after a single dose (Segura, 2023). In a study of male cats conducted by Levy et al. (2004), high antibody titers were observed following immunization with synthetic GnRH bound to a carrier protein, along with significant decreases in testosterone levels and spermatogenesis and the development of testicular atrophy. Histological examinations have revealed a reduction in interstitial cell numbers and atrophy of the seminiferous tubules (Levy, 2004). Similar studies have reported that GnRH-based vaccines can reduce testosterone concentrations in healthy male dogs (Sabeur et al., 2003; Walker et al., 2007). Antibody titers have been reported to peak within a few weeks after repeated subcutaneous injections of GnRH vaccines, leading to temporary infertility (Donovan et al., 2012; Siel et al., 2020). Immunocontraceptive methods are considered promising options in male cats and dogs due to their minimally invasive nature and potential reversibility (Kutzler and Wood, 2006; Munks, 2012). However, individual variability in antibody responses, uncertainty regarding the duration of contraceptive effects, and the relatively small sample sizes reported in available studies remain important limitations to the routine clinical use of this approach (Levy et al., 2004; Segura Ochoa et al., 2023).

GONADOTROPIN-RELEASING HORMONE ANTAGONISTS

Among non-surgical approaches to fertility suppression in male cats and dogs, GnRH antagonists are noteworthy because they directly inhibit the hypothalamic-pituitary-gonadal (HPG) axis (Gobello, 2007). GnRH antagonists bind to GnRH receptors in the anterior pituitary gland, thereby blocking the effects of endogenous GnRH and suppressing the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH; García Romero et al., 2009). The resulting decrease in LH and FSH secretion leads to reduced intratesticular testosterone levels and suppression of spermatogenesis (Baran et al., 2016). Unlike GnRH agonists, antagonists do not produce an initial transient stimulatory effect (Gobello, 2012). Unlike GnRH agonists, GnRH antagonists competitively block GnRH receptors in the anterior pituitary without initially activating them; therefore, directly suppress gonadotropin secretion without inducing an initial stimulatory flare (Gobello, 2012). The absence of an initial testosterone surge may represent a clinical advantage of GnRH antagonists by avoiding effects associated with the transient hormonal stimulation observed with GnRH agonists (Gobello, 2012). However, early GnRH antagonist formulations have been reported to cause adverse reactions associated with histamine release, significantly limiting their clinical use (Gobello, 2012). Although these side effects have been reported to occur less frequently with newer-generation GnRH antagonist analogs, their high cost limits their use in large-scale population control programs (Koechling et al., 2010). The effects of acyline, a third-generation GnRH antagonist, on fertility in male dogs have been experimentally investigated (Valiente et al., 2007; García Romero et al., 2009). A single

subcutaneous dose of 330 µg/kg has been reported to induce short-term suppression of fertility, accompanied by decreased libido, reduced scrotal size, impaired semen parameters, and significant declines in circulating gonadotropin and testosterone concentrations (Valiente et al., 2007; García Romero et al., 2009). Similarly, reversible impairment of spermiogenesis, spermatocytogenesis, and sperm motility was reported in male cats following a single subcutaneous dose of acyline (330 µg/kg; García Romero et al., 2012). However, the short duration of action of GnRH antagonists and the need for repeated administration limit their use in clinical practice compared with long-acting GnRH agonist implants (Gobello, 2012). Therefore, the ability of GnRH antagonists to suppress fertility without inducing an initial transient hormonal stimulation is considered a potential advantage (García Romero et al., 2009).

GONADOTROPIN-RELEASING HORMONE AGONISTS

GnRH agonists are agents used to reversibly suppress fertility in male cats and dogs (Goericke-Pesch et al., 2011; Romagnoli et al., 2012). GnRH, the primary regulator of the HPG axis, controls the release of LH and FSH from the anterior lobe of the pituitary gland (Baran et al., 2016). In males, LH regulates testosterone synthesis, while FSH regulates spermatogenesis (Baran et al., 2016). By 2005, more than 2,000 GnRH analogs had been developed and experimentally tested (Padula, 2005). GnRH agonists mimic the effects of endogenous GnRH but generally have greater and longer-lasting biological activity (Padula, 2005). However, the continuous release of these potent GnRH agonists initially causes a temporary increase in gonadotropin secretion. Subsequently, GnRH receptor downregulation and desensitization occur in the pituitary gland (Gobello, 2007). As a result of this process, LH and FSH release is suppressed, testosterone levels decrease, and spermatogenesis is temporarily inhibited (Trigg et al., 2006). Because GnRH receptor desensitization is temporary, GnRH agonists provide reversible contraception, with reproductive function gradually returning after the implant's drug supply is depleted (Junaidi et al., 2003). GnRH agonists such as deslorelin have been formulated as slow-release subcutaneous implants to provide sustained drug delivery and prolonged suppression of reproductive function (Driancourt and Briggs, 2020). Slow-release subcutaneous implants cause a decrease in testosterone concentration and a significant reduction in motile sperm count (Driancourt and Briggs, 2020). The most commonly used GnRH agonist in clinical practice is deslorelin (Lucas, 2014). Deslorelin is reported to have biological activity approximately 100 times greater than that of endogenous GnRH (Padula, 2005). Deslorelin implants containing 6 mg of active ingredient, when administered subcutaneously, reduce plasma LH and testosterone concentrations to undetectable levels within approximately 4 weeks and cause infertility in male dogs within 6 weeks (Junaidi et al., 2003). The suppressive effects of the deslorelin implant were reversible, with LH and testosterone concentrations returning to normal ranges by approximately 52 weeks and semen characteristics fully recovering by 60 weeks after implantation (Junaidi et al., 2003). Deslorelin doses greater than 0.25 mg/kg have been reported to suppress spermatogenesis in male dogs (Trigg et al., 2001). However, reproductive function is not suppressed immediately after implant administration (Romagnoli et al., 2012). In a study by Romagnoli et al. (2012), six adult male dogs received a single 4.7 mg deslorelin implant (Suprelorin®), and sterility was achieved after a mean of 54 ± 21 days (Romagnoli et al., 2012). The delayed onset of sterility following deslorelin implantation is associated with the initial stimulatory effect of the GnRH agonist and the gradual decline in semen parameters (Romagnoli et al., 2012). Longer-acting formulations include implants containing 9.4 mg of deslorelin (Sirivaidyapong et al., 2012). Although reproductive recovery has been reported after approximately 60 weeks, considerable individual variability exists in the timing of recovery following deslorelin treatment (Morsink, 2009; Stempel et al., 2022). Although the Gonazon® implant containing 18.5 mg azagly-nafarelin was originally developed for use in female dogs, it has also been shown to suppress reproductive function in male dogs. In a study involving eight male Beagle dogs, administration of an 18.5 mg azagly-nafarelin implant reduced LH bioavailability by approximately 70% at 11 weeks, while testosterone concentrations decreased to basal levels within 17.5 ± 8.4 days (Ludwig et al., 2009). Following an initial increase lasting four days, testosterone concentrations were reported to return to baseline within three weeks, with azoospermia developing within 2 to 4 weeks (Ludwig et al., 2009). In a larger study, testosterone concentrations were found to fall below the threshold (< 0.35 nmol/L) in the vast majority of 53 dogs, and this suppression persisted for 6 to 22 months (Goericke-Pesch et al., 2010). Approximately 8 weeks after administration, reductions of 54% and 52% in testicular and prostate volumes, respectively, were observed. The reduction in prostate volume has been reported to be more pronounced in dogs with prostatic hyperplasia, and a decrease in aggressive behavior has also been observed (Goericke-Pesch et al., 2010). Implants containing 4.7 mg have been reported to cause a decrease in testosterone concentration, testicular atrophy, and a reduction in fertility. In cats, the efficacy of deslorelin implants has been evaluated. Implants containing 4.7 mg have been reported to cause decreases in testosterone concentration, testicular atrophy, and suppression of spermatogenesis (Novotny et al., 2012). Keratinized spines on the glans penis, considered clinical indicators of testosterone activity, have been reported to disappear within approximately nine weeks (Goericke-Pesch et al., 2011; Novotny et al., 2012). Histological evaluations in cats have shown the persistence of long spermatids in testicular tissue along with atrophy (Novotny et al., 2012). In addition,

GnRH stimulation tests using buserelin indicated an increase in testosterone concentrations before deslorelin treatment; however, after administration of a 4.7-mg deslorelin implant, four of the five tomcats had basal testosterone concentrations and revealed no further increase in response to buserelin from week 4 onward (Goericke-Pesch et al., 2013).

PROGESTINS

Progestins, which are synthetic analogs of progesterone, are widely used for contraception, especially in female dogs and cats (Kutzler and Wood, 2006). In male dogs, progestins may affect reproductive function through mechanisms other than gonadotropin suppression. Their effects on semen quality have been suggested to result primarily from direct effects on the epididymis, particularly during epididymal sperm maturation. However, significant suppression of libido has not been consistently demonstrated in male dogs (England, 1997). Oral administration of megestrol acetate (MA) at a dose of 2 mg/kg for seven days has been reported to cause no significant changes in sperm quality in male dogs, whereas secondary sperm abnormalities occurred at higher doses (4 mg/kg; England, 1997). Subcutaneous administration of medroxyprogesterone acetate (MPA) at a dose of 20 mg/kg resulted in rapid and significant reductions in sperm motility and output and deterioration in sperm morphology (England, 1997). The rapid changes in sperm motility, output, and morphology were considered to result primarily from the direct effects of MPA on epididymal sperm maturation and transport rather than from gonadotropin suppression, as plasma LH concentrations were not suppressed (England, 1997). No significant effects on libido were reported (England, 1997). The duration of action of progestins varies depending on the compound used and the route of administration. Megestrol acetate is a short-acting progestin with a half-life of several hours, whereas medroxyprogesterone acetate has a reported half-life of 12-17 hours after oral administration and 40-50 days after injection. No adverse effects have been reported following MPA treatment in dogs (Romagnoli et al., 2024). However, data regarding the duration and reversibility of progestin-induced fertility suppression in males remain limited. Compared with progestins, GnRH agonists have been more extensively studied for fertility control in male dogs, with more clearly characterized durations of action and reversibility (Romagnoli et al., 2024). In contrast, information regarding the duration of contraceptive efficacy, reversibility, and long-term safety of progestins in males remains limited (Vasetska, 2020; Romagnoli et al., 2024).

CHEMICAL CASTRATION

Chemical castration is a minimally invasive contraceptive method developed as an alternative to surgical orchiectomy in male cats and dogs (Oliveira et al., 2007; 2012). In chemical castration, chemical agents are administered directly into the testis, epididymis, or ductus deferens. Intratesticular injections may disrupt spermatogenesis through testicular tissue damage, whereas injections into the epididymis or ductus deferens may induce fibrosis and obstruction of sperm transport, potentially resulting in azoospermia and infertility (Oliveira et al., 2012; Hess et al., 2024).

Chemical castration has been proposed as a potential alternative to surgical castration in population control programs because it may reduce the need for surgical facilities and anesthesia; however, its suitability for large-scale use depends on the efficacy, safety, and practicality of the chemical agent used (Baran et al., 2010; Oliveira et al., 2013; Bahçe and Karaca, 2026). A recent study indicated that the mentioned method is a cost-effective and feasible alternative, although concerns remain regarding tissue damage and reversibility (Hess et al., 2024). The volume of the injected solution should be adjusted to the testicular size to ensure appropriate distribution within the testis (Leoci et al., 2014; Rafatmah et al., 2019). Intratesticular injection can be performed by advancing the needle along the long axis of the testis and depositing the solution gradually while withdrawing the needle (Leoci et al., 2014; Rafatmah et al., 2019). Alternatively, injection into the mediastinum testis has been investigated as a technique for controlled administration of chemical agents (Bahçe and Karaca, 2026). When determining the dose, testicular volume is taken into account, and the injection is performed without compromising anatomical integrity (Canpolat et al., 2016). Zeuterin® (Ark Sciences, USA), containing zinc gluconate, is a United States Food and Drug Administration approved preparation for use in male dogs and contains 0.2 M zinc gluconate (13.1 mg zinc/ml; Oliveira et al., 2013). The same active ingredient is used in Latin American countries under the name Esterilsol® for both dogs and cats (Kutzler, 2015). In a study conducted with Zeuterin, side effects were mostly limited to the injection site and were reported in 1.1% of 270 male dogs (Kutzler, 2015). Histological examinations revealed almost complete fibrosis of the seminiferous tubules and Leydig cells at 60 and 75 days after intratesticular injection (Tepsumethanon et al., 2005). With intraepididymal injections and injections into the ductus deferens, the primary mechanism is chemical obstruction and the development of secondary testicular atrophy. Azoospermia has been reported to develop within 90 days after intraepidermal administration of 50 mg of zinc arginine (Fahim et al., 1993). Histological examination revealed normal seminiferous tubules, atrophy of the rete testis, and an absence of spermatozoa in the epididymis and ductus deferens (Fahim et al., 1993). However, temporary

azoospermia or oligospermia has been reported following formalin administration (Bahçe and Karaca, 2026). In a study comparing formalin-induced chemical castration with surgical castration, a significant decrease in serum testosterone levels, deterioration in sperm parameters, and degenerative changes in testicular tissue were observed. Activation of the cellular stress response was also demonstrated (Moustafa et al., 2023). It has been emphasized that chemical castration may cause more pronounced local tissue damage than surgery and that dose and administration technique are key determinants of outcomes (Moustafa et al., 2023). Non-surgical sterilization methods based on the intratesticular administration of calcium chloride are being considered as alternative approaches to fertility control in male cats (Jana and Samanta, 2011). In a clinical study, a single intratesticular injection of a 20% calcium chloride solution was reported to substantially suppress spermatogenesis, cause degeneration and necrosis of testicular tissue, and lead to a substantial decrease in serum testosterone concentrations (Jana and Samanta, 2011). Biochemical analyses revealed increased testicular lipid peroxidation and malondialdehyde levels, together with decreased antioxidant enzyme activities, reduced glutathione/oxidized glutathione (GSH/GSSG) ratio, and mitochondrial membrane potential, supporting the involvement of oxidative stress in calcium chloride-induced testicular damage (Jana and Samanta, 2011).

Table 1. The representative studies on non-surgical contraceptive methods in male dogs and cats

Study	Species	Contraceptive method	Application	Main findings
Levy et al. (2004)	Male cats	GnRH immunocontraception	GnRH vaccine	Anti-GnRH antibodies developed; serum testosterone declined, with testicular atrophy and suppressed spermatogenesis
Donovan et al. (2012)	Male dogs	GnRH immunocontraception	GnRH immunization	Reproductive and endocrine functions were suppressed
García Romero et al. (2009)	Male dogs	GnRH antagonist (acyline)	Subcutaneous injection	Gonadotropin (LH, FSH) and testosterone concentrations were temporarily reduced
Romagnoli et al. (2012)	Male dogs	GnRH agonist (deslorelin)	4.7-mg implant	Semen quality declined and temporary sterility developed
Goericke-Pesch et al. (2011)	Male cats	GnRH agonist (deslorelin)	4.7-mg implant	Testosterone and testicular volume decreased, and reproductive behaviour was suppressed
Oliveira et al. (2013)	Male cats	Zinc-based chemical contraception	Intratesticular injection	Azoospermia occurred in most treated cats, and testicular size decreased
Oliveira et al. (2012)	Male dogs	Zinc gluconate-based chemical contraception	Intratesticular injection	Azoospermia was observed in most treated dogs
Moustafa et al. (2023)	Male dogs	Chemical castration (formalin)	Intratesticular injection	Testosterone decreased, and degenerative changes in testicular tissue were observed
Bahçe and Karaca (2026)	Male dogs	Chemical castration (formalin and $CaCl_2$)	Testicular mediastinum injection	Semen quality declined, and testicular tissue changes were observed

GnRH: Gonadotropin-releasing hormone; LH: Luteinizing hormone; FSH: Follicle-stimulating hormone; $CaCl_2$: Calcium chloride.

CONCLUSION

In the present review, non-surgical contraceptive methods in male dogs and cats, including progestins, GnRH agonists and antagonists, immunocontraceptive approaches, and chemical castration methods, were evaluated in terms of their mechanisms of action, duration of effectiveness, reversibility, and clinical applicability. Surgical sterilization remains a widely used method today due to its high effectiveness and permanent effects. However, non-surgical approaches may provide reversible or permanent alternatives depending on the method used. GnRH agonists may provide long-term suppression of reproductive function; however, the duration of their effects and the time required for fertility to return may vary among individual animals and cannot be predicted with certainty. GnRH antagonists generally have a rapid onset of action, although their relatively short duration of effect may limit their clinical use. Immunocontraceptive methods may provide prolonged contraceptive effects, but variability in individual immune responses remains an important limitation. Chemical castration methods may represent a minimally invasive alternative to surgical castration; however, their efficacy and safety depend on the chemical agent and administration technique used.

The present review has some limitations that should be considered when interpreting the findings. The available evidence for some non-surgical contraceptive methods in male dogs and cats is based on a limited number of studies with relatively small sample sizes. In addition, differences in treatment protocols, doses, outcome measures, and follow-up periods make direct comparisons among the available methods difficult. The limited number of long-term and comparative studies also restricts definitive conclusions regarding their efficacy, reversibility, safety, and clinical applicability. Future studies should therefore focus on standardized treatment protocols, long-term safety, duration and predictability of contraceptive effects, recovery of fertility, and direct comparisons between different non-surgical contraceptive approaches.

DECLARATIONS

Author's contributions

Fikret Karaca conceived and determined the topic of the review. Zehra Coşkun Bahçe conducted the literature search, evaluated the relevant literature, and wrote the manuscript. All authors critically reviewed, checked, and approved the final version of the manuscript before publication.

Availability of data and materials

No new datasets were generated or analyzed in the present review. All information evaluated in this article was obtained from previously published studies cited in the manuscript.

Competing interests

The authors declare no conflict of interest.

Ethical consideration

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. ChatGPT (OpenAI) was used solely to assist with language editing and improving the clarity and readability of the manuscript. All AI-assisted text was critically reviewed, revised, and verified by the authors to ensure scientific accuracy, appropriate academic language, and consistency with the original sources. The authors take full responsibility for the final content of the manuscript. The originality and accuracy of the information presented in the manuscript were checked and confirmed by the authors before submission and will be reconfirmed before acceptance and publication.

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An Update on Canine Parvovirus in Ecuador and Worldwide: Pathogenesis, Diagnosis, Epidemiology, Treatment, and Control

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ABSTRACT

Canine parvovirus (CPV) remains one of the most relevant viral pathogens affecting domestic dogs worldwide, particularly in young and unvaccinated populations, due to its high environmental resistance and rapid transmission. The present study aimed to provide an updated and integrative overview of epidemiology, pathogenesis, diagnosis, and control of CPV through a systematic qualitative review. A comprehensive literature search was carried out using major academic databases, including PubMed, SciELO, and Scopus, considering studies published between 2020 and 2026. A total of 312 articles were initially identified, of which 70 met the inclusion criteria and were selected for article review. The results confirmed the global circulation of multiple variants (CPV-2a, CPV-2b, and CPV-2c), including the presence of CPV-2b in Ecuador, as well as the persistence of the virus in diverse environments and its impact on susceptible populations such as puppies between 6 weeks and 6 months of age and susceptibility, and some breeds such as Doberman Pinschers, Rottweilers, and American Pit Bull Terriers. Molecular diagnostic techniques, particularly PCR and qPCR, remain the most reliable tools for early detection. Despite advances in supportive therapy and emerging antiviral approaches, vaccination continues to represent the most effective preventive strategy. Continuous surveillance and improvements in diagnostic and immunization strategies are essential to reduce the impact of CPV infections.

Keywords: Canine parvovirus, Genoma, Pathogenesis, Transmission

INTRODUCTION

The most common viral infections that may affect various body systems are Canine parvovirus (CPV), distemper canine, and canine infections respiratory disease (Decaro et al., 2020). Although CPV is not considered a notifiable disease, it remains a significant health concern due to its high morbidity in susceptible canine populations and its high morbidity in susceptible canine populations and its high mortality in untreated puppies (90%), despite survival rates exceeding 80-90% with intensive supportive care (Zhou et al., 2024). Nevertheless, its impact can be substantially reduced through appropriate vaccination programs and biosecurity measures (López et al., 2023; WOAH, 2025).

Canine parvovirus emerged in the late 1970s and has continued to evolve in response to host adaptation (De la Torre et al., 2018). Currently, CPV is recognized as one of the leading causes of severe viral gastroenteritis in dogs worldwide (Grecco et al., 2024). The virus belongs to the genus *Protoparvovirus* within the family *Parvoviridae* (Ozaeta et al., 2025). *Canine parvovirus* is characterized as a small, non-enveloped virus containing a single-stranded DNA genome, features that confer remarkable environmental stability and facilitate its persistence and transmission (López-Astacio et al., 2023). Furthermore, CPV exhibits a relatively high mutation rate, similar to RNA viruses, which has contributed to the emergence of new variants with enhanced host-cell tropism, replication efficiency, and pathogenicity (Loza-Vega, 2025).

Despite its relatively small genome, CPV contains essential genes involved in viral replication and infectivity (Ozaeta et al., 2025). The molecular structure of parvovirus is codified by non-structural proteins such as NS1, which plays a critical role in viral replication (López-Astacio et al., 2023). On the other hand, structural proteins are encoded by the *cap* gene and form capsid proteins VP1 and VP2. These proteins are involved in key virus-host interactions, contributing to cellular invasion, viral dissemination, and persistence of infection (López-Astacio et al., 2023).

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From an epidemiological perspective, canine parvovirus continues to represent a major challenge for veterinary medicine due to its rapid spread, environmental resilience, and potential for indirect transmission through fomites (López- Astacio et al., 2023). A recent study has identified CPV-2b as the predominant antigenic variant circulating in Ecuador (Loor-Giler et al., 2025). Moreover, puppies between three weeks and six months of age have been reported to be at a higher risk of developing severe clinical disease, while infection appears to occur more frequently in males than in females (Ozaeta et al., 2025). Although adult dogs can also become infected, many remain asymptomatic and shed the virus through their feces, thereby contributing to environmental contamination and viral maintenance within susceptible populations (De la Torre et al., 2018).

Understanding the pathogenesis of CPV is essential for the development of effective prevention and control strategies, as genetic variations among viral strains may influence both virulence and host immune responses (López-Astacio et al., 2023). In parallel, advances in diagnostic technologies, including polymerase chain reaction (PCR), immunofluorescence assays, and serological tests, have enabled more sensitive and specific detection of infection, facilitating early and accurate diagnosis (Li et al., 2025). In this context, there is a growing need to integrate current knowledge on CPV in a critical and structured manner, not only to summarize existing evidence about specific aspects of the disease but also to identify gaps and emerging challenges. The present review aimed to address knowledge gaps by integrating current evidence on CPV pathogenesis, global distribution and epidemiology, and the molecular characterization of the CPV variants circulating in Ecuador. Furthermore, it systematically synthesized the limited available evidence regarding the effectiveness of the diagnostic, vaccination, and control strategies implemented in the country through a systematic qualitative review of recent literature.

MATERIALS AND METHODS

The present study was designed as a qualitative systematic review aimed at integrating current scientific evidence on CPV, with particular emphasis on its epidemiology, pathogenesis, diagnostic approaches, treatment and control strategies. The methodological approach followed established principles for systematic reviews according to PRISMA 2020, with the aim of ensuring transparency, reproducibility and rigor in the process of identifying, selecting and synthesizing available literature.

A comprehensive search was conducted in recognized academic databases, including PubMed, SciELO, and Scopus, to identify relevant studies published between 2020 and 2026. The mentioned databases were selected due to their broad coverage of veterinary literature. PubMed was prioritized as a primary source of high-quality scientific evidence, while SciELO facilitated access to regional studies, particularly from Latin America. Google Scholar was used as a complementary tool to identify additional literature not indexed in conventional databases (Page et al., 2021). Table 1 summarizes the distribution of articles retrieved from each database included in the literature search.

Table 1. Databases used for the literature search

Database	Results
PubMed	66
Scopus	231
SciELO	15
Total	312

The search strategy combined descriptors in both English and Spanish, using Boolean operators such as “AND”, “OR”, and “NOT”. The general search equation applied was (“canine parvovirus” OR “CPV” OR “canine parvoviral enteritis”) AND (epidemiology OR pathogenesis OR diagnosis OR treatment OR therapy). Filters were applied to include articles published within the selected timeframe. While open-access publications were prioritized to facilitate accessibility, this may have introduced a selection bias by excluding relevant high-quality studies available in subscription-based journals (Page et al., 2021).

Applying the filters corresponding to the year of publication, open access articles, language, and accessibility criteria, the search yielded a total of 312 records. After applying the inclusion and exclusion criteria, as well as removing duplicates, theses, and irrelevant articles, 70 studies were selected for a detailed analysis. The selection process was conducted according to the PRISMA 2020 framework, ensuring transparency in the identification, screening, eligibility, and inclusion stages, as shown in Figure 1 (Page et al., 2021).

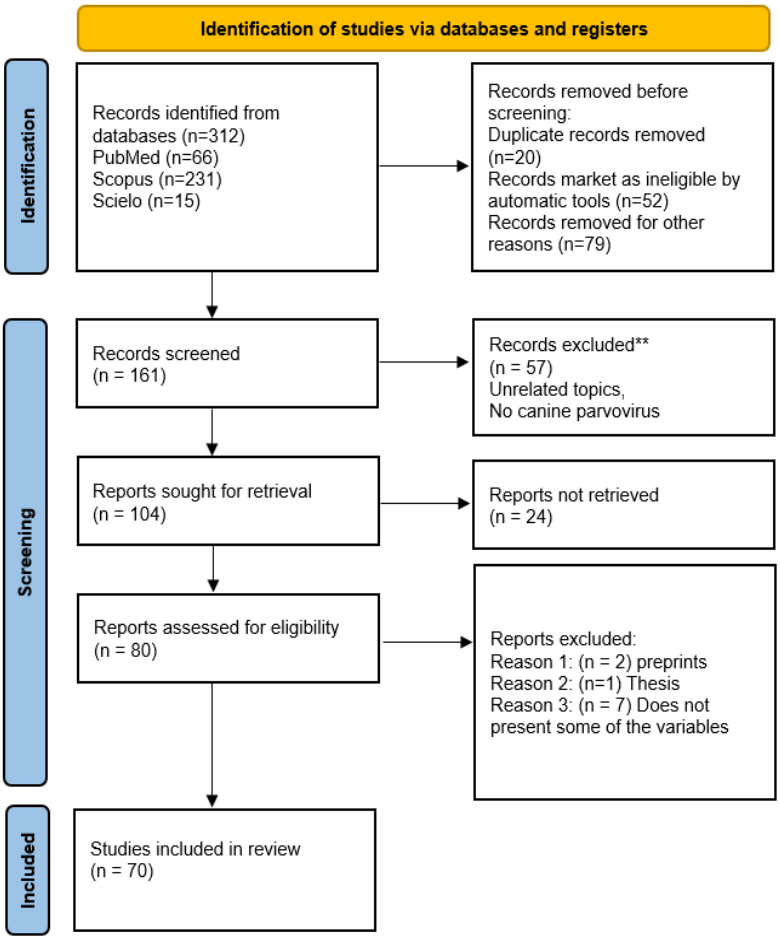


Figure 1. PRISMA flowchart for identification, screening, eligibility, and inclusion of studies about epidemiology, pathogenesis, diagnosis, treatment, and control of canine parvovirus

Inclusion and exclusion criteria

The inclusion criteria were established to ensure that the scientific relevance and methodological quality of the selected studies were assessed using the appropriate Joanna Briggs Institute (JBI) critical appraisal tools according to the study design. Each study was evaluated for methodological rigor, data collection procedures, outcome assessment, and potential sources of bias. The results of the quality assessment were considered during the synthesis and interpretation of the evidence. Original peer-reviewed articles addressing CPV in relation to its epidemiology, pathogenesis, diagnosis, treatment, disease control, and investigation conducted in Ecuador were included. Studies involving domestic dogs of any age, breed, or geographic region were considered, as well as investigations evaluating viral transmission, prevalence, risk factors, clinical manifestations, immune response, and preventive strategies such as vaccination and biosecurity measures. Additionally, studies about diagnostic methodologies, including enzyme-linked immunosorbent assays (ELISA) tests, PCR-based techniques, virus isolation, serological assays, or molecular characterization, were included.

Studies conducted exclusively on species other than canines, articles unrelated to CPV, or investigations focused solely on unrelated viral diseases were excluded. Likewise, narrative reviews, editorials, preprints, thesis, and conference abstracts without complete data, duplicate publications, and documents lacking primary scientific information or full-text access were not considered. This criterion was applied due to accessibility limitations and is recognized as a potential source of bias in the study selection process (Page et al., 2021).

Study selection process

The study selection process was carried out in different sequential stages according to the PRISMA flow guidelines by independent reviewers with conflict resolution. Initially, 312 identified records were screened in databases by title and abstract, and studies that did not meet the established thematic criteria related to CPV were excluded. Subsequently, potentially relevant articles were assessed through full-text review to determine their final eligibility.

The final selection of studies was conducted through a systematic evaluation process that identified 70 articles meeting the established inclusion criteria and considered suitable for qualitative synthesis. Although the review primarily included studies published in English within the predefined time frame, 7 articles published in Spanish were also

incorporated due to their scientific relevance and contribution to the topic. In addition, one study published in 2016 was retained despite falling outside the selected publication period because of its significant relevance and frequent citation in the literature, and one article published in 2018 because it has relevant information on Ecuador. The complete flow of the study selection process is presented in Figure 1.

RESULTS

The family *Parvoviridae* comprises a large group of viruses containing a single-stranded DNA genome ranging from 4 to 6 kilobases, enclosed within a small non-enveloped icosahedral capsid (Ozaeta et al., 2025). The virion measures approximately 18-26 nm in diameter (Yin et al., 2022). Replication occurs within the nucleus of actively dividing cells (Li et al., 2025). *Parvoviridae* comprises viruses that infect a wide range of vertebrate and invertebrate hosts and is classified into three subfamilies, including *Densovirinae*, which includes parvoviruses that infect invertebrate hosts; *Parvovirinae*, which comprises viruses infecting vertebrate hosts such as mammals, birds, and reptiles; and *Hamaparvovirinae*, is a subfamily that infects crustaceans and insects (López-Astacio et al., 2023). *Hamaparvoviridae* subfamily is classified based on nucleotide sequence homology within the helicase domain (Loza-Vega, 2025). The subfamily *Parvovirinae* has been further divided into eight genera according to the host species they infect (Table 2).

Table 2. Genera of the family *Parvoviridae*, their principal hosts, and susceptible species

Genus	Hosts	Susceptible species
Amdoparvovirus	Carnivores	Foxes (Aleutian mink disease virus), Minks (Aleutian disease virus)
Aveparvovirus	Galliform birds	Chickens (Chicken parvovirus), Turkeys (Turkey parvovirus)
Bocaparvovirus	Carnivores	Canines (Canine minute virus, Canine bocavirus 1), Felines (Feline bocavirus)
	Pinnipeds	California sea lion (Pinniped bocaparvovirus 1 and 2)
	Primates	Humans (Human bocavirus 1, 2a, 2b, 2c, 3, and 4), Gorillas (Gorilla bocavirus)
	Ungulates	Bovines (Bovine parvovirus), Swine (Porcine parvovirus 1, 2, 3, 4-1, 4-2)
	Equines	Horses (Equine parvovirus)
Dependoparvovirus	Anseriformes	Ducks (Duck parvovirus), Geese (Goose parvovirus)
	Chiroptera	Bats (Bat adeno-associated virus)
	Pinnipeds	California sea lion (Adeno-associated virus)
	Primates	Humans (Parvovirus B19), Non-human primates (Rhesus macaque, Pig-tailed macaque), Squirrels (Chipmunk parvovirus)
Erythroparvovirus	Ungulates	Bovines (Bovine parvovirus 3)
	Carnivores	Canines (Canine parvovirus), Felines (Feline parvovirus), Minks (Mink enteritis virus), Raccoons, Arctic wolves
Protoparvovirus	Primates	Humans (Bufavirus 1a, 1b, and 2)
	Rodents	Rats (Kilham rat virus, Rat parvovirus 1), Mice (Minute virus of mice, Mouse parvovirus 1-5), Hamsters
	Ungulates	Swine (Porcine parvovirus type 1, Kresse strain, NADL-2)
	Chiroptera	Fruit bats (*Eidolon helvum* parvovirus)

The present data in the table are adapted from Ozaeta et al. (2025)

STRUCTURE AND GENOME OF PARVOVIRUSES

Parvoviruses are small, non-enveloped viruses, a characteristic that contributes to their remarkable environmental stability and resistance to physical and chemical inactivation (Tuteja et al., 2022). The virion consists of a highly structured icosahedral capsid approximately 18-26 nm in diameter, which protects the viral single-stranded DNA genome and plays a critical role in host-cell recognition, attachment, and entry (Flores et al., 2020). The capsid is assembled from 60 protein subunits, predominantly composed of VP2, the major structural protein that accounts for approximately 90% of the capsid and contains the principal antigenic determinants involved in host range specificity and immune recognition (Liu et al., 2025). In addition to VP2, the capsid contains VP1, a minor structural protein that shares

most of its sequence with VP2 but possesses a unique N-terminal region essential for viral infectivity and intracellular trafficking (López-Astacio et al., 2023). A third capsid protein, VP3, has also been identified in CPV and is generally considered a cleavage product derived from VP2 during virion maturation rather than a distinct gene product (Tuteja et al., 2022).

The *parvovirus* genome consists of a linear single-stranded DNA (ssDNA) molecule (Figure 2) ranging from approximately 4 to 6 kb in length and containing palindromic terminal sequences that fold into characteristic hairpin structures (López-Astacio et al., 2023). The terminal hairpins function as self-priming structures for DNA synthesis and are essential for viral replication. During replication, the hairpin termini are processed by viral and host cellular factors, generating primers that initiate DNA synthesis (Qi et al., 2020). Replication of parvovirus allows genome replication to proceed through a unidirectional process known as rolling-hairpin replication. The terminal palindromic sequences, together with adjacent nucleotides, contain critical cis-acting elements required for viral DNA replication, packaging, and genome encapsidation (Tuteja et al., 2022). In addition to serving as origins of replication, the terminal hairpins participate in the resolution of genomic termini through the action of viral proteins such as NS1 (Loza-Vega et al., 2025). The multifunctional protein introduces site-specific nicks at defined sequences, generating new 3′-OH ends that are subsequently extended by the host cell DNA replication machinery (López-Astacio et al., 2023). Consequently, productive viral replication is highly dependent on actively dividing cells, particularly those in the S phase of the cell cycle, where the cellular factors required for DNA synthesis are abundantly available (Pénzes et al., 2023).

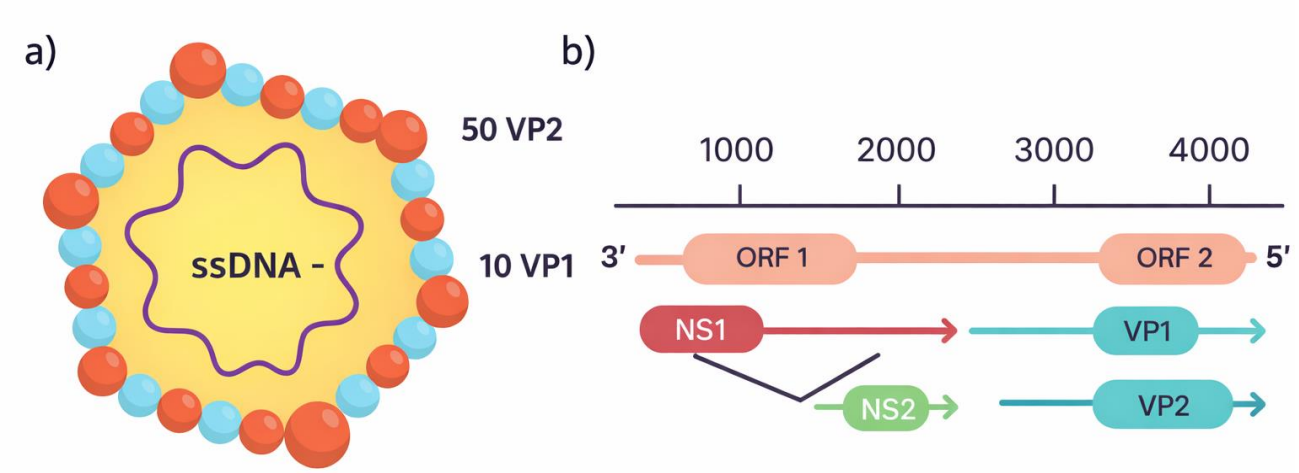


Figure 2. Representation of the structure of parvoviruses. **a:** Capsid of the virus. **b:** ORF 1 and ORF 2 with structural and non-structural proteins of the parvovirus. Source: Created by the authors based on Tuteja (2022).

The parvovirus genome contains two major open reading frames (ORFs; Chen et al., 2023). The left ORF encodes the *rep* gene, which gives rise to the non-structural proteins, including NS1 and NS2 (López- Astacio et al., 2023). NS1 is a multifunctional protein essential for viral replication, possessing sequence-specific endonuclease (nickase), helicase, and ATPase activities (Magouz et al., 2023). In addition to initiating viral DNA replication, NS1 regulates viral transcription, interacts with host cellular replication machinery, and contributes to virus-induced cytopathic effects, including cell-cycle arrest, apoptosis, and oxidative stress-mediated cell damage (Pénzes et al., 2023). NS2, although less extensively characterized, plays important roles in viral replication, intracellular trafficking, nuclear export, and efficient virion production (Magouz et al., 2023).

The second ORF encodes the *cap* gene, which contains the genetic information required for the synthesis of the structural capsid proteins VP1 and VP2 (López-Astacio et al., 2023). VP1 is a minor capsid component that contains a unique N-terminal region (VP1u) enriched in basic amino acids, including arginine and lysine residues, which contribute to nuclear localization and infectivity (Mia and Hasan, 2021). Importantly, VP1u harbors a phospholipase A2 (PLA2) domain that facilitates viral escape from the endosomal compartment following cell entry (López-Astacio et al., 2023). In contrast, VP2 constitutes approximately 90% of the capsid structure and contains the major antigenic determinants responsible for host-cell receptor recognition, tissue tropism, and host-range specificity (Tuteja et al., 2022). Mutations within VP2 have been strongly associated with the emergence of novel antigenic variants and alterations in host susceptibility (Chen et al., 2024).

Expression of the *cap* gene is regulated through alternative splicing and the use of distinct translational initiation sites, allowing precise control of the VP1-to-VP2 ratio within mature virions (López-Astacio et al., 2023). Consequently, the sequence variability and genomic organization of ORF2 are of particular importance for understanding viral

evolution, molecular surveillance, and vaccine development (Mia et al., 2021). Due to their compact genomes and limited coding capacity, parvoviruses rely on multifunctional structural and non-structural proteins to complete their replication cycle (Magouz et al., 2023). The structural proteins coordinate multiple interactions with host cells and tissues, facilitating viral replication, dissemination, persistence, and adaptation within susceptible host populations (López-Astacio et al., 2023).

CELLULAR TROPISM

Parvoviruses exhibit a highly restricted cellular tropism that depends on both the presence of specific cell-surface receptors and the proliferative status of the host cell (Chen et al., 2024). In the case of *Protoparvoviruses* such as CPV, viral attachment is primarily mediated by the transferrin receptor type 1 (TfR1), which is highly expressed on actively dividing cells, including intestinal crypt epithelial cells, hematopoietic progenitor cells, and lymphoid tissues (Uhl and Fasina, 2025). The TfR1 serves as the primary cellular receptor for CPV and is a receptor that determines host range, tissue tropism, and viral pathogenicity (Uhl and Fasina, 2025).

Viral entry is initiated by receptor binding followed by clathrin-mediated endocytosis (Afumba et al., 2022). Acidification triggers conformational changes in the capsid, leading to the exposure of the unique N-terminal region of VP1 (VP1u), which contains a PLA2 domain required for disruption of the endosomal membrane and enables the escape of the viral particle into the cytoplasm (Shi et al., 2023). Subsequently, the virion is transported to the nucleus, where partial uncoating allows the release of the single-stranded viral DNA. Genome replication proceeds through a rolling-hairpin mechanism that depends on the inverted terminal repeats (ITRs) located at both the 5′ and 3′ ends of the viral genome, as well as the multifunctional NS1 protein (Magouz et al., 2023). NS1 recognizes the terminal sequences and functions as a site-specific nickase, helicase, and recruiter of host replication factors, thereby exploiting the cellular DNA replication and repair machinery for viral genome amplification (Uhl and Fasina, 2025). Alternative transcription and RNA splicing generate multiple viral mRNAs encoding both non-structural (NS) and structural (VP) proteins, which regulate viral replication, capsid assembly, and interactions with host antiviral responses (López-Astacio et al., 2023). As viral replication progresses, CPV induces cell-cycle dysregulation, oxidative stress, apoptosis, and other cytopathic effects that contribute to tissue damage and facilitate the release and dissemination of newly formed virions (Afumba et al., 2022).

PARVOVIRUS IN WILDLIFE SPECIES

Canine parvovirus emerged as a canine pathogen in the late 1970s following a host-switch event involving a feline panleukopenia virus (FPV)-like ancestor circulating among wild carnivores (Qi et al., 2020). Although the precise evolutionary pathway remains unresolved, molecular and phylogenetic evidence supports the hypothesis that CPV originated through the adaptation of an FPV-related virus capable of infecting carnivore species (Zhou et al., 2024). This hypothesis is further strengthened by the presence of intermediate viral variants circulating in wildlife populations that share genetic features of both FPV and CPV, together with the limited ability of classical FPV strains to effectively infect dogs (Franzo et al., 2023).

Wild carnivores play an important role in the ecology and maintenance of canine parvoviruses (CPV; Sarchese et al., 2025). Studies conducted in Türkiye and Italy have shown that wild canids, particularly urban red foxes (*Vulpes vulpes*), Eurasian badgers, and wolves, act as reservoirs of CPV infection and contribute to viral transmission between wildlife and domestic dog populations (Kurucay et al., 2023; Sarchese et al., 2025). The evidence supports the importance of wildlife-domestic animal interfaces in the epidemiology of canine parvoviral disease.

Its genomic evolution has been characterized by an unusually high substitution rate for a DNA virus, estimated at approximately 10^{-4} substitutions per nucleotide site per year, a value comparable to those reported for certain RNA viruses (Jantafong et al., 2022). The accelerated mutation rate has facilitated the emergence of novel antigenic variants with substantial genetic and phenotypic differences (Zhou et al., 2024).

Serological surveys have demonstrated a high prevalence of antibodies against FPV among wild felid populations, suggesting frequent exposure to the virus, likely through interactions with domestic cats or shared environmental sources of infection (Ozaeta et al., 2025). This finding supports the existence of complex transmission networks involving both domestic and wild carnivore hosts (Ozaeta et al., 2025).

CANINE PARVOVIRUS IN ECUADOR

Molecular and epidemiological studies conducted in Ecuador have demonstrated the circulation of the three classical antigenic variants of canine parvovirus type 2 (CPV-2), namely CPV-2a, CPV-2b, and CPV-2c (De la Torre et al., 2018). Recent evidence indicated that CPV-2b is currently the predominant variant detected in clinical samples reported in a multicenter study conducted between 2022 and 2023, which analyzed samples collected from six Ecuadorian provinces,

including Carchi, Chimborazo, Guayas, Imbabura, Pichincha, and Santo Domingo de los Tsáchilas (Loor-Giler et al., 2025). The study employed quantitative PCR (qPCR) and molecular characterization of the VP2 gene, enabling both viral load quantification and the identification of mixed infections involving multiple CPV variants (De la Torre et al., 2018; Loor-Giler et al., 2025).

Prior to the study by Loor-Giler et al. (2025), investigations with smaller sample sizes and more restricted geographic coverage reported different patterns of variant distribution, with CPV-2a identified as the predominant circulating strain, followed by CPV-2b (De la Torre et al., 2018). The changes in variants may be attributed to methodological differences, including limited sampling areas, lower sample numbers, and the use of conventional sequencing approaches without large-scale qPCR-based viral quantification.

Age-related analyses of CPV prevalence in Ecuador revealed that dogs between 3 and 12 months of age represented the largest proportion of positive cases, with the probability of infection decreasing progressively as age increased (Basurto et al., 2024; Loor-Giler et al., 2024). This age-associated susceptibility is likely related to the combined effects of immunological immaturity and the progressive decline of maternally derived antibodies, which create a period of increased vulnerability to infection (Basurto et al., 2024).

The majority of cases reported in Ecuador involve domestic dogs presenting with hemorrhagic gastroenteritis, with puppies younger than six months of age exhibiting the highest risk of developing severe clinical disease (Basurto et al., 2024). The findings are consistent with the established pathogenesis of CPV infection, which primarily affects rapidly dividing cells within the intestinal crypts, lymphoid tissues, and bone marrow (Basurto et al., 2024; Loor-Giler et al., 2024).

The detection of co-circulating CPV variants and mixed infections highlights the importance of continuous molecular surveillance in Ecuador (De la Torre et al., 2018). In a recent study, 78.47% of dogs with CPV-2 showed a high prevalence of infection, elevated viral loads, and ongoing viral evolution; regular monitoring of circulating strains is essential to evaluate potential impacts on vaccine effectiveness and to support the implementation of evidence-based prevention and control strategies (Loor-Giler et al., 2025).

EPIDEMIOLOGY IN SUSCEPTIBLE HOSTS

Canine parvovirus is a widely contagious agent that affects domestic and wild canid populations globally and is recognized as a major cause of viral gastroenteritis in dogs (Qi et al., 2020). Although dogs of all ages are susceptible to infection, the highest incidence of clinical disease is observed in puppies between 6 weeks and 6 months of age, particularly those with incomplete vaccination schedules, inadequate maternal antibody protection, or no previous immunization (Squires et al., 2024). Different studies conducted in the United States have identified breed-associated susceptibility, with Doberman Pinschers, Rottweilers, American Pit Bull Terriers, Labrador Retrievers, German Shepherd Dogs, and their crossbreeds reported to be at increased risk of developing severe disease (Flores et al., 2020; Jyothi et al., 2024).

Host susceptibility may also be influenced by factors that compromise immune function, including malnutrition, stress, parasitism, and concurrent infectious diseases (Jyothi et al., 2024). Co-infections with pathogens such as canine distemper virus, canine enteric coronavirus, rotavirus, astrovirus, adenovirus, calicivirus, and emerging viruses including norovirus, kobuvirus, sapovirus, and canine circovirus have been associated with increased disease severity and poorer clinical outcomes (Jyothi et al., 2024; Li et al., 2025). These findings suggest that viral and bacterial co-infections may enhance susceptibility to CPV infection and contribute to the progression of clinical disease (Jyothi et al., 2024; Li et al., 2025).

Although adult dogs can become infected, many develop subclinical or mild infections due to pre-existing immunity acquired through vaccination or previous exposure (Muñoz et al., 2021). Even after infection, animals may persistently shed viral particles in their feces, promoting environmental persistence and further transmission of the pathogen (Serena et al., 2021). The virus is predominantly spread via the fecal–oral pathway through direct contact with infected feces or indirectly through contaminated fomites (Ozaeta et al., 2025). The epidemiological success of CPV is largely attributable to its exceptional environmental stability, as the virus can remain infectious for extended periods and exhibits considerable resistance to many commonly used disinfectants and adverse environmental conditions (Miranda and Thompson, 2016).

PATHOGENESIS

The transmission of CPV-2 is by the fecal-oral route, through the ingestion of contaminated fecal material or contact with contaminated fomites (Flores et al., 2020). In high-density environments such as kennels, shelters, and breeding facilities, infected animals may also contribute to environmental contamination through body secretions, facilitating transmission to susceptible hosts (López-Astacio et al., 2023).

Canine parvovirus type 2 exhibits a specific tropism for rapidly dividing cells (López-Astacio et al., 2023). Following oral exposure, the virus initially replicates within lymphoid tissues of the oropharynx, including the tonsils and regional lymph nodes (Tuteja et al., 2022). Viral replication in the mentioned tissues is followed by dissemination through the bloodstream, resulting in a systemic viremia that typically develops within 3-5 days post-infection, and the first symptoms appear between day 3 and day 5 (Tuteja et al., 2022). During viremia, the virus targets hematopoietic progenitor cells in the bone marrow and lymphoid tissues, causing depletion of leukocyte precursors and leading to severe leukopenia, lymphopenia, and neutropenia (Maganga et al., 2023).

In puppies born to non-immune dams, infection during the first weeks of life may result in viral myocarditis due to active replication within cardiomyocytes (Tuteja et al., 2022). However, the clinical manifestation has become relatively uncommon globally because most neonatal puppies acquire maternally derived antibodies (MDA) through colostrum, which provide protection during the early postnatal period (Zhou et al., 2024).

As the infection develops, infected leukocytes facilitate the spread of CPV-2 to the epithelial cells undergoing active proliferation within the intestinal crypts of Lieberkühn (Tuteja et al., 2022). Viral replication destroys the crypt stem cells responsible for continuous epithelial renewal, thereby disrupting the regeneration of intestinal villi (Tuteja et al., 2022). Consequently, villous atrophy, crypt necrosis, and collapse of the intestinal mucosal architecture occur, leading to malabsorption, fluid loss, and the development of acute enteritis, often accompanied by hemorrhagic diarrhea (Grecco et al., 2024).

The destruction of lymphoid and hematopoietic cells results in immunosuppression, causing sepsis characterized by lymphopenia and neutropenia (Maganga et al., 2023). Simultaneously, disruption of the intestinal epithelial barrier, together with inflammation and crypt necrosis, facilitates bacterial translocation from the intestinal lumen into the bloodstream. Canine parvovirus type 2 frequently predisposes affected animals to secondary bacterial infections, endotoxemia, and septicemia, which are major contributors to disease severity and mortality (Maganga et al., 2023).

Infected dogs begin shedding large quantities of virus in their feces approximately 4-7 days after exposure, often before the onset of clinical signs (Maganga et al., 2023). The extensive viral shedding, combined with the remarkable environmental stability of CPV-2, contributes significantly to the rapid dissemination of the virus within susceptible populations and communal environments (Magouz et al., 2023).

CLINICAL SIGNS

The incubation period of CPV infection typically ranges from 4 to 7 days (Purwitasari et al., 2022). Early clinical manifestations include lethargy, depression, anorexia, and fever, which are subsequently followed by vomiting, hemorrhagic diarrhea, dehydration, and progressive weakness (Gerlach et al., 2020; Suvethika and Kumar, 2021). Canine parvovirus infection results in loss of fluids, electrolytes, and plasma proteins through the gastrointestinal tract, leading to severe dehydration and, in advanced cases, hypovolemic shock (Purwitasari et al., 2022). The small intestinal epithelium serves as the primary site of viral replication, where infection of crypt epithelial stem cells disrupts normal epithelial renewal (Purwitasari et al., 2022). Consequently, villous atrophy, crypt necrosis, mucosal erosion, and intestinal hemorrhage develop throughout the duodenum, jejunum, and ileum, resulting in malabsorption and severe enteritis (Parrish and Sykes, 2021).

Clinically, CPV-2 infection is associated with two major disease manifestations, including enteritis and myocarditis (Sherasiya et al., 2024). The enteric form is by far the most common presentation and primarily affects puppies older than six weeks of age (Sherasiya et al., 2024). Clinical signs include acute hemorrhagic diarrhea, vomiting, pyrexia, anorexia, depression, abdominal discomfort, and rapid dehydration, which may progress to shock and death if left untreated (Zhou et al., 2024). The myocardial form of CPV-2 infection is currently less common and is generally observed in puppies infected during the neonatal period, particularly those born to non-immune dams lacking protective maternally derived antibodies (Dines et al., 2023). The viral replication within actively dividing cardiomyocytes leads to myocarditis characterized by myocardial inflammation, degeneration, and necrosis (Zhou et al., 2024). Clinically, affected puppies may present with acute heart failure, respiratory distress, arrhythmias, or sudden death (Gjurovski et al., 2023; Sherasiya et al., 2024).

From a pathological perspective, enteric CPV infection is characterized by segmental hemorrhagic enteritis, intestinal mucosal necrosis, crypt collapse, villous atrophy, and depletion of lymphoid tissues (Liu et al., 2025). Gross lesions may include gastric erosions, intestinal petechial or ecchymosis accumulation of serous to hemorrhagic intestinal contents, and enlargement of mesenteric lymph nodes (Zhou et al., 2024). In severe cases, extensive ulceration of the intestinal mucosa and marked gallbladder distension may also be observed (Ain-Fatin et al., 2020).

Recent studies investigating acute canine diarrhea have reported significant associations between CPV and canine coronavirus (CCoV) co-infections, although prevalence rates vary according to age group and geographic region (Li et al., 2025; Liu et al., 2025). Because the clinical manifestations of CPV are not pathognomonic, differential diagnoses

should include canine distemper, canine enteric coronavirus infection, hemorrhagic gastroenteritis of other etiologies, hookworm infection, coccidiosis, and other causes of acute enteritis (Chethan et al., 2023; Dines et al., 2023). In addition, intestinal parasitic infections caused by *Toxocara canis* and *Ancylostoma caninum* should be considered among the principal presumptive diagnoses, as these pathogens may produce clinical signs similar to CPV infection, including hemorrhagic diarrhea, vomiting, lethargy, and pale mucous membranes (Ain-Fatin et al., 2020).

DIAGNOSIS

Because the clinical manifestations of CPV infection may overlap with those of other causes of acute hemorrhagic gastroenteritis, early and accurate diagnosis is essential to facilitate appropriate case management and prevent viral dissemination, particularly in veterinary hospitals, shelters, breeding facilities, and other environments where multiple animals are housed together (Jantafong et al., 2022). Importantly, infected dogs may begin shedding virus in their feces before the onset of gastrointestinal signs, thereby increasing the risk of environmental contamination and transmission (Decaro et al., 2020).

Samples commonly used for CPV diagnosis include feces, rectal swabs, and whole blood (Tuteja et al., 2022). Confirmatory diagnosis can be achieved through different laboratory methods, including antigen-detection ELISA, PCR, qPCR, hemagglutination assays, electron microscopy, and virus isolation in cell culture (Decaro et al., 2020; Nandi et al., 2022). Among these approaches, PCR-based methods are considered the most sensitive and specific diagnostic tools currently available (Decaro et al., 2020).

In routine veterinary practice, rapid immunochromatographic tests and commercial ELISA kits are widely used because of their ease of application, rapid turnaround time, and high specificity, which frequently exceeds 90% (Decaro et al., 2020). However, their sensitivity is considerably more variable and has been reported to range from approximately 16% to 80% in ELISA tests when compared with molecular assays such as PCR and qPCR, which have high sensitivity and specificity, particularly during early infection or in samples containing low viral loads (Segev et al., 2022; Hyeon et al., 2023; Table 3). In addition, the rapid antigen test has 96.4% sensitivity and 88.2% specificity compared with qPCR (Abousenna et al., 2024).

Table 3. Comparison of immunological and molecular tests used in the diagnosis of canine parvovirus

Diagnostic method	Target detected	Reported diagnostic performance	Main limitations	Clinical use context	Reference
Rapid antigen test (lateral flow)	CPV viral antigen in feces	Sensitivity 96.4% and specificity 88.2% compared with qPCR and viral shedding stage.	May not accurately reflect sensitivity in clinical fecal samples; experimental kit	Rapid screening; point-of-care; useful as an initial test	Abousenna et al. (2024)
ELISA (antigen detection)	CPV viral antigen	Sensitivity ranges from 16% to 80% depending on the commercial assay and reference standard.	Lower sensitivity than molecular methods	Complementary diagnostic support; epidemiological studies	Li et al. (2025)
Conventional PCR	CPV viral DNA	High sensitivity and specificity reported across different laboratory protocols.	Does not quantify viral load; requires laboratory infrastructure	Confirmatory diagnosis when qPCR is not available	Segev et al. (2022); Li et al. (2025)
qPCR	CPV viral DNA (quantifiable)	Very high sensitivity and specificity; reference molecular method	Requires specialized equipment and trained personnel	Confirmatory diagnosis and viral load quantification	Loor-Giler et al. (2024); Li et al. (2025)
ddPCR	Viral DNA (absolute quantification)	Higher analytical sensitivity and greater precision (shown in related viruses such as CDV)	No specific clinical validation metrics for CPV; mainly used in the literature.	Advanced research; potential for detection of low viral loads	Iribarnegaray et al. (2024)

ELISA: Enzyme-linked immunosorbent assay; qPCR: Real-time qPCR; ddPCR: Droplet digital PCR. CDV: Canine distemper virus; Diagnostic performance values shown in this table correspond to representative studies or commercial assays and should not be interpreted as universal performance estimates for each diagnostic method. Test performance varies according to the assay, sample type, stage of infection, and reference standard used.

Interpretation of diagnostic results should take vaccination history into account. Modified-live virus vaccines may result in transient positive antigen or molecular test results for up to approximately 28 days following vaccination, potentially complicating diagnostic interpretation (Decaro et al., 2020). Conversely, false-negative results may occur when viral concentrations in feces are below the detection threshold of the assay or when virus particles are neutralized by intestinal antibodies, reducing antigen availability for detection (Li et al., 2025). Consequently, when animals with clinical signs suggestive of CPV infection yield negative results on rapid antigen tests, complementary molecular testing is strongly recommended to confirm or exclude infection and to minimize the risk of viral spread (Decaro et al., 2020; Nandi et al., 2022).

Among molecular techniques, qPCR is currently regarded as the diagnostic reference standard because of its high sensitivity, specificity, and ability to detect low viral loads while simultaneously providing quantitative information on viral burden (Segev et al., 2022; Li et al., 2025). In addition, qPCR is particularly valuable for confirming cases with negative antigen test results and for epidemiological surveillance of circulating strains (Table 3). Droplet digital PCR (ddPCR) has recently emerged as a promising molecular platform capable of absolute nucleic acid quantification and enhanced analytical sensitivity compared with conventional qPCR (Iribarnegaray et al., 2024). Nevertheless, despite its potential advantages, the clinical application of ddPCR for routine CPV diagnosis remains limited and has not yet been fully validated for widespread veterinary use; it is currently used only for canine distemper virus (Iribarnegaray et al., 2024). Overall, molecular diagnostic techniques enable the detection of CPV during the early stages of infection and play a critical role in disease surveillance, outbreak control, and the implementation of effective preventive measures (Table 3).

In addition to laboratory diagnosis, the identification and molecular characterization of circulating CPV-2 genotypes constitute essential epidemiological and immunological tools (Li et al., 2025). Genotyping, particularly through the analysis of the VP2 gene, provides valuable information regarding viral evolution, transmission dynamics, geographic distribution, and the coexistence of distinct antigenic variants (Li et al., 2025). Furthermore, molecular surveillance enables the detection of emerging mutations that may influence host range, viral fitness, antigenicity, and potentially vaccine performance (Muñoz et al., 2021). Recent studies conducted in Ecuador have demonstrated the concurrent circulation of multiple CPV-2 variants, as well as the occurrence of mixed-genotype infections, highlighting the importance of continuous molecular monitoring to improve disease surveillance and support evidence-based prevention and control strategies (De la Torre et al., 2018; Loor-Giler et al., 2024).

TREATMENT

The CPV infection is a globally distributed disease for which treatment remains primarily supportive and aimed at correcting the physiological disturbances associated with infection (Horecka et al., 2020). Persistent vomiting and acute hemorrhagic gastroenteritis are among the most characteristic clinical manifestations of the disease (Muñoz et al., 2021). Viral destruction of intestinal crypt epithelial cells compromises the integrity of the gastrointestinal barrier, facilitating bacterial translocation and predisposing affected animals to sepsis, endotoxemia, and systemic inflammatory responses (Gerlach et al., 2020; Dos Santos et al., 2023).

Consequently, intensive supportive care remains the cornerstone of CPV treatment (Muñoz et al., 2021). Fluid therapy is considered the most critical component of patient management, with intravenous crystalloid solutions, such as Lactated Ringer's solution, commonly administered to correct dehydration, electrolyte imbalances, and hypovolemia (Horecka et al., 2020). Potassium supplementation should be adjusted according to individual patient requirements and laboratory findings (Schmitz, 2021). In cases of severe hypoproteinemia, colloid support, plasma transfusions, or other blood products may be considered to restore oncotic pressure and improve circulatory stability (Gerlach et al., 2020).

Additional therapeutic measures include the administration of antiemetics such as maropitant-cerenia (1 mg/kg subcutaneously every 24 hours), metoclopramide-primperan (0.2-0.5 mg/kg intravenous route every 6-8 hours) to control vomiting and facilitate nutritional support, as well as broad-spectrum antimicrobial therapy to reduce the risk of secondary bacterial infections resulting from intestinal barrier disruption and neutropenia (Mazzaferro, 2020). Once emesis is adequately controlled, early enteral nutrition is generally recommended by the American Animal Hospital Association (AAHA), as it promotes intestinal mucosal recovery, maintains gastrointestinal integrity, and may improve clinical outcomes (Mazzaferro, 2020; Ozaeta et al., 2025).

Different adjunctive therapies have also been investigated, including immunomodulators, probiotics, antioxidants, hyperimmune plasma, and other supportive interventions (Chethan et al., 2023; Ozaeta et al., 2025). However, the clinical evidence supporting many therapies, such as probiotics (Schmitz, 2021) and antioxidants, is limited, and their use should be considered complementary to standard supportive treatment rather than a replacement for established therapeutic protocols (Muñoz et al., 2021; Chethan et al., 2023). Despite significant advances in supportive care, no universally accepted antiviral treatment is currently available for routine clinical management of CPV infection (Decaro

et al., 2020). Therefore, early diagnosis, aggressive supportive therapy, and appropriate monitoring remain the most effective strategies for reducing morbidity and mortality associated with this disease (Decaro et al., 2020).

Although no breed has been consistently identified as having the highest treatment failure rate in canine parvoviral enteritis, Rottweilers, Doberman Pinschers, German Shepherd Dogs, and American Pit Bull Terriers are recognized as breeds with increased susceptibility to severe disease (Decaro et al., 2020). Previous studies indicated that factors such as low body weight, disease severity at admission, and inadequate immune status are stronger predictors of mortality than breed alone (Decaro et al., 2020).

At present, the management of CPV infection relies primarily on supportive care aimed at correcting dehydration, electrolyte imbalances, and secondary bacterial complications (Mazzaferro, 2020). However, in animals presenting with high viral loads or severe systemic disease, supportive therapy alone may be insufficient to effectively control viral replication (Decaro et al., 2020). Consequently, recent studies have focused on the development of targeted antiviral strategies designed to improve clinical outcomes and reduce mortality associated with CPV infection (Decaro et al., 2020).

Current antiviral studies can be broadly categorized into three major areas. The first involves interferon-based therapies, particularly interferons (IFN I), which have demonstrated immunomodulatory and antiviral effects, including reductions in viral shedding and improvements in survival rates in some experimental and clinical studies (Reyes-Cruz et al., 2021; Kim et al., 2022). The second area focuses on antibody-based therapies, including hyperimmune plasma and monoclonal antibodies, which aim to neutralize circulating virus particles and limit viral replication during the acute phase of infection (Lopes et al., 2024). Recent investigations have reported that the administration of a human immunomodulator (0.5 mg/day × 5 days) to puppies under six months of age promises to reduce disease severity and enhance recovery in affected animals (Muñoz et al., 2021).

The third area encompasses small-molecule antiviral compounds targeting different stages of the viral replication cycle (Decaro et al., 2020). Different compounds, such as mRNA alternatives, have demonstrated antiviral activity against CPV *in vitro* by interfering with viral replication, nucleic acid synthesis, or virus-host interactions (Uhl and Fasina, 2025). Nevertheless, most small-molecule mRNA remains at the experimental stage, and additional studies are required to establish their safety, efficacy, pharmacokinetics, and clinical applicability in naturally infected dogs (Ulas et al., 2024).

Collectively, the antiviral strategies highlight the progress achieved in the search for targeted therapies against CPV. However, the magnitude of their reported benefits varies considerably among studies. While recombinant feline IFN I has demonstrated improvements in survival and clinical recovery in experimentally infected dogs (Reyes-Cruz et al., 2021), other antiviral candidates, including metoclopramide-primperan and maropitant-cerenia (Mazzaferro, 2020), and RNA interference-based approaches (Uhl and Fascina, 2025), have mainly shown reductions in viral replication under laboratory conditions, with limited evidence from clinical patients. This discrepancy suggests that promising *in vitro* antiviral activity does not necessarily translate into equivalent therapeutic efficacy *in vivo*. Consequently, despite the growing body of evidence supporting these novel interventions, robust clinical trials involving larger populations are still required to determine their therapeutic value, safety profile, and feasibility in routine veterinary medicine (Lopes et al., 2024).

PREVENTION AND CONTROL

The prevention and control of CPV rely primarily on systematic and timely vaccination, which remains the most effective strategy for reducing infection rates, disease severity, and outbreak occurrence (Ford et al., 2022). Modified-live virus (MLV) vaccines are generally considered the vaccines of choice because they induce a rapid, robust, and long-lasting immune response (Ford et al., 2022). In contrast, inactivated vaccines, although safe, typically exhibit lower immunogenicity and require multiple doses to achieve comparable protection; therefore, their use is generally restricted to specific situations or the evaluation of novel vaccine formulations (Squires et al., 2024).

Puppies should receive their first vaccination between 6 and 8 weeks of age, followed by booster doses administered every 3-4 weeks until at least 16-20 weeks of age to ensure adequate protection during the period of declining MDA (Table 4). The vaccination schedule is designed to minimize the impact of the so-called window of susceptibility, a critical period during which maternal antibodies may be insufficient to prevent infection while still interfering with active immunization (AAHA, 2022). Following completion of the primary vaccination series, a booster is generally recommended at one year of age and subsequently at intervals of up to three years, in accordance with current international vaccination guidelines (Squires et al., 2024).

The use of vaccines capable of providing broad protection against circulating CPV variants is essential for maintaining population immunity (Pearce et al., 2023). Although many commercial vaccines were originally developed using CPV-2 or CPV-2b strains, substantial evidence indicates that vaccines confer effective cross-protection against the

currently circulating CPV-2a, CPV-2b, and CPV-2c variants (Al-Saadi et al., 2025). Furthermore, novel vaccine approaches are being investigated to improve immunogenicity in the presence of high levels of maternal antibodies, potentially enabling effective immunization at earlier ages (Zhou et al., 2024). Nevertheless, sporadic cases of CPV continue to occur despite vaccine availability, often as a consequence of incomplete vaccination schedules, persistent maternal antibody interference, improper vaccine handling or storage, and exposure to high environmental viral loads in endemic settings (Ford et al., 2022).

Table 4. Canine parvovirus vaccines currently available in Ecuador, including vaccine type, vaccination schedule according to age, and recommended immunization protocols

Animal age	Commercial vaccine	Vaccine type	Protocol	Maternal antibody interference	Biosafety and contraindications
Puppies (4-6 weeks)	Nobivac Canine-1Pv	Modified live virus	Start > 6 weeks; 1 mL SC or IM; boosters every 2-4 weeks	Interference may occur due to maternal antibodies	Vaccinate only healthy animals; do not vaccinate pregnant females; store at 2-8 ˚C; do not freeze
	Nobivac Puppy DP	Modified live virus	4-6 weeks; 1 mL SC	No significant interference reported	Store at 2-8˚C; protect from light; avoid heat exposure
	Vibix C Puppy Advanced	Inactivated virus	≥ 6 weeks; 1 mL SC	No maternal antibody interference	Deworm at least 10 days before vaccination; store at 2-8 ˚C; protect from light
	Nobivac Parvo-C	Live attenuated virus	2-4 weeks; 1 mL SC; revaccinate every 3-4 weeks until 3 months	Interference may occur; recommended vaccination at 6-9 weeks	Store at 2-8˚C; protect from light
Juvenile and adult dogs (> 6 weeks)	Vanguard Plus	Modified live virus	Start at 6 weeks; boosters at 9 and 12 weeks; ≥ 12 weeks: repeat dose after 3 weeks	No significant interference reported	Avoid vaccination in parasitized, weakened, or pregnant animals; store at 2-7 ˚C; do not freeze
	Nobivac DHPPi	Modified live virus	6-9 weeks; 1 mL SC	Possible interference; final dose recommended at 12 weeks	Store at 2-8˚C; protect from light
	Parvigen	Live attenuated virus	Two doses: 8-12 weeks and 3-4 weeks later (not before 12 weeks); annual revaccination	Maternal antibodies may reduce the response	Store at 2-8˚C; do not freeze; protect from light
	Nobivac Parvo-C	Live attenuated virus	6-9 weeks; 1 mL SC	No significant interference reported	Store at 2-8˚C; protect from light
	Vanguard Plus (Zoetis)	Modified live virus	Start at 6 weeks; boosters at 9 and 12 weeks; ≥ 12 weeks: repeat dose after 3 weeks; 1 mL SC	No significant interference reported	Avoid vaccination in parasitized, weakened, or pregnant animals; store at 2-7 ˚C; do not freeze

SC: Subcutaneous; IM: Intramuscular; DP: Distemper-parvovirus

In Ecuador, where multiple CPV variants circulate and CPV-2b has been identified as the predominant genotype in recent studies, continuous monitoring of circulating strains is important to support vaccination programs and epidemiological surveillance (Loor-Giler et al., 2025).

Environmental biosecurity represents another essential component of disease control. Canine parvovirus is exceptionally resistant to environmental degradation and may remain infectious in contaminated environments for extended periods, often exceeding one year under favorable conditions (Mia and Hasan, 2021). Consequently, contaminated areas should undergo thorough cleaning followed by disinfection using sodium hypochlorite solutions at a concentration of 0.37% for 15 minutes or other disinfectants such as iodine, glutaraldehyde with proven efficacy against non-enveloped viruses (Zhang, 2026). Immediate removal of fecal material, isolation of infected animals, and strict control of personnel, equipment, and clothing movement between contaminated and clean areas are critical measures for reducing indirect viral transmission (Decaro et al., 2020; Rivera and Ayora, 2024). Infected dogs should be isolated to minimize viral transmission. Although fecal viral shedding decreases substantially after clinical recovery, dogs may continue shedding CPV for up to 2-4 weeks following infection (Ozaeta et al., 2025). Consequently, isolation for at least

14 days after the resolution of clinical signs is recommended, particularly in multi-dog environments where the risk of transmission is high (Decaro et al., 2020).

Finally, owner and veterinary staff education plays a fundamental role in the prevention of canine parvoviral disease (Horecka et al., 2020). Avoiding the exposure of unvaccinated puppies to public environments, strengthening vaccination programs in shelters and breeding facilities, and maintaining accurate health records contribute substantially to reducing disease incidence (WOAH, 2025). In addition, molecular surveillance of circulating strains, combined with timely reporting of cases, facilitates the early detection of epidemiologically relevant viral changes and supports evidence-based adjustments to local prevention and immunization strategies (Loor-Giler et al., 2025).

Collectively, vaccination, environmental disinfection, isolation of infected animals, epidemiological surveillance, and educational initiatives constitute the most effective integrated approach for the prevention and control of canine parvoviral disease in both urban and rural populations (Ozaeta et al., 2025). More recently, subunit vaccines based on the VP2 protein have shown protective efficacy against heterologous CPV2 strains, suggesting that updated vaccine formulations may enhance protection against emerging viral variants (Jantafong et al., 2022). A summary of the vaccines currently available in Ecuador is presented in Table 4.

CONCLUSION

Canine parvovirus remains a major infectious disease of dogs worldwide due to its high transmissibility, environmental persistence, and ongoing genetic evolution. The emergence and global distribution of CPV variants have important implications for epidemiological surveillance and disease control. In Ecuador, there are three variants (CPV-2a, CPV-2b, and CPV-2c), and CPV-2b was most common in 2022-2023 in different provinces. Furthermore, vaccination remains the most effective preventive strategy, with MLV vaccines providing cross-protection against circulating variants; supportive care (fluid therapy, antiemetics, antimicrobials) is the mainstay of treatment; no antiviral agent is yet approved for routine use.

The marked tropism of CPV for rapidly dividing cells explains the severity of gastrointestinal and hematological manifestations, which can rapidly progress to fatal outcomes if not diagnosed and managed in a timely manner. In this context, advances in diagnostic technologies, particularly molecular techniques such as PCR and qPCR, have significantly improved the accuracy and speed of detection, enabled earlier intervention and improved clinical outcomes.

From a preventive perspective, vaccination remains the most effective strategy for controlling CPV infection. Nevertheless, factors such as maternal antibody interference, incomplete vaccination schedules, and the circulation of multiple viral variants continue to challenge vaccine effectiveness in certain contexts. The emergence and co-circulation of CPV-2 variants highlight the need for continuous molecular surveillance and the periodic evaluation of vaccine formulations to ensure sustained protection.

Future studies should focus on characterizing CPV, the evolution of circulating variants, and identifying mutation hotspots, particularly in the VP2 gene, which are critical for antigenic variation. Such studies are essential to evaluate the potential impact of emerging strains on vaccine efficacy and to guide the development of updated, strain-matched vaccines. Additionally, expanded epidemiological studies in regions such as Ecuador are needed to improve understanding of local viral dynamics and to support the implementation of more effective national prevention and control strategies.

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Authors' contributions

Pamela Rodríguez Huera was responsible for conceptualization, investigation, writing of the original draft, visualization, and project administration. David Martinez Martinez contributed to data curation, validation, supervision, and writing, review, and editing. All authors have read and approved the final edition of the manuscript before publication in the present journal.

Availability of data and materials

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

Competing interests

The authors have declared no conflict of interest.

Ethical considerations

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Antimicrobial Resistance in Catfish Aquaculture in Indonesia: Antibiotic Use, Resistance Genes, and One Health Risks

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ABSTRACT

Catfish (*Clarias* spp.) aquaculture plays a crucial role in food security and rural livelihoods in Indonesia. Inappropriate antimicrobial use (AMU) can increase antimicrobial resistance (AMR), posing risks to aquaculture, environmental health, and food safety. The present study aimed to review AMR associated with catfish aquaculture in Indonesia within a One Health framework. A review was carried out according to structured literature review guidelines. Relevant peer-reviewed articles published between 2020 and 2026 were found in Scopus, Web of Science, PubMed, ScienceDirect, and Google Scholar. The searches employed specific combinations of keywords concerning catfish aquaculture, antimicrobial usage, antimicrobial resistance, and Indonesia. Data on AMU, resistant bacteria, antimicrobial-resistance genes (ARGs), and antibiotic residues were extracted from the included studies and categorized into these four subjects descriptively. Findings across studies were compared to identify commonly reported antimicrobials, patterns of bacterial resistance, detected ARGs, and the occurrence of antibiotic residues. Due to differences in study designs, sampling methods, bacterial species, and antimicrobial susceptibility testing methods, the findings were synthesized narratively rather than pooled statistically. The current findings indicated that oxytetracycline, tetracycline, and enrofloxacin were the most commonly used antibiotics. Resistant bacteria, particularly *Aeromonas hydrophila*, frequently exhibited resistance to tetracycline, oxytetracycline, ampicillin, and amoxicillin. Other pathogens, including *Edwardsiella tarda* and *Proteus* spp., also demonstrated multidrug resistance to tetracycline, oxytetracycline, ampicillin, and amoxicillin. The ARGs such as *tetA*, *tetE*, *qnrA*, *qnrB*, *qnrS*, *ampC*, *blaGES*, and *mexF* were detected in bacterial isolates and aquaculture environments. Antibiotic residues, including fluoroquinolones, sulphonamides, chloramphenicol, and tetracycline, were detected in aquatic environments and catfish products. Oxytetracycline residues ranging from 0.14 to 0.90 mg/kg were detected in catfish meat, with 25% of 59 samples exceeding the reported maximum residue limits. The current findings suggested that catfish aquaculture in Indonesia may contribute to the emergence and spread of AMR. The co-occurrence of antibiotic use, resistant bacteria, ARGs, and environmental contamination highlighted the need for strengthened antimicrobial stewardship, surveillance, and integrated disease management under a One Health approach.

Keywords: Antimicrobial resistance, Catfish aquaculture, Gene, One Health

INTRODUCTION

Aquaculture is an important source of animal protein and livelihoods, particularly in low- and middle-income countries (Koroma et al., 2025). In Indonesia, freshwater aquaculture plays a vital role in providing affordable nutrition, with catfish (*Clarias* spp.) as a key commodity because of its fast growth, adaptability to various environments, and strong market demand (Segaran et al., 2023). National production has remained consistently high in recent years, reflecting the economic and nutritional importance of catfish farming in both rural and urban communities (Susilowati et al., 2024).

High stocking densities, limited water exchange, and variable environmental conditions are common in intensive catfish aquaculture and collectively heighten the risk of infectious disease outbreaks. Bacterial pathogens such as *Aeromonas* spp., *Edwardsiella* spp., *Proteus* spp., and *Vibrio* spp. are commonly linked to diseases in catfish production (Ma et al., 2025; Dompreh et al., 2026). High stocking density and poor or unstable water quality can adversely affect fish health and production, leading farmers to use antibiotics to control disease (Wise et al., 2021). Appropriate antimicrobial treatments can be effective against bacterial infections; however, frequent or uncontrolled antimicrobial use (AMU) can contribute to antimicrobial resistance (AMR; Shahi et al., 2025).

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Antimicrobial resistance is a growing global health issue, and AMU in livestock contributes to the emergence of resistant microorganisms. In aquaculture, antibiotics are used primarily to treat bacterial infections, although inappropriate use may occur when treatment is administered without proper diagnosis or veterinary decision (Ye et al., 2025). The mentioned practices may exert selective pressure that promotes the survival and proliferation of resistant organisms (Tiseo et al., 2020; Shahi et al., 2025). In Indonesia, growing concerns about antimicrobial use in aquaculture have prompted regulatory requirements for veterinary oversight of antibiotic administration, complemented by recent efforts to monitor AMU and mitigate AMR (Ministry of Marine Affairs and Fisheries, 2024). Aquaculture environments provide unique ecological conditions that facilitate AMR persistence and distribution (Okeke et al., 2022). Residual antibiotics, resistant bacteria, and antimicrobial resistance genes (ARGs) can accumulate in water, sediment, and cultured organisms, enabling interactions between environmental and pathogenic microbial communities (Yuan et al., 2023; Ariyani et al., 2024). Antibiotic residues, resistant bacteria, and antimicrobial resistance genes (ARGs) may subsequently transfer across ecological compartments via water discharge, aquaculture products, and human exposure, supporting the relevance of a One Health approach that integrates animal, environmental, and public health perspectives (Al-Khalaifah et al., 2025).

Despite growing global attention to AMR in aquaculture, evidence from Indonesian catfish production remains limited and dispersed across studies. Available studies have explored different facets of AMR, such as AMU, phenotypic resistance in bacterial isolates, ARGs, and antimicrobial residues, but these results were rarely considered together (Mohammed et al., 2025; Kawsar et al., 2026). Therefore, the present review aimed to synthesize available evidence on AMR associated with catfish aquaculture in Indonesia, focusing on AMU, resistant bacteria, ARGs, and antimicrobial residues, and to interpret the findings from a One Health perspective in order to identify links among aquaculture practices, aquatic environments, food products, and potential public health concerns, as well as to highlight priorities for surveillance and antimicrobial stewardship.

SEARCH STRATEGY

A structured literature search was conducted to identify studies addressing AMR and AMU in catfish aquaculture, with particular emphasis on evidence from Indonesia and relevant international studies for contextual comparison. Literature searches were performed using Scopus, Web of Science, PubMed, and ScienceDirect, with Google Scholar and Google searches serving as supplementary sources to expand the literature retrieval. The search strategy combined terms representing the target species, production system, antimicrobial exposure, resistance, and geographical context, including catfish (catfish or *Clarias*), aquaculture, antimicrobial exposure (antibiotic or antimicrobial), resistance (resistance or antimicrobial resistance or AMR), and Indonesia. A total of 81 records were retrieved from Scopus, 113 from Web of Science, 59 from PubMed, 120 from ScienceDirect, and 195 through supplementary Google Scholar/Google searching. All retrieved references were collected, organized, and managed using Mendeley version 1.19.8, resulting in a reference library containing 217 records. The Mendeley count was not considered additional yield because it represented records imported from different databases and supplementary search sources. The collected records were reviewed for relevance to catfish aquaculture and AMR-related outcomes. Studies were considered relevant when they provided extractable information on AMU, phenotypic antimicrobial resistance, antimicrobial-resistant bacteria, ARGs, antimicrobial residues, or other AMR-related outcomes in catfish, catfish products, or aquaculture-associated environments. Indonesian studies formed the main evidence base, while international studies on catfish or relevant freshwater aquaculture systems were kept separate for contextual comparison. After selecting the literature and completing full-text assessments, 34 studies were included in the final analysis, 13 from Indonesia and 21 from international or relevant contexts. Complete numerical records of duplicate removal and the initial title-and-abstract assessment were not retained during the original literature-selection process; therefore, only verifiable numbers were reported, and no unrecorded selection data were retrospectively estimated.

Data extraction

Relevant information was extracted from the selected literature, including author, year, country or Indonesian location, production or environmental system, AMU, antimicrobial residues, bacterial species, phenotypic resistance results, and ARGs. Outcomes not reported in an article were recorded as not reported rather than interpreted as negative findings. Indonesian evidence and international contextual evidence were synthesized separately to prevent the direct extrapolation of international findings to the Indonesian context. Because the included studies differed substantially in study design, bacterial species, sampling strategies, antimicrobial panels, laboratory methods, and reported outcomes, the evidence was synthesized using a narrative and descriptive approach rather than quantitative meta-analysis.

CURRENT EVIDENCE ON ANTIMICROBIAL RESISTANCE IN CATFISH AQUACULTURE

Bacterial pathogens

Bacterial species reported in Indonesian catfish evidence included *Aeromonas hydrophila* (*A. hydrophila*) and other *Aeromonas* spp., *Edwardsiella tarda*, *Proteus mirabilis*, *Proteus vulgaris*, *Escherichia coli*, *Salmonella* spp., and *Vibrio cholerae* (Table 1). *Aeromonas* spp. have been reported in several regions of Indonesia, including Jambi, East Java, and other local production environments (Mulia et al., 2021; Dewi et al., 2023; Mawardi et al., 2023; Wulandari et al., 2024). Because the included studies did not consistently report mortality estimates, no general quantitative statement on mortality was performed.

Antibiotic usage

Antimicrobial exposure reported in Indonesian catfish studies included oxytetracycline, tetracycline, enrofloxacin, sulfamethoxazole, ciprofloxacin, furazolidone, ampicillin, erythromycin, kanamycin, and chloramphenicol (Table 1). Oxytetracycline was reported in multiple catfish studies (Mulia et al., 2021; Kurniawan, 2023; Mawardi et al., 2023; Putri and Triyanto, 2023), whereas enrofloxacin has been frequently reported (Mulia et al., 2021; Dewi et al., 2023; Kurniawan, 2023; Mawardi et al., 2023; Putri and Triyanto, 2023). The extracted data indicated which antimicrobials were reported but did not establish farmers' motivations for selecting particular medicines.

Occurrence of antimicrobial resistance bacteria

Phenotypic resistance differed according to bacterial species and study location (Table 1). In the Jambi region, Indonesia, *A. hydrophila* isolates exhibited resistance to tetracycline (100%), oxytetracycline (94.42%), ampicillin (77.70%), amoxicillin (77.72%), and ciprofloxacin (16.68%), while no resistance to enrofloxacin or streptomycin was reported (Wulandari et al., 2024). Mawardi et al. (2023) reported *A. hydrophila* resistance to oxytetracycline (54.54%), tetracycline (15.06%), and enrofloxacin (16%). Mulia et al. (2021) reported *Aeromonas* spp. resistance to ampicillin (80%), erythromycin (70%), oxytetracycline (40%), chloramphenicol (30%), and kanamycin (10%), with 0% resistance to enrofloxacin. *Edwardsiella tarda* isolates were resistant to erythromycin (88.89%), enrofloxacin (88.89%), and oxytetracycline (66.67%; Putri and Triyanto, 2023). *Proteus mirabilis* and *P. vulgaris* demonstrated significant resistance to multiple Beta-lactam antibiotics and erythromycin; however, the reported Indonesian isolates exhibited no resistance to ciprofloxacin (Wibawati et al., 2021).

Detection of antimicrobial resistance genes

The Indonesian evidence reported ARGs such as *tetA* and *tetE* (conferring tetracycline resistance), *qnrA*, *qnrB*, and *qnrS* (linked to quinolone resistance), *ampC* and *blaGES* (Beta-lactam resistance), *sul1* (sulphonamide resistance), *mexF* (related to multidrug efflux resistance), and *strA-strB* (streptomycin resistance; Table 1). Wulandari et al. (2024) detected *ampC* in 57.14% of Beta-lactam-resistant *A. hydrophila* isolates, and among the quinolone-resistant isolates, *qnrA* and *qnrS* were detected in 75%, and *qnrB* in 25%. Ikhrami et al. (2024) identified *sul1* at 67%, *tetA* at 63%, *blaGES* at 55%, and *mexF* at 25% in an irrigation canal and aquaculture pond. Kurniawan (2023) identified *tetE* in *Aeromonas* spp., while Mulia et al. (2021) reported the presence of *tetA* and *strA-strB* in *Aeromonas* species.

Antibiotic residues in aquatic environments and catfish products

Antibiotic residues were detected in different aquaculture environments and catfish products (Table 1). Ariyani et al. (2024) detected multiple antimicrobial compounds including norfloxacin (31.0 ng/L), enrofloxacin (23.0 ng/L), ofloxacin (22.0 ng/L), sulfamethoxazole (11.6 ng/L), ciprofloxacin (11.55 ng/L), chloramphenicol (4.55 ng/L), and tetracycline (3.4 ng/L), in Cirata Reservoir water. Ulkhaq et al. (2024) found chloramphenicol residues under 0.3 ppb in 16.67-36.37% of catfish samples collected from three traditional markets in Banyuwangi, Indonesia, with all concentrations below the maximum residue limit specified in their study. Hakimah et al. (2021) detected oxytetracycline residues above the maximum residue limit in 25% of 59 catfish meat samples obtained from traditional markets in Yogyakarta, Indonesia, with concentrations ranging from 0.14 to 0.90 mg/kg. The mentioned findings indicated antimicrobial exposure in the tested catfish products but should not be extended to other locations.

Integrated evidence of antimicrobial resistance

Indonesian studies identified four related types of evidence, including reported AMU or exposure, phenotypically resistant bacteria, ARGs, and antimicrobial residues (Table 1). The individual studies did not consistently measure all four components within the same farms or samples. Consequently, the synthesis demonstrated co-occurrence across the Indonesian evidence, rather than establishing a direct causal link within individual production units.

Table 1. Antimicrobial use, antimicrobial residues, antimicrobial-resistant bacteria, and antimicrobial-resistance genes reported in Indonesian catfish aquaculture and related aquatic environments from 2020 to 2026

Location	Category/system	Antibiotics used	Antibiotic residues	Resistant bacteria and genes	Resistance profile	Reference
Cirata Reservoir, West Java, Indonesia	Reservoir environment associated with intensive aquaculture and other anthropogenic inputs	Not directly quantified; fluoroquinolone occurrence was considered potentially associated with aquaculture and other human activities.	24/65 residues detected in water and/or sediment, major classes included fluoroquinolones, tetracyclines, sulphonamides, diaminopyrimidines, lincosamides, sulphones, and amphenicols.	Potential antibiotic-resistant bacteria	Potential selection pressure for antibiotic-resistant bacteria, particularly from fluoroquinolones; not phenotypically measured	Ariyani et al. (2024)
	Reservoir-based aquaculture and river-reservoir catchment	Enrofloxacin was reported by 20% of surveyed aquaculture farmers for common carp and tilapia during the wet season.	14 antibiotics were modeled from human, livestock, and aquaculture sources; antibiotic residues were also evaluated against measured concentrations in river and reservoir water.	Not investigated	Not investigated	Ariyani et al. (2025)
Palembang, South Sumatra, Indonesia	Environment / Urban River environment (Kedukan, PU, and Buah Rivers)	Tobramycin Ampicillin Tetracycline Kanamycin Ciprofloxacin Cotrimoxazole Cefixime Gentamicin Chloramphenicol	Not investigated	*Escherichia coli*, *Coliform*	Tobramycin: 95% Ampicillin: 75% Tetracycline: 18% Kanamycin: 36% Ciprofloxacin: 18% Cotrimoxazole: 36% Cefixime: 36% Gentamicin: 75% Chloramphenicol: 55%	Sunarti et al. (2022)
Kulon Progo, Yogyakarta, Indonesia	Environment / irrigation canal and aquaculture ponds	None reported by the surveyed farmers.	Not investigated	Antibiotic Resistance Genes: *sul1*, *tetA*, and *blaGES* detected in aquaculture pond water; *mexF* detected in the irrigation system.	Not investigated	Ikhrami et al. (2024)
Jambi Province, Indonesia	Catfish aquaculture ponds	Not investigated	Not investigated	*Aeromonas hydrophila*; *ampC* (57.14%), *qnrA* (75%), *qnrS* (75%), and *qnrB* (25%) among corresponding resistant isolates	Tetracycline (100%), Oxytetracycline (94.42%), Ampicillin (77.70%), Amoxicillin (77.72%) Ciprofloxacin (16.68%) Enrofloxacin (0%) Streptomycin (0%)	Wulandari et al. (2024)
East Nusa Tenggara, Indonesia	Freshwater aquaculture; tilapia and catfish farms	Oxytetracycline (80-90%) Enrofloxacin (10%) Sulfamethoxazole (10%) Ciprofloxacin (5%) Furazolidone (5%)	Not investigated	*Aeromonas veronii* *Aeromonas hydrophila* *Aeromonas caviae; tetE* detected in 13/14 Oxytetracycline-resistant strains.	39% (16/40) of *Aeromonas* strains resistant to ≥1 tested antibiotic; Oxytetracycline (20-50%) Sulfamethoxazole (0-50%) Amoxicillin (100-50) Florfenicol (0-50%) Enrofloxacin (0%)	Kurniawan (2023)

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West and Central Java, Indonesia (Sukabumi, Bogor, Banyumas, Magelang)	Freshwater aquaculture farms, including *Clarias gariepinus*.	Tetracycline Oxytetracycline Enrofloxacin	Not investigated	*Aeromonas hydrophila*	Oxytetracycline (54.54%) Tetracycline (15.06%) Enrofloxacin (16%)	Mawardi et al. (2023)
Pasuruan, East Java, Indonesia	Farmed African catfish (*Clarias gariepinus*); 25 diseased fish from five sub-districts.	Enrofloxacin; reported as commonly used in local catfish farming, but farm-level use was not quantitatively assessed	Not investigated	*Aeromonas hydrophila* (23/25), *Aeromonas salmonicida* (2/25)	No Enrofloxacin resistance reported for *A. hydrophila*; inhibition zones 24-44 mm.	Dewi et al. (2023)
Yogyakarta, Indonesia	Aquaculture / catfish system.	Erythromycin Oxytetracycline Enrofloxacin	Not investigated	*Edwardsiella tarda*	Erythromycin (88.89%) Enrofloxacin (88.89%) Oxytetracycline (66.67%)	Putri and Triyanto (2023)
Banyuwangi, East Java, Indonesia	Aquaculture / catfish system.	Not directly assessed	Not investigated	*Proteus mirabilis* (3/74)	Penicillin (100%) Ampicillin (100%) Oxacillin (100%) Cefadroxil (100%) Erythromycin (66.7%) Cefixime (33.3%) Tetracycline (0%) Ciprofloxacin (0%) Streptomycin (0%) Chloramphenicol (0%)	Wibawati et al. (2021)
				Proteus vulgaris (8/74)	Oxacillin (100%) Penicillin (75%) Ampicillin (62.5%) Cefadroxil (62.5%) Erythromycin (62.5%) Streptomycin (50%) Cefixime (25%) Tetracycline (0%) Ciprofloxacin (0%) Chloramphenicol (0%)	
Java, Indonesia (Yogyakarta, Central Java, and West Java)	Aquaculture / Diseased farmed walking catfish.	Ampicillin Erythromycin Oxytetracycline Kanamycin Enrofloxacin Chloramphenicol	Not investigated	*Aeromonas* spp. (*A. dhakensis*, *A. veronii* bv. *veronii*, *A. caviae*, and *A. hydrophila*), Antibiotic Resistance Genes: *tetA* (30%), *strA* (30%), *qnrA* not detected.	Ampicillin (80%) Erythromycin (70%) Oxytetracycline (40%) Chloramphenicol (30%) Kanamycin (10%) Enrofloxacin (0%)	Mulia et al. (2021)
Banyuwangi, East Java, Indonesia	Product / Farmed African catfish (*Clarias gariepinus*) from traditional markets (n = 90).	Not directly assessed	Chloramphenicol detected in 36.37% of samples from Kertosari, 23.33% from Banyuwangi Kota, and 16.67% from Blambangan; all detected residues < 0.3 ppb	Not investigated	Not investigated	Ulkhaq et al. (2024)
Yogyakarta City, Indonesia	Product / Marketed catfish (*Clarias* sp.); 59 samples from 59 sellers in 16 traditional markets	Not directly assessed	Oxytetracycline residues detected above the maximum residue limit in 25% of 59 samples; 0.14-0.90 mg/kg	Not investigated	Not investigated	Hakimah et al. (2021)

Table 2. Antimicrobial use, antimicrobial residues, antimicrobial-resistant bacteria, and antimicrobial-resistance genes reported in catfish aquaculture and related freshwater aquaculture systems across different countries and regions, from 2020 to 2026

Location	Category / System	Antibiotics used	Antibiotic residues	Resistant bacteria	Antibiotic resistance genes and profile	Reference
Multiple countries/regions (Global evidence)	Aquaculture systems, including freshwater aquaculture and catfish-related evidence	Ampicillin Ceftriaxone Chloramphenicol Ciprofloxacin Co-trimoxazole Erythromycin Oxytetracycline Quinolone	Antibiotic residues reported across aquatic environments; heterogeneous across reviewed studies	*Proteobacteria* *Cyanobacteria* *Actinobacteriota* *Bacteroidota* *Verrucomicrobiota*	*tetA, tetB, tetC, tetG, tetM, sul1, qnrB, ermF, cat1 and intI1*	Mohammed et al. (2025)
				Plesiomonas shigelloides	*emrD, macB, catB, ksgA, tolC, MdfA, sul1, bacA and DegP*	
				Salmonella spp.	*stn, bcfC, avrA, sodC1, ssaQ*	
				Escherichia coli	β-lactam: 92%	
Global and Bangladesh	Aquaculture; comprehensive review	Azithromycin Chlortetracycline Amoxicillin Ciprofloxacin Oxytetracycline Sulfadiazine Erythromycin Trimethoprim	Residues of Fluoroquinolones, Sulfonamides, Nitrofurans, Chloramphenicol, and other antimicrobials reported in aquaculture products and environments	*Aeromonas* spp.	Ampicillin, Amoxicillin, Cephalexin, Cephalothin, Ceftiofur, Chloramphenicol, Florfenicol, Nalidixic Acid, Oxalinic Acid, Gentamicin, Kanamycin, Erythromycin, Tetracycline, Oxytetracycline, Trimethoprim, Sulfamethoxazole, Trimethoprim-Sulfamethoxazole, Ticarcillin, Cefotaxime, Nitrofurantoin, Ciprofloxacin, Norfloxacin, Doxycycline, and Streptomycin	Afroze et al. (2025)
				Vibrio spp.	Ampicillin, Amoxicillin, Carbenicillin, Cephalexin, Cephalothin, Cefotaxime, Cefpodoxime, Ceftiofur, Chloramphenicol, Florfenicol, Colistin, Nalidixic Acid, Oxalinic Acid, Gentamicin, Kanamycin, Erythromycin, Tetracycline, Oxytetracycline, Trimethoprim, Sulfamethoxazole, Trimethoprim-Sulfamethoxazole, Streptomycin, Rifampicin, Spectinomycin, and Vancomycin	
				Escherichia coli	Ciprofloxacin, Nalidixic Acid, Tetracycline, Sulfamethoxazole, Ampicillin, Trimethoprim, Chloramphenicol, Penicillin, Kanamycin, Gentamicin, Streptomycin, Trimethoprim-Sulfamethoxazole, and Ticarcillin	
				Salmonella spp.	Ampicillin, Erythromycin, Gentamicin, Nalidixic Acid, Nitrofurantoin, Penicillin, Streptomycin, Tetracycline, and Trimethoprim	
				Staphylococcus aureus	Streptomycin, Ampicillin, Penicillin, Erythromycin, Cephalothin, Rifampicin, Nitrofurantoin, Piperacillin, Ceftriaxone, Ceftazidime, Ciprofloxacin, Nalidixic Acid, Tetracycline, Sulfamethoxazole, Trimethoprim, Chloramphenicol, Kanamycin, and Gentamicin	
				Pseudomonas spp.	Ampicillin, Amoxicillin, Amoxicillin-Clavulanic Acid, Cephalothin, Ceftazidime, Cefotaxime, Ceftiofur, Chloramphenicol, Florfenicol, Nalidixic Acid, Oxalinic Acid, Ciprofloxacin, Norfloxacin, Tetracycline, Oxytetracycline, Doxycycline, Gentamicin, Kanamycin, Streptomycin, Erythromycin, Sulfamethoxazole, Trimethoprim, Trimethoprim-Sulfamethoxazole, Nitrofurantoin, and Ticarcillin	
Global	Catfish aquaculture; review	Penicillin Tetracyclines Macrolides Sulphonamides, Enrofloxacin, and other antimicrobials reported across catfish production systems	Not specifically quantified	*Aeromonas hydrophila* *Aeromonas* spp. *Vibrio* spp. Enterobacteriaceae *Escherichia coli* *Edwardsiella tarda;* Antibiotic Resistance Genes include β-lactam, Tetracycline, Aminoglycoside, and Folate-pathway antagonist resistance genes	Resistance to multiple antimicrobial classes reported across catfish systems, including Beta-lactams (Penicillin, Cephalosporins): High resistance (Extended-spectrum beta-lactamases-mediated) Tetracyclines: High resistance Sulphonamides: Moderate to high resistance Macrolides: Variable resistance Fluoroquinolones: Variable resistance (generally lower than beta-lactams) Carbapenems: Low resistance	Shahi et al. (2025)

Global	Aquaculture; systematic review and modelling of antimicrobial consumption, including catfish as a distinct species group	Catfish: estimated mean antimicrobial-use intensity 157 mg/kg biomass (95% UI: 9-2,751); catfish accounted for an estimated 8.3% of global aquaculture antimicrobial consumption in 2017. Across aquaculture, Quinolones, Tetracyclines, Amphenicols, and Sulfonamides were the most frequently reported classes	Not investigated	Not investigated	Not investigated	Schar et al. (2020)
Africa	African aquaculture; scoping review of 41 studies, predominantly pond-based systems; tilapia and catfish were the main species studied	Tetracyclines, Penicillin, Sulphonamides, Fluoroquinolones were the most frequently reported classes; use was predominantly therapeutic	Residues of Tetracyclines, Sulfonamides, and Cephalosporins were reported in fish, sediment, and feed; concentrations of 70-450 µg/kg were reported across studies, with some exceeding MRLs	*Escherichia coli*	43% multidrug resistance; ARGs: *tet*, *sul*, *bla*	Matee (2026)
				Salmonella spp.	40% multidrug resistance; ARGs: *tet*, *sul*, *bla*	
				Aeromonas hydrophila	63% multidrug resistance, 29% Extensively Drug-Resistant; antimicrobial-resistance genes: tet, sul,	
				Vibrio spp.	multiple antibiotic resistance index: 0.3-0.7 Antimicrobial-resistance genes: tet, bla	
USA (Alabama)	Pond-based channel catfish	Not investigated	Not investigated.	*Enterococcus* spp.	Ampicillin (~44.8%) rifampicin (~42.4%) Tetracycline (~38.4%); Multidrug resistance observed in 36% of isolates antimicrobial-resistance genes included *aac(6')-Iid* (65.7%), *aac(6')-Ii* (22.9%), *efmA* (20.0%), *msr(C)* (20.0%), *lsa(A)*, *emeA*, *efrA*, *efrB*, and *dfrE*; plasmid replicons also detected.	Soku et al. (2025)
Africa (Nigeria)	Farmed catfish	Antibiotic use reported as widespread/intensive; specific agents and frequencies were not quantified	Not investigated	*Aeromonas* spp. (*A. caviae*, *A. dhakensis*, *A. enteropelogenes*, *A. hydrophila*, and *A. veronii*)	Beta-lactams: High resistance Additional resistance to Oxytetracycline, Neomycin, Sulfamethoxazole, and Colistin; Multidrug resistance prevalence 36.09% and extensive drug resistance 60.43%; Multiple Antibiotic Resistance Index 0.20-0.80; Susceptibility retained to Ciprofloxacin, Gentamicin, and Florfenicol	Adah et al. (2024)
Egypt (Dakahlia Governorate)	Freshwater fish farmers: Catfish and Tilapia	Not directly assessed	Not investigated	*Edwardsiella tarda*	Multidrug resistance to ≥6 antibiotic classes: Penicillin, Aminoglycosides, Fluoroquinolones, Tetracyclines, Sulphonamides, and Lincosamides. Antibiotic Resistance Genes: *blaTEM*, *blaCTX-M*, *sul1*, *tetA*, *aadA1*, *qnrA*, *qnrS*	Algammal et al. (2022)
Asia	Cultured and wild-caught aquatic food animals; systematic review and meta-analysis of 749 point-prevalence surveys	Antimicrobial use generally unspecified; reported on-farm use was associated with higher multidrug resistance	Not investigated	*Aeromonas* spp.	Carbapenems: ~40.3% Cephalosporins: ~69.6%) Aztreonam: ~56.6% Colistin: ~51.5%	Schar et al. (2021)
				Vibrio spp.	Colistin: ~42.7%	
				Escherichia coli	Colistin: ~5.2%	
Cambodia	Pond aquaculture of striped catfish and giant snakehead; fish, water, and sediment from 17 ponds/8 farms	Ampicillin Oxytetracycline Cefpodoxime Ciprofloxacin Trimethoprim-Sulfamethoxazole Gentamicin Florfenicol Oxalinic Acid Erythromycin	Not investigated	*Aeromonas* spp.	High resistance to Trimethoprim-Sulfamethoxazole, Oxalinic Acid, Oxytetracycline	Peng et al. (2024)
				Escherichia coli	Multidrug-resistant isolates resistant to Ampicillin, Cefpodoxime, Trimethoprim-Sulfamethoxazole, and Florfenicol	
				Enterobacteriaceae	Resistant to Ampicillin (100%); Partial resistance to Trimethoprim-Sulfamethoxazole, Oxytetracycline, Cefpodoxime, Gentamicin, and Florfenicol	
				Pseudomonas spp.	Resistant to Ampicillin (100%), Cefpodoxime, Trimethoprim-Sulfamethoxazole, and Florfenicol; Susceptible to Ciprofloxacin, Oxytetracycline, and Gentamicin.	

Global antimicrobial resistance in catfish aquaculture

Global and regional studies provided contextual evidence that AMR was widely reported in catfish and freshwater aquaculture (Table 2). Reported resistant organisms included *Aeromonas* spp., *Escherichia coli*, *Vibrio* spp., *Salmonella* spp., *Pseudomonas* spp., *Enterococcus* spp., and *Edwardsiella tarda*, with multidrug resistance observed in several settings (Schar et al., 2020, 2021; Algammal et al., 2022; Adah et al., 2024; Peng et al., 2024; Afroze et al., 2025; Mohammed et al., 2025; Shahi et al., 2025; Soku et al., 2025; Matee, 2026). Resistance to tetracyclines, Beta-lactams, sulphonamides, and other antibiotic classes was frequently reported, although resistance patterns differed substantially among bacterial taxa, countries, and antimicrobial panels (Schar et al., 2021; Algammal et al., 2022; Adah et al., 2024; Peng et al., 2024; Afroze et al., 2025; Shahi et al., 2025; Soku et al., 2025; Matee, 2026). Global studies identified ARGs including *tet*, *sul*, *bla*, and *qnr* groups and detected antimicrobial residues in aquaculture-related matrices (Algammal et al., 2022; Mohammed et al., 2025; Matee, 2026). Contextual evidence highlighted the importance of aquatic environments in AMR persistence and distribution, although global data did not specifically represent conditions on Indonesian catfish farms (Schar et al., 2020, 2021; Mohammed et al., 2025; Shahi et al., 2025; Matee, 2026).

CHALLENGES AND PRIORITIES FOR ANTIMICROBIAL RESISTANCE CONTROL IN INDONESIAN CATFISH AQUACULTURE

Indonesian studies indicated that AMR in catfish aquaculture included bacterial, genetic, environmental, and food-product domains. The most consistent finding across studies was resistance to *Aeromonas* spp., along with reports of ARGs and antimicrobial residues in aquaculture and catfish meat (Kurniawan, 2023; Ariyani et al., 2024; Wulandari et al., 2024). However, the findings were contradictory and geographically limited, and the reviewed studies did not establish a nationally representative AMR prevalence (Schar et al., 2020).

Oxytetracycline, tetracycline, and enrofloxacin were consistently reported in the Indonesian catfish studies (Mulia et al., 2021; Dewi et al., 2023; Kurniawan, 2023; Mawardi et al., 2023; Putri and Triyanto, 2023). The recurrence of these agents was consistent with broader aquaculture studies indicating extensive use of tetracyclines and other broad-spectrum antimicrobials (Schar et al., 2020; Shahi et al., 2025). The present review did not specifically investigate why Indonesian farmers chose these medicines. Accessibility, cost, diagnostic limitations, and empirical treatment may relate to farmers' choices.

Susceptibility to antibiotics differed markedly within *Aeromonas* spp., with enrofloxacin resistance ranging from 0% (Mulia et al., 2021; Dewi et al., 2023; Wulandari et al., 2024) to 16% (Mawardi et al., 2023). The differences in susceptibility profiles across studies may reflect variation in bacterial populations, sampling approaches, antimicrobial exposure, laboratory methods, and production settings (Mulia et al., 2021; Dewi et al., 2023; Mawardi et al., 2023; Wulandari et al., 2024).

ONE HEALTH IMPLICATIONS OF ANTIMICROBIAL RESISTANCE IN CATFISH AQUACULTURE

Aeromonas spp., particularly *A. hydrophila*, warrant attention as they were recurrently identified in Indonesian catfish samples and possess phenotypic resistance and ARGs (Mulia et al., 2021; Mawardi et al., 2023; Wulandari et al., 2024). Wulandari et al. (2024) detected *ampC* and *qnr* genes, while Mulia et al. (2021) detected *tetA* and *strA-strB*. Similar reports from other aquaculture settings indicated that *Aeromonas* spp. can harbor a variety of resistance genes (Hayatgheib et al., 2021; Jeamsripong et al., 2025). The presence of mobile or transferable resistance genes in aquatic bacteria is important for horizontal gene transfer and the spread of AMR in aquatic environments (Hayatgheib et al., 2021; Jeamsripong et al., 2025). However, the Indonesian studies in this review did not directly quantify gene-transfer conditions (Mulia et al., 2021; Wulandari et al., 2024).

Environmental evidence further broadened the One Health perspective. Ikhrami et al. (2024) detected *sul1*, *tetA*, *blaGES*, and *mexF* in an irrigation canal and aquaculture pond, while Ariyani et al. (2024) detected multiple antimicrobial residues in reservoir water. The findings indicated that AMR-related factors and antimicrobial compounds were found outside fish tissues and clinical isolates. Aquatic cultures may therefore serve as interfaces where resistant organisms, resistance genes, antimicrobial residues, animals, and humans interact (Okeke et al., 2022; Prata, 2022; Yuan et al., 2023).

Evidence on food safety was limited but significant. Residues of chloramphenicol were identified at low concentrations in catfish sold in Banyuwangi (Ulkhaq et al., 2024), whereas Hakimah et al. (2021) documented oxytetracycline levels exceeding the maximum residue limit in 25% of 59 samples collected from markets in Yogyakarta. Although the geographically limited evidence was insufficient to estimate national consumer exposure, the findings emphasize the importance of residue monitoring, adherence to withdrawal periods, and a more integrated approach to antimicrobial use and food safety surveillance (Hakimah et al., 2021; Ulkhaq et al., 2024). Sub-inhibitory antimicrobial concentrations can create selection pressure in environmental microbial communities, providing a plausible biological basis for integrated monitoring approaches (Chow et al., 2021).

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CONCLUSION

Findings from Indonesian studies indicated that catfish aquaculture and associated aquatic environments were affected by antimicrobial exposure and harbored resistant bacteria, ARGs, and antimicrobial residues. *Aeromonas* spp. were the most consistently detected resistant bacteria, while studies conducted in Indonesia also reported resistance to *E. tarda* and *Proteus* species. Antimicrobial resistance genes from the tetracycline, quinolone, beta-lactam, sulphonamide, streptomycin, and multidrug resistance classes were documented. Antimicrobial compounds were detected in aquatic environments and marketed catfish, including oxytetracycline above the reported maximum residue limits.

The current evidence supports the enhancement of One Health surveillance and antimicrobial stewardship initiatives. Future studies should prioritize integrated farm-level investigations that simultaneously assess antimicrobial use, phenotypic resistance, resistance genes, and antimicrobial residues to better clarify the sources and transmission pathways of AMR in Indonesian catfish aquaculture. The conclusions should, however, be interpreted with caution because the available Indonesian evidence remains geographically limited and heterogeneous, the review protocol was not prospectively registered, complete records of the early screening process were unavailable, and a formal risk-of-bias assessment was not performed.

DECLARATIONS

Authors' contributions

Kadek Karang Agustina conceptualized and designed the study, as well as contributed to the literature review, data collection, data analysis, and manuscript preparation. I Made Sukada, Ida Bagus Krisna Pradnyadana, Putu Arya Duta Adnyana, Ni Putu Dewi Setia Sari, Ni Ketut Harmini Saraswati, and Ida Bagus Kade Suardana participated in the literature review, data collection, data analysis, and manuscript preparation. All authors rigorously reviewed the manuscript, approved the final edition, and accepted responsibility for all aspects of the study.

Availability of data and material

All data synthesized in this review are derived from the cited publications and sources listed in the references of the manuscript.

Competing interests

The authors have declared no conflict of interest.

Ethical considerations

ChatGPT, created by OpenAI, was used exclusively for editing and proofreading in English to enhance grammar, clarity, readability, and academic tone. The authors carefully reviewed and edited all AI-assisted revisions and assume full responsibility for the AI-edited content. ChatGPT was not used to generate, analyze, or interpret the study data, and the authors take full responsibility for the accuracy, originality, and integrity of the final manuscript before submission, after primary acceptance, and at final stage of publication.

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Effects of Dried Tomato Pomace Dietary Supplementation on Production Performance, Egg Quality and Carotene Content in laying hens: A Narrative Review

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ABSTRACT

Tomato pomace is a rich source of protein, fiber, carbohydrates, and carotenoids for layer hens. Adding a moderate level of dried tomato pomace to laying hen diets is considered safe and has no adverse effects on performance metrics such as egg production, feed intake, egg weight, or shell thickness. The present study aimed to evaluate the effects of nutritional supplementation with dried tomato pomace on the productivity, egg quality, and carotene content of laying hens. A narrative literature search was conducted using scientific databases, including Scopus, PubMed, and Web of Science. The search primarily focused on peer-reviewed studies published from 2019 to 2026. Relevant previous studies were included to provide important background and context for the current study. Although tomatoes are rich in nutrients, such as carotenoids, fiber, vitamins, and bioactive compounds, processing industries often discard tomato pomace as waste. Improper disposal of tomato waste also poses environmental issues. Due to its nutritional content, dried tomato pomace could serve as a dietary supplement for chickens, especially in laying hens, where dietary carotenoids can influence egg yolk coloration and nutritional quality. Dietary tomato pomace, a useful delivery system for improving human health, positively affected lycopene deposition in egg yolk. Dried tomato pomace can be added to laying hens' diets at up to 5-12% (50-120 g/kg feed) to improve egg yolk color and carotenoid content without adversely affecting laying hens' productivity, while also lowering production costs and egg cholesterol content. Including dried tomato pomace at levels up to 12% (120 g/kg feed) can improve egg nutrition by increasing natural antioxidants such as lycopene and β-carotene, making it an economical and sustainable feed supplement for poultry.

Keywords: Carotene, Egg quality, Laying hen, Production performance, Tomato pomace

INTRODUCTION

Utilizing agro-industrial byproducts offers an alternative approach to addressing the shortage and rising cost of conventional feed in poultry nutrition (Georganas et al., 2023). Dried tomato pomace is a nutritionally valuable non-conventional feed ingredient that can be incorporated into laying-hen diets without adversely affecting key laying performance parameters, while improving yolk pigmentation (Mierlita et al., 2024). In commercial poultry production, feed costs represent a major component of total production costs, accounting for more than 60% of total costs (Noblet et al., 2022). Therefore, least-cost feed formulation and the use of alternative feed ingredients have received considerable attention as strategies for minimizing feed costs and improving the profitability of poultry production (Alhotan, 2021). The nutritional value of the substitute feed should allow partial replacement of conventional ingredients without adversely affecting productivity or immunity (Sultana et al., 2016).

In laying hens, molting is a normal physiological process that involves the replacement and shedding of old feathers (Glatz and Tilbrook, 2021). Egg laying is significantly reduced or stops during molting because the reproductive tract regresses and then regenerates, temporarily suppressing reproductive activity (Glatz and Tilbrook, 2021). Recent studies on fasting-induced molting in laying hens have indicated significant alterations in egg quality, production performance, and reproductive function (Ma et al., 2025). Mansoori et al. (2007) found that DTP successfully triggered molting and a decrease in ovarian weight, and hens that experienced molting induced by DTP demonstrated greater egg production after molting compared to hens that were subjected to feed withdrawal. Studies evaluating dried tomato pomace and tomato waste as dietary ingredients have generally reported no adverse effects on major laying-performance parameters, including feed intake, egg production, feed conversion ratio, egg weight, and egg mass (Salajegheh et al., 2012; Mahata et al., 2020). Additionally, DTP had no notable effects on several egg-quality parameters, including eggshell weight,

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eggshell thickness, and Haugh unit. However, DTP inclusion significantly increased the yolk color score (Salajegheh et al., 2012).

Laying hens fed diets with DTP may consume more feed due to the feed's palatability and the presence of bioactive substances including antioxidants such as ascorbic acid and carotenoids (Mierlita et al., 2024). However, because tomato pomace contains large amounts of dietary fiber, excessive supplementation may reduce feed intake by increasing intestinal fullness and bulk density; therefore, the response depends on the level of dietary inclusion (Reda et al., 2022). Lycopene, β-carotene, lutein, and other carotenoids are present in tomato pomace, which laying hens consume and incorporate into the egg yolk (Reda et al., 2022). By lowering lipid oxidation and raising provitamin A activity, the deposition of dietary carotenoids, especially lycopene, improves egg yolk coloring (a deeper yellow to orange hue), increases the yolk's carotenoid concentration and antioxidant capacity, and increases the yolk's nutritional value for consumers (Reda et al., 2022). Dried tomato pomace contains substantial levels of crude protein (15-30 g/100 g), crude fat (8-24 g/100 g of dry weight), and dietary fiber (11-66 g/100 g of dry weight, dry matter basis; Chabi et al., 2024). However, DTP composition differs with tomato type and processing conditions. Because of its nutritional value, DTP is a suitable agro-industrial by-product for partially replacing conventional feed ingredients in chicken diets (Chabi et al., 2024). Tomatoes are among the most widely produced agricultural products worldwide. Asia accounts for 62.6% of the world's 186 million tons of tomatoes, followed by the Americas (13.1%), Europe (12.2%), Africa (11.9%), and Oceania (0.2%; FAOSTAT, 2022). Juices, sauces, and canned fruits are among the processed goods that frequently use tomatoes as a raw ingredient. Due to overproduction of fresh tomatoes or processing losses, the tomato production and processing sectors generate industrial waste (Laranjeira et al., 2022).

The tomato (*Solanum lycopersicum*) is one of the most widely produced vegetable crops in Mediterranean countries. After juice extraction, tomato pomace, primarily composed of water, tomato seeds, and peels, remains (Lu et al., 2019). The high-water content of tomato pomace limits its shelf life. Because of these storage limitations, tomato pomace is often dried (Salajegheh et al., 2012). Tomato pomace has significant nutritional value, containing up to 16 g/100g of crude protein due to its diverse bioactive constituents, including vitamins, polyphenols, minerals, and carotenoids (Azabou et al., 2020). Additionally, lycopene constitutes 80-90 g/100 g dry weight of the total carotenoid content in tomato pomace (Nour et al., 2018).

Tomato pomace is rich in essential amino acids, fatty acids, flavonoids, carotenoids, and certain minerals including calcium, copper, manganese, zinc, and selenium (Azabou et al., 2020). The effects of tomato pomace on digestive enzymes, reproductive and productive capacity, and the deposition of carotenoids in laying hen egg yolks are not well understood. Tomato processing industries generate large quantities of waste pomace, which is often discarded despite being rich in nutrients such as carotenoids, fiber, vitamins, and bioactive compounds. The improper disposal of tomato waste can pose environmental risks. Considering its nutritional profile, dried tomato waste pomace could serve as a dietary supplement in poultry nutrition, particularly in laying hens, since dietary carotenoids can affect egg yolk color and nutritional value (Georganas et al., 2023). Nevertheless, the optimal inclusion levels and overall impacts of DTP on laying hen performance, egg quality, and egg carotenoid content remain largely undetermined (Georganas et al., 2023). Therefore, the present study aimed to examine the effects of dietary DTP supplementation on production performance, egg quality, and egg carotenoid content in laying hens.

SEARCH CRITERIA

The literature search strategy for this narrative review used major scientific databases, including PubMed, Scopus, and Web of Science. The evaluation focused primarily on peer-reviewed articles published between 2019 and 2026. Relevant previous studies were included as needed to provide important background and context for the current study. Search queries combined keywords such as laying hens, dried tomato pomace, production performance, egg quality, and egg yolk carotenoids. The review included 20 studies identified from scientific databases, including Scopus, PubMed, and Web of Science. Studies were eligible for inclusion when they were peer-reviewed, involved laying hens, and reported original experimental data on the effects of dietary supplementation with DTP on production performance, egg quality, or egg carotenoid content. Studies were excluded when they involved poultry species other than laying hens, did not investigate DTP supplementation, or did not report outcomes related to production performance, egg quality, or carotenoid content. The identified studies were screened based on their titles and abstracts, followed by full-text assessment of potentially eligible articles. Relevant information from the included studies was extracted and synthesized narratively according to the objectives of the review.

CHEMICAL COMPOSITION OF TOMATO POMACE

Del Valle et al. (2006) reported that tomato pomace typically contains 56 g/100 g of dry weight in pulp and peels, with seeds constituting 44 g/100 g. However, tomato pomace composition depends on the final product and the peeling

techniques used during production (Eslami et al., 2023). Tomato pomace is rich in valuable components, including lycopene, proteins, lipids, and dietary fiber (Table 1). More specifically, tomato pomace primarily consists of fiber (39.11-59.03 g per 100 g of dry weight), fats (2-16.24 g per 100 g of dry weight), and proteins (15.08-24.67 g per 100 g of dry weight). These variations depend on factors such as tomato variety, growth stage, ripening process, processing methods, extraction conditions, and analytical techniques used (Lu et al., 2019).

The proximate chemical composition of tomato pomace collected at different stages of industrial processing, on a dry-weight basis, consisted of 59.03 g/100 g neutral detergent fiber (fiber), 25.73 g/100 g of total sugars, 19.27 g/100 g of protein, 7.55 g/100 g of pectin, 5.85 g/100 g of lipids, and 3.92 g/100 g of minerals (Del Valle et al., 2006). Dried tomato wastes have high levels of lycopene (0.05106 g/100 g) and β-carotene (0.00956 g/100 g of dry weight) and substantial antioxidant properties, according to Nour et al. (2018).

The chemical composition of tomato pomace differs considerably across the studies (Lu et al., 2022; Chabi et al., 2024). The wide variation in reported protein, oil, and fiber contents in tomato pomace mainly results from differences in seed-to-skin ratio and processing techniques (Lu et al., 2022). Tomato pomace is a diverse by-product made largely of skins, seeds, and leftover pulp, with the proportions of these components fluctuating with tomato variety and industrial processing conditions (Lu et al., 2022; Chabi et al., 2024). Pomace with a higher seed percentage typically has higher protein and fat levels, as tomato seeds are naturally rich in both nutrients (Lu et al., 2022). Conversely, pomace with a higher skin percentage typically has higher fiber content (Lu et al., 2022; Chabi et al., 2024). Furthermore, the final composition can be significantly impacted by variations in processing and separation techniques, such as pressing, drying, milling, and pulp or seed removal (Chabi et al., 2024). Therefore, instead of indicating inherent inconsistencies among the studies, the reported ranges in proximate composition, especially for crude protein, crude fiber, ether extract, and ash, should be regarded as indicative of variations in raw material composition and processing conditions (Lu et al., 2022).

Table 1. Chemical composition of tomato pomace

Material	Fibres (g/100 g of DW)	Proteins (g/100 g of DW)	Fat (g/100 g of DW)	Lycopene (g/100 g of DW)	Reference
Pomace	39.11-59.03	15.08-24.67	2.00-16.24	9.82-611.105	Savadkoohi and Farahnaky (2012); Shao et al. (2013)
Peels	62.79-78.56	1.85-11.13	1.63-5.50	50-1930	Grassino et al. (2016); Wang et al. (2016); Nour et al. (2018)
Seeds	16.00	20.2-40.94	17.80-24.50	22.01-37.43	Mechmeche et al. (2017); Szabo et al. (2021)

DW: Dry weight, Source: Eslami et al. (2023)

EFFECTS OF TOMATO POMACE ON LAYING HENS' PERFORMANCE

The increases in hen-day egg production and egg mass may be attributed to the high crude fiber content and nutritional value of tomato waste (Handayani et al., 2023). However, depending on the specific tomato variety and processing method, DTP exhibited substantial levels of dietary fiber, approximately 11-66 g per 100 g on a dry matter basis (Chabi et al., 2024). While dietary fiber contributes nutritional value to tomato pomace, excessive dietary fiber levels may reduce nutrient digestibility and utilization in poultry (Jha and Mishra, 2021). Crude fiber levels were reported as 52.44 g/100 g of dry weight in tomato peels and seeds (Nour et al., 2018), 40.43 g/kg in tomato peels (Varzaru et al., 2021), and 35 g/100 g of dry weight in tomato pulp (Jafari et al., 2006).

Fiber affects anatomical, functional, and physiological changes in the gastrointestinal tract (Deehan et al., 2022). Dietary fiber affects intestinal microbiota, gastrointestinal tract development, egg quality, laying performance, and nutritional digestibility (Desbruslais et al., 2021). When laying hens were fed tomato waste at 7.1 g/100 g of dry weight or 8.6 g/100 g of dry weight, the maximum dietary fiber content was increased (Handayani et al., 2023). Chickens' carcasses demonstrated undigested feed components and nutritional deficiencies attributable to excessive dietary fiber intake (Jha and Mishra, 2021). As a result, egg yield and egg mass weight were expected to decrease (Handayani et al., 2023). According to Yannakopoulos et al. (1992), laying hens fed diets containing up to 15 g/100 g of dry weight tomato pomace for 8 weeks did not produce more eggs than chickens fed a control diet. Jafari et al. (2006) reported that egg mass and production increased in laying hens when fed up to 10 g/100 g of dry weight of DTP for 12 weeks.

In a study by Mierlita et al. (2024), laying hens were fed diets supplemented with 4% DTP and 20% hempseed cake for 10 weeks (70 days), which increased feed intake and improved the antioxidant content and oxidative stability of egg yolk. However, chickens fed the diet comprising 4% DTP and 20% hempseed cake exhibited a body weight gain of 97.5

g, whereas the control group demonstrated a gain of 116.4 g. This result showed that incorporating 4% DTP and 20% hempseed cake into the diets of laying hens did not adversely affect their weight gain, indicating that these components can be included without hindering the growth performance of the hens. Additionally, Yannakopoulos et al. (1992) reported that body weight gain, feed consumption, and overall performance metrics of laying hens were not significantly affected by the dietary inclusion of tomato meal. In laying hens under normal environmental conditions, dietary inclusion of DTP at levels up to 19 g/100 g of dry weight for 10 weeks produced no adverse or appreciable effects on overall performance parameters, including egg production, body weight, feed intake, feed conversion ratio, and egg weight traits (Salajegheh et al., 2012). Alagawany et al. (2022) reported that dietary supplementation with 3-12 g/100 g of dry weight sun-DTP for 28 days (from 1 to 5 weeks of age) improved body weight and body weight gain in growing Japanese quail, with the 6 g/100 g of dry weight inclusion level producing the greatest improvement. Similarly, Muhammad et al. (2025) reported that dietary supplementation with dried tomato pomace at 10 and 20 g/kg (1% and 2%) for 35 days improved growth performance, including body weight gain and feed conversion ratio, in Ross 308 broiler chicks exposed to heat stress.

Akyol and Denli (2023) conducted a 9-week feeding trial in laying hens and reported that dietary inclusion of 4% dried tomato pulp increased feed consumption, egg production, and egg weight compared with the control group. Salajegheh et al. (2012) reported that dietary supplementation with dried tomato pomace (DTP) at 150, 170, and 190 g/kg diet (15%, 17%, and 19%, respectively) for 10 weeks did not significantly affect average feed intake of Lohmann LSL-Lite laying hens compared with the control group. Tufarelli et al. (2022) evaluated the effects of diets with 15 g/100 g dry weight DTP on laying hens over 8 weeks and indicated that incorporating DTP into the diet did not negatively affect productive performance, egg production, egg weight, or feed conversion ratio. Previous studies have indicated that dietary inclusion of dried tomato pomace (DTP) at 5-10 g/100 g of the diet did not negatively affect feed intake in laying hens, suggesting good dietary acceptance. Jafari et al. (2006) reported similar feed consumption at DTP inclusion levels of 5% and 10% over a 12-week feeding period, while Mansoori et al. (2008) observed no significant effect on feed intake at 5% and 10% DTP inclusion over 9 weeks. Handayani et al. (2023) reported that integrating tomato waste into laying hen diets increased feed consumption and appeared to have a quadratic relationship with egg mass and daily egg production per hen, although the eggshell strength decreased significantly.

EGG QUALITY

In a meta-analysis of 22 studies involving laying hens, Handayani et al. (2023) reported that dietary tomato waste inclusion levels ranging from 0 to 20 g/100 g of dry weight of the diet improved yolk color, which was primarily attributed to the lycopene and other carotenoid pigments present in tomato waste. In addition, tomato-waste supplementation significantly reduced overall eggshell strength, suggesting that adverse effects on shell integrity may occur under some dietary conditions (Handayani et al., 2023). Lycopene, the red pigment found in fruits, vegetables, and other plant components, is one of the carotenoid molecules generated by plants (Cooperstone et al., 2016; Reboul, 2019). Dietary carotenoids have been indicated to affect egg yolk color. Previous studies indicated that carotenoid compounds in laying hens' diets significantly altered the intensity of their egg yolk color (Rakonjac et al., 2014; Kaspers, 2016). Calislar and Uygur (2010) reported that dried tomato pulp can be included in laying hen diets at levels up to 2 g/100 g of diet, improving egg yolk color without adversely affecting egg production. Similarly, Salajegheh et al. (2012) reported that dietary inclusion of DTP at 15 g/100 g, 17 g/100 g, and 19 g/100 g for 10 weeks (70 days) significantly improved egg yolk color without adversely affecting egg production, laying performance, or other egg quality traits. Ahmed et al. (2022) reported that the dietary inclusion of tomato waste meal at levels up to 26 g/100 g of diet for 84 days did not negatively impact laying performance or egg quality parameters, except for a notable enhancement in egg yolk color intensity. Karadas et al. (2006) found that adding 20 g/kg of tomato powder to adult Japanese quail diets for 23 days increased the egg yolk color score compared to a low-carotenoid control diet. Similarly, Jafari et al. (2006) observed that including dried tomato pulps at a level of up to 10 g/100 g of dry weight in the diet for 12 weeks enhanced egg production and egg mass in laying hens, without negatively impacting egg weight, eggshell weight, shell thickness, Haugh unit, or yolk color. Tufarelli et al. (2022) reported that dietary supplementation with 15 g/100 g of DTP combined with 5 g/100 g dried grape pomace for 8 weeks (56 days) significantly increased egg yolk color score, without adversely affecting egg production, egg weight, feed conversion ratio, or major egg quality traits in laying hens. In addition, Yannakopoulos et al. (1992) reported that diets containing 8 g/100 g or 15 g/100 g of tomato meal, fed to laying hens for 80 days (from 32 to 43 weeks of age), had no adverse effects on egg production or quality. Akdemir et al. (2012) indicated that dietary supplementation of laying hens with tomato powder at 0.5 g/100 g and 1 g/100 g for 90 days improved egg production persistency and increased egg yolk concentrations of carotenoids (lycopene, β-carotene, and lutein) and vitamin A. An et al. (2019) stated that supplementation of laying hen diets with synthetic lycopene (0.002 g/100 g diet) or tomato paste (1.7 g/100 g diet) for 4 weeks (28 days) affected laying performance and egg quality traits,

while increasing lycopene deposition in egg yolk and reducing lipid oxidation during egg storage. Moreover, higher amounts of DTP (up to 19 g/100 g) were included in the diets of laying hens for 8 weeks by Salajegheh et al. (2012), indicating no appreciable or significant impact on plasma total cholesterol, low-density lipoproteins, or egg yolk cholesterol content despite the substantial amount of tomato waste. Furthermore, tomato meal's high fiber content may limit its inclusion in poultry diets by reducing nutrient digestibility and utilization (Leeson and Summers, 2001).

Leke et al. (2015) indicated that dried tomato meal at inclusion levels up to 8 g/100 g for 8 weeks might be a beneficial alternative feed ingredient, since it did not negatively affect egg cholesterol content or egg quality. Leke et al. (2015) found no significant differences in egg nutrient or cholesterol content between the control diet and diets containing 2, 4, 6, or 8 g/100 g of dried tomato meal for 8 weeks. According to Reda et al. (2022), egg quality parameters, including albumen, yolk, and shell percentages, yolk index, Haugh unit, shell thickness, and egg shape index, were not affected by dietary tomato pomace inclusion up to 12 g/100 g for 8 weeks. Moreover, DTP at levels up to 12% enhanced lycopene deposition in egg yolk, suggesting its potential as a dietary strategy for producing lycopene-enriched eggs and promoting human health (Reda et al., 2022).

Seidavi et al. (2018) reported that when tomato pomace was incorporated at practical levels (typically 10 g/100 g to 19 g/100 g), no adverse or statistically significant changes in blood biochemical profiles, including serum total protein, albumin, and globulin levels, were observed. Reda et al. (2022) found that tomato pomace increased liver and kidney function by 6 g/100 g and 12 g/100 g, respectively. Panaite et al. (2019), in a 6-week feeding trial, found that dietary inclusion of dried tomato waste (2.5%, 5.0%, or 7.5%) in diets containing 5% flaxseed did not significantly affect egg weight. However, the 5.0% and 7.5% dried tomato waste treatments significantly reduced feed intake and laying percentage compared to the control. The effects of tomato by-products on eggshell quality appear to be inconsistent and may depend on inclusion level, by-product characteristics, and basal-diet composition (Table 2; Handayani et al., 2023; Mierlita et al., 2024).

Table 2. Egg quality parameters in laying hens fed dried tomato pomace

Study	Material used	Inclusion level	Egg production	Egg color	Eggshell quality
Salajegheh et al. (2012)	DTP	0, 15, 17 and 19%	No significant effect	Significant increase in yolk color (7.25 to 9.67 RYCF units)	Shell thickness not affected
Tufarelli et al. (2022)	DTP	0 and 15% DTP ± 5% DGP	No significant effect	Highest with 15% DTP plus 5% DGP	Thickness and strength not affected
Mierlita et al. (2024)	DTP plus hempseed cake	4% DTP plus 20% hempseed cake	No significant effect	Increased (RYCF 6.67 to 9.26)	Thickness not significantly affected (0.362 to 0.364 mm)
Jafari et al. (2006)	Dried tomato pulp	0, 5, 10 and 15%	5-10% increased egg production and egg mass	No significant difference	Shell thickness (0.352, 0.361, 0.362 and 0.360 mm); no significant effect
Calislar and Uygur (2010)	Dried tomato pulp	0-20%	No significant effect on egg yield	Increased yolk pigmentation	Shell weight, thickness, and strength not affected
Yannakopoulos et al. (1992)	Tomato meal	8 and 15%	No significant adverse effect	Increased yolk color	No adverse effect on shell quality
Mansoori et al. (2008)	DTP	0, 5 and 10% (0, 50 and 100 g/kg)	No significant effect on egg production, egg weight, or egg mass	Significant increase in Roche yolk color score, from 6.7 in control to 9.7 and 10.3 at 5 and 10% DTP, respectively	No significant main effect on shell thickness or shell weight/egg weight ratio
Panaite et al. (2019)	Dried tomato waste (DTW) + 5% flaxseed	2.5, 5.0 and 7.5% DTW	Laying percentage significantly decreased in some DTW-supplemented groups; egg mass was not significantly affected	Yolk Roche color score increased with increasing DTW; the reported 7.5% DTW + 5% flaxseed treatment had a 3.5-fold higher score than control after 4 weeks	-

DGP: Dried grape pomace, DTP: Dried tomato pomace, RYCF: Roche yolk color Fan, mm: Millimeters, DTW: Dried tomato waste

CAROTENE CONTENTS OF EGG IN LAYING HENS

Dietary inclusion of dried tomato waste can increase carotenoid deposition in egg yolk, particularly lutein and zeaxanthin, and enhance yolk pigmentation in laying hens (Panaite et al., 2019). Several studies have demonstrated that laying hens fed diets containing tomato powder or tomato peel have higher yolk carotenoid contents (Akdemir et al., 2012; An et al., 2019). Akdemir et al. (2012) indicated that adding 0.5-1 g/100 g of dry weight of tomato powder to the diet for four weeks increased the quantities of lycopene and β-carotene in the yolk. Egg yolk coloration and antioxidant properties can be modified when laying hens consume diets supplemented with 4.5% linseed combined with 1% dried tomato paste and 1% sweet red pepper for 47 days (Omri et al., 2019). An efficient dietary method for improving the carotenoid profile and antioxidant status of egg yolks is to feed laying hens tomato pomace (Omri et al., 2019; Mierlita et al., 2024; Table 3). Nour et al. (2017) incorporated dried tomato waste for 6 weeks into the diet and observed an increase in total yolk carotenoids from 8.44 to 12.67 µg/g.

Dietary supplementation with DTP at 4 g/100 g of the diet for 10 weeks has been shown to improve yolk color intensity and increase the deposition of natural antioxidants and carotenoid-related compounds in laying hens (Mierlita et al., 2024). As an effective substitute for artificial pigments such as canthaxanthin and apocarotenoic acid ester, DTP offers a natural source of carotenoids that may support pigmentation (Yunitasari et al., 2023; Mierlita et al., 2024). Calislar and Uygur (2010) studied the impact of incorporating dry tomato pulp at rates up to 20 g/100 g for 8 weeks and found no negative effects on overall egg production, noting enhanced yolk coloration. More recently, a meta-analysis demonstrated that tomato-waste supplementation can affect laying performance and several egg-quality characteristics (egg weight, eggshell thickness, eggshell strength, eggshell weight, and yolk weight), although the magnitude and direction of responses differ by inclusion level and experimental conditions (Handayani et al., 2023).

Tomato-derived products and byproducts contain carotenoids, such as lycopene and other pigments (Nour et al., 2018). Incorporating tomato-derived products and byproducts into chicken diets can enhance egg yolk pigmentation and intensify yolk color (Nour et al., 2018; An et al., 2019). This improvement is highly valued by customers (Nour et al., 2018; An et al., 2019). Dried tomato pulp can be included in laying-hen diets at levels up to approximately 100 g/kg without negatively affecting laying-hen performance (Jafari et al., 2006). However, a higher inclusion level of 150 g/kg decreased egg production and egg mass, which may be associated with the relatively high fiber content of tomato pulp (Jafari et al., 2006).

The relatively high fiber content and nutrient composition of DTP should be considered when formulating poultry diets because DTP measurably affects nutrient digestibility and metabolizable energy utilization (Mansoori et al., 2008).

Table 3. Effects of tomato pomace on carotene contents of eggs in laying hens

Study	Tomato material	Inclusion level	Yolk color and carotenoid outcome
Salajegheh et al. (2012)	DTP	0, 15, 17 and 19%	Yolk color score significantly increased (7.25 to 9.67)
Tufarelli et al. (2022)	DTP	0 or 15% DTP, with 0/4/8% flaxseed meal and 0/5% dried grape pomace	Yolk color was highest with the 15% DTP + 5% DGP combination.
Mierlita et al. (2024)	DTP + hempseed cake	4% DTP + 20% hempseed cake	RYCF increased from 6.67 to 9.26
Akdemir et al. (2012)	Tomato powder	5 and 10 g/kg	Yolk color and yolk lycopene, β-carotene, lutein, and vitamin A increased.
Panaite et al. (2019)	Dried tomato waste + flaxseed	7.5% dried tomato waste + 5% flaxseed	Lutein and zeaxanthin increased by 29% and 24%, respectively; yolk Roche color increased 3.5-fold
Nour et al. (2017)	Dried tomato waste	7.5%	Total yolk carotenoids increased from 8.44 to 12.67 µg/g and RYCF from 3.66 to 6.60 after six weeks.
Jafari et al. (2006)	DTP	0%, 5%, 10%, 15%	Significant linear increase in Roche yolk color Fan score as inclusion level increased, enhancing yolk pigmentation
Omri et al. (2019)	Dried tomato paste + sweet red pepper powder	1% dried tomato paste + 1% sweet red pepper, in a diet also containing 4.5% linseed	Significantly increased Roche yolk color fan score and yolk and increased yolk carotenoids
Calislar and Uygur (2010)	Dried tomato pulp	Up to 20%	Increased RYCF value and significantly increased yolk lycopene and β-carotene concentrations

DGP: Dried grape pomace, DTP: Dried tomato pomace, RYCF: Roche yolk color fan.

CONCLUSION

Dried tomato pomace is a promising agricultural by-product that contains nutrients and bioactive compounds, including carotenoids that may contribute to the nutritional value of laying-hen diets. Adding dried tomato pomace to laying hen diets improved egg yolk pigmentation and carotenoid content without negatively impacting productivity. Dietary inclusion of dried tomato pomace at levels up to approximately 12% (120 g/kg diet) has generally been reported to be safe for laying hens, without significant adverse effects on key performance parameters such as egg production, feed intake, egg weight, and shell thickness, although responses may vary depending on the composition and processing of the tomato pomace and the overall diet. Future studies should focus on finding the optimal level of dried tomato pomace inclusion for different laying hen breeds and production phases, as well as maximizing nutrient use and maintaining productivity. Additional studies are needed to identify effective strategies for reducing the negative effects of its high fiber content and improving nutrient digestibility.

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Authors' contributions

Eyayu Gobe solely designed the review topic, conducted the comprehensive literature search, synthesized and critically analyzed the published data, and literature collection and organization and, contributed to reviewing and editing the manuscript for scientific accuracy and clarity, and provided intellectual input. The final edition of the manuscript was reviewed and approved by the author before publication in the present journal.

Availability of data and materials

The present study did not produce or analyze any new data. The literature reviewed in the current study is cited in the reference list.

Competing interests

The author has declared no competing interests.

Ethical considerations

The author affirmed that this article is original, has not been published previously, and is not under consideration for publication elsewhere. The author confirmed that no artificial intelligence (AI) or AI-assisted technologies were used in the preparation, drafting, or writing of this article. The author is responsible for the accuracy, originality, and integrity of the final content of the article before submission, during the revision process, and upon final publication. The author has carefully checked and verified all review data, interpretations, and conclusions of the present study and takes full responsibility for the intellectual and scientific content of the manuscript.

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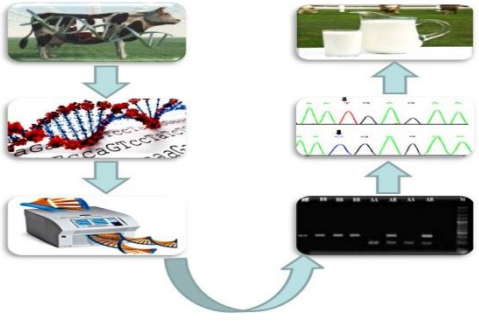
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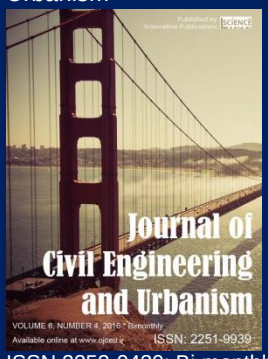
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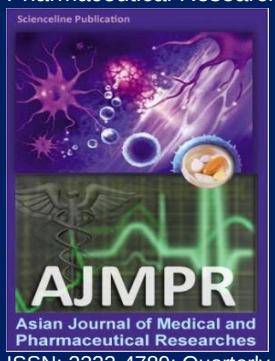
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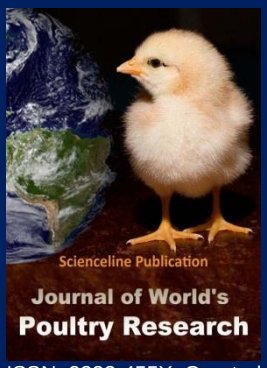
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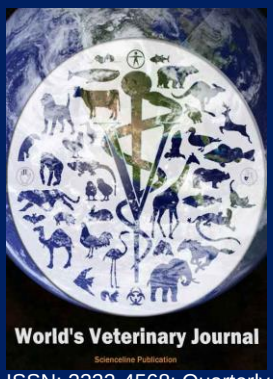
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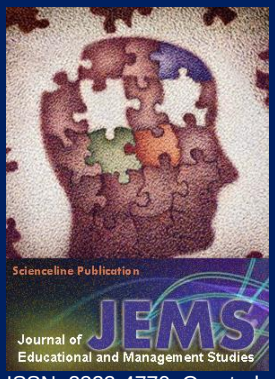
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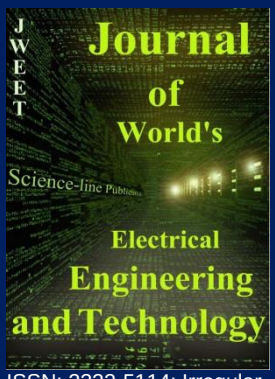
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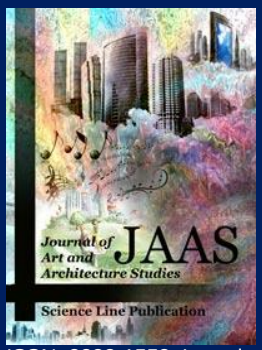
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