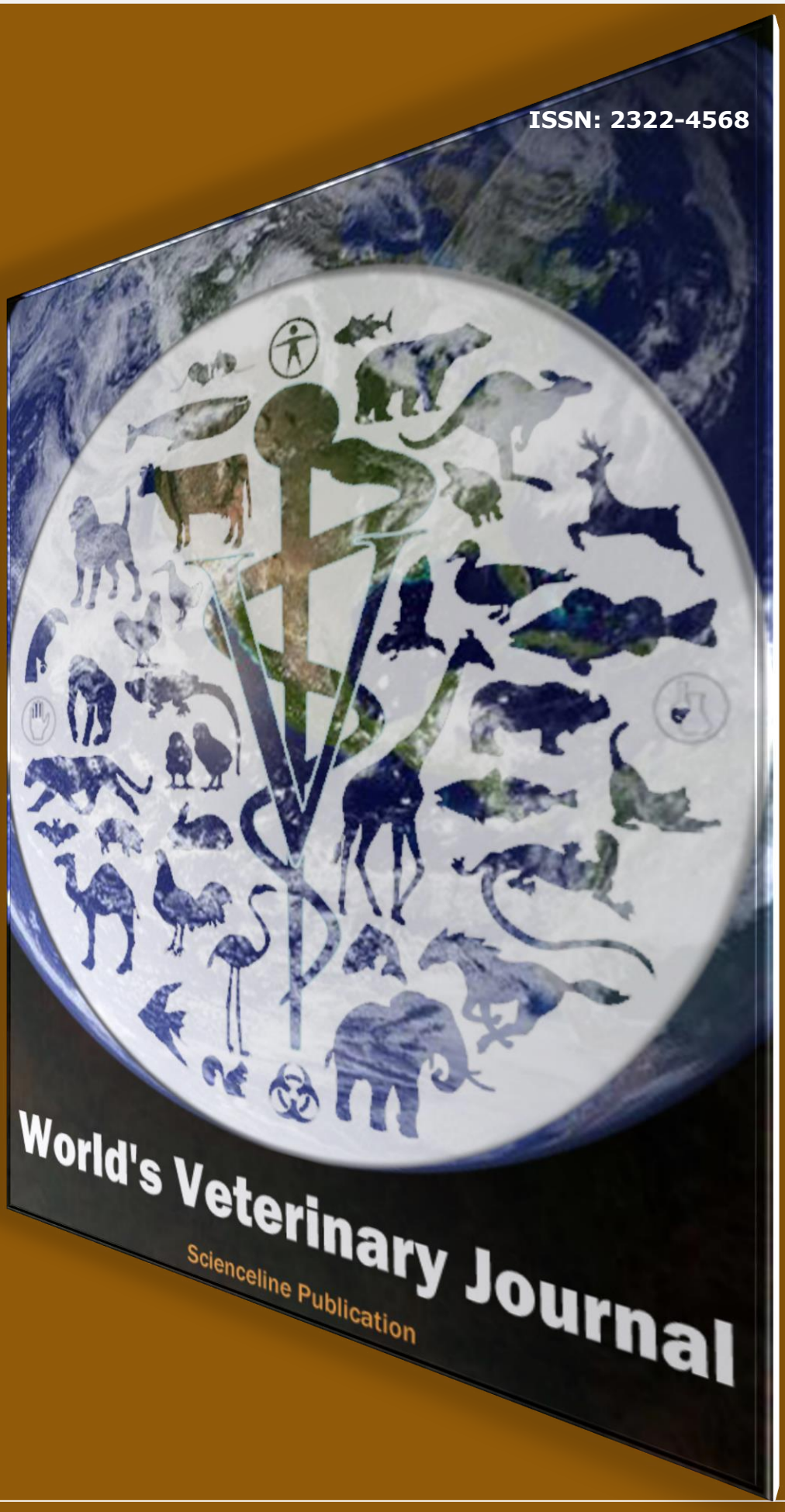
World's Veterinary Journal

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Volume 15, Issue 3, September 2025

2025, Scienceline Publication

World's Veterinary Journal

World Vet J, 15(3): 549-830, September 2025

ISSN 2322-4568

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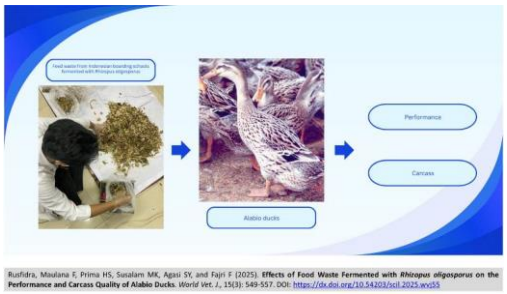
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Volume 15 (3); September 2025

Research Paper

Effects of Food Waste Fermented with *Rhizopus oligosporus* on the Performance and Carcass Quality of Alabio Ducks

Rusfidra, Maulana F, Prima HS, Susalam MK, Agasi SY, and Fajri F.
World Vet. J. 15(3): 549-557, 2025; pii:S232245682500055-15
DOI: https://dx.doi.org/10.54203/scil.2025.wvj55



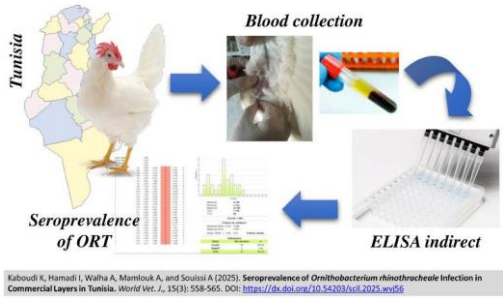
ABSTRACT: The incorporation of food waste as an alternative feed ingredient has attracted considerable academic attention, owing to its potential to decrease feed costs and promote sustainable practices within livestock production systems. The present study aimed to evaluate the effects of food waste from Pesantren (FWP), Indonesia, fermented with *Rhizopus oligosporus* (*R. oligosporus*) as feed on the performance and carcass quality of Alabio ducks. A completely randomized design with four replications was used in the present study. A total of 200 one-day-old male Alabio ducks, with an average initial body weight of 39 ± 2 grams, were raised for eight weeks and randomly divided into five dietary groups, consisting of 40 ducks per group and five ducks per replicate with different FWP inclusion levels. The treatments varied based on the inclusion levels of FWP fermented with *R. oligosporus* in the diets of Alabio ducks which included FWP 0% (Treatment A, the control group), FWP 10% (Treatment B), FWP 20% (Treatment C), FWP 30% (Treatment D), and FWP 40% (Treatment E). Different parameters were observed at 56 days of age, including feed consumption, final body weight, feed conversion ratio (FCR), protein intake, carcass percentage, and abdominal fat percentage. The current results indicated that FWP significantly affected feed consumption, final body weight, FCR, and protein intake. Treatment D resulted in the highest body weight, protein intake, and the best FCR. However, there were no significant differences among treatments in carcass and abdominal fat percentages. In conclusion, incorporating up to 30% of FWP fermented with *R. oligosporus*, based on dry matter, into the diet of male Alabio ducks does not negatively impact their performance or carcass quality. Using FWP fermented with *R. oligosporus* as part of the duck feed offered a sustainable and cost-effective method for organic waste management in Pesantren poultry systems.
Keywords: Alabio duck, Carcass quality, Fermented feed, Growth performance, *Rhizopus oligosporus*

[Full text-PDF]

Research Paper

Seroprevalence of *Ornithobacterium rhinothracheale* Infection in Commercial Layers in Tunisia

Kaboudi K, Hamadi I, Walha A, Mamlouk A, and Souissi A.
World Vet. J. 15(3): 558-565, 2025; pii:S232245682500056-15
DOI: https://dx.doi.org/10.54203/scil.2025.wvj56



ABSTRACT: *Ornithobacterium rhinotracheale* (ORT) infection is a worldwide disease in commercial poultry production. It is caused by a gram-negative bacterium, inducing respiratory and articular infections. The present study investigated the seroprevalence of ORT in commercial laying chicken farms in the Sfax region of South-eastern Tunisia. Diagnosis of ORT is based on the clinical signs such as respiratory distress, sneezing, cough, and limping, gross pathology in post-mortem examination (fibrinous tracheitis, fibrinous pneumonia, caseous arthritis, and tendinitis), and laboratory investigations, including isolation and identification, molecular and serological analysis. In the current study, 470 serum samples were collected from 25 commercial layer flocks (18 to 20 blood samples/flock) and tested using an indirect enzyme-linked immunosorbent assay (ELISA), which revealed that all 25 flocks (100%) demonstrated evidence of past or recent ORT infection. Seropositivity of the tested sera indicated the presence of antibodies against ORT, ranging widely from 7.69% to 100% within individual layer hens flocks. The average antibody levels varied considerably among flocks, from 1425.67 to 15233 in different flocks. The current findings indicated widespread ORT exposure in commercial layer farms in the Sfax region of South-eastern Tunisia, signifying the potential for horizontal transmission of ORT, particularly in multi-age

layer integrations where older flocks can act as a source of infection for younger pullets.
Keywords: Biosecurity, Commercial layer, Enzyme-linked immunosorbent assay, *Ornithobacterium rhinotracheale*

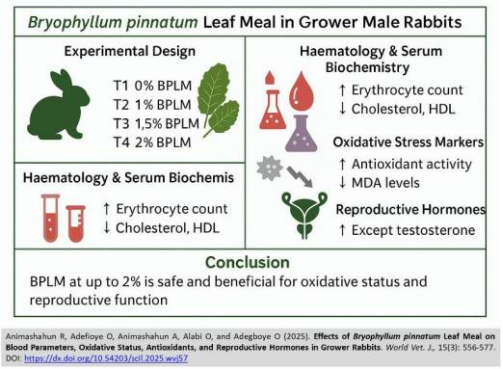
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Research Paper

Effects of *Bryophyllum pinnatum* Leaf Meal on Blood Parameters, Oxidative Status, Antioxidants, and Reproductive Hormones in Grower Rabbits

Animashahun R, Adefioye O, Animashahun A, Alabi O, and Adegboye O.
World Vet. J. 15(3): 566-577, 2025; pii:S232245682500057-15
DOI: https://dx.doi.org/10.54203/scil.2025.wvj57

ABSTRACT: In the search for safe and effective phytogenic alternatives to synthetic additives in rabbit nutrition, *Bryophyllum pinnatum* has shown promising biological potential. This study investigated the effects of dietary inclusion of *Bryophyllum pinnatum* leaf meal (BPLM) on hematological parameters, serum biochemistry, oxidative stress markers, and reproductive hormone levels in grower male rabbits. A total of 48 Hyla grower male rabbits, aged 7–8 weeks, were randomly allotted to four dietary treatments in a completely randomized design (CRD): T1 (0% BPLM), T2 (1% BPLM), T3 (1.5% BPLM), and T4 (2% BPLM), with 12 rabbits per group, further subdivided into three replicates of four animals each. After an 10-week feeding trial, blood samples were collected and analyzed for hematological indices (erythrocyte count, packed cell volume, hemoglobin concentration, erythrocyte indices, total and differential white blood cell counts), biochemical parameters (liver enzymes, total protein, glucose, cholesterol, triglycerides, low-density lipoprotein, and high-density lipoprotein [HDL]), oxidative stress markers (malondialdehyde [MDA] and 2,2-diphenyl-1-picrylhydrazyl scavenging activity [DPPH]), and reproductive hormones. The majority of hematological and biochemical parameters were not significantly affected by BPLM inclusion, indicating no adverse impact on physiological homeostasis. However, erythrocyte count, serum cholesterol, and HDL levels showed significant differences. Erythrocyte count increased progressively from 0% to 1.5% BPLM inclusion, but declined at the 2% level. Serum cholesterol decreased gradually with increasing BPLM inclusion, with the lowest value observed at 2%. Antioxidant activity improved significantly with increasing BPLM levels, as evidenced by enhanced DPPH scavenging capacity and reduced MDA concentrations, particularly at 1.5% and 2% inclusion. Furthermore, reproductive hormones, apart from testosterone, were significantly elevated in rabbits fed diets containing 1.5% and 2% BPLM, suggesting enhanced reproductive function. Overall, these findings indicate that dietary inclusion of BPLM up to 2% is safe and beneficial for growing male rabbits, with positive effects on antioxidant defense and reproductive performance. The functional phytogenic properties of *Bryophyllum pinnatum* (*B. pinnatum*) leaves highlight their potential as a natural feed additive for improving rabbit health and productivity.
Keywords: Functional nutrition, Male fertility, Medicinal plant, Oxidative stress, Phytogenic feed additive, Rabbit



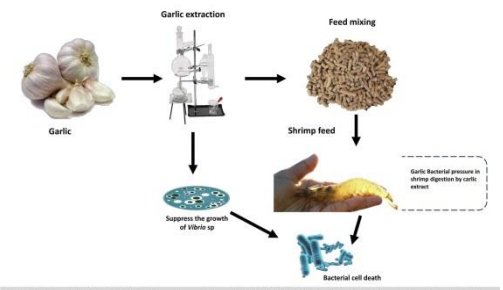
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Research Paper

Evaluation of Garlic Powder as a Control Agent of *Vibrio* sp. in the Digestive Tract of White-leg Shrimp

Saputra A, Suharyadi, Marlina E, Sektiana SP, Samsuharapan SB, and Ulfa AM.
World Vet. J. 15(3): 578-587, 2025; pii:S232245682500058-15
DOI: https://dx.doi.org/10.54203/scil.2025.wvj58

ABSTRACT: Garlic (*Allium sativum*) is a natural plant recognized for its active compounds, such as allicin, which possess antibacterial properties that can combat pathogenic microorganisms. *Vibrio* sp. bacteria can be pathogenic to vannamei shrimp (*Litopenaeus vannamei*), causing mortality marked by necrosis and melanization in the hepatopancreas cells. The present study aimed to examine the chemical compositions of garlic powder and its potential to inhibit the growth of *Vibrio* sp. bacteria in vannamei shrimp. Vannamei shrimp, with a population of 400 shrimp/m^3, were orally administered garlic powder mixed into their feed. The experimental doses were 0.1 mg of garlic powder (Group A), 0.5 mg of garlic powder (Group B), and 1 mg of garlic powder in 1 g of shrimp feed (Group C), along with shrimp that did not receive garlic powder (K, control group). Vannamei shrimp at the post-larva stage were used with a density of 1000 shrimp in each tank. Phytochemical analysis of the garlic powder revealed the presence of compounds



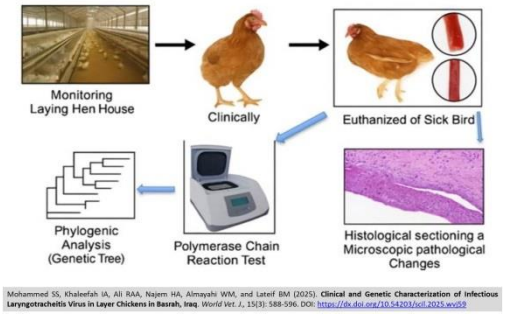
such as flavonoids, alkaloids, tannins, phenols, and saponins. Thin-layer chromatography using quercetin as the reference standard yielded a positive result, indicated by a retention factor of 0.66. Notably, there was a significant reduction in total bacterial counts of *Vibrio* sp. in 40 days of treatment. A garlic powder at a dose of 0.5 mg/g was particularly effective in inhibiting the growth of *Vibrio* sp. bacteria. The current results indicated that shrimp not given garlic powder (control group) exhibited significant atrophy and hepatopancreas infiltration.
Keywords: Garlic, Hepatopancreas, Vannamei Shrimp, *Vibrio* sp.

[Full text-PDF]

Research Paper

Clinical and Genetic Characterization of Infectious Laryngotracheitis Virus in Layer Chickens in Basrah, Iraq

Mohammed SS, Khaleefah IA, Ali RAA, Najem HA, Almayahi WM, and Lateif BM.
World Vet. J. 15(3): 588-596, 2025; pii:S232245682500059-15
DOI: https://dx.doi.org/10.54203/scil.2025.wvj59



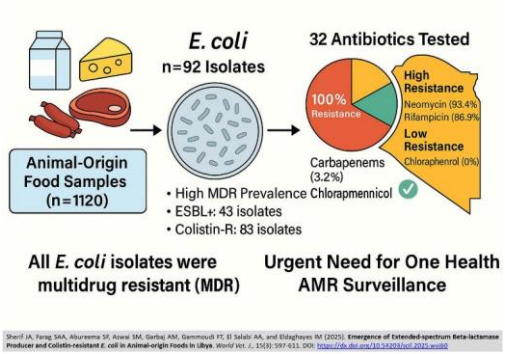
ABSTRACT: Infectious Laryngotracheitis (ILT) has been widely prevalent in numerous countries in recent years. The present study aimed to detect ILT in chickens exhibiting clinical suspicion, utilizing molecular analytical techniques in Basrah, Iraq. Clinical signs, histological changes, and polymerase chain reaction (PCR) were used to diagnose thirty-five samples collected from clinically suspected chickens, including trachea and lungs for histopathology and PCR testing for the *p32* gene. The present study demonstrated that the three isolates of ILTV, including ILT-BASRAH 1, ILT-BASRAH 2, and ILT-BASRAH 3, caused the typical signs and pathological changes of ILT in layers chickens. Phylogenetic analyses indicated that ILT-BASRAH 1 exhibited a distant relationship with other current local isolates. Conversely, the isolates ILT-BASRAH 2 and ILT-BASRAH 3 exhibited a close relationship with each other. The initial results indicated that ILT-BASRAH 1 exhibited high nucleotide similarity to MK895003.1, MK895000.1, and MK894999.1 from Australia, which are representative of recombinant strains derived from live attenuated vaccine strains, as well as PQ232589.1 from Georgia. Furthermore, ILT-BASRAH 2 and ILT-BASRAH 3 were identified as vaccine-related isolates. These isolates demonstrated a high level of genetic similarity and a close phylogenetic relationship to the *Gallid alphaherpesvirus* 1 isolate TJ2019 (GenBank accession no. PP062931.1), originating from Chinese source flocks. The current study determined that ILT-BASRAH isolates 1, 2, and 3 exhibited genetic similarity, suggesting that they likely originated from the same or closely related sources. Their difference from vaccine strains, including the minor variation in ILT-BASRAH 1, might indicate regional viral adaptation.
Keywords: *Gallid alphaherpesvirus*, Histological change, Layer chicken, Phylogenetic

[Full text-PDF]

Research Paper

Emergence of Extended-spectrum Beta-lactamase Producer and Colistin-resistant *E. coli* in Animal-origin Foods in Libya

Sherif JA, Farag SAA, Abureema SF, Azwai SM, Garbaj AM, Gammoudi FT, El Salabi AA, and Eldaghayes IM.
World Vet. J. 15(3): 597-611, 2025; pii:S232245682500060-15
DOI: https://dx.doi.org/10.54203/scil.2025.wvj60



ABSTRACT: The increasing prevalence of *Escherichia coli* (*E. coli*) infection poses significant health challenges worldwide. Understanding the genetic, pathogenic, and antimicrobial resistance profiles of *E. coli* is crucial for effective diagnosis and treatment. The present study aimed to assess the prevalence and antimicrobial susceptibility patterns of *E. coli* isolated from different samples of food products from animals, with specific attention to identifying and characterizing extended-spectrum beta-lactamase (ESBL)-producing isolates. The present study analyzed 92 *E. coli* isolates obtained from 1120 food samples, including milk, dairy products, meat, and meat products, collected randomly from retail markets in Libya. The isolates were tested for antimicrobial susceptibility, and the antibiotic resistance profiles were evaluated using 32 antibiotics from 12 different classes. Multiple antibiotic resistance (MAR) and antibiotic resistance index were calculated, with MAR ≥ 0.2 indicating high antibiotic resistance. Isolates were categorized as multidrug resistant (MDR), extensively drug-resistant (XDR), or pan drug-resistant (PDR) based on standard definitions. The ESBL production was assessed using the double-disc synergy test, and colistin resistance was tested using the agar diffusion method. Antimicrobial susceptibility testing of *E. coli* isolates revealed 100% resistance to penicillin and

cloxacillin, with high resistance rates observed against neomycin (93.4%), rifampicin (86.9%), and methicillin (75%). However, all isolates were susceptible to chloramphenicol, whereas carbapenems (imipenem, meropenem, ertapenem) indicated the lowest resistance (3.2%). Cefepime demonstrated the highest effectiveness among cephalosporins, with a resistance rate of 1.08%. The MAR ranged from 0.09 to 0.6, with the highest MAR value (0.6) observed in isolates resistant to 20 antibiotics. All isolates were MDR, but no XDR or PDR strains were detected. Among the 92 isolates, 43 were confirmed as ESBL producers, primarily originating from raw milk, Iben (fermented milk), and other dairy products. In addition, 83 isolates demonstrated phenotypic resistance to colistin. The present study highlighted the significant presence of MDR *E. coli* in food products of animal origin, particularly raw milk, fermented milk, and chicken meat in Libya, emphasizing the urgent need for antimicrobial stewardship, stronger regulatory frameworks, and integrated One Health surveillance approaches to combat AMR in Libya.
Keywords: Antimicrobial resistance, Colistin, *Escherichia coli*, Extended-spectrum beta-lactamases, Food sample

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Research Paper

Effectiveness of Guava Leaf Fraction on Phagocytosis Activity and Leukocyte Differentiation in Tilapia (*Oreochromis niloticus*) Infected with Aeromonas hydrophila

Wardani LMDK, Andayani S, and Maimunah Y.
World Vet. J. 15(3): 612-621, 2025; pii:S232245682500061-15
DOI:https://dx.doi.org/10.54203/scil.2025.wvj61

ABSTRACT: One obstacle to the sustainability of fisheries is the bacterial infection caused by *Aeromonas hydrophila (A. hydrophila)*. To overcome the sustainability issues posed by antibiotic use against *A. hydrophila* in fisheries, guava leaf (*Psidium guajava* L.) immunostimulants offer a promising natural alternative. The present study aimed to examine the impact of dynamic compounds derived from guava leaves on leukocyte differentiation and phagocytosis in tilapia infected with *A. hydrophila*. A completely randomized design was established, consisting of three treatment groups and two controls, with three replications. The groups included 150 ppm of guava leaf fraction (Group A), 125 ppm of guava leaf fraction (Group B), and 100 ppm of guava leaf fraction (Group C), which were injected into tilapia. The negative control group was infected with *A. hydrophila* bacteria without receiving the guava leaf fraction, and the positive control group was not infected with *A. hydrophila* bacteria and did not receive the guava leaf fraction. The dosage determination of the guava leaf fraction was conducted using the lethal concentration 50% test. The results demonstrated that Fraction 5 of guava leaf extract significantly enhanced the non-specific immune response of tilapia. The optimal dose of 150 ppm led to a substantial increase in lymphocyte counts and phagocytic activity following infection with *A. hydrophila*. Consequently, all treatment groups (100, 125, and 150 ppm) exhibited survival rates statistically equivalent to those of the positive control group, which was significantly higher than the 39% survival rate in the negative control group. Thus, guava leaf Fraction 5, particularly at 150 ppm, serves as an effective immunostimulant and natural antibiotic alternative for controlling A. hydrophila in tilapia.
Keywords: *Aeromonas hydrophila*, Guava leaf fraction, Immune response, Immunostimulant



Wardani LMDK, Andayani S, and Maimunah Y (2025). **Effectiveness of Guava Leaf Fraction on Phagocytosis Activity and Leukocyte Differentiation in Tilapia (*Oreochromis niloticus*) Infected with *Aeromonas hydrophila*.** *World Vet. J.*, 15(3): 612-621. DOI: https://dx.doi.org/10.54203/scil.2025.wvj61

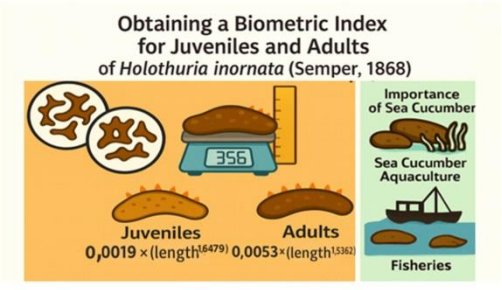
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Research Paper

Obtaining a Biometric Index for Juveniles and Adults of *Holothuria inornata* (Semper, 1868)

Zumaya-Higuera MG, Pacheco-Vega JM, Nieto-Navarro JT, Omont A, Torres-Ochoa E, and Gamboa-Delgado J.
World Vet. J. 15(3): 622-628, 2025; pii:S232245682500062-15
DOI: https://dx.doi.org/10.54203/scil.2025.wvj62

ABSTRACT: The sea cucumber *Holothuria inornata* (*H. inornata*) is a species at risk in Mexico. To help protect this species, it is important to provide information related to biological and fisheries management. The present study aimed to predict the weight of the sea cucumber (*H. inornata*) using biometric measurements such as wet weight and dry weight. A total of 267 *H. inornata* were manually collected from El Borrego Beach in the municipality of San Blas, Nayarit, Mexico. Species identification involved characterizing calcareous ladders. Each specimen was sedated using xylazine 10%, and measurements were taken out of water with a digital scale. Multiple linear regressions were performed, with size serving as the independent variable and wet weight, dry weight, and humidity percentage as dependent variables. A general equation was developed to predict the wet weight of the *H. inornata* at



Zumaya-Higuera MG, Pacheco-Vega JM, Nieto-Navarro JT, Omont A, Torres-Ochoa E, and Gamboa-Delgado J. (2025). **Obtaining a Biometric Index for Juveniles and Adults of *Holothuria inornata* (Semper, 1868).** *World Vet. J.*, 15(3): 622-628. DOI: https://dx.doi.org/10.54203/scil.2025.wvj62

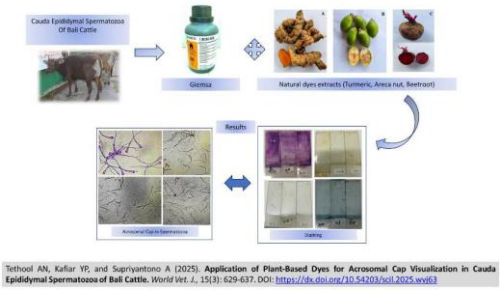
the juvenile stage. For correlations based on dry weight, the following equations were established. In immature adults, including dry weight, the equation was 0.0019 × (length^1.6479); and in mature adults, including dry weight, it was 0.0053 × (length^1.5362). The biometric parameters used for weight estimation exhibited a high degree of correlation with actual weight. Wet weight was the biometric parameter that showed the best fit among the studied parameters. Utilizing these biometric indices facilitates the recording of wet and dry weights for interested parties, thereby supporting population and biomass evaluations within the *H. inornata* fishery and aquaculture sectors.
Keywords: Biometric index, Dry weight, *Holothuria inornata*, Wet weight
[Full text-PDF]

Research Paper

Application of Plant-Based Dyes for Acrosomal Cap Visualization in Cauda Epididymal Spermatozoa of Bali Cattle

Tethool AN, Kafiar YP, and Supriyantono A.
World Vet. J. 15(3): 629-637, 2025; pii:S232245682500063-15
DOI: https://dx.doi.org/10.54203/scil.2025.wvj63

ABSTRACT: The cauda epididymis contains mature spermatozoa capable of fertilization, with acrosomal integrity being a crucial factor in their function. Damage to the acrosome impairs the viability and fertilizing ability of spermatozoa. Conventional staining methods, such as Giemsa or fluorescence microscopy, are effective but less practical in low-resource settings. The present study aimed to evaluate damage to the acrosomal cap of cauda epididymal spermatozoa in Bali cattle and to assess the integrity of the acrosomal cap using Giemsa and natural dyes such as turmeric, beetroot, and areca nut extract. The present study utilized cauda epididymal spermatozoa from Bali cattle aged 3-4 years and weighing 316-412 kg. The study employed a completely randomized design with four treatments, each replicated 20 times. The treatments were Giemsa stain 100% (Control, P0), 10% Giemsa + 90% turmeric extract (P1), 10% Giemsa + 90% beetroot extract (P2), and 10% Giemsa + 90% areca nut extract (P3). Using Giemsa and natural dyes resulted in different colors of spermatozoa acrosomes. The P0 preparations exhibited a dark purple coloration, P1 appeared yellowish, corresponding to the turmeric pigment, and P3 appeared bluish, while P2 demonstrated no detectable staining on the slides. The acrosomal caps of spermatozoa in P0 were dark purple, those in P1 were yellowish, and those in P2 and P3 were unstained (Transparent or faint). The average acrosomal cap integrity was the highest in P0, followed in order by P1, P2, and P3. Based on the current findings, it can be concluded that P1, P2, and P3 signified acrosomal cap integrity in cauda epididymal spermatozoa of Bali cattle. However, P1 produced the best results, showing a distinguishable color and the highest acrosomal cap integrity score (86.9 ± 2.0%). Beetroot and areca nut extracts produced weaker staining and lower acrosomal integrity values. Therefore, turmeric extract may serve as a practical, cost-effective, and environmentally friendly alternative for acrosome evaluation in veterinary reproductive laboratories, particularly in resource-limited settings.
Keywords: Giemsa, Intact acrosomal cap, Natural dye, Spermatozoa



Tethool AN, Kafiar YP, and Supriyantono A (2025). **Application of Plant-Based Dyes for Acrosomal Cap Visualization in Cauda Epididymal Spermatozoa of Bali Cattle.** *World Vet. J.*, 15(3): 629-637. DOI: https://dx.doi.org/10.54203/scil.2025.wvj63

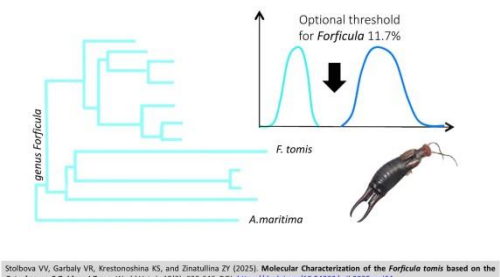
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Research Paper

Molecular Characterization of the *Forficula tomis* based on the *Cytochrome C Oxidase* I Gene

Stolbova VV, Garbaly VR, Krestonoshina KS, and Zinatullina ZY.
World Vet. J. 15(3): 638-646, 2025; pii:S232245682500064-15
DOI: https://dx.doi.org/10.54203/scil.2025.wvj64

ABSTRACT: The ovine Earwigs of the genus *Forficula* play a significant role in agriculture and beekeeping, acting as pests and vectors of honey bee pathogens. Despite the ecological and agricultural significance, nucleotide sequences of the species *Forficula tomis* (*F. tomis*) are absent in genetic databases, which complicates its molecular identification. The present study aimed to perform the molecular characterization of the earwig *F. tomis* using the *cytochrome c oxidase I* (*COI*) gene fragment as a molecular marker. A total of 18 earwig specimens were hand-collected from *Apis mellifera* hives in the Tyumen region, Russia. During the cultivation, 9 adults were obtained, which were used in the present study. Morphological analysis confirmed that *F. tomis* is the predominant species of bee hives in the Tyumen region. Sequencing of the *COI* gene fragment made it possible to obtain a reference sequence of *F. tomis*, which, in phylogenetic analysis, formed a separate clade and demonstrated a significant genetic distance (up to 22.9%) with other representatives of the genus *Forficula*. The optimal species differentiation threshold for the group was 11.7%, which significantly exceeds the standard barcode gap (3%) and may indicate long-term evolutionary isolation or the presence of cryptic diversity. The obtained results



Stolbova VV, Garbaly VR, Krestonoshina KS, and Zinatullina ZY (2025). **Molecular Characterization of the *Forficula tomis* based on the *Cytochrome C Oxidase I* Gene.** *World Vet. J.*, 15(3): 638-646. DOI: https://dx.doi.org/10.54203/scil.2025.wvj64

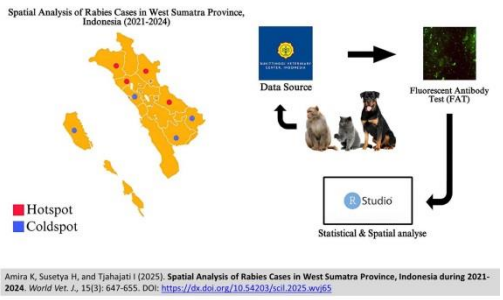
expand the present understanding of the genetic structure of the genus *Forficula* and have practical significance for monitoring apiary pests, developing methods for molecular identification of bee pests, and understanding their role in the spread of pathogens.
Keywords: Beekeeping, C*ytochrome c oxidase I* gene, DNA barcoding, Earwigs, *Forficula tomis*

[Full text-PDF]

Research Paper

Spatial Analysis of Rabies Cases in West Sumatra Province, Indonesia during 2021-2024

Amira K, Susetya H, and Tjahajati I.
World Vet. J. 15(3): 647-656, 2025; pii:S232245682500065-15
DOI: https://dx.doi.org/10.54203/scil.2025.wvj65



Amira K, Susetya H, and Tjahajati I (2025). **Spatial Analysis of Rabies Cases in West Sumatra Province, Indonesia during 2021-2024**. *World Vet. J.*, 15(3): 647-655. DOI: https://dx.doi.org/10.54203/scil.2025.wvj65

ABSTRACT: The West Sumatra Province, Indonesia, has been an endemic rabies region since its initial rabies incident in 1953. This study aimed to analyze spatial patterns for rabies cases in animals in 19 districts/cities in West Sumatra Province, Indonesia. Secondary data on rabies-positive cases in animals were identified in 198 samples through the Fluorescent Antibody Test (FAT) obtained from the Bukittinggi Veterinary Center's database from 2021 to 2024. Statistical and spatial analyses were conducted in R Software v.4.4.2. The Moran's Index revealed a cluster distribution of rabies cases throughout the study period. The highest prevalence occurs in Lima Puluh Kota District (28.79%). The index indicated four regions as high-high quadrants, three regions as low-high quadrants, 11 regions as low-low quadrants, and one region as a high-low quadrant. However, Lima Puluh Kota District was consistently found as a region with substantial local spatial autocorrelation. In conclusion, the prevalence of rabies cases in animals in West Sumatra Province fluctuated during 2021-2024. Spatial analysis with Moran's Index and Local Indicator of Spatial Autocorrelation (LISA) indicated a cluster distribution of the disease. The current study underscored the need for targeted, location-specific control measures in these high-risk clusters to effectively mitigate the spread of the disease.
Keywords: Fluorescent Antibody Test, Moran's Index, Rabies, Spatial analysis

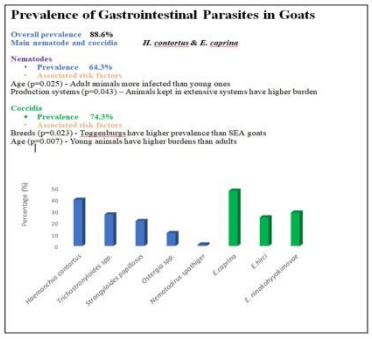
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Research Paper

Prevalence and Intensity of Gastrointestinal Parasites and Related Risk Factors in Goats Kept by Smallholder Farmers in Juja Sub-County, Kenya

Koyan OC, Kagira J, Waweru KP, Naomi M, and Osuga I.

World Vet. J. 15(3): 656-668, 2025; pii:S232245682500066-15
DOI: https://dx.doi.org/10.54203/scil.2025.wvj66



Koyan OC, Kagira J, Waweru KP, Naomi M, and Osuga I (2025). **Prevalence and Intensity of Gastrointestinal Parasites and Related Risk Factors in Goats Kept by Smallholder Farmers in Juja Sub-County, Kenya**. *World Vet. J.*, 15(3): 656-668 DOI: https://dx.doi.org/10.54203/scil.2025.wvj66

ABSTRACT: Gastrointestinal parasites (GIP) negatively impact the health and productivity of goats. Regular screening of the animals for these parasites is crucial for implementing effective control strategies. A cross-sectional study was conducted to determine the prevalence of GIP and its risk-related factors in goats owned by smallholder farmers in Juja Subcounty, Kiambu County, Kenya. A total of 210 goats consisting of both males and females, of different breeds including Boer, Toggenburg, Alpine, and Small East African were randomly sampled from 80 farms. Fresh fecal samples were collected and analyzed for parasites using McMaster techniques for egg/oocyst examination. A structured questionnaire was administered to assess the risk factors such as age, sex, breed, origin of the goats, production system, and purpose and frequency of cleaning goat houses in relation to the prevalence of the parasites. The overall prevalence of the GIP was 88.6%, with nematodes and coccidia detected in 64.3% and 74.3% of the goats, respectively. The mean eggs per gram was 67.72, while the mean oocysts per gram was 243. Goat fecal samples positive for gastrointestinal nematodes (GIN) eggs were cultured and identified after recovery of third-stage larvae (L3) using the Baermann technique, whereas the coccidian oocysts were sporulated in 2.5% potassium dichromate solution, followed by microscopic examination based on morphological features of the parasites. The identified GIN nematodes included *Haemonchus contortus*, *Trichostrongylus* spp., *Strongyloides papillosus*, *Ostertagia* spp., and *Nematodirus spathiger,* while the coccidians were *Eimeria caprina*, *Eimeria ninakohlyakimovae,* and *Eimeria hirci*. The prevalence of GIN was significantly associated with age (higher in adults), production systems (higher in intensive systems), and body condition score (higher in animals with poor body condition). For coccidia, the prevalence was associated with the animal origin (higher in goats from Murera Ward), breed (higher in small East African goats), age (higher in younger goats), body

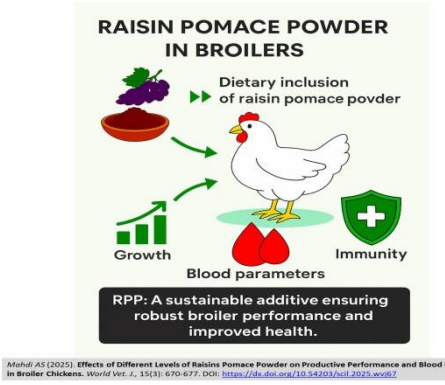
condition score (higher in goats with poor condition), and cleaning frequency (higher in goats kept in irregularly cleaned pens). In conclusion, the overall prevalence and burden of GIP reported in the study were high, with the pathogenic gastrointestinal nematodes and *Eimeria* spp. being the most prevalent parasites.
Keywords: *Coccidia*, Goat, Nematode, Prevalence, Risk factor

[Full text-PDF]

Research Paper

Effects of Different Levels of Raisins Pomace Powder on Productive Performance and Blood Parameters in Broiler Chickens

Mahdi AS.
World Vet. J. 15(3): 669-677, 2025; pii:S232245682500067-15
DOI: https://dx.doi.org/10.54203/scil.2025.wvj67



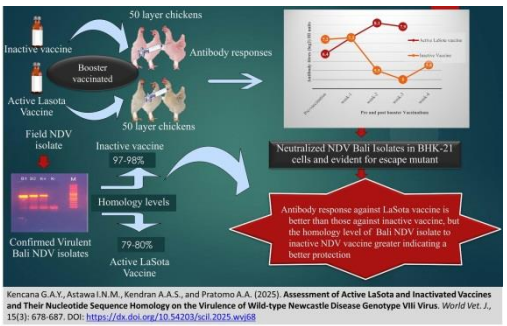
ABSTRACT: One of the primary concerns in poultry farming is the exploration of natural feed ingredient alternatives that are, concurrently, more cost-effective and environmentally sustainable. Raisin pomace powder (RPP), a by-product from grape processing, may contain numerous beneficial nutrients and natural compounds that contribute to optimal performance in chickens. The present study assessed the impact of incorporating RPP into broiler chicken diets concerning growth performance, carcass characteristics, economic efficiency, blood parameters, and immune indices. A total of 180 unsexed Ross 308 broiler chickens, all one day old with an initial average body weight of approximately 38 g, were randomly divided into four dietary treatments. The present study comprised a control group (T1) and three experimental groups, including 10 g/kg of RPP (T2), 20 g/kg of RPP (T3), and 30 g/kg of RPP (T4). The study duration spanned 35 days, covering both the initial and finisher phases. The present results demonstrated no statistically significant differences in body weight, weight gain, feed intake, dressing percentage, or economic efficiency among the treatment groups. However, the feed conversion ratio was notably higher in T2 in comparison to the control group. Hematological parameters, encompassing packed cell volume, hemoglobin concentration, as well as red and white blood cell counts, were not significantly influenced by the inclusion of RPP. Serum levels of total protein and globulin remained stable across groups, whereas albumin levels exhibited a significant decrease in T4. Additionally, cholesterol levels were reduced in T3 compared to T1. The current findings suggested that RPP can be included in broiler diets up to 30 g/kg without adverse effects on productivity, physiological status, or immune development. Utilizing RPP provided a sustainable strategy for poultry nutrition through the employment of agro-industrial by-products, thereby potentially improving feed resource efficiency and environmental sustainability.
Keywords: Broiler chicken, Bursa of Fabricius, Economic efficiency, Feed conversion ratio, Productive performance, Raisin pomace powder

[Full text-PDF]

Research Paper

Assessment of Active LaSota and Inactivated Vaccines and Their Nucleotide Sequence Homology on the Virulence of Wild-type Newcastle Disease Genotype VIIi Virus

Kencana G.A.Y., Astawa I.N.M., Kendran A.A.S., and Pratomo A.A.
World Vet. J. 15(3): 678-687, 2025; pii:S232245682500068-15
DOI: https://dx.doi.org/10.54203/scil.2025.wvj68



ABSTRACT: Many vaccines are commercially available to prevent Newcastle Disease (ND) in chickens, but the disease is still endemic in many countries. The present study aimed to evaluate the efficacy of ND vaccines *in vivo* and to analyze the nucleotide sequence similarity between the vaccines and the wild-type virus encountered in the field, as well as the underlying reasons for vaccination failures against ND in chickens. One hundred laying Isabrown hens, aged 22 weeks with an average weight of 1.75 kg, from two farms (Farm A and B) in Tabanan, Bali, Indonesia, served as experimental animals. All laying hens had previously been vaccinated four times orally with LaSota vaccines (Farm A) and alternately with LaSota and inactive ND vaccines (Farm B). They were then given a booster vaccination orally with live LaSota (Farm A) and intramuscularly with an inactive vaccine (Farm B). Sera samples were collected every week for four weeks in Farm A and for five weeks in Farm B, and the antibody titers were examined by hemagglutination inhibition (HI) test. The genotypes of the field isolates were determined through sequencing and bioinformatics analysis using MEGA 7. The mean $\log_2$ antibody titers for hens vaccinated with the LaSota vaccine were 7.2 HI units before vaccination, 7.3 HI units at one week post-vaccination, 8.1 HI units at two weeks post-vaccination, and 7.9 HI units at three weeks post-vaccination. In contrast, the mean $\log_2$ antibody titers for those vaccinated with the

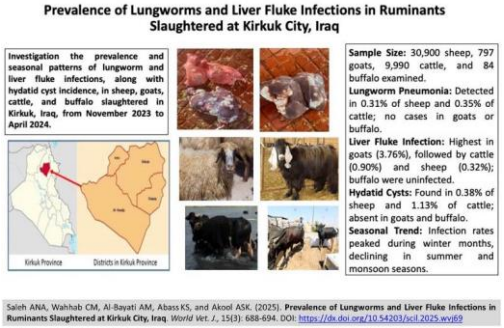
inactive NDV vaccine were 6.4 HI units at pre-vaccination, 7.3 HI units at one week, 5.5 HI units at two weeks, 5.0 HI units at three weeks, and 5.8 HI units at four weeks post-vaccination. The NDV-LaSota vaccine illustrated 80-81% similarity to a recent ND virus isolate from chickens in Bali (ND/chicken/GAYK01/Penebel Bali/2023), while inactive vaccines exhibited 96-98% similarity. Thus, vaccines were closely related to the Bali virus, whereas the active vaccine was less similar. Based on antibody responses and homology levels, the inactive NDV vaccine induced stronger protective immunity against NDV field isolates than the Active LaSota vaccine.
Keywords: Antibody titer, Field isolate, Neutralization, Newcastle disease vaccine, Sequencing

[Full text-PDF]

Research Paper

Prevalence of Lungworms and Liver Fluke Infections in Ruminants Slaughtered at Kirkuk City, Iraq

Saleh ANA, Wahhab CM, Al-Bayati AM, Abass KS, and Akool ASK.
World Vet. J. 15(3): 688-694, 2025; pii:S232245682500069-15
DOI: https://dx.doi.org/10.54203/scil.2025.wvj69



Saleh ANA, Wahhab CM, Al-Bayati AM, Abass KS, and Akool ASK. (2025). **Prevalence of Lungworms and Liver Fluke Infections in Ruminants Slaughtered at Kirkuk City, Iraq**. *World Vet. J.*, 15(3): 688-694. DOI: https://dx.doi.org/10.54203/scil.2025.wvj69

ABSTRACT: Parasitic infections represent one of the most prevalent and economically significant diseases affecting livestock worldwide. The present study aimed to investigate risk factors associated with liver fluke and lungworm infections in small ruminants, characterize the diversity of lungworm species, and determine the prevalence of infections in Kirkuk, Iraq. The study was conducted from November 2023 to April 2024 in Northeastern Iraq, examining 30,900 sheep, 797 goats, 9,990 cattle, and 84 buffalo at slaughter. All carcasses underwent thorough gross pathological examination to detect parasitic infections, including lungworms, liver flukes, hydatid cysts, and associated pulmonary lesions. Distinct pathological manifestations were particularly evident in naturally infected local sheep. The present study revealed the following disease prevalence patterns across species. Pneumonia was detected in 0.31% of sheep and 0.35% of cattle, with no cases observed in goats or buffalo. Hepatitis showed an incidence rate of 0.39% in sheep and 0.75% in cattle. Hydatid cyst occurrence in hepatic and pulmonary tissues was documented at 0.38% in sheep and 1.13% in cattle, while remaining undetectable in caprine and bovine specimens. Liver fluke infection rates varied significantly among species, with sheep exhibiting 0.32% prevalence, goats demonstrating the highest rate at 3.76%, cattle showing 0.90% infection, and buffalo populations remaining completely uninfected. The present findings revealed significantly higher disease susceptibility in ruminants during winter months, with progressively lower infection rates observed throughout summer and monsoon seasons. This seasonal pattern may reflect environmental factors influencing parasite life cycles and host immunity.
Keywords: Animal disease, Hydatid cyst, Parasitic infection, Pneumonia, Ruminant

[Full text-PDF]

Research Paper

Veterinarians' Perceptions of Competence and Communication Skills in Bali, Indonesia

Dharmawan NS, Wulanyani NMS, and Agustina KK.
World Vet. J. 15(3): 695-702, 2025; pii:S232245682500070-15
DOI: https://dx.doi.org/10.54203/scil.2025.wvj70



Dharmawan NS, Wulanyani NMS, and Agustina KK (2025). **Veterinarians' Perceptions of Competence and Communication Skills in Bali, Indonesia**. *World Vet. J.*, 15(3): 695-702. DOI: https://dx.doi.org/10.54203/scil.2025.wvj70

ABSTRACT: Effective communication is a crucial aspect of veterinary medicine. Despite its importance, this subject has not been sufficiently studied in the specific context of Bali, Indonesia, resulting in a notable gap in the existing research literature. The present study addressed a significant gap in the literature by systematically examining how communication challenges, specifically client dissatisfaction, inadequate treatment compliance, and recurrent misunderstandings between veterinarians and pet owners, affect the quality of veterinary care. The current study explored how veterinary practitioners in Bali, Indonesia, perceive their professional skills and communication abilities. Data were collected through interviews, focus group discussions, open-ended questionnaires, and surveys, involving 218 practitioners in the study area. The participants included small animal practitioners (65%), livestock veterinarians (25%), and mixed-practice clinicians (10%), with experience ranging from recent graduates to senior professionals with over 20 years in the field. The current findings revealed that 86.2% of respondents considered communication skills as equally important as clinical knowledge in facilitating critical professional outcomes, including client trust, treatment compliance, and clinical effectiveness. Effective communication was recognized as crucial for fostering confidence, increasing job satisfaction, strengthening client relationships, and supporting important activities such as anamnesis and treatment discussions. However, practitioners encountered difficulties in discussing sensitive topics, including life-threatening diagnoses, euthanasia, and costly treatment options. Notably, 86.7% of participants indicated the necessity

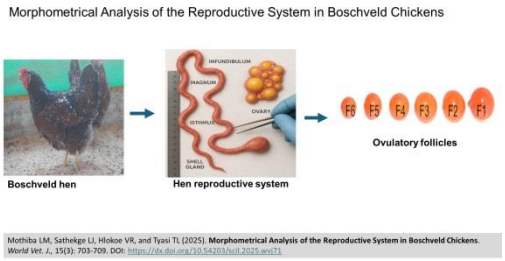
for post-employment communication training to maintain and advance their skills, highlighting the significance of ongoing professional development in Indonesia. The present study emphasized the critical role of communication in veterinary practice and highlighted the demand for targeted training programs to address existing gaps.
Keywords: Client, Communication skill, Perception, Veterinarian

[Full text-PDF]

Research Paper

Morphometrical Analysis of the Reproductive System in Boschveld Chickens

Mothiba LM, Sathekge LJ, Hlokoe VR, and Tyasi TL.
World Vet. J. 15(3): 703-709, 2025; pii:S232245682500071-15
DOI: https://dx.doi.org/10.54203/scil.2025.wvj71



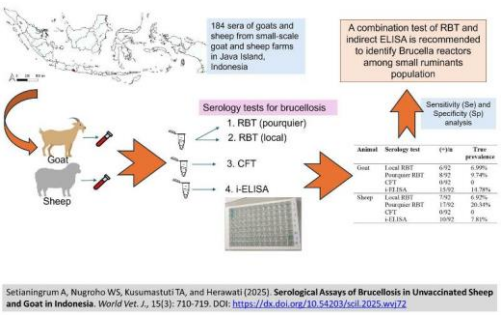
ABSTRACT: The egg yield is determined by the number of ovarian follicles that ovulate and the oviduct's capability to form a hard-shelled egg around the egg cell. However, the morphological characteristics of the reproductive organs in Boschveld chickens have not been explored. The present study aimed to evaluate the morphology of the reproductive system, including the oviduct and the developing ova in the Boschveld chicken breed. To perform a morphological analysis, ovarian follicles and oviducts were obtained from six Boschveld hens (Out of a total of twenty), aged 30 weeks, sourced from the University of Limpopo experimental farm in South Africa. The present results revealed significant differences in both the weight and number of small white (2-4 mm), large white (5-6 mm), and small yellow (7-8 mm) follicles. Among the mature follicles, Follicle 1 (F1) exhibited the highest weight, whereas F6 demonstrated the lowest weight. Additionally, the weights and lengths of the infundibulum, magnum, isthmus, and shell gland varied significantly in Boschveld chickens. The present findings indicated that the magnum was the heaviest and longest oviduct segment in Boschveld chickens, and the heaviest pre-ovulatory follicle was ovarian F1, followed by ovarian F2. The results revealed a distinct profile characterized by many small white follicles and a well-developed magnum, which is the largest part of the oviduct. These findings provided a crucial anatomical baseline for the breed, indicating that the structure of the follicular hierarchy and oviduct plays a crucial role in its egg-laying ability. Improving the development and function of these reproductive parts could boost productivity in the Boschveld breed.
Keywords: Boschveld chicken, Isthmus, Magnum, Morphology, Ovarian follicle

[Full text-PDF]

Research Paper

Serological Assays of Brucellosis in Unvaccinated Sheep and Goat in Indonesia

Setianingrum A, Nugroho WS, Kusumastuti TA, and Herawati.
World Vet. J. 15(3): 710-719, 2025; pii:S232245682500072-15
DOI: https://dx.doi.org/10.54203/scil.2025.wvj72



ABSTRACT: Brucellosis is recognized as one of the most widespread zoonotic diseases and is often overlooked, impacting livestock populations worldwide. Annual routine screenings utilize serological assays, such as the Rose Bengal test (RBT) and the complement fixation test (CFT), to identify the presence of brucellosis. The present study aimed to evaluate the diagnostic and discriminative performance of the CFT, the locally produced RBT antigen from Indonesia, the commercial RBT antigen (Pourquier), and the indirect enzyme-linked immunosorbent assay (ELISA) using sera from Indonesian goats and sheep. A total of 184 serum samples were collected, including 92 from goats and 92 from sheep. The samples were obtained through jugular venipuncture from animals on small-scale goat and sheep farms in Java Island, Indonesia. The results were analyzed to determine true prevalence (TP), accuracy, and cross-tabulation with parallel interpretation to assess the sensitivity and specificity of each test analysis. The TP of brucellosis in small ruminant serum samples using local RBT, Pourquier RBT, CFT, and indirect ELISA was 6.99%, 9.74%, 0%, and 14.78% in goats, and 6.92%, 20.34%, 0%, and 7.81% in sheep, respectively. Moderate Kappa interrater reliability was observed between the local and Pourquier RBTs for both goats (0.537) and sheep (0.440). The combined RBT sensitivity in goats and sheep was 27% and 20%, respectively, while the combined RBT specificity was 92% in goats and 80.5% in sheep. Using more than one diagnostic method is essential for screening and investigating brucellosis in goat and sheep populations. A combination of RBT and indirect ELISA tests is recommended to identify *Brucella* reactors among small ruminants in Indonesia.
Keywords: Brucellosis, Complement fixation test, Indirect enzyme-linked immunosorbent assay, Rose Bengal test, Small ruminant

[Full text-PDF]

Research Paper

Tick Fauna (Acari: Ixodidae) of Domestic Ruminants in the Hauts-Bassins Region, Burkina Faso: A Prevalence and Seasonal Survey

Sidiki BHEA, Fidèle TD, Alizèta S, Adama K, and Hamani M.

World Vet. J. 15(3): 720-733, 2025; pii:S232245682500073-15
DOI: https://dx.doi.org/10.54203/scil.2025.wvj73



ABSTRACT: As in other West African countries, ticks (Acari: Ixodidae) infesting domestic ruminants are a major constraint for livestock breeders in Burkina Faso. The present epidemiological survey study was conducted from October 2022 to September 2023 in two pastoral areas of the Hauts-Bassins region of Burkina Faso to inventory the tick genera and species present, and determine the prevalence and associated risk factors in infested ruminants, including host sex, age, and season. A total of 8,274 live ticks were collected from 800 ruminants randomly selected (300 cattle, 300 sheep, and 200 goats) across two pastoral zones (Sidéradougou and Saho pastoral zones) and three seasons (cold dry season, hot dry season, and rainy season) from the herds of 140 livestock farmers surveyed. Morphological identification identified six species within the genera *Amblyomma* (67.32%), *Hyalomma* (26.35%), and *Rhipicephalus* (*Boophilus*, 6.33%), with *Amblyomma variegatum* as the dominant species. The overall prevalence of tick infestation was 74.90%, including 37.25% in cattle, 23.50% in sheep, and 14.15% in goats, with an average infestation level of 10.34 ± 20.30 ticks per animal. Male ruminants (52.62%) were more infested than females (47.38%), and juveniles (41.37%) were more infested than adults (58.63%). Seasonal patterns revealed peak infestation during the rainy and cold dry seasons in both Pastoral zones, and ticks showed a predilection for the inguinal and pastern regions. These findings highlight the extensive tick burden in Hauts-Bassins region, Burkina Faso's pastoral systems, and underscore the need for targeted, season- and host-specific control strategies to mitigate tick-borne disease risks and improve livestock productivity.
Keywords: Pastoral area, Prevalence, Ruminant, Seasonal dynamic, Tick infestation

[Full text-PDF]

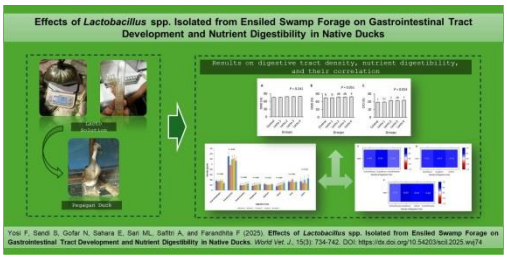
Research Paper

Effects of Lactobacillus spp. Isolated from Ensiled Swamp Forage on Gastrointestinal Tract Development and Nutrient Digestibility in Native Ducks

Yosi F, Sandi S, Gofar N, Sahara E, Sari ML, Safitri A, and Farandhita F.

World Vet. J. 15(3): 734-742, 2025; pii:S232245682500074-15

DOI: https://dx.doi.org/10.54203/scil.2025.wvj74



ABSTRACT: The application of lactic acid bacteria (LAB), particularly *Lactobacillus*, as feed additives has demonstrated benefits for poultry, including enhanced gut function and better nutrient digestion. However, studies on LAB derived from swamp grass silage remain limited. The present study aimed to evaluate the effects of supplementing *Lactobacillus* spp. (Lacto) solution derived from ensiled swamp forage (*Hymenachne acutigluma*) at different concentrations through drinking water on the relative weight and length, as well as intestinal density and nutrient digestibility of native ducks. The relationship between intestinal tract density and nutrient digestibility was assessed using the Pearson correlation coefficient. The present study involved sixty 24-week-old native ducks, divided into five groups with four replicates each. The groups included a control group and groups that received Lacto solutions in their drinking water. The Lacto solutions were at concentrations of 1×10^6 CFU/mL (Lacto 1), 1×10^7 CFU/mL (Lacto 2), 1×10^8 CFU/mL (Lacto 3), and 1×10^9 CFU/mL (Lacto 4). The current results indicated that supplementation of the Lacto solution in drinking water increased the relative weights of the proventriculus, small intestine, duodenum, jejunum, ileum, and ceca. The relative weight and density of the intestine in the Lacto groups increased linearly with higher concentrations of Lacto solution. Compared to the control group, ducks receiving Lacto supplementation showed improved crude fiber digestibility (CFD) and a tendency to enhance organic matter digestibility (OMD). In 33-week-old ducks, a higher proventriculus density was associated with increased dry matter digestibility (DMD) and OMD. Additionally, the densities of the duodenum, jejunum, and ileum were positively correlated with DMD, OMD, and CFD. The present findings indicated that the administration of Lacto solution at increasing concentrations up to 10^9 CFU/mL via drinking water effectively improved the development of the small intestines of ducks, which was indicated by an increase in the intestinal relative weight and density, as well as enhancing the OMD and CFD in the diets.
Keywords: Drinking water, Lactic acid bacteria, *Lactobacillus*, Native duck, Nutrient digestibility, Swamp forage

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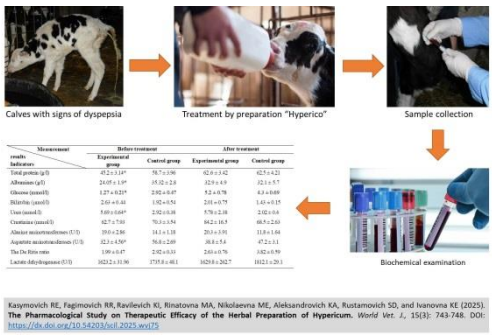
Research Paper

The Pharmacological Study on Therapeutic Efficacy of the Herbal Preparation of Hypericum

Rakhmatullin EK, Rakhmatullov RF, Kadikov IR, Makaeva AR, Mayorova EN, Korchemkin AA, Sagdeev DR, and Kurshakova EI

World Vet. J. 15(3): 743-748, 2025; pii:S232245682500075-15

DOI: https://dx.doi.org/10.54203/scil.2025.wvj75



ABSTRACT: Dyspepsia is a condition associated with digestive disorders, including diarrhea, weakness, dehydration, and intoxication. The investigation and application of therapeutic and prophylactic agents for managing gastrointestinal diseases in young cattle, including dyspepsia in newborn calves, is concerning. The present study aimed to investigate the biochemical mechanisms of action of Hypericum in the treatment of dyspepsia in newborn calves. Two groups of black and white breed bulls (five animals per group), aged 8-10 days, were assigned to assess the effects of Hypericum on biochemical parameters, including total protein, albumin, glucose, bilirubin, urea, creatinine, aspartate aminotransferase, alanine aminotransferase, and lactate dehydrogenase. Calves in the first group (n = 5), exhibiting pronounced signs of dyspepsia, were administered the treatment. This treatment consisted of 250 mL of Hypericum, combined with 500-700 mL of warm water, instead of milk, during the following two feedings. Then, milk was added to each serving of this mixture until the total volume reached 1.25-1.50 liters, and it was given to calves until clinical signs of recovery appeared. Calves of the second group (n = 5) were clinically healthy and received boiled water instead of the Hypericum. The current findings demonstrated that the biochemical parameters of the experimental calves improved following the administration of Hypericum, reaching levels comparable to those of clinically healthy calves. After prophylactic administration of Hypericum at a dose of 6.5 mL/kg during the first six feedings, the general condition of the experimental calves was satisfactory. In the experimental group of calves administered Hypericum at a dosage of 6.5 mL/kg mixed with milk over a period of two days, only one out of fifteen calves exhibited signs of dyspepsia, including liquid feces and lethargic behavior within the initial four days, representing a prevalence of 6.7%. Hypericum could prevent the occurrence of dyspepsia by 93%. The utilization of Hypericum had a therapeutic influence and positively impacted the clinical condition, behavior, and appetite of the calves.

Keywords: Albumin, Biochemical indicator, Calf, Dyspepsia, Hypericum, Total protein

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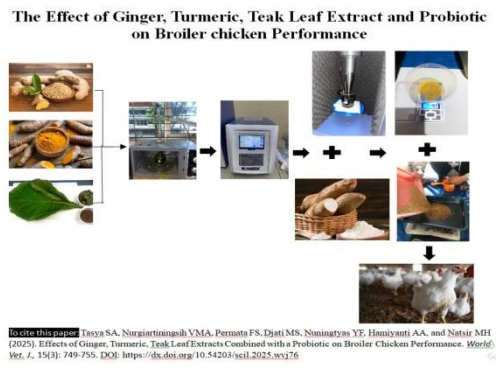
Research Paper

Effects of Ginger, Turmeric, Teak Leaf Extracts Combined with a Probiotic on Broiler Chicken Performance

Tasya SA, Nurgiartiningsih VMA, Permata FS, Djati MS, Nuningtyas YF, Hamiyanti AA, and Natsir MH.

World Vet. J. 15(3): 749-755, 2025; pii:S232245682500076-15

DOI: https://dx.doi.org/10.54203/scil.2025.wvj76



ABSTRACT: In poultry production, the transition to antibiotic-free systems has resulted in an increased demand for effective natural growth promoters. The present study aimed to evaluate the effectiveness of a mash-form feed additive made from microwave-extracted ginger, turmeric, and teak leaf phytobiotics, combined with a multi-strain bacterium as a probiotic, administered at graded inclusion levels in broiler chickens. A 35-day feeding experiment was conducted with 300 broiler chickens, arranged in a completely randomized design with five dietary treatments and five replicates per treatment. In the first group, chickens received only the basal diet and served as the control (T0). The treatment groups received the basal feed top-dressed with the phytobiotic and probiotic additive at inclusion levels of 0.2% (T1), 0.4% (T2), 0.6% (T3), and 0.8% (T4). Measurements of performance, including body weight gain (BWG), feed conversion ratio (FCR), performance index, and income over feed cost (IOFC), were collected and analyzed. High-performance liquid chromatography confirmed the presence of key bioactive compounds, such as curcumin and 6-gingerol. The inclusion of 0.2% bacterium as a probiotic additive resulted in optimal BWG, FCR, and the highest IOFC, indicating that this level was biologically effective and economically efficient. In contrast, higher inclusion levels of bacteria as probiotics led to reduced performance, likely due to polyphenol-induced mineral binding and issues

with feed palatability. The co-administration of probiotics may enhance the bioavailability of curcumin and promote a healthier gut environment. The present findings highlighted the potential of phytobiotic-probiotic combinations as alternatives to antibiotic growth promoters when used at optimal dose levels.
Keywords: Antibiotic-free, Ginger, Performance, Probiotic, Teak leaf, Turmeric

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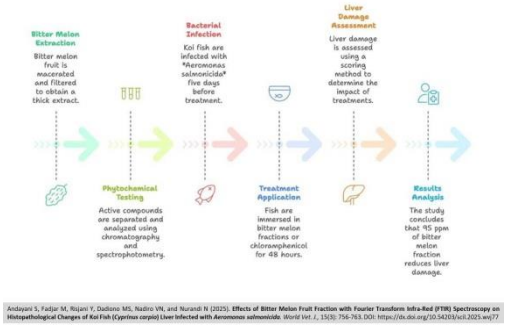
Research Paper

Effects of Bitter Melon Fruit Fraction with Fourier Transform Infra-Red (FTIR) Spectroscopy on Histopathological Changes of Koi Fish (*Cyprinus carpio*) Liver Infected with *Aeromonas salmonicida*

Andayani S, Fadjar M, Risjani Y, Dadiono MS, Nadiro VN, and Nurandi N.

World Vet. J. 15(3): 756-763, 2025; pii:S232245682500077-15

DOI: https://dx.doi.org/10.54203/scil.2025.wvj77

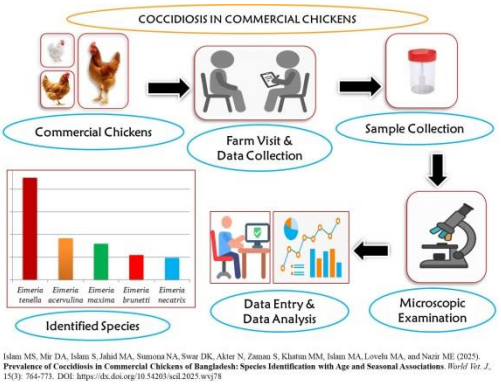


ABSTRACT: Bitter melon is widely recognized for its medicinal properties, including its potential health benefits in various species. The present study investigated the impact of the bitter melon fraction on liver histological changes of koi fish infected with *Aeromonas salmonicida* (*A. salmonicida*). Maceration of bitter melon fruit was carried out for 4 days, then filtered and evaporated to obtain a thick extract. Phytochemical testing included column chromatography for the separation of active compounds and UV-Vis spectrophotometry for the analysis of the absorbance spectra of the fractions. The *A. salmonicida* bacterial infection was performed five days prior to the fish being soaked in a bitter melon fraction, at a bacterial density of 10^7 cells/ml for 48 hours. A completely randomized design was employed, featuring five treatments, including no bitter melon fraction with no infection (K -), other groups infected with *A. salmonicida* and supplemented with 75 ppm of bitter melon (A), 95 ppm of bitter melon (B), 115 ppm of bitter melon (C), and 5 ppm chloramphenicol (antibiotic, K +). Each treatment group consisted of 15 fish (average length and weight of Koi fish were 8 cm and 12 grams) with three replications, which were immersed in their respective solutions for 48 hours. The current study adopted the maceration method with 98% ethanol for the extraction of bitter melon fruit. To determine liver histological damage in Koi fish, the authors employed the scoring method. The findings indicated slight liver damage in histology parameters with an average score of 1.2-1.4 for treatments A and B, respectively; however, treatment C and the positive control exhibited moderate damage with an average necrosis score of 2. According to the obtained data, the bitter melon fruit fraction at a concentration of 95 ppm had the highest preventive effects on the liver of the Koi fish infected with *A. salmonicida*.
Keywords: Bitter melon fruit, Fraction, Koi fish, Liver
[Full text-PDF]

Research Paper

Prevalence of Coccidiosis in Commercial Chickens of Bangladesh: Species Identification with Age and Seasonal Associations

Islam MS, Mir DA, Islam S, Jahid MA, Sumona NA, Swar DK, Akter N, Zaman S, Khatun MM, Islam MA, Lovelu MA, and Nazir ME.
World Vet. J. 15(3): 764-773, 2025; pii:S232245682500078-15
DOI: https://dx.doi.org/10.54203/scil.2025.wvj78



ABSTRACT: Coccidiosis is one of the most economically significant diseases in the poultry industry, resulting in substantial economic losses through treatment costs, reduced growth rates, and decreased body weight gain. The present study aimed to determine the prevalence of different *Eimeria* (*E.*) species in commercial chickens and to assess age-specific and seasonal patterns of coccidiosis. A total of 595 chicken fecal samples were randomly collected from three different chicken types, namely broiler breeds (n = 227), layer breeds (n = 205), and Sonali (a dual-purpose crossbred, n = 163) chickens between April 2022 and March 2023, across 27 different farms in the Pirojpur district of Bangladesh. The farms were selected for sampling based on the presence of chickens having clinical signs such as diarrhea, decreased feed consumption, impaired weight gain, lameness, and increased mortality. After sacrificing the suspected chickens, the intestine was carefully inspected and examined for gross pathological lesions. Mucosal scrapings from different intestinal segments and fecal samples were analyzed microscopically for the presence of oocysts using standard parasitological methods. The identification of different *Eimeria* species was conducted based on the morphological and morphometric characteristics of the oocysts. The result revealed that the overall prevalence of

coccidiosis was 13.78%. Among the chicken types, Sonali demonstrated the significantly highest prevalence (21.47%), followed by layers (13.66%) and broilers (8.37%). Five different *Eimeria* species were identified, with *E. tenella* (45.12%) being the most prevalent, followed by *E. acervulina* (18.29%), *E. maxima* (15.85%), *E. brunetti* (10.98%), and *E. necatrix* (9.76%). No significant influence of age or season on coccidiosis prevalence was observed in broiler and layer chickens. In Sonali chickens, the prevalence of coccidiosis increased significantly with age, while no significant effects of season were observed. It has been concluded that the total prevalence of coccidiosis was 13.78%, with Sonali chickens exhibiting the highest prevalence. Five distinct species of *Eimeria* were identified, with *E. tenella* being the most common species. Furthermore, no effects of season and age on prevalence of *Eimeria* species were observed, except for Sonali chickens, where the prevalence increased with age.

Keywords: Coccidiosis, *Eimeria*, Morphometry, Poultry, Prevalence, Sonali chicken

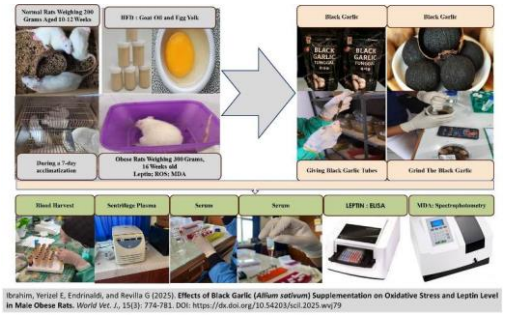
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Research Paper

Effects of Black Garlic (*Allium sativum*) Supplementation on Oxidative Stress and Leptin Level in Male Obese Rats

Ibrahim, Yerizel E, Endrinaldi, and Revilla G.

World Vet. J. 15(3): 774-781, 2025; pii:S232245682500079-15
DOI: https://dx.doi.org/10.54203/scil.2025.wvj79



ABSTRACT: Obesity is characterized by excessive fat accumulation in tissues, leading to leptin resistance and chronic inflammation. Black garlic is a well-documented natural remedy capable of improving leptin resistance and oxidative stress through its antioxidant compounds, such as S-Allyl cysteine and polyphenol flavonoids. The present study aimed to evaluate the effects of black garlic on oxidative stress and leptin levels in obese male rats. Twenty-five adult male Wistar rats, aged 10 to 12 weeks and weighing approximately 200 g, were divided into five experimental groups, each comprising five rats. These groups included a negative control group fed a standard diet (K1), a positive control group given a high-fat diet (K2), Treatment group 1 receiving a high-fat diet plus 200 mg of black garlic (P1), Treatment group 2 receiving a high-fat diet plus 400 mg of black garlic (P2), and Treatment group 3 receiving a high-fat diet plus 800 mg of black garlic (P3). The rats received black garlic orally for 28 days. The blood samples were analyzed for markers of oxidative stress, specifically malondialdehyde, as well as leptin levels. The present findings indicated that black garlic significantly decreased malondialdehyde and leptin concentrations in groups P1, P2, and P3 compared to the positive control group. Black garlic, administered at doses of 200, 400, and 800 mg, demonstrated the capacity to diminish oxidative stress and leptin levels in obese male rats.

Keywords: Antioxidant, Black garlic, Leptin, Malondialdehyde, Obesity, Oxidative stress, Rat

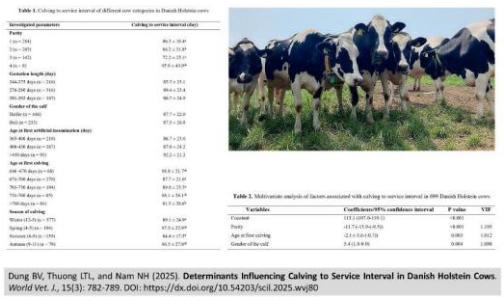
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Research Paper

Determinants Influencing Calving to Service Interval in Danish Holstein Cows

Dung BV, Thuong LTL, and Nam NH.

World Vet. J. 15(3): 782-789, 2025; pii:S232245682500080-15
DOI: https://dx.doi.org/10.54203/scil.2025.wvj80



ABSTRACT: The calving-to-first-service interval is crucial for dairy herd productivity. However, factors influencing this reproductive parameter remain understudied in modern dairy systems. The present study aimed to investigate the determinants of the calving-to-first-service interval in Danish Holstein cows, focusing on parity, age at first insemination, age at first calving, gestation length, gender of the born calf, and calving season. Data from 699 cows, managed on a single farm in Southwest Denmark, were collected and analyzed to identify risk factors associated with the calving-to-first-service interval. The mean calving-to-the-first-service interval was 87.6 ± 23.7 days. The present results indicated that parity, age at first calving, and calving season are key modifiable factors that affected the calving-to-first-service interval of the investigated Danish Holstein cows. The final multivariate linear regression model, which explained 13.7% of the variation in the calving-to-first-service interval, identified parity, age at first calving, and the gender of the born calf as significant risk factors for the interval. Parity and age at first calving were negatively related to the calving-to-first-service interval. Male calves were linked to an increased calving-to-first-service interval. Gestation length and age at the first artificial insemination indicated no association with the calving-to-first-service interval. Focusing on key factors such as parity, age at first calving, and gender of the born calf can improve reproductive success and profitability in dairy herds.

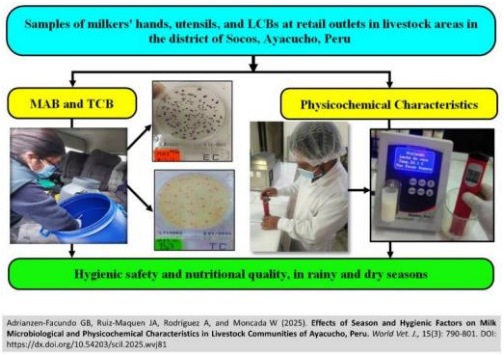
Keywords: Age, Calving, First calving, Gender, Parity, Service interval

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Research Paper

Effects of Season and Hygienic Factors on Milk Microbiological and Physicochemical Characteristics in Livestock Communities of Ayacucho, Peru

Adrianzen-Facundo GB, Ruiz-Maquen JA, Rodríguez A, and Moncada W.
World Vet. J. 15(3): 790-801, 2025; pii:S232245682500081-15
DOI: https://dx.doi.org/10.54203/scil.2025.wvj81



ABSTRACT: Climatic changes can influence milking practices and the dairy production chain, thereby influencing the microbiological safety and physicochemical quality of raw bovine milk (RBM), with potential implications for public health. The present study aimed to evaluate the effects of seasonal variability on the hygienic factors and characteristics of RBM produced in livestock communities in the Socos district, Ayacucho, Peru. For mesophilic aerobic bacteria (MAB) and total coliform bacteria (TCB), the compact dry plate method was used. Sixty random samples were collected during the rainy season (RS) and the dry season (DS). Samples were taken from milkers' hands (six in the RS and six in the DS), rags (six in the RS and six in the DS), buckets (six in the RS and six in the DS), receiving tanks (eight in the RS and eight in the DS), and distribution centers (four in the RS and four in the DS). To evaluate physicochemical characteristics, including density, fat, protein, total solids, lactose, pH, and mineral salts, eight samples of Brown Swiss cattle RBM were collected directly from the tanks (four in the RS and four in the DS) of two distribution point centers. The present results indicated that the MAB count for hygienic factors was lower in the DS than in the RS, except in the milker's hands and distribution centers. For the TCB count related to hygienic factors, the values were lower in the DS than in the RS, except in receiving tanks and distribution centers. In the RS, the physicochemical qualities complied with the Peruvian Technical Standard (PTS), except for protein content and mineral salt levels. In the DS, the physicochemical quality did not comply with the PTS; however, these properties were within acceptable limits in the receiving tanks for MAB and on the milker's hands for TCB. As a result, the bacterial load on inert surfaces and the characteristics of RBM were lower in the DS compared to the RS. Still, contamination during milking and at distribution centers was greater.
Keywords: Coliform, Hygienic safety, Mesophilic, Milk, Physicochemical quality, Season

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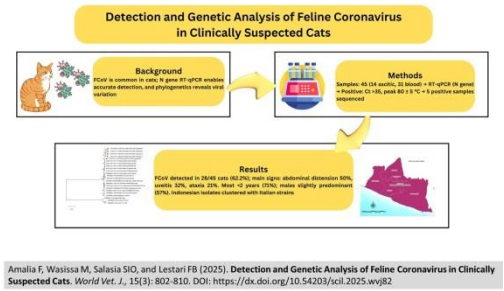
Research Paper

Detection and Genetic Analysis of Feline Coronavirus in Clinically Suspected Cats

Amalia F, Wasissa M, Salasia SIO, and Lestari FB.

World Vet. J. 15(3): 802-810, 2025; pii:S232245682500082-15

DOI: https://dx.doi.org/10.54203/scil.2025.wvj82



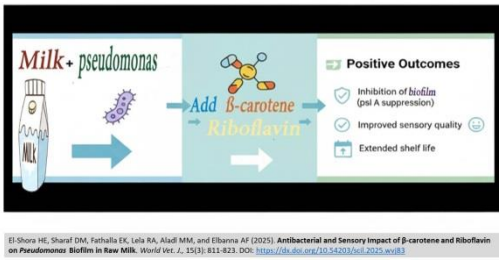
ABSTRACT: Feline coronavirus (FCoV) is a common infection in cats, producing outcomes that differ from mild intestinal disease to the fatal form known as feline infectious peritonitis (FIP). Accurate antemortem diagnosis is challenging, and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) targeting the nucleocapsid (N) gene offers high sensitivity and specificity, while phylogenetic analysis reveals viral variation. The present study aimed to detect FCoV in clinically suspected cats using RT-qPCR and to analyze the phylogenetic relationships of local isolates from Yogyakarta, Indonesia. A total of 45 clinical samples, consisting of 14 ascitic fluid and 31 blood samples, were collected from cats of different breeds (mixed, domestic shorthair, and Persian), aged 0.5-4 years, including 22 males and 23 females, that were presented to seven veterinary clinics in Yogyakarta with clinical signs such as vomiting, diarrhea, abdominal effusion, uveitis, and ataxia. The RT-qPCR revealed 62.2% (28/45) positive cases, with detection rates of 100% (14/14) in ascitic fluid and 45.2% (14/31) in blood. The most frequent clinical findings among FCoV-positive cats were abdominal distension (50%), uveitis (32.1%), ataxia (21.4%), and rhinitis (17.9%). Most infected cats (71.4%) were under two years of age, with a slightly higher prevalence in males (57.1%). Phylogenetic analysis of three isolates from ascitic fluid demonstrated a close genetic relationship with strains from Italy, the United Kingdom, and China, clustering into two distinct clades. The present study highlighted the utility of RT-qPCR targeting the N gene as a reliable diagnostic tool for clinical cases, while providing new clinicopathological and molecular data on naturally occurring FCoV infection in Indonesia. These findings contribute to the global understanding of FCoV molecular epidemiology and support future surveillance and control strategies for coronavirus infections in domestic cats.
Keywords: Cat, Feline coronavirus, Feline infectious peritonitis, Phylogenetic analysis

[Full text-PDF]

Research Paper

Antibacterial and Sensory Impact of β-carotene and Riboflavin on *Pseudomonas* Biofilm in Raw Milk

El-Shora HE, Sharaf DM, Fathalla EK, Lela RA, Aladl MM, and Elbanna AF.
World Vet. J. 15(3): 811-823, 2025; pii:S232245682500083-15
DOI: https://dx.doi.org/10.54203/scil.2025.wvj83

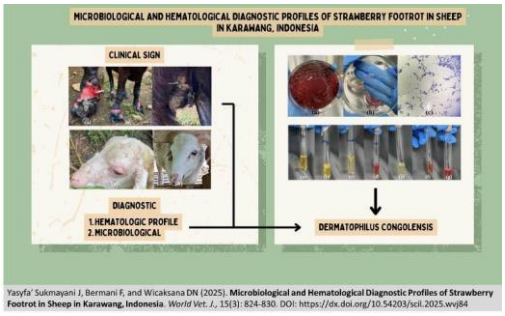


ABSTRACT: Milk is a highly nutritious yet perishable food that is susceptible to microbial contamination and spoilage, particularly by *Pseudomonas* species. The present study aimed to assess the antimicrobial and sensory effects of β-carotene and riboflavin as natural additives for preserving raw milk, with a specific emphasis on controlling biofilm-forming *Pseudomonas* strains. A total of 100 raw milk samples were collected from different markets in Gharbia, Egypt, and tested for *Pseudomonas* spp. Biofilm formation was assessed in isolates, followed by the detection of the quorum-sensing transcriptional regulator *lasR*, pellicle formation protein A (polysaccharide biosynthesis protein A) *pelA*, and polysaccharide synthesis locus protein A *pslA* genes. An *in vitro* trial was performed on eight groups of raw milk to evaluate the effects of β-carotene and riboflavin at concentrations of 1 µg/ml and 4 µg/ml, respectively, on *Pseudomonas* counts and sensory properties. The groups include positive control (G1), raw milk inoculated with *Pseudomonas* and fortified with β-carotene (G2), raw milk inoculated with *Pseudomonas* and fortified with riboflavin (G3), raw milk inoculated with *Pseudomonas* and fortified with a combination of β-carotene and riboflavin (G4), negative control (G5), milk fortified with β-carotene only (G6), milk fortified with riboflavin only (G7) and milk fortified with both β-carotene and riboflavin (G8). Reverse transcription polymerase chain reaction (RT-PCR) was performed to assess the impact of these additives on *Pseudomonas*, specifically on *16S rRNA* and *pslA* gene expression. *Pseudomonas* spp. were found in 20% of samples, with 75% forming biofilms. The *pslA* gene was detected in 83.33% of these samples. β-carotene and riboflavin indicated dose-dependent antibacterial effects, with minimum inhibitory concentration values, and *minimum bactericidal concentration* values. Milk fortified with these compounds, particularly in G4, demonstrated the most significant reduction in *Pseudomonas* during a 6-day storage period. Additionally, β-carotene maintained a preferable sensory quality compared to other groups. RT-PCR confirmed the highest *pslA* gene suppression with the combined treatment. The current findings indicated that β-carotene at a concentration of 1 µg/ml, whether used independently or in combination with riboflavin, can serve as an effective natural preservative in dairy products.
Keywords: Beta-Carotene, Biofilm, *Pseudomonas, PslA* Expression, Riboflavin
[Full text-PDF]

Case Report

Microbiological and Hematological Diagnostic Profiles of Strawberry Footrot in Sheep in Karawang, Indonesia

Yasyfa' Sukmayani J, Bermani F, and Wicaksana DN.
World Vet. J. 15(3): 824-830, 2025; pii:S232245682500084-15
DOI: https://dx.doi.org/10.54203/scil.2025.wvj84

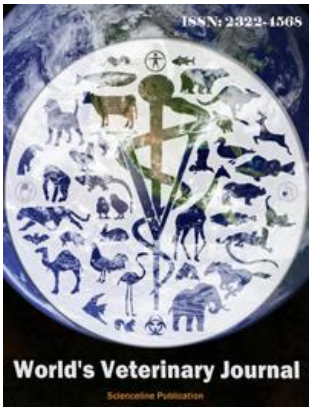


ABSTRACT: Dermatophilus congolensis (D. congolensis) is a gram-positive, coccus-shaped pathogenic bacterium that develops a filamentous structure during the infectious phase. The D. congolensis can cause scab lesions on sheep's feet, which may eventually develop into bloody tissue resembling crushed strawberries. The present study aimed to provide diagnostic, microbiological, and hematological profiles of strawberry footrot in sheep in Karawang Regency, Indonesia. Six sheep (Ovis aries), with an average age of two years and exhibiting similar signs of strawberry footrot, were sampled from community farms in the region. Blood samples and exudate swabs from scab lesions on the feet and udder were collected in August 2024 for analysis. Hematological testing and bacterial isolation using blood agar, gram staining, fermentation, and biochemical tests were performed. The hematologic results indicated thrombocytosis, macrocytic normochromic anemia, and leukocytosis in the sheep. All samples exhibited beta hemolysis zones on blood agar and were gram-positive, coccus-shaped bacteria with a railroad appearance. Biochemical tests were positive for catalase, negative for indole, negative for Voges-Proskauer (VP), and showed the ability to ferment glucose and maltose. Based on clinical signs, hematological findings, and bacterial isolation, D. congolensis was confirmed as the causative agent of strawberry footrot in sheep in Karawang Regency, Indonesia.
Keywords: Dermatophilus congolensis, Hematological profile, Microbiology, Strawberry footrot

[Full text-PDF]

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World's Veterinary Journal

E-ISSN: 2322-4568

Frequency: Quarterly

DOI Prefix: 10.54203

Current Issue: 2025, Vol: 15, Issue: 3 (September)

Publisher: SCIENCELINE

www.wvj.science-line.com

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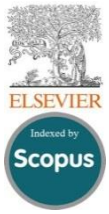
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Email: administrator@science-line.com
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2025, Scienceline Publication
World's Veterinary Journal
World Vet J, 15(3): 549-557.
ISSN 2322-4568

DOI: https://dx.doi.org/10.54203/scil.2025.wvj55
PII: S232245682500055-15

Effects of Food Waste Fermented with *Rhizopus oligosporus* on the Performance and Carcass Quality of Alabio Ducks

Rusfidra[1], Fajri Maulana[2]*, Heppy Setya Prima[3], Malikil Kudus Susalam[4], Satri Yusasra Agasi[2], and Fadhli Fajri[2]

[1]*Department of Technology and Livestock Production, Faculty of Animal Science, Universitas Andalas, West Sumatra, Padang 25163, Indonesia*
[2]*Department of Agricultural Industrial Technology, Study Program of Animal Feed Technology, Politeknik Negeri Tanah Laut, South Kalimantan 70815, Indonesia*
[3]*Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Negeri Medan, Medan, North Sumatra, Indonesia*
[4]*Department of Agroindustry, Animal Science Study Program, Faculty of Mathematics and Natural Sciences, Universitas Negeri Padang, Sijunjung, Indonesia*

*Corresponding author's Email: fajrimaulana@politala.ac.id

ORIGINAL ARTICLE
Received: June 19, 2025
Revised: July 21, 2025
Accepted: August 24, 2025
Published: September 30, 2025

ABSTRACT

The incorporation of food waste as an alternative feed ingredient has attracted considerable academic attention, owing to its potential to decrease feed costs and promote sustainable practices within livestock production systems. The present study aimed to evaluate the effects of food waste from Pesantren (FWP), Indonesia, fermented with *Rhizopus oligosporus* (*R. oligosporus*) as feed on the performance and carcass quality of Alabio ducks. A completely randomized design with four replications was used in the present study. A total of 200 one-day-old male Alabio ducks, with an average initial body weight of 39 ± 2 grams, were raised for eight weeks and randomly divided into five dietary groups, consisting of 40 ducks per group and five ducks per replicate with different FWP inclusion levels. The treatments varied based on the inclusion levels of FWP fermented with *R. oligosporus* in the diets of Alabio ducks which included FWP 0% (Treatment A, the control group), FWP 10% (Treatment B), FWP 20% (Treatment C), FWP 30% (Treatment D), and FWP 40% (Treatment E). Different parameters were observed at 56 days of age, including feed consumption, final body weight, feed conversion ratio (FCR), protein intake, carcass percentage, and abdominal fat percentage. The current results indicated that FWP significantly affected feed consumption, final body weight, FCR, and protein intake. Treatment D resulted in the highest body weight, protein intake, and the best FCR. However, there were no significant differences among treatments in carcass and abdominal fat percentages. In conclusion, incorporating up to 30% of FWP fermented with *R. oligosporus*, based on dry matter, into the diet of male Alabio ducks does not negatively impact their performance or carcass quality. Using FWP fermented with *R. oligosporus* as part of the duck feed offered a sustainable and cost-effective method for organic waste management in Pesantren poultry systems.

Keywords: Alabio duck, Carcass quality, Fermented feed, Growth performance, *Rhizopus oligosporus*

INTRODUCTION

Duck farming in Indonesia has significant potential, particularly with local breeds such as the Alabio duck (*Anas platyrhynchos Borneo*), which is native to South Kalimantan, Indonesia (Suryana et al., 2011). Alabio ducks are recognized for their exceptional environmental adaptability, high egg production, and superior meat quality (Sulaiman and Irawan, 2020). A significant challenge in duck farming is the high feed cost, making up over 60% of total expenses. Consequently, new strategies are essential to lower feed costs while maintaining production performance (Aji et al., 2022; Febrina et al., 2022). One promising approach is using organic waste as feed, which can lower feed expenses and enhance sustainability in poultry farming, especially in Pesantren-based duck production systems (Febrina et al., 2024).

Among different types of organic waste, food waste from Pesantren (FWP) offers unique advantages. Meanwhile, the nutrient content of hotel and restaurant food waste has been extensively studied, including crude protein at 21.41%, crude fiber at 5.90%, and 3,484.23 kcal/kg of metabolizable energy (Febrina et al., 2024). The use of organic waste as animal feed often relies on a centralized collection system, which smallholder farmers may not easily access. Furthermore, the supply of organic waste can be unpredictable, and transporting and processing the waste pose significant logistical challenges. In contrast, Pesantren Islamic boarding schools, where students live together, generate substantial amounts of food waste on-site, including rice leftovers, fish and meat scraps, and residues from fruit and

To cite this paper: Rusfidra, Maulana F, Prima HS, Susalam MK, Agasi SY, and Fajri F (2025). Effects of Food Waste Fermented with *Rhizopus oligosporus* on the Performance and Carcass Quality of Alabio Ducks. *World Vet. J.*, 15(3): 549-557. DOI: https://dx.doi.org/10.54203/scil.2025.wvj55

vegetables. This easily accessible and reliable waste stream offers a practical alternative feed source for duck farmers, especially in rural or Pesantren-focused communities (Rusfidra et al., 2025).

The nutritional content of FWP consists of 17.85% crude protein, 10.83% crude fat, 10.86% crude fiber, 92.89% dry matter, and 6.24% ash (Hidayat et al., 2024). To enhance FWP value, fermentation biotechnology can be utilized (Muslim et al., 2018). One of the most effective microorganisms for this purpose is *Rhizopus oligosporus (R. oligosporus)*, a fungus widely used in tempeh production (Rusfidra et al., 2025).

Rhizopus oligosporus produces protease, lipase, and amylase enzymes, which respectively break down proteins into amino acids, fats into fatty acids and glycerol, and starches into simple sugars, thereby enhancing the digestibility and bioavailability of nutrients for ducks (Djulardi et al., 2023). Fermentation with *R. oligosporus* has been shown to increase protein availability, reduce crude fiber, and diminish anti-nutritional factors (Ndego et al., 2023).

Previous studies have investigated the potential of organic waste as an ingredient in poultry feed (Muslim et al., 2018; Widjastuti et al., 2020; Febrina et al., 2024). However, studies on Pesantren-specific food waste, especially when combined with other local agro-industrial residues such as tofu dregs, remain limited. The present study expands on prior studies by employing a locally relevant feed mixture composed of 90% FWP and 10% tofu dregs, fermented with *R. oligosporus* (Rusfidra et al., 2025).

Therefore, the present study aimed to assess the potential of FWP fermented with *R. oligosporus* as an alternative feed for improving growth performance and carcass quality in Alabio ducks.

MATERIALS AND METHODS

Ethical approval

The collection of FWP in the present study was conducted with the consent of Pesantren administrators in Indonesia, who were informed about the study's objectives, procedures, and potential benefits. The current study was conducted after obtaining ethical approval under the Minister of Agriculture Decree No. 306/KPTS/TN.330/4/1994, which was approved by the Animal Research Ethics Committee of Andalas University, Padang, Indonesia, as required for the use of experimental animals in research and teaching.

Feed safety

Regarding feed safety, food waste can pose potential risks due to the presence of biological contaminants such as pathogenic bacteria or molds (Zhang et al., 2022). The waste materials used in the present study consisted of approximately 150 kg of food waste collected from the kitchen of an Indonesian Pesantren over 10 days. The waste included leftover cooked rice, vegetable peels, fruit residues, fish bones, and meat scraps. Before fermentation, all materials were manually sorted to remove non-organic contaminants such as plastic or packaging, then homogenized, mixed with 10% tofu dregs by weight. The waste materials underwent fermentation utilizing *R. oligosporus*, after which a thermal treatment was applied at 80°C for one hour to inactivate any potential undesirable microorganisms. Following fermentation, the material was sun-dried for two days under direct sunlight, with periodic turning to promote uniform drying, until it achieved a moisture content of approximately 10-12%. The dried product was subsequently subjected to proximate analysis to assess its nutritional adequacy before incorporation into feed formulations. These methodologies were meticulously employed to mitigate health risks and ensure the safe use of the fermented product in duck diets (Rusfidra et al., 2025).

Experimental design

A total of 200 one-day-old male Alabio ducks (*Anas platyrhynchos Borneo*), with an average initial body weight of approximately 39 ± 2 grams, were raised. The experiment was conducted over eight weeks, employing a completely randomized design that incorporated five distinct dietary treatments, each replicated four times, resulting in a total of 20 experimental units. The treatments varied based on the inclusion levels of FWP fermented with *R. oligosporus* in the diets of Alabio ducks which included 0% FWP (Treatment A, the control group), 10% FWP (Treatment B), 20% FWP (Treatment C), 30% FWP (Treatment D), and 40% FWP (Treatment E; Rusfidra et al., 2025). Each experimental unit consisted of 10 male Alabio ducks, resulting in a total of 200 ducks. The experimental cages were colony-type, each measuring 130 × 130 × 100 cm, with a total of 20 units used in the present study. Each cage was fitted with feeders, drinkers, a digital scale, and cleaning tools. The ducks were kept under controlled environmental conditions, with ambient temperatures ranging from 28 to 32°C and relative humidity maintained between 60% and 75%. Natural sunlight was supplemented to ensure a consistent 16-hour light and 8-hour dark cycle. Feed and water were provided *ad libitum*, allowing continuous access throughout the experimental period.

Experimental diets

The inclusion levels of FWP were determined based on previous studies that evaluated the optimal amount of food waste in poultry diet (Rusfidra et al., 2025). All experimental diets were formulated to be iso-nitrogenous (Containing the same amount of crude protein) and iso-caloric (Containing the same amount of metabolizable energy) with an average crude protein content of 17% and a metabolizable energy content of approximately 2,800 kcal/kg, based on the proximate analysis of each feed ingredient (Table 1). All diet formulations aimed to ensure equal protein and energy levels for male Alabio ducks according to established nutritional guidelines, according to NRC (1994), while utilizing modified ingredients such as corn, soybean meal, and rice bran. The feed composition for each treatment used for male Alabio ducks in the present study is listed in Table 2, and the nutritional content of the feed treatments for male Alabio ducks is displayed in Table 3.

The feed formulation was carried out to ensure all treatments met the nutritional requirements of Alabio ducks, in accordance with the guidelines provided by the Indonesian national standard (SNI 01-3931-2006) and NRC (1994), thereby eliminating any observed performance differences that could be attributed to the inclusion level of FWP rather than a nutrient imbalance.

Parameters

Feed consumption (g/head), final body weight (g/head), feed conversion ratio (FCR), and protein intake (g/head), along with carcass percentage and abdominal fat percentage, were evaluated during the present study.

Table 1. Feed ingredients and their nutritional contents used in the diets of male Alabio ducks (*Anas platyrhynchos Borneo*)

Ingredients	Crude protein (%)	Crude fat (%)	Crude fiber (%)	Calcium (%)	Phosphorus (%)	Lysine (%)	Methionine (%)	Metabolizable energy (Kkal)[a]
Ground corn[b]	8.55	2.66	3.00	0.38	0.19	0.26	0.18	3300.00
Fish meal[b]	54.83	3.01	2.29	3.92	2.03	6.43	2.43	2600.00
Soybean meal[b]	45.35	2.49	2.75	0.63	0.36	0.60	0.60	2240.00
Fermented food waste from Pesantren[b]	19.48	15.06	7.68	3.84	2.67	1.38	0.56	3100.00
Rice bran [b]	9.50	5.09	14.00	0.69	0.26	0.67	0.27	1640.00
Fish oil[b]	0	100.00	0	0	0	0	0	8600.00
Coconut oil[b]	0	100.00	0	0	0	0	0	8600.00
Bone meal[b]	0	0	0	24.00	12.00	0	0	0
CaCO3[b]	0	0	0	40.00	0	0	0	0
Top mix[c]	0	0	0	0.06	0	0.003	0	0

a: Scott et al. (1982), b: Laboratory analysis results during the study, c: Packaging label from PT Medion Indonesia

Table 2. Feed composition for each treatment used for male Alabio ducks (*Anas platyrhynchos Borneo*) in the present study

Ingredients	Treatment A (%)	Treatment B (%)	Treatment C (%)	Treatment D (%)	Treatment E (%)
Ground corn	57.00	49.00	42.00	35.00	27.00
Fish meal	10.00	10.00	10.00	10.00	10.00
Soybean meal	12.00	10.00	7.00	4.00	2.00
Fermented food waste from Pesantren	0.00	10.00	20.00	30.00	40.00
Rice bran	15.00	15.00	15.00	15.00	15.00
Fish oil	1.00	1.00	1.00	1.00	1.00
Coconut oil	1.00	1.00	1.00	1.00	1.00
Bone meal	1.00	1.00	1.00	1.00	1.00
CaCO3	2.00	2.00	2.00	2.00	2.00
Top mix	1.00	1.00	1.00	1.00	1.00
Total	100.00	100.00	100.00	100.00	100.00

Table 3. Nutritional content of the feed treatments used for male Alabio ducks (*Anas platyrhynchos Borneo*) in the present study

Nutrients	Treatment A (%)	Treatment B (%)	Treatment C (%)	Treatment D (%)	Treatment E (%)
Crude protein	17.22	17.58	17.57	17.56	17.92
Crude fat	4.88	6.12	7.37	8.61	9.86
Crude fiber	4.37	4.84	5.32	5.79	6.27
Calcium	1.83	2.17	2.51	2.85	3.19
Phosphorus available	0.88	0.76	1.00	1.24	1.49
Metabolizable energy	2827.80	2829.00	2840.80	2852.60	2853.80
Lysine	0.96	1.07	1.17	1.27	1.38
Methionine	0.46	0.49	0.51	0.54	0.57

Note: Compiled based on Table 1 and Table 2.

Fermentation of Pesantren food waste with *Rhizopus oligosporus*

A total of 500 grams of FWP was prepared and mixed with 50 grams of tofu dregs, obtained freshly from a local tofu producer in Padang, Indonesia, as an additional carbon source. The mixture was placed into heat-resistant polypropylene plastic bags (Cahayapack, Indonesia) and sterilized with an autoclave at 121°C and 15 psi for 20 minutes. After sterilization, the substrate was cooled at room temperature for approximately 45 minutes until it reached ambient temperature. The cooled substrate was then inoculated with *R. oligosporus* at a concentration of 3% of the total substrate weight (equivalent to 16.5 grams). The inoculum was prepared by suspending *R. oligosporus* spores in sterile distilled water to achieve a concentration of 10^6 spores per gram of substrate. The inoculation process was conducted under sterile conditions in a laminar airflow chamber. Following inoculation, the spores were evenly mixed into the substrate to ensure uniform distribution. This method was adapted from the established fermentation protocol described by Muslim et al. (2018) and Rusfidra et al. (2025).

The fermentation process was conducted aerobically in an incubator set at 32°C for five days. After incubation, the fermented material was heated at 80°C for one hour to inactivate the enzymatic and microbial activity of *R. oligosporus*. The final product was then dried at 60°C until the moisture content dropped below 14%, as confirmed by oven-drying (Eyela, Tokyo Rikakikai Co., Ltd., Japan) of a representative sample at 105°C for 24 hours following the standard method of proximate analysis (AOAC, 2005; Figure 1). A proximate analysis of the fermented product was performed to determine its nutritional composition, which served as the basis for designing the experimental diets in the present study (Rusfidra et al., 2025).

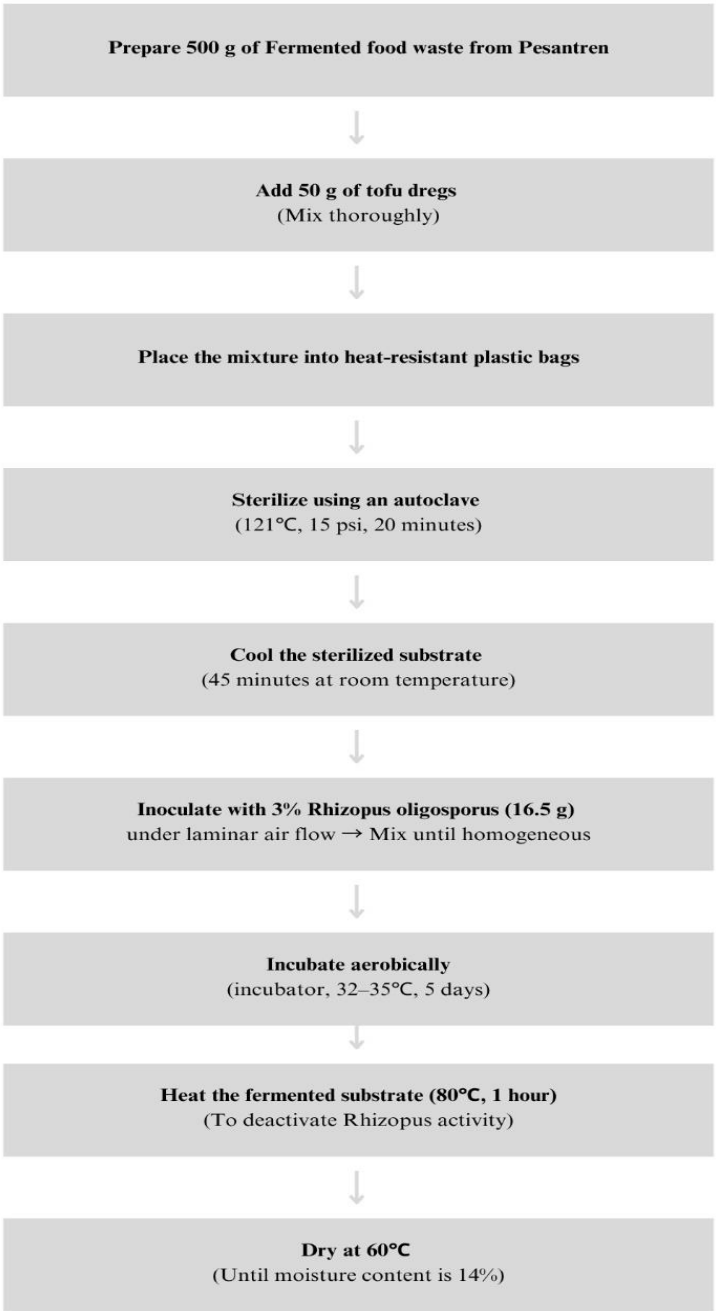


Figure 1. The preparation of Pesantren food waste fermented with *Rhizopus oligosporus*

The preparation of *Rhizopus oligosporus*

The preparation of *R. oligosporus* inoculant started with selecting high-quality soybeans, which were soaked for 6-12 hours, boiled, and manually dehulled to promote fungal growth. The soybeans were then coarsely ground to increase surface area, supporting optimal mycelial development. The substrate was sterilized in heat-resistant polypropylene bags using an autoclave at 121°C and 15 psi for 15 to 30 minutes, then cooled to 35°C, the ideal temperature for inoculation to prevent thermal damage to the spores and ensure successful fermentation (Rusfirda et al., 2025).

Inoculation was carried out under aseptic conditions inside a laminar airflow cabinet by applying a standardized spore suspension containing 10^7 to 10^8 colony-forming units (CFU) per gram to 100 g of sterile substrate. The *R. oligosporus* spores used in the present study were sourced from the Indonesian culture collection, Research Center for Biology, National Research and Innovation Agency, Indonesia (Rusfidra et al., 2023). Spore concentration was determined using a hemocytometer under a light microscope to ensure accurate and consistent inoculum levels.

The inoculated substrate was incubated at 32°C, an optimal temperature for *R. oligosporus* growth, for five days in shallow containers (1-2 cm thick) with adequate aeration to ensure proper fungal development. Following the incubation phase, the substrate that had been entirely colonized was dried at a low temperature (Not exceeding 40°C) to preserve the viability of the fungal spores. It was then finely ground and stored in sealed, light-protected containers at a temperature between 4°C and 10°C for later use in feed fermentation. The process of preparing the *R. oligosporus* inoculum is depicted in Figure 2.

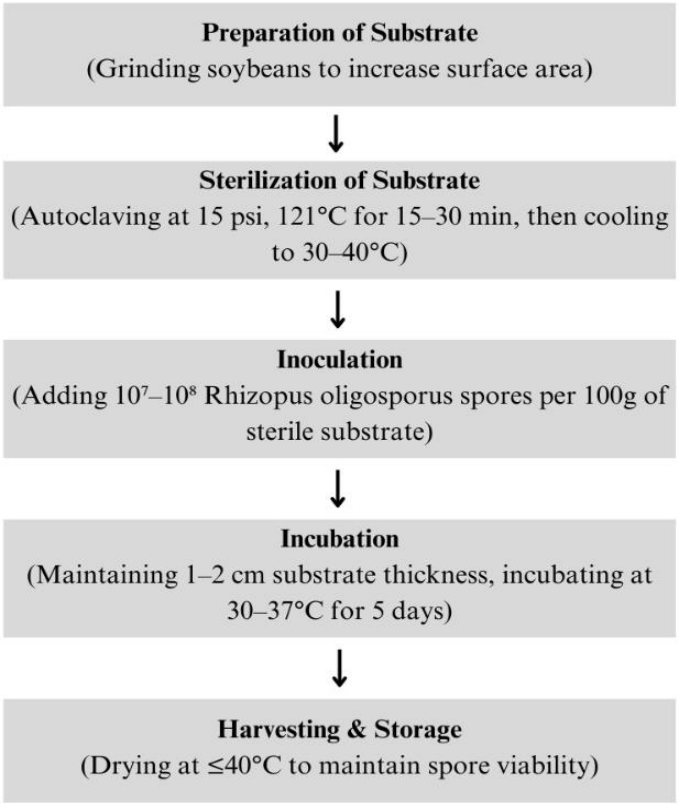


Figure 2. The preparation of *Rhizopus oligosporus*

Statistical analysis

The collected data were subjected to one-way analysis of variance (ANOVA) to assess the impact of different dietary treatments on the growth performance and carcass characteristics of Alabio ducks. Upon identifying a significant treatment effect ($p < 0.05$), Duncan's Multiple Range Test (DMRT) was used to assess mean differences among groups. The results were expressed as means ± standard error (SE), with SE representing the dispersion of the sample mean and reflecting the reliability of the reported mean estimates.

RESULTS AND DISCUSSION

The effects of fermented FPW on feed intake, final body weight, and FCR in male Alabio ducks at 8 weeks old are presented in Table 4. These performance metrics were assessed to understand how different levels of FPW in the diet influence growth and feed efficiency over the 8-week feeding period.

Table 4. Effects of fermented food waste from Pesantren on feed consumption, final body weight, and feed conversion in male Alabio Ducks (*Anas platyrhynchos Borneo*) at 8 weeks of age

Treatment	Feed consumption (g/head)*	Final body weight (g/head)*	Feed conversion ratio*
Treatment A (0% FWP)	4708.38 ± 42.93[a]	1532.42 ± 55,44[a]	3.07 ± 0.09[b]
Treatment B (10% FWP)	4650.38 ± 38.03[a]	1501.15 ± 62,68[a]	3.10 ± 0.15[b]
Treatment C (20% FWP)	4643.75 ± 45.37[a]	1495.21 ± 65,84[a]	3.11 ± 0.16[b]
Treatment D (30% FWP)	4632.94 ± 52.08[a]	1462.87 ± 68,60[a]	3.17 ± 0.17[b]
Treatment E (40% FWP)	4549.56 ± 55.90[b]	1329.94 ± 93,82[b]	3.43 ± 0.22[a]
SE	23.65	35.25	0.08
P-Value	0.005	0.009	0.042

FWP: Food waste from Pesantren fermented with *R. oligosporus*, SE: Standard error. [a,b] Different superscript letters in the same column indicate significant differences ($p < 0.05$).

Feed consumption of Alabio ducks fed FWP fermented with *R. oligosporus* ranged from 4549.56 ± 55.90 to 4708.38 ± 42.93 g per bird. Feed intake in treatments B, C, and D did not differ significantly from that of the control group ($p > 0.05$). The current results suggested that including FWP up to 30% did not decrease feed intake, indicating that the fermented feed was palatable. This may be due to the fermentation process of FWP, which improves flavor and aroma by producing glutamate compounds and B-complex vitamins (Trisna et al., 2019; Maulana et al., 2021; Wizna et al., 2025).

Moreover, all experimental diets were formulated to be iso-nitrogenous and iso-caloric, ensuring balanced nutrient profiles despite the reduced inclusion of conventional ingredients such as corn and soybean meal. This consistency across treatments A to D likely contributed to comparable feed intake and growth performance. Additionally, fermentation improved the palatability and digestibility of fermented FWP, enhancing nutrient availability and absorption in ducks.

Feed consumption was significantly decreased ($p < 0.05$) in Treatment E (4549.56 ± 55.90 g/head) compared to Treatments C (4643.75 ± 45.37 g/head) and D (4632.94 ± 52.08 g/head). This reduction may be attributed to the higher crude fiber content (6.27%), which likely increased gut fill and satiety, suppressing voluntary intake. Elevated fiber levels in poultry diets are known to slow digestion and prolong satiety (Svihus, 2011; Maulana et al., 2024).

The FWP fermented with *R. oligosporus* reduced the use of corn and soybean meal. In Treatment B, corn usage decreased by 14.04%, and soybean meal usage decreased by 16.67%. Treatment C lowered corn usage by 26.32% and soybean meal by 41.67%. Treatment D reduced the use of corn by 38.60% and soybean meal by 66.67% and Treatment E lowered corn usage by 52.63% and soybean meal by 83.33%. The final body weight among treatments A, B, C, and D showed no significant differences ($p > 0.05$), indicating that feed consumption and protein intake did not vary significantly across all treatments ($p > 0.05$). Notably, utilizing FWP up to 30% did not negatively impact feed intake or nutrient availability. Specifically, feed consumption and protein intake were recorded as Treatment A (4708.38 ± 42.93 g and 800.42 ± 7.30 g), Treatment B (4650.38 ± 38.03 g and 790.56 ± 6.47 g), Treatment C (4643.75 ± 45.37 g and 789.44 ± 7.71 g), and Treatment D (4632.94 ± 52.08 g and 792.24 ± 8.30 g).

Duck body weight gain depends on feed intake, as higher consumption boosts the chances of meeting nutritional requirements and promotes optimal growth (Zaqi et al., 2019). Additionally, weight gain is influenced by protein intake, as protein is essential for the development of new tissue (Obeidat et al., 2022). Protein intake is positively correlated with feed consumption, whereby an increase in feed intake leads to a corresponding increase in protein intake (Maulana et al., 2025).

The FCR is a crucial factor in assessing feed efficiency, especially when using waste or unconventional feed ingredients. A lower FCR signifies better efficiency, meaning less feed is needed to produce one kilogram of meat (Zhu et al., 2023). In the present study, the FCR values for each treatment were as follows: Treatment A (3.07 ± 0.09), Treatment B (3.12 ± 0.11), Treatment C (3.14 ± 0.08), Treatment D (3.17 ± 0.10), and Treatment E (3.43 ± 0.22). Treatments A to D indicated an improved feed efficiency (FCR 3.07-3.17), likely attributable to optimal feed-to-gain ratios, while Treatment E demonstrated a reduced efficiency (FCR 3.43). However, intergroup differences were not statistically significant ($p > 0.05$; Djulardi et al., 2023). The current FCR values (3.07-3.43) consistently exceeded commercial production standards (2.2-2.8), suggesting substantial scope for enhancing feed efficiency through targeted nutritional interventions (Pangeran et al., 2021). The effects of fermented FWP on protein intake, carcass percentage, and abdominal fat percentage in male Alabio ducks are presented in Table 5.

Table 5. Effect of food waste from Pesantren fermented with *Rhizopus oligosporus* on protein intake, carcass percentage, and abdominal fat percentage in male Alabio Ducks (*Anas platyrhynchos Borneo*) at 8 weeks of age.

Treatment	Protein intake (g/head)*	Carcass (%)^{NS}	Abdomen fat (%)^{NS}
Treatment A (0% FWP)	800.42 ± 7.30^{a}	63.50 ± 1.03	2.11 ± 0.07
Treatment B (10% FWP)	790.56 ± 6.47^{a}	63.17 ± 0.87	2.08 ± 0.05
Treatment C (20% FWP)	789.44 ± 7.71^{a}	62.64 ± 0.62	2.06 ± 0.03
Treatment D (30% FWP)	792.24 ± 8.30^{a}	62.21 ± 1.05	2.04 ± 0.09
Treatment E (40% FWP)	773.43 ± 9.50^{b}	61.83 ± 0.64	1.98 ± 0.04
SE	3.96	11.15	0.03
P-value	0.004	0.087	0.069

NS: Non-significant, FWP: Food waste from Pesantren fermented with *R. oligosporus*, SE: Standard error. $^{(*)a-b}$ Different superscript letters in the same column indicate significant differences ($p < 0.05$)

Adequate protein consumption is essential for optimal growth, tissue synthesis, and maintenance in poultry species (NRC, 1994). The current results indicated that Treatments A, B, C, and D did not differ significantly in protein intake values ($p > 0.05$). The current findings suggested that the feed formulations containing FWP in treatments A, B, C, and

D provided comparable protein intakes, with values of 800.42 ± 7.30 g/head (Treatment A), 790.56 ± 6.47 g/head (Treatment B), 789.44 ± 7.71 g/head (Treatment C), and 792.24 ± 8.30 g/head (Treatment D), respectively, which met the nutritional requirements of Alabio ducks as recommended by NRC (1994). Protein intake is influenced by feed consumption; higher feed consumption results in higher protein intake (Nova et al., 2022). Treatment E demonstrated a significantly lower protein intake (773.43 ± 9.50 g/head) compared to Treatment A (800.42 ± 7.30 g/head), Treatment B (790.56 ± 6.47 g/head), Treatment C (789.44 ± 7.71 g/head), and Treatment D (792.24 ± 8.30 g/head, $p < 0.05$).

The significantly lower protein intake in Treatment E (773.43 ± 9.50 g/head) likely resulted from reduced feed palatability due to the 40% inclusion of fermented Pesantren food waste. This suboptimal protein consumption may negatively impact growth performance, as feed palatability directly influences consumption and consequently protein intake in poultry (Wang et al., 2023).

Carcass percentages across all treatments; A (63.50 ± 1.03%), B (63.17 ± 0.87%), C (62.64 ± 0.62%), D (62.21 ± 1.05%), and E (61.83 ± 0.64%) showed no significant differences ($p > 0.05$) and remained within the established range (60-65%) for male Alabio ducks (NRC, 1994). The non-significant treatment differences ($p > 0.05$) demonstrated that fermented Pesantren waste can effectively replace conventional feed components while maintaining equivalent carcass yields in duck production systems. The current findings indicated that the inclusion of FWP as an alternative feed ingredient did not negatively affect key metabolic processes such as nutrient absorption and protein synthesis, nor did it impair muscle tissue development in Alabio ducks. Carcass percentage can be affected by suboptimal growth, which may be due to high crude fiber content in poultry feed (Nuraini et al., 2022).

Abdominal fat percentage is a parameter that reflects the efficiency of energy metabolism and fat accumulation in the animal's body (Ayuti et al., 2022). The present study indicated that the inclusion of FWP did not significantly ($p > 0.05$) affect the abdominal fat percentage across all treatments.

The average abdominal fat percentage was 2.11 ± 0.07 in Treatment A, 2.08 ± 0.05 in Treatment B, 2.06 ± 0.03 in Treatment C, 2.04 ± 0.09 in Treatment D, and 1.98 ± 0.04 in Treatment E. These results demonstrated that all dietary formulations maintained proper fat deposition patterns, indicating balanced nutritional conditions (Nova et al., 2022). The current findings suggest that the energy used from different feed treatments (A to D) was proportionally utilized by the ducks for both muscle growth, as shown by carcass percentages ranging from 61.83% to 63.50%, and fat deposition, with abdominal fat percentages between 2.11% and 2.03%.

The fermentation method using *R. oligosporus* on Pesantren food waste illustrated promising potential for use by small-scale duck farmers in Indonesia, especially those near Pesantren communities. Compared to other bioconversion methods, such as ensiling or enzymatic hydrolysis, the fermentation process in the present study was relatively simple, requiring only basic materials and equipment, including heat-resistant plastic containers, a household heat source (Stove or steamer), and a clean, well-ventilated space for incubation. Although the study employed an autoclave and a controlled incubator, in real-world settings, sterilization can be achieved through steaming for 30 to 60 minutes, and incubation can occur in a warm room with good airflow (Liu et al., 2022; Wang et al., 2023).

CONCLUSION

Fermenting Pesantren food waste with *Rhizopus oligosporus* was effective for Alabio duck diets at inclusion levels of up to 30%, preserving growth performance, feed efficiency, and carcass yield similar to those of conventional methods feeds. This approach provided a sustainable and cost-effective alternative that aligns with circular economy principles by valorizing local waste resources. Additionally, including up to 30% fermented Pesantren food waste could preserve nutritional quality and palatability, reduce reliance on traditional feed ingredients, and support eco-friendly poultry production in Pesantren communities. It is suggested that further refinement of processing methods or ingredient balancing may be needed to improve feed efficiency to meet commercial standards.

DECLARATIONS

Acknowledgments

The authors would like to thank the Department of Technology and Livestock Production, Universitas Andalas, the Department of Agricultural Industrial Technology, Politeknik Negeri Tanah Laut, the Department of Biology, Universitas Negeri Medan, and the Department of Agroindustry, Universitas Negeri Padang, Indonesia, for their support and collaboration in this study.

Authors' contributions

Rusfidra contributed to the conceptualization of the study and project administration. Fajri Maulana was responsible for formal analysis and writing the original draft of the manuscript. Heppy Setya Prima handled the investigation and validation of the research data. Malikil Kudus Susalam took charge of resources and visualization. Satri Yusasra Agasi provided supervision and methodology support. Finally, Fadhli Fajri was involved in data curation and funding acquisition. All authors read and approved the final edition of the manuscript for publication.

Funding

The present study did not receive any financial support.

Competing interests

The authors declared no conflict of interest.

Ethical considerations

This study was originally written by the authors and has not been published elsewhere. The authors checked the text of the article for plagiarism index and confirmed that the text of the article is written based on their original scientific results.

Availability of data and materials

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

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2025, Scienceline Publication
World's Veterinary Journal
World Vet J, 15(3): 558-565.
ISSN 2322-4568

DOI: https://dx.doi.org/10.54203/scil.2025.wvj56
PII: S232245682500056-15

Seroprevalence of *Ornithobacterium rhinothracheale* Infection in Commercial Layers in Tunisia

Khaled Kaboudi[1*], Imen Hamadi[2], Atef Walha[3], Aymen Mamlouk[4], and Adel Souissi[5]

[1]*Department of Poultry Management and Pathology, LR14-AGR03, High Institute of Research and Agricultural Education, National School of Veterinary Medicine, 2020 Sidi Thabet, University of Manouba, Tunisia*
[2]*Private Veterinary Practitioner, Tunis, Tunisia*
[3]*Pharmaceutical MEDIVET Laboratories, Soukra Ariana, Tunisia*
[4]*Department of Microbiology and Immunology, High Institute of Research and Agricultural Education, National School of Veterinary Medicine, 2020 Sidi Thabet, University of Manouba, Tunisia*
[5]*Animal Production Department, Regional District of Agricultural Development, Sfax, Tunisia*

*Corresponding author's Email: khaled.kaboudi@enmv.uma.tn

ORIGINAL ARTICLE
Received: June 22, 2025
Revised: July 24, 2025
Accepted: August 27, 2025
Published: September 30, 2025

ABSTRACT

Ornithobacterium rhinotracheale (ORT) infection is a worldwide disease in commercial poultry production. It is caused by a gram-negative bacterium, inducing respiratory and articular infections. The present study investigated the seroprevalence of ORT in commercial laying chicken farms in the Sfax region of South-eastern Tunisia. Diagnosis of ORT is based on the clinical signs such as respiratory distress, sneezing, cough, and limping, gross pathology in post-mortem examination (fibrinous tracheitis, fibrinous pneumonia, caseous arthritis, and tendinitis), and laboratory investigations, including isolation and identification, molecular and serological analysis. In the current study, 470 serum samples were collected from 25 commercial layer flocks (18 to 20 blood samples/flock) and tested using an indirect enzyme-linked immunosorbent assay (ELISA), which revealed that all 25 flocks (100%) demonstrated evidence of past or recent ORT infection. Seropositivity of the tested sera indicated the presence of antibodies against ORT, ranging widely from 7.69% to 100% within individual layer hens flocks. The average antibody levels varied considerably among flocks, from 1425.67 to 15233 in different flocks. The current findings indicated widespread ORT exposure in commercial layer farms in the Sfax region of South-eastern Tunisia, signifying the potential for horizontal transmission of ORT, particularly in multi-age layer integrations where older flocks can act as a source of infection for younger pullets.

Keywords: Biosecurity, Commercial layer, Enzyme-linked immunosorbent assay, *Ornithobacterium rhinotracheale*

INTRODUCTION

Ornithobacterium rhinotracheale (ORT) is a gram-negative bacterium that causes a highly contagious respiratory illness in poultry. This bacterium's shape can vary (pleomorphic), and it cannot move independently (nonmotile). The organism has a rod shape and does not produce spores for survival (Van Empel and Hafez, 1999). The ORT belongs to rRNA superfamily V and shares some genetic characteristics with other bacteria, such as *Cytophaga*, *Riemerella*, and *Flavobacterium* (Van Empel and Hafez, 1999; Canal et al., 2005). The severity of ORT infection depends on different factors, such as environmental conditions, including ambient temperature, humidity, and ammonia, the bacterium's ability to form protective biofilms, and the presence of co-infections with other pathogens such as *Avian Metapneumoviruses* and *Avibacterium paragallinarum* (Marien et al., 2005; De la Rosa-Ramos et al., 2015; Stępień-Pyśniak et al., 2024).

Over 18 serotypes of ORT, designated from A to R, have been identified through studies (Hafez and Sting, 1999; De la Rosa-Ramos et al., 2018). Serotype A of ORT stands out for its high dominance, infecting 94% of chickens and 57% of turkeys (Siddique et al., 2008; Kursa et al., 2022). Furthermore, several studies revealed variations in virulence and adherence properties among serotypes A, B, C, D, and E (Chansiripornchai et al., 2007; De Haro-Cruz et al., 2013).

While ORT is susceptible to common disinfectants such as chloride, phenols, and glutaraldehyde, it can become endemic in poultry houses, persistently infecting new flocks even after poor cleaning and disinfection protocols of the poultry houses. Persistence of ORT is particularly problematic in multi-age layer farms (Hafez and Schulze, 2003).

Ornithobacterium rhinotracheale primarily transmits horizontally among chickens, which means the infection spreads through inhalation of respiratory droplets, direct contact with infected chickens, or indirectly through contaminated feed, equipment, and drinking water. However, vertical transmission appears to be limited (Boulianne et al., 2020). While ORT was isolated from ovary and oviduct, infertile eggs, hatching eggs, and even dead embryos, it is unclear if this pathogen is viable or contributes significantly to chicken infection (Shahata et al., 2006; Boulianne et al., 2020).

To cite this paper: Kaboudi K, Hamadi I, Walha A, Mamlouk A, and Souissi A (2025). Seroprevalence of *Ornithobacterium rhinotracheale* Infection in Commercial Layers in Tunisia. *World Vet. J.*, 15(3): 558-565. DOI: https://dx.doi.org/10.54203/scil.2025.wvj56

The presence of ORT is confirmed in a wide range of domestic and wild birds suffering from respiratory issues, including chickens, turkeys, ducks, chukar partridges, geese, quail, ostriches, guinea fowl, rooks, pheasants, and pigeons (Boulianne et al., 2020).

Clinical signs of ORT infection are more readily apparent in meat birds (broilers and turkeys) compared to laying hens (Van Veen et al., 2000). Turkey flocks can be exposed to ORT and seroconvert without exhibiting any outward signs of illness (Back et al., 1998a). The age of susceptibility to infection differs among bird types. Broilers typically experience infection between three and four weeks of age, while turkeys are more susceptible from 14 weeks until slaughter age (Charlton et al., 1993; Back et al., 1998b; Van Veen et al., 2000). The pathogenicity of ORT as a primary pathogen in layer chickens is poorly understood. However, severe respiratory signs were described in layer hens' flocks in Japan aged from 26 to 30 weeks (Umali et al., 2018).

Outbreaks of ORT have been reported in breeder farms, causing similar clinical signs to those observed in broiler chickens and turkeys, along with secondary economic consequences (Charlton et al., 1993). Economic losses include decreased egg production, decreased eggshell quality, and reduced hatchability (Charlton et al., 1993). Breeder hens seemed to be most susceptible before or during their peak laying period (Hafez, 1996). The ORT infection in commercial laying hens has been associated with clinical illness, manifesting as increased mortality, respiratory signs, and decreased egg production (Joubert et al., 1999; Lopes et al., 2002).

Diagnosing ORT infection requires a multi-pronged approach due to the challenges associated with culturing the bacteria. While cultural methods are available for isolating and identifying the causative agent, ORT's slow growth (more than 48 hours) in liquid media, such as blood agar or soybean casein agar, incubated at 37°C for 24 to 48 hours under anaerobic or microaerophilic conditions, makes the process challenging (Mayahi et al., 2016). Serological tests offered a more practical alternative for large-scale testing and surveillance programs (Back et al. 1998a). Serotyping of ORT isolates has been carried out using specific antisera against 18 serotypes, employing an enzyme-linked immunosorbent assay (ELISA) and an agar gel precipitation test (Hassan et al., 2020). Real-time polymerase chain reaction (PCR) was also performed for the definitive diagnosis of ORT infections and/or bacterial and viral respiratory co-infections, particularly in cases where cultural or serological methods were not available or were inconclusive (Hashish et al., 2022; Nguyen et al., 2023; Krylova et al., 2024).

The scarcity of published data on ORT prevalence within Tunisia's commercial poultry sector, particularly laying hens, highlighted the need for the present study. The present study aimed to investigate the seroprevalence of ORT infections in commercial layer farms located in the Sfax region of south-eastern Tunisia.

MATERIALS AND METHODS

Ethical approval

The experiment was approved by the Institution of Agricultural Research and Higher Education, National School of Veterinary Medicine of Sidi Thabet, University of Manouba, Tunisia.

Study region

The current study was carried out on twenty-five commercial layer flocks, from November 2020 to May 2021, located in the governorate of Sfax, Tunisia. The governorate of Sfax is located in the Southeast of the country on the eastern coast of Tunisia (Latitude: 34° 44' 26.02" N; Longitude: 10° 45' 37.01" E (Figure 1). Sfax is bounded by the Mediterranean Sea to the east, the governorate of Mahdia to the north, and the governorates of Kairouan, Sidi Bouzid, and Gafsa to the west. The climate of Sfax, where the current study was conducted, is Mediterranean, characterized by mild, relatively rainy winters and hot, sunny summers. Although winter is mild, there can sometimes be cold periods, during which the temperature drops to around 0°C (32°F) and remains at around 10°C (50°F). In summer, during invasions of hot air from the desert, which have become more frequent in recent years, the temperature can exceed 40°C (104°F). The present study focused on commercial layer farms in the Sfax governorate, Tunisia. According to the latest poultry establishment census conducted by Tunisian veterinary authorities between 2015 and 2016, Sfax boasted a particularly high concentration of layer hen flocks, accounting for 70% of Tunisia's total. Furthermore, Sfax is home to an estimated 88 percent of the nation's pullet farms, representing 295 flocks of pullets (ONAGRI, 2021).

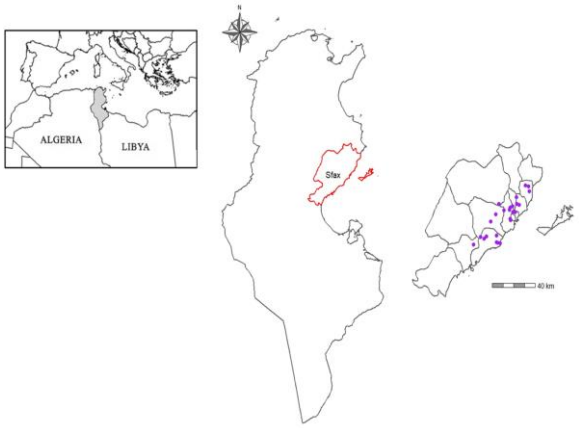


Figure 1. Geographical situation of the governorate of Sfax and distribution of sampled poultry houses. purple points represent flocks' locations.

Flocks and blood samples

The current study involved twenty-five commercial layer flocks, numbered from 1 to 25, chosen randomly across three age groups (20 to 30 weeks, 31 to 55 weeks, and over 55 weeks, Table 1). Data collection utilized a standardized survey form administered at each farm which focused on biosecurity measures implemented, including vehicle disinfection, footbath, distance between houses, pest control and results of bacteriological analysis of environmental samples for controlling disinfection (the supervising veterinarian of the farm carried out swabs of surfaces and equipment), medication history (date of treatment, molecules, method of administration, and duration of treatment), and the overall health status of the flocks (history of pathologies and cumulative mortality).

To assess seroprevalence, blood samples were randomly collected from 18 to 20 chickens in each flock, yielding 470 serum samples. Blood samples were collected from the wing vein and centrifuged at 1500 rpm for ten minutes to separate the serum. Aliquots of the whole sera were stored in clean tubes at -20°C until further analysis (Xue et al., 2020). Serological testing (indirect ELISA) to detect antibodies against ORT was subsequently performed in the Microbiology Laboratory of the National Veterinary Medicine School of Sidi Thabet, Tunisia.

Table 1. Individual seroprevalence of *Ornithobacterium rhinotracheale* infection according to age group and flock size in commercial layers in Tunisia from November 2020 to May 2021

Factor	Category	Flock (Number)	Tested sera (Number)	Positive sera (Number)	Seroprevalence (%)	P value
Age group	20-30 weeks	5	154	113	73.4%	0.001*
	31-55 weeks	10	112	76	67.9%	
	Above 55 weeks	10	204	175	85.8%	
Flock size	Small: < 10 000	7	121	86	71%	0.001*
	Medium: 10 001-30 000	13	233	172	73.8%	
	Big: Above 30,000	5	116	106	91.9%	

*: Significant difference in each column ($p < 0.05$)

Enzyme-linked immunosorbent assay

An indirect ELISA was employed to detect antibodies against ORT in the collected sera, using commercially available kits (ID-VET, ID Screen® ORT Indirect, France), and strictly adhered to the manufacturer's instructions. Following incubation and washing steps, the plates were read at a wavelength of 450 nm using an ELISA reader (Thermofisher®, USA). Samples were categorized as positive or negative based on a pre-determined sample-to-positive ratio (S/P) cut-off value. An S/P ratio less than or equal to 0.4 (titer ≤ 844) was considered negative, while values exceeding 0.4 (titer > 844) were considered positive. Flock-level ORT prevalence was determined by designating a flock as positive if at least one chicken within the flock tested positive for indirect ELISA. Notably, none of the sampled chickens in the present study had been previously vaccinated against ORT.

Statistical data

Statistical analyses were performed using R software (version 4.3.3; R Core Team, 2024) to assess group differences. Specifically, potential associations among age, flock size, and the mean ELISA titers were investigated using the Pearson chi-square test to identify statistically significant variations ($p < 0.05$).

RESULTS

The current epidemiological investigation indicated that 23 of the 25 visited flocks (92%) belonged to multi-age farms. Biosecurity measures varied across farms, with five flocks (aged from 20 weeks to 26 weeks) demonstrating satisfactory practices, 17 with acceptable measures (aged from 32 weeks to 52 weeks), and the remaining three exhibiting low biosecurity levels (aged above 55 weeks). Disinfection before pullet entry, a crucial control measure, was only implemented by 12 flocks (aged from 20 weeks to 48 weeks). The efficacy of disinfection was evaluated by 18 farms based on laboratory analyses of environmental samples (swabs of surfaces, soil, and equipment). The origin of the pullet supply was considered a potential risk for contamination. Only four flocks were sourcing pullets from a single origin (hatchery in Sfax). The remaining 21 flocks obtained pullets from multiple sources (hatcheries in Sfax and Tunis), which could introduce new pathogens.

The present serological analysis revealed a high prevalence of ORT exposure in the studied flocks. A significant majority (77.45%, n = 364) of the 470 collected serum samples tested positive for antibodies against ORT. Notably, positive samples were detected in all 25 flocks investigated (Figure 2A). Seropositivity varied considerably among

flocks, ranging from 7.69% (flock 25) to 100% (Flocks 14, 15, and 16). Furthermore, ELISA mean titers significantly varied across all flocks ($p < 0.05$). The ELISA titers ranged from 1425.67 (flock 12) to a much higher level of 15233 (flock 19; Figure 2B; $p < 0.05$). The variation in antibody levels was further substantiated by the coefficient of variation (CV) values, which ranged from 30.94% to 93.40% and indicated substantial variability in antibody responses among chickens within each flock.

Seroprevalence of ORT infection varied across different age groups (Table 1). Young hens (20-30 weeks old) illustrated a seroprevalence of 73.4%, while seroprevalence increased to 85.8% in older layers (above 55 weeks, $p < 0.05$). Additionally, flocks between 31 and 55 weeks had a slightly lower seroprevalence of 67.9%. The statistically significant difference ($p < 0.05$) exhibited a potential association between age and exposure to ORT. However, the correlation between layer age and ELISA mean titers was low positive ($r = 0.351$; Figure 3). Seroprevalence also exhibited variation based on flock size (Table 1). Smaller flocks (under 10,000 chickens) had a seroprevalence of 71%, while this seroprevalence increased to 91.4% in large flocks (over 30,000 chickens). Medium-sized flocks from 10,001 to 30,000 chickens illustrated an intermediate seroprevalence of 73.8%. The statistically significant difference ($p < 0.05$) suggested a potential link between flock size and exposure to ORT. Furthermore, a positive correlation ($r = 0.467$) was observed between ELISA mean titers and flock size (Figure 3), which indicated that larger flocks may tend to have higher average antibody levels, potentially reflecting a greater possibility of exposure in the flock.

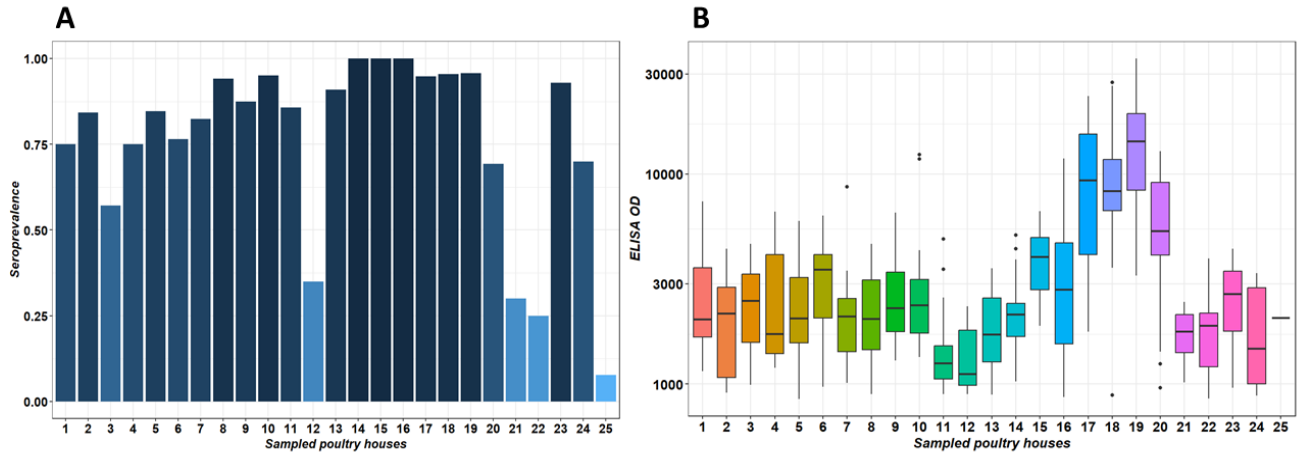


Figure 2. Seroprevalence frequency and enzyme-linked immunosorbent assay titer variation among sampled poultry houses with ages varied from 20 weeks to 107 weeks in the governorate of Sfax, Tunisia. **A**: Seroprevalence frequency, **B**: ELISA titer; each bar corresponds to a flock. Upper limit is the highest titer, lower limit is the lowest titer, horizontal line of the bar is the average antibody titer against *Ornithobacterium rhinotracheale.*

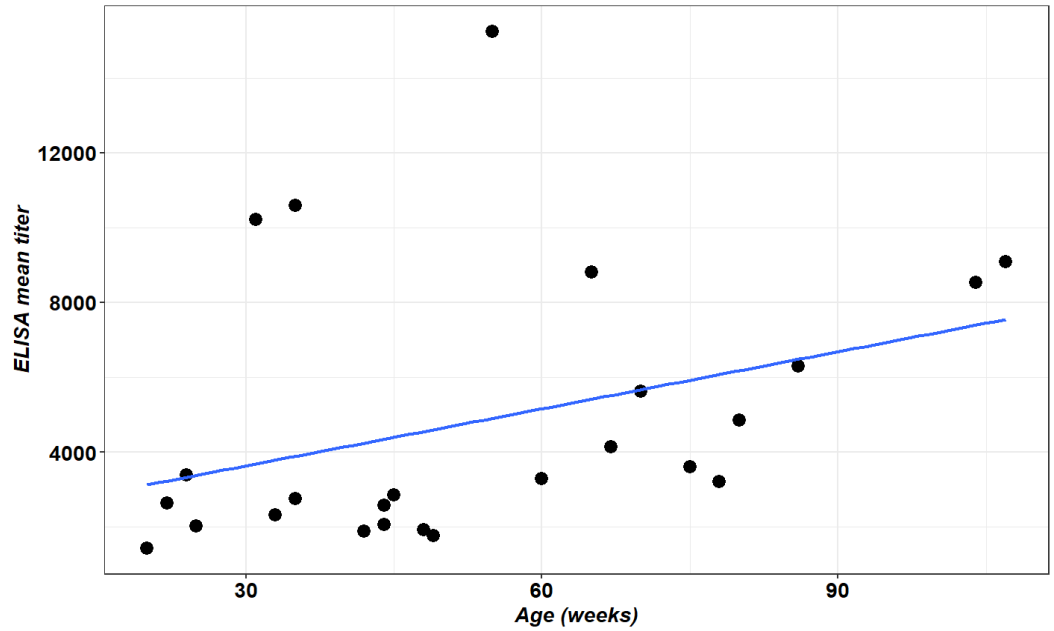


Figure 3. Correlation between layer age flocks (age varied from 20 weeks to 107 weeks) and *Ornithobacterium rhinotracheale* enzyme-linked immunosorbent assay mean titer, in the governorate of Sfax, Tunisia.

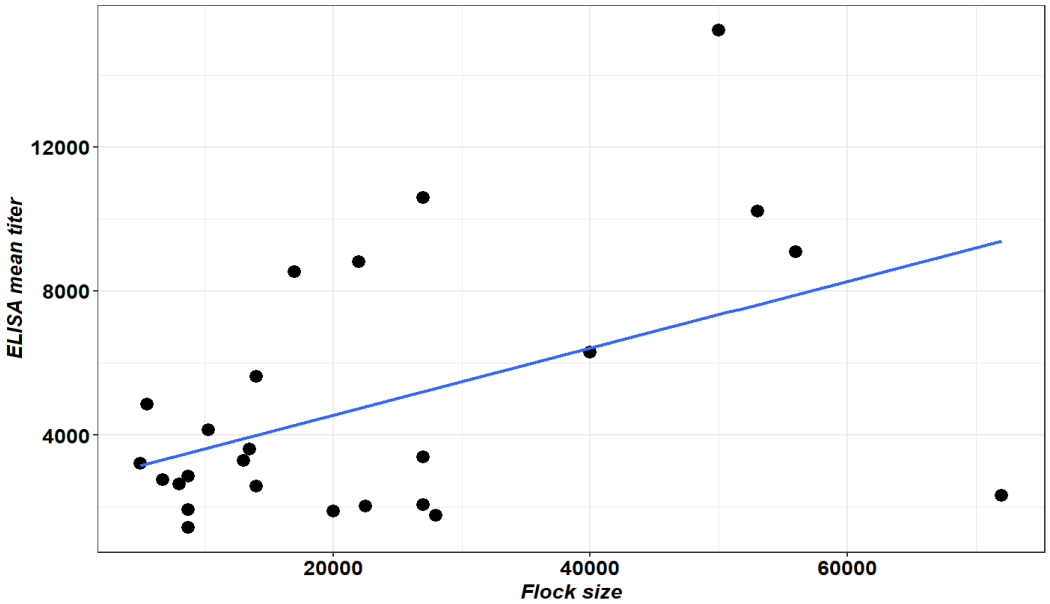


Figure 4. Correlation between flock size (age varied from 20 weeks to 107 weeks) and *Ornithobacterium rhinotracheale* enzyme-linked immunosorbent assay mean titer, in the governorate of Sfax, Tunisia.

DISCUSSION

Ornithobacterium rhinotracheale is a bacterial pathogen responsible for contagious respiratory and articular infections in poultry, particularly affecting turkeys, broilers, and laying hens (Boulianne et al., 2020). Susceptibility to ORT infection can vary depending on several factors, including bird species, the age of the animal, and overall farming conditions (Boulianne et al., 2020). The immune system typically responds to ORT exposure by producing specific antibodies (immunoglobulins M, G, and A). These antibodies can be detected as early as 5 days post-exposure through experimental aerosol inoculation (Eröksüz et al., 2006). The early detection capability of serological tests makes them valuable tools for identifying and monitoring ORT infections in poultry flocks.

The current study investigated the seroprevalence of ORT infection in commercial layer flocks located in the Sfax governorate, southeastern Tunisia. A total of 470 serum samples were collected from 25 randomly selected flocks, all of which were in the laying period. Utilizing an indirect ELISA test, found that 364 samples (77.45%) were positive for antibodies against ORT, indicating individual-level seroprevalence. Notably, positive samples were detected in all 25 flocks, resulting in a 100% flock-level prevalence. The high seroprevalence in the present study aligned with previous findings, which reported a 61.5% seroprevalence (eight seropositive flocks out of 13 flocks) in meat turkey farms within the governorate of Sfax (Ben Mbarek, 2009).

The current findings indicated a 100% flock-level seroprevalence for ORT in Tunisian layer flocks, consistent with previous reports from other countries. Similarly, 100% was reported in commercial layers from the North Central Region of the United States (Heeder et al., 2001) and South Korea (Jung, 2020). However, seroprevalence rates varied across studies. For instance, a seroprevalence of 96% was reported in India (Sumitha et al., 2015), while a lower prevalence of 17.4% in commercial layer flocks was found in different regions of Turkey (Aras et al., 2016).

These discrepancies might be attributed to several factors, including sample size and selection methods for participating farms, geographic location and potential regional variations, seasonal variations in ORT circulation patterns, management practices employed on the farms, such as biosecurity measures and vaccination protocols, the nature of the poultry farming systems, such as free-range versus intensive housing, farm density within the study area, potentially influencing transmission risks, antibiotic usage patterns on the farms, environmental factors that may influence ORT persistence and transmission, and the specific serological tests utilized (Ali et al., 2022).

The present study indicated a trend of increasing seroprevalence with flock age. The highest prevalence (85.78%) was observed in layers older than 56 weeks. Additionally, a moderate positive correlation ($r = 0.351$) was found between age and antibody titers, indicating a potential link between longer lifespans and higher exposure to ORT. The results of the current study aligned with the reports in Iran by Allymehr (2006) and in India by Baksi et al. (2017), where the highest prevalence was detected in older broiler breeders. Similarly, older chickens had higher infection rates and stronger antibody responses in China (Xue et al., 2020). However, no significant association was confirmed between age and ORT antibodies in broiler breeders in southern Brazil (Canal et al., 2003). These contrasting findings suggested that

the influence of age on ORT exposure may depend on different factors, warranting further investigation.

CONCLUSION

The high seroprevalence of *Ornithobacterium rhinotracheale* (ORT) antibodies (ranging from 1425.67 in flock 12 to 15233 in flock 19) detected in the current study, coupled with the absence of vaccination against ORT in Tunisia, strongly suggested widespread exposure to ORT in the commercial layer flocks (aged from 20 weeks to 100 weeks) of the Sfax governorate, Tunisia. The current findings highlighted the need for comprehensive prevention strategies to mitigate the impact of ORT on poultry health and productivity in the study region. Future studies could explore the most effective biosecurity measures, vaccination programs, and management practices to minimize ORT circulation and associated economic losses in different locations.

DECLARATIONS

Acknowledgments

The authors would like to thank the technical staff of all the visited farms in the governorate of Sfax, Tunisia, for their assistance in completing the present study.

Ethical considerations

This article was originally written by the authors and has not been published elsewhere. The authors checked the text of the article and confirmed that the article is written based on their original scientific results.

Authors' contributions

Khaled Kaboudi performed methodology, investigation, data analysis, and original draft writing. Imen Hamadi provided sampling, laboratory analysis, and data analysis. Atef Walha concluded experimental design, investigation, and sampling. Aymen Mamlouk provided laboratory analysis, statistical data, and review. Adel Souissi performed sampling and field supervision. All the authors confirmed the final edition of the manuscript before submission to the journal.

Competing interests

The authors have not declared any conflict of interest.

Funding

The present study did not receive any financial support.

Availability of data and materials

The data that support the findings of the present study are available from the corresponding author upon reasonable request.

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2025, Scienceline Publication
World's Veterinary Journal
World Vet J, 15(3): 566-577.
ISSN 2322-4568

DOI: https://dx.doi.org/10.54203/scil.2025.wvj57
PII: S232245682500057-15

Effects of *Bryophyllum pinnatum* Leaf Meal on Blood Parameters, Oxidative Status, Antioxidants, and Reproductive Hormones in Grower Rabbits

Razaq Animashahun[1*], Oluwabori Adefioye[1], Adedeji Animashahun[2], Olayinka Alabi[1], and Ooreoluwa Adegboye[1]

[1]*Animal Science Programme, College of Agricultural Sciences, Landmark University, P.M.B. 1001, Omu Aran, Nigeria*
[2]*Department of Animal Breeding and Genetics, Federal University of Agriculture, Abeokuta, P.M.B 2240, Abeokuta, Nigeria*

*Corresponding author's Emails: animashaun.rasaq@lmu.edu.ng; kunleannies@yahoo.com

ORIGINAL ARTICLE
Received: June 24, 2025
Revised: July 23, 2025
Accepted: August 26, 2025
Published: September 30, 2025

ABSTRACT

In the search for safe and effective phytogenic alternatives to synthetic additives in rabbit nutrition, *Bryophyllum pinnatum* has shown promising biological potential. This study investigated the effects of dietary inclusion of *Bryophyllum pinnatum* leaf meal (BPLM) on hematological parameters, serum biochemistry, oxidative stress markers, and reproductive hormone levels in grower male rabbits. A total of 48 Hyla grower male rabbits, aged 7–8 weeks, were randomly allotted to four dietary treatments in a completely randomized design (CRD): T1 (0% BPLM), T2 (1% BPLM), T3 (1.5% BPLM), and T4 (2% BPLM), with 12 rabbits per group, further subdivided into three replicates of four animals each. After an 10-week feeding trial, blood samples were collected and analyzed for hematological indices (erythrocyte count, packed cell volume, hemoglobin concentration, erythrocyte indices, total and differential white blood cell counts), biochemical parameters (liver enzymes, total protein, glucose, cholesterol, triglycerides, low-density lipoprotein, and high-density lipoprotein [HDL]), oxidative stress markers (malondialdehyde [MDA] and 2,2-diphenyl-1-picrylhydrazyl scavenging activity [DPPH]), and reproductive hormones. The majority of hematological and biochemical parameters were not significantly affected by BPLM inclusion, indicating no adverse impact on physiological homeostasis. However, erythrocyte count, serum cholesterol, and HDL levels showed significant differences. Erythrocyte count increased progressively from 0% to 1.5% BPLM inclusion, but declined at the 2% level. Serum cholesterol decreased gradually with increasing BPLM inclusion, with the lowest value observed at 2%. Antioxidant activity improved significantly with increasing BPLM levels, as evidenced by enhanced DPPH scavenging capacity and reduced MDA concentrations, particularly at 1.5% and 2% inclusion. Furthermore, reproductive hormones, apart from testosterone, were significantly elevated in rabbits fed diets containing 1.5% and 2% BPLM, suggesting enhanced reproductive function. Overall, these findings indicate that dietary inclusion of BPLM up to 2% is safe and beneficial for growing male rabbits, with positive effects on antioxidant defense and reproductive performance. The functional phytogenic properties of *Bryophyllum pinnatum* (*B. pinnatum*) leaves highlight their potential as a natural feed additive for improving rabbit health and productivity.

Keywords: Functional nutrition, Male fertility, Medicinal plant, Oxidative stress, Phytogenic feed additive, Rabbit

INTRODUCTION

The use of antimicrobial agents as growth promoters has long been a widespread practice in monogastric livestock production to improve growth performance and enhance resistance to diseases. However, concerns over antibiotic residues in animal products, their adverse effects on human therapeutics, and the emergence of antimicrobial resistance have led to strict regulations and bans on antibiotic growth promoters (AGPs) in many countries (Xu et al., 2022). The ban on AGPs has prompted increased research into safer, natural alternatives, such as plant-based leaf meals.

The use of phytogenic feed additives, such as leaf meals, in animal nutrition has gained significant attention for their potential to improve growth performance, immune function, and overall health in livestock (Wang et al., 2024). Replacing antibiotics with leaf-based additives also supports several United Nations Sustainable Development Goals (SDGs), notably SDG 1 (No Poverty), SDG 2 (Zero Hunger), and SDG 3 (Good Health and Well-being, Granato et al., 2022; Gamage et al., 2023). *Bryophyllum pinnatum* (*B. pinnatum*), a locally available and cost-effective feed additive, contributes to SDG 1 by reducing reliance on expensive synthetic inputs while enhancing rabbit health, growth, and reproductive performance (Abdelsalam and Fathi, 2023). This can increase meat yield and income for smallholder farmers, boosting food security and economic resilience in low-income communities. Overall, the adoption of plant-based additives in animal diets promotes sustainable livestock production, economic growth, and improved public health outcomes (Wang et al., 2024).

To cite this paper: Animashahun R, Adefioye O, Animashahun A, Alabi O, and Adegboye O (2025). Effects of *Bryophyllum pinnatum* Leaf Meal on Blood Parameters, Oxidative Status, Antioxidants, and Reproductive Hormones in Grower Rabbits. *World Vet. J.*, 15(3): 566-577. DOI: https://dx.doi.org/10.54203/scil.2025.wvj57

Studies on rabbits have demonstrated the efficacy of phytogenic feed additives in improving hematological parameters, oxidative stress markers, and reproductive performance (Oloruntola et al., 2018; Abdelghani et al., 2024). For instance, the inclusion of garlic (*Allium sativum*) and ginger (*Zingiber officinale*) in rabbit diets has been linked to enhanced antioxidant activity, improved lipid metabolism, and increased testosterone levels (Unigwe and Igwe, 2022). Similarly, supplementation with *Moringa oleifera* has been shown to influence blood profiles and immune responses in rabbits positively (Badawi et al., 2017). These findings highlight the potential of plant-based additives in modulating physiological functions and promoting overall health in rabbits. El-Hady et al. (2020) reported that incorporating phytogenic feed additives into broiler chicken diets led to marked improvements in hematological indices, including hemoglobin concentration, erythrocyte count, and hematocrit. These results suggest that plant leaf extracts may have potential applications in improving blood cell formation in animals. It has been discovered that some leaf meals, such as *papaya* leaf meal, have hepatoprotective effects, which means they can help safeguard the liver from harm and enhance liver function (Ugbogu et al., 2023). N'nanle et al. (2020) observed that incorporating certain leaf meals, such as fenugreek and *Moringa oleifera*, into animal diets can elevate high-density lipoprotein (HDL) levels while reducing total cholesterol. In contrast, other leaf meals like cassava leaf meal may exert minimal effects on cholesterol modulation. Several studies have examined the effects of leaf meals on lipid peroxidation in rabbits. For instance, investigations utilizing Moringa leaf meal have demonstrated a reduction in lipid peroxidation markers, such as malondialdehyde (MDA) levels. The presence of bioactive compounds in leaf meal is believed to contribute to its antioxidant effects (Vergara-Jimenez et al., 2017). Studies have also shown that certain herbs and botanicals can exhibit antibacterial, antiviral, anthelmintic, coccidiostatic, anti-inflammatory, and antioxidant properties (Rostami et al., 2017; Abd El-Hack et al., 2020). Phytogenic alternatives to antibiotics, particularly flavonoids such as quercetin and kaempferol, as well as terpenoids and phenolic acids derived from plants like *Moringa oleifera* and *Zingiber officinale*, have been reported to improve hematological parameters, reduce lipid peroxidation (as indicated by decreased malondialdehyde levels), enhance antioxidant enzyme activities (e.g., superoxide dismutase, catalase), support immune function, and modulate hormonal balance in poultry (Abdelli et al., 2021; Wang et al., 2024). Given its rich phytochemical profile, *B. pinnatum* may exhibit similar effects when included in rabbit diets, making it a promising candidate for further investigation. Livestock feed supplies essential nutrients that can influence hormonal activity, thereby supporting growth and development in animals (Cuchillo-Hilario et al., 2024). For instance, diets rich in protein can stimulate the secretion of growth hormone (GH), which plays a key role in promoting tissue growth, muscle development, and overall productivity (Firmenich et al., 2020). For lactating animals, specialized feeds are formulated to support milk production by ensuring the availability of nutrients that influence key lactogenic hormones (Pan et al., 2023). The primary hormones involved in milk synthesis and let-down include prolactin, oxytocin, estrogen, and progesterone. Deficiency in certain nutrients can disrupt hormonal balance, leading to irregular cycles and reduced fertility (Skoracka et al., 2021).

Bryophyllum pinnatum, also referred to as the “life plant” or “miracle leaf,” has been extensively studied for its medicinal potential, notably its anti-inflammatory, antioxidant, and hormone-regulating properties. Its leaves contain various phytochemicals such as flavonoids (notably quercetin, kaempferol, and rutin), alkaloids, and polyphenols, which are largely responsible for its health-promoting effects (Kumar et al., 2019). Among these, quercetin is recognized for its antioxidant capacity and has demonstrated anti-inflammatory, anticancer, and cardioprotective benefits by mitigating oxidative stress (Carrillo-Martinez et al., 2024). Kaempferol has also been shown to have anti-inflammatory, anticancer, and neuroprotective effects (Qattan et al., 2022). Owing to its high-water content and nutritional value, *B. pinnatum* leaves are utilized in some areas as an additive in feed for cattle, goats, and poultry (Omah et al., 2022). While *B. pinnatum* has indeed been studied for its pharmacological properties, including antioxidant, anti-inflammatory, and hormonal effects, most of these investigations have focused on its medicinal applications in humans or used *in vitro* or *in vivo* models using rodents (Araújo et al., 2023). However, there remains a significant knowledge gap regarding its nutritional and physiological effects in monogastric livestock, particularly rabbits. Studies in poultry and ruminants suggest that its inclusion in feed improves immune response, reduces oxidative stress, and enhances reproductive hormone secretion (Philip et al., 2023; Obianwuna et al., 2024).

However, data on *B. pinnatum* effects in monogastric animals, particularly weaned rabbits, are limited. Given the physiological challenges associated with weaning, including oxidative stress and immune suppression, investigating *B. pinnatum* as a dietary supplement may provide insights into its role in mitigating these challenges while improving blood parameters, oxidative status, and male reproductive hormones. Despite the extensive ethnomedicinal use of *B. pinnatum*, its application in livestock, particularly in weaned rabbits, remains underexplored, as well as its safety and efficacy have not been extensively studied in veterinary clinical trials. Therefore, this study aimed to evaluate the effects of dietary *B. pinnatum* leaf meal on hematological and serum biochemical parameters, oxidative stress markers, and male reproductive hormones in weaned rabbits.

MATERIALS AND METHODS

Ethical approval

The study was approved by the Landmark University Research Ethics Committee, Landmark University, Omu Aran, Nigeria. All procedures were carried out in accordance with the principles of the Declaration of Helsinki and the Interdisciplinary Principles and Guidelines for the Use of Animals in Research, Testing, and Education, as outlined by the Ad Hoc Animal Research Committee of the New York Academy of Science.

Study area

The study was conducted at the Teaching and Research Farm, as well as the Animal Science and Biochemistry Laboratories of Landmark University, Omu-Aran, Kwara State, Nigeria. According to the 1991 census, Omu-Aran had an estimated population of approximately 148,610 and covers a land area of 1,095 square kilometers. It shares borders with Ifelodun Local Government Area (LGA) to the north, Osun State to the south, and Ekiti and Offa LGAs. Geographically, Omu-Aran is positioned at approximately 8.9°N latitude and 5.061°E longitude.

Source of feed ingredients and forage

Bryophyllum pinnatum leaves were sourced from Landmark University Teaching and Research Farm, Omu-Aran, Kwara State, Nigeria. These were harvested, air dried (28°C) for 72 hours, milled to 300 micron particle sizes, and incorporated into compounded feed.

Animal management, experimental design, and diets

A total of 48 weaned male Hyla rabbits (mean age and weight of 6 weeks and 557g, respectively) were purchased from a reputable Rabbit Farm in Omu Aran, Nigeria, and used in this 10-week feeding trial. The rabbits were housed in well-ventilated cages under standard management practices, while clean drinking water was provided *ad libitum*. The study was conducted using a completely randomized design (CRD), with the rabbits allocated into four dietary treatment groups. Each group consisted of 12 rabbits, subdivided into 3 replicates of 4 rabbits each. The diets were formulated to be isonitrogenous (16.5% crude protein) and isocaloric (2700 kcal/kg metabolizable energy) in accordance with the requirements of the National Research Council (NRC, 1994), to ensure that any observed differences in performance and physiological responses were attributable to the graded inclusion levels of *B. pinnatum* rather than variations in nutrient composition. Group 1 served as the control with no *B. pinnatum* leaf meal (BPLM), while Groups 2 to 4 received BPLM at 1.0%, 1.5%, and 2.0%, respectively (Oladipupo, 2024), as shown in the composition in Table 1.

Table 1. Feed composition of the experimental diets fed to 6-week-old grower Hyla rabbits

Feed ingredient (%)	Inclusion levels of *Bryophyllum pinnatum* (%)			
	0.00	**1.00**	**1.50**	**2.00**
Corn	39.39	38.90	38.72	38.70
Wheat bran	26.29	25.94	25.84	25.60
Soya bean cake	9.89	9.87	9.81	9.75
Groundnut cake	6.59	6.58	6.66	6.52
Palm kernel cake	13.14	12.97	12.77	12.73
Bryophyllum pinnatum leaf meal	0.00	1.00	1.50	2.00
Bone meal	4.00	4.00	4.00	4.00
Premix	0.25	0.25	0.25	0.25
Methionine	0.15	0.15	0.15	0.15
Lysine	0.10	0.10	0.10	0.10
Salt	0.25	0.25	0.25	0.25
TOTAL (%)	100.00	100.00	100.00	100.00
Crude protein (%)	16.54	16.51	16.56	16.43
Metabolizable energy (kcal/kg)	2724	2725	2727	2728

Composition of Rabbit Grower Premix (per kg of premix): Vitamin A: 1,200,000 IU, Vitamin D_3: 200,000 IU, Vitamin E: 2,500 mg, Vitamin K_3: 500 mg, Vitamin B_1 (Thiamine): 300 mg, Vitamin B_2 (Riboflavin): 600 mg, Vitamin B_6 (Pyridoxine): 400 mg, Vitamin B_{12}: 2 mg, Niacin: 4,000 mg, Pantothenic acid: 1,000 mg, Folic acid: 100 mg, Biotin: 10 mg, Choline chloride: 60,000 mg, Iron (Fe): 3,000 mg, Copper (Cu): 400 mg, Zinc (Zn): 3,000 mg, Manganese (Mn): 2,000 mg, Iodine (I): 50 mg, Cobalt (Co): 20 mg, and Selenium (Se): 10 mg

Blood sampling

At 7:00 AM on the final day of the feeding trial, two sets of 5 mL blood samples were collected from the marginal ear vein using a sterile 23-gauge needle and a 5 mL syringe. One animal per replicate (average weight: 1.50 kg) was

sampled, resulting in a total of 12 blood samples per treatment group. Samples for hematological analysis were transferred into EDTA-coated tubes, while those intended for serum biochemical assays were collected into plain tubes and allowed to clot at room temperature. The clotted samples were centrifuged at 3000 rpm for 10 minutes to separate the serum, which was then stored at –20°C until analysis.

Hematological analysis

The hematological parameters were analyzed using an automated hematology analyzer (DxH 900 Beckman Coulter, Brea, California, USA). Parameters included packed cell volume (PCV), hemoglobin concentration (Hb), red blood cell count (RBC), white blood cell count (WBC), and differential leukocyte counts (neutrophils, lymphocytes, monocytes, eosinophils, and basophils). The erythrocyte indices, i.e., mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were calculated (Fan et al., 2018), using the following formula;

MCV (fl) = ([RBC (×106 / µL) / PCV]) × 10

MCH (pg) = (RBC [×106 / µL] / Hb [g/dL]) × 10

MCHC (g/dl) = (PCV / Hb [g/dL]) × 100

Serum biochemical analysis

Blood samples were collected at 7:00 a.m. and transferred into labeled plain tubes. These samples were used to assess serum concentrations of total protein, total cholesterol, triglycerides, low-density lipoprotein (LDL), high-density lipoprotein (HDL), albumin, glucose, and liver enzymes, including alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP). All analyses were performed using the Randox Evidence MultiSTAT system (Model 2017; Randox Laboratories Ltd., Crumlin, County Antrim, Northern Ireland, UK)

Hormonal profile evaluation

The hormonal profile was evaluated using the ARCHITECT® hormone assay (Abbott Laboratories, USA). Follicle Stimulating Hormone (FSH) was measured in IU/L, Luteinizing Hormone (LH) was measured in IU/mL, estradiol in pg/mL, testosterone in ng/dL, and progesterone in ng/mL.

Antioxidants assay evaluation

The total antioxidant activity of blood plasma was determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical assay, conducted in a 10 mM sodium phosphate buffer at pH 7.4. Absorbance readings of the DPPH· solution were taken at 516 nm using a Halo DB-20 Double Beam Spectrophotometer (Dynamica Scientific Ltd., UK).

Serum lipid peroxidation evaluation

The Thiobarbituric acid reactive substance, which is an indication of the oxidative stability of the meat, was analyzed by evaluating the level of malonaldehyde (MDA) in the blood samples using a commercial kit (Sigma-Aldrich, St. Louis, MO, USA) and expressed as µmol/L.

Statistical analysis

All the data from this investigation were reported as the average of three replicates. These were then subjected to one-way analysis of variance (ANOVA) using the SAS version 9.4 program (SAS, 2013). Differences at 5% ($p < 0.05$) were analyzed using Duncan's Multiple Range Test.

RESULTS AND DISCUSSION

Hematological parameters

The results (Table 2) showed that, except for RBC and erythrocyte indices (MCV and MCH), most hematological parameters were not significantly influenced ($p > 0.05$) by dietary inclusion of BPLM. Key parameters such as PCV, Hb, and WBC remained within normal physiological ranges for rabbits, 30-50% for PCV, 10-15 g/dL for Hb, and 3-12 × 10^9/L for WBC based on reference values from the reports of Merck Veterinary Manual (2023) and Lichtenberger and Lennox (2011). The findings in the present study indicated that BPLM at the tested inclusion levels did not adversely affect these parameters or the oxygen-carrying capacity. However, RBCs were slightly below the reference range of 4-7 × 10^{12}/L, particularly at the 2% inclusion level. The slightly lower level of RBC at higher inclusion levels may indicate a mild suppressive effect on erythropoiesis at higher BPLM doses. In contrast, slight numerical ($p > 0.05$) increases in PCV and Hb were observed at 1.5% and 2% inclusion levels, suggesting a possible hematinic effect. These findings align with the study of Ufelle et al. (2011), who reported that phytochemicals in *B. pinnatum*, such as flavonoids and ascorbic acid, may stimulate erythropoiesis and haemoglobin synthesis. WBC counts remained within normal limits across all treatments, with the highest values recorded at 2% inclusion. This increase may reflect an immune-stimulatory response, consistent with the immunomodulatory potential of *B. pinnatum* reported in albino rats (Bassey et al., 2021). Comparable effects have been observed with other phytobiotics, such as garlic (*Allium sativum*) and ginger (*Zingiber officinale*), which have enhanced immune parameters in rabbits (Okoye et al., 2016). The 2% BPLM inclusion may be

reaching a threshold where mild anti-nutritional effects begin to subtly suppress RBC production, while bioactive compounds continue to enhance immune parameters (e.g., via elevated WBCs). This suggests a dose-sensitive trade-off between hematopoiesis and immunostimulation.

Table 2. Haematological evaluation of 6-week-old grower Hyla rabbits fed different *Bryophyllum pinnatum* leaf meal

Parameter \ Inclusion levels of *B. pinnatum* across diets	0%	1%	1.5%	2%	SEM	p -value
PCV (%)	39.50	40.80	42.00	39.25	0.63	0.52
RBC ($\times10^{12}$/L)	3.50^{b}	3.75^{ab}	3.95^{a}	2.90^{c}	0.10	0.07
HB (g/L)	11.85	12.70	1290	11.90	0.81	0.29
WBC ($\times10^{9}$/L)	5.20	5.00	5.10	5.25	0.21	0.55
MCV (µm³)	11.29^{b}	10.88^{b}	10.63^{b}	13.53^{a}	0.32	0.05
MCH (g/dL)	33.86^{b}	33.87^{b}	32.66^{b}	41.03^{a}	0.18	0.09
MCHC (%)	30.00	31.13	30.71	30.32	1.00	0.17
LYMPH (%)	30.50	30.5	33.50	30.00	1.053	0.55
NEUT (%)	68.00	67.50	66.00	66.00	1.23	0.91
EOSPH (%)	1.50	2.00	0.50	0.00	0.31	0.23

[a,b,c]: Means within a row with different superscript letters differ significantly ($p < 0.05$). PCV: Packed cell volume, WBC: White blood cell, RBC: Red blood cell, HB: Haemoglobin, MCV: Mean corpuscular volume, MCH: Mean corpuscular haemoglobin, MCHC: Mean corpuscular haemoglobin concentration, RBC: Red blood cell, WBC: White blood cell, NEUT: Neutrophil, LYMPH: Lymphocyte, EOSPH: Eosinophil, SEM: Standard error of means.

Differential leukocyte counts further illuminate the immune and inflammatory status of the rabbits. Neutrophil, lymphocyte, and eosinophil values remained within established ranges, 20-75% for neutrophils, 25-80% for lymphocytes, and 0-5% for eosinophils (El-Sheikh et al., 2010). A mild increase in neutrophil percentage at 2% inclusion may indicate enhanced innate immunity, echoing findings in broiler chickens where *B. pinnatum* leaf extract elevated neutrophil counts and improved resistance to infections (Bassey et al., 2021). Lymphocyte levels peaked at 1.5% inclusion, suggesting a possible stimulatory effect on lymphopoiesis. Similar lymphocyte-enhancing effects have been reported with herbal additives such as *Moringa oleifera* and *Ginger* (Okoye et al., 2016; Desouky et al., 2019). Eosinophil counts remained stable across treatments, indicating no allergic or hypersensitivity reactions from BPLM. Overall, the stability and favorable modulation of these haematological indices suggest that *B. pinnatum* leaf possesses immunomodulatory properties without inducing systemic stress or inflammation.

Previous studies on *B. pinnatum* in monogastric animals have reported varied haematological outcomes, depending on species, dosage, and study conditions. For example, Datta et al. (2023) found that *B. pinnatum* extract improved antioxidant status without negatively affecting WBC counts. Similarly, Ekpo et al. (2021) reported improvements in PCV and RBC at moderate inclusion levels (≤ 1.5%) in rabbits, while higher levels (> 2%) led to mild anaemia and increased oxidative stress. These outcomes support the current observation that high BPLM inclusion might impair RBC production, possibly due to the interference of bioactive compounds with haematopoiesis.

The presence of phytochemicals such as flavonoids, alkaloids, and tannins (Kumar et al., 2019) in *B. pinnatum* leaves may account for the observed changes in RBC and WBC values. Moreover, the antioxidant properties of the leaves could protect erythrocytes from oxidative damage, contributing to improved PCV and Hb, especially at 1.5% inclusion.

An inclusion level of 1.5% BPLM in the present study appears optimal for maintaining healthy haematological profiles in rabbits, promoting erythropoiesis, and enhancing immune function without adverse effects. The 2% level may slightly suppress RBC production, though compensatory increases in MCV and MCH suggest physiological adaptation. This finding aligns with the report by Ekpo et al. (2021), who evaluated the effects of ethanolic and aqueous leaf extracts of *B. pinnatum* on haematological parameters in Wistar rats. Their study noted reductions in MCV, MCH, and MCHC, alongside changes in RBC and hemoglobin levels, indicating that *B. pinnatum* bioactives can modulate erythropoiesis and red blood cell indices in a dose-dependent manner. The compensatory increase in MCV and MCH in our study may thus be attributed to the phytochemical constituents of BPLM, such as flavonoids, polyphenols, and vitamin C, which are known to influence oxidative status and haematopoietic processes.

Serum biochemical parameters

The serum biochemical profile offers critical insights into the metabolic health and lipid metabolism of the animals. In the present study, the inclusion of BPLM positively influenced several biochemical parameters. As presented in Table 3, only serum cholesterol and high-density lipoprotein (HDL) levels were significantly affected ($p < 0.05$) by the dietary

inclusion of BPLM. While most serum parameters, such as total protein, albumin, glucose, and liver enzymes, remained within normal physiological ranges, some notable trends were observed. Serum cholesterol levels decreased significantly with higher levels of BPLM inclusion, indicating a potential hypolipidemic effect ($p > 0.05$). This finding suggests that while moderate (up to 1.5%) inclusion supports normal metabolic physiology, higher inclusion levels may exert additional lipid-lowering effects, though potentially at the expense of erythropoiesis.

Table 3. Biochemical evaluation of 6-week-old grower male Hyla rabbits fed varying levels of *Bryophyllum pinnatum* leaf meal

Parameter \ Inclusion levels of *B. pinnatum* across diets	0%	1%	1.5%	2%	SEM	p-value
TPN (g/dL)	5.70	5.75	5.88	5.65	1.21	0.90
ALB (g/dL)	2.45	2.66	2.75	2.28	1.15	0.43
GLU (mg/dL)	81.05	79.35	70.10	71.00	1.94	0.84
CHO (mmol/dL)	13.60^{a}	13.00^{a}	11.50^{ab}	8.50^{b}	0.05	0.07
TRI (mmol/L)	1.31	1.31	1.30	1.33	0.05	0.74
LDL (mmol/dL)	0.83	0.79	0.63	0.61	0.15	0.14
HDL (mmol/dL)	1.66^{a}	1.57^{a}	1.18^{ab}	1.73^{a}	0.96	0.11
ALP (µ/L)	72.90	73.50	73.05	73.20	3.95	0.95
AST (µ/L)	39.35	39.10	40.10	36.85	2.14	0.95
ALT (µ/L)	58.80	59.95	57.50	88.45	1.12	0.88

^{a,b}: Means within a row with different superscript letters differ significantly ($p < 0.05$). TPN: Total protein, GLU: Glucose, ALB: Albumin, CHO: Cholesterol, TRI: Triglycerides, LDL: Low density lipoprotein, HDL: High density lipoprotein, ALP: Alkaline phosphatase, AST: Aspartate aminotransferase, ALT: Alanine aminotransferase. SEM: Standard error of means.

Total protein, albumin, and globulin levels showed modest increases with increasing BPLM levels, peaking at 1.5%. These values remained within the normal ranges for rabbits (total protein: 5.4-7.5 g/dL; albumin: 2.5-4.0 g/dL) as reported by Cheeke (1987). Elevated protein levels may indicate enhanced liver synthetic capacity or improved nutrient assimilation, likely facilitated by the bioactive compounds in *B. pinnatum* (Andrade et al., 2020).

Serum glucose levels exhibited a consistent but slight decrease with increasing BPLM inclusion, although all values remained within the normal physiological range for rabbits (75-150 mg/dL). This pattern suggests a mild hypoglycemic effect that did not result in any clinical signs of low blood sugar, indicating that the animals maintained glucose homeostasis. The observed trend could be linked to the bioactive compounds present in BPLM, particularly flavonoids and phenolic compounds**.** These phytochemicals are known to possess insulin-sensitizing properties. Specifically, they may act by: Stimulating glucose transporter type 4 (GLUT-4) translocation in peripheral tissues (especially muscle and adipose tissues), enhancing insulin receptor sensitivity**,** reducing hepatic gluconeogenesis (production of glucose by the liver), and overall improving the efficiency of glucose uptake into cells. This proposed mechanism is supported by previous studies. For instance, Sok Yen et al. (2021) reported that plant-derived polyphenols (from *Camellia sinensis*). Enhance insulin signaling pathways, leading to improved glycemic control. Additionally, a similar hypoglycemic effect was documented by Okoduwa et al. (2017), where Albino rats supplemented with *Aloe vera* and *Ocimum gratissimum* extracts at dosages ranging from 150 to 2000 mg/kg showed a dose-dependent reduction in blood glucose levels. Therefore, the mild decline in serum glucose levels observed in this study with BPLM supplementation could be attributed to a synergistic action of its phytochemicals, mimicking insulin or improving insulin action. Importantly, since glucose values stayed within the safe range, this suggests a beneficial modulation of glucose metabolism rather than a pathological reduction, making BPLM a potential functional feed additive for glycemic regulation in monogastric animals.

Cholesterol and HDL levels were significantly reduced at higher inclusion levels of BPLM ($p < 0.05$). Notably, cholesterol concentrations decreased from 13.60 mmol/dL in the control group (0% BPLM) to 8.50 mmol/dL in the group fed 2% BPLM. This hypolipidemic effect is consistent with reports on phytogenic additives such as oregano and garlic, which lower cholesterol levels by inhibiting its biosynthesis or promoting its excretion (Kothari et al., 2019; Adjei-Mensah et al., 2024). The cholesterol-lowering activity observed in this study corroborates the findings of Singha and Lahkar (2020) and Bassey et al. (2021), who demonstrated similar effects in rats administered ethanolic extracts of *B. pinnatum* at doses ranging from 100-400 mg/kg and 180-540 mg/kg, respectively. These effects may be attributed to the presence of bioactive compounds such as saponins and flavonoids, as highlighted by Sok Yen et al. (2021).

Triglyceride levels also declined progressively with increasing BPLM inclusion, reinforcing its lipid-lowering potential. Bioactive compounds such as saponins, flavonoids, and sterols may inhibit lipid absorption or promote lipid metabolism, as observed in rats and poultry models (Azodo et al., 2021; Oladipupo, 2024). In the present study, low-density lipoprotein (LDL), often referred to as "bad cholesterol," also decreased insignificantly ($p > 0.05$) progressively

along the row, with the lowest concentration observed at the 2% inclusion level. This cardioprotective effect mirrors findings by Cardinali et al. (2015), who reported similar LDL reductions with oregano and rosemary in rabbit diets.

Interestingly, HDL, often regarded as "good cholesterol", increased slightly across treatments, peaking at 1.5% inclusion. This may reflect enhanced reverse cholesterol transport, indicating a healthier lipid profile and overall metabolic improvement. These findings underscore the phytobiotic potential of *B. pinnatum*, aligning it with other established herbal additives such as neem (*Azadirachta indica*), garlic (*Allium sativum*), and *Moringa* (*Moringa oleifera*) in rabbit nutrition (Onu, 2010; Webb et al., 2022).

No significant changes ($p > 0.05$) were observed in liver function enzymes (ALP, ALT, and AST), indicating that BPLM up to 2% does not impair hepatic function. These results aligned with earlier findings on the hepatoprotective effects of plant-based additives (Datta et al., 2023), e.g., Flavonoids, polyphenols, and vitamin C in bryophyllum, curcumin in turmeric, catechins in green tea, limonoids, allicin, sulfur-containing compounds in garlic, gingerols, and shogaols in ginger, and azadirachtin in neem

In contrast to some synthetic antibiotics or less-tolerated phytogenics that can cause hepatocellular damage, *B. pinnatum* appears safe up to 2% inclusion level in growing rabbits. Similar hepato-stability has been observed with oregano and thyme supplementation in poultry (Kothari et al., 2019).

The findings from Tables 2 and 3 suggest that *B. pinnatum* leaf meal, when included up to 2% in rabbit diets, is safe and beneficial. It enhanced haematological and serum biochemical parameters without adverse effects. Its phytogenic properties, including antioxidant, immunomodulatory, hypoglycemic, and hypolipidemic effects, make it a potential natural alternative to synthetic growth promoters and antibiotics. This supports the broader move toward antibiotic-free animal production systems, where phytobiotics play an increasingly important role in maintaining health and performance. Furthermore, *B. pinnatum*, being a locally available and underutilized plant, offers a cost-effective and sustainable solution for livestock production. Compared to other leaf meals such as neem, *Moringa*, and bitter leaf (*Vernonia amygdalina*), which are often used at higher levels (3-5%) and sometimes associated with mild haematological stress (Tokofai et al., 2020), *B. pinnatum* at 1-2% appears equally effective and better tolerated.

Serum oxidative stress markers and antioxidant status

Table 4 presents the serum oxidative stress markers and antioxidant levels of rabbits fed diets containing varying levels (0%, 1.0%, 1.5%, and 2.0%) of BPLM. The DPPH (2,2-diphenyl-1-picrylhydrazyl) assay is widely used to evaluate the free radical scavenging ability of antioxidants in biological systems. In this study, dietary inclusion of BPLM significantly enhanced serum antioxidant activity in grower rabbits, with the most notable effect observed at the 1.0% inclusion level. DPPH scavenging activity increased from 24.77 µg/mL in the control group to 34.83 µg/mL, indicating improved radical neutralization. This enhancement is likely due to the presence of bioactive compounds such as flavonoids, phenolics, alkaloids, and vitamin C, which are known to donate electrons or hydrogen atoms to counteract free radicals (Yemitan et al., 2020).

Table 4. Lipid peroxidation and antioxidant (DPPH) concentrations of 6-week-old grower Hyla rabbits fed different levels of *Bryophyllum pinnatum* leaf meal inclusion

Parameters	T1 (0%)	T2 (1%)	T3 (1.5%)	T4 (2%)	SEM (±)	p value
DPPH (µg/mL)	24.77^{d}	34.83^{a}	29.50^{b}	26.49^{c}	1.44	0.04
MDA (nmol/L)	2.52^{a}	2.05^{b}	1.93^{c}	1.80^{d}	0.10	0.04

^{a,b,c,d}: Means within a row with different superscript letters differ significantly ($p < 0.05$). SEM: Standard error of mean; DPPH: 2,2-diphenyl-1-picrylhydrazyl; MDA: Malonaldehyde.

The antioxidant potential of *B. pinnatum* has been well-documented, with evidence of its ability to inhibit lipid peroxidation and scavenge reactive oxygen species *in vitro* (Daniel et al., 2020; Bassey et al., 2021). However, the decline in DPPH activity at higher inclusion levels, 29.50 µg/mL at 1.5% and 26.49 µg/mL at 2.0% suggests a dose-dependent response. This may reflect a threshold beyond which efficacy diminishes, possibly due to phytochemical overload or the emergence of pro-oxidant effects, especially in the presence of redox-active metals (Jomova et al., 2025). Moreover, high concentrations of tannins and saponins in leaves like *B. pinnatum* can reduce antioxidant effectiveness by forming complexes with proteins or interfering with the absorption and action of beneficial compounds (Kashyap et al., 2022). Similar biphasic patterns have been observed with other phytogenic additives, such as ginger and turmeric, where antioxidant activity improves at moderate levels but plateaus or declines with excessive supplementation (Jimoh et al., 2024).

Flavonoids and polyphenols, which are abundant in *B. pinnatum*, are known for their strong antioxidant properties (Pandey et al., 2017; Luo et al., 2022), but their benefits are maximized within an optimal range. At higher inclusion levels, saturation or disruption of endogenous antioxidant systems may occur (Ogunmoyole et al., 2025). Comparable trends have been reported with *Moringa oleifera*, *Azadirachta indica*, and *Allium sativum*, which enhance antioxidant status in monogastric animals under physiological stress (Mohlala et al., 2023; Wang et al., 2024).

Malondialdehyde (MDA), a key biomarker of lipid peroxidation and oxidative stress, showed a significant reduction ($p < 0.05$) with increasing levels of BPLM inclusion. Specifically, MDA concentrations decreased from 2.52 nmol/L in the control group (T1) to 1.80 nmol/L in the group receiving 2.0% BPLM (T4). This downward trend suggests a reduction in oxidative stress, likely due to the antioxidant properties of *B. pinnatum*, which contains compounds such as flavonoids, polyphenols, and vitamin C (Sok Yen et al., 2015). The significant reduction in MDA levels at higher inclusion rates supports the hypothesis that BPLM enhances antioxidant defense and mitigates lipid peroxidation ($p < 0.05$). Similar effects have been observed in animals fed phytogenic additives such as *Oregano*, which stimulate endogenous antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT), while reducing oxidative markers such as MDA (Kothari et al., 2019). These results underscore the role of BPLM in protecting cell membranes and maintaining cellular integrity, which is vital for animal health and productivity. The observed decline in MDA may reflect not only the direct free radical scavenging action of BPLM's bioactive compounds but also the suppression of lipid peroxidation chain reactions (Daniel et al., 2020). This improvement in oxidative stability suggests that dietary BPLM could be a viable strategy for reducing oxidative damage in rabbits (Monageng et al., 2023).

The results presented in Table 4 indicate that BPLM has beneficial effects on oxidative stress parameters in grower rabbits. Moderate inclusion levels (1.0%-1.5%) significantly improved antioxidant status, as evidenced by increased DPPH activity and reduced MDA levels. While 2.0% inclusion continued to suppress oxidative damage (as shown by lower MDA levels), the antioxidant activity appeared to decline slightly, suggesting a possible upper threshold for optimal efficacy. These findings reinforce the potential of BPLM as a natural dietary antioxidant, capable of enhancing oxidative resilience and improving animal health. The inclusion of BPLM at appropriate levels offered a sustainable, phytogenic alternative to synthetic antioxidants, particularly in antibiotic-free rabbit production systems. However, further studies may be warranted to investigate long-term effects and to optimize dosage for maximum antioxidant benefit.

Reproductive hormones

Table 5 presents the potential endocrine-modulating effects of BPLM on male rabbits. The evaluated hormones, FSH, LH, estradiol, prolactin, testosterone, and progesterone, play essential roles in regulating male reproductive function and overall reproductive health. Significant increases ($p < 0.05$) in FSH, LH, estradiol, and prolactin at moderate BPLM inclusion (1.0-1.50%) levels suggest a stimulatory effect on reproductive endocrine activity. Conversely, the lack of significant changes in testosterone levels ($p > 0.05$) indicates that BPLM's effects may be mediated more through the modulation of pituitary gonadotropins than through direct stimulation of testosterone synthesis.

Table 5. Reproductive hormones of 6-week-old grower male Hyla rabbits fed diets containing varied levels of *Bryophyllum pinnatum* leaf meal

Parameters	T1 (0%)	T2 (1%)	T3 (1.5%)	T4 (2%)	SEM (±)	p value
FSH (mIU/mL)	1.79^{c}	2.22^{b}	3.33^{b}	2.22^{b}	1.11	0.050
LH (mIU/mL)	3.67^{b}	4.46^{a}	3.77^{b}	3.38^{c}	0.42	0.047
Estradiol (pg/mL)	19.56^{b}	38.50^{a}	17.43^{c}	16.32^{d}	5.04	0.010
Prolactin (ng/mL)	14.86^{d}	19.79^{a}	18.72^{b}	16.25^{c}	6.90	0.044
Testosterone (ng/dL)	7.27	7.18	6.94	6.34	1.10	0.055
Progesterone (ng/dL)	0.10^{c}	0.65^{a}	0.33^{b}	0.10^{c}	2.76	0.002

^{a,b,c,d} Values on the same row with different superscript letters are significant ($p < 0.05$). SEM: Standard error of mean; FSH: Follicle-stimulating hormone; LH: Luteinizing hormone.

Follicle stimulating hormone is essential for spermatogenesis, promoting sperm cell development and maturation in the testes (Santi et al., 2020). In the present study, FSH levels increased significantly ($p < 0.05$) with BPLM supplementation, peaking at 3.33 mIU/mL in the T3 group (1.50% inclusion), while the control group (T1) had the lowest level (1.79 mIU/mL). The increasing concentration of FSH from the control to group 3 may suggest that BPLM may enhance spermatogenic activity, potentially improving sperm quantity and quality. The present findings align with previous reports indicating that plant-derived bioactive compounds, such as flavonoids, can stimulate gonadotropin secretion, thereby creating a hormonal environment conducive to male fertility (Andrade et al., 2020).

Luteinizing hormone (LH) stimulates testosterone production by Leydig cells in the testes (Esteves and Humaidan, 2025). Significantly higher LH levels ($p < 0.05$) were observed in T2 (1.00%) and T3 (1.50%) groups compared to the control, with T2 showing the highest concentration (4.46 mIU/mL). While testosterone levels did not differ significantly, the increased LH suggests a potential stimulatory effect of BPLM on the hypothalamic-pituitary-gonadal axis ($p > 0.05$). This supports earlier findings that certain phytogenic compounds could influence LH secretion and enhance reproductive function (Swelum et al., 2021; Shai et al., 2022).

Estradiol, although primarily associated with female reproduction, also contributes to male reproductive health by modulating spermatogenesis, sexual behavior, and sperm motility (Ramya et al., 2023). Estradiol levels were significantly elevated ($p < 0.05$) in T2 (1.00% BPLM), reaching 38.50 pg/mL, the highest among all groups. This may indicate an enhanced endocrine response to moderate BPLM inclusion. Given its role in sperm maturation, the increase in estradiol could support improved fertility, though the exact mechanisms warrant further investigation (Schulster et al., 2016). Prolactin is involved in testosterone regulation, spermatogenesis, and sperm motility (Martínez-Fresneda et al., 2020). A significant increase in prolactin concentration was observed in the T2 group (1.00% BPLM), which recorded the highest level (19.79 ng/mL) compared to the control, T3, and T4 groups ($p < 0.05$). This finding suggests that BPLM may positively influence endocrine function at moderate inclusion levels (1.0-1.5%). Such modulation has been linked to improved sperm quality and fertilization potential, reinforcing the reproductive benefits of BPLM (Zurfluh et al., 2025).

Testosterone plays a crucial role in regulating libido, spermatogenesis, and the development of male secondary sexual characteristics. However, no significant differences in testosterone levels were observed among the treatment groups ($p > 0.05$). This finding indicated that while BPLM may not directly enhance testosterone production, it could influence upstream hormonal pathways such as FSH and LH that regulate its synthesis. Previous studies suggested that increases in pituitary gonadotropins may precede changes in testosterone levels, which may not be immediately detectable due to hormonal feedback mechanisms (Adeniyi et al., 2025).

Progesterone, although typically associated with female physiology, progesterone in males supports sperm motility and maturation (Kolatorova et al., 2022). A significant increase was observed in the T2 group (0.65 ng/dL) compared to the control, T3, and T4 groups, suggesting that BPLM may beneficially modulate male reproductive function at lower to moderate inclusion levels (1.0-1.5%, $p < 0.05$). Elevated progesterone has been linked to improved sperm function and viability (Nagy et al., 2021).

Overall, the significant increases in FSH, LH, prolactin, and estradiol, alongside the elevation in progesterone, suggest that BPLM may enhance spermatogenesis, sperm quality, and motility. While testosterone levels remained unchanged, the modulation of pituitary hormones implies that BPLM influences key pathways that support male reproductive performance. These findings highlight the potential of BPLM as a natural phytogenic feed additive for improving fertility in male rabbits.

CONCLUSION

The dietary inclusion of BPLM up to 2% in grower male rabbit diets proved safe, with no negative impact on hematological or biochemical parameters. Instead, it enhanced antioxidant status, shown by increased DPPH activity and reduced MDA levels, and improved reproductive hormone profiles, particularly at 1.5% and 2% inclusion, as indicated by elevated LH levels. These findings suggest that BPLM is a functional feed additive with health-promoting potential in rabbit production. Future studies should explore higher inclusion levels and long-term effects, including impacts on female reproduction. Further investigation into its mechanisms of action, tissue safety, gut health, immune response, and cost-effectiveness is also recommended.

DECLARATIONS

Acknowledgments

The authors gratefully acknowledge the management of Landmark University, Omu-Aran, Kwara State, for granting access and permission to use the university's facilities.

Authors' contributions

Razaq Animashahun and Olayinka Alabi contributed to the conceptualization of the study, developed the protocols and experimental design, and were responsible for editing and reviewing the manuscript. Oluwabori Adefioye and Ooreoluwa Adegboye carried out the experimental study and prepared the initial draft of the manuscript. Adedeji Animashahun performed the statistical analyses. All authors have read and approved the final version of the manuscript.

Availability of data and materials

The authors confirm that all data supporting the findings of this study are included within the manuscript.

Ethical considerations

This manuscript represents the authors' original study and has not been previously published. All authors have assessed the text for similarity and affirm that it is based on their original scientific research.

Funding

This research was conducted without any financial support.

Competing interests

The authors declare no conflict of interest.

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2025, Scienceline Publication
World's Veterinary Journal
World Vet J, 15(3): 578-587.
ISSN 2322-4568

DOI: https://dx.doi.org/10.54203/scil.2025.wvj58
PII: S232245682500058-15

Evaluation of Garlic Powder as a Control Agent of *Vibrio* sp. in the Digestive Tract of White-leg Shrimp

Afandi Saputra[1*], Suharyadi [1], Erni Marlina[1], Sinar Pagi Sektiana[1], Sri Budiani Samsuharapan[1], and Ade Maria Ulfa[2]

[1]*Department of Aquaculture, Jakarta Technical University of Fisheries, Ministry of Marine Affairs and Fisheries, Republic of Indonesia, Jl. Raya Pasar Minggu, Jati Padang, Jakarta-12520, Indonesia*
[2]*Pharmacy Study Program, Faculty of Health Sciences, Malahayati University, Jl. Pramuka No. 27, Bandar Lampung, Indonesia*
*Corresponding author's Email: afandi.saputra38@gmail.com

ORIGINAL ARTICLE
Received: June 26, 2025
Revised: July 28, 2025
Accepted: August 27, 2025
Published: September 30, 2025

ABSTRACT

Garlic (*Allium sativum*) is a natural plant recognized for its active compounds, such as allicin, which possess antibacterial properties that can combat pathogenic microorganisms. *Vibrio* sp. bacteria can be pathogenic to vannamei shrimp (*Litopenaeus vannamei*), causing mortality marked by necrosis and melanization in the hepatopancreas cells. The present study aimed to examine the chemical compositions of garlic powder and its potential to inhibit the growth of *Vibrio* sp. bacteria in vannamei shrimp. Vannamei shrimp, with a population of 400 shrimp/m^3, were orally administered garlic powder mixed into their feed. The experimental doses were 0.1 mg of garlic powder (Group A), 0.5 mg of garlic powder (Group B), and 1 mg of garlic powder in 1 g of shrimp feed (Group C), along with shrimp that did not receive garlic powder (K, control group). Vannamei shrimp at the post-larva stage were used with a density of 1000 shrimp in each tank. Phytochemical analysis of the garlic powder revealed the presence of compounds such as flavonoids, alkaloids, tannins, phenols, and saponins. Thin-layer chromatography using quercetin as the reference standard yielded a positive result, indicated by a retention factor of 0.66. Notably, there was a significant reduction in total bacterial counts of *Vibrio* sp. in 40 days of treatment. A garlic powder at a dose of 0.5 mg/g was particularly effective in inhibiting the growth of *Vibrio* sp. bacteria. The current results indicated that shrimp not given garlic powder (control group) exhibited significant atrophy and hepatopancreas infiltration.

Keywords: Garlic, Hepatopancreas, Vannamei Shrimp, *Vibrio* sp.

INTRODUCTION

Endemic diseases of vannamei shrimp (*Litopenaeus vannamei)* include white spot syndrome virus (Balakrishnan et al., 2011), taura syndrome virus (Flegel, 2012), early mortality syndrome, and acute hepatopancreatic necrosis disease (AHPND; Saputra et al., 2023a). Somboon et al. (2012) found that vannamei shrimp infected with white feces syndrome (WFS) contained *Vibrio* (*V.*) species of different types, including *V. parahaemolyticus*, *V. cholerae*, and *V. alginolyticus. Vibrio parahaemolyticus*, at a density of 10^4 CFU $ml^{-1,}$ can be pathogenic in vannamei shrimp (Saputra et al., 2023a). *Vibrio parahaemolyticus* exhibits pathogenic effects, including necrosis, hemocyte infiltration, and melanization, in shrimp hepatopancreas cells (Lai et al., 2015). Saputra et al. (2023a) found that a concentration of 10^6 CFU ml^{-1} resulted in 100% mortality in shrimp within 73 hours after challenge. Conversely, Ananda Raja et al. (2017) reported that the lethal dose (LD) of *V. parahaemolyticus* in vannamei shrimp was 2.6×10^4 CFU ml^{-1} under different conditions.

Recently, efforts to prevent shrimp diseases by using herbal ingredients such as *Piper crocatum*, *Citrus limon*, *Zingiber officinale*, and *Borassus flabellifer* as an alternative to antibiotics have been reported by Reverter et al. (2014), Palanikumar et al. (2020), and Saputra et al. (2023b). Antibiotics act selectively but can lead to the development of bacterial resistance (Haifa-Haryani et al., 2022). Antibiotics can harm fish and the environment; therefore, residue-free treatment should be used, incorporating environmentally friendly, natural herbal ingredients. Garlic (*Allium sativum*) is one of the candidates for natural antibacterial ingredients that function as a medicine, reported to have an inhibitory effect of 20 µL on *Erwinia carotovora* and *Xanthomonas campestris* bacteria (Chen et al., 2018; Chirawithayaboon et al., 2020). Chen et al. (2018) stated that allicin is the main component responsible for the onion aroma and is one of the active substances that can eliminate pathogenic bacteria or exhibit antibacterial properties. Vinyl-1,2-dithiacyclopent-3-en-1,2-dithiacyclohex-5-en- and 2-vinyl-4H-1,3-dithin in *Allium sativum* exhibit broad pharmacological effects with low levels of toxicity (Mikaili et al., 2013).

To cite this paper: Saputra A, Suharyadi, Marlina E, Sektiana SP, Samsuharapan SB, and Ulfa AM (2025). Evaluation of Garlic Powder as a Control Agent of *Vibrio* sp. in the Digestive Tract of White-leg Shrimp. *World Vet. J.*, 15(3): 578-587. DOI: https://dx.doi.org/10.54203/scil.2025.wvj58

The application of herbal treatments is most effective in preventing issues in shrimp farming (Saputra et al., 2023b). Monitoring the clinical condition of shrimp during maintenance is crucial for assessing their health before outbreaks of pathogenic diseases, such as AHPND, and potential mortality (Tran et al., 2013). Low cell necrosis levels in the hepatopancreas and intestines of vannamei shrimp are indicators of shrimp health (Li et al., 2018). The present study evaluated the chemical compounds of garlic powder and its ability to prevent the growth of *Vibrio* sp. bacteria in orally administered vannamei shrimp, focusing on measuring the total bacterial count, the level of *Vibrio* sp. in the hepatopancreas, and observing histopathological changes.

MATERIALS AND METHODS

Ethical approval

The present study was conducted in June and August 2020 at the Microbiology and Chemistry Laboratory of the Ahli Usaha Perikanan (AUP) Polytechnic, Serang, Banten, Indonesia. In the present study, the shrimp were raised according to the standard operating procedures for good management practices in vannamei shrimp aquaculture, as outlined by the Ministry of Marine Affairs and Fisheries of the Republic of Indonesia.

Study design

The study employed a completely randomized design with four treatment groups, each having three replications. The treatments involved adding garlic powder to commercial feed at different concentrations, including a control group with no garlic powder (Group K). The second group received 0.1 mg of garlic powder per 1 g of vannamei shrimp feed (Group A). The third group was given 0.5 mg of garlic powder per 1 g of feed (Group B), and the fourth group received 1 mg of garlic powder per 1 g of vannamei shrimp feed (Group C). Vannamei shrimp (*Litopenaeus vannamei*) were used at the post-larva stage with a density of 1000 shrimp in each tank. These shrimps were obtained from the Cultivation Laboratory at Politeknik AUP in Serang, Banten, Indonesia. During the maintenance period, vannamei shrimp were fed at a dose of 3% four times a day (Zainuddin et al., 2019). The commercial feed (Gold Coin Marine Shrimp Feed, Vietnam) has a particle size and nutritional composition that range from 0.5 to 2.2 mm, with crude protein at 36%, crude fat at 5%, crude fiber at 4%, ash at 15%, moisture at 12%, and a metabolic energy content of 3.200 kcal/kg. For the 40-day maintenance of vannamei shrimp, garlic powder was prepared at approximately 500 to 1000 grams using a kinetic method originating from farmers in Banten province, Indonesia (Pathak et al., 2022). Before the experiment, the shrimp were acclimated to a temperature of 28-30°C in a 2.5 m^3 round plastic tub for one month (Zhao et al., 2024).

Container preparation

Vannamei shrimp were housed in a circular container with a diameter of two meters and a height of one meter. A total of 12 circular tanks made of tarpaulin were sterilized using chlorine (Tjiwi-Kimia, Sidoarjo, East Java, Indonesia) at a dose of 0.3 mg/l. The sterilization process for equipment, including containers, aeration devices, and water, required a 24-hour sterilization period. Cleaning was performed with sterile water until the chlorine was gone. Aeration was provided at nine points per tank, each holding one cubic meter, to ensure proper oxygen diffusion during shrimp maintenance (Meidiana et al., 2023).

Garlic phytochemicals

Garlic extraction was carried out by dissolving 50 grams of garlic powder into 250 mL of ethanol 99.9%. The kinetic extraction process was performed for 24 hours with a magnetic stirrer on a hot plate (Sundang et al., 2012). A phytochemical analysis was performed on ethanol extracts of *Allium sativum*, including tests for flavonoids, alkaloids, tannins, terpenoids, phenols, and saponins, using the method described by Ahmad et al. (2015). For thin-layer chromatography (TLC; Sigma-Aldrich, St. Louis, Missouri, USA), the procedure described by Matysik et al. (2016) was employed. The garlic flavonoids were screened via TLC using ethanol as the solvent. The retardation factor (RF) value on silica gel (Sigma-Aldrich, St. Louis, Missouri, USA) helped to identify the quercetin component (Patel et al., 2017). The silica gel used for TLC had a density of approximately 0.5 to 0.6 g/mL, with the silica content reaching up to 99.9% silicon dioxide (Pathak et al., 2022). The ethanol extract of garlic was identified separately on the TLC plate and then developed in the solvent system. The lethal concentration 50 (LC_{50}) of garlic powder given to vannamei shrimp was 1 mg/g in shrimp feed (Syngai et al., 2016).

High-performance thin-layer chromatography analysis

High-performance thin-layer chromatography (HPTLC; CAMAG, Switzerland) analysis was performed using the method described by Patel et al. (2017). The standard solution was prepared by dissolving 2 mg each of quercetin and gallic acid in 10 mL of methanol 99.9%. For application on the TLC plate, 1-6 µL of this standard solution was used. For

the extraction and hydrolysis of the garlic sample, garlic (10 g) was subjected to ethanol extraction for two hours per cycle. The resulting extract was dried using an evaporator, and the residue was re-dissolved in 80 mL of methanol 70%. This solution was then hydrolyzed for two hours with 40 mL of 2 M ammonium chloride (NH_4Cl) and 40 mL of toluene. The hydrolyzed mixture was neutralized with NaOH 5% and refluxed for one hour. The mobile phase from this reaction was subsequently extracted twice with 30 mL of toluene, followed by four extractions with 50 mL of ethyl acetate. The combined ethyl acetate extracts were evaporated to dryness. The resulting residue (10 mg) was dissolved in 5 mL of methanol to create the test sample. A volume of 15 µL of this sample solution was spotted onto the TLC plate. The stages in thin-layer chromatography column (CO-TLC) were performed on silica gel (Sigma-Aldrich, St. Louis, Missouri, USA) using a solvent ratio of 5:4:4 (v/v/v). In the mobile phase, toluene, ethyl acetate, and formic acid solvents were dissolved in a ratio of 5:4:1 (v/v/v). Then, the TLC reading was scanned at a wavelength of 254 nm.

Gastrointestinal sampling and bacterial observation

The collection of hepatopancreatic organs was carried out exclusively from deceased shrimp. Bacteria collection was carried out by inoculating the hepatopancreas using the modified method of Supono et al. (2019). Samples of bacterial abundance and *Vibrio* sp. were collected on days 0, 20, and 40 during post-treatment garlic powder exposure maintenance. Samples of vannamei shrimp hepatopancreas and intestines, each weighing 1.0 g, were collected to test for total bacteria and total *Vibrio* sp. bacteria. At the same time, histology testing of hepatopancreas cells was performed on day 40 of rearing. The total plate count method was used to determine the bacterial count in the shrimp digestive tract (Ananda Raja et al., 2017). The digestive tract, including the hepatopancreas and intestines, was placed into sterile microtubes for weighing. Then, the sample was crushed using a sterile micro-pestle, and 1mL of sterile physiological solution (NaCl 0.9%) was added. The sample solution was then diluted stepwise with sterile physiological solution (Supono et al., 2019).

After incubation for 24 hours, the bacterial colonies were counted using a colony counting device, with the petri dish placed on top. The bacterial colonies were then counted using the following formula.

$$N = C \times \frac{1}{P} \times \frac{1}{V}$$

N means colony count (CFU mL^{-1}), C means the number of bacterial colonies on the petri dish (Colonies mL^{-1}), P means dilution factor, and V means sample volume (mL; Supono et al., 2019).

Histological analysis

The method of Ananda Raja et al. (2017) was used to perform histopathological examination of the hepatopancreas in vannamei shrimp infected with *Vibrio* sp. bacteria. Shrimp health was assessed by visually inspecting the color and structure of the hepatopancreas and intestines. After determining the shrimp's health condition, the hepatopancreas and intestines were collected and stored in Davidson's fixative (Sigma-Aldrich, St. Louis, Missouri, USA) for 24 hours (Ananda Raja et al., 2017). For observation and differentiation of hepatopancreas and intestinal cells, staining was performed using hematoxylin and eosin (H&E; Sigma-Aldrich, St. Louis, Missouri, USA).

Statistical analysis

A logarithmic transformation was used to process the data on bacterial abundance in the digestive tract. The SPSS program was employed to analyze the abundance of *Vibrio* sp. in each treatment. The transformed data were then analyzed using analysis of variance (ANOVA) with Minitab 26 for Windows at a confidence level of 95% ($p < 0.05$). After identifying a significant difference in the current results, the test was conducted using the least significant difference (LSD) method. The RF value on silica gel was used to identify the quercetin component. The data processing outcomes were then displayed in graphs to clearly illustrate the results of each treatment.

RESULTS AND DISCUSSION

Phytochemical screening

The *Allium sativum* phytochemical test results (Table 1) indicated that it contains flavonoids, saponins, tannins, phenols, terpenoids, and alkaloids, as reported by Dalhat et al. (2018). The phytochemical content in *Allium sativum* exhibited diverse secondary metabolite properties, making it a potential treatment for vannamei shrimp farming (Palanikumar et al., 2020). The phenolic compound (quercetin), reported by Rempe et al. (2017), functions as an antibacterial by disrupting the cell membrane (membrane rigidification), intercalating into DNA, inhibiting DNA gyrase, inactivating type III secretion, and inhibiting bacterial zinc (HPFABZ), dehydratase, hydroxyphenyl-phenylalanine ammonia-lyase, and protein kinase. In addition to phenol, other compounds with antibacterial properties were found in tannins. Tannin, a multidentate ligand, can bind to proteins primarily through hydrophobic interactions and hydrogen bonds, which can inhibit bacterial metabolism (Jöbstl et al., 2006). A relationship exists between the activity of tannic

acid against bacteria and its inhibition of receptor binding, which affects bacterial cell wall damage (Theisen et al., 2014). Pandey and Negi (2018) observed that at concentrations below the minimum inhibitory level, tannins possess antivirulence effects, alter the expression of virulence factors, and reduce bacterial pathogenicity, which prevents bacteria from attaching to cell receptors, thereby blocking their entry onto different surfaces.

Table 1. Phytochemical test results of *Allium sativum*

Compound identification	Color indicator parameters	Results
Flavonoids	Fading yellow color	Positive
Alkaloids	Orange deposition	Positive
Tannins	Blackish brown, blackish blue	Positive
Terpenoids	Orange, orange-brown	Positive
Phenol	Blackish brown, blackish blue	Positive
Saponins	Permanent foam	Positive

High-performance thin-layer chromatography analysis

The HPTLC chromatogram of the ethanol extract from *Allium sativum* was confirmed through chromatography and absorption spectrum analysis, with the reference standard scanned at a wavelength of 254 nm (Figure 1). The peak appeared at an Rf value of 0.66, with the peak area calculated as 0.039% (w/w). Patel et al. (2017) reported that the HPTLC screening of *Lagerstroemia indica* extract yielded an Rf value of 0.63 with a peak area of 0.041%. Pure compounds can be isolated when the ratio of solvents and eluents used has the correct polarity (Saputra et al., 2016). Pathak et al. (2022) employed HPTLC screening of quercetin and gallic acid standards using a toluene-ethyl acetate-formic acid (v/v/v) eluent mixture.

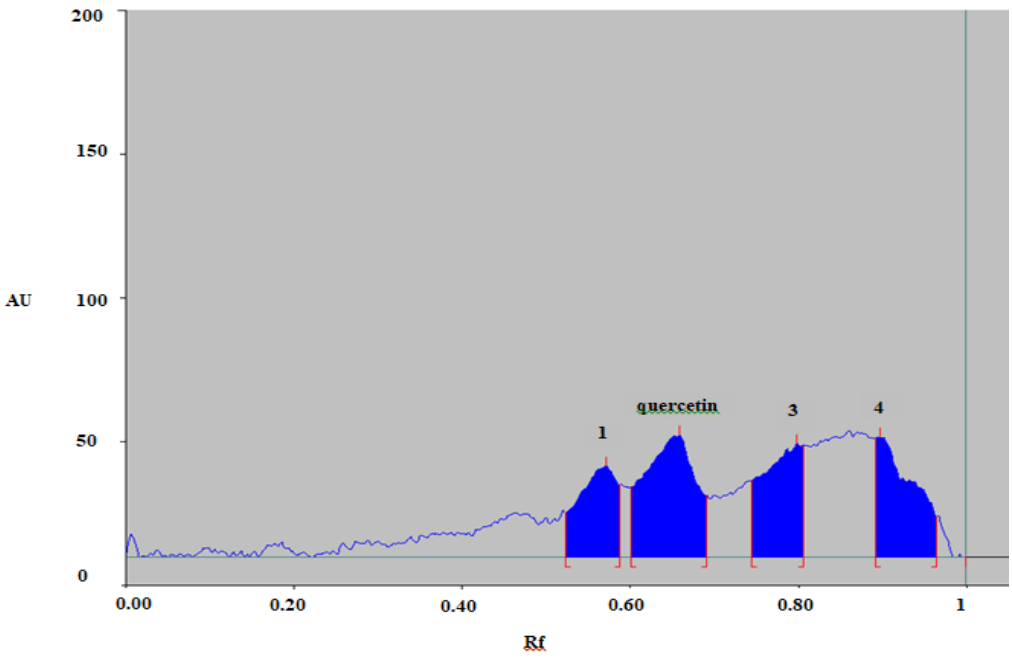


Figure 1. *Allium sativum* ethanol extract using quercetin standard, scanned at 254 nm. Rf: Retardation factor, AU: Absorbance units. The peak appeared at an Rf value of 0.66, and the AU was 55 for the *Allium sativum* ethanol extract.

Total bacterial abundance and total *Vibrio* species

The levels of bacteria and *Vibrio* sp. in the digestive tract of vannamei shrimp fed with *Allium sativum* powder, as well as in the control group, indicated an increasing pattern ($p \leq 0.05$) throughout the shrimps' rearing period. This present study demonstrated a correlation between the administration of *Allium sativum* powder and the proliferation of bacteria, including *Vibrio* sp., indicating that *Allium sativum* powder can inhibit the growth of *Vibrio* sp. bacteria for up to 40 days of culture (DOC). The results of the data analysis indicated that the total bacterial count after applying *Allium sativum* powder in DOC 40 significantly decreased ($p \leq 0.05$) in both the treatment and control groups. The total bacteria in DOC 40 in the digestive tract of vannamei shrimp in the control group had a value of 8.5×10^6 CFU g^{-1}, and in the treatment groups were 2.4×10^6 CFU g^{-1} (Group A), 3.4×10^6 CFU g^{-1} (Group B), and 2.1×10^6 CFU g^{-1} (Group C).

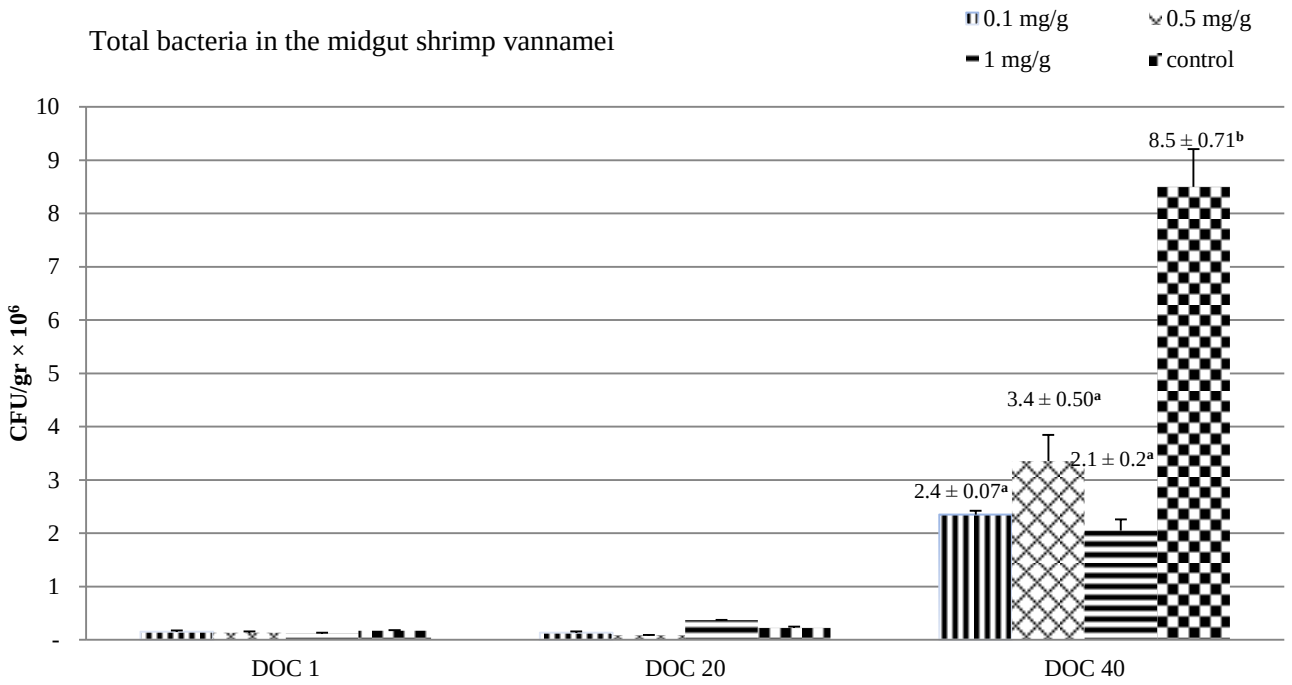


Figure 2. Bacterial abundance in the digestive tract of vannamei shrimp fed with *Allium sativum* powder for 40 days of culture. DOC: Day of culture. [a,b] The difference in letters indicates a significant difference ($p < 0.05$).

The total bacterial population in DOC 40 in the shrimp intestine in the control group, a value of 8.5×10^6 CFU g^{-1} ($p \leq 0.05$), was similar to the total number of bacteria in shrimp digestion found in nature (5×10^5 CFU g^{-1}) as reported by Buntin et al. (2008). The current results indicated that all doses of *Allium sativum* powder had the same effect in suppressing the growth of *Vibrio* sp. bacteria. Administering *Allium sativum* powder has been demonstrated to be effective in maintaining the total bacterial population during the digestive process of vannamei shrimp, with bacterial counts ranging from $2.1 \pm 0.2 \times 10^6$ to $3.4 \pm 0.5 \times 10^6$ CFU g^{-1}, showing statistically significant differences ($p \leq 0.05$). Therefore, this dosage can be considered an acceptable and effective level of application. Kongnum and Hongpattarakere (2012) reported a decrease (5.0 ± 0.14 CFU g^{-1} to 3.34 ± 0.21 CFU g^{-1}; $p \leq 0.05$) in the total bacterial count in vannamei shrimp after giving *Lactobacillus plantarum* during the 6-week treatment. In DOC 40, the bacterial abundance percentages between Gram-negative and Gram-positive bacteria indicated that the control group had a ratio of 49.4% to 50.6%. In comparison to the present study, Group A had a ratio of 12.5% to 87.5%, Group B displayed a ratio of 8.82% to 91.18%, and Group C had a ratio of 14.3% to 85.7% ($p \leq 0.05$). Figure 2 illustrates that administering *Allium sativum* powder to all treatment groups effectively suppressed total bacterial growth in vannamei shrimp maintenance compared to the control group. *Allium sativum* has been reported to function as a secondary metabolite, such as an antibacterial agent, in preventing WFS (Palanikumar et al., 2020) and also acts as an immunostimulant (Breyer et al., 2015).

The current results indicated that the abundance of *Vibrio* sp. after applying *Allium sativum* powder in DOC 40 was significantly different ($p \leq 0.05$) among treatments compared to the control group. Based on Figure 3, the total amount of *Vibrio* sp. in DOC 40 within the digestive tract of vannamei shrimp in the control group reached the highest level at 4.2×10^6 CFU g^{-1} ($p \leq 0.05$). Meanwhile, the treatment groups receiving garlic powder had an abundance of *Vibrio* sp. of 0.290×10^6 CFU g^{-1} (Group A), 0.275×10^6 CFU g^{-1} (Group B), and 0.280×10^6 CFU g^{-1} (Group C).

As shown in Figure 3, the *Vibrio* sp. abundance graph at DOC 40 demonstrated that the control group exhibited a natural increase in *Vibrio* sp. levels within the hepatopancreas of vannamei shrimp. Several factors contributed to these conditions, including the high content of organic matter in the vannamei shrimp farming environment. In the maintenance of vannamei shrimp, the duration, the size of the shrimp, and the condition of the aquaculture environment are increasingly critical, particularly the organic matter content (Ariadi et al., 2019). Organic matter plays a significant role in increasing the abundance of Gram-negative bacteria such as *Vibrio* sp. Sun et al. (2016) stated that synergistic conditions with organic sediments in vannamei shrimp culture indicated that organic sediments could significantly influence the abundance of shrimp gut bacteria. Supono et al. (2019) reported that in the vannamei shrimp farming environment, the abundance of *Vibrio* sp. ranged from 1.3 to 2.2×10^5 CFU $ml^{-1,}$ including *V. parahaemolyticus* and *V. alginolyticus*. Saputra et al. (2023a) added that *V. parahaemolyticus* found in the vannamei shrimp farming environment infected with AHPND in Serang District, Banten Province, Indonesia, had a pathogenic abundance value of 1×10^4 CFU

ml^{-1}. Similar findings were reported by Ananda Raja et al. (2017), indicating that the LD value of *V. parahaemolyticus* infection in vannamei shrimp is 2.6×10^4 CFU ml^{-1}. The total abundance of *Vibrio*, including specific *Vibrio* species, exhibited different pathogenicity. In the case of *V. parahaemolyticus* and *V. harveyi*, as reported by Chen et al. (2017), these species produce toxins that can affect the shrimp's immune system, leading to hepatopancreas damage, mortality, and luminescent vibriosis (Subagiyo et al., 2020).

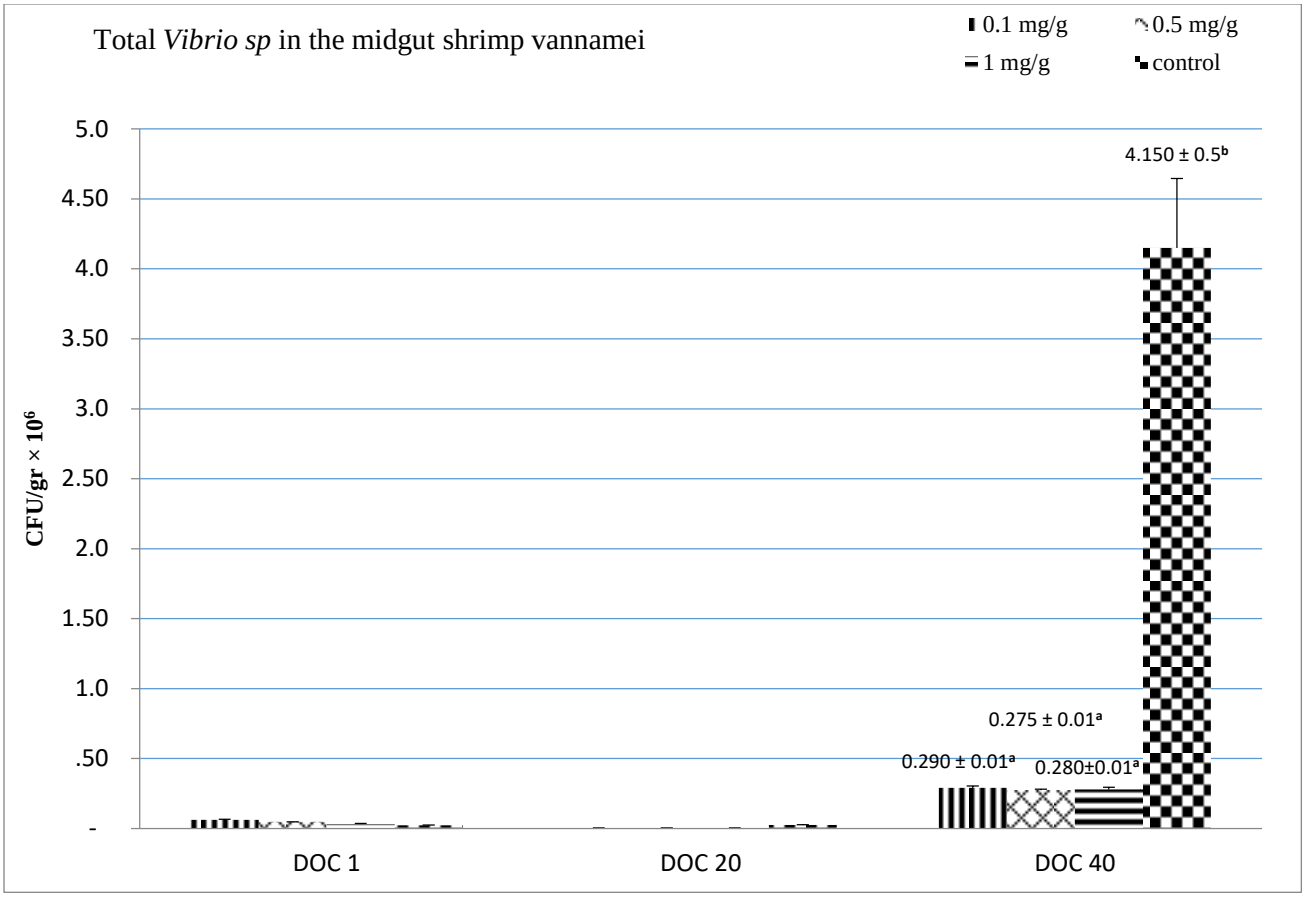


Figure 3. The abundance of *Vibrio* sp. in the digestive tract of vannamei shrimp fed with *Allium sativum* powder for 40 days of culture. DOC: Day of culture. [a,b] The difference in letters indicates a significant difference ($p < 0.05$).

The total abundance of *Vibrio* indicated a high potential for infection caused by *V. parahaemolyticus* (Saputra et al., 2023a). Previous studies have shown that the abundance of *Vibrio* in shrimp farming can affect shrimp health, where high numbers of *Vibrio* colonies can contribute to increased incidence of diseases such as AHPND in shrimp (Gustilatov et al., 2024). In a study conducted by Ananda Raja et al. (2017), it was found that *V. parahaemolyticus* was one of the most common pathogenic species among isolates from shrimp ponds, suggesting that the high abundance of these colonies may increase the risk of disease exposure in the farming environment (Ananda Raja et al., 2017). Amoah et al. (2021) observed that dietary supplementation with black garlic positively affected the immune response and health of *L. vannamei*. The current results exhibited an increase in beneficial bacteria and a decrease in *Vibrio* in the intestines of treated shrimp (groups A, B, and C), supporting the idea that garlic can help maintain the balance of the shrimp gut microbiota, which is essential for overall health. Adding *Allium sativum* powder to shrimp feed helped to keep *Vibrio* bacteria lower in groups A (0.290×10^6 CFU g^{-1}), B (0.275×10^6 CFU g^{-1}), and C (0.280×10^6 CFU g^{-1}) compared to the control group (4.150×10^6 CFU g^{-1}). *Allium sativum*, widely recognized for its antimicrobial properties, has the potential to positively modulate the intestinal microbiota of shrimp, thereby enhancing health and resistance to infections (Chen et al., 2018). Several studies have indicated that dietary garlic can significantly reduce pathogenic bacterial populations while supporting beneficial microbial communities, which are essential in regulating *Vibrio* growth (Amoah et al., 2021). Specifically, a study by Maneesin et al. (2014) reported that shrimp treated with garlic oil exhibited a decrease in total viable count, reinforcing the evidence that garlic is effective in suppressing the growth of harmful microorganisms, including *Vibrio* spp.

Histopathology of hepatopancreas

Histopathological examination of the hepatopancreas in vannamei shrimp treated with garlic powder, compared to the control group, provided insights into how *Vibrio* sp. infection affects the health of the hepatopancreatic tissue. The

hepatopancreas of vannamei shrimp indicated a level of damage that did not differ in groups A, B, and C compared to the control group ($p \leq 0.05$; Table 2). Based on general histology, vannamei shrimp with the control treatment on DOC 40 demonstrated a high abundance of *Vibrio* sp. ($4.2 \pm 0.5 \times 10^6$ CFU g^{-1}) compared to groups A, B, and C ($p \leq 0.05$). The histopathology findings in the control group revealed hemocyte infiltration in the tubular space and atrophy of the tubular epithelium in the hepatopancreas tissue. Figures 4a and 4b depict the hepatopancreas with hemocyte infiltration on DOC 40, while Figures 4c and 4d show atrophy of the tubule epithelium in the control group on the same day.

Table 2. Hepatopancreas damage level of vannamei shrimp by *Vibrio* pathogenicity

Treatments	Average level of damage	Damage (%)	Damage rates
control	1.98 ± 0.15	25.87	Medium
0.1 mg/g	0.29 ± 0.11	5.13	Light
0.5 mg/g	0.18 ± 0.09	3.44	Light
1 mg/g	0.20 ± 0.16	4.17	Light

Notes: Classification of hepatopancreas tissue damage levels; Normal (Grade 0): At this level, the structure of the hepatopancreas tubules appears intact with tightly arranged epithelial cells without any signs of degeneration or necrosis. Light (Grade 1): Mild vacuolization is seen in the epithelial cells, as well as slight changes in the structure of the tubules that are still functioning. Medium (Grade 2): Vacuolization is seen moderately to severely, with epithelial cells beginning to atrophy. Severe (Grade 3): At this level, extensive cell necrosis and total disintegration of the hepatopancreas tubules are evident. Very Severe (Grade 4): Complete damage to the hepatopancreas tissue with an unrecognizable organ structure. Source: Aguilar-Rendón et al. (2020); Saputra et al. (2023a).

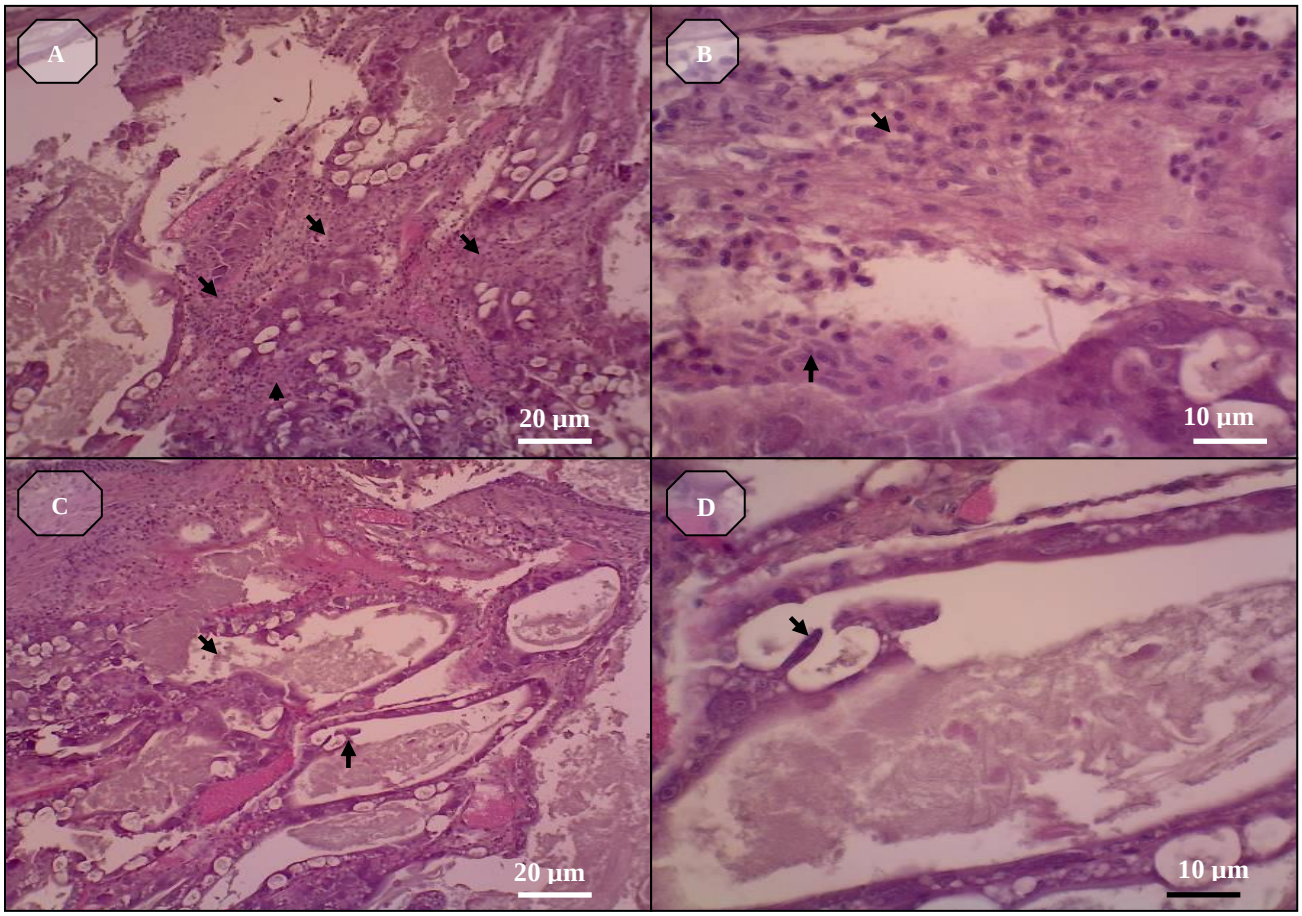


Figure 4. Hepatopancreas of *Litopanaeus vannamei* in the control treatment after 40 days of culture. **A** and **B:** Hemocyte infiltration in the inter-tubular space (arrow), **C** and **D**: Tubule atrophy (arrows). H&E staining, 10 and 20 µm.

Histopathological examination of the shrimp hepatopancreas was performed after 40 days of maintenance. Observations focused on the effects of *Allium sativum* powder administration and the control group. Acute disease occurred in the control group after DOC 40, characterized by hemocyte infiltration between tubules and atrophy. Some peeling cells were observed in the tubular lumen, and hemocytes caused infiltration around the tubules, likely due to the bacterial toxin *Vibrio* sp., as reported by Han et al (2017). *Vibrio* sp. damaged the hepatopancreas by affecting the tubules and causing hemocyte infiltration. Saputra et al. (2023a) noted that pathogens, including *V. parahaemolyticus*, can severely harm the hepatopancreas tissue in vannamei shrimp, leading to the appearance of inflamed tubules, atrophy, and peeling of tubule cells. The current results indicated that as the maintenance time of vannamei shrimp increased,

there was a corresponding rise in the total abundance of *Vibrio* sp. bacteria, both in all treatment groups and the control group. A high abundance of *Vibrio* affected the health of vannamei shrimp. Soto-Rodriguez et al. (2015) and Saputra et al. (2023a) reported a significant correlation between maintenance time and the *Vibrio* sp. virulence.

CONCLUSION

Adding *Allium sativum* powder to vannamei shrimp feed can inhibit the growth of *Vibrio* sp. bacteria at an effective dose of 0.5 mg g^{-1}, with a corresponding abundance of *Vibrio* sp. at $0.275 \pm 0.01 \times 10^6$ CFU g^{-1}. Further studies should focus on optimizing the application of garlic flour for large-scale use in shrimp ponds by farmers.

DECLARATIONS

Availability of data and materials

All data generated during the study are relevant and included in this published article.

Funding

The present study was supported by the Jakarta Technical University of Fisheries (Politeknik Ahli Usaha Perikanan), Jakarta 12520, Indonesia, which has a research program in Aquaculture Technology as of 2020.

Authors' contributions

Afandi Saputra and Suharyadi conducted the research, administration, conceptualization, and writing. Erni Marlina has done the methodology. Sinar Pagi Sektiana, Sri Budiani Samsuharapan, and Ade Maria Ulfa wrote the draft and prepared the article. All authors checked and approved the last edition of the manuscript for processing and publication in this journal.

Competing interests

All authors affirmed that they have no competing interests.

Ethical considerations

The authors declared that the manuscript is original, has not been published elsewhere, and confirmed the latest version before publication.

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2025, Scienceline Publication
World's Veterinary Journal
World Vet J, 15(3): 588-596.
ISSN 2322-4568

DOI: https://dx.doi.org/10.54203/scil.2025.wvj59
PII: S232245682500059-15

Clinical and Genetic Characterization of Infectious Laryngotracheitis Virus in Layer Chickens in Basrah, Iraq

Sara Salim Mohammed[1], Isam Azeez Khaleefah[2], Rajaa Abd Alzahra Ali[2], Harith Abdulla Najem[2*], Waleed Majeed Almayahi[2], and Budoor Muhammad Lateif[2]

[1]*Department of Veterinary Public Health; College of Veterinary Medicine; University of Basrah, Basrah, Iraq*
[2]*Department of Pathology and Poultry Diseases, College of Veterinary Medicine, University of Basrah, Iraq*

*Corresponding author's Email: harith.najem@uobasrah.edu.iq

ORIGINAL ARTICLE
Received: June 24, 2025
Revised: July 26, 2025
Accepted: August 28, 2025
Published: September 30, 2025

ABSTRACT

Infectious Laryngotracheitis (ILT) has been widely prevalent in numerous countries in recent years. The present study aimed to detect ILT in chickens exhibiting clinical suspicion, utilizing molecular analytical techniques in Basrah, Iraq. Clinical signs, histological changes, and polymerase chain reaction (PCR) were used to diagnose thirty-five samples collected from clinically suspected chickens, including trachea and lungs for histopathology and PCR testing for the *p32* gene. The present study demonstrated that the three isolates of ILTV, including ILT-BASRAH 1, ILT-BASRAH 2, and ILT-BASRAH 3, caused the typical signs and pathological changes of ILT in layers chickens. Phylogenetic analyses indicated that ILT-BASRAH 1 exhibited a distant relationship with other current local isolates. Conversely, the isolates ILT-BASRAH 2 and ILT-BASRAH 3 exhibited a close relationship with each other. The initial results indicated that ILT-BASRAH 1 exhibited high nucleotide similarity to MK895003.1, MK895000.1, and MK894999.1 from Australia, which are representative of recombinant strains derived from live attenuated vaccine strains, as well as PQ232589.1 from Georgia. Furthermore, ILT-BASRAH 2 and ILT-BASRAH 3 were identified as vaccine-related isolates. These isolates demonstrated a high level of genetic similarity and a close phylogenetic relationship to the *Gallid alphaherpesvirus* 1 isolate TJ2019 (GenBank accession no. PP062931.1), originating from Chinese source flocks. The current study determined that ILT-BASRAH isolates 1, 2, and 3 exhibited genetic similarity, suggesting that they likely originated from the same or closely related sources. Their difference from vaccine strains, including the minor variation in ILT-BASRAH 1, might indicate regional viral adaptation.

Keywords: *Gallid alphaherpesvirus*, Histological change, Layer chicken, Phylogenetic

INTRODUCTION

Chickens are primarily affected by many respiratory diseases. Infectious Laryngotracheitis (ILT) was first identified in the United States in 1925. It continues to cause substantial economic damage to the worldwide poultry sector (Fuchs et al., 2007). The ILT is caused by *Gallid herpesvirus 1*, which belongs to the Herpesviridae family (Ou and Giambrone, 2012). Clinical signs of ILT include nasal discharge, conjunctivitis, coughing, gasping, reduced egg production, and bloody mucus discharge. The severity of ILT varies; in certain cases, it may lead to severe respiratory complications that could potentially result in suffocation (Bagust et al., 2000). Diseases caused by ILT result in significant economic losses. These losses occur as they have a direct impact on growth, mortality, and egg production (Oldoni et al., 2008). Reduced egg production is also a primary indicator in laying hens infected with infectious laryngotracheitis virus (ILTV), as the disease impacts the overall health of the chickens and results in significant stress (Tadese et al., 2007). ILTV is mainly transmitted horizontally through direct contact with infected chickens or by inhaling secretions and droplets. Additionally, contaminated tools, food, and water supplies help spread the infection within poultry flocks. However, no vertical or transovarial transmission through eggs has been documented (Pajić et al., 2022). Following the infection, the virus begins to replicate in the trachea and conjunctiva. Clinical signs of illness in hens generally manifest after five days (Dufour-Zavala, 2008). Infected chickens may begin to transmit the virus within two days of infection. Additionally, sick hens could act as a source of ILTV (Chukiatsiri and Pohuang, 2018). Despite vaccination of chicken flocks, ILT disease remains a threat, particularly in areas with a high poultry industry presence (Yan et al., 2016). Therefore, a strategy of careful and regular clinical monitoring and ongoing immunological assessment of chicken flocks should be implemented as part of early diagnosis and control (Mo and Mo, 2025). Primary diagnosis of ILT depended on clinical signs, macroscopic changes, and then laboratory testing, as well as histopathological and molecular identification. Histopathological examination can identify common lesions such as syncytial cells and intranuclear inclusion bodies,

To cite this paper: Mohammed SS, Khaleefah IA, Ali RAA, Najem HA, Almayahi WM, and Lateif BM (2025). Clinical and Genetic Characterization of Infectious Laryngotracheitis Virus in Layer Chickens in Basrah, Iraq. *World Vet. J.*, 15(3): 588-596. DOI: https://dx.doi.org/10.54203/scil.2025.wvj59

and it is frequently employed for diagnostic purposes. Molecular testing is regarded as the fastest, most dependable, and precise method for confirming the diagnosis (Coppo et al., 2013). Recently, many studies have highlighted the significance of molecular screening technology in monitoring poultry flocks for disease control and diagnosis (Gatica et al., 2023; Priya et al., 2023). Furthermore, different poultry diseases, particularly respiratory infections such as Avian Influenza (AI), Infectious Bronchitis (IB), and Newcastle Disease (ND), are challenging to diagnose through clinical assessment due to the similar signs exhibited by AI, IB, and ND. Consequently, differentiating between clinical and molecular diagnoses proved to be challenging (Kamal et al., 2024). Therefore, the most effective method for diagnosis and monitoring is to use molecular techniques, which should be employed to identify the specific type of ILTV and its origin, as well as to accurately characterize the genotypic virus through the sequencing of particular gene segments (Abebe et al., 2024).

Therefore, the present study aimed to detect ILTV in commercial layer farms in Basrah, Iraq, through the examination of clinical, pathological, histological, and phylogenetic data based on the *p32* gene.

MATERIALS AND METHODS

Ethical approval

The current study was conducted with the permission of the Ethics Committee at the College of Veterinary Medicine, University of Basrah, Iraq (Ref. No. 36/2023).

Clinical signs, gross lesions, and sample collection

The present study lasted one year, from October 2023 to October 2024. Seven poultry farms under observation housed flocks of white Lohmann layers. The farms had a population ranging from 60,000 to 90,000 laying hens, each 20 weeks of age and with an average weight of 1,405 grams. The laying hens were at the end of their incubation and rearing stages. Suspected chickens exhibiting tentative clinical signs consistent with ILT were observed during the study period and underwent a meticulous clinical examination to document all signs. Subsequently, the chickens were humanely euthanized in accordance with ethical guidelines and then subjected to a thorough necropsy procedure at the Department of Poultry Diseases and Veterinary Pathology, College of Veterinary Medicine, University of Basrah, Iraq (Najem et al., 2024). Eight tissue samples from the trachea and lung were taken from chickens that exhibited typical signs of ILT.

Each sample was divided into two sections. The first section was kept in a container with 5 mL of glycerol phosphate buffer 50% solution and stored at -20°C for polymerase chain reaction (PCR). The second section was fixed in a formalin 10% solution for histopathological analysis. These fragments were then preserved in a neutral buffered formalin 10% solution, dehydrated using a progressive series of alcohol solutions, cleared using a series of xylene solutions, and infiltrated with different grades of melted paraffin in an oven set at 56°C (Yavuz et al, 2018). The tissues were further preserved in paraffin using a rotary microtome. The sections were subsequently stained using the hematoxylin and eosin staining method (H and E), as described by Lateif et al. (2024).

DNA extraction and PCR amplification

Following the manufacturer's guidelines, viral DNA was extracted from layer hens suspected of having ILTV using a commercial kit (Wizard® Genomic DNA Purification Kit, Promega, USA). The primers ILTU2 5-CTA CGTG CTGGG TGGCT ACTGC-3 and ILT2 5-AAACTCT CGGG TGGCT ACTGC-3 were utilized to amplify a segment of the *p32* gene associated with ILTV (Alaraji et al. 2019), resulting in a 588 bp product. The total volume of the reaction was established at 50 µL, with the PCR mix (InvitrogenTM, USA) containing 5 µL of DNA to initiate the amplification. The reaction was conducted using a multigene thermal cycler (Labnet, USA). Denaturation occurred at 95°C for five minutes, followed by 35 cycles of one minute at the same temperature. The extension phase lasted for ten minutes at 72°C. Following electrophoresis on a 1.5% agarose gel, the PCR results were visualized under UV light.

Sequencing

By using the maximum likelihood method with the Tamura-Nei model (Tamura and Nei, 1993), the evolutionary history can be inferred. The tree with the highest log probability (-1080.35) is the one that was displayed. The Tamura-Nei model generated a matrix of pairwise distances, which were subsequently employed to automatically construct the initial tree(s) for the heuristic search. The Neighbour-Joining method was utilized, and the topology with the optimal log likelihood value was selected. The present study examined fourteen nucleotide sequences. The codons were located in the first, second, third, and noncoding regions. The entire dataset comprised a total of 543 sites. The MEGA11 version 11 development team in the United States carried out the evolutionary analyses (Tamura et al., 2021).

RESULTS

Clinical signs, gross lesions, and histopathology findings

During the study, one poultry house was suspected of being infected with ILT. This infected poultry farm housed a total of 60,000 laying hens. Some laying hens exhibited severe clinical signs, including bloodied beaks, coughing, gasping, bulging eyes accompanied by wet rales, extended heads, and mouth-breathing. However, a decrease in egg production and conjunctivitis were commonly observed. Additionally, sudden death due to asphyxiation resulting from complete obstruction of the trachea and larynx was observed (Figures 1 and 2).

The tracheal mucosa, which showed noticeable congestion with mucoid exudate, was among the overall gross lesions. Additionally, hemorrhage on the tracheal mucosal surface was observed in the deceased chickens (Figures 3 and 4). The histopathological analysis exhibited necrotic epithelial cells mixed with blood and tracheal mucus. This material can solidify, resulting in the formation of a caseous plug in the trachea, which may potentially lead to asphyxia. Microscopic examination of the eight samples taken from chickens with respiratory distress revealed lesions in four of them. These included epithelial erosion, sloughing, and infiltration of different inflammatory cells, such as lymphocytes, along with the presence of apoptotic and necrotic cells in the subepithelial layer. In addition, there was vascular congestion and the creation of a pseudomembrane (Figures 5 and 6). The lesions in the lung samples were characterized by a thickening of the alveolar wall and the presence of inflammatory cells. Additionally, swelling of capillaries in the parabronchus and congestion of veins were observed (Figures 7 and 8).



Figure 1. Trachea of a layer chicken infected with infectious laryngotracheitis virus in Basrah, Iraq. Tracheal hemorrhage is obvious.



Figure 2. Trachea of a layer chicken infected with infectious laryngotracheitis virus in Basrah, Iraq. Tracheitis is obvious.



Figure 3. Trachea of a layer chicken infected with infectious laryngotracheitis virus in Basrah, Iraq. Blood-tinged mucous is obvious.



Figure 4. Trachea of a layer chicken infected with infectious laryngotracheitis virus in Basrah, Iraq. A clot in the trachea is obvious.

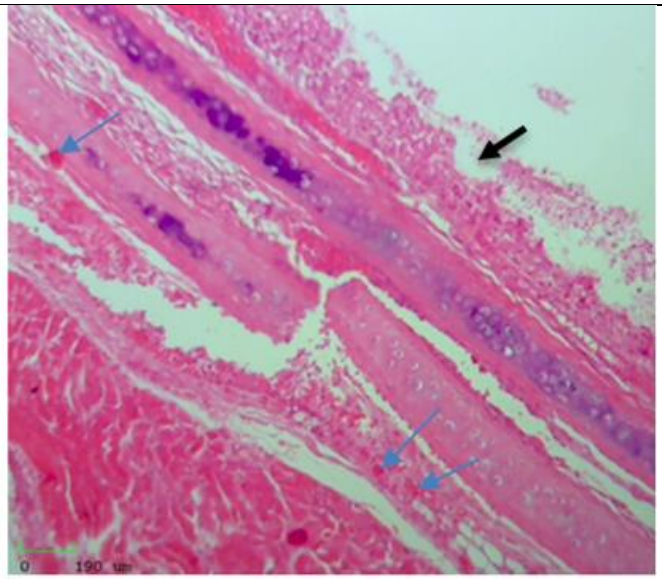


Figure 5. Trachea of a layer chicken infected with infectious laryngotracheitis virus in Basrah, Iraq. Hemorrhagic tracheitis, mucosal damage (black arrow) with congestion in blood vessels (blue arrows, H&E x10).

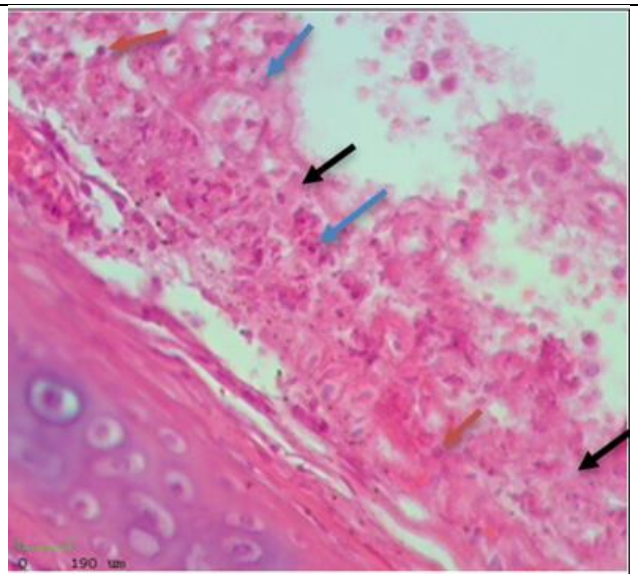


Figure 6. Trachea of a layer chicken infected with infectious laryngotracheitis virus in Basrah, Iraq. Trachea mucosal layer with inflammatory cells (blue arrows), severe epithelial necrosis (black arrows), and intranuclear inclusion bodies (red arrows, H&E x40).

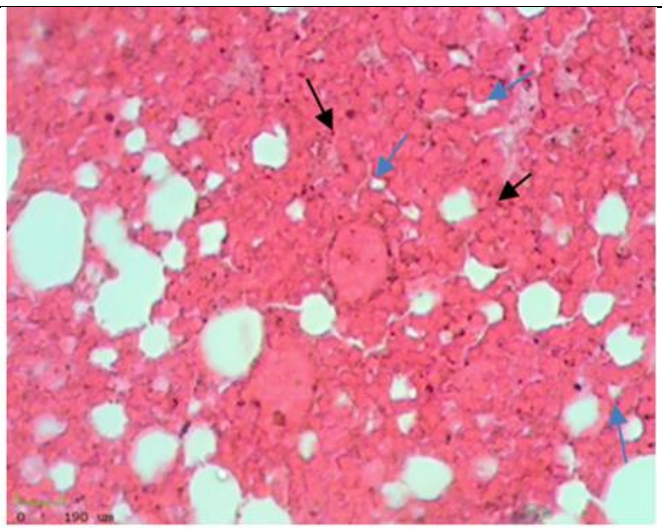


Figure 7. Lung of a layer chicken infected with infectious laryngotracheitis virus in Basrah, Iraq. Lung thickening in the alveolar wall (blue arrows) with inflammatory cells in interstitial tissue (black arrows, H&E x40).

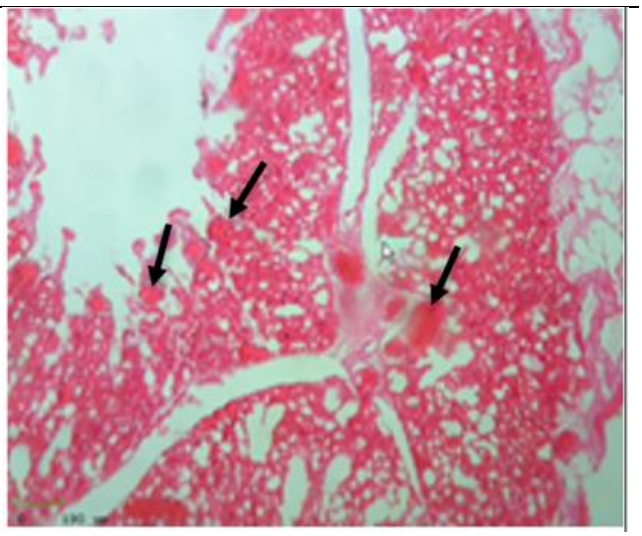


Figure 8. Lung of a layer chicken infected with infectious laryngotracheitis virus in Basrah, Iraq. Lung engorgement of capillaries in para bronchus and venous congestion (black arrows, H&E x10).

PCR amplification

The PCR was performed to detect ILTV, which targeted the *p32 gene*, with a 588 bp product. Suspected samples were analyzed using a 1.5% agarose gel, stained with ethidium bromide, and subjected to UV light exposure. The favorable outcomes were labeled as 1, 2, and 3 (Figure 9). The bands signified successful amplification of the target gene. The positive control yielded precise results at the corresponding band level, thus corroborating the reaction's efficacy.

Sequence and bioinformatics analysis of the *p32* gene

Sequences in the current investigation were lined up and compared to those of reference strains, as shown in the phylogenetic tree isolates using the MEGA X program software (Figure 10). Creating a phylogenetic tree by analyzing the targeted gene sequence of the ILTV in three local isolates from Basrah, Iraq, and comparing them to strains listed in the GenBank database worldwide. The three local strains sent to the gene bank and assigned these names PP860566.1 (ILT-BASRAH 1), PP860567.1 (ILT-BASRAH 2), and PP860568.1 (ILT-BASRAH 3) had different genetic

relationships with the other strains. The ILT-BASRAH 1 was categorized within the same group as vaccine strains due to its substantial nucleotide similarity with specific global isolates reported in recent years, namely MK895003.1, MK895000.1, and MK894999.1. There was no genetic divergence among these isolates, indicating their close genetic relationship. This discovery suggested that the isolate may be closely related to vaccination strains or the result of a strain that has been spreading globally in the region recently. On the other hand, isolates ILT-BASRAH 2 and ILT-BASRAH 3 established a new evolutionary branch, with genetic distances of 0.034 and 0.010, respectively, from the other recorded strains in GenBank.

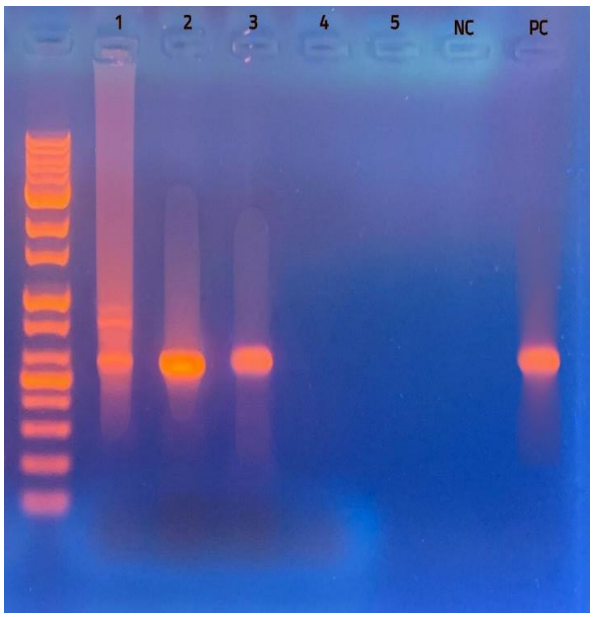


Figure 9. PCR results of the *p32* gene of Infectious Laryngotracheitis Virus. (1,2, and 3 field strains from layer chickens) were positive at the 588 base pair fragments. M=100bp DNA ladder; NC: Negative control; PC: Positive control

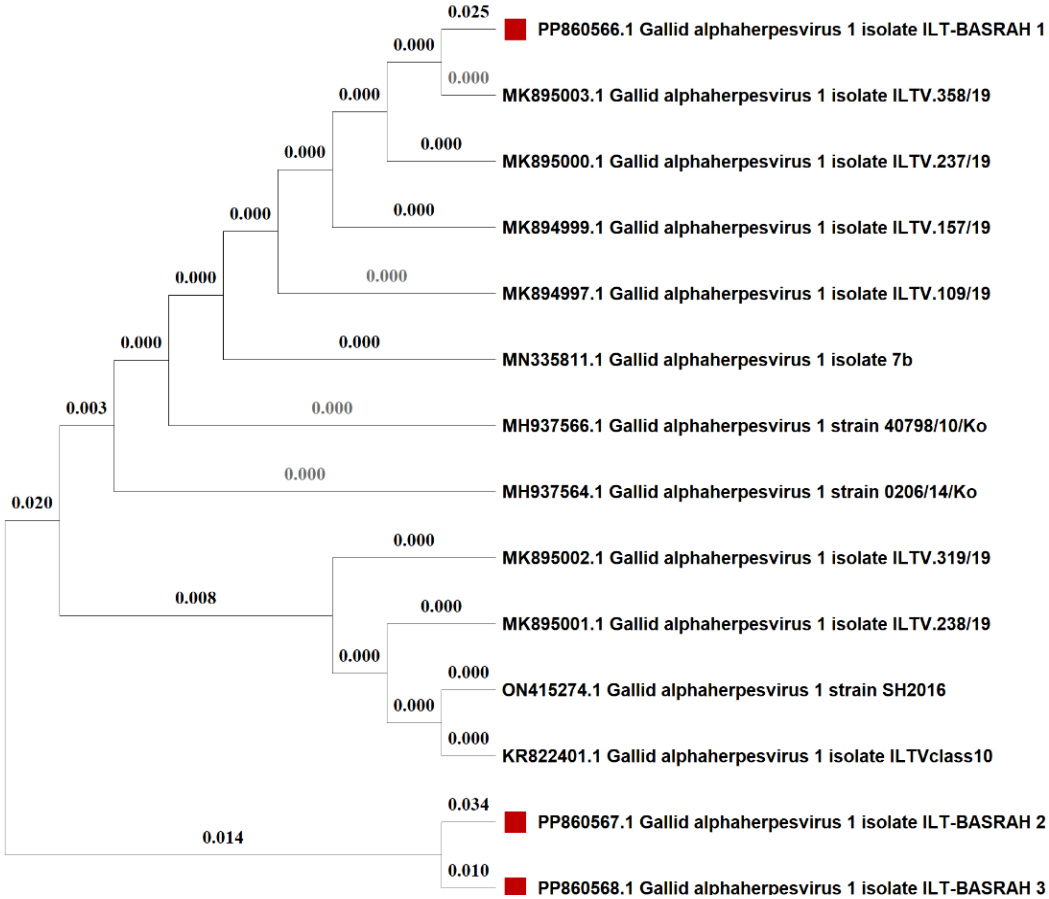


Figure 10. The phylogenetic tree of the infectious laryngotracheitis virus was isolated from a layer chicken's trachea and lung in Basrah, Iraq. Their accession numbers are used to express them in international nucleotide databases (Isolates using the MEGA X program software)

DISCUSSION

Avian ILT was identified as a specific viral disease affecting chickens within the global poultry industry (Marga et al., 2024). In Iraq, particularly in the southern region, such as Basrah Province, there is no available information regarding ILT.

The results of clinical monitoring demonstrated that one poultry house out of seven was diagnosed with ILTV. A similar result was mentioned by Gowthaman et al. (2020) and Kamal et al. (2024), who noted that the rate of infection depends on several factors, including the severity of the virus, the route of transmission among chickens, and the phase of the disease, such as acute or sub-acute infections. Another study on broiler chickens reported an infection rate of about 2% (Ali et al., 2023). The clinical observations in the present study included coughing, gasping, watery eyes with moist rales, blood in the beak, dyspnea accompanied by expectoration of blood-stained mucus, and conjunctivitis. These clinical observations were consistent with findings of García et al. (2013) and Rojs et al. (2021). Traditional descriptions of acute ILTV infection often mention rales, tracheitis, and gasping, which are linked to the involvement of the deeper layers of the upper respiratory tract and can cause tracheal obstruction.

The mortality rate in the present study was recorded as 1% which was in line with the findings of Roberts et al. (2011), who documented the virulence strain of the virus, causing death, resulting from mechanical asphyxiation caused by complete airway obstruction. The present results, which indicated that ILTV targets the respiratory system, agreed with those of Timurkaan et al. (2003) and Kaboudi et al. (2016), who reported that the virus causes pathological changes, including inflammatory changes, in the respiratory tract of infected chickens with ILTV. Similar lesions were observed by Kirkpatrick et al. (2006), who found hemorrhagic lesions in the trachea, tracheal blood clots, and congestion of the infra-orbital sinuses. These pathological changes result from rapid viral replication within the respiratory epithelium, leading to cell destruction, acute inflammatory reaction, and infiltration of inflammatory cells.

The microscopic alterations in the trachea demonstrated epithelial erosion and sloughing, along with the infiltration of many inflammatory cells, such as lymphocytes and apoptotic cells, in the subepithelial layer. Vascular congestion and the production of a pseudomembrane, which was caused by fibrin and dead cells, were observed. This finding aligned with the results reported by Carnaccini et al. (2022) in the trachea, larynx, sinuses, and conjunctiva. The appearance of lesions in the lungs exhibited that the ILT has progressed to a more advanced stage, as the virus spreads into the deeper respiratory tissues.

The severity of lesions differed based on the tissue affected and the virus's tropism. Certain ILTV strains demonstrated a high propensity to invade the trachea (tracheotropic strains), whereas their capacity to infect the lungs was less prominent. Genetic variations among strains may result in different virulence and preferred sites of replication within the organism (Perez-Contreras et al., 2021; Mo and Mo, 2025).

The molecular analysis of suspicious samples indicated positive solid PCR bands. The present study provided molecular information on the *Gallid alphaherpesvirus* strains that are currently disseminating within the poultry industry and contributed to a deeper understanding of the transmission dynamics of ILTV in Basrah, Iraq. All data regarding local strains indicated that the *p32* gene-targeted PCR test was a reliable and accurate method for determining the presence of *Gallid alphaherpesvirus* in poultry 1. These findings are consistent with those of Callison et al. (2007) and Oldoni and García (2007), who demonstrated the potential use of virus-specific genes for rapid and reliable diagnosis. The results of the phylogenetic analysis indicated that the ILT-BASRAH 1 did not exhibit close relatedness to any other existing local isolates (Figure 10).

ILT-BASRAH 2 and ILT-BASRAH 3 demonstrated a close relationship with each other. The local isolates in the present study exhibited varying genetic structures and are classified into two distinct clusters. The initial results indicated that the recombinant strain, ILT-BASRAH 1, showed high nucleotide similarity to MK895003.1, MK895000.1, and MK894999.1 from Australia, which represent recombinant strains derived from live attenuated vaccine strains, and PQ232589.1 from Georgia. It is noteworthy that recombinant strains of ILT have been documented in laying hens currently being studied for the first time in Iraq. Live attenuated vaccines have proven to be highly effective in lowering mortality rates, alleviating clinical signs, and preventing ILTV outbreaks (Fulton et al., 2000). In addition, a wide range of recombinant vaccines have been developed or are currently under investigation. These vaccines include ILTV strains with targeted deletions in virulence-associated open reading frames (Schneiders et al., 2018), as well as recombinant vectors such as fowlpox virus and turkey herpesvirus engineered to express specific ILTV glycoprotein genes (Johnson et al., 2010). In the present study, the second cluster of ILTV, ILT-BASRAH 2 and ILT-BASRAH 3, was identified as vaccine-related isolates. These isolates exhibited high genetic similarity and a close phylogenetic relationship to the *Gallid alphaherpesvirus* 1 isolate TJ2019 (GenBank accession no. PP062931.1), which was from China. *Gallid alphaherpesvirus* 1 was grouped within the chicken embryo origin and vaccine strain lineage. Furthermore, ILT-BASRAH 2 and ILT-BASRAH 3 indicated a phylogenetic association with the Georgia isolate MF417811.1. According

to Zhang et al. (2025), high-throughput sequencing conducted in China between 2015 and 2019 revealed that six ILTV isolates (SD2015, GD2017, SYB2018, HB201812, HB201806, and TJ2019) clustered together with chicken embryo origin vaccine strains. There have been significant concerns regarding the virulence and proportional risk of altering the virulence of classical live attenuated vaccines, despite their potential to provide high protection. Live attenuated vaccine strains, particularly CEO strains, can cause slight clinical signs in chickens, depending on the vaccine strain and the age of the chickens. Additionally, in poultry breeding facilities with a lot of chickens, live attenuated vaccinations can become virulent again (Guy et al., 1991).

Whole-genome sequencing technology can assist in mapping genomic feature differences and may explain these complex phylogenetic differences. The current findings are consistent with previous studies by Ojkic et al. (2006) and Moreno et al. (2010), showing that genetic sequence analysis can distinguish between vaccine-derived and field strains and reveal their evolutionary backgrounds. In the present study, phylogenetic analysis indicated that specific local ILTV isolates exhibited genetic variations distinct from those available in GenBank. These differences highlighted the potential for local viral evolution within Basrah, Iraq.

CONCLUSION

The present study concluded that the ILT-BASRAH 1, 2, and 3 isolates were genetically similar and likely originated from the same or closely related sources. These isolates are geographically separated from certain global vaccine strains, indicating minimal recent recombination or genetic variation from vaccines in these local isolates. The ILT-BASRAH 1 exhibited a slightly higher divergence from some of the reference isolates, suggesting possible unique mutations or evolution in local flocks. Comprehensive epidemiological surveillance across additional Iraqi provinces and border areas is essential to map the distribution and dynamics of ILTV strains for further studies.

DECLARATIONS

Ethical considerations

The authors have checked the ethical considerations, including issues such as plagiarism, consent for publication, misconduct, data fabrication and/or falsification, as well as double publication and/or submission redundancy.

Funding

The authors have borne all expenses. The initial research and genomic isolation procedures were carried out at the laboratories of the University of Basrah, Iraq.

Availability of data and materials

The data collected during this study are available from the corresponding author upon reasonable request.

Acknowledgments

The authors thank the staff of the University of Basrah's College of Veterinary Medicine for providing the necessary assistance and resources for the study project.

Authors' contributions

Sara Salim Mohammed reviewed and edited the manuscript. Isam Azeez Khaleefah contributed to the molecular detection. Rajaa Abd Alzahra Ali wrote the original draft. Harith Abdulla Najem contributed to the research design and field diagnosis of the disease. Waleed Majeed Almayahi performed the genetic analysis of the results. Budoor Muhammad Lateif conducted the histopathological study. All the authors read and approved the final version of the manuscript.

Competing interests

The authors declared no conflict of interest.

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2025, Scienceline Publication
World's Veterinary Journal
World Vet J, 15(3): 597-611.
ISSN 2322-4568

DOI: https://dx.doi.org/10.54203/scil.2025.wvj60
PII: S232245682500060-15

Emergence of Extended-spectrum Beta-lactamase Producer and Colistin-resistant *E. coli* in Animal-origin Foods in Libya

Jihan Ali Sherif[1], Samira Abd Allateef Farag[1], Salem Farhat Abureema[1], Salah Mohamed Azwai[2], Aboubaker Mohamed Garbaj[1], Fatim Taher Gammoudi[2], Allaaeddin Ali El Salabi[3], and Ibrahim Mohamed Eldaghayes[2*]

[1]*Department of Food Hygiene and Control, Faculty of Veterinary Medicine, University of Tripoli, Tripoli, Libya*
[2]*Department of Microbiology and Parasitology, Faculty of Veterinary Medicine, University of Tripoli, Tripoli, Libya*
[3]*Department of Public Health, Faculty of Medical Technology, University of Tripoli, Tripoli, Libya*

*Corresponding author's Email: ibrahim.eldaghayes@vetmed.edu.ly

ORIGINAL ARTICLE
Received: June 24, 2025
Revised: July 27, 2025
Accepted: August 30, 2025
Published: September 30, 2025

ABSTRACT

The increasing prevalence of *Escherichia coli* (*E. coli*) infection poses significant health challenges worldwide. Understanding the genetic, pathogenic, and antimicrobial resistance profiles of *E. coli* is crucial for effective diagnosis and treatment. The present study aimed to assess the prevalence and antimicrobial susceptibility patterns of *E. coli* isolated from different samples of food products from animals, with specific attention to identifying and characterizing extended-spectrum beta-lactamase (ESBL)-producing isolates. The present study analyzed 92 *E. coli* isolates obtained from 1120 food samples, including milk, dairy products, meat, and meat products, collected randomly from retail markets in Libya. The isolates were tested for antimicrobial susceptibility, and the antibiotic resistance profiles were evaluated using 32 antibiotics from 12 different classes. Multiple antibiotic resistance (MAR) and antibiotic resistance index were calculated, with MAR $\geq$ 0.2 indicating high antibiotic resistance. Isolates were categorized as multidrug resistant (MDR), extensively drug-resistant (XDR), or pan drug-resistant (PDR) based on standard definitions. The ESBL production was assessed using the double-disc synergy test, and colistin resistance was tested using the agar diffusion method. Antimicrobial susceptibility testing of *E. coli* isolates revealed 100% resistance to penicillin and cloxacillin, with high resistance rates observed against neomycin (93.4%), rifampicin (86.9%), and methicillin (75%). However, all isolates were susceptible to chloramphenicol, whereas carbapenems (imipenem, meropenem, ertapenem) indicated the lowest resistance (3.2%). Cefepime demonstrated the highest effectiveness among cephalosporins, with a resistance rate of 1.08%. The MAR ranged from 0.09 to 0.6, with the highest MAR value (0.6) observed in isolates resistant to 20 antibiotics. All isolates were MDR, but no XDR or PDR strains were detected. Among the 92 isolates, 43 were confirmed as ESBL producers, primarily originating from raw milk, lben (fermented milk), and other dairy products. In addition, 83 isolates demonstrated phenotypic resistance to colistin. The present study highlighted the significant presence of MDR *E. coli* in food products of animal origin, particularly raw milk, fermented milk, and chicken meat in Libya, emphasizing the urgent need for antimicrobial stewardship, stronger regulatory frameworks, and integrated One Health surveillance approaches to combat AMR in Libya.

Keywords: Antimicrobial resistance, Colistin, *Escherichia coli*, Extended-spectrum beta-lactamases, Food sample

INTRODUCTION

Food safety is a crucial component of public health and has been recognized as an effective measure for preventing foodborne diseases for over a century, owing to advancements in food production and the adoption of modern management philosophies, such as hazard analysis. However, several issues continue to persist, one of which is the high prevalence of foodborne illnesses caused by specific pathogens, which appear to have increased in recent decades.

Worldwide, foodborne diseases caused by contaminated food are rapidly spreading and can arise at any point in the food chain, from food production to delivery to consumption. Foodborne diseases may originate from a variety of environmental contaminants, such as unsafe or improper food processing, storage practices, and air, water, or soil pollution. According to the World Health Organization, 1 in 10 people falls ill each year due to contaminated food. Many of these illnesses are often linked to the consumption of contaminated undercooked meat and raw milk (Chaves et al., 2015). Furthermore, Faour-Klingbeil and Todd (2019) indicated that international commerce is impacted by food laws and regulations, which are implemented by nations as part of their control tactics against foodborne illnesses, which is a worldwide public health concern that affects people's health, livelihoods, and healthcare systems. *Listeria monocytogenes*, *Salmonella* spp., *Campylobacter* spp., and *Escherichia coli* (*E. coli*) are some of the key bacterial

To cite this paper: Sherif JA, Farag SAA, Abureema SF, Azwai SM, Garbaj AM, Gammoudi FT, El Salabi AA, and Eldaghayes IM (2025). Emergence of Extended-spectrum Beta-lactamase Producer and Colistin-resistant *E. coli* in Animal-origin Foods in Libya. *World Vet. J.,* 15(3): 597-611. DOI: https://dx.doi.org/10.54203/scil.2025.wvj60

pathogens responsible for causing foodborne illnesses, according to Habib and Mohamed (2022). Although Africa bears the highest burden of foodborne disease, there is a lack of understanding of the disease's prevalence in Africa due to the limited studies and surveillance programs (Desta, 2020), and Libya is not exceptional, where many cases are either dismissed as minor and self-limiting or go unconfirmed because of reporting, detection, and monitoring gaps.

Escherichia coli is considered a dangerous pathogen in dairy farm operations worldwide because it leads to significant economic losses (Allocati et al., 2013). There are several strains of *E. coli*, most of which are not hazardous, but a few cause serious foodborne illnesses in humans. Additionally, *E. coli* has emerged as an essential model organism for researching the spread of drug resistance within bacterial populations and serves as an indicator of the selective pressure caused by the widespread and unregulated use of antibiotics in animal agriculture (Petty et al., 2014). This has positioned *E. coli* as a key reference in global drug resistance monitoring programs (Sheikh et al., 2012). The insights derived from drug resistance monitoring can contribute to the development of strategies aimed at reducing the risk of these infections among the population (Guiral et al., 2011).

Currently, numerous antimicrobial drugs are utilized in food animal production to manage infections and serve as growth promoters, a practice that is increasingly contributing to the human food chain, raising substantial health concerns for both humans and animals (Hao et al., 2014). Common antibiotics such as tetracycline and amoxicillin are used to treat foodborne infections, but their overuse has led to rising resistance in bacteria such as *E. coli* (Miranda et al., 2014; Gwida and El-Gohary, 2015; Kapoor et al., 2017). Contributing factors to antibiotic resistance include misuse, incorrect prescription, extensive agricultural use, and inadequate regulation have been highlighted by Johnson et al. (2007) and Peterson and Kaur (2018). Developing nations, particularly those in Africa, do not have strong regulations for the use or management of antibiotics (Maron et al., 2013). According to the World Organization for Animal Health, many developing countries do not yet have legislation that addresses the proper use of antimicrobials in veterinary practices.

Bacteria have evolved numerous resistance mechanisms to oppose the effects of antibiotics. Certain antibiotics contain chemical bonds, including amides and esters, that are susceptible to hydrolytic cleavage. Certain enzymes, such as extended-spectrum beta-lactamases (ESBLs), weaken antibiotic effectiveness by breaking key chemical bonds. The ESBLs confer resistance to all penicillins, third-generation cephalosporins, and aztreonam, but not to cephamycins or carbapenems (Ćirić et al., 2018). In India, the rise of ESBL-producing strains has led to increased carbapenem use, potentially accelerating the spread of carbapenem-resistant bacteria (Nagshetty et al., 2021). In addition to the consequences of bacterial infection, the susceptibility of the causative agent to antibiotics is considered a risk factor. Drug-resistant *E. coli* strains were found in humans and food in Salvador, Brazil, showing resistance to multiple antibiotics (Melo et al., 2015). Similarly, 65 strains of *E. coli* from animals and animal products in Tunisia demonstrated resistance to eight antibiotics, including kanamycin, gentamicin, and amoxicillin (Badi et al., 2022). These strains may spread to humans via direct contact, contaminated food, or the environment.

Libya faces significant challenges in tackling bacterial food contamination due to limited awareness of food safety and security. Previous studies did not adequately cover the contamination of food from animal origin (meat and dairy) by pathogenic bacteria and their antibiotic resistance patterns.

Infections and diseases caused by antimicrobial drug-resistant pathogens have led to increased morbidity and mortality rates in health care facilities (Alonso et al., 2017). As this problem has grown dramatically, standardized definitions that classify and explain bacteria resistant to different types of antimicrobial drugs are necessary so that epidemiological data can be gathered and analyzed reliably across countries. Since this issue has grown substantially, the term multidrug-resistant (MDR) refers to bacteria that are unsusceptible to at least one agent in three or more antimicrobial categories (Magiorakos et al., 2012; Al-Hasani et al., 2023).

In Libya, data on antimicrobial resistance (AMR) of bacteria isolated from food and dairy products are lacking. Nevertheless, a key review article, conducted during the period from 1970 to 2011 and 2002 to 2021, revealed a scarcity of data on AMR, attributing it to the lack of surveillance studies (Ghenghesh et al., 2013; Atia et al., 2022). Another finding from a review of published data over 20 years is that urinary tract infection (UTI) is among the most frequently diagnosed clinical conditions. Notably, the predominant bacterial pathogen causing UTIs is *E. coli*, which displayed a significant resistance rate to commonly prescribed first-line treatments, such as nitrofurantoin and cotrimoxazole. This resistance raises a concern about potential complications, as patients with UTIs face an increased risk of developing renal damage (Hemolytic Uremic Syndrome) and future complications such as renal failure or hypertension, if their treatment is ineffective (Atia et al., 2022).

Developed nations typically have stringent regulations and thorough documentation regarding antibiotic use. However, in many African countries, veterinary antimicrobials are readily available without prescription (Mainda et al., 2015). Recent studies have increasingly focused on the epidemiology of AMR bacteria, especially those producing plasmid-mediated AmpC β-lactamases, carbapenemases, and ESBLs and these studies centered on *E. coli*, a key

indicator of antibiotic resistance due to its wide host range and clinical relevance. Studying *E. coli* helped track resistance patterns across populations and the transfer of resistance between animals and humans (van den Bogaard and Stobberingh, 2000). Consumption of animal-derived food remains a significant pathway for spreading antibiotic-resistant pathogens. Therefore, the present study aimed to assess the antimicrobial susceptibility patterns of *E. coli* isolates obtained from different foods of animal origin, with particular focus on ESBL-producing isolates.

MATERIALS AND METHODS

Ethical approval

The current study was conducted according to the guidelines of the Faculty of Veterinary Medicine, University of Tripoli, Tripoli, Libya.

Sampling, isolation, and identification

A total of 92 isolates of *E. coli* were utilized in the present study, which was a laboratory-based descriptive investigation conducted during the year 2024. Isolates of *E. coli* had been isolated previously from 1120 food samples and stored at -80°C (Garbaj et al., 2016, 2017, 2022; Eshamah et al., 2020). Among the 1120 food samples, 500 were milk and dairy products, and 620 were meat and meat products, as illustrated in Table 1. The samples of food from animal origin were collected randomly from several retail markets from different Libyan cities, including Tripoli, Sabha, Tobruk, and Regdalin. The isolates were revived from storage at -80°C by removing one cryobead from each cryovial, placing it in 5 mL of peptone water, and then incubating it overnight at 35-37°C for further testing.

Table 1. Samples cultured on agar media for the detection of *Escherichia coli* in Libya during 2024

Sample type	No. of samples	No. of positive samples
Raw cows' milk	139	21
Fermented milk (Lben)	86	28
White soft cheese (Massora)	57	13
White soft cheese (Ricotta)	36	4
Goats milk	8	2
She camels' milk	15	2
Butter	4	3
Milk powder	36	2
Ice cream	24	4
Labanh	11	1
Skim milk powder	9	0
Cereal baby food	16	0
UHT milk	8	0
Yogurt	5	0
Growing up formula	18	0
Ready to feed baby formula	10	0
Full-cream milk powder	18	0
Chicken Meat	51	4
Chicken kabab	17	0
Chicken Burger	70	1
Chicken sausage	38	0
Ground chicken	42	0
Chicken liver	5	0
Ground beef	68	0
Beef	73	2
Camel's meat	107	1
Beef Burger	84	2
Beef Sausage	47	1
Beef Kabab	18	1
Total	1120	92

Table 2. Different antibiotic categories used in the present study

Categories	Antibiotics
Penicillin	Amoxicillin 30 µg
	Ampicillin 30 µg
	Amoxicillin Clavunate
	Penicillin 10 µg
	Methicillin 5 µg
	Piperacillin/Tazobactam
	Cloxacillin 5 µg
	Ticarcillin + Clavunate
Cephalosporins	Cefepime 30 µg
	Cefoperazone75µg
	Cefotaxime 30 µg
	Ceftriaxone 30µ
	Cefoxitin 30 µg
Carbapenems	Imipenem 10 µg
	Meropenem 10µ
	Ertapenem 10 µg
Aminoglycosides	Neomycin 10 µg
	Kanamycin 30 µg
	Gentamycin 10 µg
	Tobramycin 10 µg
	Strepromycin 10 µg
Tetracyclines	Tetracycline 30 µg
	Doxycycline 30 µg
	Oxytetracycline 30 µg
Fluoroquinolones	Levofloxacin 5µ
	Ciprofloxacin 5 µg
Glycopeptides	Polymixin 300units
Phenicols	Chloramphenicol 30 µg
Sulfonamides	Sulphamethoxazole/Trimethoprim
Monobactum	Aztreonam 30 µg
Nitrofurantoin	Nitrofurantoin 300 µg
Rifampicin	Rifampicin 5 µg

Antimicrobial susceptibility profile

Antibiotic susceptibility of the *E. coli* isolates was assessed using the Kirby-Bauer disc diffusion method on Mueller-Hinton agar (MHA), following the guidelines established by the Clinical and Laboratory Standards Institute (CLSI, 2024). Thirty-two antibiotics from 12 classes (Antrim Technology Park, Antrim BT41, England) were tested, as described in Table 2. A sterile nutrient broth was first inoculated with 3-5 colonies from each isolate and then incubated at 37°C for 2-4 hours until it reached an optimum log phase. The suspension was standardized to a 0.5 McFarland turbidity level, then evenly spread onto an MHA plate. After allowing it to dry and absorb for five minutes, the plate was inoculated with a range of commercially available antibiotic discs.

Multiple antibiotic resistance index and antibiotic resistance index

The multiple antibiotic resistance **(**MAR) index and antibiotic resistance index (ARI) indices were estimated and interpreted according to Hinton and Linton (1983) as follows.

MAR index = a/b,

Antibiotic resistance index (ARI) = y / nx,

Where a is the number of antibiotics to which the isolates are resistant, b is the total number of antibiotics exposed, y is the number of resistant isolates, n is the number of isolates, and x is the number of antibiotics. A MAR value ≥ 0.2 indicated that antibiotics were ineffective.

Identification of multidrug resistance, extensively drug-resistant, and pan-drug-resistant

The isolates were considered as multidrug resistant (MDR) if they appeared resistant to at least one antimicrobial agent in three or more antimicrobial different classes, extensively drug-resistant (XDR) was defined as resistance to at least one agent in all except two or fewer antimicrobial categories, and pan drug-resistant **(**PDR) was defined as non-susceptibility to all agents in all antimicrobial categories according to Magiorakos et al. (2012).

Detection of extended-spectrum beta-lactamase producers

The incidence of antimicrobial resistance and ESBL-producing *E. coli* was phenotypically assessed by culture and antibiotic susceptibility testing of the isolates. The ESBL-producing *E. coli* was determined by double-disc synergy tests using amoxicillin-clavulanate, cefotaxime, ceftazidime, ceftriaxone, and cefoxitin (Amare et al., 2022).

Colistin susceptibility testing

Colistin resistance was phenotypically assessed using the agar diffusion method, with 4 mg/dL of colistin incorporated into the culture medium (Tartor et al., 2021).

RESULTS

Antimicrobial susceptibility profile of *Escherichia coli* isolates

A total of 32 commonly used antibiotics from 12 categories, including penicillin, cephalosporins, carbapenems, monobactams, aminoglycosides, tetracyclines, fluoroquinolones, glycopeptides, phenicols, sulfonamides, nitrofurantoin, and rifampicin, were used to evaluate the susceptibility of the isolates. The overall isolated *E. coli* susceptibility profiles are shown in Table 3. All *E. coli* isolates showed complete resistance (100%) to penicillin and cloxacillin. A high resistance rate was also observed for neomycin (93.4%), followed by rifampicin (86.9%) and methicillin (75%). In contrast, all isolates were fully susceptible to chloramphenicol. The lowest resistance was noted against carbapenems, imipenem, meropenem, and ertapenem, with a combined resistance rate of only 3.2%. Among the tested cephalosporins, cefepime was the most effective, exhibiting a resistance rate of just 1.08%. Additionally, low resistance was observed against the piperacillin/tazobactam combination (3.2%) and cefoxitin (4.3%). Resistance to nitrofurantoin, levofloxacin, ticarcillin/clavulanate, and kanamycin was similarly low, each at 5.4% (Figures 1, 2, and 3).

Multiple antibiotic resistance index and antibiotic resistance index

All tested isolates exhibited resistance to the antibiotics administered. Table 4 displays the MAR index values. The MAR index for *E. coli* isolates ranged from 0.09 to 0.6. The minimum MAR index value for *E. coli* was 0.09, corresponding to three antimicrobial agents. Conversely, the maximum MAR index value of 0.6 was associated with twenty antimicrobial agents. The calculated ARI values are provided in Table 3.

Multidrug resistance patterns of *Escherichia coli*

The prevalence of MDR bacteria was high based on the antibiotic classification. In the present study, all *E. coli* isolates were resistant to three or more antibiotic categories. For instance, *E. coli* isolate number E315 derived from raw milk was resistant to three categories (Figure 4). The current results indicated that E260 isolated from lben (fermented milk) and E52 isolated from chicken meat were resistant to nine antibiotic categories (Figures 5 and 6). However, XDR and PDR were not detected in *E. coli* isolates.

Phenotypic detection of extended-spectrum beta-lactamase production in *Escherichia coli*

Among the 92 isolates, 43 were phenotypically confirmed to be positive for ESBL. Among all types of food, 17 isolates were detected in raw milk, 10 were isolated from lben, and nine isolates were from dairy products. A total of 7 isolates were detected from meat and its products. All phenotypically ESBL-positive isolates are listed in Table 5.

Colistin-resistant isolates

A total of 83 *E. coli* were identified as phenotypically colistin-resistant isolates using the agar diffusion method.

Table 3. Antibiotic susceptibility profile and antibiotic resistance index of *Escherichia coli* isolates in Libya during 2024

Antibiotic	Susceptible (n)%	Intermediate (n)%	Resistant (n)%	ARI
Imipenem 10 µg	(63) 68.5	(26)28.2	(3) 3.2	0.0010
Meropenem 10 µg	(71) 77.2	(18)19.5	(3) 3.2	0.0010
Ertapenem 10 µg	(67) 72.8	(22)23.9	(3) 3.2	0.0010
Cefepime 30 µg	(64) 69.5	(27) 29.3	(1)1.08	0.0003
Cefoperazone 75 µg	(66) 71.7	(12) 13	(14) 15.2	0.0047
Cefotaxime 30 µg	(25) 27.1	(24) 26	(43) 46.7	0.0146
Ceftriaxone 30 µg	(49) 53.2	(29) 31	(14) 15.2	0.0047
Cefoxitin 30 µg	(76) 82.6	(12) 13	(4) 4.3	0.0013
Amoxicillin 30 µg	(41) 44.5	(32) 34.7	(19) 20.6	0.0064
Ampicillin 30 µg	(17) 18.4	(51) 55.4	(24) 26	0.0081
Amoxicillin Clavunate20/10 µg	(39) 42.3	(41) 44.5	(12) 13	0.0040
Ticarcillin + Clavunate75/10 µg	(58) 63	(29) 31	(5) 5.4	0.0016
Penicillin 10 µg	(0) 0	(0) 0	(92) 100	0.0312
Cloxacillin 5 µg	(0) 0	(0) 0	(92) 100	0.0312
Methicillin 5 µg	(2) 2.1	(21) 22.8	(69) 75	0.0234
Piperacillin/Tazobactam100/10µg	(51) 55.4	(38) 41.3	(3) 3.2	0.0010
Aztreonam 30 µg	(64) 69.5	(10)10.8	(18) 19.5	0.0061
Neomycin 10 µg	(1)1.08	(5) 5.4	(86) 93.4	0.0292
Kanamycin 30 µg	(38) 41.3	(49) 53.2	(5) 5.4	0.0016
Gentamycin 10 µg	(72) 78.2	(13) 14.1	(7) 7.6	0.0023
Tobramycin 10 µg	(46) 50	(36) 39.1	(10) 10.8	0.0033
Streptomycin 10 µg	(36) 39.1	(45) 48.9	(11) 11.9	0.0037
Levofloxacin 5 µg	(83) 90.2	(4) 4.3	(5) 5.4	0.0016
Ciprofloxacin 5 µg	(70) 76	(12) 13	(10) 10.8	0.0033
Oxytetracycline 30 µg	(1)1.08	(62) 67.3	(29) 31.5	0.0089
Tetracycline 30 µg	(74) 80	(1) 1.08	(17) 18.4	0.0057
Doxycycline 30 µg	(63) 68.4	(21) 22.8	(8) 8.6	0.0027
Sulphamethoxazole/Trimethoprim 25 µg	(80) 86.9	(1) 1.08	(11) 11.9	0.0037
Chloramphenicol 30 µg	(89) 96	(3) 3.2	0	0
Nitrofurantoin 300 µg	(73) 79.3	(14) 15.2	(5) 5.4	0.0016
Rifampicin 5 µg	(9) 9.7	(3) 3.2	(80) 86.9	0.0271
Polymixin 300units	(6) 6.5	(23) 25	(63) 68.4	0.0213
Colistin	(9) 9.7	0	(83) 90	0.0281

ARI: Antibiotic resistance index.

Table 4. Multiple antibiotic-resistant index of *E. coli* isolates in Libya during 2024

Antibiotics	Resistant isolates	MARI
20	1	0.6
16	2	0.5
15	1	0.4
14	5	0.4
12	3	0.3
11	4	0.3
10	5	0.3
9	7	0.2
8	17	0.2
7	15	0.2
6	16	0.1
5	4	0.1
4	2	0.1
3	4	0.09

MAR: Multiple antibiotic-resistant index

Table 5. Extended-spectrum beta-lactamase-positive isolates

ESBL-positive *E. coli* isolates	Sources of isolates
E49	Minced meat
E55	Beef kebab
E56	Beef burger
E57	Chicken burger
E59	Goats milk
E61	Maasorra
E63	Cow's milk
E65	Lben
E66	Lben
E67	Lben
E75	Beef burger
E166	Cow's milk
E168	Cow's milk
E169	Cow's milk
E170	Cow's milk
E171	Cow's milk
E173	Lben
E174	Maasorra
E180	Lben
E182	Lben
E188	Cow's milk
E189	Cow's milk
E194	Cow's milk
E195	Cow's milk
E199	Ricotta
E202	Cow's milk
E203	Cow's milk
E204	Butter
E209	Lben
E213	Lben
E221	Chicken meat
E222	Chicken meat
E223	Milk powder
E228	Cow's milk
E238	Ricotta
E250	Camels' milk
E252	Cow's milk
E253	Cow's milk
E260	Lben
E299	Maasorra
E317	Lben
E321	Ricotta
E323	Milk powder

ESBL: Extended-spectrum beta-lactamase.

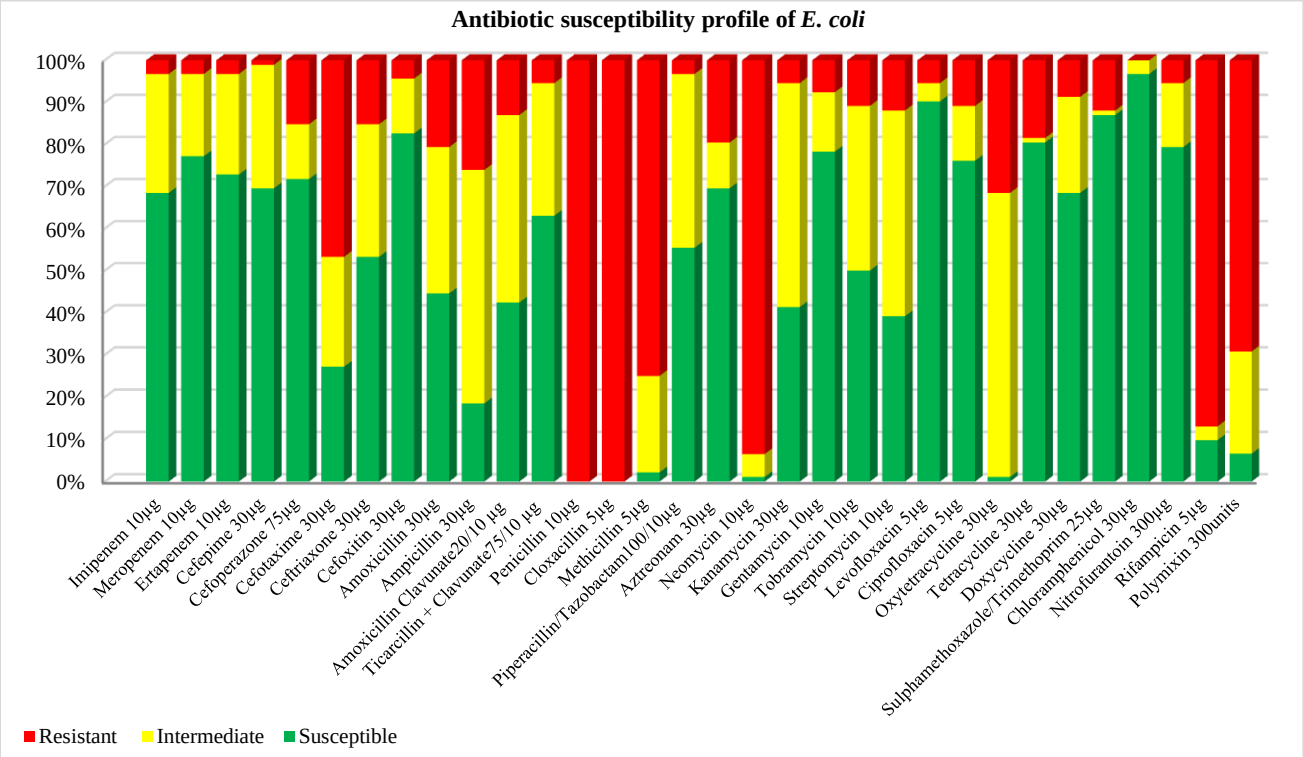


Figure 1. Antimicrobial resistance profiles of *Escherichia coli* isolates against 32 antimicrobial agents in Libya during 2024

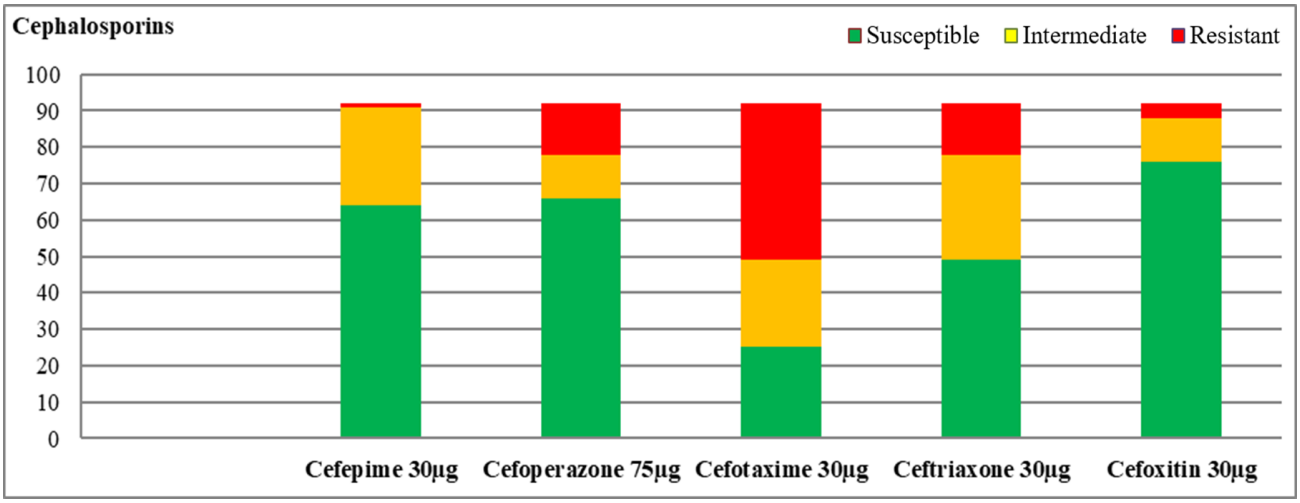


Figure 2. Antimicrobial resistance profiles of all *Escherichia coli* isolates against cephalosporins in Libya during 2024

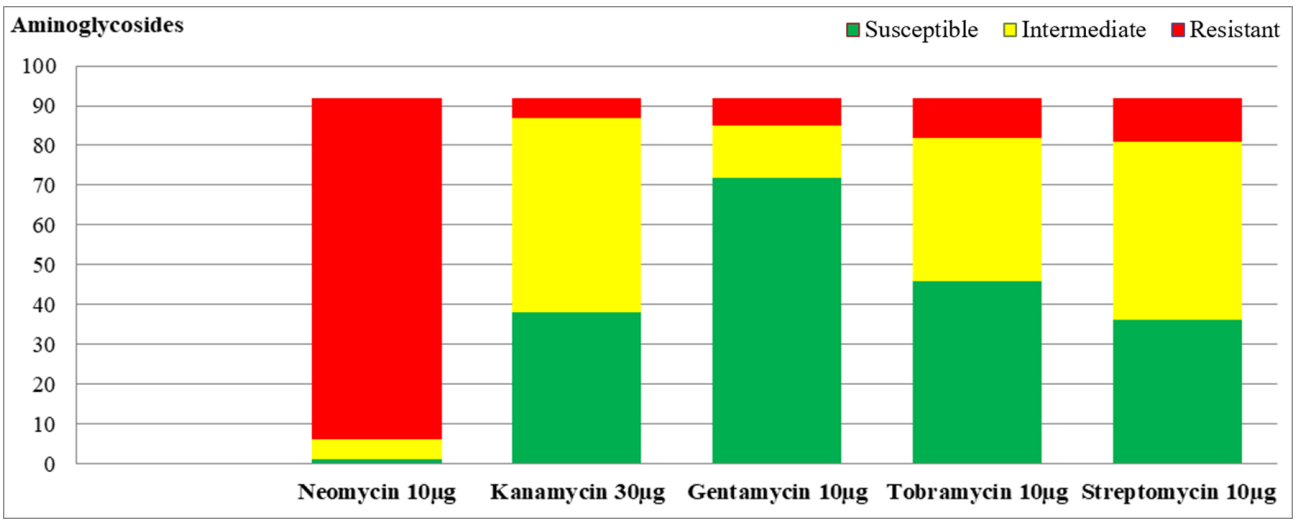


Figure 3. Antimicrobial resistance profiles of all *Escherichia coli* isolates against aminoglycosides in Libya during 2024

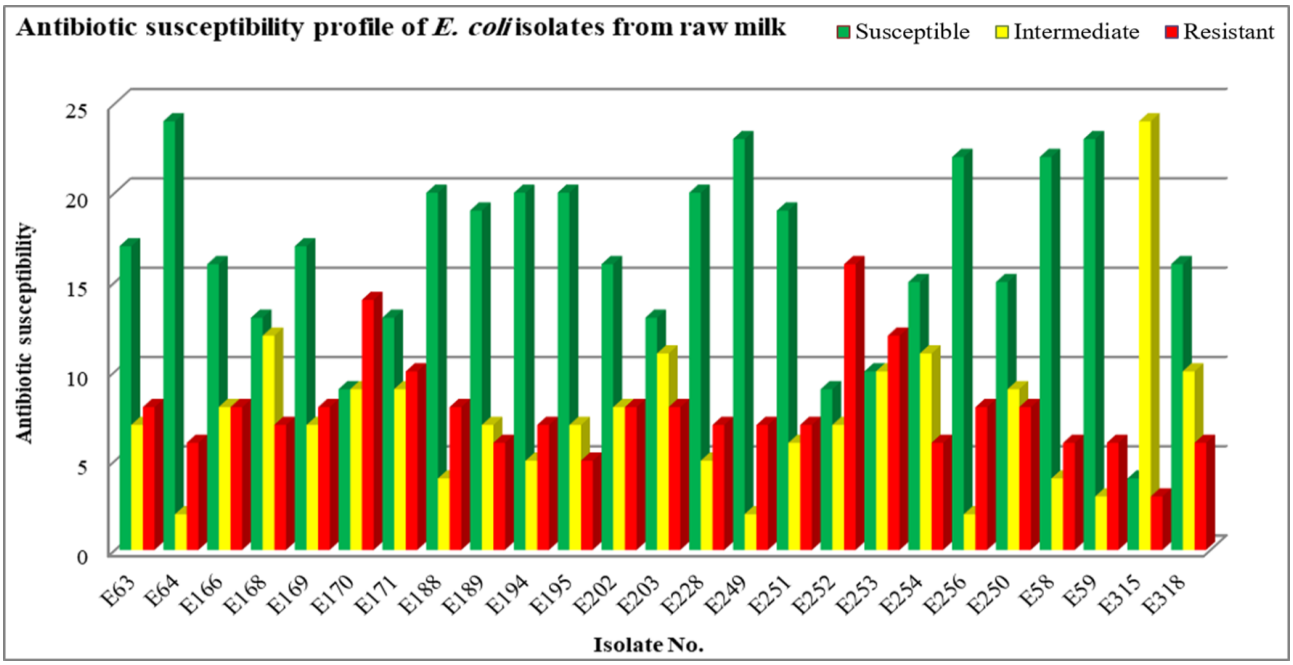


Figure 4. Antimicrobial resistance profiles of *Escherichia coli* isolated from raw milk in Libya during 2024

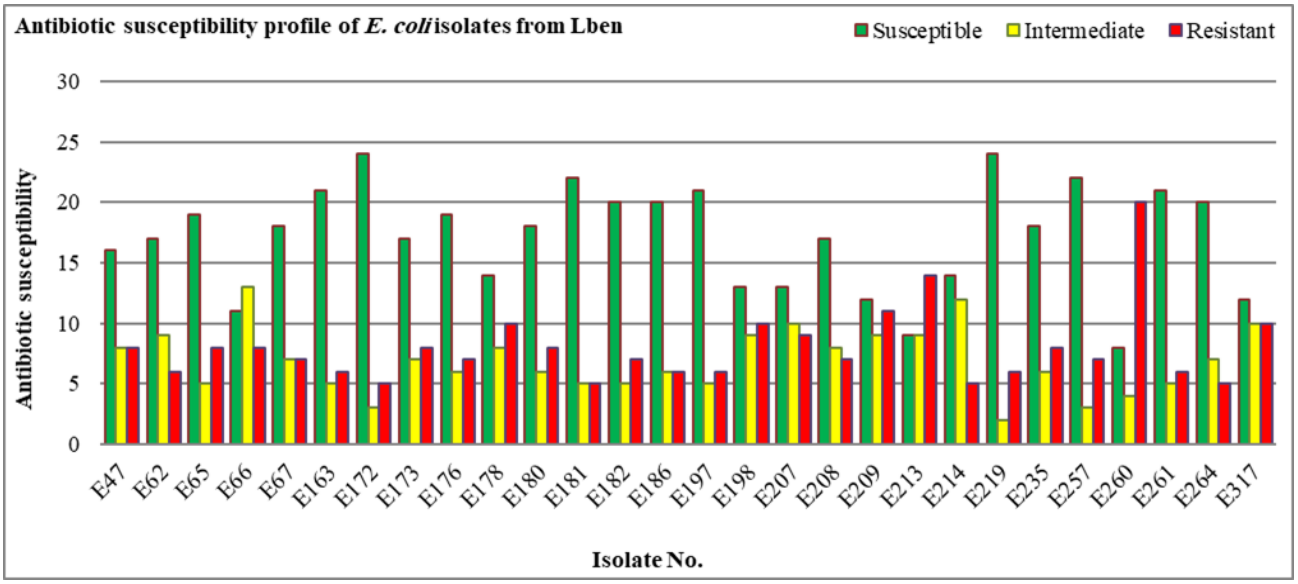


Figure 5. Antimicrobial resistance profiles of *Escherichia coli* isolated from Lben (fermented milk) in Libya during 2024

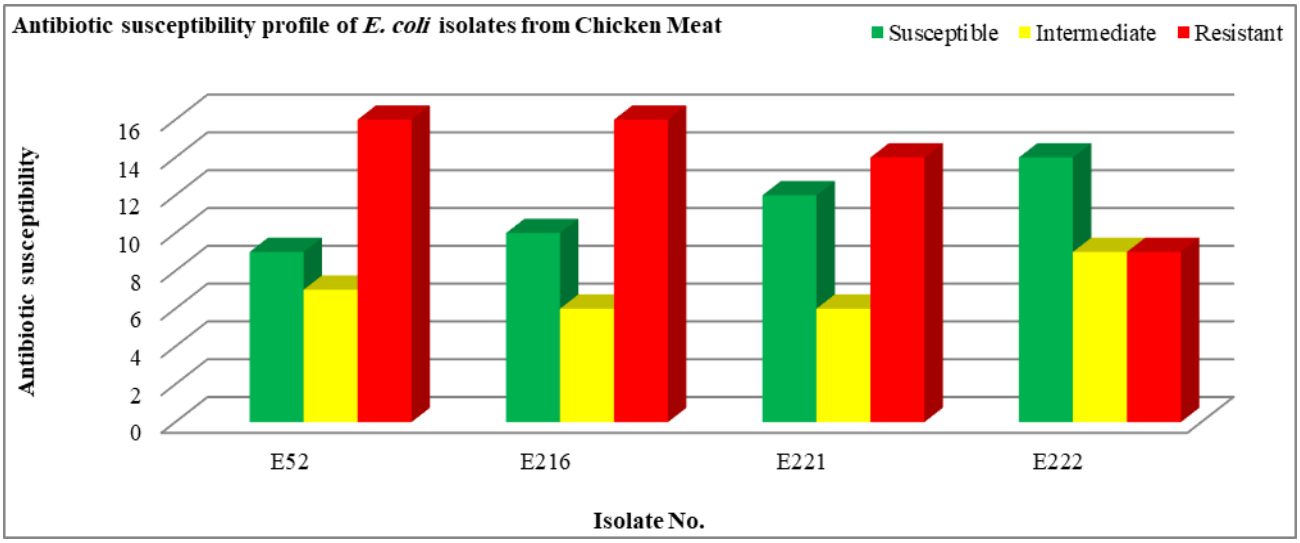


Figure 6. Antimicrobial resistance profiles of *Escherichia coli* isolated from chicken meat in Libya during 2024

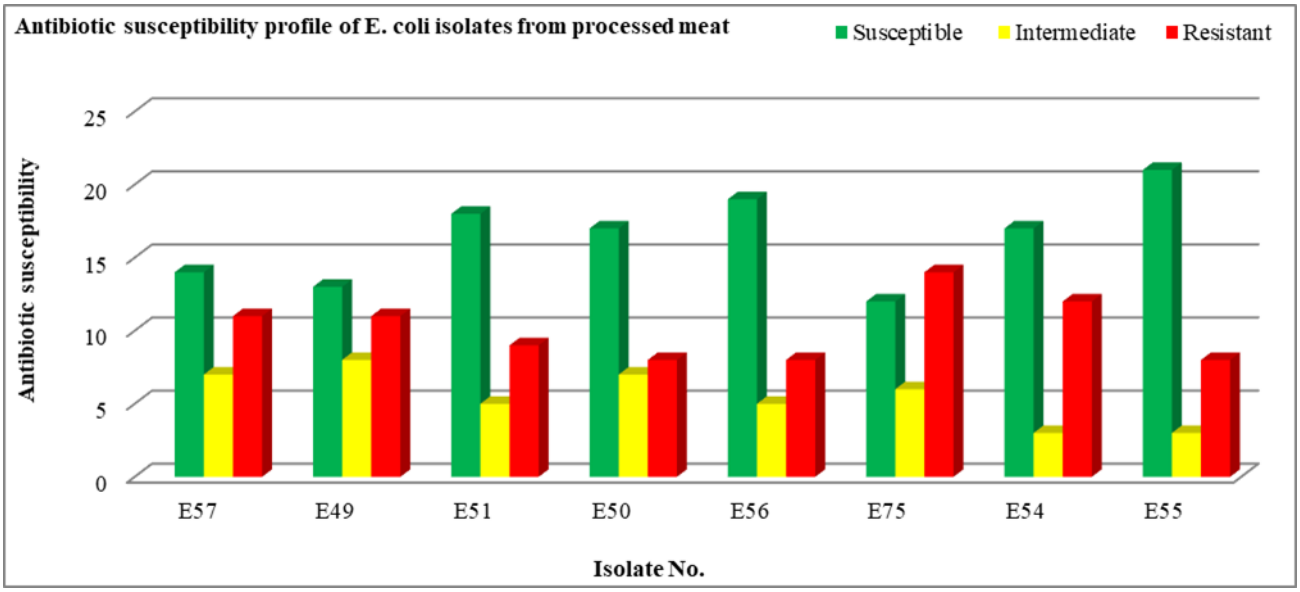


Figure 7. Antimicrobial resistance profiles of *Escherichia coli* isolated from processed meat in Libya during 2024

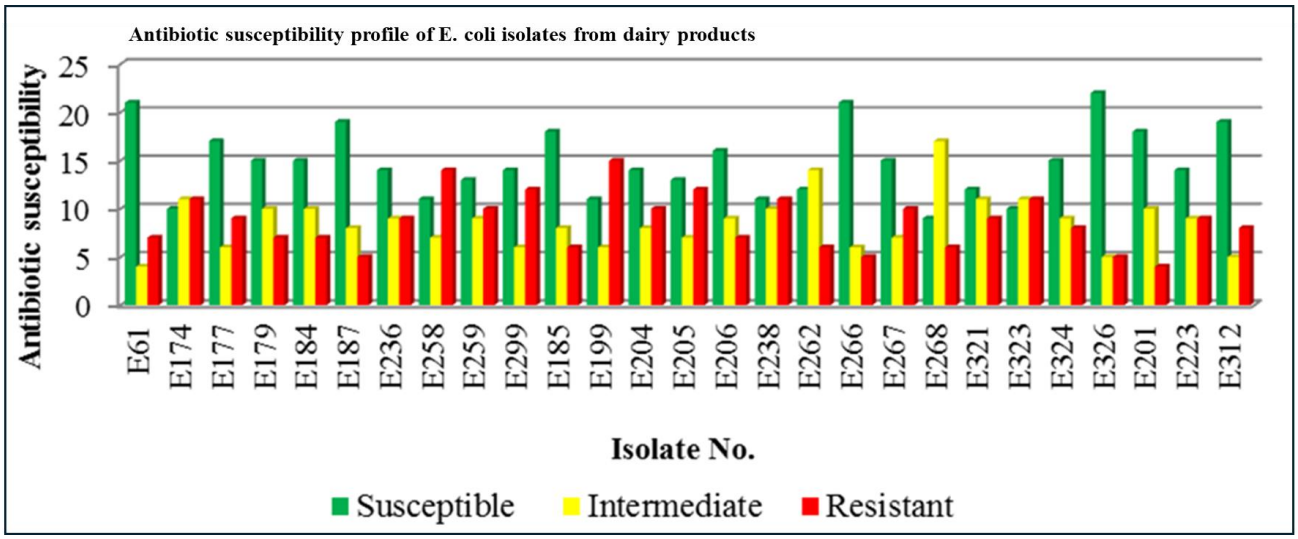


Figure 8. Antimicrobial resistance profiles of *Escherichia coli* isolated from dairy products in Libya during 2024

DISCUSSION

The isolation, antibiotic susceptibility, and molecular sequencing of foodborne bacteria such as *E. coli* from milk, meat, and their products have been the subject of several studies worldwide. Microbes are becoming an increasingly serious problem, and understanding their transmission, resistance mechanisms, and genetic relationships requires a holistic approach to public health. However, there has been a dearth of studies into the prevalence of *E. coli*-resistant strains in animal-origin foods such as milk and meat in Libya. Thus, the present study investigated the incidence of *E. coli* in milk, meat, and their associated products, in addition to examining their antibiotic susceptibility. It should be mentioned that this investigation is part of a series of studies carried out in Libya (Garbaj et al., 2016, 2017; Naas et al., 2017, 2019; Azwai et al., 2016, 2024) on locally isolated microorganisms, probably for the first time, aiming to establish the Libyan Integrated Program for Antimicrobial Resistance Surveillance.

The association of *E. coli* contamination between milk, meat, and their products is primarily due to shared sources and similar handling practices throughout the food production chain (Oliver et al., 2005). Since *E. coli* is a common inhabitant of the intestinal tract of animals, contamination can easily occur during milking or slaughter if fecal matter comes into contact with the product (Callaway et al., 2003). Cross-contamination is also common during processing, especially when hygiene practices are poor or equipment is shared between raw products. Environmental factors such as contaminated water, feed, or farm surroundings, along with inadequate refrigeration and storage, can further promote bacterial growth and spread (Wang et al., 2017). In many cases, small-scale or backyard farming systems where animals

for both milk and meat are kept together increase the risk of widespread contamination. Altogether, these factors contribute to the frequent detection of *E. coli* in both dairy and meat products (Omar et al., 2024).

Escherichia coli is commonly used as an indicator of human fecal contamination due to its constant presence in human waste. Its presence in food can result from animal, environmental, or human sources throughout the farm-to-table chain, potentially leading to foodborne illnesses and outbreaks in both humans and animals.

As shown in Table 3 and Figure 1, *E. coli* isolates exhibited the highest resistance to penicillin and cloxacillin (100%), followed by neomycin (93.4%), rifampicin (86.9%), and methicillin (75%). This may reflect the widespread antimicrobial use in the country. All 92 isolates (100%) were MDR, particularly those from lben and chicken, which demonstrated resistance to nine antibiotic classes. No extensively XDR or PDR strains were detected. As the first study in this region, no previous data are available for comparison.

Several studies have examined antimicrobial resistance in *E. coli* from animal-derived food in neighboring countries (Faour-Klingbeil and Todd, 2019). Jouini et al. (2009) analyzed 98 isolates of *E. coli* from 40 food samples in Tunisia using disk diffusion and agar dilution methods. High resistance was observed in over 43% of isolates to antibiotics such as tetracycline, sulfonamides, nalidixic acid, ampicillin, streptomycin, and trimethoprim-sulfamethoxazole. However, all isolates remained susceptible to cefotaxime, ceftazidime, cefoxitin, aztreonam, and amikacin. The emergence of such resistant strains poses a serious global health threat by limiting treatment options for bacterial infections.

Compared to previous studies in North Africa and the Middle East, the antibiotic resistance rates observed in the present study indicated some variation. In Algeria, *E. coli* from retail chicken meat showed higher resistance to sulfamethoxazole-trimethoprim and tetracycline (96.6%) and amoxicillin (65.5%) than reported here (Laarem et al., 2017). In contrast, findings from Egypt demonstrated similar resistance patterns, with moderate rates in *E. coli* from raw milk and cheese, including tetracycline (27.5%), ampicillin (18.9%), and streptomycin (18.5%). Lower resistance rates were observed for cefotaxime (4.5%) and ciprofloxacin (1.4%). These findings highlight significant antibiotic resistance in the region (Ombarak et al., 2018).

Although the resistance rate to carbapenems among different food samples was very low, the findings of the current study were much higher than those of clinical samples (0.22%-0.64%) reported in China (Li et al., 2023). Carbapenems are highly effective against *E. coli* infections; however, their use should be cautious to prevent the sporadic emergence of resistance.

Antibiotics frequently administered in food animal production for prophylaxis, therapeutic purposes, and growth promotion include sulfamethoxazole, enrofloxacin, benzylpenicillin, tylosin, amoxicillin, trimethoprim, oxytetracycline, ampicillin, and streptopenicillin. In poultry production, agents such as amikacin, neomycin, enrofloxacin, doxycycline, tetracycline, tilmicosin, and colistin sulfate are commonly employed as growth promoters (Chattopadhyay, 2014). Antibiotics and growth promoters are often applied indiscriminately to entire flocks, frequently without the guidance of veterinary professionals or public health authorities. Poultry producers or farmers commonly neglect the recommended antibiotic withdrawal period before selling products like meat, milk, dairy products, eggs, and fish (Khalifa et al., 2024). In broiler chickens, antimicrobials are routinely added to feed and water for disease prevention, leading to residues in meat and other products, posing significant public health risks. Antibiotics, including doxycycline, colistin sulfate, neomycin, tetracycline, enrofloxacin, ciprofloxacin, and amikacin, are commonly used in food animals for treatment, prophylaxis, and growth promotion. However, residues of these antibiotics can enter the human food chain, particularly if poor record-keeping leads to the sale of animal products before the required withdrawal periods are observed (Roess et al., 2013).

All isolates from different food samples were highly resistant to commonly used antibiotics, particularly the medicines belonging to the penicillin class (penicillin, methicillin, and cloxacillin). Likewise, these present results were close to the findings of Shrestha et al. (2019), who concluded that *E. coli* isolates from clinical samples exhibited high resistance against penicillin (100%). In China, the prevalence of resistant *E. coli* in both animals and foods has notably increased; studies indicate that ampicillin has documented the highest resistance rates compared to other antibiotics (60.5%; Sun et al., 2021; Liu et al., 2024). It is not surprising that the penicillin class is the most common and extensively used antibiotic, not only in humans but also in food-producing animals.

During the present study, *E. coli* isolates indicated lower resistance to amoxicillin and ampicillin (20.6%-26%). However, this contrasted with Rafiq et al. (2024), who reported 100% resistance to amoxicillin in *E. coli* from chicken meat. Similarly, Al Azad et al. (2019) and Islam et al. (2024) found high ampicillin resistance (94%-100%) in *E. coli* from broiler chickens.

The current results demonstrated that all *E. coli* isolates were sensitive to nitrofurantoin and carbapenems (imipenem, meropenem, and ertapenem), with only 3.2% showing resistance to carbapenems. These findings align with Moglad (2020), who reported low resistance (1.4%) in clinical isolates, and are consistent with results from Zanzibar, Tanzania, reported by Omar et al. (2024).

In the present study, the MAR index of *E. coli* ranged from 0.09 to 0.6. An MAR index above 0.2 suggested a high risk of MDR infection. Notably, *E. coli* from chicken meat showed resistance to 9 of 12 tested antibiotic classes, likely due to the overuse of antimicrobials by Libyan poultry farmers.

Out of all *E. coli* isolates, 43 were confirmed as ESBL producers, with a higher prevalence in raw milk than in meat products. This contrasted with the findings of Omar et al. (2024), who reported more ESBL *E. coli* in broiler chicken organs, but aligns with the findings of Mgaya et al. (2021) from Tanzania. Differences may stem from differences in farm hygiene and biosecurity practices (Zhang et al., 2015). The high prevalence of ESBL-producing *E. coli* observed in the present study may be attributed not only to the widespread use of antibiotics as prophylactics in food animals but also to horizontal gene transfer mechanisms. The routine use of antibiotics such as beta-lactams for disease prevention in livestock creates selective pressure that favors the survival and proliferation of resistant strains (Chattopadhyay, 2014). Additionally, ESBL genes commonly carried on mobile genetic elements such as plasmids, integrons, and transposons can be rapidly transferred between bacterial populations via conjugation, transformation, or transduction, facilitating the spread of resistance even across different species and environments (Carattoli, 2013; Rozwandowicz et al., 2018). This dual mechanism of antibiotic pressure and genetic exchange accelerates the dissemination of ESBL *E. coli* in both animal and human reservoirs, particularly in settings with limited biosecurity and antimicrobial regulation.

The current study revealed different antibiotic resistance rates across different sample types. Chicken meat isolates showed higher resistance than processed meat isolates, which was linked to a greater prevalence of ESBL-positive strains and prolonged antibiotic use for poultry treatment. Resistance was lower in dairy product isolates compared with raw milk and lben isolates, and the reason could be related to heat treatment. In addition, the presence of *E. coli* in meat, combined with low sanitary management, highlighted potential health risks for consumers. While cooking generally destroys *E. coli*, risks arise from undercooking, poor hygiene, cross-contamination, and improper handling.

An alternative explanation for the high *E. coli* contamination in raw and fermented milk was the poor hygiene practices during milking, inadequate equipment sanitation, and a lack of pasteurization. *E. coli* is commonly present in bovine feces, and contamination can occur when udders, hands, or milking tools are not adequately cleaned (Oliver et al., 2005). In many traditional dairy settings, raw milk is handled without refrigeration, allowing bacteria to multiply rapidly, especially in warm climates (FAO/WHO, 2006). Additionally, water used to clean equipment or animals may be contaminated, serving as another source of *E. coli* introduction. While fermentation is often perceived to reduce microbial load, it does not ensure safety if initial milk quality is poor or if fermentation conditions (time, temperature, starter cultures) are not properly controlled (Beukes et al., 2001). In regions where fermented milk products such as lben are traditionally consumed, cross-contamination and storage in unsterilized containers further increase the risk (Gran et al., 2003). Moreover, the absence of the pasteurization process that effectively kills *E. coli* makes both raw and fermented milk products more susceptible to contamination (Chye et al., 2004).

The food chain serves as the primary pathway for the transmission of antibiotic-resistant bacteria between animals and humans. Traditional fermented dairy products, which are not heat-treated before consumption, act as a vehicle for antibiotic-resistant bacteria, directly connecting the animals' natural microbiota to the human gastrointestinal tract (Hao et al., 2014).

To mitigate the emergence and spread of ESBL-producing *E. coli*, a One Health approach is essential, recognizing the interconnectedness of human, animal, and environmental health. This integrated strategy involves coordinated efforts among veterinarians, medical professionals, farmers, environmentalists, and policymakers to reduce antimicrobial misuse, improve surveillance, and strengthen biosecurity practices across sectors. Promoting responsible antibiotic stewardship in livestock production, improving food hygiene, and implementing routine screening of animal-derived foods for resistant pathogens are critical components. Additionally, strengthening laboratory capacities and data-sharing frameworks across human and veterinary health systems can improve early detection and response to antimicrobial resistance threats. Adoption of the One Health framework is particularly important in regions with high resistance burdens and limited regulatory enforcement, such as North Africa and the Middle East, to safeguard both public health and food safety (FAO, OIE, WHO, 2022).

CONCLUSION

The present study highlighted the significant presence of multidrug-resistant (MDR) *E. coli* in food products of animal origin, particularly raw milk, fermented milk, and chicken meat in Libya. All isolates indicated resistance to at least three antibiotic classes, with some demonstrating resistance to up to nine, indicating a high prevalence of MDR. Notably, 100% of isolates exhibited resistance to penicillin and cloxacillin, while 43 isolates were phenotypically confirmed as ESBL producers, with higher prevalence in raw milk samples. However, no extensively drug-resistant or pan-drug-resistant *E. coli* were detected, possibly due to limited exposure to last-resort antibiotics such as carbapenems and

nitrofurantoin, to which most isolates remained susceptible. The high multiple antibiotic resistance index values further suggested intense antibiotic selection pressure, likely due to unregulated and prophylactic use of antimicrobials in animal farming. The present findings underscore the need for stricter control measures on antibiotic use in agriculture, proper hygiene practices across the food production chain, and enhanced public awareness about antimicrobial resistance. Implementing the One Health approach integrating animal, human, and environmental health, is critical for curbing the spread of resistant *E. coli* and safeguarding food safety and public health. Future restudies should employ genomic analysis to characterize the resistance genes and mobile genetic elements responsible for the observed MDR in Libyan food products.

DECLARATIONS

Authors' contributions

Salah Mohamed Azwai, Aboubaker Mohamed Garbaj, Allaaeddin Ali El Salabi, and Ibrahim Mohamed Eldaghayes designed and planned the study. Salah Mohamed Azwai, Aboubaker Mohamed Garbaj, Jihan Ali Sherif, Samira Abd Farag, Salem Farhat Abureema, and Fatim Taher Gammoudi did the laboratory work. Salah Mohamed Azwai, Aboubaker Mohamed Garbaj, Ibrahim Mohamed Eldaghayes, Jihan Ali Sherif, Samira Abd Farag, Salem Farhat Abureema, and Fatim Taher Gammoudi contributed equally in the preparation of the manuscript. All authors contributed to the revision of the final manuscript.

Funding

This study was not supported financially and did not receive any funding budget.

Competing interests

The authors declare that there is no conflict of interest.

Acknowledgments

The authors are grateful for the support of the Faculty of Veterinary Medicine, University of Tripoli, Tripoli, Libya.

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.

Availability of data and materials

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

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2025, Scienceline Publication
World's Veterinary Journal
World Vet J, 15(3): 612-621.
ISSN 2322-4568

DOI: https://dx.doi.org/10.54203/scil.2025.wvj61
PII: S2322456825000061-15

Effectiveness of Guava Leaf Fraction on Phagocytosis Activity and Leukocyte Differentiation in Tilapia (*Oreochromis niloticus*) Infected with *Aeromonas hydrophila*

Lailatul Munawaroh Dewi Kusuma Wardani[1]*, Sri Andayani[2], and Yunita Maimunah[2]

[1] *Graduate Student of Aquaculture, Faculty of Fisheries and Marine Science, Brawijaya University, Jalan Veteran, Malang, 65145, Indonesia*
[2]*Aquaculture Department, Faculty of Fisheries and Marine Science, Brawijaya University, Jalan Veteran, Malang, 65145, Indonesia*
*Corresponding author's Email: lailatulmdk@gmail.com

ORIGINAL ARTICLE
Received: June 20, 2025
Revised: July 24, 2025
Accepted: August 26, 2025
Published: September 30, 2025

ABSTRACT

One obstacle to the sustainability of fisheries is the bacterial infection caused by *Aeromonas hydrophila (A. hydrophila)*. To overcome the sustainability issues posed by antibiotic use against *A. hydrophila* in fisheries, guava leaf (*Psidium guajava* L.) immunostimulants offer a promising natural alternative. The present study aimed to examine the impact of dynamic compounds derived from guava leaves on leukocyte differentiation and phagocytosis in tilapia infected with *A. hydrophila*. A completely randomized design was established, consisting of three treatment groups and two controls, with three replications. The groups included 150 ppm of guava leaf fraction (Group A), 125 ppm of guava leaf fraction (Group B), and 100 ppm of guava leaf fraction (Group C), which were injected into tilapia. The negative control group was infected with *A. hydrophila* bacteria without receiving the guava leaf fraction, and the positive control group was not infected with *A. hydrophila* bacteria and did not receive the guava leaf fraction. The dosage determination of the guava leaf fraction was conducted using the lethal concentration 50% test. The results demonstrated that Fraction 5 of guava leaf extract significantly enhanced the non-specific immune response of tilapia. The optimal dose of 150 ppm led to a substantial increase in lymphocyte counts and phagocytic activity following infection with *A. hydrophila*. Consequently, all treatment groups (100, 125, and 150 ppm) exhibited survival rates statistically equivalent to those of the positive control group, which was significantly higher than the 39% survival rate in the negative control group. Thus, guava leaf Fraction 5, particularly at 150 ppm, serves as an effective immunostimulant and natural antibiotic alternative for controlling A. hydrophila in tilapia.

Keywords: *Aeromonas hydrophila*, Guava leaf fraction, Immune response, Immunostimulant

INTRODUCTION

Tilapia (*Oreochromis niloticus*) is a key aquaculture commodity in Indonesia, widely cultivated due to its relatively low price, resulting in higher demand for domestic consumption. The farming of tilapia in Indonesia is increasing at a rate of 23.8% per year (Hadie et al., 2018). However, one of the obstacles to the development and sustainability of aquaculture, especially in tilapia, is bacterial infections (Saputra and Indaryanto, 2018). A significant concern is the infection caused by *Aeromonas hydrophila* (*A. hydrophila*), which frequently affects tilapia and can result in mortality rates of up to 85% in aquaculture (Tondi et al., 2023).

Aeromonas hydrophila is a bacterium that is widespread in fresh, brackish, and saline waters, and it can infect a variety of freshwater fish. Fish affected by *A. hydrophila* typically exhibit signs of abdominal swelling and bleeding on the outer surface or skin, as well as around the anus (Nahar et al., 2016). Infected fish may develop ulcers, red spots on the body surface, inflamed skin, decayed fins and tail, abdominal swelling, and bulging eyes (Arwin et al., 2016). Other clinical signs include detached scales from the body, a dark body color, a pale liver, skin bleeding, excessive mucus production, and tail damage (Pattipeiluhu et al., 2022).

Motile *Aeromonas hydrophila* (MAS) can result from several stress factors, including overcrowding in ponds, changes in environmental conditions, and inadequate water quality. Clinical signs of MAS include ulcers, bloating, fin irritation, and exposed scales (Tondi et al., 2023). Infected fish experience blood cell damage as a result of the immune response, and blood examination is used as an indicator of disease severity, highlighted by changes in leukocyte count values (Pattipeiluhu et al., 2022). The abundance of *A. hydrophila* tends to increase with declining water quality and stressed fish conditions (Saputra and Indaryanto, 2018). Furthermore, high levels of *A. hydrophila* in water can contribute to environmental pollution and toxicity (Hidayaturohman et al., 2021). While chemicals and antibiotics can control *A. hydrophila*, their use is frequently costly and may harm the environment and human health (Cao et al., 2012).

The control of *A. hydrophila* infections in fish often involves the use of several effective antibiotics, including enrofloxacin, ciprofloxacin, and fluoroquinolones. Enrofloxacin, in particular, is notable for its effectiveness in treating

To cite this paper: Wardani LMDK, Andayani S, and Maimunah Y (2025). Effectiveness of Guava Leaf Fraction on Phagocytosis Activity and Leukocyte Differentiation in Tilapia (*Oreochromis niloticus*) Infected with *Aeromonas hydrophila. World Vet. J.*, 15(3): 612-621. DOI: https://dx.doi.org/10.54203/scil.2025.wvj61

these infections. Additional antibiotics such as tetracycline, sulfonamides, trimethoprim, sulfamethoxazole, and ciprofloxacin can be employed to address skin infections caused by *A. hydrophila* (Perkasa and Dhewantara, 2019). However, the use of antibiotics in aquaculture is often inefficient, highlighting the need for alternative control measures. One promising approach is to enhance fish immunity through the use of immunostimulants derived from natural medicinal plants. These plants provide a safe and environmentally friendly alternative to traditional antibiotics. Guava leaves (*Psidium guajava* L.) are particularly noted for their potential as immunostimulants in fish, as they contain active compounds such as tannins, flavonoids, saponins, and phenolics, which can bolster defenses against diseases (Sine and Fallo, 2016; Amelia et al., 2021).

Immunostimulants serve to replace antibiotics and vaccines by activating or enhancing the components of the immune system in a non-specific manner. Immunostimulants have been shown to stimulate non-specific immune responses in fish, such as tilapia, improving the fish's defense against diseases (Sine and Fallo, 2016). The immune system includes B cells and T cells. B cells can differentiate into plasma cells that produce antibodies, while T cells play a crucial role in mediating immunity and regulating immune responses. T cells can become cytolytic T cells, which target and destroy pathogens (Setyowati and Astriana, 2020). Immunostimulants have been shown to increase T cell lymphocytes in numerous animals, including fish, enhancing immunity against intracellular bacteria and viruses (Setyowati and Astriana, 2020). Evidence suggested that compounds found in guava leaves can enhance the activity of immune cells such as macrophages, T cells, and B cells, leading to improved phagocytosis (Rochmasari, 2011). These leaves can boost the function of neutrophils, basophils, monocytes, lymphocytes, and eosinophils (Kullu et al., 2013). In healthy fish, lymphocytes typically constitute about 71.12% to 82.88% of total leukocytes, outnumbering neutrophils and monocytes. An increase in lymphocyte production is significant in enhancing the immune response, thereby improving the fish's resistance to disease and infection (Setyowati and Astriana, 2020).

The use of guava leaf fraction is expected to serve as an immunostimulant, enhancing the nonspecific immune system, as indicated by leukocyte differential and phagocytosis activity in tilapia infected with *A. hydrophila*. Based on the description provided, it can be inferred that one of the obstacles in tilapia aquaculture is infection by *A. hydrophila*. Therefore, the present study aimed to provide guava leaf fractions as immunostimulants in tilapia to prevent *A. hydrophila* bacterial infections in aquaculture.

MATERIALS AND METHODS

Ethical approval

The authors have designed the study in accordance with the ethical guidelines of Brawijaya University, Indonesia. The study was carried out in accordance with the applicable regulations for the master's program at the Faculty of Fisheries and Marine Science of Brawijaya University, Indonesia, as outlined in Rector's Regulation Number 427/PER/2012 concerning academic regulations.

Study area

The present study was conducted at the Laboratory of the Faculty of Fisheries and Marine Science, Brawijaya University, Malang, Indonesia, from September 2024 to February 2025. The animals used were tilapia strain Sultana, measuring 10 to 12 cm (± 18 grams/head), with a density of 18 fish per aquarium, sourced from Surowono, Kediri, Indonesia.

Parameters

The primary parameters measured were phagocytosis activity and leukocyte differentials in tilapia. Additional factors included the survival rate (%) and water quality, which covered temperature (°C), pH, and dissolved oxygen (DO, %).

Survival rate

The survival rate (SR) represents the percentage of organisms that remain alive at the end of a rearing period compared to those alive at the beginning. Daily data collection included recording the number of surviving tilapia fish both at the beginning and the end of the maintenance period. Subsequently, the data was calculated using the following formula.

Survival rate = Number of live tilapia at the beginning of rearing / Number of tilapia at the end of rearing (Fahrizal and Nasir, 2017).

Water quality

To measure the temperature, the thermometer was submerged at least 10 cm beneath the water's surface and kept in the water for five minutes. For pH measurement, a piece of litmus paper was placed in the water, and the resulting color change was matched against a pH chart to assess the pH level. To evaluate DO, the DO Meter's pen was immersed in the water, and the dissolved oxygen reading was automatically shown on the DO Meter's display (Nursobah et al., 2022).

Experimental design

A completely randomized design was employed, consisting of five treatment groups and three replicates. The negative control group consisted of tilapia that were not infected with *A. hydrophila* and were treated with a guava leaf fraction. The positive control group comprised tilapia infected with *A. hydrophila* and not administered the guava leaf fraction. Group A received the guava leaf fraction at a dose of 150 ppm. Group B received guava leaf fraction at a dose of 125 ppm, and Group C received guava leaf fraction at a dose of 100 ppm. On day 0, prior to the first counting day, the fish's body weight was recorded, blood was drawn, and the first injection of guava leaf fraction was given in all treatment groups except the negative control group. Injections were administered to the fish at the base of the pectoral fin or the tip of the dorsal fin, after which all groups (Group A, B, and C) were allowed to recover for 72 hours. Blood was drawn intramuscularly from the pectoral fins and dorsal fins into a small tube and examined under a microscope (ReHaZe, Indonesia) at 1000x magnification in the laboratory (Haditomo, 2017). On day three, blood was drawn from all groups for observation before infection, followed by the second injection of guava leaf fraction. On day six, blood was taken for observation before infection, after which the fish were infected with *A. hydrophila*. On days 9 and 12, blood was drawn for observation after *A. hydrophila* infection.

Lethal concentration 50% and lethal dose 50%

The toxicity profile of guava leaf fraction in freshwater fish has been documented to range from 250 ppm to 3250 ppm, demonstrating its potential utility in managing pathogenic bacterial infections in aquaculture (Rosidah and Afizia, 2012). A detailed examination of the lethal concentration 50% (LC_{50}) is essential in ascertaining the optimal dosage of the guava leaf fraction. In the relevant study, guava leaf fraction was administered intramuscularly at dosages of 125, 150, 175, 200, and 225 mg/kg of fish body weight. Results from the bacteriostatic inhibition assays indicated that the guava leaf fraction effectively suppressed the growth of *A. hydrophila*, with a significant effect observed at a concentration of 175 ppm. The fractions designated for further application were identified at concentrations of 150 ppm, 125 ppm, and 100 ppm.

To evaluate the lethal dosage 50% (LD_{50}), an experimental approach was utilized. Initially, pure cultures of *A. hydrophila*, at a concentration of 10^{10} cells/ml, were subjected to dilution, resulting in concentrations of 10^9 cells/ml, 10^8 cells/ml, 10^7 cells/ml, and 10^6 cells/ml in tryptone soy broth (TSB; Merck, Germany) for LD_{50} assessment (Sulastra and Khaerati, 2020). The incubation of these dilutions occurred over a 24-hour period at temperatures ranging from 27°C to 30°C (Telaumbanua et al., 2019). In the experimental setup, 18 tilapias were infected with *A. hydrophila* and kept in 10 liters of media water, following established treatment protocols, and monitored at four-hour intervals. Mortality rates among the fish were carefully documented over durations of 24 to 72 hours, with the initial occurrence of 50% mortality serving as the criterion for determining the LD_{50} threshold. The LC_{50} calculation used the previously mentioned dosages of guava leaf fraction administered through intramuscular injection. These dosages were based on inhibition tests that assessed their effects on *A. hydrophila* proliferation. Each test container contained 18 fish, with observations made throughout 24 to 72 hours. The first observation of 50% mortality was crucial for determining the LC_{50} dosage.

Thin-layer chromatography and column chromatography

Fractionation analysis was carried out using thin-layer chromatography (TLC; Merck, Germany) and column chromatography, following the method of Fasya et al. (2018) to purify fractions from guava leaves. After obtaining the fractions, non-specific immune response tests were performed on tilapia. Before focusing on the most promising fraction, LD_{50} and LC_{50} tests were conducted to ensure safety and effectiveness. As noted in Rusmiyati's study (2023), this fractionation process was designed to separate compound groups by their polarity, using n-hexane and ethyl acetate as solvents. For the TLC plates, strips were cut to be 1 cm wide and 10 cm tall, with a 0.5 cm margin at the top and a 1 cm margin at the bottom. The plates were then placed in an oven (Memmert, Germany) with a capacity of 55L, set to a temperature of 100°C to 120°C for approximately 15 minutes to evaporate any moisture. The guava leaf fraction was dissolved in a sufficient amount of ethanol. Approximately 5 µl of the dissolved fraction was then applied to the prepared TLC plate using a microsyringe. The plate was positioned upright in a 250 ml chamber containing 10 ml of eluent solvent and was covered with cling wrap to prevent evaporation. Once the eluent reached the upper limit, the TLC plate was carefully removed using tweezers. The staining agent used was ultraviolet (UV) light, which made the stain visible under near-UV (254 nm) and far-UV (366 nm) illumination.

Blood collection

Leukocyte differentiation was performed by taking anticoagulated fish blood with a capillary pipette and placing it on the tip of an object glass. The object glass was fixed with methanol for two minutes. Following this, the blood smear was stained with Giemsa solution (Larutan Giemsa 100ml Indo Reagen, Indonesia) and left for 20 minutes. The glasses were rinsed with distilled water and allowed to dry. Object glasses were observed using a microscope (ReHaZe, Indonesia) at 1000x magnification in the laboratory. Fish phagocytosis activity was initiated by treating fish blood with anticoagulants, then taking 50 µL, placing it into a microtiter plate, and adding 50 µL of *A. hydrophila* suspension in FBS (10^8 cells/mL). The blood was incubated at 27°C for 20 minutes (Haditomo, 2017).

Statistical analysis

The data obtained were then statistically analyzed using analysis of variance (ANOVA) with a significance level of 95% ($p > 0.05$). This analysis was followed by Duncan's test using the IBM SPSS program application version 25.

RESULTS AND DISCUSSION

Thin-layer chromatography analysis

The TLC was conducted to determine the optimal solvent ratio for the mobile phase eluent in the fractionation analysis of the guava leaf fraction. The primary objective of this analysis was to identify the most effective eluent for producing the guava leaf fraction. The results of the analysis indicated that the combination of n-hexane and ethyl acetate in a 6:4 ratio was the most effective at generating color on silica gel plates (Darmstadt, Germany) under UV light at 365 nm.

Column chromatography analysis

Column chromatography was conducted to yield pure fractions in substantial quantities. The solvent utilized in this process was a mixture of n-hexane and ethyl acetate in a 6:4 ratio, serving as the mobile phase, with silica gel powder functioning as the stationary phase. The outcomes were derived from the column chromatography test, using the plate that exhibited the most intense stain color. The present study successfully produced five distinct fractions. All fractions were tested on the same species of fish, tilapia, at concentrations of 150 ppm (Group A), 125 ppm (Group B), and 100 ppm (Group C). The fish in all groups were identical to precisely evaluate how these fractions affect non-specific immune responses, especially leukocyte activity.

Non-specific immune response test

The non-specific immune response test of tilapia against all guava leaf fractions was conducted to identify one candidate fraction from five types of fractions, based on the specific immune response (Leukocytes) of tilapia *in vivo*. The current results highlighted the best candidate fraction of guava leaves for enhancing tilapia leukocytes (Table 1).

The leukocyte count across all guava leaf fractions indicated that fraction five was significantly different ($p < 0.05$) from the leukocyte count measured on day seven, which was 26.31 ± 0.13 cells/mL, as observed in groups A, B, and C. the results for fraction five in groups A, B, and C demonstrated a significant increase, with a value of 25.46 ± 0.37 cells/ml on day three and 26.31 ± 0.13 cells/ml on day seven, marking it as the highest value among the other fractions. The rise in leukocyte values suggested that exposure to fraction five of guava leaves may enhance the body's ability to defend against foreign bodies (Lestari et al., 2017). Thus, it can be concluded that fraction five was the most promising candidate for assessing the non-specific immune response of tilapia at the LC_{50} and LD_{50} stages. On day three, the leukocyte test showed a mean value of 0.286 and a median value of 0.489 ($p < 0.05$) across all groups. By day seven, the leukocyte test revealed a mean value of 0.129 and a median value of 0.621 ($p < 0.05$).

Table 1. Tilapia leukocyte test results from five guava leaf fractions on days 3 and 7 after infection

Faction (25 ppm) \ Day (After infection)	Day 3	Day 7
Result of fraction 1	15.37 ± 0.17[b]	16.01 ± 0.45[b]
Result of fraction 2	16.36 ± 0.20[b]	17.21 ± 0.11[b]
Result of fraction 3	18.18 ± 0.06[b]	18.50 ± 0.34[b]
Result of fraction 4	20.40 ± 0.16[c]	21.42 ± 0.19[b]
Result of fraction 5	25.46 ± 0.37[d]	26.31 ± 0.13[c]
Control	11.28 ± 0.12[a]	11.40 ± 0.15[a]
Total	17.84 ± 4.53	18.47 ± 4.75

[a,b,c, and d] Mean the same superscript letters in the same column are not significantly different ($p > 0.05$).

The lethal concentration 50% test of fraction five of the guava leaf

The LC_{50} test for fraction five of guava leaves was conducted to determine the dose that kills 50% of the tilapia individuals exposed over a specified timeframe. The concentrations tested included 125 ppm, 150 ppm, 175 ppm, 200 ppm, and 225 ppm, as part of an assessment evaluating the inhibitory effects of guava leaf fraction on bacteria. These concentrations were used to determine the LC_{50} of fraction five of guava leaves in terms of tilapia mortality over a 72-hour period. The current results indicated an effective dosage of 175 ppm. Consequently, the dosage levels for subsequent divisions in the present study were 150 ppm, 125 ppm, and 100 ppm.

The lethal dosage 50% test of *Aeromonas hydrophila*

The LD_{50} test for *A. hydrophila* was conducted to determine the dose necessary to kill 50% of the tested tilapia population, serving as a preliminary assessment for groups A, B, and C. In the present study, the total bacterial densities used were 10^6 cells/mL, 10^7 cells/mL, and 10^8 cells/mL. The current findings indicated that the LD_{50} of *A. hydrophila* against tilapia was 10^7 cells/mL. As microbial doses rise, mortality rates also increase. Higher concentrations administered to the fish may lead to more significant infections, particularly as their immune systems become compromised (Susandi et al., 2017).

Leukocyte differential

The leukocyte differential values consisted of lymphocytes, monocytes, and neutrophils (Table 2). Lymphocytes are responsible for secreting antibodies in response to antigens, while neutrophils play a vital role in the immune response to

infections caused by pathogenic organisms and possess phagocytic capabilities. Monocytes contribute to fighting infections and removing dead cells from the fish's body tissues. The differentiation in leukocyte functions facilitates the elimination of infectious and toxic materials through phagocytosis (Ilmayati et al., 2015). According to Abdillah et al. (2022), lymphocytes are a key indicator in antibody formation. A decrease in lymphocyte count in fish leads to a reduction in antibody levels, whereas an increase in lymphocytes corresponds to an increase in antibodies. Observations conducted on days 9 and 12 following *A. hydrophila* infection revealed a significant increase in lymphocyte values ($p < 0.05$) across all groups, indicating that the fish had entered a phase of nonspecific immune response and had developed a more robust self-defense mechanism.

The analysis of leukocyte differentiation percentages across treatment doses of 150 ppm, 125 ppm, and 100 ppm, as well as the positive and negative control groups, revealed a significant increase ($p < 0.05$) in the percentage of lymphocytes in each treatment group. In groups A, B, and C, fluctuations in lymphocyte values accordingly influenced monocyte levels, though neutrophil values remained unaffected. Notably, the highest lymphocyte percentage before infection was recorded in Group A, ranging from 70.36% to 73.34%, followed by Group B, which ranged from 69.48% to 71.35%, and Group C, which exhibited values between 61.58% and 69.36%. Post-infection, lymphocyte values increased, with the highest percentage ($p < 0.05$) observed in Group B, ranging from 78.50% to 90.48%.

Before infection, monocyte values in Group A ranged from 30.23% to 28.40%, in Group B, fell from 31.32% to 29.40%, and in Group C, ranged from 32.45% to 31.31%. Following infection, the percentages of monocytes decreased from 24.47% to 12.32% in Group A, 21.35% to 8.20% in Group B, and 27.58% to 14.58% in Group C. The monocyte values recorded on the twelfth day of observation were significantly lower than those in the control groups ($p < 0.05$).

Table 2. The tilapia (*Oreochromis niloticus*) leukocyte differentiation value during the study from day 3 to day 12, before and after infection by *Aeromonas hydrophila*

Treatment (ppm)	**Day (Before infection by *Aeromonas hydrophila*)**		**Day (After infection by *Aeromonas hydrophila*)**	
	3	6	9	12
Lymphocytes				
0 (Negative control group)	61.35 ± 0.11^{a}	65.49 ± 0.14^{a}	65.56 ± 0.18^{a}	85.29 ± 0.15^{b}
150 ppm of guava leaf fraction (Group A)	70.36 ± 0.24^{c}	73.34 ± 0.23^{c}	75.32 ± 0.14^{b}	88.46 ± 0.11^{b}
125 ppm of guava leaf fraction (Group B)	69.48 ± 0.16^{b}	71.35 ± 0.98^{c}	78.50 ± 0.14^{c}	90.48 ± 0.03^{c}
100 ppm of guava leaf fraction (Group C)	61.58 ± 0.14^{a}	69.36 ± 0.20^{b}	72.38 ± 0.17^{b}	87.29 ± 0.17^{b}
0 (Positive control group)	64.29 ± 0.17^{a}	67.38 ± 0.27^{a}	67.44 ± 0.28^{a}	71.56 ± 0.08^{a}
Total	65.41 ± 3.97	69.38 ± 2.88	71.84 ± 4.97	84.62 ± 6.97
Monocytes				
0 (Negative control group)	35.33 ± 0.80^{c}	31.36 ± 0.10^{c}	31.32 ± 0.10^{d}	14.67 ± 0.13^{c}
150 ppm of guava leaf fraction (Group A)	30.23 ± 0.14^{a}	28.40 ± 0.20^{a}	24.47 ± 0.14^{b}	12.32 ± 0.20^{a}
125 ppm of guava leaf fraction (Group B)	31.32 ± 0.13^{b}	29.40 ± 0.13^{b}	21.35 ± 0.12^{a}	8.20 ± 080^{a}
100 ppm of guava leaf fraction (Group C)	32.45 ± 0.12^{a}	31.31 ± 0.22^{c}	27.58 ± 0.14^{c}	14.58 ± 0.10^{b}
0 (Positive control group)	34.19 ± 0.80^{c}	32.41 ± 0.75^{c}	43.17 ± 0.75^{e}	27.26 ± 0.14^{d}
Total	32.70 ± 1.92	30.57 ± 1.51	21.58 ± 7.82	15.40 ± 6.59
Neutrophil				
0 (Negative control group)	0.85 ± 0.03^{a}	0.56 ± 0.58^{a}	0.75 ± 0.03^{a}	0.78 ± 0.04^{a}
150 ppm of guava leaf fraction (Group A)	1.46 ± 0.22^{a}	0.84 ± 0.04^{c}	1.65 ± 0.07^{c}	0.96 ± 0.03^{d}
125 ppm of guava leaf fraction (Group B)	1.11 ± 0.07^{a}	0.77 ± 0.05^{b}	0.95 ± 0.03^{b}	0.88 ± 0.04^{b}
100 ppm of guava leaf fraction (Group C)	0.89 ± 0.02^{a}	0.57 ± 0.05^{b}	0.85 ± 0.03^{b}	$0.83 \pm 0,03^{b}$
0 (Positive control group)	0.61 ± 0.03^{a}	0.34 ± 0.03^{a}	0.64 ± 0.04^{a}	0.92 ± 0.03^{c}
Total	0.98 ± 0.31	0.62 ± 0.18	0.97 ± 0.36	0.87 ± 0.07

The numbers in the same column followed by the same superscript letters are not significantly different ($P < 0.05$). [a,b,c,d, and e] Mean the same superscript letters in the same column are not significantly different ($p < 0.05$).

Neutrophil values, both before and after infection, were influenced by the treatment dosages applied and demonstrated a direct correlation with increasing dosage levels. Notably, the percentage of neutrophil values was significantly lower ($p < 0.05$) in comparison to lymphocytes and monocytes, a finding likely attributable to the fundamental role of neutrophils as the initial line of defense against pathogenic invaders (Ilmayati et al., 2015). Lymphocytes are specialized cells that facilitate the production of antibodies and serve as effector cells in response to antigenic challenges. Conversely, neutrophils play a crucial role in the immune response against pathogenic organisms, exhibiting critical phagocytic capabilities. Similarly, macrophages function comparably to neutrophils, utilizing phagocytosis to eliminate foreign entities that infiltrate the organism. The efficiency of phagocytic activity in these cells depends on the type of material ingested (Wulandari et al., 2018). Materials that are easily digested by lysosomal enzymes, which include different digestive and bactericidal compounds, are quickly broken down and neutralized (Ilmayati et al., 2015).

The monocyte values recorded on day 12 of the observation period were significantly lower ($p < 0.05$) compared to control values. The neutrophil counts obtained before and after infection demonstrated a direct proportionality to the sequential increases in treatment dosage ($p < 0.05$). Observational results recorded on days 9 and 12 post-infection indicated a significant increase ($p < 0.05$) in lymphocyte values, suggesting that the subjects had entered a phase of nonspecific immune response characterized by enhanced self-protective mechanisms. The percentage of monocytes in the treatment group before infection ranged from 29.40% to 32.45%, which subsequently diminished post-infection, falling within the range of 8.20% to 27.58% ($p < 0.05$). This reduction was likely due to damage and the depletion of monocyte cells. The observed percentage of neutrophils remained lower ($p < 0.05$) than that of both lymphocytes and monocytes, reinforcing the understanding of neutrophils as the primary defense against foreign agents. Furthermore, the guava leaf fractions were found to influence leukocyte values, as evidenced by significant increases ($p < 0.05$) on days 3, 6, 9, and 12. It is noteworthy that guava leaf fractions influenced both monocyte and neutrophil levels in groups A, B, and C, despite a decrease in their values from day 3 to day 12 ($p < 0.05$).

Based on the results of the leukocyte differential, dosing with guava leaf fraction significantly affected the leukocyte differential value, where the average dose of fraction treatment increased the number of lymphocytes during maintenance and became the first line of defense against antigens that enter the fish's body. According to Siagian et al. (2014), lymphocytes act as antibody producers in fighting antigens that enter the fish's body, monocytes function as macrophages, and neutrophils function as phagocytes.

Phagocytic activity

Phagocytosis is a cellular process through which foreign substances entering a fish's body are engulfed and subsequently broken down. This mechanism initiates with the identification and attachment of these foreign particles, which are then internalized and digested (Prakarsa et al., 2024). Macrophages are the primary cells responsible for phagocytosis, given their superior ability to engulf compared to other phagocytic cells. Once a macrophage engulfs foreign material, its membrane seals around the particles, transporting them into the cytoplasm. Here, the particles are enclosed within phagocytic vacuoles for digestion (Wulandari et al., 2018). Enhanced phagocytic cell activity in fish blood indicates increased immunity in fish (Utami et al., 2013; Table 3).

The results of the phagocytosis activity assessment indicated that the administration of fraction five of guava leaves at doses of 150, 125, and 100 ppm resulted in a significantly higher phagocytosis activity index ($p < 0.05$) compared to the control group that did not receive fraction five of guava leaves. This enhancement was attributed to the capacity of fraction five of guava leaves to better recognize related antigens entering the tilapia's body. Based on these findings, it is evident that increasing the doses of guava leaves had a significant impact on the percentage of phagocytosis activity in tilapia, which facilitated regression calculations to explore the relationship between dosing and phagocytosis activity (Figure 1).

On days 9 and 12, the calculations indicated a quadratic relationship, with R^2 values of 0.9998 on day 9 and 0.9990 on day 12, which led us to conclude that the measurements taken had a significant impact on the phagocytosis activity of tilapia ($p < 0.05$) at levels of 98% and 99%. The decreases noted on days 6 and 9 were linked to the highest dosage of 150 ppm, whereas the peak on day 12 corresponded to a dosage of 100 ppm. On day 12, phagocytosis activity indicated that fish treated with the guava leaf extract were healthy, as their immune defense began to build against the incoming antigen three days after infection. This increase in phagocytosis activity demonstrated an improvement in the fish's immunity (Ilimu and Saparuddin 2023).

Table 3. The values of phagocytosis activity of tilapia (*Oreochromis niloticus*) during the study from day 6 to day 12

Treatment (ppm)	Day (After infection by *Aeromonas hydrophila*)		
	6	9	12
0 (Negative control group)	20.30 ± 0.73^{b}	31.35 ± 0.50^{b}	31.16 ± 0.52^{a}
150 ppm of guava leaf fraction (Group A)	63.52 ± 1.16^{e}	69.40 ± 0.90^{e}	43.36 ± 0.41^{c}
125 ppm of guava leaf fraction (Group B)	57.53 ± 0.07^{d}	66.23 ± 0.95^{d}	58.28 ± 0.06^{d}
100 ppm of guava leaf fraction (Group C)	39.38 ± 0.14^{c}	56.36 ± 0.61^{c}	59.20 ± 0.08^{d}
0 (Positive control group)	18.26 ± 0.06^{a}	24.17 ± 0.80^{a}	37.52 ± 0.06^{b}
Total	39.80 ± 19.21	49.50 ± 19.05	45.90 ± 11.56

The numbers in the same column followed by the same superscript letters are not significantly different ($P < 0.05$). $^{a,b,c,d, \text{ and } e}$ Mean the same superscript letters in the same column are not significantly different ($p < 0.05$).

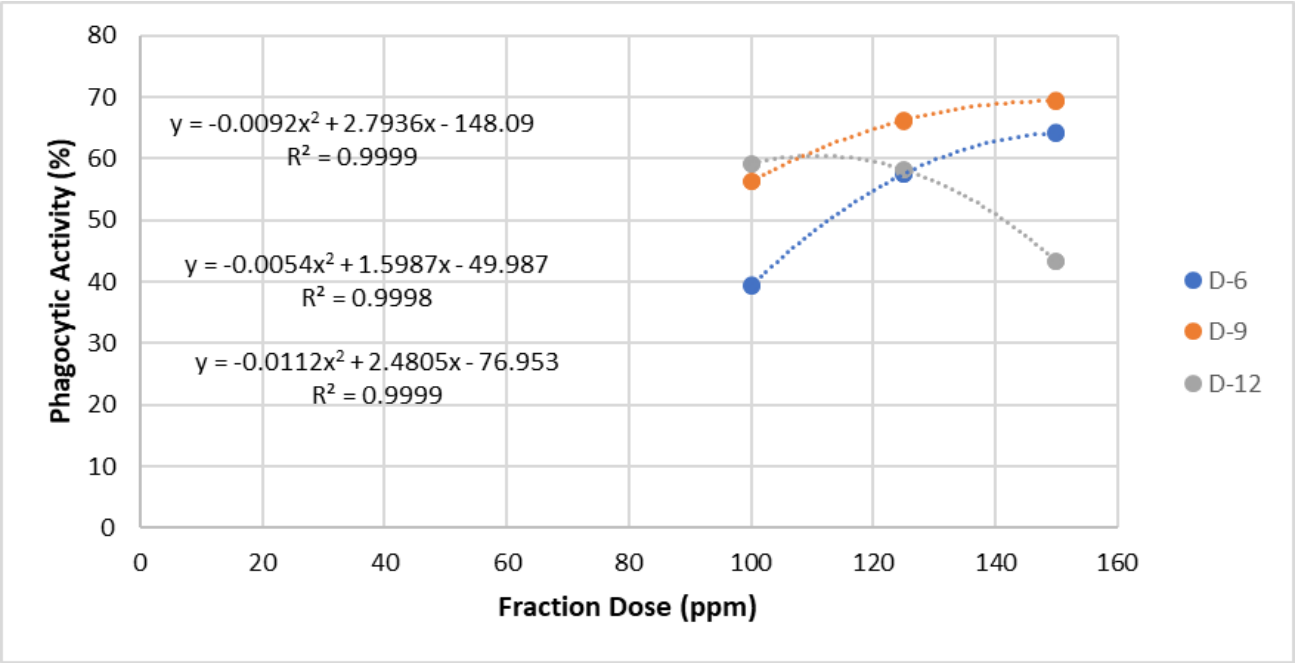


Figure 1. The dose-response relationship of guava leaf fraction five with total phagocytosis activity in tilapia from day 6 to 12.

Survival rate

The SR refers to the percentage of biota that remain alive after a specified period (Niode et al., 2017). According to Kuswanto et al. (2023), a survival rate over 50% is considered suitable, while rates from 30% to 50% are moderate, and those below 30% are regarded as poor. The survival rate is influenced by different biotic factors, including competition, parasites, age, predators, density, and human handling. In contrast, abiotic factors pertain to the physical and chemical characteristics of water. Key factors affecting fish survival include stocking density, feeding practices, disease prevalence, and water quality, which encompasses temperature, ammonia and nitrite levels, DO, and pH (Arzad et al., 2019). To ensure optimal survival rates and fish growth, it is crucial to provide food that meets the fish's nutritional needs. Feed lacking the necessary nutritional content for fish seed life is believed to compromise the physiological condition of the fish seeds. Insufficient feed nutrients can slow growth and negatively impact the survival of cultured biota (Sutedja et al., 2019). The survival rate during maintenance is illustrated in Figure 2. According to the SR graph, groups A, B, and C did not produce a pathogenic effect ($p > 0.05$), as the treatment values were nearly identical to those observed in the negative control group. In contrast, the positive control group achieved an infection rate of 39%. Thus, it can be concluded that tilapia treated with fraction five of guava leaf and infected with *A. hydrophila* experienced a higher infection rate ($p > 0.05$) compared to those treated with other fractions.

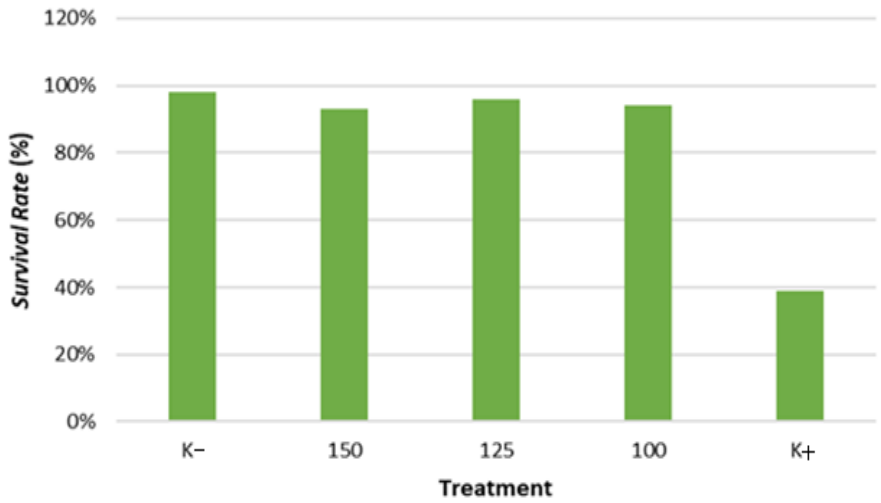


Figure 2. The survival rate of Tilapia (Oreochromis niloticus) during the 12-day study. K-: Negative control group, 150 ppm of guava leaf fraction (Group A), 125 ppm of guava leaf fraction (Group B), 100 ppm of guava leaf fraction (Group C), K+: Positive control group.

Water quality observations

Optimal water quality promotes a thriving environment for life, whereas poor water quality leads to the opposite effect. When water quality is not ideal, it can result in inadequate development (Scabra and Setyowati, 2019). During the

present study, water quality measurements were conducted, including temperature, pH, and DO, in the morning and evening for approximately one hour. Water changes were performed every three days, replacing up to 30% of the aquarium's volume. Data collected over a 12-day observation period indicated that the water quality parameters remained within optimal ranges, suggesting no adverse effects on the physiological condition of the tilapia. The results of the water quality measurements are presented in Table 4.

Table 4. Water quality parameter data during maintenance

Parameter	During maintenance	Standard range*
Temperature	25°C-27°C	25°C-30°C
pH	7-7.5	7-8
dissolved oxygen (DO)	6.5 mg/l-7 mg/l	5 mg/l-7 mg/l

***** Source of standard range: Indriati and Hafiludin (2022)

CONCLUSION

The present study identified an optimal guava leaf fraction dose of 175 ppm, with experimental doses at 150 ppm, 125 ppm, and 100 ppm. The LC_{50} calculations used doses of 125, 150, 175, 200, and 225 mg/kg fish body weight, based on inhibition tests against *Aeromonas hydrophila*. Eighteen fish per container were monitored for 24 to 72 hours, and the LC_{50} was determined from the initial mortality rates. The ideal dosage for enhancing non-specific immune responses in tilapia was found to be 150 ppm, which raised lymphocyte counts post-treatment, unlike the control group, which experienced a decline. By day three post-infection, treated fish demonstrated increased phagocytosis activity, indicating a strengthened immune response. Future research can investigate the long-term effects of guava leaf fractions on different fish species and aquatic environments.

DECLARATIONS

Acknowledgments

The authors would like to thank Professor Sri Andayani and Dr. Yunita Maimunah for their guidance in the present study and all parties involved in its implementation.

Authors' contributions

Lailatul Munawaroh Dewi Kusuma Wardani contributed to the data analysis and manuscript write-up. Sri Andayani and Yunita Maimunah contributed to writing the manuscript, as well as supervising the study. All authors read and approved the final edition of the manuscript.

Competing interests

The authors declared no conflict of interest.

Funding

This study did not receive any financial support.

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.

Availability of data and materials

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

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2025, Scienceline Publication
World's Veterinary Journal
World Vet J, 15(3): 622-628.
ISSN 2322-4568

DOI: https://dx.doi.org/10.54203/scil.2025.wvj62
PII: S232245682500062-15

Obtaining a Biometric Index for Juveniles and Adults of *Holothuria inornata* (Semper, 1868)

Miriam Guadalupe Zumaya-Higuera[1], Juan Manuel Pacheco-Vega[1]*, José Trinidad Nieto-Navarro[1], Alexia Omont[2], Erika Torres-Ochoa[3], and Julián Gamboa-Delgado[4]

[1]*Doctoral program on Biological, Agricultural Sciences, Fisheries Sciences Area, Academic unit of Agriculture, Nayarit Autonomous University, Km 9, Tepic-Compostela Highway, Xálisco, P.C. 63780, Nayarit, México*
[2]*Aquaculture Program, Northwest Center of Biological Research S.C. (CIBNOR) Av. Instituto Politécnico Nacional No. 195, Colonia Playa Palo de Santa Rita Sur, La Paz, P. C. 23096, Baja California Sur, México*
[3]*Department of Fisheries, Autonomous University of Baja California Sur, Road south Km 5.5, La Paz, P.C. 23080, Baja California Sur, México*
[4]*Mariculture Program, Faculty of Biological Sciences, Autonomous University of Nuevo León, Monterrey, P. C. 66450, Nuevo León, México*

*Corresponding author's Email: juan_pacheco@uan.edu.mx

ORIGINAL ARTICLE
Received: July 05, 2025
Revised: August 03, 2025
Accepted: August 31, 2025
Published: September 30, 2025

ABSTRACT

The sea cucumber *Holothuria inornata* (*H. inornata*) is a species at risk in Mexico. To help protect this species, it is important to provide information related to biological and fisheries management. The present study aimed to predict the weight of the sea cucumber (*H. inornata*) using biometric measurements such as wet weight and dry weight. A total of 267 *H. inornata* were manually collected from El Borrego Beach in the municipality of San Blas, Nayarit, Mexico. Species identification involved characterizing calcareous ladders. Each specimen was sedated using xylazine 10%, and measurements were taken out of water with a digital scale. Multiple linear regressions were performed, with size serving as the independent variable and wet weight, dry weight, and humidity percentage as dependent variables. A general equation was developed to predict the wet weight of the *H. inornata* at the juvenile stage. For correlations based on dry weight, the following equations were established. In immature adults, including dry weight, the equation was 0.0019 × (length^1.6479); and in mature adults, including dry weight, it was 0.0053 × (length^1.5362). The biometric parameters used for weight estimation exhibited a high degree of correlation with actual weight. Wet weight was the biometric parameter that showed the best fit among the studied parameters. Utilizing these biometric indices facilitates the recording of wet and dry weights for interested parties, thereby supporting population and biomass evaluations within the *H. inornata* fishery and aquaculture sectors.

Keywords: Biometric index, Dry weight, *Holothuria inornata*, Wet weight

INTRODUCTION

Sea cucumbers (Holothurians) have been harvested from Pacific islands for over 170 years (Purcell, 2014). Market prices exhibit considerable variation depending on factors such as species, product quality, size, and location (Perez and Brown, 2012). Among the twenty sea cucumber species sold in Hong Kong, China, *Holothuria inornata* (*H. inornata*) is the most valuable, with prices reaching 294 USD per kilogram (Purcell et al., 2025). Sea cucumbers are classified within the phylum *Echinodermata*, which represents one of the most diverse phyla within the animal kingdom (González-Navarro, 2012). The central Pacific region of Mexico and the Gulf of California demonstrated a significant diversity of echinoderms (Ríos-Jaras et al., 2013). Echinoderms are found in all the world's oceans and are mainly distributed along the seabed (Benthic) from the littoral to abyssal zones, with a particularly high abundance in coral reefs (Calcagno, 2014). Echinoderms play crucial roles in maintaining the structure and function of ecosystems (Alvarado and Chiriboga, 2008). Echinoderms possess a tegument consisting of a delicate cuticle and epidermis; beneath this lies the dermis. The dermis of holothurians is similar to that of other echinoderms in both structure and composition (Liu et al., 2025). The ossicles of the endoskeleton are tiny and embedded in the dermis, and they do not provide support in fish. Because ossicles are so diverse and detailed, they have taxonomic importance. The longitudinal muscle bands located beneath the dermis in the interambulacral zones are responsible for the body's stretching and contraction (Calcagno, 2014). Echinoderms' muscle bands or mutable collagenous tissues can rapidly change their mechanical properties, including elasticity and viscosity, within minutes under nervous system control (Siadat et al., 2021). The muscle tissues contain a large amount of extracellular matrix, mainly composed of collagen fibrils, proteoglycans, and microfibrils. The dermis of the holothurian body wall is a typical connective tissue that can quickly and reversibly change its mechanical properties in response to different stimuli (Tan et al., 2015). Therefore, an indirect method should be used to measure its weight.

Extensive studies on the dynamic mechanical properties of the sea cucumber (*Actinopyga mauritiana*; Quoy and Gaimard, 1833) dermis have demonstrated that the tissue can exist in at least three different states. These are rigid, standard, and soft states, which can be distinguished based on elastic and viscous properties as well as deformation-dependent behaviors. The mechanical parameters of the standard state are not just intermediate values between the stiff and soft states, suggesting that different mechanisms are responsible for transforming the soft state into the standard state and for converting the standard state into the rigid state (Yamada et al., 2010). The body weight of sea cucumbers

To cite this paper: Zumaya-Higuera MG, Pacheco-Vega JM, Nieto-Navarro JT, Omont A, Torres-Ochoa E, and Gamboa-Delgado J. (2025). Obtaining a Biometric Index for Juveniles and Adults of *Holothuria inornata* (Semper, 1868). *World Vet. J.*, 15(3): 622-628. DOI: https://dx.doi.org/10.54203/scil.2025.wvj62

exhibited significant variability attributable to differences in the water content within the respiratory tree and intestinal contents across different species. Liu et al. (2015) introduced a computational approach to estimate weight in *Apostichopus japonicus*, based on a method using r^2 correlation analysis, but only applicable to this species. Regarding *H. inornata*, the methods used include live biometric measurements such as weight and length, which can produce different results depending on the method applied. The precise method for collecting biometric data remains unclear, especially given the sea cucumber's ability to expel water, escape, or change its form or size. Numerous protocols are available for the biometric sampling of deceased organisms; however, there is no protocol nor a biometric index applicable to living organisms. Therefore, the present study aimed to conduct biometric sampling to establish correlations that facilitate the development of equations for estimating the live weight of juvenile, mature, and immature adult stages of *H. inornata* sea cucumbers based on their size.

MATERIALS AND METHODS

Ethical approval

Wild sea cucumber was collected with prior written permission (SPARN/DGVS/02181/24) from the Mexican Ministry of the Environment and Natural Resources (SEMARNAT). The samples were processed at the Wildlife Conservation Management Unit (UMA), Mexico, with registration number SEMARNAT, DGVS-UMA-IN-2025-NAY/22, which authorized research activities involving *H. inornata*. All relevant international, national, and institutional guidelines for animal care and use were adhered to authors.

Species identification

Initially, the verification of the species *H. inornata* was conducted through the analysis of external characteristics and the types of ossicles present in the tissue. For identification purposes, ten specimens were examined, comprising five juveniles and five adults. A 0.5 cm tissue fragment was carefully extracted from the mid-ventral region of each individual using a scalpel, serving as the basis for ossicle observation and identification. Each tissue sample was placed in a 15 mL test tube, and 5 mL of sodium hypochlorite 6% was added for tissue digestion. After five minutes, the specimens were prepared for microscopic examination using a compound microscope (Optika E-pL, Italy) equipped with a camera (P'Tkam B5, Italy), under which the ossicles were photographed. The identification of *H. inornata* was accomplished utilizing the taxonomic keys developed by the original description by Semper (1868), Solís-Marín et al. (2009), Santos-Beltrán and Salazar-Silva (2011), and Pacheco-Vega et al. (2022).

Biometrics

To find a correlation between biometric variables in *H. inornata*, sampling was conducted on different-sized organisms ranging from 10 to 255 mm, including juveniles, mature, and immature adults. A total of 267 *H. inornata* were collected from February 2023 to March 2024. The collection was performed through autonomous diving in sandy rocky areas at El Borrego beach in the municipality of San Blas, Nayarit, Mexico (Latitude 21.52545, Longitude 105.27849). A total of 267 *H. inornata*, with a minimum sample size of 157, were utilized, and biometrics were conducted. The measurements included length, measured from the mouth to the anus, and fresh weight, with classification into juveniles (Less than 15 cm) and adults (Over 15 cm; Tagliafico et al., 2011). The lengths were measured while the specimens were relaxed, without reacting to external stimuli, using a metric ruler (mm). Each specimen was then sedated with 1 mL of xylazine 10% (Aranda®, Mexico) for juveniles or 2 mL for adults, administered intramuscularly. After 30 minutes to ensure the anesthetic had fully taken effect, the procedure was then executed. This method had been previously standardized for the present study species.

Sea cucumber tissue processing

The sea cucumbers were removed from the tank, and excess water was eliminated using absorbent paper. Their weight and body mass were measured immediately thereafter (Less than one minute) employing a digital scale (Ohaus Scout® Pro, Germany). The analysis did not consider the water loss from the external surface area of the sea cucumbers. The methods employed to accurately determine the stages of maturity (Mature and immature) involved visual (Macroscopic) identification of the gonad maturity stage (Vitale et al., 2006). While the specimens were still sedated, they were sacrificed for gonadal separation according to the rules of the internal committee of the Autonomous University of Nayarit, Mexico, and following guidelines for animal slaughter. Each of the selected parts was placed in a forced convection oven (NOVATECH ®, HS45-AIA, Argentina) at 70°C for 48 hours (Rasul et al., 2022). The water content (%) was determined using the standard gravimetric method with the following formula.

$$\text{Humidity (\%)} = \left(\frac{(\text{Ww}-\text{Ws})}{\text{Wt}}\right) \times 100$$

Ww is the wet weight, Ws is the dry weight, and Wt means the sample weight.

Statistical analysis

A Shapiro-Wilk test for normality was conducted with a significance level of $p \geq 0.05$, along with a Kruskal-Wallis hypothesis test. The analysis focused only on the length, wet weight, and dry weight of each sampled organism, and the length-weight relationship was evaluated using a potential regression model ($Y = axn$). All statistical analyses were performed using Python software version 3.4.0 for Windows.

RESULTS

Based on the current observations and following the taxonomic keys, the specimens were identified as *H. inornata*. The collected specimen had a cylindrical body with scattered ambulacral feet on the dorsal surface. The dorsal surface was marked by small reddish papillae (Figure 1). The specimens had a table, a rod, and plate-shaped ossicles (Figure 2).
Based on the present findings from 128 juvenile and 139 adult *H. inornata* specimens, it appeared that the relationship between biometric variables may differ according to developmental and gonadal maturation stages (Table 1). The length-weight relationship helped create an equation that describes how parameters such as length, dry weight, and wet weight in *H. inornata* are related to their developmental stage. The biometric data for *H. inornata* indicated that using the equations is feasible for determining wet or dry weight from the measurement lengths.

The analysis of variance demonstrated statistically significant differences ($p < 0.05$) in at least one group that did not conform to a normal distribution. The current findings indicated significant differences ($p < 0.05$) in wet weight measurements among the groups (Immature, mature, and juvenile). The present data revealed that wet weight values in the mature group were significantly higher ($p < 0.05$) than those in the immature and juvenile groups. Additionally, wet weight values in the immature group were significantly higher ($p < 0.05$) than those in the juvenile group.

Table 1. Comparison of Pearson's and Spearman's coefficients of correlation for selected variables of sea cucumber with data collected from February 2023 to March 2024 in the El Borrego beach of San Blas, Nayarit, México

Base: Wet/dry	Development stage	Weight (g)	Wet weight (%)	Size (mm)	Equation	Correlation coefficients
Wet	Juveniles	$10.7^{c} \pm 9.3$	$86.2^{a} \pm 2.4$	$77.8^{c} \pm 27.5$	WW = 0.0016 × (talla^2.1664)	$r^2 = 0.84$
	Immature adult	$76.0^{b} \pm 30.8$	$84.0^{a} \pm 14.2$	$141.4^{b} \pm 37.7$	WW = 0.006 × (talla^1.8873)	$r^2 = 0.89$
	Mature adult	$171^{a} \pm 49.2$	$85.7^{a} \pm 10.3$	$199^{a} \pm 30.1$	WW = 0.5935 × (talla^1.0878)	$r^2 = 0.90$
Dry	Immature adult	$7.5^{b} \pm 4.2$	$84.0^{a} \pm 14.2$	$141.4^{b} \pm 37.7$	DW = 0.0019 × (talla^1.6479)	$R^2 = 0.76$
	Mature adult	$18.8^{a} \pm 7.9$	$85.7^{a} \pm 10.3$	$199.0^{a} \pm 30.1$	DW = 0.0053 × (talla^1.5362)	$r^2 = 0.90$

WW: Wet weight, DW: Dry weight, ^{abc} Different superscript letters within a column indicate statistically significant differences between base wet or dry groups ($p < 0.05$).

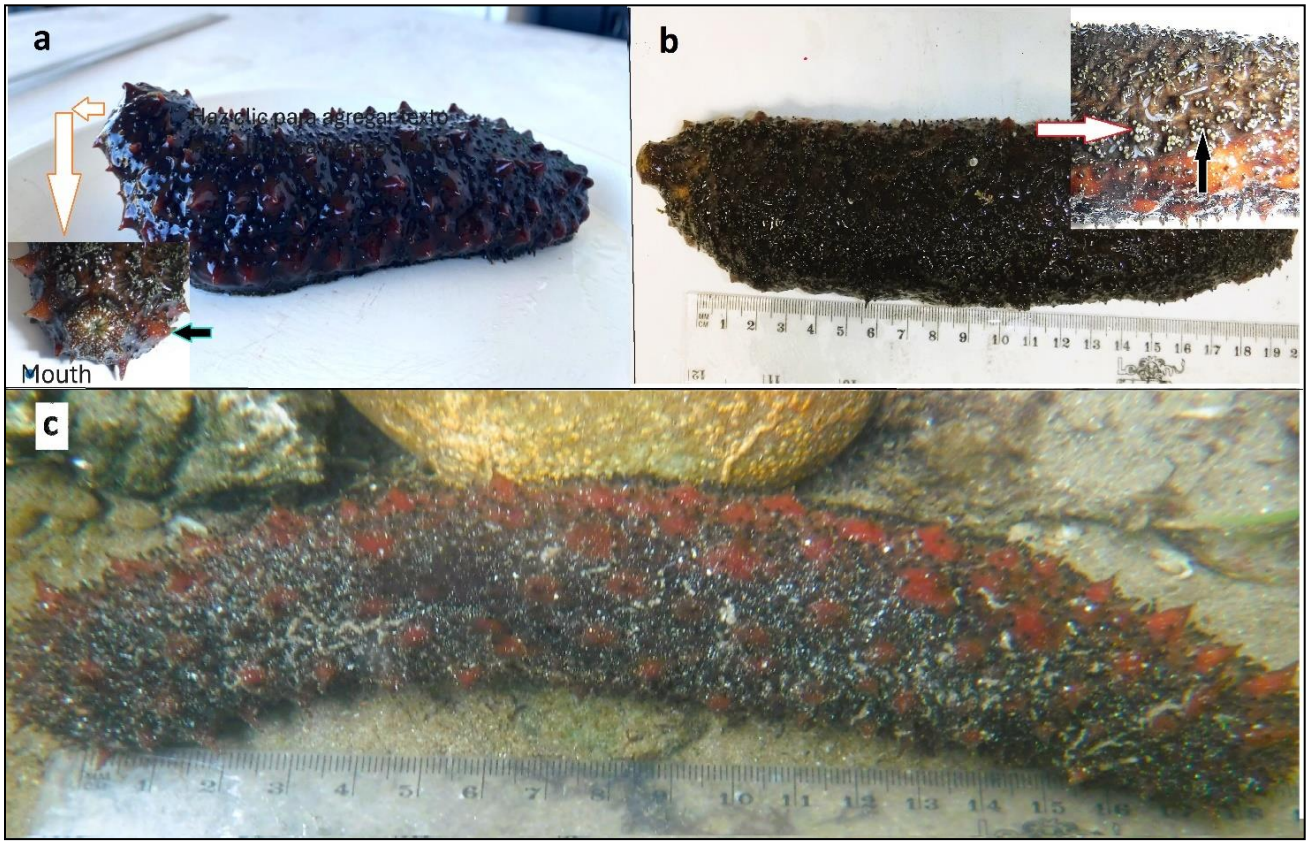


Figure 1. External morphology of sea cucumber *Holothuria inornata* (Semper, 1868). **a**: Dorsal region of the specimen with reddish conical papillae (black arrow) and circle-shaped tentacles externally bordering an area of the buccal membrane in the center of which the circular mouth is located. **b**: Ventral region with ambulacral feet, fairly of 3-5 mm in length. **c**: Adult sea cucumber.

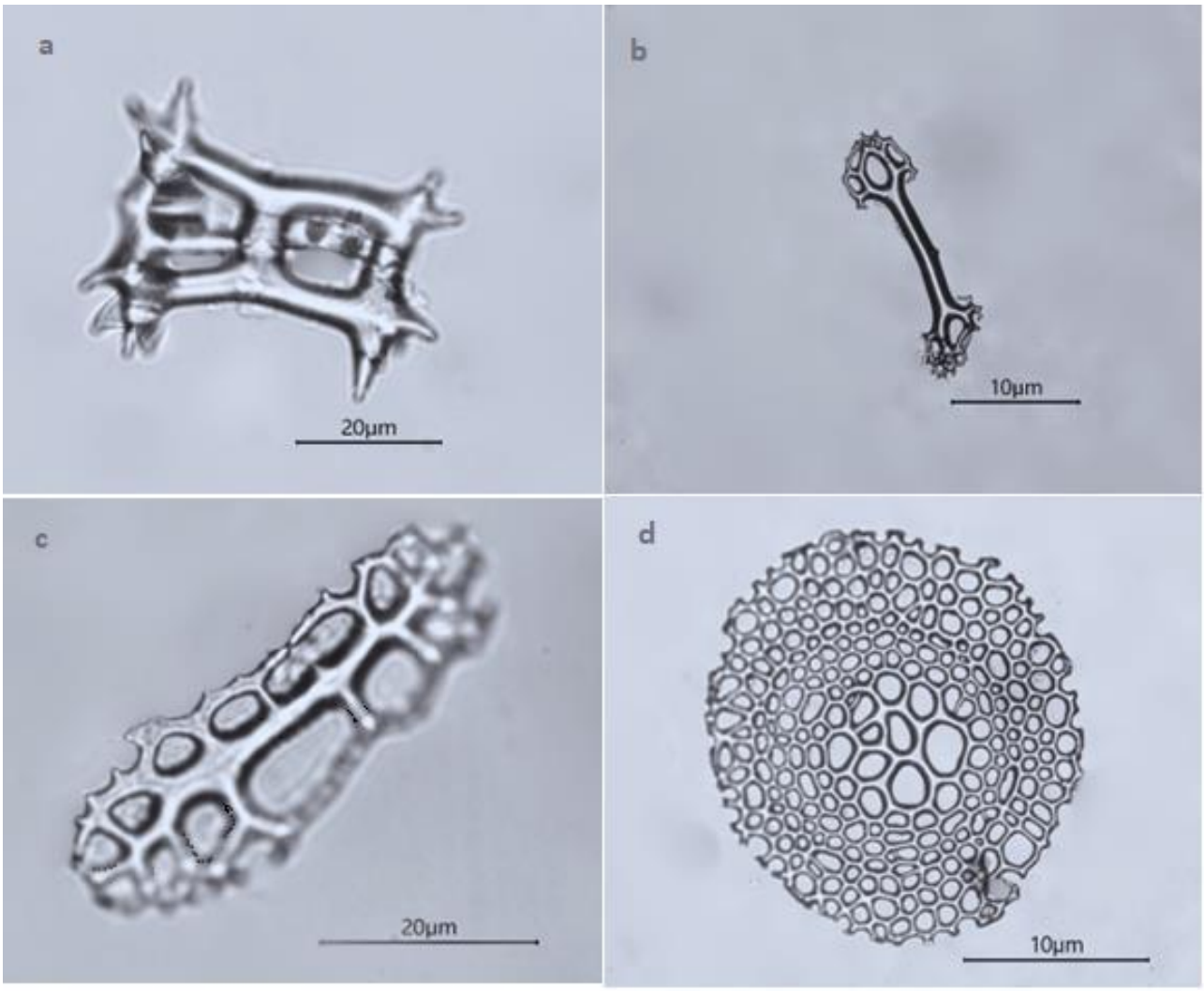


Figure 2. Micrographic images of ossicles observed in the adult stage of the sea cucumber. **a**: Table-shaped ossicles observed from the dorsal region. **b**: Rod-shaped and plate-shaped. **c**: Table-shaped ossicles. **d**: Perforated plate of ambulacral feet. The characteristics of external morphology and ossicles of *Holothuria inornata*.

DISCUSSION

The importance of biometric relationships lies in their ability to determine whether there is a correlated growth of the organism (Hammond and Purcell, 2024). Live weight across different sea cucumber species can be predicted using biometric data measurements. Hammond and Purcell (2024) highlighted the importance of biometric indices in fisheries and the findings related to *Pearsonothuria graeffei* (Semper, 1868), as these enabled the prediction of the *Pearsonothuria graeffei* length or weight based on morphometric variables. Additionally, Aydin (2019) indicated a low correlation of length and weight in *H. mammata* Grube, 1840, while Aydın (2020) reported negative allometric growth in *H. tubulosa Gmelin*, 1791, *Holothuria* (*Roweothuria*) *poli Delle Chiaje*, 1824, *H. mammata*, and *Holothuria* (*Platyperona*) *sanctori Delle Chiaje*, 1823. In previous studies, biometric data were not distinguished by development stage or gonadal maturation, which is important in species where fishing occurs, as it helps in establishing management plans that include closed seasons. According to the current results, the developmental stage (Juveniles and adults) and maturation stage exhibited differences in weight-length morphometric correlations.

In *H. inornata*, the variation in the correlation between weight and length may be linked to ontogenetic changes occurring from larval to adult stages. Additionally, biometric relationships in the sea cucumber *Cucumaria frondosa* are influenced by factors such as species, water temperature, salinity, food availability, light intensity, and substrate (Gianasi et al., 2018). Although *H. inornata* is both commercially valuable and endangered, no assessments have been conducted. This data insufficiency hinders the creation of more effective management strategies for the sea cucumber fishery in the Mexican Pacific (Herrero-Pérezrul and Chávez, 2024).

Generally, species within the Holothuroidea class demonstrated notable phenotypic plasticity, resulting in considerable variation in weight and length (Trenholm et al., 2024). This plasticity makes them sensitive to changes in the traits related to growth or gonadal maturity. The conventional method of conducting biometric assessments involves physically handling the specimen to determine its weight. Nonetheless, collecting this data from organisms such as *H. inornata* is challenging, as manipulation induces stress, prompting the organism to alter its size by depleting water and

releasing intestinal contents (Jobson et al., 2021). In addition, they species are considered to have negative allometric growth (Berrú-Paz and Ríos-Peña, 2020; Aydın, 2020). Therefore, a different equation was created for juveniles and adults. Liu et al. (2015) proposed utilizing photographic images to estimate body weight in *Apostichopus japonicus*, establishing a correlation coefficient of $r^2 = 0.96$ between length and weight, with no significant differences observed among organisms exhibiting varying degrees of gonadic development maturation. However, in the present study, the equation that described the correlation between length and weight in *H. inornata* changed due to the effect of their maturation stage. The impacts of environmental factors, including seasons, water temperature, and food availability, influence sexual maturation and the reproductive period in sea cucumbers (Hammond and Purcell, 2024), as well as the correlation between length and weight (Ramili et al., 2024). Consequently, an alternative equation should be employed depending on the respective reproductive periods.

CONCLUSION

Based on the current findings, it can be concluded that distinct length-weight indices should be employed for juveniles and adults. Additionally, the gonadic maturation stage in *H. inornata* affects biometric parameters; thus, a specific index corresponding to its reproductive stage should be used. Using these indices and their associated equations that define the length-weight relationship, accurate weight data can be derived from length for *H. inornata* juveniles, immature adults, and mature adults. These biometric indices will assist in recording wet and dry weights, supporting population and biomass assessments in the *H. inornata* fishery and its aquaculture. Based on the current insights, it is recommended to implement similar procedures for other sea cucumber species.

DECLARATIONS

Acknowledgments

The authors express their gratitude to the postgraduate program (Posgrado en Ciencias Biológico Agropecuarias-UAN) and extend their appreciation to CONACYT (Mexico) for the postgraduate scholarship. Mariana Díaz-Santana-Iturrios reviewed and refined the English language version.

Authors' contributions

Mirian Guadalupe Zumaya-Higuera and Alexa Omont collected specimens and processed the data. Juan Manuel Pacheco-Vega was the principal investigator of the study, wrote the manuscript, and posed the original idea. José Trinidad Nieto-Navarro critically reviewed the study. Erika Torres-Ochoa and Julian Gamboa-Delgado contributed to the literature review and interpretation of results. All authors contributed to the analysis of data, read and confirmed the final edition of the manuscript.

Funding

This study received partial support from the Environmental Management Unit (UMA), registered under number DGVS-UMA-IN-2025-NAY/22, which was awarded by the offices of the Ministry of Environment and Natural Resources (SEMARNAT) in Mexico, as well as from the Cooperative Societies of production Piripichi and Western Marine Gardens.

Competing interests

The authors have no conflict of interest to declare.

Ethical considerations

This paper was originally written by the authors and has not been published.

Availability of data and material

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

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2025, Scienceline Publication
World's Veterinary Journal
World Vet J, 15(3): 629-637.
ISSN 2322-4568

DOI: https://dx.doi.org/10.54203/scil.2025.wvj63
PII: S232245682500063-15

Application of Plant-Based Dyes for Acrosomal Cap Visualization in Cauda Epididymal Spermatozoa of Bali Cattle

Angelina Novita Tethool[1]*, Yoppy Rivaldo Kafiar[1], and Andoyo Supriyantono[2]

[1]*Physiology and Reproduction Laboratory, Faculty of Animal Science-Papua University, Manokwari, West Papua 98314, Indonesia*
[2]*Animal Breeding and Genetic Laboratory, Faculty of Animal Science-Papua University, Manokwari, West Papua 98314, Indonesia*

*Corresponding author's Email: a.tethool@unipa.ac.id

ORIGINAL ARTICLE
Received: June 29, 2025
Revised: August 13, 2025
Accepted: September 08, 2025
Published: September 25, 2025

ABSTRACT

The cauda epididymis contains mature spermatozoa capable of fertilization, with acrosomal integrity being a crucial factor in their function. Damage to the acrosome impairs the viability and fertilizing ability of spermatozoa. Conventional staining methods, such as Giemsa or fluorescence microscopy, are effective but less practical in low-resource settings. The present study aimed to evaluate damage to the acrosomal cap of cauda epididymal spermatozoa in Bali cattle and to assess the integrity of the acrosomal cap using Giemsa and natural dyes such as turmeric, beetroot, and areca nut extract. The present study utilized cauda epididymal spermatozoa from Bali cattle aged 3-4 years and weighing 316-412 kg. The study employed a completely randomized design with four treatments, each replicated 20 times. The treatments were Giemsa stain 100% (Control, P0), 10% Giemsa + 90% turmeric extract (P1), 10% Giemsa + 90% beetroot extract (P2), and 10% Giemsa + 90% areca nut extract (P3). Using Giemsa and natural dyes resulted in different colors of spermatozoa acrosomes. The P0 preparations exhibited a dark purple coloration, P1 appeared yellowish, corresponding to the turmeric pigment, and P3 appeared bluish, while P2 demonstrated no detectable staining on the slides. The acrosomal caps of spermatozoa in P0 were dark purple, those in P1 were yellowish, and those in P2 and P3 were unstained (Transparent or faint). The average acrosomal cap integrity was the highest in P0, followed in order by P1, P2, and P3. Based on the current findings, it can be concluded that P1, P2, and P3 signified acrosomal cap integrity in cauda epididymal spermatozoa of Bali cattle. However, P1 produced the best results, showing a distinguishable color and the highest acrosomal cap integrity score (86.9 ± 2.0%). Beetroot and areca nut extracts produced weaker staining and lower acrosomal integrity values. Therefore, turmeric extract may serve as a practical, cost-effective, and environmentally friendly alternative for acrosome evaluation in veterinary reproductive laboratories, particularly in resource-limited settings.

Keywords: Giemsa, Intact acrosomal cap, Natural dye, Spermatozoa

INTRODUCTION

Slaughterhouse operations involving cattle not only produce meat but also generate several by-products, including reproductive organs, which are often discarded as waste. The waste products generated in slaughterhouses include the testis and epididymis, which represent reproductive tissues with untapped potential, particularly as sources of viable spermatozoa that remain functional for 24 to 48 hours when stored appropriately in a refrigerator at 5°C (Abu et al., 2016). The epididymis is anatomically divided into three regions, including caput, corpus, and cauda, with the cauda epididymis serving as the primary reservoir for mature spermatozoa before ejaculation (James et al., 2020). The success of fertilization within the female reproductive tract is heavily dependent on the quality of the spermatozoa, particularly the structural and functional integrity of the acrosomal cap (He et al, 2022). The acrosome, which overlays the spermatozoa nucleus, contains hydrolytic enzymes such as hyaluronidase and acrosin, which are essential for penetrating the zona pellucida during fertilization (He et al., 2022). Preservation of acrosomal integrity is vital; damage to the acrosomal spermatozoa structure impairs spermatozoa function and may compromise fertilization, ultimately affecting conception rates (Sun et al., 2020).

Acrosomal damage may result from alterations in the lipid composition of the spermatozoa membrane, disrupting its stability and increasing susceptibility to structural degeneration (Tethool et al., 2022). Moreover, defects in acrosome formation may arise from abnormalities in spermatogenesis, particularly due to the failure of the Golgi apparatus in spermatids to produce a functional acrosome. For successful fertilization, spermatozoa should possess an intact acrosome capable of undergoing a timely acrosome reaction, which facilitates the release of enzymes such as acrosin, hyaluronidase, and corona-penetrating enzymes, thereby enabling spermatozoa penetration through the zona pellucida (Khan et al., 2024). Assessment of acrosomal integrity is commonly performed using the chlortetracycline fluorescence

To cite this paper: Tethool AN, Kafiar YP, and Supriyantono A (2025). Application of Plant-Based Dyes for Acrosomal Cap Visualization in Cauda Epididymal Spermatozoa of Bali Cattle. *World Vet. J.*, 15(3): 629-637. DOI: https://dx.doi.org/10.54203/scil.2025.wvj63

staining technique (Baňas et al., 2024). While effective, the evaluation of acrosomal integrity using chlortetracycline fluorescence requires advanced laboratory equipment, such as a fluorescence microscope, and is therefore less suitable for routine or field-based applications (Kumar et al., 2019). Giemsa staining is a widely used cytological technique for visualizing cellular components, including the acrosomal structure. While Giemsa is considered a reliable agent for assessing acrosomal integrity, its application may be constrained by high costs and limited availability, particularly in remote or resource-limited laboratory settings (Kútvölgyi et al., 2006). The limitations associated with Giemsa staining have encouraged the search for alternative staining solutions utilizing natural pigments that are more accessible, cost-effective, and environmentally sustainable (Chowdhury et al., 2014; Alegbe and Uthman, 2024).

Natural dyes such as those extracted from turmeric (*Curcuma longa*), areca nut (*Areca catechu*), and beetroot (*Beta vulgaris*) have demonstrated potential as biological stains (Nirmala and Puspitasari, 2017; Obeta et al., 2022). Turmeric contains curcuminoids, primarily curcumin, desmethoxycurcumin, and bisdemethoxycurcumin, which provide an intense yellow pigment and have been used to stain plant tissues effectively (Amalraj et al., 2017; Manasa et al., 2023). Areca nut is rich in flavonoid pigments that impart a deep orange color, and its extract has yielded clear and contrasting tissue staining results (Sharan et al., 2012). Beetroot contains betalain pigments, specifically betacyanin and betaxanthins, which offer vibrant red to purple coloration and possess potent antioxidant properties, including high vitamin C content (Liliana and Oana-Viorela, 2020). Given their biochemical properties and natural pigment content, these plant-based extracts have the potential to serve as alternative staining agents for acrosomal evaluation. The application of natural dye extracts could facilitate the development of simpler, safer, and more sustainable staining protocols that are better suited for use in veterinary laboratories, particularly in resource-limited settings (Alegbe and Uthman, 2024). The present study aimed to evaluate the effectiveness of turmeric, areca nut, and beetroot as natural dyes in identifying the acrosomal cap and assessing the acrosomal integrity of cauda epididymal spermatozoa in Bali cattle, to establish a more practical and environmentally friendly alternative to conventional staining methods such as Giemsa.

MATERIALS AND METHODS

Ethical approval

All procedures carried out in the present study complied with the operational protocols of the slaughterhouse located in Manokwari Regency, West Papua, Indonesia. The study was conducted with official research approval under permit number 061/UN42.3/PP/2023. Cauda epididymal tissues were obtained following the completion of the slaughter process.

Animals and materials

The present study utilized cauda epididymal spermatozoa from Bali cattle aged 3-4 years and weighing 316-412 kg. Cauda epididymal samples were obtained from 20 Balinese cattle slaughtered at the slaughterhouse in Manokwari Regency, West Papua, Indonesia. The cauda epididymis was placed in a thermos containing warm water (30-40 °C) and immediately transported to the laboratory for further observation. The materials used included Giemsa stain (Merck Millipore, Germany), methanol 70% (Merck Millipore, Germany), phosphate buffer (Bioenno Tech, USA), ethanol 90% (Merck Millipore, Germany), eosin-nigrosin (Merck Millipore, Germany), distilled water, and warm water. Natural staining agents were derived from turmeric (*Curcuma xanthorrhiza* Roxb.), areca nut (*Areca catechu* L.), and beetroot (*Beta vulgaris* L.; Figure 1). Each plant material, weighing ± 500 grams, was procured from local sources in Manokwari, West Papua, Indonesia.



Figure 1. The used plants as natural dyes for acrosomal cap visualization in cauda epididymal spermatozoa of Bali cattle. **A**: Turmeric (*Curcuma xanthorrhiza* Roxb), **B**: Areca nut (*Areca catechu* L), and **C**: Beetroot (*Beta vulgaris* L.).

Experimental design

An experimental study was conducted using a completely randomized design with 20 Bali cattle aged 3-4 years. The study employed four treatments, each replicated 20 times, including 100% Giemsa stain solution (Control, P0), 10% Giemsa stain solution + 90% turmeric extract (P1), Giemsa stain solution 10% + beetroot extract 90% (P2), and Giemsa stain solution 10% + areca nut extract 90% (P3). The percentage of plant extract used was based on the studies of Hartika et al. (2021), Shankar et al. (2022), and Mulia et al. (2022), which demonstrated that the optimal staining for each spermatozoa preparation using plant-based dyes was achieved with a composition of 90% natural extract combined with 10% Giemsa solution. This percentage was applied to facilitate the preparation of the staining solution, with a total volume of 200 mL composed of 10% Giemsa staining solution and 90% of each respective natural dye.

Preparation of staining solutions

Giemsa solution

The Giemsa staining solution was prepared by slowly adding 3 mL of Giemsa to a standard phosphate buffer solution, followed by the addition of 35 mL of distilled water. The mixture was stirred until homogenized (Tethool et al., 2022).

Natural dye extracts

The natural dye solutions were prepared based on preliminary trials conducted by the study of Tethool et al. (2022) using the following steps. Initially, fresh turmeric and beetroot (each 500 grams) were peeled, washed, and cut into small pieces. Then, each was ground using a chopper, and the juice was extracted. A total of 20 mL of each extract was mixed with 10 mL of methanol, 10 mL of phosphate buffer, and 35 mL of distilled water. The areca nuts were crushed, added to 100 mL of distilled water, and heated. A 30 mL aliquot of areca nut extract was then combined with 10 mL phosphate buffer and 30 mL distilled water. The extraction process of each natural material resulted in a final volume of ± 40 mL.

Evaluation of cauda epididymal spermatozoa

Spermatozoa motility

To evaluate motility (%), a 5 µL aliquot of spermatozoa from the cauda epididymis was dispensed onto a microscope slide, followed by the addition of one drop of sodium chloride solution (Widarta Bhakti, Indonesia). The preparation was covered with a coverslip and examined under a light microscope (Olympus CX 31, Japan) at 400x magnification (Gustina et al., 2022).

Spermatozoa concentration

A volume of 10 µL semen was mixed with 990 µL of sodium chloride solution in a microtube to achieve dilution. From the semen and sodium chloride mixture, 10 µL was drawn and placed into both chambers of a hemocytometer (Marienfeld, Germany). Spermatozoa concentration ($x10^7$/mL) was determined by averaging the counts from two separate grid areas (Santoso et al., 2021; Cipriani et al., 2023).

Spermatozoa viability

Assessment of spermatozoa viability (%) was carried out using eosin-nigrosin staining. A 5 µL semen sample was combined with 20 µL of eosin-nigrosin solution on a glass slide, mixed evenly, and then smeared into a thin layer for evaluation (Tethool et al., 2022). Spermatozoa viability assessment was performed using a microscope (Olympus CX 31, Japan) set at 400x magnification.

Spermatozoa abnormality

A thin smear was prepared using eosin-nigrosin-stained semen and examined under a microscope (Olympus CX 31, Japan) at 400x magnification. Spermatozoa abnormalities (%) were calculated as the percentage of abnormal spermatozoa from a total of 200 spermatozoa, including abnormalities of the head and tail (Suhardi et al., 2020).

Acrosomal integrity

Spermatozoa were collected from the dissected cauda epididymis, smeared onto glass slides, and air-dried for approximately five minutes. Slides were fixed in formalin 5% (Merck, Germany) for 30 minutes at 37°C, rinsed with distilled water, air-dried again, and then immersed in Giemsa stain for 4 hours at 37°C (Chowdhury et al., 2014; Prihantoko et al., 2020).

Statistical analysis

All data were analyzed using SPSS software version 25.0 (IBM). A one-way ANOVA was performed to assess differences among treatments, followed by Duncan's post hoc test. Statistical significance was set at a p-value less than 0.05 ($p < 0.05$).

RESULTS AND DISCUSSION

Color characteristics of spermatozoa stained by natural dyes

Samples that were stained using Giemsa and natural dyes exhibited distinct coloration (Figure 2). The control group (P0) stained with Giemsa appeared dark purple (Figure 2A), while preparations treated with turmeric extract (P1) were yellowish, consistent with the natural pigment content of turmeric (Figure 2B). Group P2, which used beetroot extract, demonstrated no detectable coloration (Figure 2C), while P3 preparation appeared bluish (Figure 2D). According to Nemzer et al. (2011), beetroot contains the red pigment betacyanin; however, in the present study, the combination of beetroot extract and Giemsa resulted in a diluted base color, namely red. Furthermore, the concentration of beetroot extract used was relatively low, approximately 31 mL, leading to a translucent appearance. The coloration observed in each preparation, such as turmeric extract stain (P1), beetroot extract stain (P2), and areca nut stain (P3), reflected the presence of active compounds in the dye materials. The yellowish tint in P1 is attributable to curcuminoids in turmeric (Ciuca and Racovita, 2023). These phenolic compounds, comprising curcumin, monodesmethoxycurcumin, and bisdemethoxycurcumin, are responsible for the yellow staining (Basha et al., 2024).

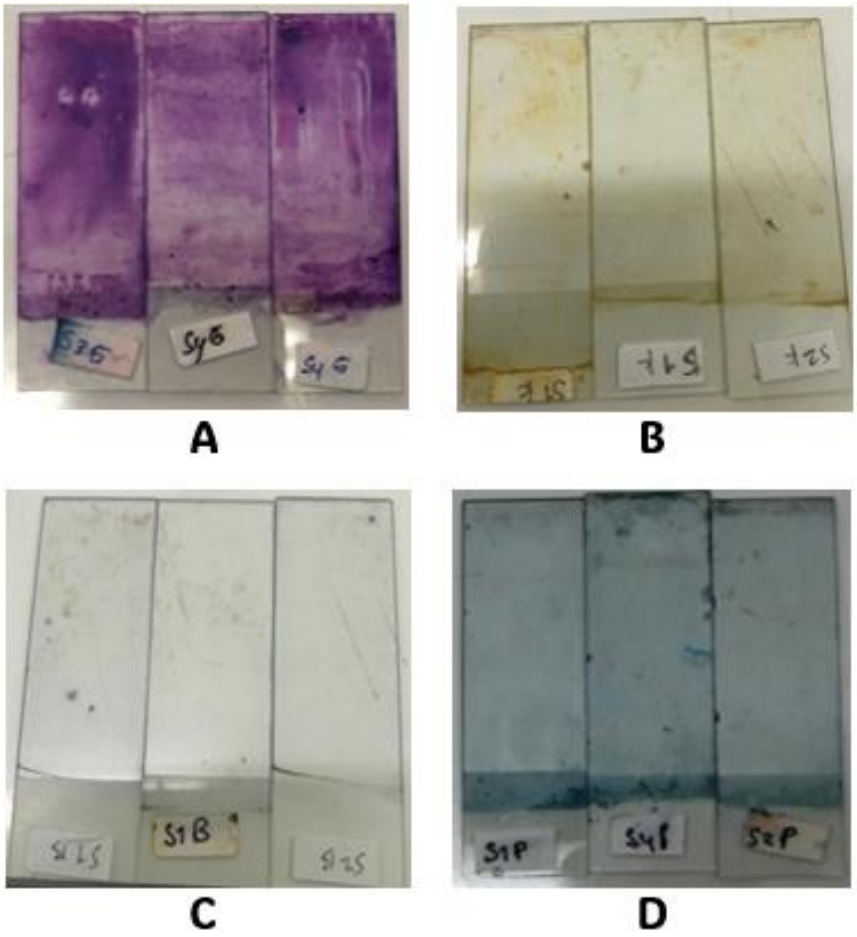


Figure 2. Staining of a 3-4-year-old Bali cattle sperm using natural dyes. **A**: Giemsa staining, **B**: Turmeric extract staining, **C**: Beetroot extract staining, and **D**: Areca nut extract staining.

According to Rautela et al. (2020), preparations stained with Giemsa for acrosomal cap evaluation showed a similar purple color as observed in the present study (Figure 2A). Preparation P3 exhibited a bluish hue, which contrasts with the findings of Ati et al. (2010), who reported that areca nut pigments (Flavonoids) produce an orange coloration. The observed difference is presumably due to the dominant staining effect of Giemsa, which overrides the natural pigment of areca nut. Additionally, preparations stained with beetroot extract demonstrated only a faint and transparent coloration. Acrosomal cap coloration varied among treatments in P0 and P1 (Figure 3A and 3B). Preparations stained with Giemsa (P0) produced a clearly defined purple acrosomal cap (Figure 3A), as Giemsa binds to membrane proteins of the sperm (Prihantoko et al., 2020). Spermatozoa in preparation P1 exhibited a yellowish acrosomal cap (Figure 3B), resulting from the turmeric extract. The acrosomal cap was distinctly visible when stained with turmeric dye in P1 treatment. In contrast, P2 and P3 produced pale or transparent caps (Figures 3C and 3D), indicating possible acrosomal damage. This

is supported by Tethool et al. (2022), who noted that damage to the spermatozoa acrosome results in faded or pale staining.

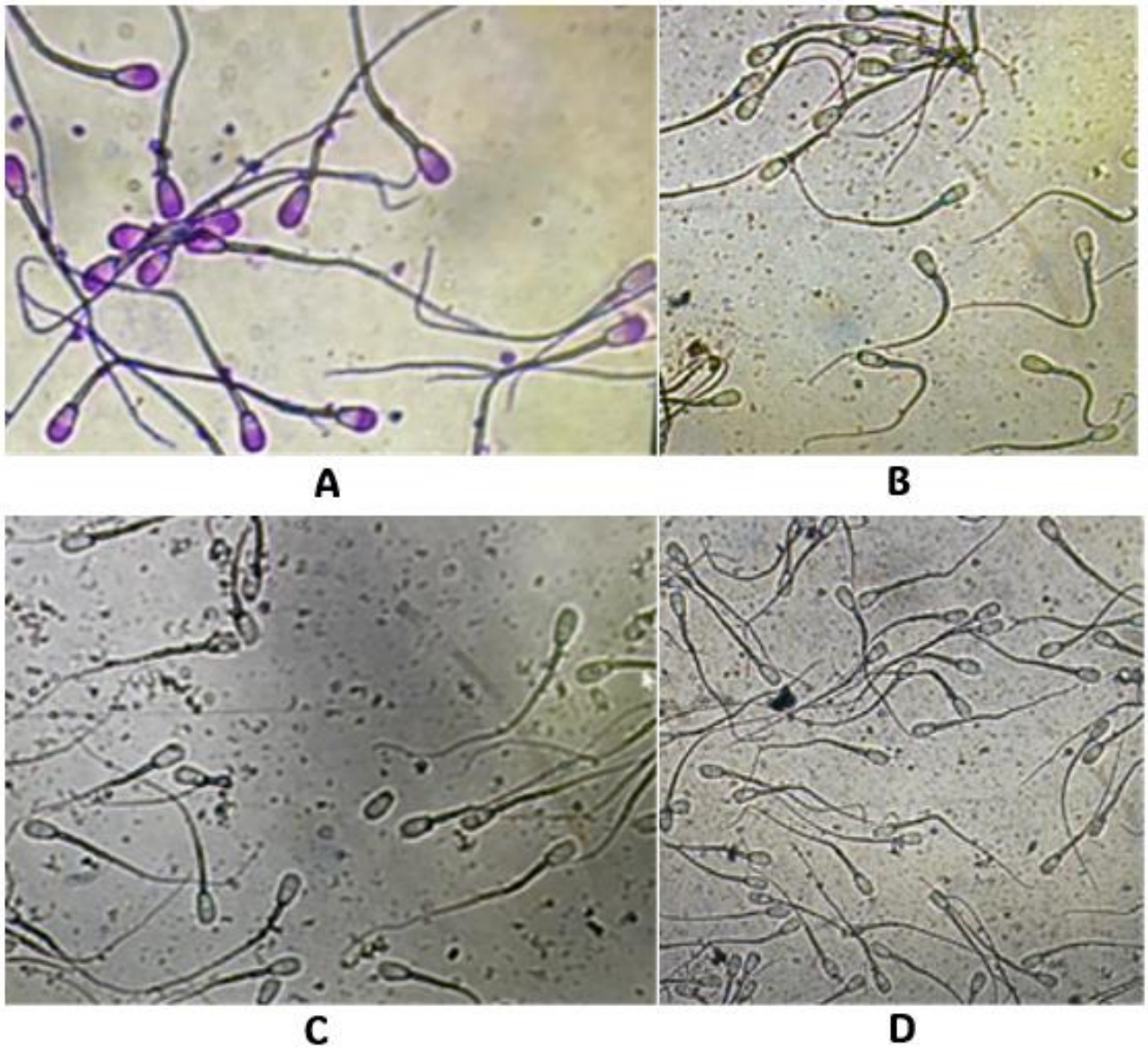


Figure 3. Acrosomal cap staining of a 3-4-year-old Bali cattle spermatozoa using natural dyes. **A**: Giemsa staining, **B**: Turmeric extract staining, **C**: Beetroot extract staining, **D**: Areca nut staining.

Integrity value of the spermatozoa acrosomal cap

An intact acrosomal cap plays a vital role in spermatozoa functionality, as it reflects their competence to recognize and adhere to the zona pellucida of the oocyte, trigger the acrosome reaction, and facilitate the enzymatic processes required for fertilization (Leung et al., 2023). Fertilizing capability is influenced by parameters such as motility, membrane integrity, and acrosome status (Bernecic et al., 2021). The acrosome plays a critical role in fertilization, as its interaction with the zona pellucida triggers the release of enzymes such as acrosin, hyaluronidase, and corona-penetrating enzymes, allowing spermatozoa to penetrate (Hirohashi and Yanagimachi, 2018).

The percentage of intact acrosomal caps detected with natural stains showed a significant difference between P1 and P3 ($p < 0.05$). Among all treatment groups, the Giemsa-stained group (P0) indicated the highest mean percentage ($87.4 \pm 2.7\%$), with a slightly lower value observed in P1 ($86.9 \pm 2.0\%$). In contrast, the mean percentages in P2 and P3 were comparatively lower, at $84.5 \pm 5.6\%$ and $84.4 \pm 4.3\%$, respectively (Figure 4). Giemsa is known to have a strong affinity for proteins, enabling it to stain the acrosome of spermatozoa effectively. The specific proteins, such as serine protease, the acrosin-binding protein (Sp32), and β-galactosidase (Nadgas et al., 2016), in the spermatozoa membrane bind to the dye, enabling clear visualization and differentiation. The acrosome, located at the anterior portion of the spermatozoa head, is essential as it contains key enzymes required for oocyte penetration during fertilization. Intact acrosomal membranes are crucial for maintaining the fertilization potential of spermatozoa (Fannessia et al., 2015).

Damage to the acrosome, particularly to its membrane, can compromise fertilization capability, reduce motility, and lead to cell death.

A cost-effective and straightforward method to evaluate acrosomal integrity involves Giemsa staining (Kumar et al., 2018). In spermatozoa with intact acrosomes, Giemsa produces a more intense (Dark purple) staining in the acrosomal region, indicating membrane integrity and preserved protein structure. Conversely, spermatozoa with damaged acrosomes exhibit fainter or unclear staining (Light purple), reflecting compromised membrane integrity (Prihantoko et al., 2020). In contrast, the staining ability of natural dyes is influenced by their varied chemical composition, such as carbohydrates, proteins, minerals, essential oils (Phellandrene, sabine, cineol, borneol, zingiberene, and sesquiterpenes; Athala, 2021; Hartika et al., 2021). In cases of acrosomal damage, staining efficiency may be compromised, leading to weak or inconsistent coloration. Additionally, morphological abnormalities, particularly in the spermatozoa head region, can indicate disruptions in the plasma membrane and acrosomal integrity (Esteves, 2007).

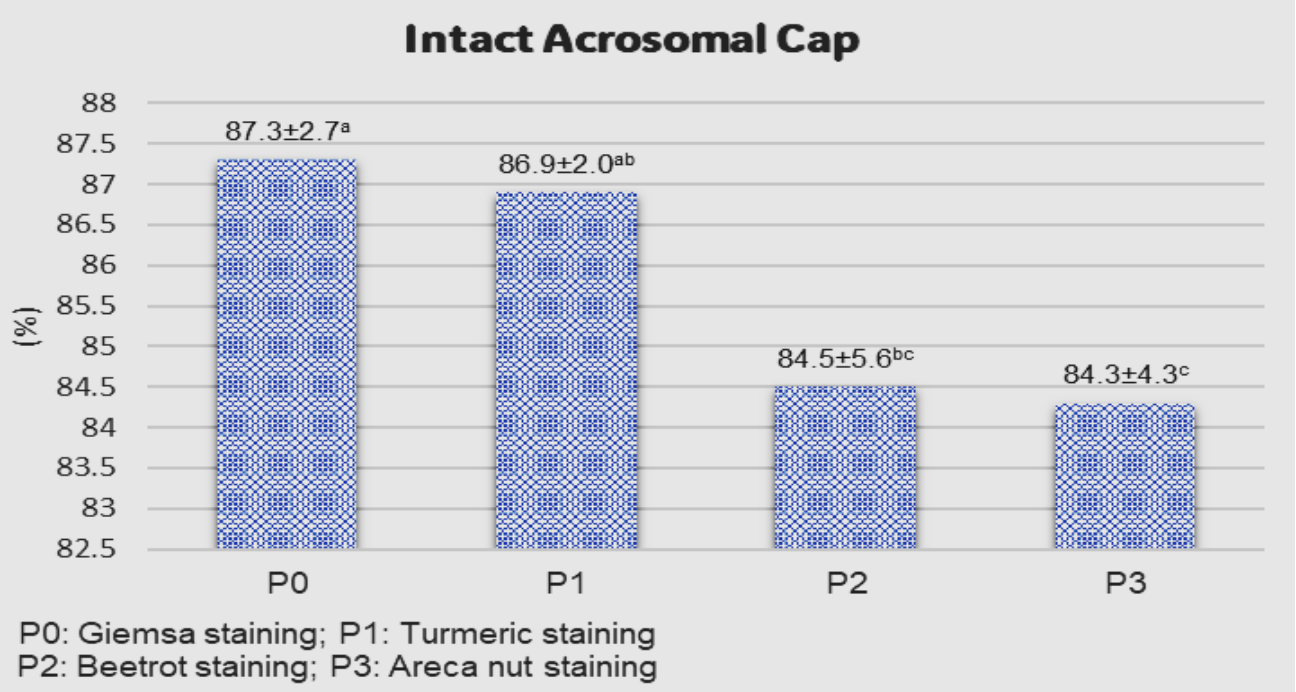


Figure 4. The percentage of intact acrosomal caps in Bali cattle (3-4 years old) across different treatments. [a,b,c] Different superscript letters indicate significant differences ($p < 0.05$) among treatments.

Characteristics of cauda epididymal Bali cattle spermatozoa

The characteristics of Bali cattle cauda epididymal spermatozoa observed in the present study included motility, concentration, viability, and abnormality (Table 1). These parameters are crucial for successful fertilization (Tanga et al., 2021).

Table 1. Characteristics of Bali cattle cauda epididymal spermatozoa aged 3-4-year-old

No	Parameter	Value
1.	Motility (%)	69.0 ± 0.02
2.	Concentration (x10^7/mL)	386.0 ± 19.61
3.	Viability (%)	86.1 ± 2.08
4.	Abnormality (%)	14.12 ± 2.13

According to the findings of Rombe et al. (2023), three-year-old Bali cattle demonstrated a higher spermatozoa concentration from all experimental groups before the application of natural dye treatment ($p < 0.05$) compared to younger (2-2.5 years old) bulls, indicating that age influences spermatozoa concentration. Motility is a common indicator of fertility in males (Santoso et al., 2021). The observed motility represented the overall motility value across all experimental groups before the application of natural dyes. The motility value in all experimental groups before the application of natural dye treatments was 69.0 ± 0.02%, slightly lower than that documented by Rombe et al. (2023), which was 71.2 ± 6.2%, and Wattimena et al. (2009), which was 75% ($p < 0.05$). This discrepancy may be due to differences in age, energy storage (ATP), and spermatogenesis maturity (James et al., 2020; Park and Pang, 2021).

Higher spermatozoa concentration correlates with higher conception rates (Keihani et al., 2021). The present study reported that the spermatozoa concentration of all experimental groups before the application of natural dye treatments was 386.0 ± 19.61 × 10^7/mL, which was higher than 310.1 ± 60.7 × 10^7/mL reported by Rombe et al. (2023; $p < 0.05$), likely due to differences in rearing systems and feeding (Kudratullah et al., 2024). Viability, indicating the percentage of live spermatozoa post-dilution (Komariah et al., 2023). In all experimental groups, before the application of natural dye treatment, the percentage of cells was recorded at 86.1 ± 2.08%, lower than the 95.9 ± 1.1% reported by Rombe et al. (2023; $p < 0.05$). These differences may result from variation in the collection time of the cauda epididymal and cattle type. Early collection post-slaughter yields better sperm quality (Sarsaifi et al., 2013; Harissatria et al., 2018; James et al., 2020). Live spermatozoa exhibited clear, unstained heads, whereas nonviable ones absorbed dye and appeared purple in all groups, consistent with Ducha (2012) and Ugur et al. (2019), who reported that compromised membrane permeability allows dye penetration. Spermatozoa abnormalities in all groups before the application of natural dye treatments were lower (14.12 ± 2.13%) than those reported by Rombe et al. (2023), at 20.6 ± 8.8% ($p < 0.05$). The low level of spermatozoa abnormalities may be due to reduced cold shock and osmotic imbalance, which cause structural damage (Gustina et al., 2023). Abnormalities can arise from primary factors, such as age, physical condition, and breed, or secondary factors, including handling and technical errors during laboratory procedures (Harissatria et al., 2018).

CONCLUSION

The combination of Giemsa with turmeric extract, beetroot extract, and areca nut extract was capable of identifying intact acrosomal caps in cauda epididymal spermatozoa of Bali cattle aged 3-4 years old. However, Giemsa combined with turmeric extract produced the most promising results, as reflected by the highest percentage of intact acrosomal caps (86.9 ± 2.0%) and clear staining outcomes. The Giemsa preparations exhibited a dark purple coloration, turmeric extract appeared yellowish, corresponding to the turmeric pigment, and areca nut extract appeared bluish, while beetroot extract demonstrated no detectable staining on the slides. Therefore, turmeric extract may serve as a practical, cost-effective, and environmentally friendly alternative for acrosome evaluation in veterinary reproductive laboratories, particularly in resource-limited settings. Further investigations are necessary to evaluate the effectiveness of turmeric extract in assessing acrosomal integrity in spermatozoa from different livestock species, as well as under different semen preservation conditions, including fresh, diluted, and cryopreserved samples. In addition, the biochemical interactions between turmeric pigments and sperm cell components should be explored to elucidate the underlying staining mechanism.

DECLARATIONS

Acknowledgments

The authors sincerely acknowledge the Department of Agriculture and Food Security, Manokwari Regency, Papua Barat, Indonesia, for the permission to collect research samples at the slaughterhouse, and extend deep appreciation to the Reproductive Physiology Laboratory Team, Faculty of Animal Science, University of Papua, Manokwari, Indonesia, for their technical assistance and support.

Authors' contributions

Angelina Novita Tethool, Yoppy Rivaldo Kafiar, and Andoyo Supriyantono conceived and designed the experiment. Angelina Novita Tethool and Yoppy Rivaldo Kafiar performed the laboratory experiments. Angelina Novita Tethool, Yoppy Rivaldo Kafiar, and Andoyo Supriyantono analyzed the data, interpreted the findings, drafted the manuscript, and revised the article. All authors have read and approved the final edition of the manuscript for submission to the journal.

Funding

The present study did not receive any financial support.

Ethical considerations

The authors affirmed that the present manuscript is their original work and has not been previously published. All authors have reviewed the content for plagiarism and verified that it reflects their own scientific findings.

Availability of data and materials

All data generated during this study are included in this article. Additional information is available from the authors upon reasonable request.

Competing interests

The authors declared no competing interests.

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2025, Scienceline Publication
World's Veterinary Journal
World Vet J, 15(3): 638-646.
ISSN 2322-4568

DOI: https://dx.doi.org/10.54203/scil.2025.wvj64
PII: S232245682500064-15

Molecular Characterization of the *Forficula tomis* based on the *Cytochrome C Oxidase I* Gene

Victory Vladimirovna Stolbova, Vladislava Ruslanovna Garbaly, Kseniya Sergeevna Krestonoshina, and Zimfira Yakubovna Zinatullina*

All-Russian Scientific Research Institute of Veterinary Entomology and Arachnology, Branch of Federal State Institution Federal Research Centre Tyumen Scientific Centre of Siberian Branch of the Russian Academy of Sciences (ASRIVEA – Branch of Tyumen Scientific Centre SB RAS) Institutskaya st. 2, Tyumen, 625041, Russian Federation, Russia

*Corresponding author's Email: zimfirazi@mail.ru

ORIGINAL ARTICLE
Received: July 06, 2025
Revised: August 08, 2025
Accepted: September 02, 2025
Published: September 25, 2025

ABSTRACT

The ovine Earwigs of the genus *Forficula* play a significant role in agriculture and beekeeping, acting as pests and vectors of honey bee pathogens. Despite the ecological and agricultural significance, nucleotide sequences of the species *Forficula tomis* (*F. tomis*) are absent in genetic databases, which complicates its molecular identification. The present study aimed to perform the molecular characterization of the earwig *F. tomis* using the *cytochrome c oxidase I* (*COI*) gene fragment as a molecular marker. A total of 18 earwig specimens were hand-collected from *Apis mellifera* hives in the Tyumen region, Russia. During the cultivation, 9 adults were obtained, which were used in the present study. Morphological analysis confirmed that *F. tomis* is the predominant species of bee hives in the Tyumen region. Sequencing of the *COI* gene fragment made it possible to obtain a reference sequence of *F. tomis*, which, in phylogenetic analysis, formed a separate clade and demonstrated a significant genetic distance (up to 22.9%) with other representatives of the genus *Forficula*. The optimal species differentiation threshold for the group was 11.7%, which significantly exceeds the standard barcode gap (3%) and may indicate long-term evolutionary isolation or the presence of cryptic diversity. The obtained results expand the present understanding of the genetic structure of the genus *Forficula* and have practical significance for monitoring apiary pests, developing methods for molecular identification of bee pests, and understanding their role in the spread of pathogens.

Keywords: Beekeeping, C*ytochrome c oxidase I* gene, DNA barcoding, Earwigs, *Forficula tomis*

INTRODUCTION

The global expansion of invasive insect species, facilitated by human activity, poses significant threats to agriculture, native ecosystems, and managed pollinators. Understanding the biology and spread of these synanthropic pests is critical for developing effective monitoring and control strategies, particularly for cryptic species complexes that are often overlooked (Garnas et al., 2016). Earwigs of the genus *Forficula* are a prime example of such widespread species that have significantly expanded their range. As synanthropic insects, they thrive in human-modified environments, including agricultural fields, gardens, and urban areas, where they are known as pests of agricultural garden (potatoes, beets, soybeans, etc.) and fruit crops (Quarrell et al., 2021; Riedle-Bauer et al., 2024), grain crops (Binns et al., 2021), melons, tobacco, and floriculture (Bey-Bienko, 1936). Beyond their impact on agriculture, earwigs have also become a persistent problem in apiculture. The earwigs are a common component of the fauna of honey bee hives, and during periods of high activity, they can cause significant damage to bee colonies (Domatsky and Domatskaya, 2020). In the hives, they feed on the food reserves of honeybees and bee bread, wax, and bee bread crumbs, small arthropods, and bee corpses (Atakishiyev, 1969; Banaszak, 1980). When present in large numbers, earwig infestations lead to colony weakening and reduced honey production (Domatsky and Domatskaya, 2020). Earwigs are carriers of infectious diseases of honey bees, such as American and European foulbrood (Sidorov, 1968) and honeybee viruses (Atakishiyev, 1969; Domatsky and Domatskaya, 2020). Thus, 5 types of viruses affecting *Apis mellifera* have been isolated from *Forficula auricularia*: deformed wing virus (DWV), Kashmir bee virus (KBV) (Dobelmann et al., 2020), black queen cell virus (BQCV), Israeli acute paralysis virus (IAPV), and sacbrood virus (SBV) (Levitt et al., 2013).

The genus *Forficula* belongs to the family Forficulidae and is the most numerous in the order Dermaptera. The genus includes at least 68 species (Steinmann, 1993). The most widespread and most important species of earwigs from this genus is *F. auricularia*, the common or European earwig. The species has a cosmopolitan range, which includes Europe, North Africa, Asia, North America, South America, Australia, and New Zealand (Bey-Bienko, 1936; Kirkland et

To cite this paper: Stolbova VV, Garbaly VR, Krestonoshina KS, and Zinatullina ZY (2025). Molecular Characterization of the *Forficula tomis* based on the *Cytochrome C Oxidase I* Gene. *World Vet. J.*, 15(3): 638-646. DOI: https://dx.doi.org/10.54203/scil.2025.wvj64

al., 2020; Markova and Maslov, 2024). Numerous studies have been devoted to the study of this species (Bhattarai et al., 2022; Freda et al., 2025; Pasquier et al., 2025). Recent molecular studies have shown that *F. auricularia* is a cryptic species complex (González-Miguéns et al., 2020; Freda et al., 2025). The finding raises a critical question regarding whether similar cryptic diversity occurs in the other species within the genus, particularly in *F. tomis*. It is very important not only from a theoretical but also from a practical point of view, since a recent study found *F. auricularia* DNA in metagenomic analysis of honey from Estonia (Paluoja et al., 2025). Given the high importance of earwigs in bee hives and the emerging opportunity to identify bee pathogens using molecular methods, it is important to know the full composition of the hive's synanthropic fauna, including the presence of cryptic species that carry the main pathogens.

Forficula tomis the garden earwig, is a species close to the common earwig, but has been studied to a much lesser extent, even though its role in agriculture and, in particular, in bee hives is no less important (Atakishiyev, 1969). *Forficula tomis* has a much more limited range, distributed in the Southern and central parts of Europe to the West as far as Moldova (Elisovetcaia et al., 2014), in the middle and Southern strip of the European part of Russia (Aleksanov et al., 2023), in Armenia and Turkey (Anlaş, 2012), in the mountains of Central Asia (Bey-Bienko, 1936), in Siberia (Dorzhieva, 2011), Korea, and Japan (Steinmann, 1993). The species was probably brought to Siberia at the beginning of the 20th century by settlers (Bey-Bienko, 1936). Following its introduction, *F. tomis* became the most common earwig species in Siberia and successfully spread to Eastern Siberia, where it was recorded in Transbaikalia in 2006 (Dorzhieva, 2011). In Russia and the former Soviet Union, *F. tomis* is regularly found in bee hives, and has been recorded in hive samples from the Eastern European part of Russia (Volga-Kama region), the Republic of Bashkortostan, and Azerbaijan (Sidorov, 1968; Atakishiyev, 1969; Bakalova, 2011). In Siberia, this species is the only earwig reported to inhabit honeybee hives to date (Stolbova, 2023). Previous studies indicate that *F. tomis* is a recognized pest in apiculture across both Eastern Europe (Atakishiyev, 1969; Banaszak, 1980) and Siberia (Domatsky and Domatskaya, 2020). At the same time, unlike the common earwig *F. auricularia*, this species has been much less studied; in particular, there are no DNA sequences of this species in published scientific articles or the Genbank and Barcode of Life (BOLD) databases (Ratnasingham, 2007). Species identification presents particular challenges when dealing with nymphal-stage earwigs collected from hives, as morphological characteristics are often insufficient for reliable determination. Consequently, comprehensive DNA reference data for all bee-associated pest species are needed to enable accurate molecular identification.

As a molecular marker, the *cytochrome c oxidase I* (*COI*) gene was selected, which is widely used and considered the most convenient molecular marker for identifying insect species (Wang et al., 2017; Kaur and Singh, 2020). The *COI* gene does not contain introns and has a variety of regions, both highly variable, which provide a high level of interspecies polymorphism and low intraspecies polymorphism, and conserved regions that are often used to develop universal PCR primers, which simplifies its use and amplification (Boehme et al., 2012). In addition, the advantage of the *COI* gene is its presence in a large number of copies in the mitochondrial genome, as well as a high rate of evolution, approximately 2-9 times higher than the rate of nuclear genes (Hebert et al., 2003). Taken together, these characteristics allow the analysis of both close and distant phylogenetic relationships and make the *COI* gene an ideal candidate for DNA barcoding, providing rapid, reliable, and cost-effective species identification, even in the case of cryptic and very closely related species, which is difficult with morphological analysis (Kaur and Singh, 2020). The present study aimed to perform the molecular characterization of the earwig *F. tomis* using the *COI* gene fragment as a molecular marker.

MATERIALS AND METHODS

Ethical approval

The present study was conducted ethically in accordance with the guidelines of the All-Russian Scientific Research Institute of Veterinary Entomology and Arachnology and national regulations.

Earwig cultivation

Earwigs were collected from *Apis mellifera* hives in the spring of 2024 from apiaries in the Tyumen region, Russia. A total of 18 specimens at the nymphal stage of development were collected. To obtain adults, earwigs were kept in plastic containers (14×21 cm) on a moistened coconut substrate at a temperature of 20°C and relative humidity of 60%. Dry oatmeal, fresh carrots, and high-protein food for aquarium goldfish of the Goldfish brand (38% protein, 7% fat, 3% plant fibre) were used as a food source (Tomkins, 1999; Markova and Maslov, 2024). During the cultivation, some earwigs (9 specimens) reached the imago stage, and 2 clutches of eggs were obtained. The remaining earwigs died before reaching adulthood due to unspecified causes. Species identification of earwig adults was carried out using a guideline of Bey-Bienko (1936).

Preparation of the target gene

A total of 9 adult *F. tomis* specimens (3 males, 6 females) were collected for molecular genetic analysis. Specimens were homogenized whole using a Bioprep-24 device (Hangzhou Allsheng Instruments Co., LTD, China) at +4°C using a diaGene kit (diaGene, Russia) for DNA extraction from animal tissues on spin columns according to the manufacturer's standard instructions provided in the protocol. DNA quantity and quality were determined spectrophotometrically using a Nano-300 (Hangzhou Allsheng Instruments Co., LTD, China). Sample purity was assessed spectrophotometrically by measuring the absorbance ratio at 260 and 280 nm, with ratios between 1.9 and 2.1 indicating high-quality DNA preparations. Amplification was performed on a GeneExplorer GE-96G device (Bioer, China). Amplification conditions for the gene fragment primer: preincubation at 95°C for 5 minutes, then 35 cycles of denaturation for 20 seconds at 95°C, annealing for 20 seconds at 51°C, and elongation for 20 seconds at 72°C. Composition of the reaction mixture (20 µl), including 14.4 µl of deionized water, 4 µl of 5X ScreenMix-HS mix (Evrogen, Russia), and 0.3 µl of forward and reverse primer. In the present study, universal *COI* primers originally designed by Hebert et al. (2003) were employed, as they have become standard markers in DNA barcoding studies (Kim et al., 2012; Mifkova et al., 2023). The primer sequence was taken from the study by Archana et al. (2015), including COIuF - 5'-GGTCAACAAATCATAAAGATATTGG-3' and COIuR 3'-TAAACTTCAGGGTGACCAAAAAATCA-5'. Electrophoresis in a 1.5% agarose gel was used to visualize the amplification results.

Analysis of sequencing results

Sequencing of the obtained nucleotide sequences was performed by Evrogen LLC (Russia) using the Sanger method. Electropherogram quality control and base calling were performed by the service provider. The forward and reverse reads were assembled into consensus sequences using the *de novo* assembly algorithm (CAP3) implemented in Unipro Ugene v.52.1. Bioinformatics analysis of the sequences included checking raw data in the Finch TV 1.4.0 program for forward and reverse readings in ABI format. Signal quality and artifacts were visually assessed. During the assembly process, low-quality base calls at the sequencing termini were automatically trimmed. The resulting consensus sequence was manually inspected; no ambiguous bases (N) were present in the final sequences, confirming high read quality. To construct a phylogenetic tree in the MEGA program, 38 nucleotide sequences of various lengths were selected from the NCBI database, representing different *Forficula* species and coinciding with the *COI* gene fragments sequenced by us. The sequences underwent multiple alignment (ClustalW) and trimming to 587 bp. The uniform length of 587 bp was determined by the region flanked by the universal *COI* primers and represents the high-quality, unambiguous consensus sequence obtained after trimming low-quality base calls from the sequencing termini. The approach ensured a consistent and comparable alignment across all taxa, which was a fundamental requirement for robust phylogenetic reconstruction. The selected fragment encompassed a sufficiently variable region of the *COI* gene suitable for resolving interspecific relationships within the genus *Forficula*.

Phylogenetic tree construction

The outgroup was selected to root the phylogenetic tree and provide a reference point for inferring evolutionary relationships within the ingroup (*Forficula* spp.). Anisolabis maritima (family *Anisolabididae*) was chosen as it represents a well-established external lineage to the family *Forficulidae*, to which the genus *Forficula* belongs. The taxonomic distance ensures that the outgroup is unequivocally outside the clade of interest, a fundamental criterion for accurate rooting, while remaining sufficiently phylogenetically close to allow for reliable sequence alignment and inference of homology. The species *Anisolabis maritima*, Bonelli, 1832, was consequently used as an outgroup. The phylogenetic tree was constructed using the Neighbor-Joining (NJ) method under the p-distance model, with node support assessed by 1000 bootstrap replicates.

To quantitatively assess the genetic divergence between *Forficula* species, pairwise genetic distances were calculated based on multiple nucleotide sequence alignments. The resulting matrix of genetic distances was analyzed to determine the optimal threshold for species differentiation. The optimal threshold was identified using the threshOpt algorithm, which implements the silhouette coefficient maximization method (Rousseeuw, 1987) to iteratively select the threshold value that maximizes the average silhouette coefficient. The method is recommended for threshold-based insect species delimitation using *CO I* barcodes, as it effectively minimizes identification errors compared to arbitrary fixed thresholds (Zhang and Bu, 2022). Furthermore, local minima in the distribution of genetic distances were identified using the localMinima function, which inverts the input data to treat local minima as peaks and applies the sciPy.signal.find_peaks algorithm (Virtanen et al., 2020). The prominence parameter was set to 12.0 to define the minimum height a valley must have relative to its surrounding peaks to be considered significant, effectively filtering out noise-related artifacts.

RESULTS AND DISCUSSION

Based on morphological criteria, all earwigs collected from apiaries belonged to the species *F. tomis*. Males of *F. tomis* are morphologically quite well distinguished from other species of the genus *Forficula* by the structure of the paired appendages of the last abdominal segment - cerci. In males of *F. tomis*, the cerci (forcipes) do not have an isolated tooth on the inner side, are expanded, and touch in the basal half. The outer sides of the cerci are almost parallel to each other along their entire length; the apical parts of the cerci are moderately thin, slightly bent at the very end (Bey-Bienko, 1936; Figure 1). However, in females of most species of the genus *Forficula*, the cerci are uniform (simple, thin, close together) and therefore have no diagnostic value (Bey-Bienko, 1936; Freda et al., 2025). Morphological species identification of nymphs and females of the genus *Forficula* can be carried out only presumably by additional criteria (color of the head, pronotum, elytra, and ratio of sizes of different body parts), which have wide variability depending on age, ecological, and biological conditions of the habitat. For this reason, to date, there are no identifiers of the genus *Forficula* for females and preimaginal stages of development. Such specimens can be identified only using molecular diagnostic methods.

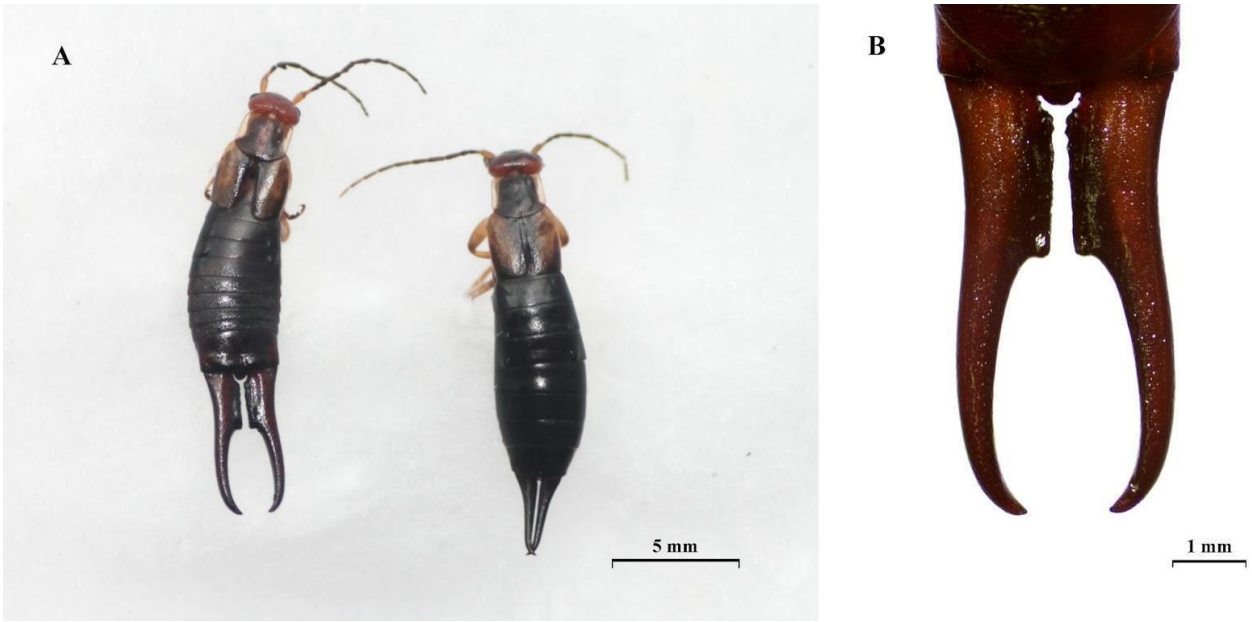


Figure 1. *Forficula tomis*, collected from an apiary in the Tyumen region, Russia. **A**: Male (left) and female (right); **B**: Cerci of the male.

Since earwig species are highly morphologically similar and often represent cryptic species complexes, their classification has been revised several times. Initial study by Wirth et al. (1998) revealed that *F. auricularia* comprises a complex of at least two cryptic species. Subsequent study (González-Miguéns et al., 2020) has expanded this number, and the complex is now considered to include at least four species (*F. auricularia*, *F. mediterranea*, *F. aeolica*, *F. dentata*). Given this precedent, it is plausible that the morphologically defined species *F. tomis* may likewise constitute a cryptic species complex. However, to date, there is insufficient information in the databases to verify this assumption, since out of 68 species listed in the identification guides, sequenced DNA sequences are available for only 16 species.

In the present study, as a result of sequencing, the sequences of the *F. tomis COI* gene fragment were obtained for the first time. The combined forward and reverse reads of the samples were subjected to multiple alignment, after which a consensus sequence of 589 bp in length was formed:

5`-
GTCCGGGATGGTTGGGACATCGCTAAGTCTGTTGATCCGAGCCGAGTTGGGGCAACCTGGGGCATTAATTG
GGGATGACCAAATTTACAACGTAATTGTAACCGCACATGCGTTTGTAATGATTTTTTTTATGGTAATGCCA
ATCATGATTGGAGGGTTTGGGAATTGACTGGTTCCCCTGATGCTTAGCGCCCCCGATTGGCATTCCCTCGTA
TAAACAACATAAGCTTTTGATTGCTTCCCCCTTCTTTGATGTTACTACTTTCAGGTAGAATGGTGGATAGCG
GGGCAGGGACAGGGTGAACGGTTTACCCCCCTCTGTCCGGGGCCATTGCTCACGCAGGGGCTTCGGTGGA
TTTGAGAATCTTTTCCCTGCATTTGGCAGGAATTTCCTCTATTTTAGGTGCTGTTAACTTTATCACAACCGTA
ATCAACATGCGCCCAGTAGGATTAAGCCTAGAACGGATGCCTTTGTTTGTTTGGTCAGTAGCTATCACTGC
TTTGTTGTTATTGTCTTTGCCCGTATTGGCGGGGGCCATTACTATGCTTTTAACCGATCGAAACCTAAATAC
CTCCTTTTTTGACCCTG-3`

Analyses with ORFfinder confirmed the absence of stop codons and indels, supporting the conclusion that the obtained sequence represents a functional mitochondrial *COI* gene and not a nuclear pseudogene (numt). In BLAST alignment, the highest similarity was with *F. dentata* (80.44%). The surprisingly low sequence identity for congeneric species could be attributed to several non-exclusive factors. These include the potential presence of extensive cryptic

diversity within the genus *Forficula* that remains underrepresented in genetic databases, a long evolutionary history of divergence between *F. tomis* and other sequenced congeners, or the possibility that current taxonomic boundaries within the genus do not fully reflect the deep genetic divergences. The notably high genetic distance observed supports the hypothesis that *F. tomis* represents a highly divergent lineage. The consensus amino acid sequence for the *COI* gene fragment:

>ORF 585 nt / 194 aa

MVGTSLSLLIRAELGQPGALIGDDQIYNVIVTAHAFVMIFFMVMPIMIGGFGNWLVPLMLSAPDMAFPRMN
NMSFWLLPPSLMLLLSGSMVDSGAGTGWTVYPPLSGAIAHAGASVDLSIFSLHLAGISSILGAVNFITTVINMRP
VGLSLERMPLFVWSVAITALLLLLSLPVLAGAITMLLTDRNLNTSFFDP

The consensus sequence of *F. tomis* obtained in the present study formed a distinct clade on the phylogenetic tree, which appeared as a sister group to the clade comprising *F. ruficollis* (Portugal) and *F. mikado* (Japan). Bootstrap support for this node was 77% (Figure 2), which is generally considered moderately high support in phylogenetic analyses. The formed clade was positioned distantly from both *F. auricularia* and *F. dentata*. The tree was rooted using *Anisolabis maritima* (*Anisolabididae*), which, as expected, formed a long external branch (branch length = 0.1506), confidently separating it from all representatives of the family *Forficulidae*. The substantial genetic divergence confirms the correct choice of an outgroup and provides a reliable reference point for interpreting the internal topology of the genus *Forficula*.

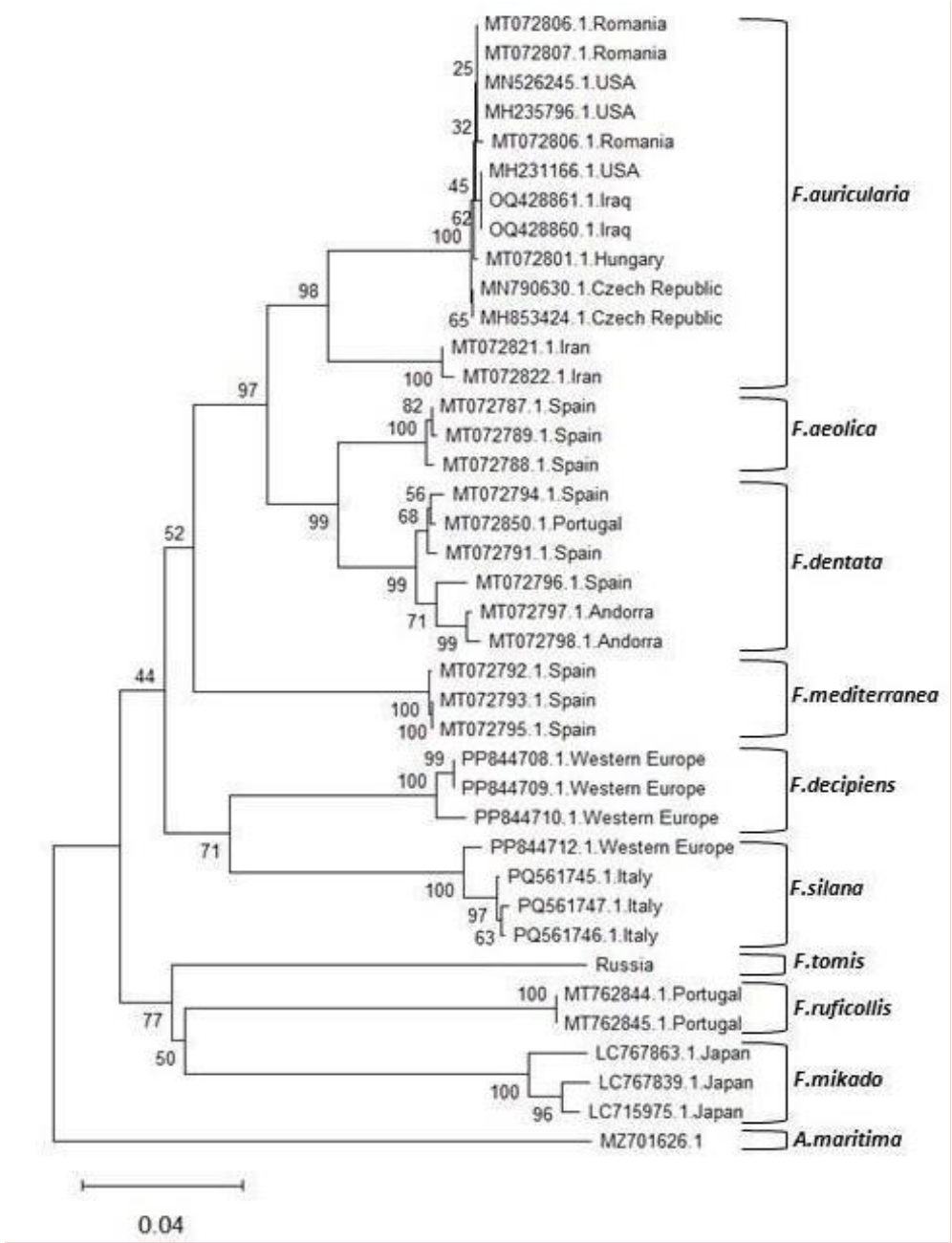
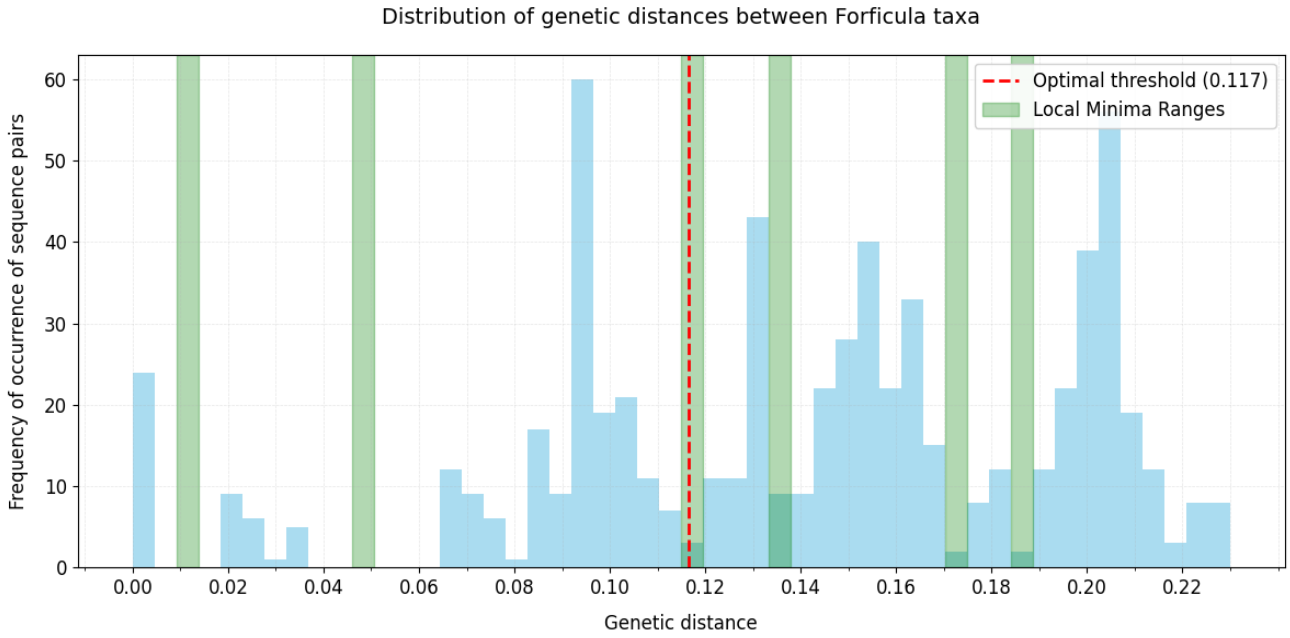


Figure 2. Phylogenetic tree of the genus *Forficula*, with the species *A. maritima* used for the outgroup. Construction method: Neighbor-Joining, evolutionary distance model: P-distance, node stability assessment: 1000 bootstrap replicates.

The substantial genetic gap, evidenced by both the BLAST results and the phylogenetic reconstruction, is likely caused by the low representation of suitable nucleotide sequences from relevant geographic regions in the NCBI database and the generally low level of study of earwigs in Russia and adjacent areas. It is probable that with expanded geographic sampling and a more comprehensive database, the phylogenetic relationships within the genus will be resolved with greater accuracy.

The analysis of genetic distances showed that the maximum distance (0.2290) was observed between the sequence *F. tomis* (Russia) and the sequence *F. silana* (Western Europe) with NCBI accession number PP844712.1. The median of distances was 0.1482, and the standard deviation was 0.0550. The threshOpt algorithm determined the optimal threshold at 0.117, which corresponded to the silhouette index of 0.606 (> 0.5 stable values). The "localMinima" algorithm identified 6 local minima, which can serve as potential thresholds for separating species (Graph 1). The six local minima identified in the genetic distance distribution likely correspond to genetic boundaries between major lineages within the genus *Forficula*. Given the limited taxonomic sampling (9 species) in the current analysis, these minima cannot be unambiguously assigned to known species complexes without additional data. However, their positions are consistent with the deep genetic divergences observed in the phylogenetic tree (Figure 2) and may represent both described species and potential cryptic diversity. For instance, the minima at the lower end of the range (~ 0.01-0.08) may reflect intraspecific variation or recent speciation events within complexes such as *F. auricularia*, while the minima at higher values (~ 0.11-0.19) likely correspond to divergence between well-established species (e.g., *F. auricularia* and *F. dentata*). The pattern underscores the complex phylogenetic structure of the genus and highlights the need for a more comprehensive taxon sampling to validate these preliminary boundaries.



Graph 1. The distribution of pairwise genetic distances among *Forficula taxa*. Blue bars show the frequency of sequence pairs in ranges of genetic distances, and green bars indicate ranges of local minima. The red dotted line at 0.117 indicates the optimal threshold.

The success of DNA barcoding is often related to the presence of a barcoding gap. If intraspecific divergence does not overlap with interspecific divergence, DNA barcodes can effectively identify specimens. DNA barcoding loses its effectiveness when the gap in the barcode becomes small or absent (Meyer and Paulay, 2005). Thresholds of sequence divergence, such as 2% or 3%, have been proposed to group specimens into preliminary species (Hebert et al., 2004; Smith et al., 2005). The obtained threshold of 11.7% (Graph 1) was significantly higher than the standard threshold (barcode gap) of 3%, but it is not always reliable in delimiting species boundaries, especially in groups with cryptic species such as *Forficula* and other insects. The presence of cryptic species and high levels of divergence mean that the gap is often greater than 3%, making the use of a universal threshold problematic. For example, the interspecific genetic distance in Molytinae (Coleoptera: Curculionidae) was exactly 11% in (Ren and Zhang, 2024), and the average interspecific divergence in scale insects (Hemiptera: Diaspididae) was 10.07% in (Niu et al., 2024). It should be noted that about a quarter of insect species have high intraspecific genetic variability (> 3%). Due to the high intraspecific genetic variability in insects, false positive results can easily occur when determining species boundaries based on the *COI* gene thresholds (Zhang and Bu, 2022). To obtain reliable results for phylogeny reconstruction, it is necessary to supplement studies with genetic markers such as *COII*, *16s rRNA*, *ITS II*, *28Sr RNA*, and others, increase the sample size,

and expand studies to other regions (Huemer and Wieser, 2023). Therefore, the high threshold observed in the present study is not anomalous but rather underscores the limitations of applying a universal distance threshold for species delimitation in genetically structured groups (Ren and Zhang, 2024). It highlights the necessity of integrative taxonomic approaches for *Forficula*, combining multiple molecular markers (e.g., nuclear genes, transcriptomes), morphology, ecology, and other data sources to achieve robust species boundaries (Janzen et al., 2017).

CONCLUSION

Morphological analysis confirmed that *F. tomis* is the sole earwig species inhabiting hives in Tyumen apiaries. Tyumen *F. tomis* specimens form a distinct clade, with BLAST analysis revealing significant genetic distance between *F. tomis* and other representatives of the *Forficula* genus. The identified optimal species differentiation threshold (11.7%) significantly exceeds the generally accepted barcode gap value of 3%. While *COI* variability suffices for genus-level identification, species-level phylogenetic resolution requires supplemental markers. Adaptive methods ("threshOpt" and "localMinima") uncovered complex genetic differentiation patterns, underscoring the necessity of integrative approaches for understudied insect groups, especially in such poorly studied regions as Siberia. Future studies should prioritize comparative morphology of *F. tomis*, nuclear marker analysis, and expand sampling across Northern Eurasia.

DECLARATIONS

Authors' contributions

All authors participated in writing the article, searching for information, and editing the text. Victory Vladimirovna Stolbova collected the earwigs and performed morphological identification of species, Vladislava Ruslanovna Garbaly performed bioinformatics analysis, translation, and editing of the publication, Kseniya Sergeevna Krestonoshina conducted molecular genetic studies, was responsible for strategic planning, Zimfira Yakubovna Zinatullina was engaged in project administration and its conceptualization, and collected materials. All authors checked and approved the final version of the manuscript.

Competing interests

The authors have not declared any conflicts of interest.

Funding

The study was supported by a grant from the Russian Science Foundation under the reference No. 24-26-00065, https://rscf.ru/project/24-26-00065/.

Ethical considerations

All authors declare that this paper is original, conducted solely by the authors, and it has not been submitted and published elsewhere.

Availability of data and materials

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

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2025, Scienceline Publication
World's Veterinary Journal
World Vet J, 15(3): 647-655.
ISSN 2322-4568

DOI: https://dx.doi.org/10.54203/scil.2025.wvj65
PII: S232245682500065-15

Spatial Analysis of Rabies Cases in West Sumatra Province, Indonesia during 2021-2024

Kartika Amira[1], Heru Susetya*[2], and Ida Tjahajati[3]

[1]*Master Program in Veterinary Sciences, Universitas Gadjah Mada, Sleman 55281, Yogyakarta, Indonesia*
[2]*Department of Epidemiology and Veterinary Public Health, Faculty of Veterinary Medicine, Universitas Gadjah Mada, Sleman 55281, Yogyakarta, Indonesia*
[3]*Department of Internal Medicine, Faculty of Veterinary Medicine, Universitas Gadjah Mada, Sleman 55281, Yogyakarta, Indonesia*
*Corresponding author's Email: herususetya@ugm.ac.id

ORIGINAL ARTICLE
Received: June 25, 2025
Revised: July 29, 2025
Accepted: August 29, 2025
Published: September 25, 2025

ABSTRACT

The West Sumatra Province, Indonesia, has been an endemic rabies region since its initial rabies incident in 1953. This study aimed to analyze spatial patterns for rabies cases in animals in 19 districts/cities in West Sumatra Province, Indonesia. Secondary data on rabies-positive cases in animals were identified in 198 samples through the Fluorescent Antibody Test (FAT) obtained from the Bukittinggi Veterinary Center's database from 2021 to 2024. Statistical and spatial analyses were conducted in R Software v.4.4.2. The Moran's Index revealed a cluster distribution of rabies cases throughout the study period. The highest prevalence occurs in Lima Puluh Kota District (28.79%). The index indicated four regions as high-high quadrants, three regions as low-high quadrants, 11 regions as low-low quadrants, and one region as a high-low quadrant. However, Lima Puluh Kota District was consistently found as a region with substantial local spatial autocorrelation. In conclusion, the prevalence of rabies cases in animals in West Sumatra Province fluctuated during 2021-2024. Spatial analysis with Moran's Index and Local Indicator of Spatial Autocorrelation (LISA) indicated a cluster distribution of the disease. The current study underscored the need for targeted, location-specific control measures in these high-risk clusters to effectively mitigate the spread of the disease.

Keywords: Fluorescent Antibody Test, Moran's Index, Rabies, Spatial analysis

INTRODUCTION

Rabies is a fatal zoonotic disease caused by the rabies virus (RABV), a neurotropic virus classified within the genus *Lyssavirus*, from the *Rhabdoviridae* family (Kumar et al., 2023). It is a part of the *Mononegavirales* (MNV) order, which comprises single-stranded RNA viruses with non-segmented genomes. RABV is highly neurotrophic in mammals and causes encephalomyelitis after the virus infects the animal and reaches the brain (Marston et al., 2018). Rabies is one of the major zoonotic diseases, classified as a strategic priority disease in Indonesia due to its significant impact on public health and the socio-economic sector (Kemenkes RI, 2017). The overall yearly economic losses from rabies were projected to be 84.1 billion Rupiahs in Bali (Batan et al., 2015). This virus not only attacks animals such as dogs, bats, monkeys, foxes, and cats but is also zoonotic (Samad et al.,2024). This disease is mainly transmitted through dog bites (Kumar et al., 2023). Rabies has an incubation period of approximately 2 weeks to 6 years, with an average of 2 to 3 months (Samad et al., 2024).

The mortality rate of rabies in Indonesia remains relatively high, with 100 to 156 deaths in humans reported annually and a Case Fatality Rate (CFR) approaching 100%. Indonesia has experienced an average of 80,861 cases of rabies animal bites and 105 deaths per year (Kemenkes RI, 2020). From January to July 2024, Indonesia reported 71 human rabies fatalities (WHO, 2024). This report illustrated that rabies is still a serious threat to public health in Indonesia, making it one of the rabies-endemic countries; only 12 out of 34 provinces are free of rabies (Kementan RI, 2024).

The incidence of rabies initially occurred in 1953 in West Sumatra, which is still not free from rabies (Kemenkes RI, 2024). The total number of human casualties in 2024 was 7.369 cases, 3 of whom died of rabies in West Sumatra (Dinkes, 2024). Rabies-positive cases were identified through laboratory confirmation using the Fluorescent Antibody Test (FAT), the gold standard technique recommended by the World Health Organization (WHO) for rabies diagnosis in animals (Rupprecht et al., 2018). West Sumatra Province, Indonesia, is an endemic region with limited spatial epidemiological data on rabies cases. There have been no reports of spatial analysis of rabies cases applied to both global and local spatial autocorrelation. Therefore, this study aimed to analyze the spatial pattern and clustering trends of laboratory-confirmed rabies cases in West Sumatra Province from 2021 to 2024 using Moran's Index and Indicator of Spatial Autocorrelation (LISA).

To cite this paper: Amira K, Susetya H, and Tjahajati I (2025). Spatial Analysis of Rabies Cases in West Sumatra Province, Indonesia during 2021-2024. *World Vet. J.*, 15(3): 647-655. DOI: https://dx.doi.org/10.54203/scil.2025.wvj65

MATERIALS AND METHODS

Ethical approval

This study has been conducted in accordance with institutional guidelines and has passed ethical clearance from the Medical and Health Research Ethics Committee (MHREC), Universitas Gadjah Mada, Indonesia, under certificate number KE/FK/0551/EC/2025.

Data source

This study used 198 samples submitted for rabies testing at the Bukittinggi Veterinary Center, Indonesia, from 2021 to 2024, obtained from 19 districts/cities in West Sumatra Province, Indonesia (Figure 1). The samples were animal brain specimens collected by district veterinary officers using passive surveillance and outbreak reporting systems. The Fluorescent Antibody Test is considered the gold standard for rabies diagnosis, providing consistent results in more than 95% of the cases (Rupprecht et al., 2018; Kachhawaha and Tanwar, 2024). This study was conducted from April to May 2025.



Figure 1. The location of study in West Sumatra Province, Indonesia

Statistical analysis

The data required for this autocorrelation analysis included information on rabies-positive cases in animals and official maps of West Sumatra Province. The first step was to merge the West Sumatra Province map with the rabies case dataset. The data integration process generated georeferenced rabies surveillance data, combining laboratory-confirmed case counts with their spatial distribution across the infected areas. The second stage was to create a spatial weighting matrix to determine the observed weights between locations based on the relationship between locations (Kosfeld et al., 2006). The statistical and spatial analysis of Moran's Index and Local Indicator of Spatial Autocorrelation were fed into R Software (v.4.4.2). Spatial autocorrelation measurements were calculated using Moran's Index. Spatial randomness can indicate the presence of clustering patterns or forming trends in space. The Moran's Index method was employed with a standardized spatial weighting matrix W through the following equation (Moraga, 2023; Wibowo et al., 2024).

$$I = \frac{n \sum_{i=1}^{n} \sum_{j=1}^{n} w_{ij} \, (x_i - \bar{x})(x_j - \bar{x})}{\sum_{i \neq j}^{n} w_{ij} \, \sum_{i=1}^{n} (x_i - \bar{x})}$$

The LISA statistic for each region *i* was formulated as follows (Wibowo et al., 2024).

$$I_i = \frac{z_i}{m_2} \sum_{j=1}^{n} w_{ij} \, z_j$$

$$z_i = (x_i - \bar{x})$$

$$z_j = (x_j - \bar{x})$$

$$m_2 = \sum_{i=1}^{n} \frac{(x_i - \bar{x})^2}{n}$$

Moran's Index (I) measures spatial autocorrelation, indicating whether similar values cluster or disperse geographically. It compares values (x_i, x_j) against the mean ($\bar{x}$), weighted by proximity (w_{ij}) with values ranging from -1 to 1. Positive values ($0 < I \leq 1$) show clustering of similar values, negative values ($-1 \leq I < 0$) indicate dispersion, and $I \approx 0$ suggests randomness. Moreover, extreme values ($|I| \geq 1$) reveal strong patterns (Moraga, 2023; Wibowo et al., 2024).

The Moran's Index results were visualized using a Moran Scatterplot to analyze rabies case patterns. The scatterplot plots each location's Z-score against its neighbors' average Z-scores, revealing spatial correlations (Zhukov, 2010). The Z-score was computed as $Z(I) = [I - E(I)]/\sqrt{VAR(I)}$, where I is the observed Moran's Index, E(I) is its expected value (null hypothesis), and VAR(I) is its variance. The regions were classified into four quadrants.

While Moran's Index measures overall spatial autocorrelation, it cannot identify localized patterns between individual locations and their neighbors. The LISA addresses this limitation by analyzing spatial associations at each specific observation point and its immediate surroundings to better understand the local spatial relationships at each location. Moreover, information derived from LISA is essential. The LISA statistic I used to measure local spatial autocorrelation at location i, computed from n observations (x_i, x_j), their mean $\bar{x}$, and their spatial weights w_{ij}. The null hypothesis (H_0: $I_i = 0$) was rejected when $|Z(I_i)| > Z_{a/2}$, indicating significant local clustering/dispersion (Lee and Wong, 2001).

RESULTS

During the period from 2021 to 2024, 198 positive rabies cases were identified in the animals under study. Details of annual positive rabies cases in West Sumatra Province are presented in Table 1. Based on Table 1, the proportion of rabies-infected cases confirmed by the Bukittinggi Veterinary Center is an average of 58.22%. In 2021, there were 47 positive cases of rabies (61.04%); in 2022, there were 37 cases (59.68%); in 2023, there were 54 cases (50.94%); and in 2024, the highest number of cases was 60 (61.22%).

Rabies cases in West Sumatra Province during the observation period showed an uneven distribution in each district/city. Lima Puluh Kota District recorded the highest number of cases, namely 57 cases, which is approximately 28.79% of the confirmed cases. Agam District and West Pasaman District ranked second, each with 31 cases (15.66%), while Sijunjung District recorded 22 cases (11.11%). Several other areas, such as Tanah Datar District, Payakumbuh City, Padang Pariaman District, and Bukittinggi City, indicated a lower number of infected cases, ranging from 3 to 6 cases. However, there are several areas with very low incidence rates or no cases at all, such as the South Pesisir District and Solok District, as well as the Kepulauan Mentawai District, which possesses a rabies-free status.

The value of Moran's Index designates a clustered pattern, including 0.2202 in 2021, 0.327 in 2022, 0.3212 in 2023, and 0.2893 in 2024. The pattern of rabies cases in West Sumatra Province, Indonesia, based on Moran's Index (0.2805) from 2021 to 2024 has the same pattern, i.e., the clustered pattern (positive autocorrelation).

Table 1. The prevalence of rabies-infected cases in animals during 2021-2024 in West Sumatra Province, Indonesia

Year	Number of Samples	Positive	Prevalence (%)
2021	77	47	61.04 %
2022	62	37	59.68 %
2023	106	54	50.94 %
2024	98	60	61.22 %
Total	343	198	58.22 %

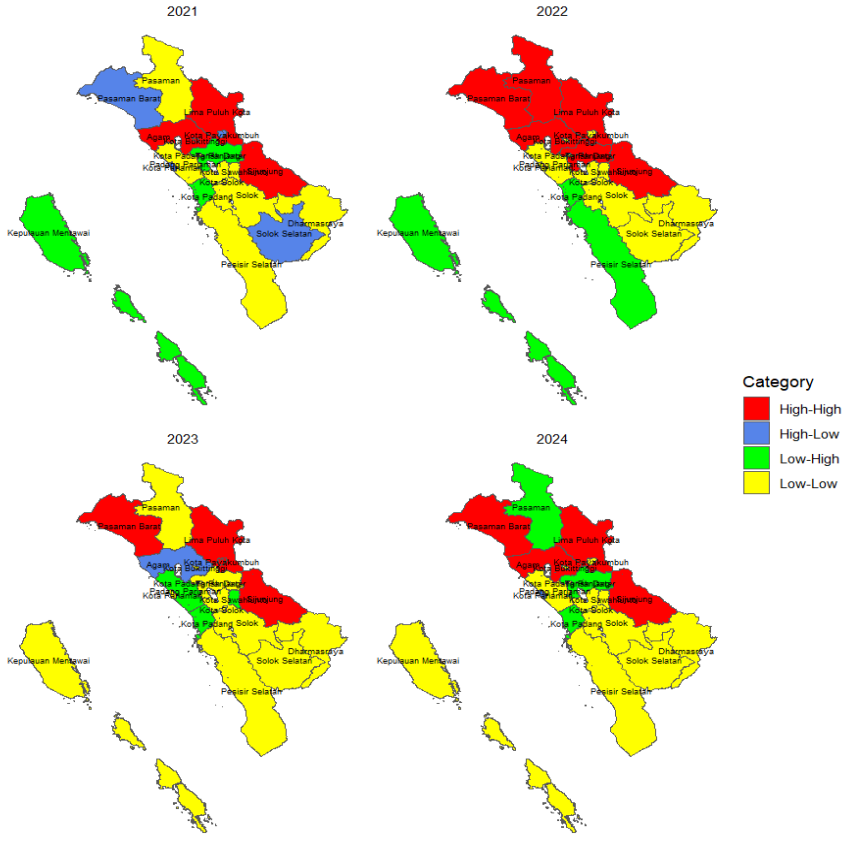


Figure 2. Moran Scatterplots showing global spatial autocorrelation of rabies cases during 2021-2024 in West Sumatra, Indonesia

Figure 2 shows the dynamics of the spatial distribution of rabies cases based on Moran's I quadrant classification during the 2021-2024 period. The region was classified into 4 quadrants, including high-high (HH), low-high (LH)/coldspot, low-low (LL), and high-low (HL)/hotspot, based on their spatial patterns (Shrestha et al., 2023). The Hotpot quadrant is an area with high cases surrounded by areas with low cases, while a coldspot is an area with low cases surrounded by areas with high cases (Ekowati et al., 2021). The number of districts/cities classified as high-low (hotspots) and low-high (coldspots) fluctuates annually. Overall, the results indicated that the rabies hotspot (high-low) areas most often appear in Pariaman City in 2021 and 2024. In 2023, only the Agam District was included in the hotspot, and no region was identified as a hotspot area in 2022. However, Padang city was consistently a coldspot area with Kepulauan Mentawai and Tanah Datar Districts as areas where a coldspot could most often be identified.

The results of the analysis of the spread of the spatial pattern of annual rabies cases are presented in Figure 2. A comparison of these results with those of cumulative observations over four years, presented in Figure 3, shows that there is only one district (Payakumbuh), which is included in the hotspot category, and there are three districts identified in the coldspot category (Padang, Pasaman, and Tanah Datar) from 2021 to 2024. The areas classified as hotspots, coldspots, and those in Quadrant I and Quadrant III for rabies cases in West Sumatra Province are shown thematically in figures 2 and 3, and further detailed in Table 2.

The identification of spatial autocorrelation at the local level was carried out using the LISA method, with the LISA value of each district/city in West Sumatra presented to show the strength and pattern of spatial relationships between districts/cities. High and positive LISA values in the Lima Puluh Kota District suggest the presence of local spatial autocorrelation. According to this research, there is a geographical clustering pattern in the frequency of rabies cases in these locations, which tends to be comparable to that of nearby places. In 2022, such trends were also noted in the districts of Agam and Lima Puluh Kota (Figure 4). Overall, Lima Puluh Kota District demonstrated consistency as an area with notable local spatial autocorrelations between 2021 and 2024 (Figure 5).

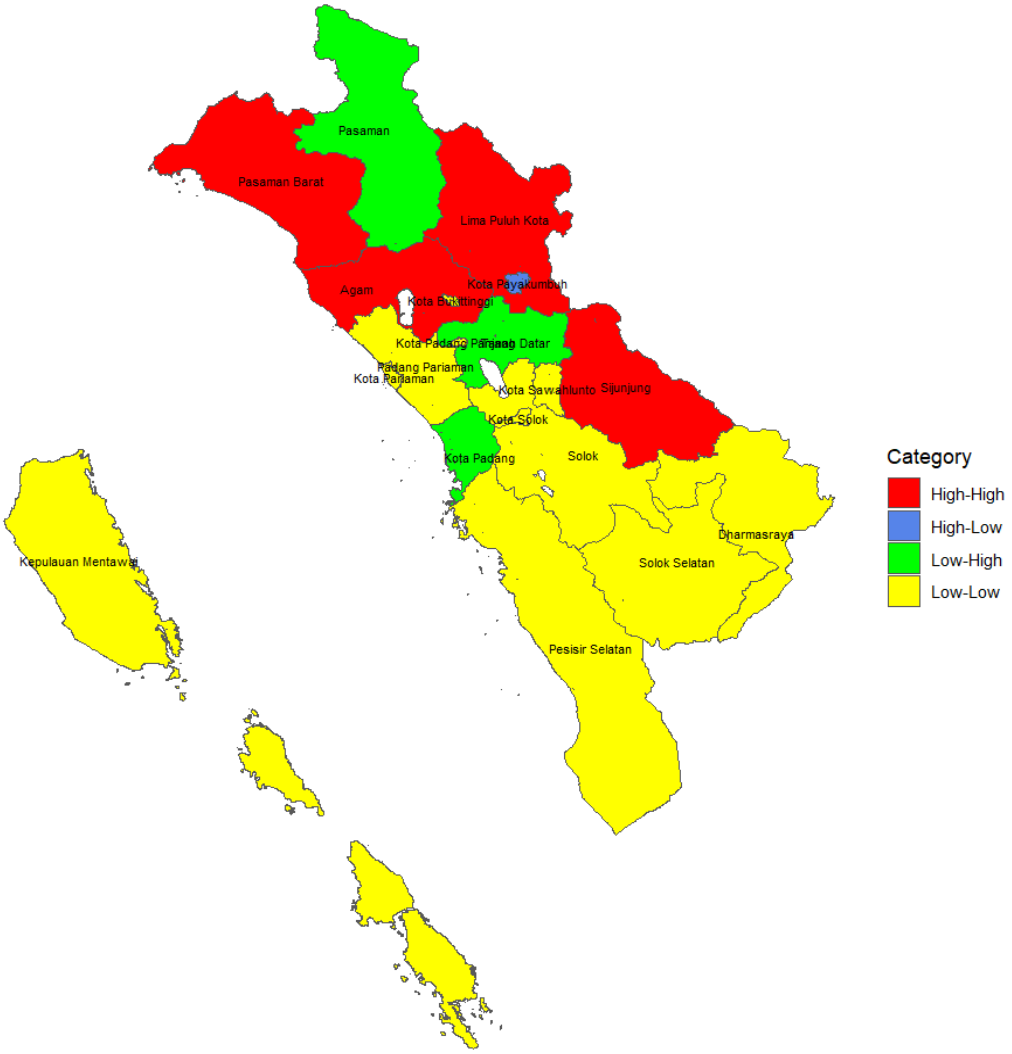


Figure 3. The distribution of rabies cases for four years of observation (2021-2024) in West Sumatra Province, Indonesia, based on Moran's Scatterplot.

Table 2. Districts/cities that are hotspots, coldspots, quadrant I, and quadrant III of rabies cases during 2021-2024 in West Sumatra Province, Indonesia

Year	Quadrant	Districts/Cities
2021	Quadrant I (High-High)	Bukittinggi, Agam, Lima Puluh Kota, Sijunjung
	Quadrant II (Coldspot)	Kepulauan Mentawai, Padang, Tanah Datar
	Quadrant III (Low-Low)	Dharmasraya, Padang Panjang, Sawahlunto, Solok, Padang Pariaman, South Pesisir, Pasaman, Kota Solok
	Quadrant IV (Hotspot)	Payakumbuh, South Solok, Pariaman, Pasaman Barat
2022	Quadrant I (High-High)	Pasaman, Tanah Datar, Agam, Lima Puluh Kota, Pasaman Barat, Sijunjung
	Quadrant II (Coldspot)	Kepulauan Mentawai, South Pesisir, Padang
	Quadrant III (Low-Low)	Dharmasraya, Bukittinggi, Padang Panjang, Payakumbuh, Kota Solok, Sawahlunto, Padang Pariaman, Solok, South Solok, Pariaman
	Quadrant IV (Hotspot)	-
2023	Quadrant I (High-High)	Payakumbuh, Lima Puluh Kota, Pasaman Barat, Sijunjung
	Quadrant II (Coldspot)	Sawahlunto, Padang Pariaman, Padang
	Quadrant III (Low-Low)	Dharmasraya, Kepulauan Mentawai, Bukittinggi, Padang Panjang, Solok, South Pesisir, South Solok, Pasaman, Tanah Datar, Pariaman, Kota Solok
	Quadrant IV (Hotspot)	Agam District
2024	Quadrant I (High-High)	Agam, Lima Puluh Kota, West Pasaman, Sijunjung
	Quadrant II (Coldspot)	Padang, Pasaman, Tanah Datar
	Quadrant III (Low-Low)	Dharmasraya, Kepulauan Mentawai, Bukittinggi, Kota Solok Padang Panjang, Payakumbuh, Sawahlunto, Padang Pariaman, South Pesisir, Solok, South Solok
	Quadrant IV (Hotspot)	Pariaman
2021-2024	Quadrant I (High-High)	Agam, Lima Puluh Kota, West Pasaman, Sijunjung
	Quadrant II (Coldspot)	Padang, Pasaman, Tanah Datar
	Quadrant III (Low-Low)	Dharmasraya, Kepulauan Mentawai, Bukittinggi, Padang Panjang, Sawahlunto, Solok, Padang Pariaman, South Pesisir, South Solok, Pariaman, Kota Solok
	Quadrant IV (Hotspot)	Payakumbuh

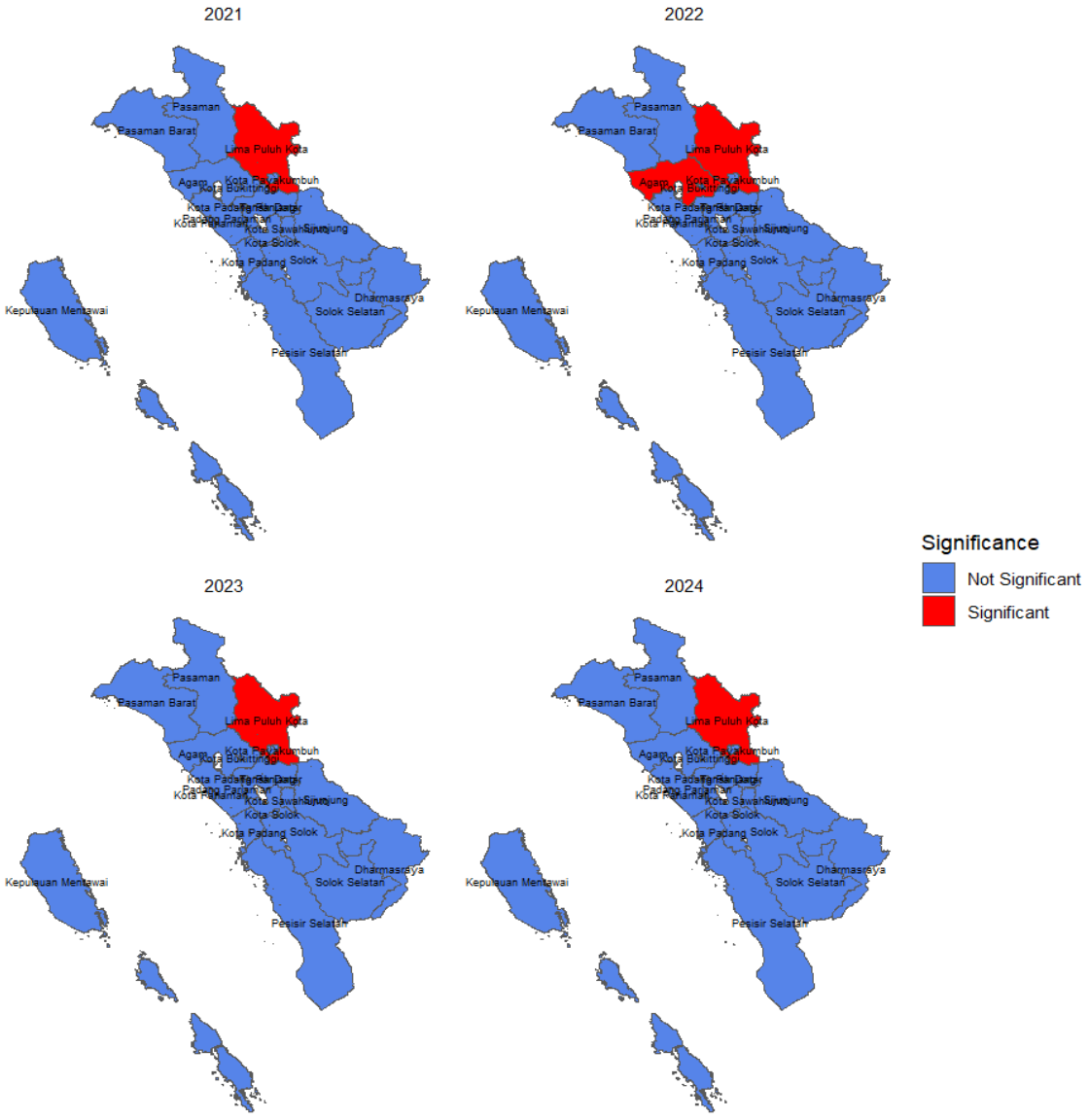


Figure 4. The LISA Moran's I rabies cases in West Sumatra Province, Indonesia during 2021-2024



Figure 5. The LISA Moran's I rabies cases in West Sumatra Province, Indonesia, during 2021-2024

DISCUSSION

Based on Statistics West Sumatra Province (2023), there were 19 regencies/cities in West Sumatra Province, Indonesia, and only Kepulauan Mentawai Regency is currently rabies-free (Kementan RI, 2024). Based on the results of the present study (2021-2024), the pattern of rabies cases in West Sumatra was a cluster pattern, where the districts with high positive cases were the neighboring districts. These findings aligned with existing study demonstrating clustered spatial distributions of rabies cases in Indonesia, particularly along Sumatra's Western and Eastern coasts, West Java, East Java, West Nusa Tenggara, and Kalimantan (Ward, 2014). The clustering pattern shown by both Moran's I and LISA statistics suggests spatial dependence among the districts. This means that districts with high case numbers are often surrounded by similarly high-case neighbors, possibly due to shared ecological, cultural, and human-animal interface factors (Ekowati et al., 2021). Spatial analysis of rabies in Bali and North Tapanuli, Indonesia, revealed important patterns and factors influencing disease spread. The studies in Bali and North Tapanuli showed that rabies cases in both regions had a spatially clustered pattern, with high-risk areas surrounded by low-risk areas, and vice versa (Ekowati et al., 2021; Simanjuntak et al., 2024).

Moran's Index data revealed that the most prevalent rabies hotspots were in Lima Puluh Kota District, Sijunjung District, Agam District, and the west Pasaman District. Lima Puluh Kota and Sijunjung Districts had the maximum number of cases of bites and were the hotspots for rabies cases in West Sumatra province from 2021 to 2024. The LISA method showed consistency in the Lima Puluh Kota District as a region with significant local spatial autocorrelations. These findings indicated that Lima Puluh Kota District and its surroundings are rabies cluster areas that require special attention in the formulation of spatial disease control and prevention strategies. According to Fadillah et al. (2021), the practice of dog owners in Lima Puluh Kota District reflected a lack of socialization and communication, information, and education activities. Further emphasis on rabies control programs is needed for dog owners who focus on responsibility for dog ownership. Targeted vaccination and movement control programs should prioritize these high-risk clusters, particularly in districts like Lima Puluh Kota, where rabies has persisted for several years. Risk factors associated with rabies incidence include animal care practices, vaccination status, and human behavior (Saepudin et al., 2022).

Direct contact with infected dogs is the only way to determine if rabies is being transmitted in West Sumatra. Although bites and scratches are two definite ways that rabies is spread, animal licking can also cause instances (WHO, 2023). The high frequency of infectious rabies in West Sumatra is intimately related to the community's long-standing custom of raising dogs to hunt wild boars (Rahmah et al., 2017; Dalimunthe et al., 2025). Community traffic activities and the buying and selling of dogs are contributing factors to the rise in rabies cases. Most dogs from outside West Sumatra province came from Sukabumi Regency in West Java Province, which remains rabies-free (Wicaksono et al., 2018).

Various efforts have been made to suppress the incidence of rabies. The primary goal of rabies eradication targeted the vaccination and elimination of dogs, but this initiative is hindered by the lack of public awareness regarding pet health. For instance, many people have never vaccinated their pets, the management system remains ineffective and is inadequately supervised, and there is insufficient community outreach on rabies prevention.

CONCLUSION

The prevalence of rabies-infected cases in animals from 2021 to 2024 in West Sumatra Province was 58.22 %, identified from 198 rabies-positive samples in animals. Spatial analysis of rabies cases in West Sumatra Province revealed significant clustering patterns (positive autocorrelation) based on Moran's Index. Hotspot areas with high case concentrations were identified in Agam, Lima Puluh Kota, West Pasaman, and Sijunjung Districts. LISA analysis (2021-2024) particularly highlighted Lima Puluh Kota District as a persistent area of significant local spatial autocorrelation, confirming its status as a core rabies transmission zone. These results underscore the need for targeted, location-specific control measures in such high-risk clusters to mitigate disease spread effectively.

DECLARATIONS

Funding

This study is not funded by any external entity.

Availability of data and materials

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

Acknowledgments

The authors would like to express my sincere gratitude to my research supervisor for their invaluable guidance, expertise, and continuous support throughout the course of this study. Special appreciation is also extended to the Bukittinggi Veterinary Center, the Department of Animal Husbandry and Animal Health of West Sumatra Province, and the Department of Public Health of West Sumatra Province for their valuable insights, data access, and collaborative contributions.

Authors' contributions

Kartika Amira was responsible for the overall design and execution of the research, data collection, analysis, and preparation of the draft of the manuscript. Heru Susetya and Ida Tjahajati assisted in the conceptualization of the research. Kartika Amira, Heru Susetya, and Ida Tjahajati revised the manuscript after corrections from the reviewer. All authors have read and approved the final version of the manuscript before publication in the present journal.

Competing interests

There is no conflict of interest in this study.

Ethical considerations

All the authors have read and approved the final manuscript. All authors checked the originality of data and sentences via plagiarism checkers.

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2025, Scienceline Publication
World's Veterinary Journal
World Vet J, 15(3): 656-668.
ISSN 2322-4568

DOI: https://dx.doi.org/10.54203/scil.2025.wvj66
PII: S232245682500066-15

Prevalence and Intensity of Gastrointestinal Parasites and Related Risk Factors in Goats Kept by Smallholder Farmers in Juja Sub-County, Kenya

Odinga Christopher Koyan[1,2], John Kagira[1]*, Kamundia Patrick Waweru[2], Maina Naomi[3], and Osuga Isaac[1]

[1]*Department of Animal Sciences, Jomo Kenyatta University of Agriculture & Technology, P.O. Box 62000-00200, Nairobi, Kenya*
[2]*Department of Animal Health and Production, Maasai Mara University, P.O. Box 861-20500, Narok, Kenya*
[3]*Department of Biochemistry, Jomo Kenyatta University of Agriculture & Technology, P.O. Box 62000-00200, Nairobi, Kenya*
*Corresponding author's Email: jkagira@jkuat.ac.ke

ORIGINAL ARTICLE
Received: June 28, 2025
Revised: July 29, 2025
Accepted: August 29, 2025
Published: September 25, 2025

ABSTRACT

Gastrointestinal parasites (GIP) negatively impact the health and productivity of goats. Regular screening of the animals for these parasites is crucial for implementing effective control strategies. A cross-sectional study was conducted to determine the prevalence of GIP and its risk-related factors in goats owned by smallholder farmers in Juja Subcounty, Kiambu County, Kenya. A total of 210 goats consisting of both males and females, of different breeds including Boer, Toggenburg, Alpine, and Small East African were randomly sampled from 80 farms. Fresh fecal samples were collected and analyzed for parasites using McMaster techniques for egg/oocyst examination. A structured questionnaire was administered to assess the risk factors such as age, sex, breed, origin of the goats, production system, and purpose and frequency of cleaning goat houses in relation to the prevalence of the parasites. The overall prevalence of the GIP was 88.6%, with nematodes and coccidia detected in 64.3% and 74.3% of the goats, respectively. The mean eggs per gram was 67.72, while the mean oocysts per gram was 243. Goat fecal samples positive for gastrointestinal nematodes (GIN) eggs were cultured and identified after recovery of third-stage larvae (L3) using the Baermann technique, whereas the coccidian oocysts were sporulated in 2.5% potassium dichromate solution, followed by microscopic examination based on morphological features of the parasites. The identified GIN nematodes included *Haemonchus contortus*, *Trichostrongylus* spp., *Strongyloides papillosus*, *Ostertagia* spp., and *Nematodirus spathiger*, while the coccidians were *Eimeria caprina*, *Eimeria ninakohlyakimovae*, and *Eimeria hirci*. The prevalence of GIN was significantly associated with age (higher in adults), production systems (higher in intensive systems), and body condition score (higher in animals with poor body condition). For coccidia, the prevalence was associated with the animal origin (higher in goats from Murera Ward), breed (higher in small East African goats), age (higher in younger goats), body condition score (higher in goats with poor condition), and cleaning frequency (higher in goats kept in irregularly cleaned pens). In conclusion, the overall prevalence and burden of GIP reported in the study were high, with the pathogenic gastrointestinal nematodes and *Eimeria* spp. being the most prevalent parasites.

Keywords: *Coccidia*, Goat, Nematode, Prevalence, Risk factor

INTRODUCTION

Goats are highly versatile livestock species, capable of flourishing in a wide range of environments, including arid deserts, semi-arid areas, and mountainous regions. The global goat population stands at approximately 1.1 billion (Ahari and Waiz, 2024) and is largely concentrated in Asia (57.7%) and Africa (35.7%; FAO, 2018). Rearing of goats is quite popular in countries such as Kenya, where the goat population is 28 million (Park, 2022), comprising of exotic goat breeds such as Toggenburg, Saanen, and Alpine (Shivairo et al., 2013) and indigenous goats that include Galla and small East Africa and the crossbred of exotic and indigenous (Kiura et al., 2020). In rural and peri-urban Kenya, goat rearing plays a vital role in the economy, providing employment and income, and it is crucial for people's livelihoods, food security, and nutrition (Lokhit et al., 2005; Kipserem, 2011; KARLO, 2020). However, despite their benefits, goats are vulnerable to various parasitic diseases, such as gastrointestinal helminthosis and coccidiosis.

Goats reared in tropical countries suffer from a heavy burden of helminthosis and coccidiosis due to favorable conditions for parasite-host interactions, poor control strategies, and the emergence of drug resistance (Mpofu et al., 2022; Hussein et al., 2023). Infection usually occurs when animals ingest infective larvae or eggs and oocysts from contaminated environments, and transmission continues through excretion into the environment. The economic impacts

To cite this paper: Koyan OC, Kagira J, Waweru KP, Naomi M, and Osuga I (2025). Prevalence and Intensity of Gastrointestinal Parasites and Related Risk Factors in Goats Kept by Smallholder Farmers in Juja Sub-County, Kenya. *World Vet. J.*, 15(3): 656-668 DOI: https://dx.doi.org/10.54203/scil.2025.wvj66

of the two parasitic diseases include reduced meat, milk, and wool production, reduced fertility, increased mortality, and treatment costs (Shimelis et al., 2011; Siyoun and Kitaw, 2014). Previous studies have reported the prevalence of internal parasites in goats across various geographical regions, including Cameroon, 94.4% (Ntonifor et al., 2013), Zimbabwe, 92.5% (Pandey et al., 1994), South Africa, 82.25% (Gwaze et al., 2009), Ethiopia, 54.17% (Hussein et al., 2023), Nigeria 35.5% (Nwosu et al., 2007), 33% Namibia (Kumba et al., 2003), and India, 25.2% (Sharma and Mandal, 2013). In Kenya, the prevalence of gastrointestinal nematodes (GIN) in goats has been reported, with overall helminth prevalence ranging from 75% (Waruiru, 2004) to 82% (Maichomo et al., 2004) in goats reared under an extensive production system. The most common gastrointestinal tract nematode species in small ruminants include *Hemonchus contortus*, *Oesophagostomum* spp., *Trichostrongylus* spp., *Ostertagia* spp., *Nematodirus* spp., and *Cooperia* spp. (Waruiru et al., 2000; Maichomo et al., 2004). Recent epidemiological data on helminths in goats kept by smallholder farmers in Kenya are scanty.

Several studies have described the high prevalence of coccidiosis in goats across Africa. For instance, Maichomo et al. (2004), Ikpeze et al. (2010), Mohamaden et al. (2018), and Etsay et al. (2020) documented rates of 60%, 73%, 85%, and 45% in Egypt, Nigeria, Ethiopia, and Kenya, respectively. Caprine coccidiosis is caused by protozoa of the *Eimeria* genus, with the common species identified in Africa being *E. ninakohlyakimovae*, *E. hirci*, *E. arliongi*, and *E. caprina*, (Kusiluka et al.,1996; Harper and Penzhorn, 1999; Etsay et al., 2020). Investigations conducted in Kenya have reported moderate to high prevalence of coccidiosis, with infection rates ranging from 45% to 48% in goats (Maichomo et al., 2004; Kanyari et al., 2009). The main risk factors associated with gastrointestinal tract parasites include breeds, sex, age, body condition scores, production systems, and frequency of cleaning (Maichomo et al., 2004; Kanyari et al., 2009; Hussein et al., 2023).

To control these parasites, farmers keeping goats rely on symptoms and advice from animal health practitioners. However, most of the clinical signs are not pathognomonic for gastrointestinal tract parasitosis, and indeed, the proportions of asymptomatic cases are quite high. In spite of this, farmers have opted for regular use of anthelmintics (Mickiewicz et al., 2021; Potârniche et al., 2021), which led to the emergence of anthelmintic resistance and increased burden of the disease. In Kenya, there is no recent published information on helminthosis and coccidiosis, especially in areas where there are mixed intensive and extensive systems of production. Thus, the outcome of this investigation will provide baseline data on the prevalence, intensity, and risk factors associated with gastrointestinal parasites, hence informing proper control strategies among the goat farmers and policymakers. In recent years, there have been changes in production systems, the introduction of goats across different regions, the emergence of climate change and anthelmintic resistance, amongst other factors. For example, in Kenya, rearing of goats has increased in urban and peri-urban areas, and there has been a recent introduction of dairy goats in high-potential areas such as Kiambu County. A recent farmer-based study in Kiambu County, Kenya, highlighted diarrhea and helminthosis as the major conditions affecting these goats (Kagucia et al., 2020). However, no laboratory study was conducted to verify this information. The present study aimed to assess the prevalence, intensity, and associated risk factors of gastrointestinal parasites in goats reared by smallholder farmers in Juja Sub-county, Kiambu County, Kenya.

MATERIALS AND METHODS

Ethical approval

All animal and laboratory procedures were reviewed and ratified by Jomo Kenyatta University of Agriculture and Technology Institutional Scientific and Ethical Review Committee (JKU/ISERC/02317/1331).

Study area

The study was conducted in Juja Sub-County, Kiambu County, Kenya, which has five administrative wards, including Juja, Witeithe, Kalimoni, Theta, and Murera. Juja Sub-County lies between latitude 1°10'59" S and longitude 37°07'00"E**.** It has a human population of 139,853, which is growing rapidly due to proximity to urban areas such as Nairobi City, Juja Town, and Thika Town. The climate of the study area is tropical with an average annual rainfall of 840 mm (range from 468 mm to 900 mm) and average temperatures of 19.8^{o}C (ranging between 14°C-26^{o}C). Peri-urban livestock farming is common in the sub-county, with the latest census showing a population of 136,251 cattle, 17,300 pigs, 63,031 chickens, 32,000 sheep, and 12,699 goats (KNBS, 2019).

Study design and sample size

A cross-sectional survey was conducted between June and October 2024. The sample size for this study was determined using the Thrusfield formula and adjusted for small populations (Thrusfield, 2018). The expected prevalence of 75% based on the previous investigations (Waruiru, 2004) was used in the calculation. The minimum sample size was

162. In the current study, a total of 210 goats were sampled. The 210 goats were sampled using a snowballing technique from the 80 farms. The initial group of farmers provided by the local animal health providers and extension officers helped the investigators identify other farmers until the sample size was achieved. A maximum of 5 goats per farm were sampled.

Questionnaire survey

A structured questionnaire was administered to the household head to describe the farm characteristics and risk factors associated with the prevalence of GIT parasites. The questionnaire included questions on the origin of goats, sex, breed, age, goats' body condition score, and production system (intensive and extensive). However, the questionnaire of this study did not take into goats' diet. The investigators and animal health professionals assessed the body condition scores of the sampled goats. The questionnaire also covered aspects of farm management, history of helminthosis and reported clinical signs, the frequency of pen cleaning, deworming intervals and personnel involved, the types of anthelmintics used for treatment and their effectiveness, as well as the demographic details of the respondents. Animals were grouped as young (< 1 year) and adult (1 year and above). The body conditions of the goats were assessed as described by Kripali et al. (2010) and were grouped as good, medium, and poor.

Sample collection and analysis

Approximately 25 g of fresh fecal samples were collected per rectum following the procedure by Terfa et al. (2023). The fecal sample collected was labelled accordingly and placed in a cool box with ice and transported to the parasitology laboratory at Jomo Kenyatta University of Agriculture and Technology (JKUAT) for parasitological analysis. Briefly, the fecal samples were analyzed following standard parasitological procedures. Saturated sodium chloride (SG 1.20; Analytical grade, Sigma-Aldrich, MO, USA) was used as the flotation medium. Eggs/oocysts counts were analyzed using a modified McMaster technique (Whitlock Universal Slide, Australia) and examined under 10× magnification using an Olympus B 201 microscope (Optical element Corporation, Milvile, USA). Samples containing strongyle eggs were subjected to fecal culture, and the resulting larvae, which were harvested using the Baermann technique, were identified to the species level based on morphological features as described by MAFF (1986). Additionally, oocysts in the fecal samples were sporulated using 2.5% (w/v) potassium dichromate and identified to species level based on morphological features of the sporulated oocysts (Abunemeh, 2016).

Statistical analysis

The collected data were entered and managed in an MS Excel (Microsoft, USA) worksheet, and the analysis was conducted using SPSS statistical software version 17.0. Descriptive analysis was conducted, and thereafter, data were provided in the form of tables. The relationship between the independent factors and the prevalence of various GIT parasites was assessed using the Pearson Chi-square. Significance level was set at $p < 0.05$.

RESULTS

Characteristics of the respondents and farms

The farmers who participated in the study originated from Kalimoni (37.5%), Juja (20.0%), Theta (16.25%), Witeithie (13.75%), and Murera (12.5%) wards. In the wards, all participating farmers raised goats (100%), and also kept cattle (53.8%), sheep (67.5%), and poultry (56.25%) in their farms. The goats were mainly kept under an extensive system of production (73.8%). The farmers owned between 5 and 110 cattle and 2 to 260 sheep per farm. Most of the respondents were married men (70.0%), aged between 18-35 years (55.0%) in Table 1. The small East African (SEA) goat was the most common breed kept by farmers. Most farmers (46.3%) owned between 1 and 20 goats, while a few (25.0%) had 51 to 100 goats. The goats were housed in simple wooden structures (82.5%), and pens were cleaned with varying frequency, with 43.8% of farmers reporting irregular cleaning schedules.

History of helminthosis, deworming, and clinical signs

Over half (55%) of respondents reported a history of worm infections in their goats, with the most frequently observed symptoms being the presence of worms/segments in the feces (25.1%) and diarrhea (21.25%). In contrast, death and mucus presence were much less reported, each cited by only 2.5% of respondents (Table 2). The majority (83.8%) of farmers administered deworming treatments by themselves, while only 10% sought help from animal health professionals. When determining drug dosages, 52.8% of respondents estimated body weight visually, and 38.75% followed the instructions indicated on the drug bottle label or sachet. Farmers primarily relied on synthetic dewormers, including Nilzan® (Levamisole HCI and Oxyclozanide, Cooper Kenya Ltd; 38.75%), Ivermectin® (Avermectins,

Boehringer Ingelheim, Germany, 28.75%), Alben® (Albendazole, AdvaCare Pharma, India) (25%), Force One® (Levamisole HCI and Oxyclozanide, HighChem, Kenya; 16.25%), Levafas® (Levamisole HCI and Oxyclozanide, Norbrook, Kenya; 6.25%), Diamond® (3.0% Levamisole HCI and Oxyclozanide, Norbrook, Kenya; 5%) and Nefluk ® (Albendazole, Cooper Kenya; 3.75%).

Prevalence, spectrum, and intensity

The overall animal-level prevalence of GIN and coccidia was 64.29% and 74.29%, respectively. The farm-level prevalence of GIN and coccidia was 56.25% and 70.00%, respectively. The mean eggs per gram (EPG) was 67.72 (0-5600) and oocysts per gram (OPG) was 243.19 (0-7000**),** respectively. The infection rate reported for GIN in the sampled goats includes *Haemonchus contortus* (40.0%), *Trichostrongylus* spp. (28.6%), *Strongyloides papillosus* (19.5%), *Ostertagia* spp. (9.5%), and *Nematodirus spathiger* (2.4%), while the coccidia species included Eimeria *caprina* (43.3%), *Eimeria ninakohlyakimovae* (29.1%), and *Eimeria hirci* (27.6%), as shown in Table 3. The mixed infection rate recorded was 67.6%. In goats**,** the proportional distribution of the larvae from the five identified gastrointestinal nematode genera was *Haemonchus contortus* (39.4%), *Trichostrongylus* spp. (26.9%), *Strongyloides papillosus* (21.3%), *Ostertagia* spp. (11.1%), and *Nematodirus spathiger* (1.3%), while the coccidia species included *Eimeria caprina* (47.1%), *Eimeria ninakohlyakimovae* (28.5%), and *Eimeria hirci* (24.4%) presented in Figure 1. Among the GINs, *H. contortus* was the most frequently identified species in larval cultures, whereas *E. caprina* was the predominant species among the infective oocysts.

Table 1. Characteristics of farmers and goats' management in Juja Subcounty, Kenya during 2024

Variable	Category	Frequency	Percentage
Origin (ward) of farmers	Kalimoni	30	37.5
	Juja	16	20.0
	Theta	13	16.25
	Witeithie	11	13.75
	Murera	10	12.5
Gender of farmers	Male	56	70.0
	Female	24	30.0
Marital status of the respondent	Married	67	83.8
	Single	13	16.3
Age of farmers	18-35 years	44	55.0
	36- 50 years	22	27.5
	51 years and above	14	17.5
Production systems	Extensive	59	73.8
	Intensive	21	26.3
Goats' breeds	SEA	146/210	69.5
	Toggenburg	52/210	24.8
	Galla	2/210	0.95
	Boer	7/210	3.3
	Alpine	3/210	1.4
Number of goats	1-20	37	46.3
	21-50	23	28.8
	51-100	20	25.0
Housing (floor) for goats	Concrete	3	3.8
	Slighted	11	13.8
	Other	66	82.5
Frequency of pen cleaning	Daily	6	7.5
	Every 2-3 days	16	20.0
	Weekly	23	28.8
	Other	35	43.8

SEA: Small East African goat

Table 2. Occurrence of helminthosis, clinical signs, and goats management as reported by respondents in Juja Sub-County, Kenya during 2024

Variables	Categories	Frequency	Percentage
History of helminthosis	Yes	44	55.00
	No	36	45.00
Clinical signs reported	Diarrhea	17	21.25
	Weight loss	3	3.75
	Worm in the stools	20	25.1
	Presence of mucus	2	2.5
	Loss of appetite	4	5.0
	Death	2	2.5
	Rough haircoat	14	17.5
	No clinical signs	18	22.5
History of deworming	Yes	75	93.75
	No	5	6.25
Deworming interval	Every 3 months	46	57.50
	4< 6 months	13	16.25
	>6 months	11	13.75
	Every month	5	6.25
	Could not remember	5	6.25
Person responsible for deworming	Farmers (owner)	67	83.75
	Animal health professionals	8	10.00
	Could not remember	5	6.25
Determination of the dose to administer to the animals	By estimation of weights	36	45.00
	By the indications on the bottle/sachet	31	38.75
	Other methods	13	16.25
Anthelmintics used in treatment	Nilzan® (levamisole HCI and 6.0% Oxyclozanide)	26	38.75
	Ivermectin® (Avermectins)	18	28.75
	Alben® (Albendazole)	15	25.00
	Force One® (Levamisole HCI and Oxyclozanide)	11	16.25
	Levafas® (Levamisole HCI and Oxyclozanide)	5	6.25
	Diamond® (Levamisole HCI and Oxyclozanide)	3	5.00
	Nefluk ® (Albendazole)	2	3.75
Effectiveness of the anthelmintic	Effective	65	81.25
	Not effective	7	8.75
	Not sure	8	10.00

Table 3. Prevalence of gastrointestinal nematodes and coccidia in goats kept by smallholder farmers in Juja Sub-County, Kenya during 2024

Parasites	Affected goats (n)	Prevalence (%)	EPG Range	Mean	SEM
Nematodes species					
Haemonchus contortus	84(84/210)	40.0	0-5600	130.24	32.328
Trichostrongylus spp.	60(60/210)	28.6	0-3100	148.81	22.773
Strongyloides papillosus	41(41/210)	19.5	0-1000	53.33	10.719
Ostertagia spp.	20(20/210)	9.5	0-600	15.71	4.769
Nematodirus spathiger	5(5/210)	2.4	0-300	3.42	2.270
Mixed infections	142(142/210)	67.6			
Coccidians species			**OPG Range**	**Mean**	**SEM**
Eimeria caprina	91(91/210)	43.3	0-7000	462.43	60.736
Eimeria ninakohlyakimovae	61(61/210)	29.1	0-3500	143.86	27.487
Eimeria hirci	58(58/210)	27.6	0-4000	127.14	25.932

EPG: Eggs per gram; OPG: Oocysts per gram; SEM: Standard error of the mean

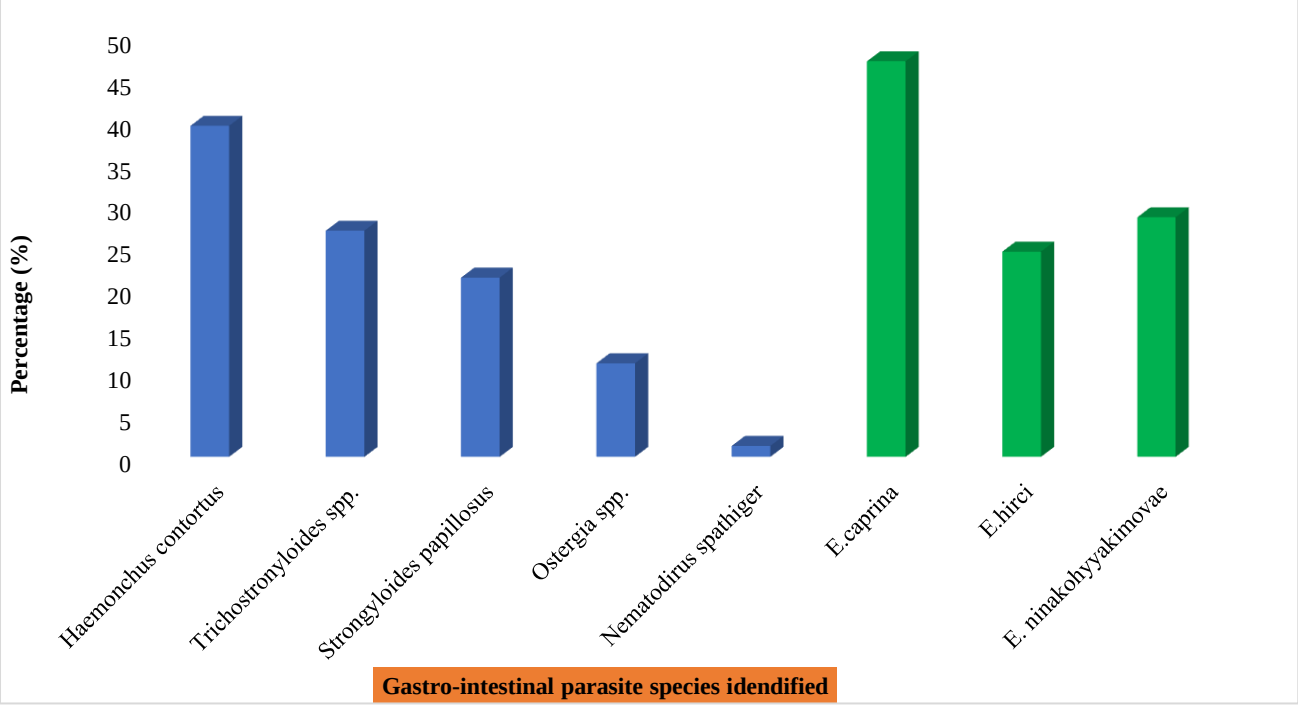


Figure 1. Proportion of the gastrointestinal tract identified in the sampled goats in Juja Subcounty, Kenya, during 2024

Assessment of caprine gastrointestinal parasites

The intensity of gastrointestinal parasite infection in goats was categorized into four levels and is presented in Table 4. Among the infected goats, 55.71% exhibited a light infection, followed by 29.05% with a moderate, and 3.81% with a heavy GIP infection. Additionally, 11.43% of the goats showed no signs of infection.

The prevalence and risk factors associated with gastrointestinal tract nematodes

The prevalence of GIN was significantly associated with age, production systems, and body condition score (Table 5). Adult goats had a significantly higher (70.1%) prevalence of GIN compared to the young ones (54.2%, p = 0.025). Further, goats kept under the extensive system of production had a significantly lower (63.1%) prevalence of GIN compared to those kept intensively (66%) (p = 0.043). Goats with good body condition scores had a significantly lower prevalence of GIN compared to those with poor body condition scores (83.8%, p = 0.023). The occurrence of GIN was not significantly influenced by the ward of origin (p = 0.405), sex (p = 0.664), production purpose (p = 0.106), or cleaning frequency (p = 0.678, Table 4).

The prevalence of gastrointestinal tract coccidia and the associated risk factors

The prevalence of coccidia was significantly associated with the origin of the goat (p = 0.049), being lowest in Kalimoni Ward (70.69%) and highest in Murera Ward (85.70%). Among breeds, SEA goats had a significantly (p= 0.023) lower prevalence (73.1 %) compared to Alpine and Galla (100.00%), Toggenburg (77.43%), but higher than Boer goats (57.1%). Adult goats had a significantly (p = 0.007) lower prevalence (67.7 %) compared to younger goats (84.3%, Table 6). Goats with a good body condition score had a significantly (p = 0.02) lower prevalence (45.6%) compared to those with poor body conditions (97.3%). Additionally, goats housed in pens cleaned daily had a significantly (p = 0.049) lower prevalence of coccidia (33.1 %) than those in pens cleaned less frequently (80.2%, Table 5).

Table 4. Assessment of the infection degree of caprine gastrointestinal parasites

Infection degree	Frequency	Percentage	Mean	Std. deviation
No infection	24	11.43	0.25	0.68
Light	117	55.71	1.66	0.49
Medium	61	29.05	3.28	0.45
Heavy	8	3.81	5.25	.89
Total	210	100		

Std. deviation: Standard deviation

Table 5. The association between prevalence and risk factors of gastrointestinal nematodes in goats kept by farmers in Juja sub-county, Kenya

Risk factors	Categories	Prevalence	p-value
Ward of origin of goats	Kalimoni	66.38	0.405
	Theta	60.00	
	Witeithie	58.33	
	Juja	80.95	
	Murera	42.86	
Age	Adult	70.10	0.025*
	Young	54.20	
Production systems	Extensive	63.10	0.043*
	Intensive	66.00	
Body condition Score	Good	47.40	0.023*
	Medium	65.50	
	Poor	83.80	
Sex	Males	62.20	0.664
	Females	65.63	
Production purpose	Milk	72.92	0.106
	Meat	63.11	
	Dual purpose	57.50	
Frequency of cleaning	Daily	33.33	0.678
	Every 2-3 days	54.29	
	Weekly	64.44	
	Others	70.69	

***** Statistically significant at $p < 0.05$

Table 6. The association between prevalence and risk factors of coccidiosis in goats kept by farmers in Juja Sub-County, Kenya

Risk factor	Categories	Prevalence	p value
Ward of origin of goats	Kalimoni	70.7	0.049*
	Theta	71.4	
	Witeithie	83.3	
	Juja	81.0	
	Murera	85.7	
Breeds	SEA	73.1	0.023*
	Galla	100.0	
	Boer	57.1	
	Toggenburg	77.4	
	Alphine	100.0	
Age	Adult	67.7	0.007*
	Young	84.3	
Sex	Males	74.4	0.574
	Females	71.1	
Production purpose	Milk	79.6	
	Meat	74.4	0.932
	Dual purpose	70.0	
Production systems	Extensive	72.5	0.292
	Intensive	80.0	
Body condition Score	Good	45.6	0.020*
	Medium	81.0	
	Poor	97.3	
Frequency of cleaning	Daily	33.3	0.049*
	Every 2-3 days	68.6	
	Weekly	75.6	
	Other	80.2	
	Could not remember	50.0	

***** Statistically significant at $p < 0.05$.

DISCUSSION

In Kenya, goat rearing offers numerous advantages, including being a valuable source of food security, income, employment, and for socio-cultural purposes in rural areas. However, GIT parasites such as helminths and coccidia affect the health and productivity of these goats. To ensure effective livestock health management, it is essential to continuously monitor the epidemiology of these parasites to enable proper intervention strategies.

Goat rearing in the area was small-scale, with the male gender being the main owners of the animals. Further, the current study noted that a significant proportion of the farmers were young, suggesting a shift toward youth engagement in agriculture, possibly driven by unemployment or agribusiness opportunities in peri-urban areas. Most farmers were married farmers, and this may reflect the role of livestock as a family livelihood asset. Like other farmers in most developing countries, the small farmers in the study area also kept cattle, sheep, and poultry for the provision of food and income. The most commonly kept goat breed by respondents was the SEA. This could be attributed to its resilience to diseases, tolerance to harsh environmental conditions, affordability, availability, and relatively high reproductive performance (Odoi et al., 2007). However, as shown in other studies (Shivairo et al., 2013; Kagucia et al., 2020; Mutunga et al., 2023), a substantial number of farmers also kept exotic goats such as Toggenburg, Galla, Boer, and Alpine, possibly for milk production.

Goats were typically housed in simple wooden structures, with pen cleaning occurring irregularly for most farms. This irregular cleaning can provide a good environment for the growth and development of parasites observed in the study. Some farmers also kept the goats under intensive production systems, where goats were confined and fed cut forages and crop residues, and supplemented with concentrates. Kagucia et al. (2020) also observed that intensive systems were favored by dairy goat farmers involved in commercial milk production. Farmers in this study reported that their goats had a history of helminth infections, showing symptoms such as diarrhea, weight loss, rough coats, and, in some cases, death. While these signs are commonly linked to parasitic diseases, they are not definitive indicators and can be compounded by poor nutrition (Kagucia et al., 2020). Additionally, farmers are generally unaware of the availability of laboratory services for fecal screening to assess worm burdens. Poor nutrition or resource constraints can weaken an animal's immune response, making them more susceptible to higher helminth burdens (Sweeny et al., 2021).

The current study shows that the prevalence of GIN was comparatively higher than that reported in Kenya at 62% (Maingi et al., 2001), Ethiopia at 54.17% (Hussein et al., 2023), Uganda at 43.0% (Nsereko et al., 2015), and Nigeria at 26.6% (Adamu and Kyari, 2023). Further, the prevalence was lower than that reported by Waruiru (2004) and Maichomo et al. (2004) in extensively reared goats from Kenya. Elsewhere, in Uganda, a high prevalence of 74.5% has been reported, with gastrointestinal nematodes being the predominant parasites (Namutosi and Higenyi, 2019). In another study in Tanzania, Haule (2015) reported a moderate prevalence (58.1%) of helminths such as nematodes, trematodes, and cestodes. Similarly, Hussein et al. (2023) reported a slightly higher prevalence (54.2%) of gastrointestinal parasite infection in Ethiopian goats. The persistently high prevalence of GIN in the current study area, in general, can be attributed to inadequate health management practices, such as poor deworming protocols, the co-grazing of ruminants, and variations in agro-ecological conditions (Maichomo et al., 2004). Additionally, suboptimal nutritional practices in the study area (Kagucia et al., 2020) may exacerbate the infection burden, since inadequate nutrition can impair the animals' ability to withstand the adverse effects of worm infestations (Sweeny et al., 2021).

The spectrum of strongyles species that were reported included *H. contortus*, *Trichostrongylus* sp., *Strongyloides papillosus*, and *Ostertagia* spp. A similar range of parasites has been reported by Waruiru et al. (2000) and Maichomo et al. (2004) from Kenya and Hussein et al. (2023) from Ethiopia. The most common gastrointestinal nematode identified in the present study was *H. contortus*, which previous studies have also cited as a major nematode affecting goats in the tropics (Bakunzi et al., 2013; Ntonifor, 2013; Zvinorova et al., 2016). The high prevalence could be attributed to the parasite's prolific egg-laying capacity, which facilitates rapid pasture contamination and subsequent transmission among grazing animals (Roeber et al., 2013). Additionally, *H. contortus* is well recognized for its strong propensity to develop resistance to anthelmintic drugs, posing significant challenges to effective parasite control (Kotze and Prichard, 2016). Generally, strongyle infections in goats contribute to substantial economic losses, primarily through reduced productivity and increased mortality (Arsenopoulos et al., 2021). Reduced productivity is associated with decreased feed intake, poor weight gain, diminished work performance, and the added financial burden of treatment and parasite control efforts (Ayaz et al., 2018). Species, such as *Haemonchus* and *Trichostrongylus* are particularly pathogenic, causing anemia and hypoproteinemia in goats due to chronic intestinal blood loss and their blood-feeding behavior (Githigia et al., 2001; Paul et al., 2020).

Identifying risk factors associated with the occurrence of gastrointestinal (GIT) parasites is crucial for developing effective intervention strategies. In this study, the prevalence of GIN was significantly linked to age, body condition score, and production system. Age-related analysis showed that adult animals had significantly higher GIN infestations

compared to young animals. This could be due to lower infections in young ones who were still suckling. Similar findings were reported in studies conducted in Ethiopia (Dabasa et al., 2017), Malaysia (Yusof and Isa, 2016), and Lesotho (Matsepe et al., 2021). However, the studies in Kenya (Githigia et al., 2005), Zimbabwe (Zvinorova et al., 2016), Ethiopia (Dagnachew et al., 2011), and Pakistan (Ayaz et al., 2013) contradicted this observation, reporting a higher prevalence of GIN in young animals compared to adults. These studies attributed the lower prevalence in adults to acquired immunity developed through repeated exposure, which enables them to expel new infestations more effectively.

In this study, goats with poor body condition exhibited a higher prevalence of GIN compared to those in good body condition, aligning with the findings from previous studies (Fayisa et al., 2020; Hussein et al., 2023). Malnourished animals are less capable of fighting infections, whereas well-fed animals are unlikely to succumb to the disease under ideal environmental conditions (Johnson et al., 2020). Further, infection of small ruminants with strongyles can result in gastroenteritis, protein-losing enteropathy, poor weight gain, and loss of body condition. Poor nutritional status compromises the animal's immune response, making malnourished goats more susceptible to parasitic infections. In contrast, well-nourished animals are generally more resilient and less likely to develop severe disease, particularly under favorable environmental conditions (Johnson et al., 2020). Moreover, strongyle infections in small ruminants are known to cause gastroenteritis, protein-losing enteropathy, reduced weight gain, and deterioration in body condition (Paul et al., 2020). Farmers and animal health professionals should be encouraged to use body scoring, among other tools, to diagnose helminthosis and other conditions.

In the current study, goats in extensive systems had a high worm burden, and this could be due to increased infections from contaminated pastures. In some cases, extensive production systems have been characterized by poor nutrition, inadequate hygiene conditions, frequent exposure to contaminated grazing areas, and insufficient health control measures (Zvinorova et al., 2016). The lower infection rates in intensive systems can be attributed to better management practices, including improved housing, feeding, and healthcare (Kagucia et al., 2020).

The present study recorded a high prevalence of coccidian infections in the examined goats, above the rates reported by Rehman et al. (2011) in Pakistan (47.09%) and Kimbita et al. (2009) in Tanzania (64.2). However, the prevalence observed was lower than that reported by Wang et al. (2010) in China (87.9%). The coccidian species identified in the study included *E. caprina*, *E. ninakohlyakimovae*, and *E. hirci.* There is a wide variation in the species of *Eimeria* found in goats in different countries. In Turkey, Deger et al. (2003) found *E. ninakohlyakimovae* (36.00%), *E. hirci* (23.42%), and *E. caprina* (18.28%) were detected. Similarly, in Turkey, Goz et al. (2006) reported *E. ninakohlyakimovae* (18.9%) and *E. hirci* (7.5%). In Iran, Kheirandish et al. (2012) identified *E. ninakohlyakimovae* (41.8%), *E. caprina* (31.7%), and *E. hirci* (11.05%) were detected. In Brazil, Cavalcante et al. (2012) detected *E. hirci* (18%), *E. ninakohlyakimovae* (16.2%), *E. caprovina* (2.8%), and *E. caprina* (1%) were detected.

Coccidiosis is recognized as an economic disease affecting goats (Yusof and Isa, 2016), often leading to chronic diarrhea, resulting in dehydration and loss of essential electrolytes and nutrients, further impairing growth and productivity. It also damages the gut lining, causing protein loss through leakage into the intestinal lumen (Agrawal et al., 2024). This disease results in reduced muscle mass and poor overall growth performance in goats.

In the present study, the prevalence of coccidia was significantly associated with origin, age, breed, body condition score, and cleaning frequency. A significant variation in coccidiosis infection rates was observed based on wards. Goats in areas closer to urban centers had higher infection rates compared to those farther away. For instance, Kalimoni Ward, situated farther from Juja Town, exhibited a lower infection rate compared to Juja, Witeithie, and Murera Wards, which are located closer to the town. This is possibly because most farming production systems in urban Juja are intensive, driven by limited land availability. These systems are often associated with poor sanitation and low levels of hygiene, which can contribute to higher coccidiosis infection rates. The higher prevalence in young animals compared to adult animals has been reported previously (Negasi et al., 2012). Young animals are highly vulnerable to coccidian and face a greater risk of exposure to oocysts shed by the dams. This increased susceptibility is likely due to their immature and underdeveloped immune systems. In contrast, adult animals typically develop immunity to coccidia, allowing them to thrive despite exposure (Paul et al., 2020).

In this study, the prevalence of coccidia infection was higher in Toggenburg breeds compared to other breeds, like SEA goats. Disparities in gastrointestinal parasitic infections among dairy goats have also been documented in Brazil, Tanzania, and Kenya (Costa et al., 2000; Odoi et al., 2007; Shija et al., 2014). The higher prevalence of coccidia among Toggenburg in this study could be attributed to the increased susceptibility of this exotic breed to coccidiosis. This study revealed a significant association between coccidia infection and the body condition of goats, with a higher prevalence observed in animals with poor body condition compared to those in better condition. Poor body condition could be attributed to factors, such as malnutrition, concurrent diseases, or the parasitic infection itself, which weakens the immune response to the parasite's infective stages. Farms in the study had varying hygiene standards, which impacted coccidia abundance. Pens that were cleaned daily had lower coccidiosis infection rates, while those cleaned less

frequently exhibited higher rates. Effective management practices, such as maintenance of cleanliness, are essential preventative measures to control coccidiosis.

In the current study, a large proportion of goats had combined helminth and coccidia infections, which was also observed in an earlier study in Kenya (Waruiru et al., 2000). It is possible that the combined infections of nematodes and *Eimeria* spp. affected the animals negatively in terms of health and productivity. It would be important for extension officers to advise farmers on the combined control of co-infections of helminthosis and coccidiosis.

CONCLUSION

The current study indicated that the prevalence and intensity of GIT helminthosis and coccidiosis are high in the study area and were closely associated with age, sex, body condition score, production purpose, breeds, production systems, and frequency of cleaning. The high infection rate of gastrointestinal nematodes (64.29%) and coccidia (74.29%) highlights potential shortcomings in current parasite control programs, as well as issues related to the animals' nutrition and management in the study area. Since helminthosis and coccidiosis often manifest similar clinical signs, farmers and animal health professionals often manage the condition as helminthosis, and this can lead to a high burden of the disease in goats. Understanding the specific species of gastrointestinal parasites and related epidemiological factors will aid in developing targeted control strategies, potentially reducing production losses and enhancing rural livelihoods. There is a need for improved collaboration between livestock extension officers and farmers to enhance effective health care programs that support regular parasite control practices on farms.

DECLARATIONS

Funding

This study was funded by the Bioinnovate Africa Project.

Ethical considerations

The author has checked the ethical issues, including plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy.

Authors contributions

John Kagira conceptualized the study design, supervised the laboratory analyses, and proofread the final manuscript. Christopher Odinga conducted the study, collected data, and performed data analysis. Patrick Kamundia performed data analysis and contributed to the preparation of the first draft of the manuscript. Naomi Maina and Isaac Osuga contributed to the manuscript writing, proofreading, and preparation, supported the study design, data interpretation, and manuscript drafting. All authors reviewed and approved the final version of the manuscript for submission.

Acknowledgments

The authors of the article are grateful to the Bio Innovate Africa Project for funding this study. Much appreciation also to all of the farmers of Juja subcounty for voluntarily participating in the study.

Availability of data and materials

Inquiries regarding data availability can be directed to the corresponding author.

Competing interests

The authors declare there is no conflict of interest.

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2025, Scienceline Publication
World's Veterinary Journal
World Vet J, 15(3): 669-677.
ISSN 2322-4568

DOI: https://dx.doi.org/10.54203/scil.2025.wvj67
PII: S232245682500067-15

Effects of Different Levels of Raisins Pomace Powder on Productive Performance and Blood Parameters in Broiler Chickens

Atheer Salih Mahdi

Department of Animal Production, Faculty of Agriculture, University of Kufa, Iraq

*Corresponding author's Email: Atheers.mahdi@uokufa.edu.iq

ORIGINAL ARTICLE
Received: June 27, 2025
Revised: July 26, 2025
Accepted: August 31, 2025
Published: September 25, 2025

ABSTRACT

One of the primary concerns in poultry farming is the exploration of natural feed ingredient alternatives that are, concurrently, more cost-effective and environmentally sustainable. Raisin pomace powder (RPP), a by-product from grape processing, may contain numerous beneficial nutrients and natural compounds that contribute to optimal performance in chickens. The present study assessed the impact of incorporating RPP into broiler chicken diets concerning growth performance, carcass characteristics, economic efficiency, blood parameters, and immune indices. A total of 180 unsexed Ross 308 broiler chickens, all one day old with an initial average body weight of approximately 38 g, were randomly divided into four dietary treatments. The present study comprised a control group (T1) and three experimental groups, including 10 g/kg of RPP (T2), 20 g/kg of RPP (T3), and 30 g/kg of RPP (T4). The study duration spanned 35 days, covering both the initial and finisher phases. The present results demonstrated no statistically significant differences in body weight, weight gain, feed intake, dressing percentage, or economic efficiency among the treatment groups. However, the feed conversion ratio was notably higher in T2 in comparison to the control group. Hematological parameters, encompassing packed cell volume, hemoglobin concentration, as well as red and white blood cell counts, were not significantly influenced by the inclusion of RPP. Serum levels of total protein and globulin remained stable across groups, whereas albumin levels exhibited a significant decrease in T4. Additionally, cholesterol levels were reduced in T3 compared to T1. The current findings suggested that RPP can be included in broiler diets up to 30 g/kg without adverse effects on productivity, physiological status, or immune development. Utilizing RPP provided a sustainable strategy for poultry nutrition through the employment of agro-industrial by-products, thereby potentially improving feed resource efficiency and environmental sustainability.

Keywords: Broiler chicken, Bursa of Fabricius, Economic efficiency, Feed conversion ratio, Productive performance, Raisin pomace powder

INTRODUCTION

The increasing global demand for poultry meat and eggs, combined with intensified competition for traditional feed ingredients such as soybean meal and maize, underscores the importance of exploring sustainable alternatives (Alshelmani et al., 2021). In poultry farming, natural feed additives are increasingly employed to enhance growth performance and overall productivity (Kairalla et al., 2022a). Furthermore, concerns regarding the residual effects of antibiotic growth have led to the development of alternative additives designed to improve chicken performance (Kairalla et al., 2022b; Kairalla et al., 2023). Grapes (*Vitis vinifera*) represent one of the most economically significant crops worldwide, with an annual production exceeding 78 million tons (OIV, 2019; Čech et al., 2021). The food processing industry produces significant waste, with fruit processing alone generating large amounts of pomace, peels, and seeds (Muzaffar et al., 2022). The processing of grapes for juice, wine, and raisin production generates substantial by-products globally (Spanghero et al., 2009; Mewa-Ngongang et al., 2019). By-products are rich in beneficial compounds, such as carotenoids, polyphenols, and vitamins, which can be extracted to use as functional foods and nutraceuticals (Ritika et al., 2024; Boruah and Ray, 2024). Sustainable utilization of these by-products can create new industry revenue streams, promote a circular economy, and reduce waste (Foti et al., 2022; Boruah and Ray, 2024). Several compounds, such as antioxidants, flavonoids, and polyphenols, can enhance oxidative stability and promote beneficial gut bacteria in poultry, contributing to overall health (Azizi et al., 2018; Kumanda et al., 2019). The presence of tartaric acid and prebiotic fructooligosaccharides in raisin by-products may positively influence the gut microbiota, supporting their use as functional feed additives in poultry diets (Al-taee et al., 2014). Due to increasing concerns regarding synthetic antioxidants in poultry diets, attributable to their potential toxicity, carcinogenicity, and accumulation in edible tissues, there is a rising interest in investigating safer and natural alternatives (Mousa et al, 2020; Garg, 2024).

To cite this paper: Mahdi AS (2025). Effects of Different Levels of Raisins Pomace Powder on Productive Performance and Blood Parameters in Broiler Chickens. *World Vet. J.*, 15(3): 670-677. DOI: https://dx.doi.org/10.54203/scil.2025.wvj67

Among these, phenolic compounds derived from grape pomace have garnered significant attention, not only for their antioxidant properties but also as an environmentally sustainable solution for the management of grape processing waste, which comprises approximately 20% of the total grape weight (Llobera and Canellas, 2007). Improper disposal of raisin pomace in landfills can cause environmental pollution by producing greenhouse gases and leachate, increasing the risk of soil and water contamination (Limeneh et al., 2022).

The bioactive composition of grape pomace, including high levels of polyphenolic compounds, along with 25-35% crude cellulose, 4-10% hemicellulose, 5-6% pectin, 8-14% crude protein, 4-10% crude fat, and 30-45% non-nitrogenous substances, supports its potential as a functional ingredient in poultry feed, providing an eco-friendly and cost-effective way to manage pomace waste (Kara et al., 2016; Kasapidou et al., 2016).

Despite increasing interest in the utilization of fruit by-products in animal nutrition, studies particularly evaluating the use of raisin pomace in poultry diets remain limited. The present study aimed to assess the effects of incorporating different levels of raisin pomace into broiler chickens' diets on growth performance as well as hematological and biochemical blood parameters.

MATERIALS AND METHODS

Ethical approval

The present study received approval from the Central Committee of Research Ethics at the University of Kufa, Iraq, pursuant to the decision made during the fifth session held on 12/07/2023. Ethical clearance was obtained for the project, and the study was carried out in strict accordance with the approved institutional ethical standards (Official Approval Code: 7121).

Raisin pomace preparation

Raisin pomace, which includes skins, seeds, and pulp residues, was collected daily from juice shops in Baghdad, Iraq, until enough quantity was gathered. Samples were immediately air-dried on clean concrete floors in a well-ventilated room at ambient temperature to preserve heat-sensitive compounds such as polyphenols. After drying, the material was sealed in airtight plastic bags and stored at approximately 5°C to maintain nutritional and microbial quality. Before use, the dried pomace was ground into a fine powder to ensure uniform mixing in the experiment diets.

Study area

The field experiment was conducted at the poultry farm of the Department of Animal Production, Faculty of Agriculture, University of Kufa, Iraq. The study commenced on July 20, 2023, and lasted for 35 days, concluding on August 24, 2023. A total of 180 unsexed one-day-old Ross 308 broiler chickens were obtained from Al-Anwar Hatchery in Babil Province and randomly assigned to the experimental treatments.

Experimental design

A total of 180 unsexed Ross 308 broiler chickens were assigned to the experimental design, which consisted of four dietary treatments, each replicated three times with 15 chickens per replicate, totaling 12 pens. The chickens were divided into four treatment groups, with T1 serving as the control group and receiving a basal diet without any additives, T2 was administered the basal diet supplemented with 10 g/kg raisin pomace powder (RPP), T3 was given the basal diet supplemented with 20 g/kg RPP, and T4 was given the basal diet supplemented with 30 g/kg RPP. Raisin pomace powder, after being dried and finely ground, was accurately weighed and manually mixed into the respective feed mixtures for treatments T2, T3, and T4. The supplemented diets were thoroughly blended to ensure homogeneous distribution of the additive throughout both the starter and finisher rations.

Chickens' management

With a total of 12 pens, each measuring 3 m^2, the stocking density was 5 birds/m^2, which complied with the commercial and welfare standards recommended for Ross 308 under moderate environmental conditions. The chickens were reared for a period of 35 days, during which they were subjected to a two-phase feeding regimen; an initial diet from day 1 to 21 and a subsequent finisher diet from day 22 to 35. Throughout the study, feed and clean water were provided *ad libitum*. Environmental conditions were managed following established protocols for broiler chicken management. The temperature started at 33-34°C during the first week and was gradually decreased by 2-3°C each week until reaching 24°C by the end of the study. Relative humidity was kept between 55% and 65%. A lighting schedule of 23 hours of light and 1 hour of darkness was used for the first three days, then shifted to 20 hours of light and 4 hours of darkness from day four onward to enhance feed intake and growth performance.

The experimental diets were formulated in accordance with the National Research Council (NRC, 1994) nutrient requirements for broiler chickens. All diets were designed to be isocaloric and isonitrogenous, thereby ensuring a consistent nutritional intake throughout the duration of the trial.

Routine veterinary health management was applied uniformly across all groups. Vaccination was administered against Newcastle disease and infectious bronchitis on days 7 and 21. In addition, all diets were supplemented with the anticoccidial drug Decoquinate (Deccox®, Netherlands) at 30 ppm in the feed, provided from the first day of age until day 35 as a preventive measure against coccidiosis. Chickens were monitored daily for their health status and behavior. The control group was administered a basal diet, as delineated in Table 1.

Table 1. Percentage composition and chemical analysis of starter and finisher diets in broiler chickens

Ingredients	Starter (1-21 days)	Finisher (22-35 days)
Cracked yellow corn	50.50	58.00
Soybean meal	36.00	27.50
Cracked wheat	8.00	7.00
Premix*	2.50	2.50
Oil	1.50	3.50
Dicalcium phosphate**	0.1	0.1
Limestone	1.1	1.1
Salt	0.3	0.3
Total	100	100
Calculated chemical composition		
Metabolizable energy (kcal/ kg feed)	3015	3210
Crude protein (%)	21.11	19.58
Calcium (%)	1.102	1.08
Available phosphorus (%)	0.74	0.71
Energy: Protein	130.4	163.9

* A Jordanian-manufactured premix containing 4900 kcal/kg metabolizable energy, 18% crude protein, 1.1% fat, 15-19% calcium, 9.4% lysine, 6.8% phosphorus, 4.8% sodium, 5.8% chlorine, 7.8% methionine, 7.8% methionine + cystine, and 0.55% threonine. It contains vitamins and trace minerals to meet the bird's requirements. **Dicalcium phosphate contains 22% non-phytate calcium and 8% non-phytate phosphorus.

Data collection and trait determination

Growth performance

Data were collected at two key time points: The end of the starter phase (Day 21) and the end of the experiment on Day 35. A total of 12 chickens per treatment group (4 chickens per replicate) were randomly chosen at each interval for growth performance assessment. Live body weight (g) was recorded by weighing each chicken individually with a digital precision scale. Weight gain (g) was determined by the difference in body weight over specific intervals (0-21, 22-35, and 0-35 days). Feed intake (g) was calculated as the difference between the total feed offered and the residual feed per replicate, expressed on a per-chicken basis. Feed conversion ratio (FCR) was computed by dividing feed intake by weight gain.

Carcass traits

On day 35, 4 chickens per replicate were utilized for carcass evaluations. The dressing percentage (%) was determined after the chickens had undergone an 8-hour fasting period, followed by weighing, slaughtering, defeathering, and evisceration. The dressing yield was computed as (eviscerated carcass weight / live body weight) × 100.

Economic efficiency

The present study evaluated the economic efficiency by combining three key factors, including production efficiency, feed utilization, and rearing duration. For economic efficiency, the following formula was used. Economic efficiency = (Total marketed chickens' weight in kg × 10,000) / (Number of chickens reared × rearing period in days × FCR). This indicator demonstrates how well production inputs are converted into live body weight output. Higher values indicate better overall production and financial performance (Naji and Hanna, 1999).

Blood parameters

Blood samples were collected from the brachial vein into EDTA tubes for measuring hematological parameters, including hemoglobin (Hb, g/dL) using Sahli's method, packed cell volume (PCV, %) via microhematocrit centrifugation, and red blood cell (RBC) and white blood cell (WBC) counts employing Natt and Herrick's solution under a hemocytometer. For biochemical analysis, blood collected in plain tubes was subjected to centrifugation at 3000 rpm for 10 minutes, after which the serum was stored at -20°C until subsequent analysis. Parameters assessed with commercial kits (Biolabo, France) and a UV-Vis spectrophotometer included total protein and albumin (g/dL), measured

respectively by biuret and bromocresol green methods. Globulin (g/dL) was derived by subtracting albumin from total protein, while cholesterol (mg/dL) was determined using the CHOD-PAP enzymatic technique. All procedures adhered to veterinary clinical guidelines as outlined by Jain (1993).

Immune indices

On day 35, the chickens were humanely euthanized for examination of the bursa of Fabricius. The organ was removed, cleaned, and weighed using a digital scale (± 0.01 g). The relative weight of the bursa of Fabricius was calculated as (bursa weight / live body weight) × 100, and the bursal index was calculated using the same formula following the method of Lucio and Hitchner (1979).

Statistical analysis

The data were analyzed using one-way analysis of variance (ANOVA) to evaluate the effects of dietary treatments. When significant differences ($p < 0.05$) were observed, Duncan's multiple range test (Duncan, 1955) was applied to separate means. Statistical analyses were performed using SAS software, version 9.0 (SAS, 2012).

RESULTS

Live body weight

Body weight was measured individually at day 21 (Week 3) and day 35 (Week 5). No statistically significant differences were observed among the treatment groups at either time point ($p > 0.05$). The mean final body weights at day 35 for treatments T1, T2, T3, and T4 were 2318 g, 2253 g, 2303 g, and 2307 g per broiler chicken, respectively. Although there were minor numerical differences, statistical analysis indicated no significant treatment effects on the final live outcomes weight.

Weight gain

The present results indicated that there were no statistically significant differences in average weight gain among broiler chickens across all treatment groups during the intervals day 0 to 21 (Starter phase), day 22-35 (Finisher phase), and day 0-35 (Overall period; $p > 0.05$; Table 2). The total weight gains, which represent the overall increase in body weight from the start to the end of each specified period, were 2280 g, 2215 g, 2265 g, and 2269 g per chicken for treatments T1, T2, T3, and T4, respectively, by the end of the study (Day 35).

Dressing percentage

At the end of the experiment (Day 35), no statistically significant differences were observed in dressing percentage among broiler chickens across treatment groups ($p > 0.05$). The recorded dressing percentages were 73.0% for T1, 73.7% for T2, 73.6% for T3, and 74.0% for T4.

Table 2. Effects of using different levels of raisin pomace powder on live body weight, weight gain, and dressing percentage during the 35-day trial period in broiler chickens

Treatments	Live body weight (g)		Weight gain (g)			Dressing percentage
	Week 3	Week 5	0-3 weeks	4-5 weeks	0-5 weeks	
T1	1027 ± 161.00	2318 ± 44.94	989 ± 161.00	1292 ± 122.09	2280 ± 44.94	73.0 ± 10.2
T2	1045 ± 166.36	2253 ± 178.44	1007 ± 166.36	1208 ± 73.79	2215 ± 178.44	73.7 ± 8.0
T3	1053 ± 185.15	2303 ± 681.55	1016 ± 185.15	1249 ± 496.41	2265 ± 681.55	73.6 ± 5.9
T4	1025 ± 120.08	2307 ± 379.68	987 ± 120.08	1282 ± 259.61	2269 ± 379.68	74.0 ± 1.00
Significant	NS	NS	NS	NS	NS	NS

T1: Control (without addition), T2: 10g raisins pomace powder/ kg feed, T3: 20g raisins pomace powder/ kg feed, T4: 30g raisins pomace powder/ kg feed, NS: Non-significant

Feed intake

The current findings indicated that there were no statistically significant differences in feed intake among broiler chickens across all experimental periods by the end of the study ($p > 0.05$; Table 3). Treatment T3 recorded the highest total feed intake at 3760 g per chicken, while the control group (T1) indicated the lowest intake at 3573 g per chicken.

Feed conversion ratio

Broiler chickens during the starter phase (0-3 weeks) across all groups exhibited no significant differences ($p > 0.05$). However, during the finisher phase (4-5 weeks), a significant decline in FCR was observed in treatments T2 and T3 compared to the control group ($p < 0.05$), whereas treatment T4 did not demonstrate any significant difference compared to the control and other treatment groups ($p > 0.05$). Over the entire period (0-5 weeks), Treatment T2 demonstrated the lowest feed efficiency, with an FCR of 1.69 g feed/g weight gain, significantly higher than that of the control group (1.57 g feed/g weight gain; $p < 0.05$). Treatments T3 and T4 recorded FCR values of 1.66 and 1.63 g feed/g weight gain, respectively, and indicated no significant differences compared to the control group ($p > 0.05$).

Economic efficiency

The incorporation of different levels of RPP into broiler chickens' diets did not yield a statistically significant impact on the economic outcome across all treatment groups compared to the control group by the end of the study ($p > 0.05$; Table 3).

Hematological parameters

Based on the current results, no statistically significant differences were observed among the experimental treatments in PCV, Hb concentration, RBC, or WBC count in broiler chickens at 35 days of age ($p > 0.05$; Table 4).

Biochemical blood

The current findings indicated that there were no significant differences in serum total protein and globulin concentrations among broiler chickens across experimental groups ($p > 0.05$). However, serum albumin levels in treatment T4 were significantly lower compared to the control group and T2 ($p < 0.05$); no significant differences were detected among treatments T2, T3, and T4 ($p > 0.05$). Concerning serum cholesterol levels, no statistically significant differences were observed between treatments T2 and T4 in comparison to the control group ($p > 0.05$). Conversely, Treatment T3 demonstrated a significant decrease compared to the control group ($p < 0.05$; Table 5).

Relative weight of the bursa of Fabricius and bursal index

Based on the current results, there were no statistically significant differences in the relative weight of the bursa of Fabricius among all groups ($p > 0.05$). The recorded values were 0.142%, 0.182%, 0.169%, and 0.142% for treatments T1, T2, T3, and T4, respectively. Similarly, no significant differences were observed in the bursal index among broiler chickens across all groups at 35 days of age ($p > 0.05$). The bursal index values were 1.00, 1.29, 1.19, and 1.00 for treatments T1, T2, T3, and T4, respectively (Table 6).

Table 3. Effects of using different levels of raisin pomace powder in diets on feed intake, feed conversion ratio, and economic figures during the 35-day trial period in broiler chickens

Treatments	Feed intake (g)			Feed conversion ratio (g feed/ g weight gain)			Economic efficiency
	0-3 weeks	4-5 weeks	0-5 weeks	0-3 weeks	4-5 weeks	0-5 weeks	
T1	1494 ± 201.25	2079 ± 321.99	3572 ± 248.20	1.51 ± 0.07	1.61 ^{b} ± 0.13	1.57 ^{b} ± 0.07	423 ± 17.58
T2	1599 ± 588.98	2135 ± 194.54	3734 ± 583.61	1.58 ± 0.27	1.77 ^{a} ± 0.07	1.69 ^{a} ± 0.27	382 ± 11.27
T3	1601 ± 223.38	2159 ± 321.99	3760 ± 509.82	1.58 ± 0.13	1.74 ^{a} ± 0.40	1.66 ^{ab} ± 0.27	397 ± 183.80
T4	1532 ± 771.44	2169 ± 321.99	3701 ± 409.20	1.55 ± 0.13	1.69 ^{ab} ± 0.20	1.63 ^{ab} ± 0.20	404 ± 112.03
Significant	NS	NS	NS	NS	*	*	NS

T1: Control (without addition), T2: 10g raisins pomace powder/ kg feed, T3: 20g raisins pomace powder/ kg feed, T4: 30g raisins pomace powder/ kg feed. *$^{a, b}$ Different superscript letters vertically indicate a significant difference ($p < 0.05$). NS: Non-significant.

Table 4. Effects of using different levels of raisin pomace powder on haematological indices during the 35-day trial period in broiler chickens

Treatments	Packed cell volume (%)	Hemoglobin (g/dl)	Red blood cells ($\times 10^6$/ mm^3)	White blood cells ($\times 10^3$ / mm^3)
T1	29.23 ± 3.42	10.20 ± 6.98	2.28 ± 2.21	25.96 ± 32.74
T2	33.26 ± 36.22	8.97 ± 2.41	2.86 ± 3.09	21.80 ± 46.15
T3	32.30 ± 17.64	9.60 ± 5.43	2.79 ± 1.81	28.30 ± 8.39
T4	31.25 ± 1.34	9.20 ± 1.54	2.75 ± 0.07	26.05 ± 14.09
Significant	NS	NS	NS	NS

T1: Control (without addition), T2: 10g raisins pomace powder/ kg feed, T3: 20g raisins pomace powder/ kg feed, T4: 30g raisins pomace powder/ kg feed, NS: Non-significant.

Table 5. Effects of using different levels of raisin pomace powder on the blood biochemical indices during the 35-day trial period in broiler chickens

Treatments	Total protein (g/dL)	Albumin (g/dL)	Globulin (g/dL)	Cholesterol (mg/ dL)
T1	3.81 ± 0.47	2.28 [a] ± 0.87	1.53 ± 0.40	159.04 [a] ± 21.47
T2	3.74 ± 2.75	2.25 [a] ± 0.74	1.49 ± 3.09	128.80 [ab] ± 130.94
T3	3.30 ± 0.80	2.02 [ab] ± 1.01	1.28 ± 1.41	106.58 [b] ± 95.59
T4	3.33 ± 0.40	1.77 [b] ± 1.01	1.55 ± 0.60	114.35 [ab] ± 90.69
Significant	NS	*	NS	*

T1: Control (without addition), T2: 10g raisins pomace powder/ kg feed, T3: 20g raisins pomace powder/ kg feed, T4: 30g raisins pomace powder/ kg feed. *[a, b] Different superscript letters vertically indicate a significant difference ($p < 0.05$). NS: Non-significant.

Table 6. Effect of using different levels of raisin pomace powder on the relative weights of the Bursa of Fabricius and bursal index during the 35-day trial period in broiler chicken

Treatments	Relative weights (%)	Bursal index
T1	0.142 ± 0.10	1.00 ± 0.000
T2	0.182 ± 0.09	1.29 ± 0.60
T3	0.169 ± 0.07	1.19 ± 0.55
T4	0.142 ± 0.13	1.00 ± 0.93
Significant	NS	NS

T1: Control (without addition), T2: 10g raisins pomace powder/ kg feed, T3: 20g raisins pomace powder/ kg feed, T4: 30g raisins pomace powder/ kg feed, NS: Non-significant.

DISCUSSION

The incorporation of RPP into the diet at a level of 10-30 g/kg did not have a significant impact on body weight gain in broiler chickens. The present findings are consistent with those of Jonathan et al. (2021) and Şen and Başalan (2022), which indicated that adding grape pomace at levels up to 60 g/kg did not adversely affect growth. Furthermore, Aditya et al. (2018) and Dupak et al. (2021) reported no adverse effects at a dosage of 10 g/kg. In contrast, Romero et al. (2022) identified growth inhibition at elevated inclusion levels, which was attributed to the potential tannin or fiber overload. Conversely, the consistent growth observed in the current study implied that raisin pomace, characterized by lower levels of anti-nutritional factors, may be more beneficial for the broiler chickens' performance. During the current study, feed intake remained statistically unchanged among all groups, which was consistent with the findings of Aditya et al. (2018) and Şen and Başalan (2022). Notably, FCR markedly improved in T2 (10 g/kg), in contrast to the findings of Kumanda et al. (2019), who found a decrease in FCR as grape pomace levels increased. The improvement might be due to enhanced digestibility or nutrient absorption, possibly linked to the bioactive compounds found in raisin pomace. Based on the current findings, the inclusion of RPP in broiler chickens' diets did not lead to significant changes in dressing percentage or carcass yield across all treatment groups. The present findings aligned with the results reported by Aditya et al. (2018) and Kumanda et al. (2019), who noted minimal impacts of grape pomace inclusion on carcass characteristics. Additionally, Aljumaili et al. (2023), who examined grape seed oil, an alternative grape by-product, observed no adverse effects on growth performance or carcass attributes. These results further supported the potential benefits of grape derivatives in poultry nutrition. However, Bennato et al. (2020) observed that slight improvements in carcass traits with grape pomace supplementation, which was attributed to increased antioxidant levels in the diet, and these differences may result from variations in the type and processing of the pomace used. Hematological parameters, including PCV, Hb, RBC, and WBC, remained stable across all groups. The present hematological finding was consistent with the findings of Pascariu et al. (2017) and Jonathan et al. (2021), who reported no significant changes in RBC, WBC, and PCV values when broiler chickens were fed grape pomace at different inclusion levels. These results closely align with the present findings using RPP, which confirmed that polyphenol-rich by-products such as grape or raisin residues do not negatively affect hematological stability, especially RBS, WBC counts, and PCV, even at higher dietary levels. Additionally, Aditya et al. (2018) reported consistent levels of glucose, HDL, and triglycerides with the use of grape pomace. Furthermore, no significant differences were observed in the weight or index of the bursa of Fabricius across different treatments during the present study. While Mavrommatis et al. (2021) indicated that grape pomace influenced immune gene expression, they did not examine its anatomical effects. Therefore, the present study assessed the anatomical impact of raisin pomace on the bursa of Fabricius, offering new evidence for its safety in supporting the immune system development. The inclusion of RPP at different dietary levels did not lead to significant

differences in the calculated economic efficiency among treatment groups. These findings aligned with those of Reyes et al. (2020), who reported similar results when assessing grape pomace in broiler chickens' diets, and the current findings indicated that RPP inclusion up to 30 g/kg did not negatively impact feed efficiency or growth performance, nor did it alter overall production efficiency. Notably, the present study was the first investigation to document the effect of RPP on performance assessment grounded in economic efficiency within poultry production, thereby presenting a novel contribution to the scholarly literature on functional feed additives.

CONCLUSION

Raisin pomace powder can be safely included in broiler chickens' diets at levels of up to 30 g/kg without negative effects on performance, health, or economic efficiency. The current findings not only supported the safe use of agro-industrial by-products in poultry nutrition but also offered new insights into the application of RPP, particularly concerning the bursal index and economic evaluation. Future studies are warranted to explore raisin pomace's functional roles in gut health, meat quality, antioxidant status, and large-scale economic implications.

DECLARATIONS

Authors' contributions

Atheer Salih Mahdi was responsible for developing the research idea, designing the experiment, collecting and analyzing the data, drafting the manuscript, and preparing the final edition for publication. All stages of the study and manuscript preparation were carried out independently by the author. The author has read and approved the final edition of the manuscript.

Funding

This research was not supported by any external funding agency or institution. The study was conducted entirely using personal funds provided by the author.

Availability of data and materials

All data generated or analyzed during the present study are included in the manuscript. Additional datasets, if required, are available from the corresponding author upon reasonable request.

Competing interests

The author has declared any conflict of interest.

Ethical considerations

The author has checked the ethical issues, including plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy.

Acknowledgments

The author wishes to extend sincere gratitude to the Faculty of Agriculture at the University of Kufa for granting access to its laboratories to facilitate this research. Additionally, the author is profoundly appreciative of Dr. Ali J. Hammod and Dr. Ali H. Mohammed for their invaluable guidance and unwavering support throughout the course of the study.

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2025, Scienceline Publication
World's Veterinary Journal
World Vet J, 15(3): 678-687.
ISSN 2322-4568

DOI: https://dx.doi.org/10.54203/scil.2025.wvj68
PII: S232245682500068-15

Assessment of Active LaSota and Inactivated Newcastle Vaccines and Their Nucleotide Sequence Homology on the Virulence of Wild-type Newcastle Disease Genotype VIIi Virus

Gusti Ayu Yuniati Kencana[1], I Nyoman Mantik Astawa[1], Anak Agung Sagung Kendran[2], and Adityar Ahkami Pratomo[3]

[1]*Veterinary Virology Laboratory, Faculty of Veterinary Medicine, Udayana University, Denpasar 80234, Indonesia*
[2]*Clinical Pathology Laboratory, Faculty of Veterinary Medicine, Udayana University, Denpasar 80234, Indonesia*
[3]*PT Sanbio Laboratories, Gunung Putri, Bogor, West Java, Indonesia*

*Corresponding author's Email: yuniati_kencana@unud.ac.id

ORIGINAL ARTICLE
Received: June 17, 2025
Revised: July 23, 2025
Accepted: August 27, 2025
Published: September 25, 2025

ABSTRACT

Many vaccines are commercially available to prevent Newcastle Disease (ND) in chickens, but the disease is still endemic in many countries. The present study aimed to evaluate the efficacy of ND vaccines *in vivo* and to analyze the nucleotide sequence similarity between the vaccines and the wild-type virus encountered in the field, as well as the underlying reasons for vaccination failures against ND in chickens. One hundred laying Isabrown hens, aged 22 weeks with an average weight of 1.75 kg, from two farms (Farm A and B) in Tabanan, Bali, Indonesia, served as experimental animals. All laying hens had previously been vaccinated four times orally with LaSota vaccines (Farm A) and alternately with LaSota and inactive ND vaccines (Farm B). They were then given a booster vaccination orally with live LaSota (Farm A) and intramuscularly with an inactive vaccine (Farm B). Sera samples were collected every week for four weeks in Farm A and for five weeks in Farm B, and the antibody titers were examined by hemagglutination inhibition (HI) test. The genotypes of the field isolates were determined through sequencing and bioinformatics analysis using MEGA 7. The mean $\log_2$ antibody titers for hens vaccinated with the LaSota vaccine were 7.2 HI units before vaccination, 7.3 HI units at one week post-vaccination, 8.1 HI units at two weeks post-vaccination, and 7.9 HI units at three weeks post-vaccination. In contrast, the mean $\log_2$ antibody titers for those vaccinated with the inactive NDV vaccine were 6.4 HI units at pre-vaccination, 7.3 HI units at one week, 5.5 HI units at two weeks, 5.0 HI units at three weeks, and 5.8 HI units at four weeks post-vaccination. The NDV-LaSota vaccine illustrated 80-81% similarity to a recent ND virus isolate from chickens in Bali (ND/chicken/GAYK01/Penebel Bali/2023), while inactive vaccines exhibited 96-98% similarity. Thus, vaccines were closely related to the Bali virus, whereas the active vaccine was less similar. Based on antibody responses and homology levels, the inactive NDV vaccine induced stronger protective immunity against NDV field isolates than the Active LaSota vaccine.

Keywords: Antibody titer, Field isolate, Neutralization, Newcastle disease vaccine, Sequencing

INTRODUCTION

Newcastle disease (ND) is a significant viral disease in poultry caused by Avian Paramyxovirus type 1, a genus within the *Avulavirus* family (ICTV, 2019; Rima et al., 2019). The Newcastle disease virus (NDV) infects the respiratory tract, digestive tract, and nervous system, causing clinical signs such as anorexia, diarrhea, respiratory and neurological disorders with high morbidity and mortality rates (Samad et al., 2022). Although vaccinations to prevent the disease in poultry have been carried out regularly, the disease remains endemic in Indonesia, resulting in significant economic losses for the poultry industry. The ND cases continue to persist in poultry, including vaccinated chickens (Kencana et al., 2018), indicating that such vaccination cannot fully protect chickens from ND. Several factors, including genetic and antigenic differences between the vaccines and field isolates, as well as improper vaccination procedures, may contribute to vaccination failure (Hu et al., 2022).

The NDV is an enveloped virus with negative-sense ssRNA consisting of six open reading frames (ORF) encoding for nucleocapsid protein (NP), phosphoprotein (P), matrix (M) protein, fusion (F) protein, hemagglutinin/neuraminidase (HN), and large (L) RNA-directed RNA polymerase (Adi et al., 2010; Zhao et al., 2018; Song et al., 2019; Nath et al., 2020). The F protein plays an essential role in the host's immunity against the virus, as it contains the neutralizing epitope of NDV (Zhao et al., 2019). Additionally, the amino acid sequence of the F protein at its cleavage site plays a crucial role in determining the pathogenicity of the virus in hosts (Kim et al., 2013; Wang et al., 2017). A vaccine with a high homology level to the virulent field NDV is likely to induce a highly protective immune response against the field virus.

To cite this paper: Kencana G.A.Y., Astawa I.N.M., Kendran A.A.S., and Pratomo A.A. (2025). Assessment of Active LaSota and Inactivated Vaccines and Their Nucleotide Sequence Homology on the Virulence of Wild-type Newcastle Disease Genotype VIIi Virus. *World Vet. J.,* 15(3): 678-687. DOI: https://dx.doi.org/10.54203/scil.2025.wvj68

Based on their virulence, NDV is classified into three pathotypes, including lentogenic, mesogenic, and velogenic NDV (Ashraf et al., 2014). Field NDV, which causes significant economic losses to breeders, is the virulent ND virus, known as very virulent Newcastle disease (VVND), resulting in high mortality rates. Currently, active NDV vaccines are generally created using low- to moderate-virulence NDV, referred to as lentogenic and mesogenic NDV, respectively. Nevertheless, the inactive NDV vaccine made from vvNDV offers a higher similarity to field vvNDV strains. Infection with NDV in chickens causes diseases with clinical signs that range from subclinical to severe clinical syndromes. Infection with lentogenic strains typically results in mild clinical signs, including sneezing and slight nasal discharge. Moderate clinical signs, such as respiratory and neurological disorders, can occur in chickens infected with mesogenic NDV. Meanwhile, velogenic NDV causes severe clinical signs with high morbidity and mortality rates (Ghiamirad et al., 2010; Samad et al., 2022).

Different vaccines are commercially available to prevent NDV infection in chickens and other avian species, with several vaccination programs. In Indonesia, active NDV vaccines prepared using low-virulent viruses, such as La Sota and B1, have been proven safe and capable of inducing humoral, cellular, and mucosal immunity in chickens. However, antigenic mismatch between the vaccine and virulent NDV is often found in the field and is believed to be the cause of outbreaks. Therefore, an inactive vaccine using homologous NDV is also prepared to improve the efficacy of the NDV vaccine in the field (Damayanti et al., 2023). The NDV vaccines can be administered as either single active or inactive vaccines, or as combined active and inactive vaccines (Kencana et al., 2016). Although different vaccination regimes have been implemented to prevent ND in poultry, cases of NDV remain prevalent among chickens in Indonesia. The presence of antibodies against NDV among unvaccinated chickens indicated that NDV is still circulating among the chicken population in the field (Kencana et al., 2018). In highly susceptible chickens, infection with this virus can cause severe disease with a high mortality rate (Dharmayanti et al., 2024). Additionally, changes in the antigenicity and pathogenicity of low virulent NDV, such as the La Sota strain, can lead to ND outbreaks (Xiao et al., 2009; Ke et al., 2010; Wibowo et al., 2017). The level of nucleotide sequence homology between neutralization epitopes and the cleavage site of the F gene, as well as between the vaccine and the field virus, is crucial for the success of vaccination in preventing outbreaks of the disease (Ke et al., 2010). Therefore, the present study aimed to determine the ability of antibodies against the vaccine to neutralize field NDV isolates and to investigate the nucleotide sequence homology between the vaccine and field isolates, and identifying the cause of NDV vaccination failure in chickens.

MATERIALS AND METHODS

Ethical approval

The present study was approved by the Animal Ethics Committees of the Faculty of Veterinary Medicine, Udayana University, Indonesia, with the Animal Ethics Approval Certificate number: B/284/UN14.2.9/PT.01.04/2023

Experimental animals

A total of 100 laying hens from two poultry farms in Tabanan regency, consisting of 50 animals from farm A and 50 animals from farm B, were used. Farm A, with a population of 5500 laying hens, was raised in an open house pen setting in an environment with 65% humidity and an average daily temperature of 28 °C. Meanwhile, Farm B, with a population of 8000 laying hens, was raised in an open house pen setting in an environment with 60% humidity and an average daily temperature of 22 °C. The Isa brown laying hens aged 22 weeks with an average body weight of 1.7 kg were obtained from PT Charoen Pokphand, Indonesia. Laying hens in Farm A were previously vaccinated four times monthly with active LaSota vaccine, whereas those in Farm B were vaccinated four times monthly with alternately with LaSota and inactive vaccines. Laying hens in farm A were then booster vaccinated once orally with active La Sota vaccine (Sanavac LS/H120, Indonesia), whereas those in farm B were booster vaccinated once intramuscularly with inactive vaccine (Medivac ND-IB, Indonesia). All laying hens from selected farms used in field trials of the active Sanavac LS/H120 and the inactive Medivac ND-IB vaccines were fed *ad libitum* with feed concentrate for laying chickens (Charoen Pokphand Indonesia). Sera samples were collected every week for four weeks in Farm A and for five weeks in Farm B. Two mL of blood was collected from each animal via brachialis vein, and sera were then collected by centrifugation at 1000xg for five minutes (Abdi et al, 2016).

Examination of the antibody against Newcastle disease virus

The antibody against NDV in serum samples was tested by hemagglutination inhibition (HI) test, following the procedure described by the OIE (2012). A serial two-fold dilution of sera samples was performed in U-bottomed 96-well microtitration plates. Each sera dilution was mixed with 4 hemagglutination (HA) units of NDV and incubated at room temperature for 60 minutes. After adding 1% chicken red blood cells in phosphate-buffered saline, the samples were incubated for 30 minutes at room temperature, and the dilution endpoint of each serum sample was determined. The antibody titer was expressed as the antilog of the highest dilution of the serum sample that completely inhibited NDV from hemagglutinating 1% red blood cells. Each serum sample was tested in triplicate.

Isolation of wild-type Newcastle disease virus

Two wild-type NDV isolates were obtained from field cases, which were isolated and identified using a method similar to that described by Alazawy and Al Ajeeli (2020). The two NDV isolates were collected from infected chickens in the area's proximity to the vaccine trials. The virus was inoculated into chicken embryonated eggs, and they were subjected to sequencing analysis to determine their genotypes at PT Sanbio Laboratories, Indonesia. The virus isolates were inoculated into 10-day-old chicken embryos and incubated for three days at 37℃. Chorioallantoic fluid was collected, and the HA test confirmed the presence of NDV.

Adaptation and titration of wild-type Newcastle disease virus in BHK-21 cells

One wild-type NDV isolate was adapted to replicate in BHK-21 cell culture. The virus (0.1 mL) at the titer of 2^7 HA units was initially inoculated into chicken embryos as described by Alazawy and Ajeeli (2020). The virus was then inoculated into BHK-21 cell culture at a multiplicity of infection of one. After three passages in BHK cells, the media were collected in aliquots and stored at -80 °C until further use. A ten-fold dilution of the virus was prepared in a 96-well tissue culture plate. Each dilution of the virus was added to five wells of BHK-21 cells, which were grown in 96-well TC plates. The presence of cytopathic effects (CPE) was observed in each well. The presence of CPE was characterized by the formation of syncytia and detachment from the surface of the flasks or plates where the cells were grown. The tissue culture infective dose ($TCID_{50}$) of the virus was determined using the method of Reed and Muench (1938).

Polymerase chain reaction and nucleotide sequencing of wild-type Newcastle disease virus isolates

Total RNA was extracted from the infected chorioallantoic fluid of chicken embryonated eggs using TRIzol LS (Thermo Fisher Scientific, USA) methods, as described by the manufacturer's instructions. The extracted RNA samples were subjected to a reverse-transcription polymerase chain reaction (RT-PCR) assay. A 5 µL RNA-extracted sample was reverse-transcribed into cDNA using random primers and amplified using primers specific to the NDV *F1* gene. Primers used for RT-PCR were forward 5′-ATCCAAGCAGGTACCCAACG-3′ and reverse 5′-AAGTCGGAGGATGTTGGCAG-3′, with an expected amplicon of 732 bp (Putri et al., 2021). The RT-PCR procedures were conducted as follows. Firstly, viral RNA was reverse-transcribed into cDNA at 50°C for 30 minutes, followed by the first denaturation at 94°C. Forty amplification cycles were performed, consisting of denaturation at 94°C for 15 seconds, annealing at 56°C for 30 seconds, and elongation at 68°C for 60 seconds. Finally, the cycles were terminated by incubation at 68°C for five minutes. The PCR products were analyzed using 1% agarose gel and stained with ethidium bromide (Putri et al., 2021).

Phylogenetic analysis

Nucleotide sequencing was performed using the PCR product isolated from the electrophoresis gel. Sequencing was performed at PT Sanbio Laboratory, Bogor, Indonesia. Based on the nucleotide sequence of the *F1* gene, phylogenetic analysis was conducted using the MEGA7 software (Kumar et al., 2016). The *F1* gene coding sequence of NDV Bali isolates was compared with the sequence of the *F1* gene of NDV isolated from other genotypes available in GenBank, as listed in Table 1.

Table 1. Newcastle disease virus isolates from the Gene Bank as references for alignment of NDV Bali-Penebel isolates

No	Accession number of ND isolates	No	Accession number of ND isolates
1.	AB465607.1|Japan/Ishii/62	21.	JX854452.1|Pheasant/MM20/Pakistan/2011
2.	AF079323.1|DB5	22.	KC906188.1|VG/GA-AVINEW
3.	AF534997.1|ZJ/2000	23.	KF026013.1|UPM-IBS/002/2011
4.	AY861659.1|SP13	24.	KF792018.1|chiken/Israel/2011/1115_818
5.	D00243.1|NDVFHN	25.	KF792019.1|chicken/KY-Israel/2013/50_826
6.	DQ195265.1|Lasota	26.	KF792020.1|parrot/Israel/2012/841_824
7.	EF464163.1|JL01	27.	KM056356.1|ndv60/Avinew
8.	EU239663.1|JL-1	28.	M24693.1|NDVFPB
9.	EU258665.1|NDV027344	29.	M24696.1|NDVFPE
10.	EU289028.1|VG/GA	30	JN872151.1_Hitchner
11.	EU330230.1|D58	31	PP695101.1_MHW-G-VII-i/19-A
12.	FJ600542.1|HN0801	32.	PP695102.1_MHW-G-VII-i/19-C
13.	FJ608340.1|MQ/Liaoning/05	33.	MT988380.1_NDV/Duck/M147/19
14.	HQ697255.1_chicken/Sukorejo/019/10	34.	MT988381.1_NDV/Duck/A74/19
15.	HQ697256.1|chicken/Makassar/003/09	35.	MZ488460.1_Broiler-Indonesia-GunungKidul-ISW19-2019
16.	HQ697257.1|strain_chicken/Gianyar/013/10	36.	MZ488462.1_Chicken-Indonesia-BandarLampung-P032001007-2020
17.	HQ697258.1|strain_chicken/Sragen/014/10	37.	MZ488463.1_Chicken-Indonesia-LampungTengah-BRS20-2020
18.	HQ697259.1|strain_chicken/Kudus/017/10	38.	MZ488464.1_Chicken-Indonesia-MuaraEnim-A031909005-2019
19.	HQ697260.1|strain_chicken/Kudus/018/10	39.	MZ488467.1_Layer-Indonesia-Yogyakarta-DOCHTN20-2020
20.	HQ697261.1|chicken/bali/020/10	40.	MZ488468.1_Peacock-Indonesia-Palembang-P032105010-2021

Cross-neutralization assay

The antibody raised against NDV vaccines was examined for neutralizing activity against wild-type NDV. Wild NDV at 100 $TCID_{50}$ was added to each 2-fold dilution of serum samples and incubated for 60 minutes at 37°C. The virus-serum mixture was inoculated into five wells of BHK-21 monolayers grown in 96-well tissue culture plates (Lu et al., 2018). The cells were then incubated at 37°C, and the presence of CPE in each well was observed. The neutralizing titers of the antibody were expressed as the protective dose (PD_{50}), calculated using the method of Reed and Muench (1938).

Statistical analysis

The data on antibody titers of chickens following booster vaccinations with LaSota and inactivated ND vaccines were presented descriptively. Phylogenetic analysis was conducted using Mega7 software.

RESULTS

Antibody titers following booster vaccinations

Prior to the present study, laying hens on Farm A had been vaccinated 4 times orally with both active La Sota, and those in Farm B were vaccinated 4 times alternately with LaSota and inactive vaccines. Following booster vaccinations once with active LaSota and inactive vaccines respectively for Farm A and Farm B, the log_2 mean antibody titers of laying hens after booster vaccinations in Farm A were 6.4 HI units at pre-booter vaccination, 7.3 HI units at the first week, 8.1 HI units at the second week, and 7.9 HI units at the third week post-vaccination. The log_2 mean antibody titers for those following booster vaccination with the inactive NDV vaccine in Farm B were 7.2 HI units at pre-vaccination, 7.3 HI units at the first week, 5.5 HI units at the second week, 5.0 HI units at the third week, and 5.8 HI units at the fourth week post-vaccination (Figure 1).

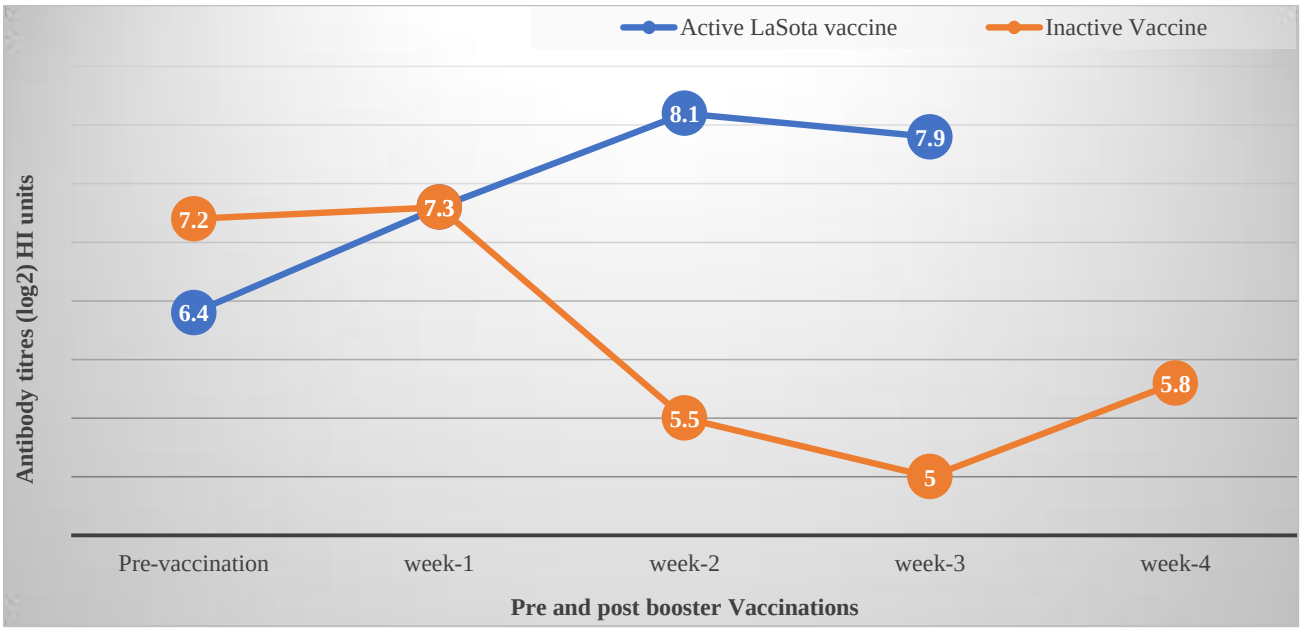


Figure 1. Serum antibody titers of Isabrown chickens following booster vaccination with active LaSota and inactive Newcastle disease virus vaccines

Adaptability of Bali NDV isolates in BHK-21 cell culture

Two Bali NDV isolates were adaptable in BHK-21 cell culture, characterized by the presence of CPE. Initially, the infected cells appeared as small syncytia, which were visible at 24 hours post-infection. At 48 hours post-infection, the syncytia enlarged, and the cells began to detach from the surface of the flask where they were grown (Figure 2).

Genotype characteristics of field wild-type NDV isolates

Using primers specific to the F1 gene, the RT-PCR assay yielded a single 732 bp amplicon in both NDV isolates, corresponding to the *F1* gene. Two NDV Bali isolates, B1 (ND/chicken/GAYK01/PenebelBali/2023 and B2 (ND/Chicken/GAYK02/Penebel Bali/2023), were subjected to RT-PCR and both resulted in 732 bp DNA bands (Figure 3).

Sequencing analysis demonstrated that two wild-type NDV of Bali isolates, B1 (ND/chicken/GAYK01/PenebelBali/2023 and B2 (ND/Chicken/GAYK02/PenebelBali/2023), were both grouped into genotype VIIi. Compared with those of genotype VII, genotype VIIi of two NDV Bali isolates has two amino acid changes, R78K (Neutralizing epitope) and R114Q (Cleavage site; Figures 4 and 5).

Sequencing analysis revealed that the identity level of the amino acid sequence for the F1 gene among field NDV Bali isolates and the inactive vaccine used in this booster vaccination was 96-98%. These identity levels were 19-20% higher compared to those among field NDV Bali isolates and LaSota, which was 80-81%, used for booster vaccination (Table 2).

Phylogenetic tree analysis indicated that two virulent wild-type NDV of Bali isolates belong to genotype VIIi. Both isolates were closely related to virulent NDV circulating among chickens in Indonesia, specifically those identified as NDV/ck/West Java/S081/2023 and NDV/ck/East Java/S102/2023 (Figure 5). Nucleotide sequence analysis indicated a homology level of active LaSota and inactive with two field ND isolates, ND/chicken/GAYK01/Penebel Bali/2023 were 80-81% and 96-98%, respectively.

Sequencing analysis demonstrated that the identity level of the amino acid sequence for the *F1* gene among field NDV Bali isolates and the inactive vaccine used in this booster vaccination was 96-98%. These identity levels were 19-20 % higher than those among the field NDV of Bali isolates and LaSota (Table 2), which was used for booster vaccination. Amino acid sequence of 112RRQKR↓F117 and 112RRRKR↓F117 at the cleavage site of the F protein showed that the NDV isolates used in the present study were velogenic NDV belonging to VIIh.

Phylogenetic tree analysis demonstrated that two virulent wild-type NDV Bali isolates belong to genotype VIIi. Both isolates were closely related to virulent NDVs circulating among chickens in Indonesia, specifically those identified as NDV/ck/West Java/S081/2023, NDV/ck/East Java/S102/2023 (Figure 6). Nucleotide sequence analysis indicated a homology level of active LaSota and inactive with two field ND isolates, ND/chicken/GAYK01/Penebel Bali/2023 were 80-81% and 96-98%, respectively.

Neutralizing activities of the antibody against the field wild-type NDV were examined using BHK-21 cell culture. Only the antibody against the active LaSota vaccine was tested against one field NDV isolate. The collected antibodies were pooled and tested using the HI test. The Geometric mean titer of antibody obtained from pooled serum was 7.4 (log_2) HI units. The neutralizing titer of the antibody obtained was 9.4 (log_2) PD_{50}. The evidence for the presence of an escape mutant was observed in some wells at dilutions of 6 and 7 (log_2), showing the presence of CPE (Figure 1), which was not expected to occur in the neutralization assay (Table 3).

The antibody against field wild-type NDV was examined using BHK-21 cell culture. Only the antibody against the active LaSota vaccine was tested against one field NDV isolate. The collected antibodies were pooled and tested using the HI test. The Geometric mean titer of antibody obtained from pooled serum was 7.4 (log_2) HI units. The neutralizing titer of the antibody obtained was 9.4 (log_2) PD_{50}. Evidence of an escape mutant was observed, as some wells at dilutions of 6 and 7 (log_2) showed the presence of CPE (Figure 6), which was not expected to occur in the neutralization assay (Table 3). The presence of such an escape mutant requires further investigation.

Table 2. Amino Acid and nucleotide sequence homology of *F1* gene between Newcastle disease virus of Genotype VII and Genotype II

	Amino Acid sequence				**Nucleotide sequence**			
	Seed GVIIi	Seed GVIIh	Seed GII	Bali	Seed GVIIi	Seed GVIIh	Seed GII	Bali
Bali	96-98	89-90	80-81	97-100	97-99	87	79-80	97-100

Table 3. Schematic representation of neutralization assay at various dilutions of serum against LaSota vaccine and field Newcastle disease virus isolate in BHK-21 cells

Assay replicates / **Serum dilution**	**1**	**2**	**3**	**4**	**5**
2^{-6}	-	+	-	+	-
2^{-7}	-	-	-	-	+
2^{-8}	-	-	-	+	-
2^{-9}	-	-	+	+	-
2^{-10}	+	+	+	+	+
2^{-11}	+	+	+	+	+
2^{-12}	+	+	+	+	+
Uninfected BHK-21	-	-	-	-	-

Legend (-): No cytopathic effect was observed; (+): Cytopathic effect was observed

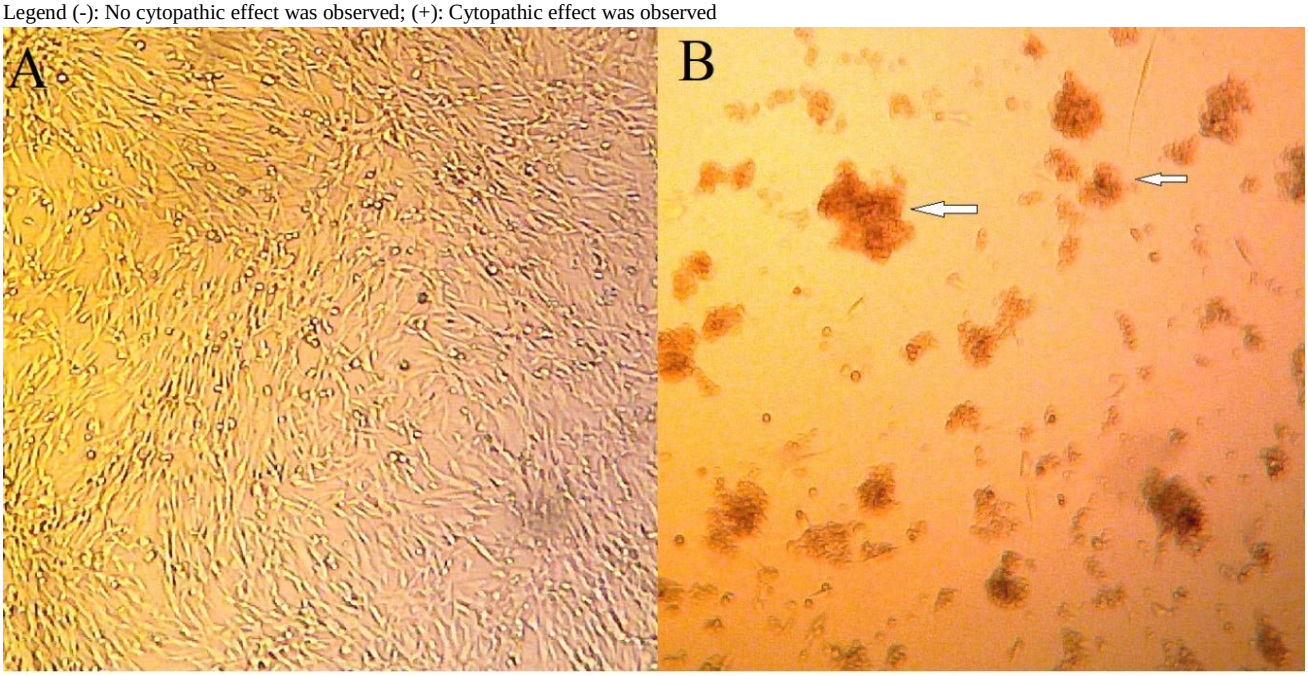


Figure 2. Cytopathic effect induced by Newcastle disease virus in BHK-21 cells. No cytopathic effect was observed in normal uninfected cells (**A**). Syncytium and detachment of cells were observed in the infected cells (**B**)

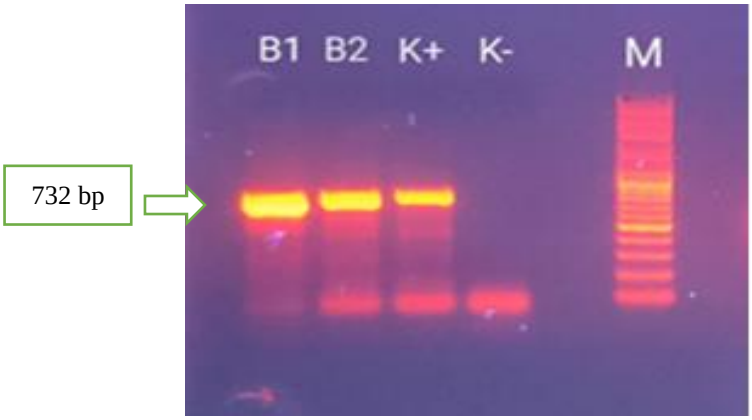


Figure 3. Profiles of F1 amplicons of virulent Newcastle disease virus isolates analyzed by polymerase chain reaction assay. Lanes **B1**: ND/chicken/GAYK01/PenebelBali/2023, **B2**: ND/Chicken/GAYK02/PenebelBali/2023, **K+**: Positive control, **K-**: Negative control, **M**: Markers



Figure 4. Mutation sites of Newcastle disease virus at K78R (neutralizing epitope) and Q114R (cleavage site) as markers of virulent Newcastle disease virus. The mutations were determined based on alignment and comparison of Genotype II with those of Genotype VII.

	58	89	91	113	138	189	195	228	239	259	278	287	307	308	309	401	411
ND/WS/G7H	S	R	V	T	P	R	I	F	M	A	I	R	S	T	S	G	R	I
ND/WS/G7I	P	.	I	V	L	W	.	Y	L	T	V	K	A	A	P	R	Q	V
NDV/Chicken/GAYK01/PenebelBali/2023	P	.	I	V	L	W	.	Y	L	T	V	K	A	.	.	R	Q	V
NDV/Chicken/GAYK02/PenebelBali/2023	P	K	T	V	L	W	V	Y	L	T	V	K	A	.	.	R	Q	V

Figure 5. Amino acid polymorphisms at the neutralizing epitope and cleavage sites of the F gene among Newcastle disease virus of genotype VII.

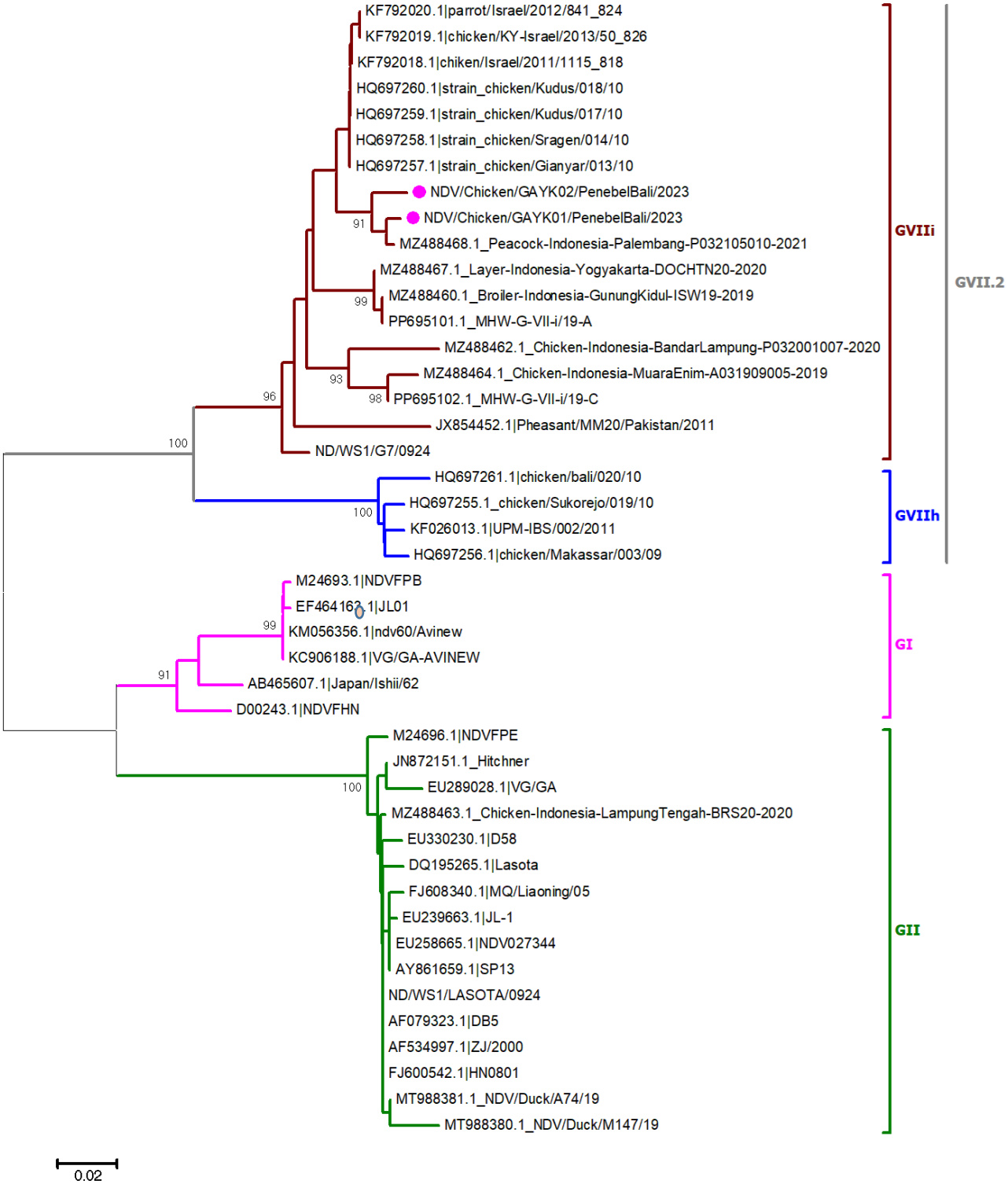


Figure 6. Phylogenetic relationships of the F1 gene of two virulent Newcastle disease virus Bali isolates with those previously published GenBank sequences for reference. The virulent Newcastle disease virus isolates used in this study were marked with a purple circle (●).

DISCUSSION

Booster vaccination in Layer chickens with active LaSota vaccine appeared to induce a higher antibody response as compared to inactive vaccine (Figure 1). Live NDV vaccines, such as LaSota, are prepared using a virus strain with low virulence (lentogenic) and have been widely used for chicken vaccination (Li et al., 2020). However, compared to the inactive vaccine prepared with local virulent NDV, it was expected to exhibit a higher level of homology to the field wild-type NDV. Therefore, it was anticipated that the antibody response against the inactive NDV vaccine would provide better protection of chickens against NDV infection in the field. Nucleotide and amino acid sequences were then analyzed to determine the genetic distance of the vaccine strain and field wild-type NDV isolates.

To explore the homology levels of both active LaSota and inactive vaccine against field NDV isolates, a portion of the *F1* gene, approximately 732 bps long, from wild-type NDV Bali isolates, B1 (ND/chicken/GAYK01/PenebelBali/2023) and B2 (ND/Chicken/GAYK02/Penebel/Bali/2023), was sequenced. As expected, compared to the LaSota vaccine, the *F1* gene of the inactive vaccine used in the present study exhibited higher homology with field wild-type NDV isolates. The F1 region is a part of the *F* gene that includes the neutralizing epitope K78R and the cleavage site Q114R (Liu et al., 2008; Samal et al., 2011). These elements are vital for the host's immune response to NDV infection. Changes in amino acids within this neutralizing epitope can result in a new escape mutant variant, enabling the virus to evade the neutralizing activities of antibodies induced by vaccines. Additionally, changes in the amino acid sequence at the cleavage site of the F protein can alter the pathogenicity of the virus, as it serves as a determinant of virulence and membrane fusion activity. As shown in Figure 3, a change in amino acid sequence Q114R was found at the cleavage site, which can reduce the replication ability and pathogenicity of the virus (Samal et al., 2011). Kim et al. (2013) reported that while the current NDV vaccines remain effective in preventing the disease, they are not entirely protective against infection, as the vaccines are made from viruses with different genotypes than those of field wild-type NDV. Only vaccines prepared using closely related genotypes will be able to prevent infection and effectively stop the spread of the virus. The use of a chimeric LaSota virus, which involves replacing its *F* and *H*/*N* genes with those from genotype VII, has been shown to effectively prevent NDV infection (Qiao et al., 2021).

The role of the F protein in immunity and protection of the host against NDV infection has been reported (Umali et al., 2013). A LaSota vaccine containing F protein cleavage sites (FPCS) of APMV-8 (Genotype VII) was shown to induce high neutralizing antibodies and protect chickens from challenge with wild-type genotype VII. The cleavage site of the F protein of NDV is vital in vaccine preparation, and the vaccine containing FPCS of NDV genotype VII is expected to be more effective in preventing NDV infection in chickens. As the two wild-type NDVs of Bali isolates used in the present study were grouped into genotype VIIi (Figure 4) and exhibited higher homology levels to the inactive vaccine than to the active LaSota vaccine, the inactive NDV vaccine was expected to induce a better protective immune response.

In Indonesia, it was found that the genetic distances among circulating NDV isolates, based on their *F* gene, have been reported to range from 0.4 to 9.6%, with a homology level of 90.4-99.5% (Wibowo, 2017). All virulent NDV strains have the F protein cleavage site motif of R-R-Q-R/K-R-F and are generally associated with high pathogenicity indexes, ranging from 1.05 to 1.87 in day-old chicks (Bogoyavlenskiy et al., 2009).

The antibody raised against LaSota was shown to be able to neutralize the wild-type NDV isolates belonging to serotype VII. This demonstrated that the antibody against LaSota remained capable of inducing protective immunity against the field NDV isolate. In Indonesia, virulent NDV typically belongs to genotypes VIIh and VIIi. The primary differences in the *F1* gene among VII (VIIh and VIIi) and genotype II (LaSota) occur at the neutralizing epitope K78R and at Q114R (Cleavage site; Liu et al., 2008; Samal et al., 2011). As shown in the neutralization test, a new escape mutant may exist, as the raised antibody against LaSota was not able to completely neutralize the wild-type NDV isolates. Escape mutants can arise from amino acid alterations in the neutralizing epitope and the cleavage site of the *F1* gene.

Kim et al. (2013) reported that although the current NDV vaccines are still effective in preventing ND, they are not fully protective against infection, as the vaccines are prepared using viruses with different genotypes than those of wild-type NDVs found in the field. Only a vaccine prepared with closely related genotypes can prevent infection and the spread of the virus. The approach of using chimeric LaSota by replacing its *F* and *H*/*N* genes with those of Genotype VII has been proven effective in preventing NDV infection (Qiao et al., 2021).

CONCLUSION

The active LaSota NDV vaccine was able to induce a protective immune response in laying hens after booster vaccination, with steadily increasing titers following the first, second, and third immunizations. However, with the

booster vaccination using the inactive vaccine, the antibody titers declined until the third vaccination and only increased after the fourth vaccination. Antibodies induced by the active LaSota vaccine were capable of neutralizing field virulent NDV isolates. The presence of escape mutants was detected in association with the CPE observed in several wells exhibiting low antibody levels. Sequencing analysis of the *F1* gene was conducted on two NDV isolates collected from the field, identified as ND/chicken/GAYK01/PenebelBali/2023 and ND/chicken/GAYK02/PenebelBali/2023. The results indicated that both isolates belong to the NDGVIIIi (Newcastle Disease Virus Genotype VIIi) and demonstrated homology levels of 80-81% with the active LaSota vaccine and 96-98% with the inactive NDV vaccine. Further studies are required to explore the presence of the escape NDV mutant.

DECLARATIONS

Funding

The present study was funded by the Institute of Research and Community Service, Udayana University, Indonesia (Hibah Unggulan Udayana Scheme) under the grant number B/1.486/UN14.4.A/PT.01.03/2023.

Authors' contributions

Gusti Ayu Yuniati Kencana conceived and designed the experiment, collected samples, and performed data analysis. I Nyoman Mantik Astawa and Anak Agung Sagung Kendran helped in conceiving and designing the experiment. All authors conducted laboratory testing. All authors read and approved the final edition of the manuscript for publication.

Competing Interests

The authors declared that they have no competing interests.

Ethical considerations

The authors declare that this manuscript is original and is not being considered elsewhere for publication. Other ethical issues, including consent to publish, misconduct, data fabrication, and redundancy, have been reviewed by the authors.

Availability of data and materials

All data from this study are available upon reasonable requests from the authors.

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2025, Scienceline Publication
World's Veterinary Journal
World Vet J, 15(3): 688-694.
ISSN 2322-4568

DOI: https://dx.doi.org/10.54203/scil.2025.wvj69
PII: S232245682500069-15

Prevalence of Lungworms and Liver Fluke Infections in Ruminants Slaughtered at Kirkuk City, Iraq

Abbas Najm Aldin Saleh[1*], Cheia Majeed Wahhab[2], Almas M. Al-Bayati[3], Kasim Sakran Abass[4], and Al Salihi Karima Akool[5]

[1]*Department of Pathology and Poultry Disease, College of Veterinary Medicine, University of Kirkuk, Kirkuk, Iraq*
[2]*Department of Nursing, Kirkuk Technical Institute, Northern Technical University, Iraq*
[3]*Department of Medicine and Preventive, College of Veterinary Medicine, University of Kirkuk, Kirkuk, Iraq*
[4]*Department of Physiology, Biochemistry and Pharmacology, College of Veterinary Medicine, University of Kirkuk, Kirkuk, Iraq*
[5]*Department of Basic Science, College of Dentistry, Al-Iraqia University, Baghdad, Iraq*

*Corresponding author's Email: abbasalbeaty@uokirkuk.edu.iq

ORIGINAL ARTICLE
Received: July 02, 2025
Revised: August 06, 2025
Accepted: September 03, 2025
Published: September 30, 2025

ABSTRACT

Parasitic infections represent one of the most prevalent and economically significant diseases affecting livestock worldwide. The present study aimed to investigate risk factors associated with liver fluke and lungworm infections in small ruminants, characterize the diversity of lungworm species, and determine the prevalence of infections in Kirkuk, Iraq. The study was conducted from November 2023 to April 2024 in Northeastern Iraq, examining 30,900 sheep, 797 goats, 9,990 cattle, and 84 buffalo at slaughter. All carcasses underwent thorough gross pathological examination to detect parasitic infections, including lungworms, liver flukes, hydatid cysts, and associated pulmonary lesions. Distinct pathological manifestations were particularly evident in naturally infected local sheep. The present study revealed the following disease prevalence patterns across species. Pneumonia was detected in 0.31% of sheep and 0.35% of cattle, with no cases observed in goats or buffalo. Hepatitis showed an incidence rate of 0.39% in sheep and 0.75% in cattle. Hydatid cyst occurrence in hepatic and pulmonary tissues was documented at 0.38% in sheep and 1.13% in cattle, while remaining undetectable in caprine and bovine specimens. Liver fluke infection rates varied significantly among species, with sheep exhibiting 0.32% prevalence, goats demonstrating the highest rate at 3.76%, cattle showing 0.90% infection, and buffalo populations remaining completely uninfected. The present findings revealed significantly higher disease susceptibility in ruminants during winter months, with progressively lower infection rates observed throughout summer and monsoon seasons. This seasonal pattern may reflect environmental factors influencing parasite life cycles and host immunity.

Keywords: Animal disease, Hydatid cyst, Parasitic infection, Pneumonia, Ruminant

INTRODUCTION

Slaughterhouses serve as critical surveillance points for detecting economically significant and zoonotic diseases, playing a pivotal role in ensuring food safety throughout the production chain (Raji et al., 2010; Mohammed and Abass, 2021). Systematic ante-mortem and post-mortem inspections are essential for identifying meat hygiene issues and potential public health risks associated with carcass consumption (Nevas and Lundén, 2014). Meat inspection records serve as a valuable epidemiological tool for veterinary public health surveillance and disease prevention (Edwards et al., 1997; García-Díez et al., 2023). Microbial pathogens such as *Streptococcus pyogenes*, *Staphylococcus aureus*, *Mycobacterium tuberculosis*, *Klebsiella*, *Proteus*, *Pseudomonas*, and *Entamoeba histolytica* are suspected of causing lung and hepatic abscesses in humans and animals (Webb et al., 2014). *Fusobacterium necrophorum* is a Gram-negative, obligately anaerobic, rod-shaped bacterium that primarily colonizes the bovine rumen, where it adheres to the ruminal epithelium. Rumen concentrations of this pathogen are significantly elevated in grain-fed cattle compared to forage-fed animals (Neves et al., 2025). These bacterial microorganisms enter the portal circulation from the rumen and become trapped in the liver, leading to abscesses caused by bacterial pathogens, which represent a primary etiological factor in hepatic abscess development (Tadepalli et al., 2009).

Pneumonia and bronchitis are signs of lungworm infections, which typically strike young calves on permanent or semi-permanent pastures during the first grazing season (Holzhauer et al., 2011; Forbes, 2018). The majority of significant clinical signs are caused by primary parasite pneumonia and manifest during the prepatent and patent stages (Abass et al., 2020). Chakraborty et al. (2014) identified *Dictyocaulus filaria* (*D. filaria*), *Protostrongylus rufescens*, and

To cite this paper: Saleh ANA, Wahhab CM, Al-Bayati AM, Abass KS, and Akool ASK. (2025). Prevalence of Lungworms and Liver Fluke Infections in Ruminants Slaughtered at Kirkuk City, Iraq. *World Vet. J.*, 15(3): 688-694. DOI: https://dx.doi.org/10.54203/scil.2025.wvj69

Muellerius capillaris as the primary etiological agents of ovine lungworm infection, clinically manifested as verminous pneumonia or verminous bronchitis.

The life cycle of hydatid cyst consists of two stages, with a definitive host (Canids) that carry the adult worm in the small intestine, and livestock serving as an intermediate host, in which livestock and humans can accidentally become infected, where the larval stage (Hydatid cyst) develops in liver (Eckert and Deplazes, 2004, Stojković et al., 2018). The infection of hydatid cyst in the intermediate host occurs through the accidental ingestion of *Echinococcus* eggs, which are passed through the feces of a dog and contaminated water or food (Lopes et al., 2017). The economic significance of echinococcosis in livestock production stems primarily from carcass degradation and contamination of consumable visceral organs, including lungs, liver, and heart (Hussein et al., 2022).

Fascioliasis represents one of the most globally prevalent parasitic diseases of livestock, with significant economic impacts on cattle and sheep production systems worldwide (Mehmood et al., 2017). Current estimates indicate infection rates exceeding 250 million sheep and 300 million cattle globally. The two primary species of *Fasciola (F.)* spp., which are *F. gigantica* and *F. hepatica*, can infect humans and are widespread parasites in ruminants, particularly in sheep, goats, cattle, buffalo, and pigs (Periago et al., 2006). These ruminants are typically distinguished by their morphological features, including body length and form (Periago et al., 2008; Mazeri et al., 2017). Liver fluke infection is recognized as an economically significant parasitic disease in livestock production systems, primarily due to its global prevalence and the substantial tissue damage caused by hydatid cyst formation in affected organs, particularly the liver and lungs (Jawad et al., 2018). Furthermore, *fasciola*, which affects over 2.6 million people globally, is becoming a significant human health concern. Hence, *fasciola* has been regarded as a neglected illness that is resurfacing (Jaja et al., 2018). The adult bisexual stage of the *fasciola* settles in the bile ducts of the host and releases thousands of eggs per day during sexual reproduction (Fürst et al., 2012). These eggs pass into the intestines through the bile ducts before being dispersed into the fecal stream (Mohammed and Abass, 2021). The present study aimed to assess the risk factors associated with lungworm infections in small ruminants, characterize the prevalence of different lungworm species, and evaluate the incidence of these parasitic infections in Kirkuk, Iraq.

MATERIALS AND METHODS

Ethical approvals

The animal experimentation was approved by the Ethical Clearance Committee of the College of Veterinary Medicine, Kirkuk University, under registration number Ki. Vet. 5 (2025). The present study was conducted from November 2023 to April 2024 at the Al-Asiriyah slaughterhouse in Kirkuk city, Iraq

Study area

The study was conducted in Kirkuk, a city in the Northeastern part of Iraq, with a population of 1.5 million people (Abass et al., 2020). The majority of people in Kirkuk are farmers, and farming is the primary driver of the city's economy (Abass et al., 2020). The majority of livestock are raised using a semi-extensive system, which involves tethering and seasonal confinement (Abass et al., 2019). The current study was carried out at the University of Kirkuk College of Veterinary Medicine and approved by the scientific committee in Iraq.

Animals

The study was conducted at the Kirkuk Al-Asiriyah slaughterhouse in Iraq, which comprises two primary processing halls; one dedicated to sheep with a capacity of 30,900 heads and another for cattle with a capacity of 9,990 heads. The investigation included multiple livestock species (Cattle, goats, sheep, and buffalo) of local Northern Iraqi breeds. All animals were aged 6 months to 2 years, with the following weight ranges. Cattle (150-350 kg), goats (10-40 kg), sheep (15-35 kg), and buffalo (100-400 kg). Both sexes were represented, and all slaughter procedures followed Islamic (Halal) protocols (Abass et al., 2020).

The study population consisted primarily of locally sourced goats. In contrast, sheep and cattle specimens were predominantly acquired from neighboring cities, including Mosul, Tikrit, Sulaymaniyah, and the Northern Iraqi provinces, via the investigated slaughterhouse's supply chain. Routine meat inspection procedures for all four livestock species were primarily conducted by qualified animal health technicians, with veterinary intervention reserved for complex pathological findings (Kadir et al., 2012). Tissue samples from the livers and lungs of 315 sheep, 30 goats, 238 cattle, and 84 buffalo were collected from November 2023 to April 2024. All carcasses underwent thorough gross pathological examination by certified veterinary inspectors to detect hepatic fascioliasis, hydatidosis, and pulmonary lesions, following established protocols (Abass et al., 2019).

Sample collection

During sample collection, liver and lung tissues from cattle, sheep, goats, and buffalo were systematically examined for signs of infection, with a particular focus on bile duct abnormalities. A thorough macroscopic inspection was conducted to assess organ size, color, texture, shape, and the presence of parasitic lesions or other pathological changes. Following visual examination, organs were sectioned into smaller portions and further dissected into thin slices for detailed analysis (Chauke et al., 2014).

Statistical analysis

The statistical analysis was performed using Sigma Plot, version 11. Differences among groups were determined by one-way analysis of variance (ANOVA) followed by Fisher's LSD test. All results were presented as means, with statistical significance set at ($p < 0.05$).

RESULTS

A total of 30,900 sheep, 797 goats, 9,990 cattle, and 84 buffalo were killed at the Kirkuk Alsiriyah slaughterhouse, Iraq. Goat slaughter numbers differed significantly in April compared to other species ($p < 0.05$), whereas buffalo and sheep slaughter rates indicated a marked seasonal decrease in December and November, respectively ($p < 0.05$; Table 1).

Among the 30,900 sheep examined, 95 cases of pneumonia were detected (0.31%), while 35 cases were identified in 9,990 cattle (0.35%). No pneumonia cases were reported in goats or buffaloes at the Kirkuk slaughterhouse (Table 2).

A total positivity of sheep pneumonitis was significantly higher ($p < 0.05$) in April (0.41%) than in November (0.38%), December (0.28%), February (0.26%), March (0.19%), and January (0.3%). Similarly, in cattle, the positivity rate remained high (5%) in April compared to other months ($p < 0.05$; Table 3). Total positivity of pneumonitis in sheep was non-significantly higher in April (0.41%) than in November (0.38%) and January (0.30%), but it was significantly higher compared to the rest of the months (Table 4).

Table 1. The total number of sheep, goat, cattle, and buffalo from November 2023 to April 2024, in Kirkuk city, Iraq

Month	Sheep	Goat	Cattle	Buffalo
November	3900^{a}	108^{b}	1270^{a}	10^{ab}
December	5200^{b}	134^{c}	1560^{b}	7^{a}
January	5000^{b}	134^{c}	1700^{c}	17^{c}
February	5600^{c}	180^{d}	1700^{c}	15^{bc}
March	5200^{b}	194^{d}	1960^{d}	14^{bc}
April	6000^{d}	47^{a}	1800^{d}	21^{d}
Total	30900	797	9990	84

Different superscript letters in a column indicate significant differences ($p < 0.05$) among months.

Table 2. Incidence of lungworm (*Dictyocaulus filaria*) causing pneumonia in sheep, goat, cattle**,** and buffalo from November 2023 to April 2024 in Kirkuk city, Iraq

Animal species	Slaughtered animals (Number)	Pneumonia infections (Number)	Positive cases (%)
Sheep	30900	95	0.31
Goat	797	0	0
Cattle	9990	35	0.35
Buffaloes	84	0	0

Table 3. General survey of lungworm (*Dictyocaulus filaria*) pneumonia infections in sheep, goat, cattle, and buffalo from November 2023 to April 2024, in Kirkuk city, Iraq

Month / Infected animals (Number)	November	December	January	February	March	April
Sheep (%)	15^{b}	15^{b}	15^{b}	15^{b}	10^{c}	25^{a}
Goat (%)	0	0	0	0	0	0
Cattle (%)	5^{b}	5^{b}	5^{b}	5^{b}	5^{b}	10^{a}
Buffalo (%)	0	0	0	0	0	0
Total	20	20	20	20	15	35

Different superscript letters in a row indicate significant differences ($p < 0.05$) among months.

Table 4. Prevalence of pneumonia in sheep from November 2023 to April 2024 in Kirkuk city, Iraq

Month	Examined animals (Number)	Positive lungs (Number)	Total positive cases (%)
November	3900	15	0.38[a]
December	5200	15	0.28[b]
January	5000	15	0.30[ab]
February	5600	15	0.26[b]
March	5200	10	0.19[c]
April	6000	25	0.41[a]

Different superscript letters in a column indicate significant differences ($p < 0.05$) among months.

While a total positivity of pneumonitis in cattle was significantly higher ($p < 0.05$) in April (0.50%) than in November (0.39%), December (0.32%), January, and February (0.29%; Table 5), no significant difference were recorded among goats and buffaloes in the Kirkuk slaughterhouse, Iraq ($p > 0.05$). Moreover, the incidence of sheep pneumonitis in November differed significantly from that in other recorded months ($p > 0.05$; Table 5). As shown in Table 6, a total of 120 hydatid cysts were found in 30,900 sheep (0.38%) and 113 hydatid cysts were found in 9,990 cattle (1.13%), whereas there were no hydatid cysts found in goats or buffaloes. A total positivity of Hydatidosis in sheep was significantly higher ($p < 0.05$) in April (0.41%) than in December (0.40%), January (0.38%), November (0.38%), February (0.35%), and March (0.19%), as shown in Table 7. Moreover, a total positivity of hydatidosis in cattle was significantly higher ($p < 0.05$) in December (1.28 %) than in November (1.18 %), February (1.17 %), April (1.11 %), March (1.02 %), and January (0.88 %), as shown in Table 8.

Table 5. Prevalence of pneumonia in cattle from the beginning of November 2023 to the end of April 2024 in Kirkuk city, Iraq

Month	Total number of examined animals	Positive in Lungs (Number)	Total positive cases (%)
November	1270	5	0.39
December	1560	5	0.32
January	1700	5	0.29
February	1700	5	0.29
March	1960	5	0.25
April	1800	10	0.50

Table 6. The hydatid cyst infection rate among sheep, goat, cattle, and buffalo from November 2023 to April 2024 in Kirkuk city, Iraq

Animal species	Slaughtered animals (Number)	Hydatid cyst infections (Number)	Positive cases (%)
Sheep	30900	120	0.38
Goat	797	0	0
Cattle	9990	113	1.13
Buffaloes	84	0	0

Table 7. Distribution of endemic hydatid cysts in sheep from November 2023 to April 2024 in Kirkuk city, Iraq

Month	Examined animals (Number)	Positive in liver and lungs (Number)	Total positive cases (%)
November	3900	15	0.38
December	5200	21	0.40
January	5000	19	0.38
February	5600	20	0.35
March	5200	20	0.19
April	6000	25	0.41

Table 8. Distribution of endemic hydatid disease in cattle from November 2023 to April 2024 in Kirkuk city, Iraq

Months	Examined animals (Number)	Positive in liver and lungs (Number)	Total positive (%)
November	1270	15	1.18
December	1560	23	1.28
January	1700	15	0.88
February	1700	20	1.17
March	1960	20	1.02
April	1800	20	1.11

DISCUSSION

Cystic echinococcosis and liver fluke infections significantly impair livestock productivity by reducing growth rates, increasing mortality and morbidity, and predisposing animals to secondary bacterial infections. Additionally, these conditions lead to organ condemnation and diminished meat and milk production, resulting in substantial economic losses (Najjari et al., 2020). Hydatid disease and *Echinococcus* spp. further compromise production efficiency through visceral damage and carcass wastage (Jahed Khaniki et al., 2013). To mitigate these risks, implementing a reliable meat inspection system is crucial for detecting animal diseases, particularly chronic conditions that may go undetected by farmers or veterinarians. Although fascioliasis, which is caused by *Fasciola* spp., has been extensively documented for its substantial veterinary impacts (Mas-Coma et al., 2009), numerous zoonotic bacterial diseases remain underrecognized despite their significant public health consequences. These findings align with the current results, which demonstrated high detection rates of *Fasciola* spp. and *Dictyocaulus* spp. infections. Additionally, *D. filaria* in sheep and goats, and *D. viviparous* in cattle, can cause lungworm infections (Chakraborty et al., 2014). Hydatidosis is a zoonotic parasitic illness caused by *Echinococcus* (Wen et al., 2019). The current study revealed an elevated prevalence of lungworm infections in slaughtered ruminants, with detection rates of 0.35% in cattle and 0.31% in sheep. In Afghanistan, Nangarhar, 21.8% of sheep examined at a slaughterhouse were found to be infected with lungworms (Samadi et al., 2019), although the type of lungworm was not specified. In the current study, the incidence of lungworm infection in small ruminants in April was higher than in other months, which is consistent with the findings of Alemu et al. (2006), Regassa et al. (2010), and Borji et al. (2012), who reported an increased spread of lungworm in small ruminants during spring. Thus, the incidence of lungworms in sheep and cattle is influenced by seasonal dynamics (Fesseha and Mathewos, 2021).

The occurrence of different infections in a given area is influenced by a multifactorial system comprising parasites, hosts, environmental factors, and physical and physiological stressors that can alter the immune status of animals (Kadir et al., 2012). The current study identified hydatid cyst infections in 1.13% of cattle (113 cases) and 0.38% of sheep (120 cases), representing significantly lower prevalence rates than those reported by Saeed et al. (2000) in Erbil Governorate, Iraq (22.3% in cattle and 27.4% in goats). Moreover, Wadood (2005) reported higher organ-specific prevalence rates of hydatid cysts in Basra, Iraq, with liver involvement in 65.4% of cattle and 52.9% of sheep, and lung infection in 52.72% of cattle and 47.01% of sheep. The present study indicated lower hepatic fascioliasis rates compared to the findings of Wadood (2005). Additionally, Jawad et al. (2018) reported a 1.84% prevalence of hydatidosis in cattle from Karbala, Iraq.

CONCLUSIONS

The present study indicated a reduction in the prevalence of fascioliasis, lungworms, hydatid disease, and associated parasites in sheep, goats, cattle, and buffalo. The present study highlighted the critical role of meat examination records in monitoring disease incidence and identifying potential long-term trends. The current findings identified winter as the highest risk season for parasitic infections in livestock, with elevated prevalence of fascioliasis (Liver fluke), hydatidosis, and pulmonary nematodiasis (Lung worms). Moreover, the present study on liver fluke, hydatid cyst, and lungworm infections provided baseline data for future surveillance of these potentially significant parasitic diseases in Kirkuk, Iraq. It is crucial to undertake additional studies on ruminants to thoroughly investigate the effects of parasites, lungworms, and liver flukes, as well as to clarify their potential impact on health and productivity. Therefore, it is advisable to implement strengthened measures for controlling these infections in Kirkuk of Iraq. To accomplish this, stricter treatment protocols and more rigorous monitoring of ruminants should be established.

DECLARATIONS

Acknowledgments

The authors express their gratitude to the staff of the College of Veterinary Medicine's Department of Public Health, University of Kirkuk, Kirkuk, Iraq, for their support. The authors sincerely thank the personnel of Kirkuk Asiriyah Slaughterhouse and the Meat Inspection Department for their diligent efforts in gathering the necessary information for the present study.

Authors' contributions

Abbas Saleh designed and analyzed the study. Cheia Wahhab and Almas Al-Bayati collected samples, wrote the manuscript, and analyzed the data. Kasim Abass and Al Salihi Karima supported the implementation of the study. All authors have read and approved the data and the final edition of the manuscript.

Funding

The present study did not receive any financial support.

Competing interests

The authors of the present study declared no conflict of interest.

Ethical considerations

The present study was originally written by the authors and has not been published elsewhere. The authors checked the text of the article for plagiarism index and confirmed that the text of the article is written based on their original scientific results.

Availability of data and materials

Data availability is available according to the request.

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2025, Scienceline Publication
World's Veterinary Journal
World Vet J, 15(3): 695-702.
ISSN 2322-4568

DOI: https://dx.doi.org/10.54203/scil.2025.wvj70
PII: S232245682500070-15

Veterinarians' Perceptions of Competence and Communication Skills in Bali, Indonesia

Nyoman Sadra Dharmawan[1]*, Ni Made Swasti Wulanyani[2], and Kadek Karang Agustina[3]

[1]*Veterinary Medicine Study Program, Faculty of Veterinary Medicine, Udayana University, Denpasar, Bali, 80234 Indonesia*
[2]*Psychology Study Program, Faculty of Medicine, Udayana University, Denpasar, Bali, 80234 Indonesia*
[3]*Department of Public Health, Faculty of Veterinary Medicine, Udayana University, Denpasar, Bali, 80234 Indonesia*
*Corresponding author's Email: nsdharmawan@unud.ac.id

ORIGINAL ARTICLE
Received: June 26, 2025
Revised: July 27, 2025
Accepted: August 30, 2025
Published: September 30, 2025

ABSTRACT

Effective communication is a crucial aspect of veterinary medicine. Despite its importance, this subject has not been sufficiently studied in the specific context of Bali, Indonesia, resulting in a notable gap in the existing research literature. The present study addressed a significant gap in the literature by systematically examining how communication challenges, specifically client dissatisfaction, inadequate treatment compliance, and recurrent misunderstandings between veterinarians and pet owners, affect the quality of veterinary care. The current study explored how veterinary practitioners in Bali, Indonesia, perceive their professional skills and communication abilities. Data were collected through interviews, focus group discussions, open-ended questionnaires, and surveys, involving 218 practitioners in the study area. The participants included small animal practitioners (65%), livestock veterinarians (25%), and mixed-practice clinicians (10%), with experience ranging from recent graduates to senior professionals with over 20 years in the field. The current findings revealed that 86.2% of respondents considered communication skills as equally important as clinical knowledge in facilitating critical professional outcomes, including client trust, treatment compliance, and clinical effectiveness. Effective communication was recognized as crucial for fostering confidence, increasing job satisfaction, strengthening client relationships, and supporting important activities such as anamnesis and treatment discussions. However, practitioners encountered difficulties in discussing sensitive topics, including life-threatening diagnoses, euthanasia, and costly treatment options. Notably, 86.7% of participants indicated the necessity for post-employment communication training to maintain and advance their skills, highlighting the significance of ongoing professional development in Indonesia. The present study emphasized the critical role of communication in veterinary practice and highlighted the demand for targeted training programs to address existing gaps.

Keywords: Client, Communication skill, Perception, Veterinarian

INTRODUCTION

Communication in veterinary medicine involves complex interactions among veterinarians, clients, animals, and colleagues. While perceptions of communication skills among veterinary practitioners might differ, their significance in daily practice is broadly recognized (Mabin and Taylor, 2023). Recently, communication has become increasingly recognized as a vital skill for veterinary professionals. This adjustment is reflected in the increasing focus on communication training in veterinary education, especially in developed countries (McDermott et al., 2017). Effective communication is essential not only in human healthcare professions but also as a fundamental skill in veterinary medicine (Gaida et al., 2018). Nevertheless, having only scientific and technical knowledge is not sufficient to succeed in this field. Veterinarians need to develop skills such as teamwork, entrepreneurship, cultural awareness, and effective communication with clients, staff, and colleagues (Gaida et al., 2021). Moreover, these skills are particularly important for veterinary academics, as they are crucial for handling interpersonal relationships and other professional responsibilities (Mabin and Taylor, 2023).

Effective communication is essential for successful interactions among veterinary practitioners and their clients, as well as among practitioners themselves. However, a lack of proficient communication often leads to different challenges, including misunderstandings, unclear cost explanations, ineffective dialogue, insufficient client engagement, and ethical concerns (Hernandez et al., 2018). To effectively address these challenges, veterinary practitioners should prioritize improving their communication skills, including providing clear and transparent information to clients and making a dedicated effort to understand the diverse needs and expectations of their clients. Therefore, veterinary professionals can foster stronger client relationships and improve overall service delivery within the field by enhancing their communication skills, providing clear and transparent information, and striving to understand their clients' needs and expectations better (Pun, 2020).

To cite this paper: Dharmawan NS, Wulanyani NMS, and Agustina KK (2025). Veterinarians' Perceptions of Competence and Communication Skills in Bali, Indonesia. *World Vet. J.*, 15(3): 695-702. DOI: https://dx.doi.org/10.54203/scil.2025.wvj70

From a psychological perspective, communication in veterinary practice can significantly impact practitioners' mental and emotional well-being. Positive interactions, such as effectively explaining treatment plans or receiving client gratitude, can enhance job satisfaction and alleviate stress and burnout. Conversely, difficult conversations, such as delivering unfavorable and bad news, discussing euthanasia, or managing client conflicts, may contribute to anxiety, compassion fatigue, and emotional exhaustion (Pun, 2020). Given these psychological factors, it is essential to develop strong communication skills for both professional success and protecting veterinarians' mental health in this challenging field (Steffey et al., 2023).

Although communication is recognized as vital in veterinary practice, no published studies have examined veterinary practitioners' perceptions of their competence and communication skills in Indonesia, especially in Bali. The present study aimed to explore the perceptions of veterinary practitioners in Bali regarding their professional competencies and communication skills, focusing on their perspectives about the importance and role of communication in their interactions with clients.

MATERIALS AND METHODS

Ethical approval

The present study employed solely interview methods with veterinary colleagues and focus group discussions (FGD). All participants have provided consent for the data they provided to be published. The present study did not involve the use of experimental animals, and all the procedures were conducted in compliance with the applicable ethical guidelines from the Ethical Commission of Udayana University, Indonesia.

Study design

The present study employed a mixed-methods approach involving 218 veterinary practitioners in Bali, Indonesia (Figure 1), conducted from May to August 2024. Data were collected through in-depth interviews, FGD, open-ended questionnaires, and attitudinal scale questionnaires. Qualitative analysis was utilized to examine responses from interviews, FGD, and open-ended questions, whereas quantitative methods were employed to assess data from the attitude scales. This questionnaire was created based on the study of McDermott (2018). Before implementation, a multidisciplinary panel of experts conducted an extensive validation of the questionnaire by specialists. The review panel included academic experts from Udayana University's Faculty of Veterinary Medicine, senior veterinary practitioners with at least 15 years of clinical experience, government officials from animal health regulatory agencies, industry representatives from veterinary employers, and executive board members of the Indonesian Veterinary Association, including its Bali branch. The experts conducted a comprehensive evaluation of the questionnaires and attitude scales, assessing them for content validity, specifically considering each item's relevance to the constructs being assessed. Additionally, the experts evaluated face validity related to clarity and contextual appropriateness for Balinese veterinary practice, cultural sensitivity, technical accuracy of clinical information, and practical applicability of tools. The pre-testing phase involved 30 veterinary practitioners to assess the instrument's feasibility under field conditions.



Figure 1. The location of the study, Bali Island, Indonesia

Sample size

According to the Indonesian Veterinary Medicine Association data, the total number of veterinarians in Indonesia as of June 2024 was 15,131, with approximately 900-1000 practicing in Bali province (IVMA, 2024). The inclusion criteria for participants in the present study consisted of practicing veterinarians employed in animal hospitals, clinics, or private practices across different locations in Bali. Using Slovin's formula with a 5% margin of error (Khalaf et al., 2019), the minimum required sample size was calculated to be 109 respondents to adequately represent the target population, including a proportional representation of Bali practitioners. To improve the validity and reliability of the current findings, the sample size was doubled, resulting in a total of 218 participants. This expanded sample size increased statistical power and provided a better representation of the target population of veterinary practitioners in Bali, Indonesia. $n = \frac{N}{1+Ne^2}$

which n is the required sample size, N is the total population size, and e is the tolerable margin of error (5%).

Data collection and analysis

The validity of the veterinary communication skills scale (VCSS) in the present study was evaluated using content validity procedures. Content validity was established through expert evaluation to ensure the scale's items accurately represented essential veterinary communication skills. A panel of veterinary communication specialists reviewed each item for relevance, clarity, and comprehensiveness in measuring key competencies such as empathy, active listening, and client education. Items that did not align with communication competencies were improved or removed to enhance the scale's accuracy (Hecker et al., 2012). The content validity index was employed to evaluate the degree of agreement among experts regarding the scale. The current findings indicated that the results met or exceeded established acceptable thresholds, thereby confirming the strength of the measurement tool (Dalawi et al., 2023). This approach ensured that the VCSS effectively captures the critical aspects of veterinary communication, supporting its use as a valid assessment tool in both educational and clinical settings (Artemiou et al., 2014).

The validity of the statements in the present study was assessed by expert evaluations, quantified with Aiken's V index. A panel of subject-matter experts rated each statement for relevance, clarity, and appropriateness in measuring the intended constructs. Aiken's V, a statistical metric for content validity, was used to assess expert agreement, with scores from 0 to 1; higher values signify greater agreement on an item's validity. Only statements meeting a predefined threshold (Typically $V \geq 0.70$) were retained, ensuring the instrument's robustness. This method provided a rigorous and objective way to validate content, improving the credibility of the assessment tool (Timbi-Sisalima et al., 2024).

To examine the underlying structure of the perception variables, the data were analyzed using exploratory factor analysis with Jeffreys's amazing statistics program, an open-source statistics software, which enables robust factor extraction and interpretation. Principal axis factoring with oblique rotation, including promax, was employed to identify latent constructs, allowing for correlations between factors when theoretically justified. The number of retained factors was determined using multiple criteria, including Kaiser's eigenvalue-greater-than-one rule, parallel analysis, and scree plot inspection, providing a method grounded in data while remaining a theoretically relevant solution. Items with factor loadings below 0.40 or significant cross-loadings were removed to improve scale clarity and discriminant validity. This method provided empirical support for the dimensionality of the measured constructs while enhancing the scale's psychometric qualities (Traymbak et al., 2022).

Survey responses assessing the perceived importance of communication skills in clinical activities and practice factors were analyzed using a weighted Likert scale (1-5). Response frequencies for each category (1 means strongly disagree to 5 means strongly agree) were multiplied by their respective numerical values and summed to yield total weighted scores. Mean scores were calculated by dividing each variable's total weighted score by the number of responses. Results were categorized as 1.00-1.80 (Very Low), 1.81-2.60 (Low), 2.61-3.40 (Neutral), 3.41-4.20 (High), and 4.21-5.00 (Very High; Joo et al., 2024).

The current data were presented in both descriptive and qualitative formats to ensure comprehensive interpretation and clarity. This dual-method approach provided a deeper understanding of the results, as the numerical patterns were complemented by detailed narrative explanations. By combining both methods, the present study ensured that the results were statistically relevant within the specific context, thereby improving the overall validity and practical usefulness of the findings.

RESULTS AND DISCUSSION

A total of 218 veterinarians, whose ages ranged from 24 to 65 years, participated in the current study by completing a structured questionnaire. The participants included small animal practitioners (65%), livestock veterinarians (25%), and mixed-practice clinicians (10%), with experience spanning from recent graduates to senior professionals with over 20 years of experience in the field. The gender distribution of the participants in the present study revealed a slight majority

of males (51.8%) in comparison to females (48.2%). This distribution stands in contrast to the findings of McDermott et al. (2015), which reported a predominance of female respondents (57.3%) over males (42.7%) in their study conducted across the United States and the United Kingdom. This disparity may be indicative of underlying regional sociocultural norms, as well as differences in veterinary specialty selections, where large-animal practice has historically drawn a higher proportion of male practitioners (Morello et al., 2021). Additionally, varying expectations regarding work-life balance may contribute to these disparities. Noteworthy studies have demonstrated that female veterinarians encounter distinct challenges, including an elevated incidence of burnout and client bias, factors that could significantly affect their career sustainability within the specific context of Bali (Steffey et al., 2023).

Communication training

The findings regarding respondents' participation in communication training are detailed in Table 1. The distribution of participants who engaged in communication training within the context of the current study was notably balanced, with 109 respondents (Representing 50% of the total) indicating their involvement in such training. The current findings indicate that 50% of respondents received communication training during their veterinary education, with lecture-based instruction being the most common format (72.5% of trained participants). Following graduation, only 43.6% of practitioners pursued further training in communication skills. The present data revealed a significant decline in participation in communication training after the completion of formal education. A significant majority of respondents (86.7%, n = 189/218) from Bali expressed a keen interest in pursuing additional communication training opportunities. Among these practitioners, 70.4% (n = 133/189) showed a strong preference for consultation and simulation-based training formats, highlighting the critical demand for practical learning methodologies that remain unaddressed, emphasizing the significant local need for educational approaches.

Table 1. Respondent data on communication training among veterinary practitioners in Bali, Indonesia, in 2024

Time period	Participation	Number (%)	Type of Training			
			A	B	C	D
During the study	Yes	109 (50%)	14 (12.8%)	2 (1.8%)	79 (72.5%)	14 (12.8%)
	Not	109 (50%)				
After graduation	Yes	95 (43.6%)	34 (35.8%)	36 (37.9%)	18 (18.9%)	7 (7.4%)
	Not	123 (56.4%)				

A: Consultation/Simulation, B: Online, C: Lecture, D: No response

The current findings are consistent with McDermott et al. (2015), who emphasized the importance of enhancing communication training for practicing veterinarians, especially through more practical and accessible approaches such as simulation and online methods training. The present study revealed a significant gap between veterinarians' awareness and training in communication skills. Although the majority acknowledged the importance of communication competencies, only 41% had received formal instruction during veterinary school. Notably, among veterinary practitioners who engaged in post-graduation training, 61% reported a significant increase in their preparedness for client interactions. Effective communication skills are essential in veterinary practice, as they not only facilitate the understanding of client concerns but also contribute to the delivery of high-quality patient care. These communication skills may be equally, if not more, essential than clinical knowledge on their own (Kourkouta and Papathanasiou, 2014).

Simulation-based and online training offer flexible solutions for busy veterinarians. Simulation allows practitioners to engage in realistic scenarios, refining their communication skills in a clinical context without risk to patients or clients (Englar, 2017). Online training provides accessibility and convenience, facilitating integration into challenging work schedules (Spruijt et al., 2023). Pun (2020) emphasized that many veterinary practitioners lack specialized training in communication and suggested that veterinary curricula should incorporate more comprehensive communication training. Communication training in veterinary schools has been demonstrated to equip graduates for professional challenges, boost their clinical outcomes, and foster better team collaboration (Mills, 2024). In developed countries such as the United States, the United Kingdom, Germany, and Canada, communication skills training is already a standard part of healthcare curricula (Gaida et al., 2021). In veterinary medicine, effective communication is a key skill that greatly impacts professional success (Bell et al., 2019). Effective communication is linked to higher pet owner satisfaction and improved veterinarian well-being. Developing strong relationships and showing empathy play crucial roles in enhancing owner compliance (Brown, 2018). For veterinarians, strong communication skills enhance job satisfaction and foster effective teamwork (Stackhouse et al., 2020). Mills (2024) noted that many veterinary colleges have integrated

communication skills training into their curricula, often incorporating client interaction simulations and advanced communication techniques.

The strong preference for simulation-based training (70.4%) highlights its proven effectiveness in closing the knowing-doing gap in clinical communication (Spruijt et al., 2023). Unlike conventional lectures, simulations provide a safe environment for deliberate practice of high-stakes situations such as euthanasia discussions, offering repetitive exposure along with immediate feedback. This approach directly addressed the main challenges identified by Bali practitioners through different mechanisms (Table 1). First, role-playing exercises improve emotional readiness by lowering anxiety related to difficult conversations, while allowing for the integration of local client-practitioner dynamics. Additionally, the hands-on aspect of simulation promotes better skill retention than passive learning methods, leading to more lasting competency development. These advantages collectively explain why clinical simulation has become the preferred training method among respondents (Favier et al., 2021).

Perception of communication skills

The current findings revealed that the majority of respondents (86.2%) perceive communication skills as equally important as clinical knowledge, while 13.8% believe communication skills are even more important than clinical knowledge. The strong agreement among respondents on the importance of communication skills reflected the findings of McDermott et al. (2015), where 98% of surveyed veterinarians in the United States and the United Kingdom rated communication skills as at least equally important to clinical practice knowledge. Pun (2020) further supports the perspective by underscoring the significance of effective communication as a vital professional skill within the practice of veterinary medicine. Effective communication plays a pivotal role in strengthening veterinarian-client relationships, enhancing the efficacy of medical care, and facilitating a smoother transition for new graduates into clinical practice. Moreover, effective communication is integral to fostering professionalism and promoting collaborative teamwork within veterinary practice (Mills, 2024).

In the present study, communication skills were rated as highly important, with very high scores, across multiple aspects, including confidence, job satisfaction, time management, income/profit, client relationships, and colleague relationships. Additionally, communication skills were deemed highly important in most of the clinical activities, such as obtaining medical histories, making diagnoses, explaining diagnoses and prognoses, discussing treatment plans, obtaining client consent, managing client expectations, optimizing client compliance, encouraging follow-up visits, and discussing treatment costs (Table 2). Mills (2024) highlighted that effective communication is essential for establishing and maintaining long-term relationships between veterinarians and clients. Veterinarians who dedicate time to understanding client concerns and expectations are more likely to build client loyalty and foster lasting relationships (Abood, 2007). Clients who perceive that their veterinarian actively listens to and values their contributions demonstrate a greater propensity to seek subsequent services. This phenomenon is vital for the sustainability and long-term viability of veterinary practices (Pun, 2020). The present findings are consistent with prior studies, which have explored the significance of communication skills, their impact on client satisfaction, the role of training and education, factors influencing communication proficiency, the effect of communication on clinical decision-making, and communication risks related to zoonoses (Arnecke et al., 2024).

Communication challenges in veterinary practice

Regarding the communication challenges faced by veterinary practitioners in Bali, Indonesia, the current findings are summarized in Table 3. The present findings indicated that discussions surrounding euthanasia constituted the most challenging communication topic, as evidenced by 102 respondents rating it as very difficult. This was followed by communications regarding life-threatening conditions (54 respondents), costly treatments (53 respondents), difficult diagnoses (53 respondents), and complex treatment protocols (44 respondents). In contrast, discussions concerning prolonged care were perceived as moderately complex, yielding an average score of 3.39.

Multiple peer-reviewed studies have documented the professional challenges veterinarians face when conveying unfavorable news, explaining complex diagnoses, or guiding end-of-life care decisions (Artemiou et al., 2014; Hernandez et al., 2018; Gaida et al., 2021). According to Pun (2020), discussing euthanasia is often one of the most emotionally challenging parts of veterinary practice. Many veterinarians experience significant emotional stress when recommending or performing euthanasia, particularly when clients are hesitant to accept or fully understand the decision (Cooney and Kipperman, 2023). Veterinarians often have to balance the animal's welfare with the emotional needs and expectations of the owner (Quain et al., 2021). Another significant challenge is handling client expectations, particularly in cases where clients hold unrealistic expectations regarding treatment outcomes or the prognosis for critically ill animals (Christiansen et al., 2016). These professional challenges become particularly pronounced when practitioners are required to clarify the limitations of therapeutic interventions or to justify euthanasia as the most ethically defensible option (Pun, 2020).

Table 2. Veterinary practitioners' perceptions of communication skill importance across clinical activities and practice factors in Bali, Indonesia, in 2024

Variable	Number of Responses					Number of Responses × Score	Average	Category
	1	2	3	4	5			
Factors								
Confidence	0	0	9	22	187	1050	4.82	Very High
Job satisfaction	0	0	9	47	162	1025	4.70	Very High
Time management	0	0	13	58	147	1006	4.61	Very High
Income/profit	0	0	18	53	147	1001	4.59	Very High
Client relationships	0	0	6	17	194	1056	4.86	Very High
Colleague relationships	0	1	5	25	187	1052	4.83	Very High
Types of Activities								
Obtaining medical history	0	0	6	22	190	1056	4.84	Very High
Diagnosing a condition	0	0	12	26	180	1040	4.77	Very High
Explaining the diagnosis	0	0	4	11	203	1071	4.91	Very High
Discussing prognosis	0	0	8	30	180	1044	4.79	Very High
Discussing treatment	0	1	8	28	181	1041	4.78	Very High
Obtaining client consent	0	0	4	25	189	1057	4.85	Very High
Managing client expectations	0	0	6	25	187	1053	4.83	Very High
Optimizing client compliance	0	0	8	41	169	1033	4.74	Very High
Encouraging follow-up visits	0	0	7	35	176	1041	4.78	Very High
Discussing treatment costs	0	0	10	39	169	1031	4.73	Very High

1: Very Low, 2: Low, 3: Moderate, 4: High, 5: Very High

Table 3. Perceptions of practicing veterinarians in Bali regarding communication difficulties in clinical case discussions with clients, in 2024

Variable	Number of Responses					Number of Responses × Score	Average	Category
	1	2	3	4	5			
Conditions where diagnosis is difficult	9	24	82	49	53	764	3.53	Difficult
Conditions where treatment is difficult	9	28	85	52	44	748	3.43	Difficult
Life-threatening patient conditions	13	34	64	53	54	755	3.46	Difficult
Conditions requiring Euthanasia	7	16	52	41	102	869	3.99	Difficult
Conditions requiring expensive treatment	11	28	70	56	53	766	3.51	Difficult
Conditions requiring prolonged treatment	17	33	69	47	52	738	3.39	Moderate

1: Very easy, 2: Easy, 3: Moderate, 4: Difficult, 5: Very difficult

Implications for veterinary education

Previous studies in veterinary communication have highlighted the importance of a structured approach to teaching effective communication skills within veterinary curricula (Pun, 2020; Mabin and Taylor, 2023). Haldane et al. (2017) found that both veterinary practitioners and students consider verbal communication and interpersonal skills to be the most essential competencies for aspiring professional veterinarians. In English-speaking countries, the Calgary-Cambridge model has been widely adopted by veterinary institutions to develop students' communication skills, leading to improved client interactions (Bard et al., 2017). However, Agne et al. (2024) emphasize that despite the recognized importance of teaching communication-based clinical reasoning skills, current educational approaches for teaching these skills remain inadequate. Consequently, there is an urgent requirement for integrated communication skills training within veterinary education programs across higher education institutions globally.

Study limitations

While the present study contributed a valuable understanding of veterinary communication training in Bali, several limitations should be acknowledged. The self-reported nature of the data could lead to response bias, particularly regarding individuals' evaluations of their communication skills (Rosenman et al., 2011). Focusing on Bali as a regional example may restrict the relevance of these findings to other Indonesian provinces that have different cultural or practical traits (Muhtar et al., 2025). Regarding gender differences, the current findings of a slight male predominance (51.8%) contrast with Western studies (McDermott et al., 2015) and may reflect Bali's unique veterinary workforce dynamics. This warrants further investigation into cultural, educational, or professional factors influencing gender distribution in Indonesian veterinary medicine. The high preference for simulation-based training, indicated by 70.4% of respondents, can be attributed to its established efficacy in improving clinical communication skills through intentional practice in secure environments, as evidenced by the findings of Spruijt et al. (2023). This method, designed to evaluate communication issues in veterinary practice, provided instant feedback and allowed repeated practice of complex situations such as euthanasia discussions, directly targeting the specific challenges highlighted in the current results.

CONCLUSION

The present study confirmed that communication skills are equally vital as clinical expertise in veterinary practice, with 86.2% of practitioners acknowledging their importance for both clinical outcomes and professional achievement. While 50% of individuals received training primarily delivered through lectures, a notable 86.7% expressed a strong desire for additional educational opportunities. Among these respondents, a significant preference for simulation-based learning was indicated, with 70.4% highlighting it as a particularly valuable mode of instruction. The primary challenges discussed include ethical concerns surrounding euthanasia, the high costs of medical treatments, and difficulties in achieving accurate diagnoses. Veterinary curricula should incorporate mandatory communication training through realistic methods and simulations. Findings should be interpreted within Bali's context, as the unusual gender distribution needs further exploration. Implementing these changes would enhance patient care, strengthen client relationships, and increase practitioner satisfaction. Future studies should incorporate standardized client evaluations to validate self-reported data and assess regional differences. Additionally, the impact of gender on communication styles and challenges in client interactions across Indonesia's different veterinary practices should be investigated.

DECLARATIONS

Acknowledgments

The authors would like to thank the Indonesian Veterinary Medicine Association, Bali Branch, and the study respondents for their support.

Authors' contributions

All authors were actively involved in every stage of the study and manuscript preparation process. Nyoman Sadra Dharmawan designed the theoretical framework and research concept, supervised data collection, and contributed to manuscript preparation. Ni Made Swasti Wulanyani contributed to the study methodology, data analysis and interpretation, and manuscript preparation. Kadek Karang Agustina participated in study design, organized focus group discussions, coordinated data collection, and assisted in manuscript preparation. All authors have reviewed and approved the final edition of the manuscript.

Funding

The present study was funded by Udayana University through the Institute for Research and Community Service under contract number B/255.106/UN14.4.A/PT.01.03/2024.

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.

Availability of data and materials

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

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2025, Scienceline Publication
World's Veterinary Journal
World Vet J, 15(3): 703-709.
ISSN 2322-4568

DOI: https://dx.doi.org/10.54203/scil.2025.wvj71
PII: S232245682500071-15

Morphometrical Analysis of the Reproductive System in Boschveld Chickens

Leah Mmolaoa Mothiba, Lindiwe Johannah Sathekge, Victoria Rankotsane Hlokoe, and Thobela Louis Tyasi*

School of Agriculture and Environmental Sciences, Department of Agricultural Economics and Animal Production, University of Limpopo, Private Bag X1106, Sovenga 0727, Limpopo, South Africa

*Corresponding author's Email: louis.tyasi@ul.ac.za

ORIGINAL ARTICLE
Received: June 17, 2025
Revised: July 28, 2025
Accepted: August 31, 2025
Published: September 30, 2025

ABSTRACT

The egg yield is determined by the number of ovarian follicles that ovulate and the oviduct's capability to form a hard-shelled egg around the egg cell. However, the morphological characteristics of the reproductive organs in Boschveld chickens have not been explored. The present study aimed to evaluate the morphology of the reproductive system, including the oviduct and the developing ova in the Boschveld chicken breed. To perform a morphological analysis, ovarian follicles and oviducts were obtained from six Boschveld hens (Out of a total of twenty), aged 30 weeks, sourced from the University of Limpopo experimental farm in South Africa. The present results revealed significant differences in both the weight and number of small white (2-4 mm), large white (5-6 mm), and small yellow (7-8 mm) follicles. Among the mature follicles, Follicle 1 (F1) exhibited the highest weight, whereas F6 demonstrated the lowest weight. Additionally, the weights and lengths of the infundibulum, magnum, isthmus, and shell gland varied significantly in Boschveld chickens. The present findings indicated that the magnum was the heaviest and longest oviduct segment in Boschveld chickens, and the heaviest pre-ovulatory follicle was ovarian F1, followed by ovarian F2. The results revealed a distinct profile characterized by many small white follicles and a well-developed magnum, which is the largest part of the oviduct. These findings provided a crucial anatomical baseline for the breed, indicating that the structure of the follicular hierarchy and oviduct plays a crucial role in its egg-laying ability. Improving the development and function of these reproductive parts could boost productivity in the Boschveld breed.

Keywords: Boschveld chicken, Isthmus, Magnum, Morphology, Ovarian follicle

INTRODUCTION

Poultry production is a vital part of the agricultural sector, significantly contributing to the global food system by supplying meat and eggs as primary protein sources for consumers (Vlaicu et al., 2024). The demand for poultry production has increased alongside the growth of the global population in KwaZulu-Natal, South Africa (Kleyn and Ciacciariello, 2021). The Boschveld chicken, a breed developed in South Africa, is well-known for its adaptability to free-range farming and its dual-purpose utility for both meat and egg production (Manyelo et al., 2020). Boschveld chicken is known for its robust characteristics, including disease resistance and strong brooding ability, making Boschveld hens well-suited to harsh environmental conditions (Hlokoe and Tyasi, 2022). Despite these advantages, the Boschveld breed has relatively low egg production, which limits its profitability in intensive or semi-intensive farming systems (Okoro et al., 2017). Consequently, if the sequential growth and development of ovarian follicles remain stagnant at the developmental stage, egg production in chickens will decline (Xu et al., 2024). The morphology and function of the chicken's reproductive system, particularly the oviduct and ovarian follicles, are directly related to egg productivity (Chen et al., 2021). Investigations into ovarian follicle development and oviduct characteristics in different breeds, such as Potchefstroom Koekoek and hybrids of White Leghorn, have highlighted significant morphological variations influencing egg-laying performance (Nie et al., 2022). Therefore, the present study aimed to determine the morphological variations of the ovarian follicles and identify the weights and lengths of the oviduct in Boschveld chickens.

MATERIALS AND METHODS

Ethical approval

The Animal Research Ethics Committee (AREC), certified by the National Health Research Ethics Council of South Africa, approved the present study (Registration Number: AREC-290914-017).

To cite this paper: Mothiba LM, Sathekge LJ, Hlokoe VR, and Tyasi TL (2025). Morphometrical Analysis of the Reproductive System in Boschveld Chickens. *World Vet. J.*, 15(3): 703-709. DOI: https://dx.doi.org/10.54203/scil.2025.wvj71

Study area

The current study was conducted at the Experimental Farm of the Limpopo University, situated approximately 10 km northwest of the Turfloop campus in South Africa. Summer temperatures (November to January) differs from 20°C to 36°C, whereas winter temperatures (May to July) ranged from 5°C to 25°C. The farm is positioned at a latitude of 27.55ºS and a longitude of 24.77ºE, with an average annual rainfall of less than 400 mm (Hlokoe et al., 2023).

Study design

A total of 20 Boschveld hens, each weighing approximately 1.7 kg, were acquired at 20 weeks of age from Boschveld Farm, South Africa and subsequently reared at the Experimental Farm of the University of Limpopo, South Africa. The hens were housed in an intensive production system under consistent environmental conditions. The interior temperature of the poultry house ranged from 20 to 21°C, with relative humidity maintained between 50% and 60% to ensure the health and well-being of the poultry (Alabi et al., 2012). The Boschveld hens were raised within an enclosed poultry house under a controlled production system. The side openings were fitted with PVC vinyl curtains to regulate ventilation, which were kept open during daylight hours and closed at night to safeguard the hens from rodents and predators. During the laying period, a lighting schedule of sixteen hours of light and eight hours of darkness was maintained in accordance with the recommendations of Nassar et al. (2017). One week before the arrival of the chickens, the poultry house was meticulously cleaned and disinfected utilizing Virokill disinfectants (Dalton, UK) at a concentration of 100 grams per 10 liters of water, to prevent the occurrence of diseases. Biosecurity protocols were stringently implemented, including the use of footbaths with Glutaraldehyde-based disinfectants (Deluxe Chemicals, South Africa) at the entrance to ensure appropriate sanitation prior to entry house, as described by Alabi et al. (2012). The hens were provided with laying mash (Driehoek Feeds, South Africa) starting at 20 weeks and had unrestricted access to water. The diet consisted of the following nutritional components, including crude protein (16%), crude fats and oils (4.3%), crude fibres (4.8%), crude ash (13.6%), calcium (4.3%), phosphorus (0.6%), sodium (0.15%), lysine (0.7%), methionine (0.35%) and metabolizable energy (2453.60 Kcal/kg). The diet was formulated in accordance with NRC (1994) standards. Chickens received vaccinations against Marek's disease (Prevexxion® RN vaccine, Boehringer Ingelheim Animal Health Ltd, South Africa) on the first day via subcutaneous injection, against Newcastle disease with B1 strain (LaSota) vaccine (BioMedical International Pvt Ltd, India) through oral administration on day one, and against Gumboro disease (Nobilis® Gumboro 228E vaccine, Merck Sharp and Dohme Animal Health, Netherlands) on day fourteen through drinking water following the procedure by Iqbal and Khan (2023). At thirty weeks of age, six laying hens were randomly selected and euthanize to facilitate the collection of ovarian follicles and oviducts for morphological analysis.

Collection of ovarian follicles and oviducts

The selected 30-week-old Boschveld hens were euthanized, following the method described by (Jacobs et al., 2019). Before euthanizing, the hens underwent fasting for eight to twelve hours to ensure that their digestive tracts were emptied, as noted by Mfoundou et al. (2021). The chickens were euthanized by initially performing cervical dislocation, which is a method of animal euthanasia involving the rapid dislocation of the neck, leading to death through the disruption of the spinal cord and blood circulation to the brain (Jacobs et al., 2019). The pharynx, neck arteries, cervical veins, oesophagus, and windpipe were then sliced without severing the head. The carcasses were deplumed and dissected by cutting from the oesophagus down to the stomach, exposing the reproductive system to collect the oviduct and ovarian follicles, as described by Nassar et al. (2017). Moreover, the oviducts were collected following the method described by Mohammadpour et al. (2012). The ovarian follicles were collected from the reproductive system of the Boschveld hens by dissecting the hen into two halves. The ovarian follicles were then categorized according to their size (Table 1) The ovarian follicles were then categorized according to their size (Table 1). The largest pre-ovulatory follicles were hierarchically ranked and designated as Follicle 1 (F1) to F6. The weights of the small yellow follicle (SYF), large white follicle (LWF), and small white follicle (SWF) were measured using an electronic weighing scale (Medidata®, USA) with a precision of 0.01 g, while the lengths and widths of the SYF, LWF, and SWF were determined with vernier calipers (Mitutoyo® digital, Japan) with an accuracy of 0.01 mm. The number of large yellow follicles (LYF), SYF, LWF, and SWF were evaluated and recorded. The length of the ovarian follicles was measured with a vernier caliper. The total weight and length of the oviduct were measured with an electronic scale and vernier calipers, respectively. The oviducts from the six Boschveld carcasses were removed from their reproductive systems and dissected into four parts, including the infundibulum, isthmus, magnum, and shell glands. The weight of each oviduct section was recorded with an electronic scale, and the lengths and widths of the segments were measured with vernier calipers and a tape measure calibrated in centimeters.

Table 1. Follicle categories of 30-week-old Boschveld chickens

Follicle type	Size range (mm)
Small yellow follicle (SYF)	6 - 8
Large yellow follicles (LYF)	9 - 37
Follicle 1 (F1)	> 37
Follicle 2 (F2)	32 - 36
Follicle 3 (F3)	29 - 31
Follicle 4 (F4)	25 - 28
Follicle 5 (F5)	20 - 24
Follicle 6 (F6)	>20

Source: Wang et al. (2017). mm: Millimeters. The largest pre-ovulatory follicles were hierarchically ranked from F1 (largest) to F6 (smallest).

Statistical analysis

The current findings were analyzed using the Statistical Package for Social Sciences (SPSS) version 29.0 (IBM SPSS, 2023). Descriptive statistics, presented as Mean ± Standard Error (SE), were used to summarize the data regarding the morphology of ovarian follicles and oviducts. A one-way Analysis of Variance (ANOVA) was applied to examine variations among ovarian pre-hierarchical follicles and between the morphologies of ovarian follicles and oviducts. Statistical significance was set at $p < 0.05$.

RESULTS

Weight of chickens, oviducts, and pre-hierarchical follicles

Table 2 presents the differences among ovarian pre-hierarchical follicles, including the weights of SWF, SYF, and LWF. The current findings indicated significant differences in the weights of pre-hierarchical follicles, specifically revealing that the weight of SWF was substantially more than that of SYF and LWF ($p < 0.05$). However, no significant difference was found between the weights of SYF and LWF ($p > 0.05$). The average weight of the chickens was 3.50 kg, while the average weight of the oviducts was 43.53 g.

Table 3 indicates the differences in the number of ovarian pre-hierarchical follicles of 30-week-old Boschveld chickens. The present results indicated a significant difference in the number of SWF being greater than that of LWF and SYF ($p < 0.05$).

Table 2. Weight of chickens, oviducts, and pre-hierarchical follicles of 30-week-old Boschveld chickens

Parameters	Mean ± SE
Weight of small white follicles (SWF)	$6.27^{a} \pm 0.24$
Weight of small yellow follicles (SYF)	$0.55^{b} \pm 0.08$
Weight of large white follicles (LWF)	$0.56^{b} \pm 0.09$
Average weight of chickens	3.50 ± 0.76
Weight of oviducts	43.53 ± 0.12

SE: Standard error of means. [a and b] Means with the different superscript letters in the same column are significantly different ($p < 0.05$).

Table 3. Number of pre-hierarchical ovarian follicles of the 30-week-old Boschveld chickens

Parameters	Mean ± SE
Number of small white follicles (SWF)	$53.83^{a} \pm 1.05$
Number of large white follicles (LWF)	$2.50^{c} \pm 0.22$
Number of small yellow follicles (SYF)	$4.50^{b} \pm 0.22$

SE: Standard error of means. [a, b, and c] Means with the different superscript letters in the same column are significantly different ($p < 0.05$).

Number and weight of pre-ovulatory follicles

Table 4 illustrates the weights of pre-ovulatory follicles (F1 to F6). Significant differences were found among pre-ovulatory follicles, with F1 having the highest weight compared to other pre-ovulatory follicles ($p < 0.05$; Figure 1).

Table 4. Weights of pre-ovulatory follicles in 30-week-old Boschveld chickens

Parameters	Weight (Mean ± SE)
Follicle 6 (F6)	$12.51^{f} \pm 0.09$
Follicle 5 (F5)	$21.28^{e} \pm 0.09$
Follicle 4 (F4)	$27.42^{d} \pm 0.05$
Follicle 3 (F3)	$29.44^{c} \pm 0.12$
Follicle 2 (F2)	$35.44^{b} \pm 0.12$
Follicle 1 (F1)	$39.37^{a} \pm 0.09$

SE: Standard error of means. [a, b, c, d, e, and f] Means with the different superscript letters in the same column are significantly different ($p < 0.05$).

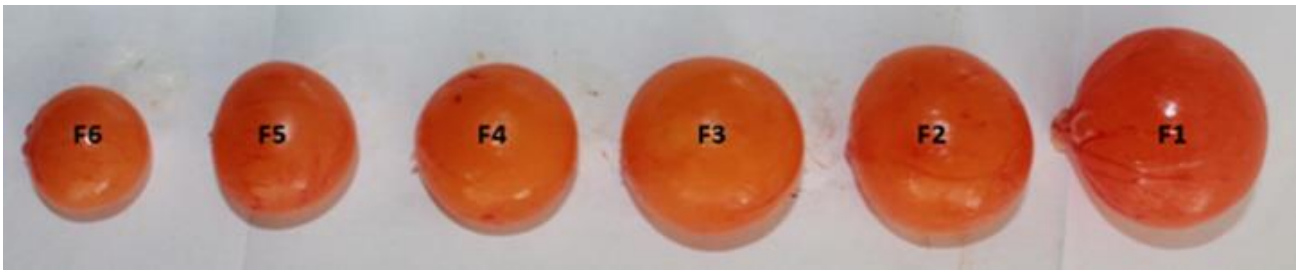


Figure 1. Classification of pre-ovulatory follicles (F1- F6) of 30-week-old Boschveld chickens

Weights and lengths of the oviduct segments

Table 5 presents the differences between the weights and lengths of oviduct segments. The weights of the infundibulum, magnum, isthmus, and shells gland were significantly different ($p < 0.05$). The magnum segment weighed more than the shell gland, isthmus, and infundibulum. The current findings exhibited that the lengths of the infundibulum, magnum, isthmus, and shell gland varied significantly ($p < 0.05$), with the magnum segment being larger than the infundibulum, isthmus, and shell gland. Figure 2 illustrates the different oviduct segments of the 30-week-old Boscveld chicken.

Table 5. Oviduct segments of 30-week-old Boschveld chickens

Parameters	Mean ± SE
Weight of the infundibulum	$9.70^{c} \pm 0.11$
Weight of magnum	$32.26^{a} \pm 0.12$
Weight of the isthmus	$3.52^{d} \pm 0.18$
Weight of the shell gland	$20.36^{b} \pm 0.16$
P-value	$p < 0.05$
Length of the infundibulum	$12.18^{b} \pm 0.96$
Length of magnum	$34.56^{a} \pm 0.08$
Length of isthmus	$7.30^{d} \pm 0.09$
Length of the shell gland	$9.39^{c} \pm 0.09$
P-value	$p < 0.05$
Length of oviduct	63.42 ± 0.12

SE: Standard error of means. [a, b, c, and d] Means with the different superscript letters in the same column are significantly different ($p > 0.05$).

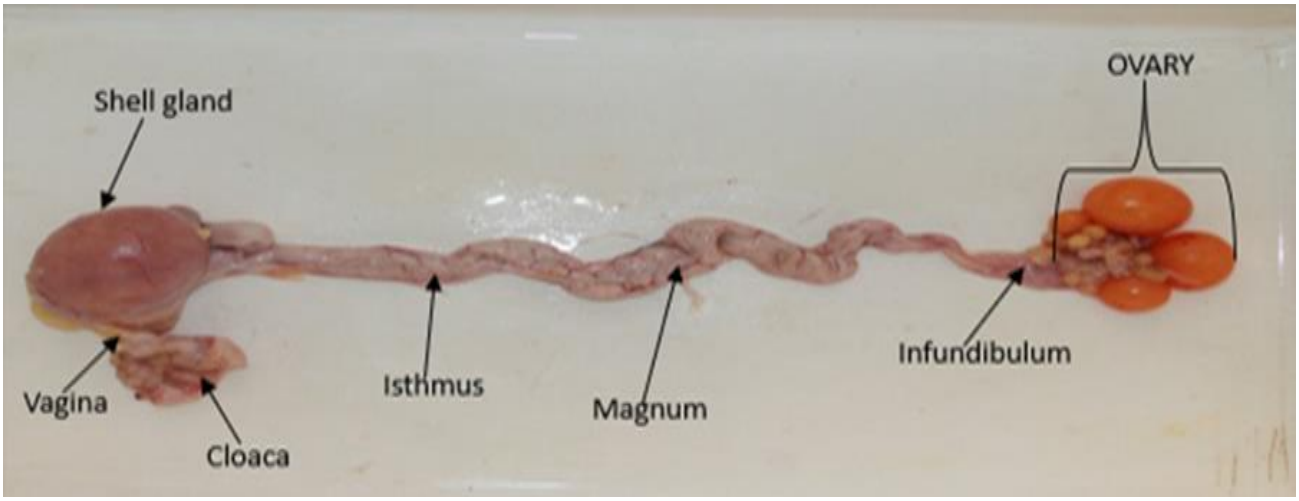


Figure 2. Oviduct anatomy of the Boschveld chicken. Source of the image: captured by the authors of the present study.

DISCUSSION

The number of ovarian follicles designated for ovulation or atresia, along with the oviduct's capacity to effectively convert ova into a hard-shelled egg, serve as indicators of optimal productivity (Yang et al., 2019). The present study revealed significant variation in the weights of pre-hierarchical ovarian follicles, with SWF weighing more than SYF and LWF. The current findings illustrated that there was no significant difference between the weights of SYF and LWF. Moreover, the number of SWF exceeded those of both LWF and SYF. Additionally, significant differences were observed in the weights of pre-ovulatory follicles, with F1 being the heaviest among all pre-ovulatory follicles. The results of the current study contrast with the findings of Nie et al. (2022), who reported that, in yellow-bearded chickens, LYF were more numerous and heavier than both SYF and LWF. Such discrepancies could be attributed to differences in chicken breeds. Nassar et al. (2017) identified significant differences in the number of LYF and SYF in chickens of the Cairo L-2 strain and the LBL strain. The current findings implied that SWF may play a vital role in understanding the reproductive potential of the breed, as a higher number of SWF could indicate an increased capacity for ovulation. Wang et al. (2017) and Li et al. (2019) asserted that the different stages of ovarian follicle growth and development influence the efficiency of egg production in chickens.

In addition, the current study characterized the weights and lengths of oviduct segments in Boschveld chickens using descriptive statistics. The present findings indicated that there were significant differences in the weights of the infundibulum, magnum, isthmus, and shell gland segments. The weight of the magnum segment was higher than the weight of the infundibulum, isthmus, and shell gland. The lengths of the infundibulum, magnum, isthmus, and shell gland differed significantly in the current study. The magnum was longer than the infundibulum, isthmus, and shell gland segments. The current results are consistent with the findings of Mahajan and Joshi (2020) in White Leghorn chickens and those of Beaulah et al. (2024) in Siruvidai chickens, who found that the magnum was the longest segment of the oviduct and the weight was higher than that of the infundibulum, isthmus, and shell gland. The results of Mahmud et al. (2017) on Nigerian local chickens indicated that the mean values and lengths of the infundibulum, magnum, and isthmus were statistically similar among the three studied genotypes. Kheawkanha et al. (2021), on the other hand, observed no statistically significant differences in oviductal weight and length among mature and old hens. The current findings suggested that a well-developed oviduct, particularly a magnum with considerable weight and length, is essential for proper egg formation and shell development.

CONCLUSION

The Boschveld chicken breed exhibited distinctive characteristics in the weights and numbers of small white, large white, and small yellow follicles. Furthermore, the current findings indicated substantial variance among the weights of pre-ovulatory follicles. It is concluded that the magnum was the longest and heaviest segment of the oviduct, with Follicle 1 ovarian follicle being the heaviest pre-ovulatory follicle. It is recommended to implement strategies that enhance follicle development and improve oviduct function to potentially increase egg production rates in Boschveld chickens. Additionally, conducting further research on reproductive organ morphology across different chicken breeds could provide valuable insights into their reproductive biology.

DECLARATIONS

Availability of data and materials

Data supporting the study's findings are available upon reasonable request from the corresponding author.

Acknowledgements

The authors gratefully acknowledge the University of Limpopo's Experimental Farm and Animal Laboratory, South Africa, for their assistance in obtaining study materials.

Authors' contributions

Tyasi Thobela Louis conducted the conceptualisation, original design, resource provision, and supervision, also contributing to data processing, interpretation, writing, and manuscript revision. Mothiba Mmolaoa Leah contributed by providing images, collecting samples, processing and interpreting data, as well as writing and revising the manuscript. Hlokoe Victoria Rankoatse contributed to the project by providing images, collecting samples, interpreting data, writing,

and revising the manuscript. Sathekge Lindiwe Johannah contributed to data interpretation, writing, and revising the manuscript. All authors checked and approved the final edition of the manuscript.

Ethical considerations

All authors have reviewed and confirmed the originality and final version of the manuscript. The study adhered to ethical standards, ensuring no instances of plagiarism, data fabrication, or redundant publication.

Funding

The study received no funding support.

Competing interests

The authors disclosed no potential conflicts of interest.

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2025, Scienceline Publication
World's Veterinary Journal
World Vet J, 15(3): 710-719.
ISSN 2322-4568

DOI: https://dx.doi.org/10.54203/scil.2025.wvj72
PII: S232245682500072-15

Serological Assays of Brucellosis in Unvaccinated Sheep and Goat in Indonesia

Ani Setianingrum*[1,4], Widagdo Sri Nugroho[2], Tri Anggraeni Kusumastuti[3], and Herawati[4]

[1]*Sain Veteriner Doctoral Programme, Faculty of Veterinary Medicine, Gadjah Mada University, Yogyakarta, 55281, Indonesia*
[2]*Department of Veterinary Public Health, Faculty of Veterinary Medicine, Gadjah Mada University, Yogyakarta, 55281, Indonesia*
[3]*Department of Socio-Economic, Faculty of Animal Science, Gadjah Mada University, Yogyakarta, 55281, Indonesia*
[4]*Department of Veterinary Public Health, Faculty of Veterinary Medicine, Brawijaya University, Malang, 65151, Indonesia*

*Corresponding author's Email: ani.setia@ub.ac.id

ORIGINAL ARTICLE
Received: July 04, 2025
Revised: August 09, 2025
Accepted: September 05, 2025
Published: September 30, 2025

ABSTRACT

Brucellosis is recognized as one of the most widespread zoonotic diseases and is often overlooked, impacting livestock populations worldwide. Annual routine screenings utilize serological assays, such as the Rose Bengal test (RBT) and the complement fixation test (CFT), to identify the presence of brucellosis. The present study aimed to evaluate the diagnostic and discriminative performance of the CFT, the locally produced RBT antigen from Indonesia, the commercial RBT antigen (Pourquier), and the indirect enzyme-linked immunosorbent assay (ELISA) using sera from Indonesian goats and sheep. A total of 184 serum samples were collected, including 92 from goats and 92 from sheep. The samples were obtained through jugular venipuncture from animals on small-scale goat and sheep farms in Java Island, Indonesia. The results were analyzed to determine true prevalence (TP), accuracy, and cross-tabulation with parallel interpretation to assess the sensitivity and specificity of each test analysis. The TP of brucellosis in small ruminant serum samples using local RBT, Pourquier RBT, CFT, and indirect ELISA was 6.99%, 9.74%, 0%, and 14.78% in goats, and 6.92%, 20.34%, 0%, and 7.81% in sheep, respectively. Moderate Kappa interrater reliability was observed between the local and Pourquier RBTs for both goats (0.537) and sheep (0.440). The combined RBT sensitivity in goats and sheep was 27% and 20%, respectively, while the combined RBT specificity was 92% in goats and 80.5% in sheep. Using more than one diagnostic method is essential for screening and investigating brucellosis in goat and sheep populations. A combination of RBT and indirect ELISA tests is recommended to identify *Brucella* reactors among small ruminants in Indonesia.

Keywords: Brucellosis, Complement fixation test, Indirect enzyme-linked immunosorbent assay, Rose Bengal test, Small ruminant

INTRODUCTION

Brucellosis remains a major global health issue, affecting livestock quality, farmers' economic prosperity, and public health (Zhang et al., 2018). Bacterial disease caused by the genus *Brucella* (*B.*) infects a wide range of species and can potentially be transmitted to humans (Franc et al., 2018). The *Brucella* spp. belong to the facultative intracellular α-2 division of proteobacteria (Poetsch and Marchesini, 2020). Small ruminants are vulnerable to infection by *B. ovis* and *B. melitensis*. Infection with *B. ovis* in sheep leads to decreased fertility, while *B. melitensis* is the most dangerous to humans (Moreno, 2014). Human cases of brucellosis mainly occur from consuming raw milk and dairy products from infected animals, as well as through direct and indirect contact with these animals (Adetunji et al., 2020). *Brucella* spp. can contaminate the carcasses of cattle, sheep, and goats (Casalinuovo et al., 2016). Meat derived from animals infected with *Brucella* poses a risk of disease transmission, particularly if the contaminated product is not cooked thoroughly (Potter, 2013).

Indonesia is a tropical country with a suitable climate for small ruminant farming. According to statistics from Indonesia, in 2022, Indonesia's goat population was 18,560,835, and the sheep population was 14,063,214 (BPS, 2022). The small ruminant population is mainly concentrated in Java Island, accounting for 52.83% of the country's goat population and 91.04% of the sheep population (BPS, 2022). In Indonesia, brucellosis remains endemic in regions such as East Nusa Tenggara, Sulawesi, Java, Sumatra, and Kalimantan (Padaga et al., 2018). Benkirane (2006) suggested that in regions, such as the Mediterranean, as well as Central and Western Asia, where *B. abortus* infection has a high prevalence, *B. melitensis* infections have also been reported in small ruminants. The exact prevalence of goat and sheep brucellosis in Indonesia remains uncertain, likely due to the ongoing focus on brucellosis eradication and control efforts mainly targeting cattle brucellosis. Additionally, the primary reason for this undetected incidence is the limited understanding of the diagnostic methods established for brucellosis in small ruminants.

To cite this paper: Setianingrum A, Nugroho WS, Kusumastuti TA, and Herawati (2025). Serological Assays of Brucellosis in Unvaccinated Sheep and Goat in Indonesia. *World Vet. J.*, 15(3): 710-719. DOI: https://dx.doi.org/10.54203/scil.2025.wvj72

Small ruminant brucellosis not only affects animals and public health but also causes significant financial losses due to clinical signs such as reproductive issues, abortions, stillbirths, decreased milk production, and the costs associated with treating and culling infected animals (Abedi et al., 2020). Additionally, managing and treating infected herds requires considerable investment in veterinary care and biosecurity measures. Large-scale vaccination campaigns and test-and-slaughter programs have successfully helped countries, including the United States, Canada, the European Union, and Norway, eliminate brucellosis, particularly in small ruminants (Zhang et al., 2018). However, the program is less effective in areas with high brucellosis prevalence that lack financial resources or veterinary services. Godfroid et al. (2013) noted that low-resource and developing countries need to expand their efforts in brucellosis control. One suggested approach for eradicating the disease is a one-health integrated system for monitoring and surveillance, aiming to prevent brucellosis in both humans and animals.

The prevalence of brucellosis in goats and sheep in Indonesia has been insufficiently studied. A study in 2015 in the Gunung Kidul district, Yogyakarta, Indonesia, revealed that the brucellosis rate in goats was as high as 9.6% (25/260) at the animal level, using serial testing with the Rose Bengal test (RBT) followed by the complement fixation test (CFT; Primatika et al., 2016). Mujiatun et al. (2016) reported a seroprevalence of 0.84% (1/119) for brucellosis in goats from slaughterhouses in Jakarta, Indonesia, as determined by CFT. Additionally, a recent study reported the seroprevalence of brucellosis in a private farm in Bogor, Indonesia, using RBT and CFT, with 22.81% (26/114) in goats and 19.61% (20/102) in sheep (Martindah et al., 2023).

The reference test for diagnosing brucellosis involves directly culturing *Brucella* from tissue samples, which can be challenging due to lengthy cultivation times and the potential for blood culture failure (Geresu and Kassa, 2015). These procedures require advanced biosafety measures. Serological testing is the most common method used to monitor antibodies against *Brucella* spp. in goat and sheep populations to determine the prevalence of brucellosis in these animals (Godfroid et al., 2010). Diagnostic methods, including the RBT and CFT, are widely accepted for identifying brucellosis reactors in cattle. However, few comparative studies have employed different serological tests, such as RBT, CFT, and enzyme-linked immunosorbent assay (ELISA), to distinguish brucellosis in local goat and sheep populations (Ridlo et al., 2024). To identify effective detection techniques for improving brucellosis prevention and control efforts in Indonesia, it is essential to compare different methods for accurately diagnosing brucellosis in goat and sheep herds. Thus, the present study aimed to evaluate the ability to diagnose brucellosis and the discriminative capacity of CFT, as well as the effectiveness of two RBT antigens, an Indonesian-manufactured *Brucella* antigen and a commercial *Brucella* antigen from Pourquier-IDEXX, in distinguishing positive results compared to indirect ELISA.

MATERIALS AND METHODS

Ethical approval

For the present study, goat and sheep owners provided their informed consent. This investigation was approved under ethical clearance code 93/EC-FKH/Int./2023 by the Institutional Review Board, Faculty of Veterinary Medicine, Gadjah Mada University, Indonesia.

Study area and animals

A total of 184 serum samples were obtained, comprising 92 samples from goats and 92 from sheep, collected from October 2023 to February 2024 in East Java Province and Yogyakarta, Indonesia. The animals were kept in a mixed farming system, with sheep and goats housed near cattle. Serum samples were collected from 48 small ruminant farms, including 23 goat farms and 25 sheep farms. Herds and flocks were selected randomly, with 3 to 5 samples collected from each farm. Data were gathered regarding the different breeds of goats, including Peranakan Etawah, Saanen, Sapera, Senduro, Jawa breed, and mixed breeds. In the case of sheep, the predominant breed was identified as the thin-tailed breed. Both male and female animals aged from 6 to 12 months and over one year were included in the study. The average body weight of local goats in Indonesia was reported to be approximately 20-30 kg for females and 25-40 kg for males. For local sheep, the average weight of males and females was found to be approximately 20-30 kg (Susilorini and Kuswati, 2019).

Sample collection

Approximately 4 mL of blood was collected from the jugular vein using a single-use vacutainer tube. The samples were stored at 4°C overnight to facilitate clotting. After centrifuging for ten minutes at 3000 rpm, the serum was separated into three aliquots and transferred to microcentrifuge tubes. Before serology analysis, the serum samples were stored at -20°C (Shabbir et al., 2013). The serum samples underwent serological testing using CFT, RBT, and indirect ELISA. The CFT analysis was conducted at the Disease Investigation Centre in Wates, Yogyakarta, Indonesia. Rose Bengal tests were conducted at the Veterinary Public Health Laboratory, and the indirect ELISA procedure was carried

out at the Veterinary Physiology Laboratory, Faculty of Veterinary Medicine, Gadjah Mada University, Yogyakarta, Indonesia.

Complement fixation test

The CFT serology assay was performed following the method described by the World Organization for Animal Health (WOAH, 2022). The process of inactivating undiluted serum involved immersing it in a water bath at 58°C for 30 minutes. A 25 µL aliquot of CFT buffer was added to all wells of the U-shaped microplate. Serum samples, positive control, and negative control were added to row A, 25 µL each. Serum dilution was performed by transferring 25 µL of inactivated serum from row A to row B, then mixing with buffer in row B wells, and subsequently transferring 25 µL from each row to the next (rows C to H). *Brucella* antigen suspension (BBVET Wates, Indonesia) 25 µL was added to each well in rows B to H. Row A wells received 25 µL of CFT buffer. Next, 25 µL of complement suspension was added to all wells (A to H). Then, the microplate was covered and incubated for 30 minutes at 37°C. Each well received up to 25 µL of sensitized sheep red blood cells 3% (RBCs). Sensitization was achieved by adding hemolysin to coat the sheep RBCs (WOAH, 2022). After covering, the microplate was incubated at 37°C for 30 minutes and then stored in the refrigerator overnight. A positive result was indicated by complete inhibition of hemolysis.

The Rose Bengal test

The local RBT was manufactured in Indonesia, specifically for the *Brucella* antigen (Pusvetma, Indonesia). The antigen was prepared using a suspension of *Brucella* strain 1119 at a concentration of 8% in buffer with a pH of 3.65. Carefully, 30 µL of serum was mixed with 90 µL of *Brucella* antigen (1:3). The plates were manually rotated for four minutes. Any level of clumping or agglutination identified positive results, whereas a negative result indicated no clumping or agglutination (WOAH, 2022). The commercial *Brucella* antigen, Pourquier RBT (Pourquier® Rose Bengal antigen, IDEXX, France), comprised a suspension of *B. abortus* (Weybridge 99 strain). For the test, approximately 25 µL of serum was mixed with 25 µL of antigen on a white plate. The mixture was gently tilted for four minutes at room temperature and then observed. Agglutination signified a positive result.

Indirect enzyme-linked immunosorbent assay

The indirect ELISA was selected as the reference standard for this study due to its high reported sensitivity and specificity for small ruminant brucellosis. Therefore, the commercial goat *Brucella* antibody immunoglobulin-G (IgG) ELISA kit (Bioenzy, Cat. No BZ-08080000-QLEB, China) and a Sheep *Brucella* antibody IgG ELISA kit (Bioenzy, Cat. No BZ-08190000-QLEB, China) were used. According to the manufacturer's guidelines, the test was performed on microtiter plates pre-coated with *Brucella* antigen. Positive and negative controls, each with a volume of 50 µL, were included. Serum samples were diluted in a sample diluent at a 1:50 ratio. Then, 50 µL of each sample was added to the wells and incubated for 30 minutes at 37°C, followed by aspiration and five washes with PBS. Horseradish peroxidase was added to each well, and the plates were incubated for another 30 minutes at 37°C. After five rinses, the tetramethylbenzidine substrate solution was added, and the plates were sealed for 10 minutes at 37°C in the dark. The reaction was stopped by adding 50 µL of acidic stop solution, resulting in a color change from blue to yellow. The optical density (OD) was measured at 450 nm using a spectrophotometer (AMR-100, Allsheng, China). The cutoff values were 0.293 for sheep and 0.448 for goats, calculated using the formula (Cutoff value = average negative control value + 0.15), as recommended by the kit manufacturer (Bioenzy, China). A positive result was indicated when the OD value was equal to or greater than the cutoff.

Data analysis

The sero-prevalences were calculated by dividing the number of positive results from RBT, CFT, and indirect ELISA tests by the total number of serum samples. The apparent prevalence (AP) and true prevalence (TP) for each serological test result were determined using the online calculator Epitools (Sergeant, 2018). Comparison analysis among the serological tests was performed using the receiver operating characteristic (ROC) curve, the area under the curve (AUC) was calculated along with its standard error (SE) and 95% confidence interval (CI; MedCalc Software Ltd, Ostend, Belgium, 2024), and the Kappa value (JASP, 2024). The AUC measures how well a test can distinguish between positive and negative results, where 1 signifies perfect separation and 0.5 indicates no ability to differentiate (Çorbacıoğlu and Aksel, 2023). The interpretation of the Kappa values was as follows. 0 - 0.20 indicated poor agreement, 0.21 - 0.40 indicated fair, 0.41-0.60 indicated moderate, 0.61 - 0.80 indicated proper, and 0.81 - 1.00 indicated perfect agreement. A 2 × 2 contingency table was employed to determine sensitivity (Se) and specificity (Sp). The Se was calculated as p (T+/D+), and Sp as p (T-/D-), according to Trevethan (2017). Both Se and Sp were derived through parallel interpretation. The animals were considered positive for brucellosis if they tested positive on one or both

tests.

RESULTS

Serum samples were collected from dairy goats (Peranakan Etawah, Saanen, Sapera, and Senduro) and meat breeds (Jawa and mixed breeds). The distribution of goat breeds was as follows. Peranakan Etawah (13/92), Saanen (5/92), Sapera (22/92), Senduro (34/92), Jawa (10/92), and mixed breed (8/92). Female goats were more common (68/92) than males (24/92), with fewer young goats (Less than 1 year, 20/92) compared to adults (More than 1 year, 72/92). All sheep serum samples were collected from meat-breed sheep, including thin-tailed sheep (87/92) and mixed-breed sheep (5/92). The number of female sheep was higher than that of males (56/92 and 36/92, respectively), with more adult sheep (71/92) than young sheep (21/92).

The serological analyses revealed that the RBT with Pourquier antigen and IgG indirect ELISA produced the highest positive results, followed by the local RBT. Table 1 displays the estimated TP for each serological test. The highest detection of *Brucella* in goats (14.78%) was achieved using indirect ELISA, while in sheep (20.34%), it was achieved using Pourquier RBT.

The Kappa analysis was conducted for two RBTs and an indirect ELISA. Table 2 showed a moderate level of agreement between the two RBT antigens used (Local and Pourquier) for goats and sheep, with values of 0.537 and 0.440, respectively. A Kappa value between 0.41 and 0.61 indicated moderate reliability and inconsistency between Local RBT and Pourquier RBT results. Negative Kappa value of -0.098 for Local RBT in sheep indicated poor diagnostic accuracy. The area under the ROC curve between CFT and indirect ELISA results indicated no agreement between goats and sheep (0.500). The CFT demonstrated limited discrimination capacity, potentially attributed to low Se in small ruminants or testing during a chronic infection phase (Sadhu et al., 2015). Furthermore, the combined results of the RBT exhibited poor agreement compared to the indirect ELISA, as the standard reference for brucellosis diagnosis in sheep and goats, in goats and sheep (Table 3, Figure 1). The AUC values for combined RBT in goats and sheep were 0.594 and 0.502, respectively, indicating poor diagnostic accuracy. The Se and Sp of the two RBT antigens were determined using cross-tabulation compared to indirect ELISA (Table 4). The Se of the combined RBT increased in goats and sheep, by 27% and 20%, respectively. In contrast, the Sp decreased for goats and sheep by 92% and 80%, respectively.

Table 1. True prevalence estimation of two RBT antigens, CFT, and indirect ELISA for the sheep and goat sera

Animal	Serology test	(+)/samples	Apparent prevalence		True prevalence	
			Estimate	95% CI	Estimate	95% CI
Goat	Local RBT	6/92	6.52%	0.0148 - 0.1157	6.99%	0.0256 - 0.1583
	Pourquier RBT	8/92	8.7%	0.0294 - 0.1445	9.74%	0.044 - 0.1928
	CFT	0/92	0	0	0	0 - 1.002
	Indirect ELISA	15/92	16.3%	0.0876 - 0.2385	14.78%	0.0793 - 0.2464
Sheep	Local RBT	7/92	7.61%	0.0219 - 0.1303	6.92%	0.0214 - 0.1613
	Pourquier RBT	17/92	18.48%	0.1055 - 0.2641	20.34%	0.1218 - 0.3162
	CFT	0/92	0	0	0	0 - 0.4175
	Indirect ELISA	10/92	10.87%	0.0451 - 0.1732	7.81%	0.0229 - 0.1689

True prevalence was calculated using the online calculator Epitools, source: Sergeant (2018), (+)/samples: Indicates positive results per total sample tested, CI: Confidence intervals, RBT: Rose Bengal test, CFT: Complement fixation test.

Table 2. Kappa value between local RBT, Pourquier RBT, and indirect ELISA results of brucellosis in sheep and goat sera

Animal	Test category	Kappa value	95% CI	Agreement
Goat	Local RBT - Pourquier	0.537	0.211 - 0.863	Moderate
	Local RBT- indirect ELISA	0.107	-0.124 - 0.339	Poor
	Pourquier - indirect ELISA	0.166	-0.084 - 0.416	Poor
Sheep	Local RBT - Pourquier	0.440	0.188 - 0.691	Moderate
	Local RBT- indirect ELISA	-0.098	-0.148 - -0.048	Poor
	Pourquier - indirect ELISA	0.013	-0.187 - 0.213	Poor

RBT: Rose Bengal test, CI: Confidence intervals. The CFT results were excluded from the analysis due to zero results.

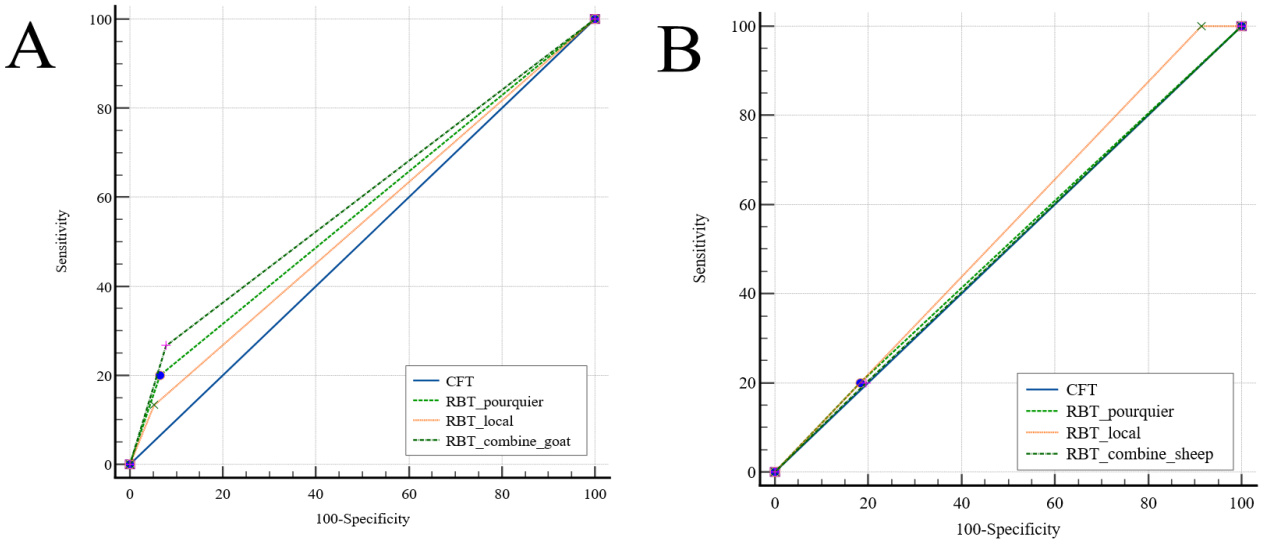


Figure 1. The receiver operating characteristic curve reflects the discriminative ability of CFT, local RBT, and Porquier RBT tests against indirect ELISA for brucellosis in goats (**A**) and sheep (**B**). Both curves show that CFT and RBT have no predictive capability for diagnosing brucellosis in small ruminants.

Table 3. Area under the curve of the receiver operating characteristic curve for CFT, Local RBT, and Pourquier RBT compared to indirect ELISA of brucellosis in sheep and goat serum

Animal	Variable	AUC	SE	95% CI
Goat	CFT	0.500	0.000	0.394 - 0.606
	Pourquier RBT	0.568	0.0553	0.460 - 0.670
	Local RBT	0.541	0.0472	0.434 - 0.645
	RBT goat combined	0.594	0.0611	0.487 - 0.696
Sheep	CFT	0.500	0.000	0.394 - 0.606
	Pourquier RBT	0.509	0.0700	0.402 - 0.614
	Local RBT	0.543	0.0155	0.436 - 0.647
	RBT sheep combined	0.502	0.0702	0.396 - 0.608

AUC: Area under the curve, SE: Standard error, CI: Confidence intervals, RBT: Rose Bengal test, CFT: Complement fixation test, RBT combined: Number of positive results by local RBT, Porquier RBT, or both tests.

Table 4. Sensitivity and specificity of serial interpretation and parallel interpretation of local RBT and Pourquier RBT results compared to the Indirect ELISA of brucellosis in sheep and goat sera

Animal	Serology test	Sensitivity	95% CI	Specificity	95% CI
Goat	Local RBT	13%	0.02 - 0.04	95%	0.87 - 0.99
	Pourquier RBT	20%	0.04 - 0.48	94%	0.85 - 0.98
	RBT combined	27%	0.08 - 0.55	92%	0.84 - 0.97
Sheep	Local RBT	0.0%	0.00 - 0.31	91%	0.81 - 0.96
	Pourquier RBT	20%	0.03 - 0.56	82%	0.72 - 0.89
	RBT combined	20%	0.03 - 0.56	80%	0.70 - 0.88

CI: Confidence intervals, RBT: Rose Bengal test, RBT combined: Positive results by local RBT, Porquier RBT, or both tests.

DISCUSSION

Brucella bacteria can persist for months in materials such as internal organs, abortion tissues, animal excreta, placenta, and products, potentially contaminating the environment (Marvi et al., 2018). Provinces in Indonesia, including Jakarta, West Java, East Java, Yogyakarta, Central Java, Banten, North Sumatra, Aceh, Lampung, Riau, East Nusa Tenggara, West Papua, Central Sulawesi, South Sulawesi, and Maluku, have reported cases of bovine brucellosis (Ridlo et al., 2024). However, there is limited information about brucellosis in sheep and goats, as well as the effectiveness of serology tests. Diagnostic tools for small ruminant brucellosis in Indonesia are limited and often rely on a single test (Martindah et al., 2023). To accurately distinguish between infected and non-infected animals and prevent unnecessary culling due to test errors, highly sensitive and specific tests are necessary. Moreover, technical issues and challenging

biological, epidemiological, and economic factors influence the performance of available tests (Ducrotoy et al., 2018). The current findings provided valuable insights for improving diagnostics in the brucellosis control program among small ruminant populations.

The Peranakan Etawah breed is the most common dairy goat in Indonesia, originating from the crossbreeding of indigenous Kacang breeds with the Etawah breed from India (Sudrajat et al., 2021). The Sapera breed resulted from crossing a female Peranakan Etawah with a Saanen buck (Hermawati and Nuraeni, 2024). The Senduro breed originates from the Senduro district in Lumajang, East Java, and is cultivated for both its milk and meat (Ciptadi et al., 2019). Etawah crossbreed and Sapera goats represented significant advancements in Indonesian goat breeding, focusing on improving milk production and adaptability. Most sheep in Indonesia are meat-type, particularly Javanese thin-tailed sheep, which are essential to the local livestock system (Sodiq and Tawfik, 2004). The present study reported the first case of brucellosis seropositivity in the Senduro goat breed, with the majority of the serum samples analyzed originating from this breed and the thin-tailed sheep. The present investigations, using indirect ELISA, indicated a higher seroprevalence in goats (16.30%) than in sheep (10.87%). False positive results were caused by the RBT using two antigens (Local RBT and Pourquier) in goat and sheep sera, which the indirect ELISA did not detect. Furthermore, the CFT results were negative in all serum samples, which most likely resulted from incomplete or insufficient host immunity caused by early-stage infection or latent infection at the time of sampling (Rossetti et al., 2022). A modified protocol (3:1 ratio of serum to antigen) was used for the local RBT antigen to enhance test sensitivity. Akhter et al. (2014), Tulu et al. (2020), and Šerić-Haračić et al. (2022) indicated that sheep and goats infected with *Brucella* may possess lower antibody levels to the main *Brucella* epitopes compared to cattle. According to Sadhu et al. (2015), the proportions of seropositive small ruminants measured by RBT are frequently higher than those measured by ELISA. Some conditions of the serum samples, particularly contamination and hemolysis, are anti-complementary; therefore, complement cannot be detected in the CFT, and is considered negative despite the presence of antibodies, which supported the findings of the present study as well as Anochie et al. (2023). These anti-complementary reactions produce invalid CFT test results. Additionally, the prozone effect can cause false-negative CFT results in serum with very high antibody titers by forming small, soluble immune complexes that do not precipitate (Thrusfield et al., 2018). Occasionally, blocking antibodies inhibit antigen-antibody interactions (Praud et al., 2012). This frequently happens during CFT for *B. abortus* in cattle, as elevated levels of excess IgG1 can disrupt IgG2, which is essential for complement fixation (Frischauf et al., 2024).

In surveillance studies, serology tests are beneficial for disease screening and detection. However, using a single serology test often results in both false-positive and false-negative outcomes. The TP of the disease is typically not reflected by the seropositive rate, also known as AP (Habibzadeh et al., 2022). The AP, Se, and Sp can all be used to estimate the TP. The results of TP indicated an increase in both RBT antigens used for investigating brucellosis in goats (6.99% and 9.74%), while the AP of local RBT in sheep was lower than the TP (6.92%), and the TP of Pourquier RBT increased (20.34%). Goats exhibited a notably higher prevalence of detected *Brucella* strains than sheep, which aligned with the findings of Hamada et al. (2017), suggesting that immunological responses might differ significantly between species. The TP estimate for CFT in the current study was identical for both sheep and goats, resulting in a zero value. The reference Se for CFT in both goats and sheep was considered low, while the Sp for CFT was 100%, indicating a high Sp, which was consistent with the findings of Rahman et al. (2013) and Legesse et al. (2023). In the present study, indirect ELISA indicated the highest TP rates among serological tests, with 14.78% for brucellosis in goats. The current findings indicated the presence of *Brucella* infection in small ruminants from Lumajang Regency in East Java and Sleman Regency in Yogyakarta, Indonesia. The indirect ELISA offers advantages such as reproducibility, ease of use with minimal equipment, and the availability of commercial kits. Based on the present results, indirect ELISA is a proper alternative to CFT for laboratories with limited resources, which was aligned with the finding of Padilla et al. (2010).

The indirect ELISA result served as the reference test for evaluating the Se and Sp of the RBT and CFT tests. The analysis of CFT, RBT, and indirect ELISA results indicated a lack of agreement between goats and sheep. However, the combined RBT produced the highest AUC score for distinguishing brucellosis in goats. In the present study, the strongest agreement among serology tests was found between two RBT antigens used to detect brucellosis in goats and sheep, with values of 0.537 and 0.440, reflecting a moderate level of agreement. A low Kappa value indicated that the correlation between the two tests was not strong, suggesting that only one test was practical or that both tests were effective but exhibited opposing correlations (Nofuentes and Luna, 2007). The current findings align with those of Praud et al. (2012) and Šerić-Haračić et al. (2022), demonstrating consistency in the accuracy of the testing methods employed, as both tests emphasized the importance of Se and Sp in accurately determining the disease status of animals. This consistency underscores the value of indirect ELISA as a complementary screening tool to RBT, particularly in small ruminant populations, where it has been shown to maintain high sensitivity in detecting brucellosis.

In Asia and Europe, the most commonly used serological tests for official brucellosis control programs are the RBT and the CFT (Godfroid et al., 2010). In the present study, *Brucella* strain 1119 at a concentration of 8% was used. In contrast, in the study by Minas et al. (2008), the whole bacteria, *B. abortus* 19 or 1119-3 cells, served as the antigen in both RBT and CFT tests, while a smooth lipopolysaccharide (LPS) *Brucella* antigen was used in the indirect ELISA test. The indirect ELISA test was selected as the reference test in the present study to determine the Se and Sp of RBT. Similarly, both RBT antigens exhibited higher Se and Sp in detecting brucellosis in goats compared to sheep. The current findings are consistent with Hamada et al. (2017), who indicated that the parallel interpretation method enhances accuracy and effectively manages diagnostic uncertainties. Similarly, Smith et al. (2019) confirmed that the parallel interpretation method significantly boosts confidence in diagnostic results. The current results indicated that combining RBT antigens with cross-tabulated indirect ELISA yielded higher Se for both goat and sheep brucellosis. Parallel testing increased Se but decreased Sp compared to individual RBT, reducing the likelihood of missing a diagnosis of a disease. Several studies by Godfroid et al. (2010) and Chenais et al. (2012) indicated that the RBT may not reliably differentiate between agglutinating reactions caused by *Brucella* species and those elicited by cross-reactive antigens from *Yersinia enterocolitica* O:9. The LPS molecules with similar antigenic epitopes exhibit cross-reactivity among different Gram-negative bacteria, including *Escherichia coli* O116 and O157, *Salmonella enterica* serovar, and *Vibrio cholerae* (Pappas, 2010).

The indirect ELISA may be highly accurate because it can detect minimal amounts of antibodies present during the early stages of infection, whereas RBT was unable to detect them, which was consistent with the study conducted by Erdenebaatar et al. (2004), who demonstrated that indirect ELISA helps to prevent false-positive results caused by non-specific reactor sera that show RBT-positive. Non-clumping antibodies become more dominant than clumping antibodies in the later stages of infections, which may cause them to be undetectable by RBT and CFT (Sha et al., 2025). These non-clumping antibodies can be detected through the addition of an IgG conjugate in an indirect ELISA test. The combination of indirect ELISA and RBT allows investigators to identify every *Brucella* reactor that is seropositive. Using this combined serology test aligns with a study by Padilla et al. (2010), who found that it was not practical to identify every *Brucella* reactor with a single agglutination test. Due to the reliability and reproducibility of the ELISA test, a combination of RBT and ELISA was advised.

Vaccination against small ruminant brucellosis and identifying infected animals through culling or slaughter are effective strategies for reducing its occurrence, spread, and adverse effects (Godfroid et al., 2014). Factors associated with brucellosis risk in small ruminants, according to surveillance data from multiple countries, include breeders' limited knowledge of clinical signs and the absence of a Rev-1 vaccination program for goats and sheep (Coelho et al., 2019). The present results emphasized the importance of utilizing multiple diagnostic methods to accurately identify true positives in livestock populations. These findings are consistent with a study by Elrashedy et al. (2022), who advocated for a multifaceted approach in diagnostic testing to enhance accuracy in livestock health assessments. Such consistency across studies highlighted the critical need for comprehensive diagnostic strategies in veterinary practices.

CONCLUSION

In the current study, indirect ELISA for the local goat and sheep populations in Indonesia was found to be more reliable and sensitive than RBT and CFT. The current results indicated that brucellosis was highly prevalent in the serum samples of goat (16.3%) and sheep (10.87%). It is recommended that a combination of RBT and indirect ELISA be used for the brucellosis surveillance program in small ruminants in Indonesia, as the reliability and reproducibility of the ELISA test enhance the effectiveness of the RBT. The current findings recommend further studies to explore the genetic diversity of *Brucella* spp. in small ruminants in Indonesia and to understand their molecular epidemiology at the national level, thereby guiding the development of economically viable prevention and control strategies.

DECLARATIONS

Acknowledgments

The present study resulted from doctoral research conducted under the Sain Veteriner program at the Faculty of Veterinary Medicine, Universitas Gadjah Mada, Indonesia. The authors sincerely thank the Disease Investigation Centre Wates (BBVet Wates), the Department of Agriculture, Food, and Fisheries of Sleman Regency, Indonesia, and the veterinary practitioners who helped with sample collection for this study.

Authors' contributions

Ani Setianingrum designed the study, conducted the laboratory analysis, wrote the manuscript, and analyzed the data. Widagdo Sri Nugroho assisted with the conceptualization of the study and interpretation of results. Tri Anggraeni

Kusumastuti revised the manuscript, and Herawati contributed to the literature review. All authors reviewed and approved the last edition of the manuscript.

Competing interests

All authors declared that there is no conflict of interest in the publication of the present study.

Ethical considerations

All authors have confirmed adherence to ethical standards, including topics related to plagiarism, misconduct, fabrication or falsification of data, consent for publication, duplicate publication and/or submission, and redundancy.

Funding

The present study was funded by the Doctoral scholarship from Brawijaya University, Indonesia.

Availability of data and materials

The authors declare that all the necessary data will be provided upon request.

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2025, Scienceline Publication
World's Veterinary Journal
World Vet J, 15(3): 720-733.
ISSN 2322-4568

DOI: https://dx.doi.org/10.54203/scil.2025.wvj73
PII: S232245682500073-15

Prevalence and Seasonal Survey of Tick Fauna (Acari: Ixodidae) of Domestic Ruminants in the Hauts-Bassins Region, Burkina Faso

Bamba Hubert Eloi Aboubacar Sidiki*[1,4], Tianhoun Denté Fidèle[2], Sawadogo Alizèta[3], Kaboré Adama[2], and Marichatou Hamani[4]

[1]*National Center for Scientific and Technological Research (CNRST)/Institute of the Environment and Agricultural Research (INERA)/Plant Production Department/Cotton Program, 01 BP 208 Bobo- Dioulasso 01, Burkina Faso*
[2]*National Center for Scientific and Technological Research (CNRST)/Institute of the Environment and Agricultural Research (INERA)/Animal Production Department, BP 10 Koudougou, Burkina Faso*
[3]*National Center for Scientific and Technological Research (CNRST)/Institute of the Environment and Agricultural Research (INERA)/Plant Production Department/Regional Center of Excellence in Fruits and Vegetables (CRE), 01 BP 910 Bobo- Dioulasso 01, Burkina Faso*
[4]*Abdou Moumouni University (UAM)/Regional Center of Excellence on Pastoral Productions (CERPP), BP 10960 Niamey, Niger*

*Corresponding author's Email: bheas2000@yahoo.fr

ABSTRACT

As in other West African countries, ticks (Acari: Ixodidae) infesting domestic ruminants are a major constraint for livestock breeders in Burkina Faso. The present epidemiological survey study was conducted from October 2022 to September 2023 in two pastoral areas of the Hauts-Bassins region of Burkina Faso to inventory the tick genera and species present, and determine the prevalence and associated risk factors in infested ruminants, including host sex, age, and season. A total of 8,274 live ticks were collected from 800 ruminants randomly selected (300 cattle, 300 sheep, and 200 goats) across two pastoral zones (Sidéradougou and Saho pastoral zones) and three seasons (cold dry season, hot dry season, and rainy season) from the herds of 140 livestock farmers surveyed. Morphological identification identified six species within the genera *Amblyomma* (67.32%), *Hyalomma* (26.35%), and *Rhipicephalus* (*Boophilus*, 6.33%), with *Amblyomma variegatum* as the dominant species. The overall prevalence of tick infestation was 74.90%, including 37.25% in cattle, 23.50% in sheep, and 14.15% in goats, with an average infestation level of 10.34 ± 20.30 ticks per animal. Male ruminants (52.62%) were more infested than females (47.38%), and juveniles (41.37%) were more infested than adults (58.63%). Seasonal patterns revealed peak infestation during the rainy and cold dry seasons in both Pastoral zones, and ticks showed a predilection for the inguinal and pastern regions. These findings highlight the extensive tick burden in Hauts-Bassins region, Burkina Faso's pastoral systems, and underscore the need for targeted, season- and host-specific control strategies to mitigate tick-borne disease risks and improve livestock productivity.

Keywords: Pastoral area, Prevalence, Ruminant, Seasonal dynamic, Tick infestation

ORIGINAL ARTICLE
Received: June 18, 2025
Revised: July 21, 2025
Accepted: August 31, 2025
Published: September 25, 2025

INTRODUCTION

In Burkina Faso, the livestock sector plays an important role in the national economy. It accounts for around 40% of agricultural value added and 30% of export earnings (Ouédraogo, 2021). With a numerically large and varied herd, this activity generates almost 20% of Gross Domestic Product (GDP) and is the third-largest source of foreign currency after gold and cotton in this country (FAO, 2019). It alone employs nearly 80% of the working population of Burkina Faso (MRA, 2011; FAO, 2019). This sector strengthens the food and nutritional security (animal protein intake) of rural and urban households, while also reducing poverty by providing them with appreciable income, especially the most vulnerable societies (MRA, 2010). Unfortunately, tick infestation and the diseases they transmit are a major constraint on livestock farming worldwide, especially in tropical locations. These zoonosis-carrying mites remain the main health problems limiting or restricting livestock productivity in Burkina Faso (Heylen et al., 2023). The skin lesions induced by direct physical damage from the tick's bite and attachment, the inflammatory and irritating effects of their saliva, allergic reactions in some individuals, and secondary infections due to skin damage from scratching (Biguezoton et al., 2016; Rodriguez-Vivas et al., 2018) can cause reduced production, weight losses, and livestock mortalities (Jongejan and Uilenberg, 2004; Ocaido et al., 2019). Indeed, these parasites are vectors of numerous pathogens responsible for many animal infections, such as babesiosis, theileriosis, anaplasmosis, and cowdriosis (CIRDES, 2014; Kasaija et al., 2021). Annual losses due to tick-borne diseases are estimated at $17.33 billion worldwide (Yéo et al., 2017; Balinandi et al., 2020; Ghafar et al., 2020).

Ecological control methods (Stachurski and Adakal, 2010; Nicaretta et al., 2020; Nava, 2024), genetic control (Shyma et al., 2015; Mandara and Maodzeka, 2023), anti-tick vaccination (Bonnet and Richardson, 2018; De la Fuente

To cite this paper: Sidiki BHEA, Fidèle TD, Alizèta S, Adama K, and Hamani M (2025). Tick Fauna (Acari: Ixodidae) of Domestic Ruminants in the Hauts-Bassins Region, Burkina Faso: A Prevalence and Seasonal Survey. *World Vet. J.*, 15(3): 720-733. DOI: https://dx.doi.org/10.54203/scil.2025.wvj73

and Estrada-Peña, 2019; Muhanguzi et al., 2022; Pereira et al., 2022), the action of parasitoids (Gaye et al., 2019), and entomopathogenic agents (Ebani and Mancianti, 2021), the action of natural predators and biopesticides (Samish et al., 2004; Ojeda-Chi et al., 2010; Hüe et al., 2015; Dorla et al., 2019) can help reduce the incidence of ticks, but are difficult to implement. However, it must be acknowledged that chemical control is the conventional method advocated through the use of synthetic acaricides. However, to control ticks, chemical control is the conventional method advocated through the use of synthetic acaricides. Unfortunately, this method has shown its limitations with the emergence of resistance in recent years (Cossío Bayúgar et al., 2020; Heylen et al., 2024). Furthermore, the management of tick resistance to acaricides is becoming more problematic as global warming is accompanied by an increase in the number of annual generations of certain predators (Bouchard et al., 2019; Yessinou et al., 2022). It has been shown that 59% of 1,598 studied predator species (representative of different ecosystems in temperate and tropical zones belonging to many animal and plant taxa) already exhibit changes in their phenotypes and/or their distribution ranges due to climate change (Brodeur et al., 2013). Numerous studies (Yoda et al., 2015; Biguezoton et al., 2016; Adjou Moumouni et al., 2021; Ouédraogo et al., 2021; Compaoré et al., 2022; Heylen et al., 2023) have studied tick diversity and explored their prevalence in Burkina Faso. It is crucial to point out that these studies do not specifically target the Hauts-Bassins region in its entirety to evaluate the prevalence of infestations at all levels. The majority of these studies focus on particular tick or ruminant species, or indeed on distinct regions within Burkina Faso.

The present study aimed to fill this information gap by identifying the most prevalent tick genera and species in the study area, determining their frequency in the pastoral areas of the Hauts-Bassins region, estimating the prevalence of infestations in ruminants within this area, and analyzing the risk factors associated with these infestations.

MATERIALS AND METHODS

Ethical approval

The present study employed a simple protocol that is accessible and understandable to livestock farmers to ensure transparency and contribute to research aimed at applying knowledge in the field of zoonotic vector mites on domestic ruminants. Farmers from the two pastoral zones were informed and consulted regarding the use of the data, and gave their consent. By adhering to the ethical research standards, this approach promotes data-driven analyses that can improve animal care practices.

Study area

The epidemiological survey study was carried out in the Hauts-Bassins region of Western Burkina Faso, covering an area of 25,479 km² (geographical coordinates: 11° 15' North and 04° 30'). The region is subject to a tropical climate of the North Sudanian type, with average annual rainfall ranging from 800 mm to 1,100 mm, with high inter-annual variability and highly variable temperatures (25-30°C). It is characterized by two alternating seasons, including the long dry period (October to April) and the rainy period (May to September; MRA, 2010). The plant formations encountered are wooded savannahs characterized by woody and herbaceous strata (perennial and annual grasses) as well as gallery forests with a lowland herbaceous stratum and reconstituted natural areas such as fallow land (MRA, 2010). This region includes two pastoral zones where the study's epidemiological survey work took place, namely the Saho and Sidéradougou pastoral zones located in the Houet and Tuy provinces, respectively (Figure 1). These two areas were selected on the basis of their accessibility and the availability of breeders open to innovations in livestock production techniques. The resident population in these areas is essentially made up of Mossi, Dioula, Peulh, Bobo, Toussian, and Bwaba ethnic groups, who make their living from livestock farming and agriculture (Kéré et al., 2021; INSD, 2022).

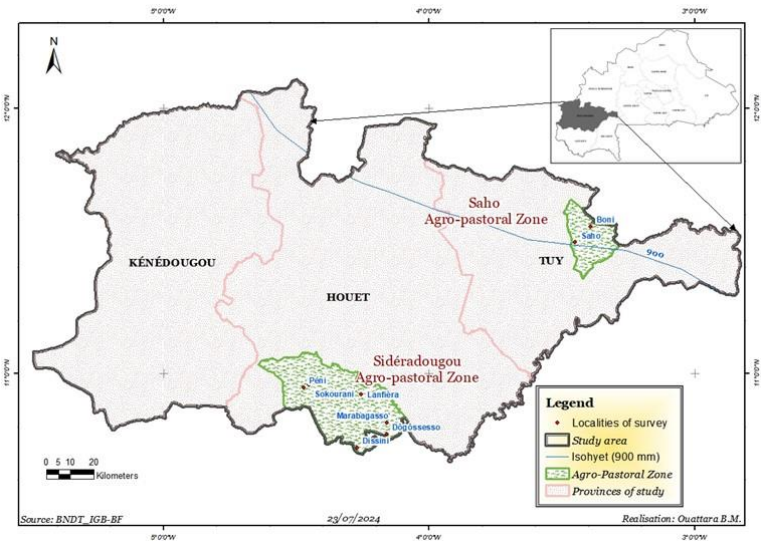


Figure 1. Geographical location of the two pastoral study areas in the Hauts-Bassins region of Burkina Faso

Sampling techniques and sample size

To select the ruminants for the study, a simple random sampling technique was employed. The necessary sample size, calculated using Thrusfield's equation (2018), was determined based on an expected prevalence (Pexp) of 90% (derived from a preliminary survey conducted in the study area and currently under publication), with a 95% confidence interval (corresponding to a Z of 1.96) and a desired absolute precision (d) of 5%. Thus, the following formula is used.

$$N = \frac{Z^2 Pexp(1-Pexp)}{d^2}$$

Applying these values, the sample size was determined to be N = 138. To enhance representativeness, 12 additional ruminants were added, bringing the total to 150 individuals, equally distributed among 50 cattle, 50 sheep, and 50 goats.

Design of the study

The study was carried out from October 2022 to September 2023, with tick samples taken from 800 domestic ruminants (300 cattle, 300 sheep, and 200 goats) randomly selected from the pastoral zones of Saho and Sidéradougou. For this purpose, the three seasons defined by Kaboré-Zoungrana et al. (2008) were considered with slight modifications, namely the rainy season (RS; June-September), the cold dry season (CDS; October-February), and the hot dry season (HDS; March-May). These ruminants, composed of both sexes (females and males), consisted of young animals (under 1 year for sheep and goats, and under 3 years for cattle) and adults (over 1 year for sheep and goats, and over 3 years for cattle). In each season, ticks were collected manually by simply pulling on the preferential tick attachment sites on the ruminant body (ears, dewlap, armpits, abdomen, inguinal region, perineal region, and pasterns) after restraining the animal. At the same time, individual information was collected on the rearing method, sex, breed, and age of the animals (with reference to the dentition of small ruminants, young - 1 year and adult + 1 year, and cattle: young - 3 years and adult + 3 years (Farougou et al., 2006). The vials used to collect the ticks were labelled with the date of collection, locality, and ruminant species.

Identification of the various tick species collected was carried out using an optical stereomicroscope (brand Olympus™ SFX - 31, Tokyo, Japan) at the entomology laboratory of the cotton program of the Institut de l'environnement et de recherches agricoles (INERA) in Bobo-Dioulasso. Ticks are immobilized by being mounted on a microscope slide in mounting medium (gelatin glycerin) and simply covered with a coverslip to facilitate their observation and identification. Ticks were identified by considering the morpho-anatomical characteristics described by Walker et al. (2003) and Barker and Walker (2014).

Statistical analysis

The data collected were used to calculate means, which were expressed as percentages and means ± standard deviation, before being subjected to descriptive and quantitative analyses. All statistical analyses were performed using R software (version 4.3.3), and graphs were produced using Excel. A Kruskal-Wallis or Wilcoxon test (5% significance level) was used to analyze variations in tick burden based on ruminant demographic characteristics, seasons, pastoral study area, and tick attachment sites on the animals' bodies. A P-value less than 0.05 was considered statistically significant.

RESULTS

Analysis of the data collected during the study reveals that the livestock farming system practised in the two pastoral zones is extensive. The animal breeds encountered were essentially Sahelian zebu peulh (286) and crossbreeds (14) in cattle, and Mossi (319), Sahelian (53), and Djallonke (128) small ruminants (goats and sheep).

Identification of tick species

A total of 8,274 ticks were collected from cattle (91.83%; n = 7598 ticks) and small ruminants (8.17%; n = 676 ticks), including goats (6.32%; n = 523 ticks) and sheep (1.85%; n = 153 ticks, Figure 2). Among the ticks collected, three genera were identified, namely *Amblyomma*, *Hyalomma*, and *Rhipicephalus* (*Boophilus*) with 67.32% (n = 5570), 26.35% (n = 2180), and 6.33% (n = 524) of individuals, respectively. These ticks were more frequently observed in the rainy season (RS; 51.81%) than in the hot dry season (HDS; 34.85%) and cold dry season (CDS; 13.34%; Figure 3). The *Amblyomma* genus was the most represented in the rainy and hot dry seasons, while the *Hyalomma* genus was dominant in the cold dry season. During the study (Figure 4), the tick development stasis observed was dominated by adults (75.70%), followed by nymphs (20.92%) and larvae (3.38%). *Amblyomma* sp. is the most represented (84.43%) among adults of all genera (n = 5570 individuals). With a total of 524 individuals, the genus *Rhipicephalus* (*Boophilus*) sp. is most represented under pupal stasis (50.57%). As for the *Hyalomma* sp. genus, it is represented with 3.12% of

individuals in larval stasis out of a total of 2180 individuals identified. Regarding the Hyalomma sp. genus, it accounts for 3.12% of individuals in larval stasis from a total of 2,180 identified individuals.

Identification of the adult tick species collected revealed a total of six species, listed in Table 1, with the genus *Hyalomma* comprising three species, followed by the genus *Rhipicephalus* (*Boophilus*) with two species. Tick imagos (n = 7994 individuals) identified contained more females (50.60%) than males (49.40%) in the overall sample (Figure 5). However, only *Amblyomma variegatum* had more males (52.60%) than females (47.40%).

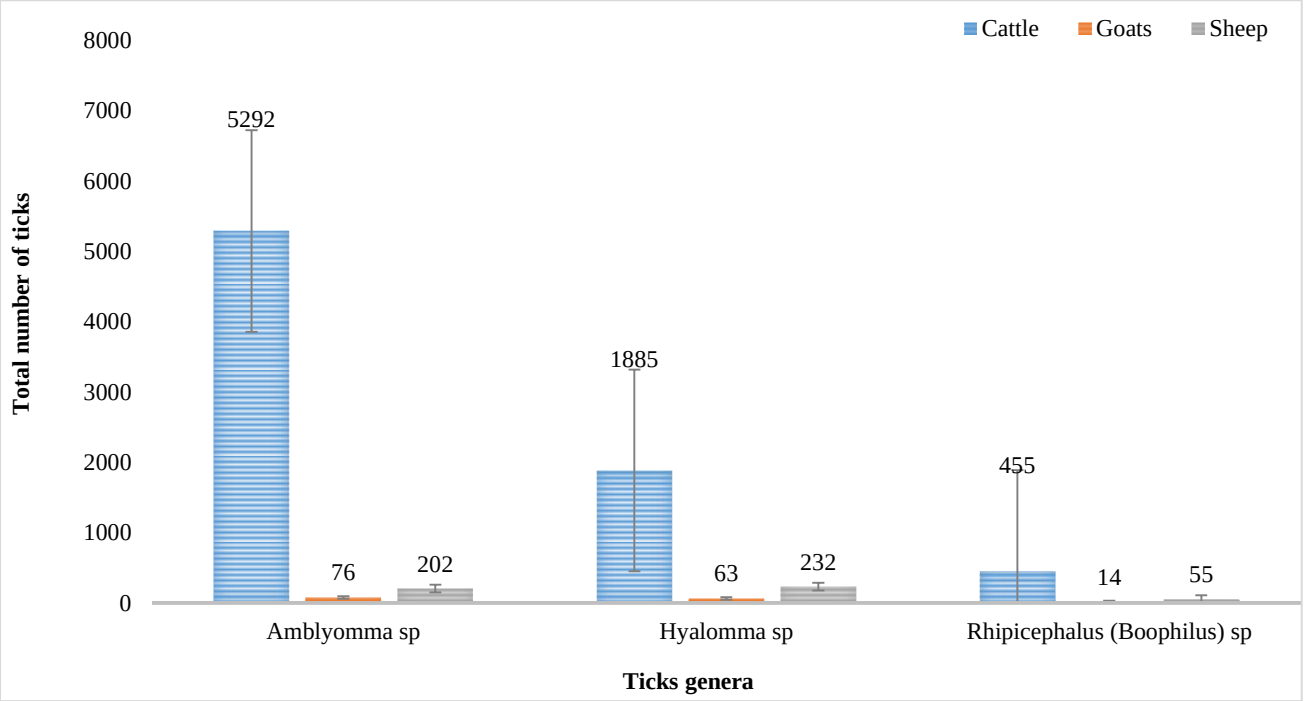


Figure 2. Tick genera encountered according to ruminant species surveyed in two pastoral areas in the Hauts-Bassins region of Burkina Faso during 2022-2023

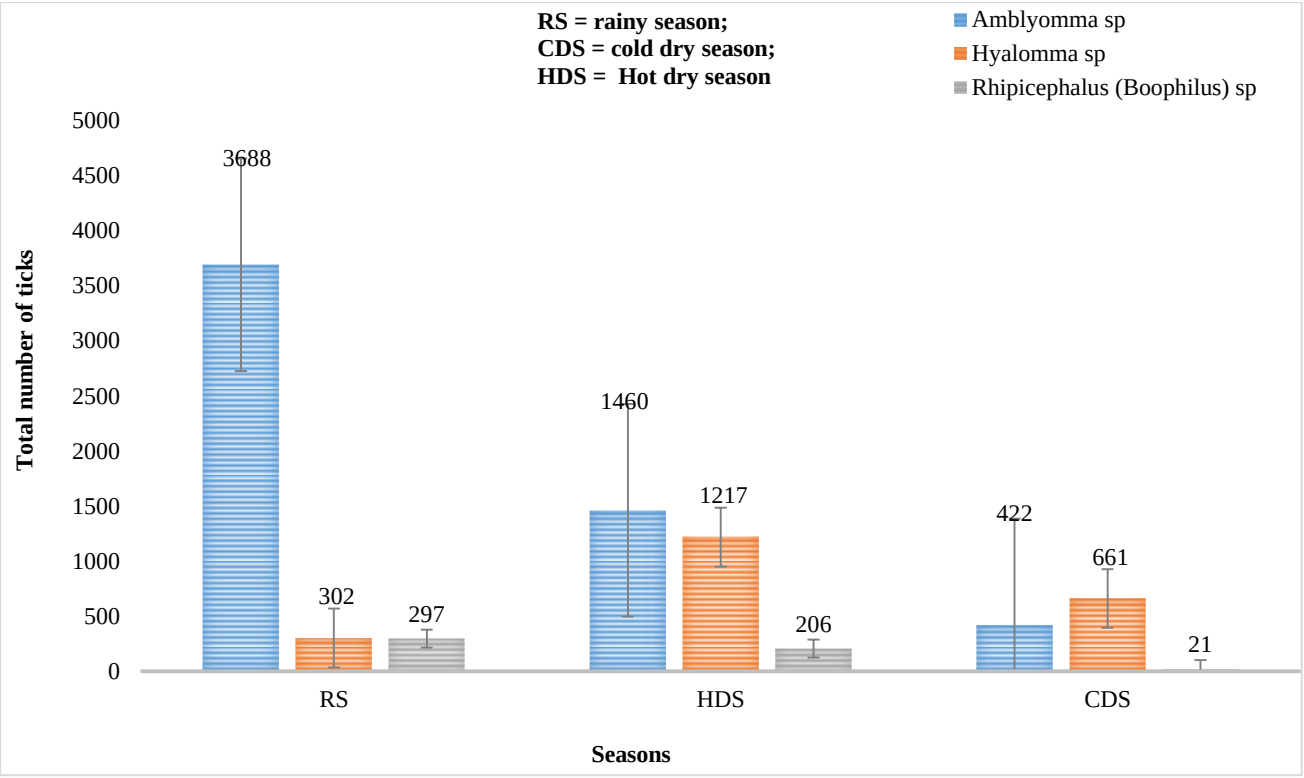


Figure 3. Seasonal distribution of tick genera in two pastoral areas in the Hauts-Bassins region of Burkina Faso during 2022-2023

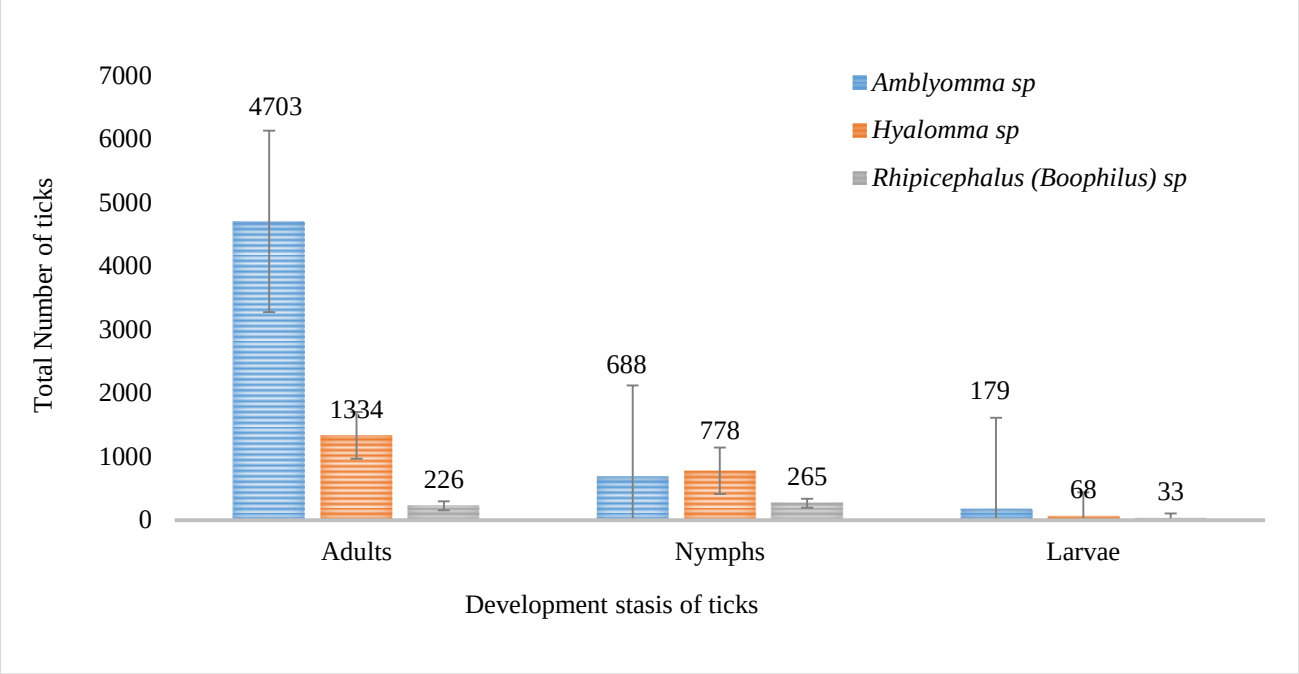


Figure 4. Development stasis of ticks according to tick genera encountered in two pastoral areas in the Hauts-Bassins region of Burkina Faso during 2022-2023

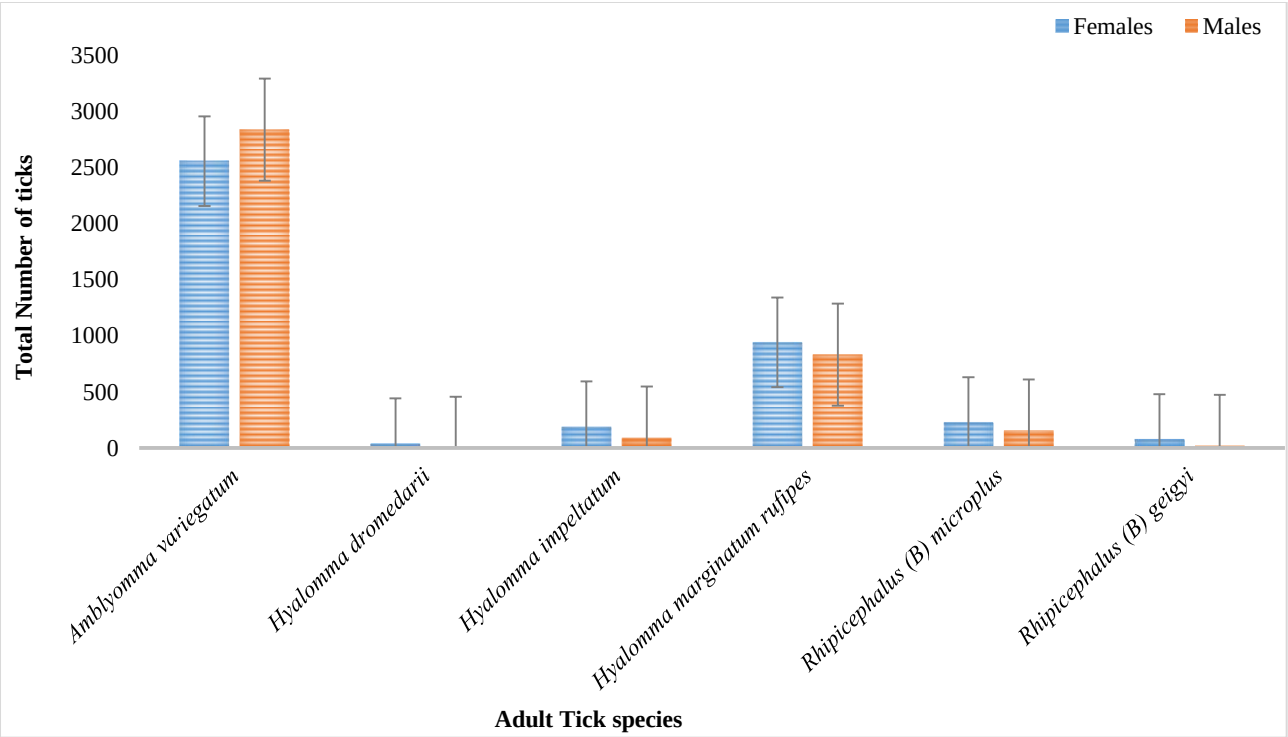


Figure 5. Tick species observed by sex during the study in two pastoral areas in the Hauts-Bassins region of Burkina Faso during 2022-2023

Table 1. Different tick species identified by tick genus in two pastoral areas in the Hauts-Bassins region of Burkina Faso during 2022-2023

Genus	Species
Hyalomma	*Hyalomma marginatum rufipes* *Hyalomma dromedarii* *Hyalomma impeltatum*
Amblyomma	*Amblyomma variegatum*
Rhipicephalus (Boophilus)	*Rhipicephalus (Boophilus) microplus* *Rhipicephalus (Boophilus) geigyi*

Prevalence and level of infestation

A total of 800 ruminants were sampled at the study sites (300 cattle, 300 sheep, and 200 goats). Of these, 52.62% (n = 421) were males and 47.38% (n = 379) females, comprising 41.37% juveniles (n = 331) and 58.63% (n = 469) adults.

For all the ruminants studied, the prevalence rate was 74.90% (n = 599), including 37.25% in cattle (n = 298), 23.50% in sheep (n = 188), and 14.15% in goats (n = 113). In the Saho pastoral zone, among all the surveyed ruminants, the overall prevalence rate was 37.40% (n = 299), with 18.75% of cattle (n = 150), 12% of sheep (n = 96), and 6.65% of goats (n = 53). In Sidéradougou, the obtained rate was 37.50% (n = 300), including 18.50% for cattle (n = 148), 11.50% for sheep (n = 92), and 7.50% for goats (n = 60).

For all ruminants surveyed, the average level of infestation was 10.34 ± 20.30 ticks per animal. By species, cattle (24.96 ± 1.57 ticks) were more infested than sheep (1.87 ± 0.17 ticks) and goats (1.11 ± 0.09 ticks). Depending on the season, the average infestation levels of the animals surveyed varied within the study area. In cattle, mean infestation levels ranged from 9.47 ± 0.87 to 40.09 ± 3.73 ticks/head, while in sheep and goats, they varied from 1.11 ± 0.19 to 2.93 ± 0.40 ticks/head and from 1.00 ± 0.14 to 1.23 ± 0.13 ticks/head, respectively. Cattle and sheep were more infested in the rainy season than goats, which were only infested in the hot dry season (HDS). Cattle were significantly more infested in the rainy season (RS, $p < 0.05$) than in the cold and hot dry seasons (CDS). In sheep, tick infestation showed no significant difference ($p > 0.05$) between the different seasons ($p = 0.07$). Similarly, in goats, the average level of infestation in the hot dry season (HDS) was not significantly higher ($p > 0.05$) than in the rainy season (RS). Comparative analysis of infestation levels between the two pastoral zones revealed no significant differences ($p > 0.05$). However, compared with the Sidéradougou pastoral zone, the Saho pastoral zone recorded a significant difference in the average infestation level ($p < 0.05$) for cattle ($p = 0.01$) and sheep ($p = 0.03$), in the cold dry season (CDS) and the rainy season (RS), respectively.

According to the sex of the animals, the average infestation level in males was significantly higher ($p < 0.05$) than in females. In goats and sheep, there was no variation in these infestation averages between the sexes. In cattle, on the other hand, the tick load in females was significantly higher ($p < 0.05$) than in males.

Concerning age, young ruminants (12.49 ± 21.34 ticks/head) were significantly more infested than adults (9.13 ± 19.61 ticks/head) overall ($p < 0.05$). However, no significant difference was observed between the two age groups for each of the three ruminant species surveyed ($p > 0.05$).

Infested anatomical regions

The preferential attachment sites of ticks on the ruminants surveyed, presented in Table 2, show that their average infestation levels varied according to the data collection periods corresponding to the three seasons of the study. Inguinal and pastern sites were the most significantly infested in ruminants surveyed during the study ($p < 0.05$).

Table 2. Average infestation levels of preferential tick attachment sites collected from surveyed ruminants according to season in two pastoral areas in the Hauts-Bassins region of Burkina Faso during 2022-2023

Seasons	Average infestation levels						
	Ears	Dewlap	Armpits	Abdomen	Inguinal region	Perineal region	Pasterns
CDS	0.43 ± 1.05 b	1.04 ± 2.59 b	0.25 ± 0.82 b	0.65 ± 1.92 b	1.45 ± 3.25 b	0.87 ± 1,72 b	0.58 ± 1.34 c
HDS	1.66 ± 2.83 a	1.17 ± 2.89 b	1.47 ± 3.54 b	1.01 ± 2.41 b	1.06 ± 2.53 b	1.26 ± 2.54 ab	1.75 ± 3.36 b
RS	0.75 ± 1.76 b	2.17 ± 6.11 a	1.90 ± 5.28 a	1.96 ± 5.31 a	3.45 ± 9.27 a	1.61 ± 4.16 a	2.84 ± 5.71a
Total	1.01 ± 2.17 C	1.51 ± 4.37 BC	1.32 ± 3.96 C	1.28 ± 3.73 C	2.05 ± 6.20 A	1.29 ± 3.12 C	1.87 ± 4.20 AB

Data are expressed as mean±SD; ^{a,b,c} Lower-case letters compare means between columns at 5%; ^{ABC} Upper-case letters compare means between rows at 5%; CDS: Cold dry season; HDS: Hot dry season; RS: Rainy season.

DISCUSSION

The results of this study indicated that the pastoral zones of Saho and Sidéradougou are exposed to infestations of parasitic ticks on ruminants, and consequently to the diseases they can transmit. Given the abundance of ticks and the high infestation of ruminants in these pastoral areas, it could be said that the Hauts-Bassins region of Burkina Faso offers highly favorable conditions for the development and spread of these mites. Indeed, the distribution, survival, and abundance of ticks in a given region are dependent on certain environmental and eco-climatic factors such as habitats, temperature, hygrometry, soil and atmospheric humidity, rainfall, and photoperiod (Sajid et al., 2017; Bouchard et al., 2019; Gray et al., 2021).

Of the 8274 ticks collected during the study, three main tick genera were identified, namely *Amblyomma*, which was the dominant genus, followed by *Hyalomma* and *Rhipicephalus* (Boophilus). Within these, six species were identified, namely *Amblyomma variegatum*, *Hyalomma marginatum rufipes*, *Hyalomma impeltatum*, *Hyalomma dromedarii*, *Rhipicephalus (Boophilus) microplus*, and *Rhipicephalus (Boophilus) geigyi*. According to ruminant host species, the most dominant tick genera by order were *Amblyomma* and *Hyalomma* in cattle and goats, versus *Hyalomma* and *Amblyomma* in sheep. The load of the genus *Rhipicephalus (Boophilus)* was lower in goats and higher in cattle. As in previous studies carried out in Burkina Faso, *Amblyomma* and *Hyalomma* genera encountered in the present work were also observed in cattle in the Sahelian and South Sudanian zones (Yao et al., 2020). The same findings have been observed in cattle in the northern Sudanian zone of Burkina Faso (Kaboré et al., 1998; Biguezoton et al., 2016; Ouédraogo et al., 2021) and in sheep by Farougou et al. (2006) in the Sudanian zone of Benin. However, the latter authors noted the predominance of *A. variegatum* followed by *Rhipicephalus (Boophilus) geigyi* in cattle in the North Sudanian region of Burkina Faso. This result contradicts the observations of the present study, which revealed a predominance of the *Hyalomma* genus followed by *Amblyomma* on small ruminants. In other countries, the predominance of the three-host tick *A. variegatum* has been reported in Cameroon, Ethiopia, Gambia, Ghana, Ivory Coast, Mali, Kenya, Nigeria, Senegal, Somalia, Tanzania, and Uganda (Jongejan et al., 2020; Tawana et al., 2022; Heylen et al., 2023; Girma et al., 2024).

Throughout this research, the overall prevalence rate of tick infestation on ruminants was 74.90% in the study area. The study of Heylen et al. (2023) reported prevalence rates of 37.60% in West Africa and 50.57% in East Africa. In Côte d'Ivoire, Yapi (2007) reported that the *A. variegatum* tick accounted for 69.5% of all ticks collected in Azaguié, Dabou, Sikensi, and Alépé (Brofodoume). In Benin, in Atacora and Donga, this tick species accounted for 69% of the ticks collected by Farougou et al. (2006). The prevalence of this tick species may be attributed to the semi-arid to warm climates of the studied pastoral zones. This aligns with findings by Walker et al. (2003) and Beati et al. (2012), who noted *A. variegatum*'s adaptability to diverse climates, including humid and hot conditions. In the study, all six tick species identified were observed in both pastoral zones and all seasons. The average infestation levels recorded were generally higher in the rainy season (RS) than in the cold and hot (HDS) dry seasons (HDS), inducing significant variability in the proportions and frequencies of occurrence of these tick species depending on the season. This result is consistent with the work of Kaboré et al. (1998) and Biguezoton et al. (2016) in Burkina Faso, who also reported a seasonal variation in tick infestation in cattle, with a predominance of ticks during the rainy season. Furthermore, as highlighted by these previous studies, the climatic conditions, notably the more favorable humidity and temperature during the rainy season, create an environment conducive to the survival and activity of ticks, thus explaining the observed increase in infestation levels. Also, these observations made during the study confirm that within a biogeographical zone, the distribution of a tick population depends on the availability of preferred hosts, the biotope, and bioclimatic conditions favourable to their survival (Estrada-Peña, 2003; Bouchard et al., 2019; Bregnard et al., 2020). The spatio-temporal distribution of the six tick species identified during the study in the Hauts-Bassins region could be explained by these factors. The two pastoral zones are characterized by a virtual absence of rainfall and high dry-season temperatures. Such conditions are unfavorable to the development and evolution of ticks, particularly immature stages, which exhibit greater sensitivity to drought compared to adults. This may account for the relatively low number of ticks collected during the study period.

Generally, all developmental stages (larvae, nymphs, and adults) of all identified tick genera infested the surveyed ruminants during the study. Similar observations on cattle in several countries were reported by Yessinou et al. (2022) and Onyiche and MacLeod (2023) in their comprehensive reviews of hard tick species and associated hosts. Furthermore, previous studies carried out in Burkina Faso reported that all developmental stages of *A. variegatum*, *H. marginatum rufipes*, and *R. Boophilus geigyi* species were collected from cattle, sheep, and goats (Adakal et al., 2013; Biguezoton et al., 2016; Ouédraogo et al., 2020). Among the ticks collected, the study recorded adults and immatures of *H. impeltatum* on all ruminants and also adults and immatures of *H. dromedarii* on cattle only. Rates of 4.34% of adult *H. impeltatum* were also recorded by Adakal (2009) in cattle and small ruminants in Bekuy, a peri-urban area of Bobo-Dioulasso in the Hauts-Bassins region of Burkina Faso. The stasis of all the species identified was dominated by adults, followed by nymphs and larvae. The same observations were made by Shuaib et al. (2020) on small ruminants in Sudan, with densities of nymphs and larvae considerably lower than those of adult ticks. This difference in densities observed between developmental stages could be explained by environmental conditions (high sensitivity of eggs and larvae to high temperatures and desiccation) and, above all, the probability of an immature stasis (larva and nymph) encountering a host. Jongejan et al. (2020), Wongnak et al. (2022), and Nimpaye et al. (2023) have also noted that tick larvae generally appear later in the season than nymphs and adults, given their differential sensitivity to environmental conditions. Clearly, temperature and desiccation influence the feeding activities and survival of ticks. Some species have

an early cycle due to the fact that they live in high-temperature environments (warm climates), boosting their development processes and thus favoring an increase in their numbers (Agoulon et al., 2015).

In all three seasons, the presence of the genera *Amblyomma*, *Hyalomma*, and *Rhipicephalus (Boophilus)* in the study area denotes the perennial nature of their activities, and consequently, the potential danger they represent as perennial vectors propagating zoonoses. Reye et al. (2012) and Balinandi et al. (2020) reported that the greatest impact on the health of livestock and humans is caused by species belonging solely to these three genera. Indeed, *Amblyomma variegatum* imagos are vectors of the *Cowdriose ruminatum* parasite, the agent responsible for the greatest damage to hides and skins in cases of heavy infestation (Behailu, 2017). This tick also promotes dermatophilosis (*Dermatophilus congolensis*) in cattle (Kaufnann, 1989) and African fever (*Rickettsia africae*) in humans (Parola et al., 2002; Tomassone et al., 2018). Ticks of the genus *Hyalomma* also transmit pathogens affecting ruminant production (Jongejan and Uilenberg, 2004) and Crimean-Congo hemorrhagic fever (CCHF) virus (Gargili et al., 2017; Chitimia-Dobler et al., 2019). Ticks of the genus *Rhipicephalus (Boophilus)* are vectors of bovine babesiosis (Rodrigues and Leite, 2013) and zoonotic pathogens (Diarra et al., 2017; Ternovoi et al., 2020).

Depending on sex, infestations by females of all tick genera in the study were higher than those caused by males. Only the level of infestation by male ticks of the genus *Amblyomma* was higher than that of females. This result is contrary to that of Yoda et al. (2015), who recorded greater infestation by females of the genus *Boophilus* than by males. The high occurrence of infestations by *Amblyomma* males could be explained by the fact that fecundated females generally detach from their host after a blood meal, fall to the ground for digestion and oviposition. Grooming of the animal can also cause them to detach (Mooring et al., 2000; Mekonnen et al., 2001). Furthermore, male ticks of the *Amblyomma* genus feed on a limited quantity of blood and can remain attached to their hosts for several months. Females will only attach if a male is already attached, due to the aggregation-attachment pheromones produced only by males (Socolovschi et al., 2008).

Adult and immature stages of *H. dromedarii* and *H. impeltatum* species were collected for the first time from ruminants in the Saho and Sidéradougou pastoral zones of the Hauts-Bassins region of Burkina Faso. However, *Hyalomma dromedarii* is adapted to Sahelian and Sahelo-Saharan climates (Al-Deeb and Muzaffar, 2020; Perveen et al., 2021), while semi-arid biotopes and dry seasons are suitable for *H. impeltatum* (Yao-Acapovi et al., 2018). The authors of the current study results then show that these tick species are colonizing savannah areas characterized by woody, herbaceous (upright grasses), and gallery forest strata. The *Hyalomma impeltatum* tick has been recorded in Côte d'Ivoire and Togo (countries bordering Burkina Faso) on sheep, goats, and cattle (Yessinou et al., 2022; Onyiche and MacLeod, 2023). Also, the study of Abdullah et al. (2018) reported a reduced prevalence of infestations of the *Hyalomma dromedarii* tick in cattle, goats, and sheep, despite it being mainly camel-infested. In addition to the warm climate this biotope offers, living conditions are optimal for these ticks in the Hauts-Bassins region. These results are in line with those of Apanaskevich et al. (2009), who reported colonization by *H. impeltatum* in forests and grasslands on the Arabian Peninsula and in several Afro-tropical regions. Furthermore, in addition to the upsurge in livestock trade between African countries (Chand, 2020), the presence and dispersal of tick species in an area depend on other factors, including the movements of their hosts (Randolph et al., 2002). These movements of livestock by herders, due to conflicts (Zannou et al., 2021) and in addition to being a resilience strategy against harsh climates (Motta et al., 2018), are also the cause of the security crisis currently experienced by the Liptako gourma countries (including Burkina Faso) in West Africa. They are also potential phenomena for the exchange of ectoparasites between animals and the transmission of pathogens to animals by ticks (Diarra et al., 2023). These observations could explain the presence of these tick species in the current study region.

In domestic ruminants, the majority of ticks were collected from the ears in the hot dry season (HDS), but tick infestation was particularly high in the armpits, pasterns, and peri and ano-genital areas (tail, perineal, and inguinal areas) in the rainy season (RS). These observations are in line with those of Kaboré et al. (1998) in Burkina Faso, who reported that these areas are the preferred attachment sites for ticks at this time of year. According to some studies, the udder/scrotum of cattle has been identified as the main preferential attachment zone for *Amblyomma variegatum* in Ghana (Addo et al., 2023). Similar observations have also been made in Burkina Faso and Cameroon (Stachurski, 2000; 2006). Indeed, Hurtado and Giraldo-Ríos (2018) believe that ticks prefer areas of an animal's body with thin skin and abundant blood flow, such as the inguinal and external genital regions. The strong preference for these peri- and ano-genital regions could be due to their vascularization and moist nature, which enables ticks to prevent desiccation during feeding. The authors of the current study's point of view is in line with Addo et al. (2023), who believe that acquiring a blood meal in the anal region would be safer and more propitious for ticks. Similarly, Similarly, the authors of the current study, like Addo et al. (2023), believe that the animal's tail, which often covers the anus, protects these mites from the sun's rays and further conceals them from the view of animal trainers who sometimes remove them. These observations could also justify the high occurrence of ticks on ruminant ears in hot weather.

The present study also indicates that all age and sex categories of the ruminants surveyed are threatened by ticks. In addition, the results revealed a dependency between tick burden and the sex of the animal. A significantly higher tick infestation was noted in male ruminants than in females. In general, male animals, as opposed to females, are used extensively in agricultural activities. This could justify this result, in addition to the fact that male ruminants often move long distances to exploit pastures and watering points, consequently remaining more exposed to ticks (Opara and Ezeh, 2011; Musa et al., 2014). Nevertheless, gender is not a discriminating factor in infestation prevalence, considering the results obtained in the small ruminants (sheep and goats) in this study. This corroborates the study of Yoda et al. (2015), who found no link between tick infestation levels and ruminant sex; however, Shahid et al. (2022) found that female small ruminants (15.56%) were more infested than males (9.20%) in Pakistan. Riaz et al. (2017) also recorded a higher tick infestation rate among females (48.52%) than males (45.16%). Sajid et al. (2017) reported a prevalence of 66.44% in males and 80.33% in females of ovine and caprine species. However, the study revealed that female cattle, unlike females of small ruminants, were more prone to tick infestations than males, an observation that could be explained by the inhibitory and stimulatory effects of hormones such as estrogens and androgens on immune responses observed in females than in males (Hughes et al., 2001; Klein, 2004).

Seasonally, the study found tick infestation levels to be higher during the wet, rainy period than during the cold, dry season. These results are in line with the study of Bekele (2002) in Ethiopia, Farougou et al. (2007) in Benin, and Yoda et al. (2015) in Burkina Faso. This observation could be explained by the climatic meteorological data during the wintering season (humidity/hygrometry and ambient temperatures), which are more favorable to tick survival and proliferation.

Generally, the age of ruminants proved to be a discriminating factor, contrary to the results reported in the study of Yoda et al. (2015) and Biguezoton et al. (2016) carried out in the Centre region of Burkina Faso. Indeed, in the study, tick infestation presented a greater occurrence in young animals than in adults. These observations are in line with those made by Rehman et al. (2017). In contrast, Sajid et al. (2009) in Pakistan, Singh and Rath (2013) in India, Rahali et al. (2016) in Morocco, and Addo et al. (2023) in Ghana reported a higher prevalence of ticks in calves compared with young and adult cattle. The high tick burden in young ruminants could be explained by their high susceptibility to infection due to their limited immunity to these parasites. In addition, young ruminants generally receive less attention on smallholdings in farming environments (Burrow et al. 2019). Furthermore, Okello-Onen et al. (1999) and Lew-Tabor et al. (2017) reported that adult animals have acquired immunity through repeated exposure to ticks.

CONCLUSION

The results of the study indicated infestation by parasitic mites in all seasons in the study region, with a significant abundance in the rainy season (RS; 51.81%) than in the hot dry season (HDS; 34.85%) and cold dry season (CDS; 13.34%). Ruminant host factors such as age and sex significantly influenced tick infestation levels. A high occurrence of these parasites was found mainly in the inguinal region, and on the pasterns and dewlaps of ruminants. The tick species identified are known to cause significant damage to animal productivity, necessitating continuous monitoring of tick populations and the diseases they transmit in the region. In order to sustainably improve the productivity of the ruminants surveyed and protect farmers against possible diseases, the results obtained on the spatio-temporal and seasonal distribution of ticks could help to formulate appropriate control strategies in the study area. However, future studies should incorporate molecular identification techniques to clarify tick species diversity and genetic variability, investigate the presence and epidemiology of tick-borne pathogens affecting livestock, evaluate the effectiveness and resistance patterns of current and alternative tick control methods, assess the influence of environmental and climatic factors on tick population dynamics, and examine the socioeconomic impact of infestations alongside farmers' knowledge and practices to guide more effective and sustainable management strategies.

DECLARATIONS

Funding

This study was funded by the Regional Center of Excellence on Pastoral Productions (CERPP) of Abdou Moumouni University (UAM), Niamey, Niger.

Ethical considerations

The authors declare that all ethical aspects of the publication of an original article have been considered in the preparation of this article.

Acknowledgments

The authors would like to thank the Regional Center of Excellence on Pastoral Productions (CERPP) of Abdou Moumouni University (UAM) Niamey/Niger, for funding the study, Mister Salif Sanou, and the herders of the two pastoral zones for their participation.

Authors' contributions

All authors participated in the study. The protocol was developed by Bamba Hubert Eloi Aboubacar Sidiki, Kaboré Adama, and Marichatou Hamani. Data collection and trial execution were conducted by Bamba Hubert Eloi Aboubacar Sidiki, Tianhoun Denté Fidèle, and Sawadogo Alizèta. All authors contributed to data analysis. The first manuscript draft was written by Bamba Hubert Eloi Aboubacar Sidiki and revised by all co-authors. All authors approved the final manuscript.

Competing interests

The authors declare that no conflict of interest has been presented.

Availability of data and materials

The authors state that they will provide the related data of this study if requested.

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2025, Scienceline Publication
World's Veterinary Journal
World Vet J, 15(3): 734-742.
ISSN 2322-4568

DOI: https://dx.doi.org/10.54203/scil.2025.wvj74
PII: S232245682500074-15

Effects of *Lactobacillus* spp. Isolated from Ensiled Swamp Forage on Gastrointestinal Tract Development and Nutrient Digestibility in Native Ducks

Fitra Yosi[1]*, Sofia Sandi[1], Nuni Gofar[2], Eli Sahara[1], Meisji L. Sari[1], Aryantini Safitri[1], and Farin Farandhita[1]

[1]*Department of Animal Science, Faculty of Agriculture, University of Sriwijaya, Indralaya 30662, South Sumatra, Indonesia*
[2]*Department of Soil Science, Faculty of Agriculture, University of Sriwijaya, Indralaya 30662, South Sumatra, Indonesia*

*Corresponding author's Email: fitrayosi@unsri.ac.id

ORIGINAL ARTICLE
Received: July 24, 2025
Revised: August 23, 2025
Accepted: September10, 2025
Published: September 25, 2025

ABSTRACT

The application of lactic acid bacteria (LAB), particularly *Lactobacillus*, as feed additives has demonstrated benefits for poultry, including enhanced gut function and better nutrient digestion. However, studies on LAB derived from swamp grass silage remain limited. The present study aimed to evaluate the effects of supplementing *Lactobacillus* spp. (Lacto) solution derived from ensiled swamp forage (*Hymenachne acutigluma*) at different concentrations through drinking water on the relative weight and length, as well as intestinal density and nutrient digestibility of native ducks. The relationship between intestinal tract density and nutrient digestibility was assessed using the Pearson correlation coefficient. The present study involved sixty 24-week-old native ducks, divided into five groups with four replicates each. The groups included a control group and groups that received Lacto solutions in their drinking water. The Lacto solutions were at concentrations of 1×10^6 CFU/mL (Lacto 1), 1×10^7 CFU/mL (Lacto 2), 1×10^8 CFU/mL (Lacto 3), and 1×10^9 CFU/mL (Lacto 4). The current results indicated that supplementation of the Lacto solution in drinking water increased the relative weights of the proventriculus, small intestine, duodenum, jejunum, ileum, and ceca. The relative weight and density of the intestine in the Lacto groups increased linearly with higher concentrations of Lacto solution. Compared to the control group, ducks receiving Lacto supplementation showed improved crude fiber digestibility (CFD) and a tendency to enhance organic matter digestibility (OMD). In 33-week-old ducks, a higher proventriculus density was associated with increased dry matter digestibility (DMD) and OMD. Additionally, the densities of the duodenum, jejunum, and ileum were positively correlated with DMD, OMD, and CFD. The present findings indicated that the administration of Lacto solution at increasing concentrations up to 10^9 CFU/mL via drinking water effectively improved the development of the small intestines of ducks, which was indicated by an increase in the intestinal relative weight and density, as well as enhancing the OMD and CFD in the diets.

Keywords: Drinking water, Lactic acid bacteria, *Lactobacillus*, Native duck, Nutrient digestibility, Swamp forage

INTRODUCTION

In recent years, the use of probiotics in poultry has risen as a substitute for antibiotics, which have been banned in several European countries and other nations, including Indonesia (Sihombing and Fajri, 2024). Probiotics are microbes that are beneficial to poultry when consumed, as they improve nutrient digestion and gut health (Ding et al., 2021). For example, several studies have shown that probiotic strains, particularly *Lactobacillus* spp., can modify gut development (Olnood et al., 2015), improve small intestine morphology (Wang et al., 2019), and increase nutrient digestibility and availability in broiler chickens, such as dry matter, fiber, protein, and minerals (Poberezhets et al., 2021). However, information on the effects of probiotics on the digestive tract density of poultry is still limited. In addition, most studies have used chickens as animal models to observe the effect of probiotics, while those on ducks are still rare.

As lactic acid bacteria (LAB), *Lactobacillus* strains have become popular and widely used as probiotics in poultry production (Halder et al., 2024). Several studies have utilized different sources to collect different LAB strains for probiotics, including digestive tract digesta and feces from healthy chickens (Ahmed et al., 2019; Chen et al., 2022) and traditional fermented foods (Poornachandra Rao et al., 2015; Suwannaphan, 2021). However, the use of LAB isolated from forage silage as a potential probiotic is still rare. There are several advantages to using forage silage as a source of LAB. Forage is relatively easy to obtain and abundant, particularly in tropical regions such as Indonesia, ensuring its sustainability (Perdinan et al., 2024). A previous study has shown that fermented swamp forage (*Hymenachne acutigluma*) produces *Lactobacillus* that have the potential to be used as probiotics, with properties such as resistance to low pH and the ability to inhibit the development of pathogenic bacteria after *in vitro* tests (Sandi et al., 2018). Previous studies suggested that administering *Lactobacillus* spp. (Lacto) solutions, isolated from ensiled swamp grass through

To cite this paper: Yosi F, Sandi S, Gofar N, Sahara E, Sari ML, Safitri A, and Farandhita F (2025). Effects of *Lactobacillus* spp. Isolated from Ensiled Swamp Forage on Gastrointestinal Tract Development and Nutrient Digestibility in Native Ducks. *World Vet. J.*, 15(3): 734-742. DOI: https://dx.doi.org/10.54203/scil.2025.wvj74

drinking water at concentrations up to 1 × 10^9 CFU/mL, can influence the physical qualities of duck meat and eggs. These effects include increased cooking loss, enhanced water-holding capacity of meat, and a higher egg yolk index (Yosi et al., 2021). It is assumed that the improvement in egg and meat quality is closely related to improved digestive tract function and nutrient digestibility (Obianwuna et al., 2022). The Lacto supplementation would enhance digestive tract development, thereby improving nutrient digestibility. The present study aimed to assess how Lacto supplementation influences the density of the proventriculus, small intestine, and ceca, as well as the nutrient digestibility in the intestines and ceca of ducks. Additionally, the relationship between intestinal density and nutrient digestibility was examined.

MATERIALS AND METHODS

Ethical approval

The present experiment was conducted at the University of Sriwijaya, South Sumatra, Indonesia, following Regulation 18/2015 on Livestock, Animal Health and Welfare in Indonesia and ethical standards.

Experimental design

The experimental setup and methodology followed the description provided by Yosi et al. (2021). Sixty local female Pegagan laying ducks, aged 24 weeks and a mean weight of 1,341 ± 104.1 g, were obtained from a duck farm in Indralaya District, South Sumatra, Indonesia. After a 3-day acclimatization period, ducks were randomly assigned to 20 plots with daily air temperatures ranging from 24 to 35°C and relative humidity of 70 to 85%. Each plot measured 1 m x 1 m × 1 m. A round drinker and a round feeder were used for their setup, and they were maintained for 60 days. Treatments were divided into five groups, with four replicates assigned to each group. The control group did not receive the Lacto solution. The Lacto concentrations were 1×10^6 CFU/mL (Lacto 1), 1×10^7 (Lacto 2), 1×10^8 CFU/mL (Lacto 3), and 1×10^9 CFU/mL (Lacto 4). During the first 30 days of the experiment, the Lacto solutions were added to the drinking water at a dose of 10 mL per bird per day (Yosi et al., 2021). A diet based on corn, noodle meal, and concentrate was formulated to meet or exceed the nutritional requirements of laying ducks in accordance with Indonesian national standards. The diet was provided *ad libitum* throughout the experiment. Table 1 shows the nutrient composition and ingredients of one experimental diet, in accordance with a previous study of Yosi et al. (2021).

Table 1. Ingredients and chemical composition of the experimental diet for Pegagan laying ducks for 60 days

Ingredients (g/kg diet as fed basis)	Composition (%)
Corn meal	16
Dried noodle waste meal	40
Concentrate[a]	32
Bran	10
Premix[b]	1.0
Lysine	0.4
Methionine	0.6
Total	100
Calculated nutrient content[c]	
Metabolizable energy (Kcal/kg)	3007.2
Crude fiber (%)	4.60
Crude protein (%)	18.74
Calcium (%)	4.22
Available phosphorus (%)	0.46

[a]: A mixture of fish meal, soybean meal, meat and bone meal, coconut meal, peanut meal, wheat flakes, leaf meal, canola, vitamins, calcium, phosphate, and trace minerals. [b]: Provided per kilogram of diet, including Calcium (32.5%), Phosphorus (1%), Iron (6 g), Zinc (3.75 g), Manganese (4 g), Copper (0.3 g), Iodine (0.075 g), vitamin D3 (50,000,000 IU), and vitamin B12 (0.5 mg). [c]: Calculated according to the recommendation of the Indonesian National Standard.

The preparation of the *Lactobacillus* spp. solution

The preparation of the Lacto solution started with the preparation of swamp grass silage. The detailed procedure for the preparation of swamp grass silage was the same as previously described by Yosi et al. (2021). The freshly cut grass was chopped into pieces ranging from 2 to 5 centimeters in length. It was then allowed to wilt without direct sunlight at 27-30°C for at least 24 hours. Next, 500 g of the withered grass was mixed with 10 mL of molasses and 5 mL of water. The mixture was placed in a triple-layer plastic bag and stored at room temperature for 21 days under anaerobic

conditions. After preparing the silage, the next step was to make the Lacto solutions and determine their concentrations according to Yosi et al. (2021). The *Lactobacillus* isolates were cultured in Man-Rogosa-Sharpe (MRS) broth (Oxoid CM0359B, England) at 37°C for 48 hours (Sandi et al., 2018). The isolates were then mixed with a peptone solution (Buffer Peptone Water, Oxoid, UK) in 90 mL until they matched the turbidity levels of McFarland reference solutions. The concentrations of the Lacto solutions in Lacto 1, Lacto 2, Lacto 3, and Lacto 4 corresponded to McFarland standards 1, 2, 3, and 4, respectively. These concentrations reflect the Lacto levels in the digestive systems of laying ducks, which range from 10^6 to 10^9 CFU/g (Rehman et al., 2007).

Sampling and measurements of the digestive tract

A total of two ducks of average weight (1,590 ± 81.6 g) in each group were randomly selected at the end of the experiment (33 weeks of age). The ducks were fasted for six hours before slaughter and given only drinking water. The ducks were euthanized by severing their throats and jugular veins with a sharp knife at the first vertebra (Nielsen et al., 2019). The contents of the gastrointestinal tract were removed after cutting it into individual segments. The digestive tract segments measured included the crop, esophagus, proventriculus, duodenum, jejunum, ileum, total small intestine, ceca, and colon. The length of the duodenum was measured from the gizzard outlet to the end of the pancreatic loop using a measuring tape. Jejunum length was measured from the end of the pancreatic loop to Meckel's diverticulum, while ileum length was measured from Meckel's diverticulum to the beginning of the cecal junction. The density of the digestive tract was assessed by dividing the empty weight of the digestive tract by its length (g/cm; Alshamy et al., 2018). The relative length and weight percentages of the digestive tract were calculated using the following formulas (Yosi et al., 2017).

Relative length of digestive tract (%) = Length of digestive tract segment (cm)/body weight (g) × 100

Relative weight of digestive tract (%) = Weight of digestive tract segment (g)/body weight (g) × 100

Measurement of nutrient digestibility

At the end of the experiment, at 33 weeks of age, one duck from each plot was placed in a metabolic cage. The excreta of each duck were collected over three days. Dry matter digestibility (DMD), organic matter digestibility (OMD), and crude fiber digestibility (CFD) were initially assessed using the AOAC (2016) procedure, using the following formulas (Yosi et al., 2016).

DMD (%) = Dry matter consumed - dry matter excreta /dry matter consumed × 100

OMD (%) = Organic matter consumed - organic matter excreted/organic matter consumed × 100

CFD (%) = Crude fiber consumed - crude fiber excreta /crude fiber consumed × 100.

Statistical analysis

SPSS statistical software package (IBM SPSS version 26) was used to analyze all experimental data. One-way analysis of variance (ANOVA) was applied to analyze the data. Duncan's multiple comparison test was used to determine differences between treatment groups. Orthogonal comparisons were performed using polynomial regression to determine the linear and quadratic effects of increasing concentrations of Lacto solution administered via drinking water. The Pearson correlation coefficient (r) between digestive tract density and nutrient digestibility was also calculated. The analyzed data were then presented in tables as means with pooled standard errors of the mean (SEM). To visualize the obtained correlations, heat maps were generated using the level plot++ function of the lattice package in R Studio (version 2023.06.0). Statistical difference was defined as $p < 0.05$.

RESULTS

Relative weight, length, and density of the digestive tract

Adding Lacto solution to drinking water in Lacto groups (Lacto 1 to 4) significantly affected the relative weights of the proventriculus, small intestine, duodenum, jejunum, ileum, and ceca ($p < 0.05$; Table 2). The relative weight of the small intestine was higher in the Lacto supplement groups (Lacto 1 to 4) compared to the control group ($p < 0.05$). Additionally, the relative weight of the small intestine in the Lacto groups showed a linear increase with higher Lacto solution concentrations ($p < 0.05$). The proventriculus and ceca weights were greater in Lacto 2 compared to the control group ($p < 0.05$), but then decreased in Lacto 4 ($p < 0.05$). The duodenum relative weight increased in Lacto 2, Lacto 3, and Lacto 4 Groups, respectively, compared to the control group ($p < 0.05$). The ileum relative weight increased in Lacto 3 and Lacto 4 Groups, respectively, compared to the control group ($p < 0.05$). The jejunum's relative weight was higher with Lacto supplementation than in the control group ($p < 0.05$), but no significant difference was found among the Lacto groups ($p > 0.05$).

It was found that the density of the small intestine, particularly the duodenum and ileum segments, was significantly impacted by supplementing drinking water with Lacto solutions ($p < 0.05$; Figure 1). The overall small intestine density was higher in Lacto 4 than in the control and Lacto 1 Groups ($p < 0.05$). Duodenal density in Lacto 2 Group exceeded that of the control group ($p < 0.05$), though it was similar to Lacto 3 and Lacto 4 Groups. Furthermore, ileal density was higher in Lacto 3 than in the control group ($p < 0.05$).

Table 2. Relative weight and length of the digestive tract of 33-week-old Pegagan laying ducks after supplementation with *Lactobacillus* spp. solutions from ensiled *Hymenachne acutigluma* via drinking water

Variable		Control	Lacto 1	Lacto 2	Lacto 3	Lacto 4	SEM
Crop-esophagus (%)	W	0.52	0.37	0.53	0.48	0.36	0.053
	L	1.50	1.10	1.45	1.28	1.35	0.135
Proventriculus (%)	W	0.39^{ab}	0.34^{b}	0.43^{a}	0.38^{ab}	0.37^{b}	0.018
	L	0.30	0.41	0.37	0.32	0.33	0.036
Small intestine (%)	W	1.54^{c}	1.80^{bc}	2.11^{ab}	2.16^{ab}	2.32^{a}	0.139
	L	10.49	9.20	10.34	9.87	9.32	0.609
Duodenum (%)	W	0.28^{b}	0.32^{ab}	0.37^{a}	0.34^{a}	0.36^{a}	0.016
	L	1.69	1.64	1.80	1.66	1.58	0.133
Jejunum (%)	W	0.59^{b}	0.77^{a}	0.78^{a}	0.80^{a}	0.82^{a}	0.047
	L	4.36	3.65	3.99	3.75	3.64	0.329
Ileum (%)	W	0.68^{b}	0.71^{b}	0.96^{ab}	1.03^{a}	1.14^{a}	0.092
	L	4.43	3.91	4.55	4.46	4.10	0.290
Ceca (%)	W	0.31^{ab}	0.27^{b}	0.38^{a}	0.26^{b}	0.30^{ab}	0.024
	L	0.98	0.80	0.97	0.92	0.96	0.097
Colon (%)	W	0.16	0.12	0.12	0.11	0.14	0.020
	L	0.47	0.43	0.37	0.39	0.38	0.085

Lacto: *Lactobacillus* spp. isolated from ensiled *Hymenachne acutigluma*, SEM: Standard error of mean, W: Weight, L: Length, Control: Group without Lacto solution, Lacto 1, 2, 3, and 4: Groups supplemented with Lacto solution at concentrations of 1×10^6 CFU/mL, 10^7, 10^8, and 10^9, respectively. [a,b, and c] Means without common superscript letters in the same row differ significantly ($p < 0.05$).

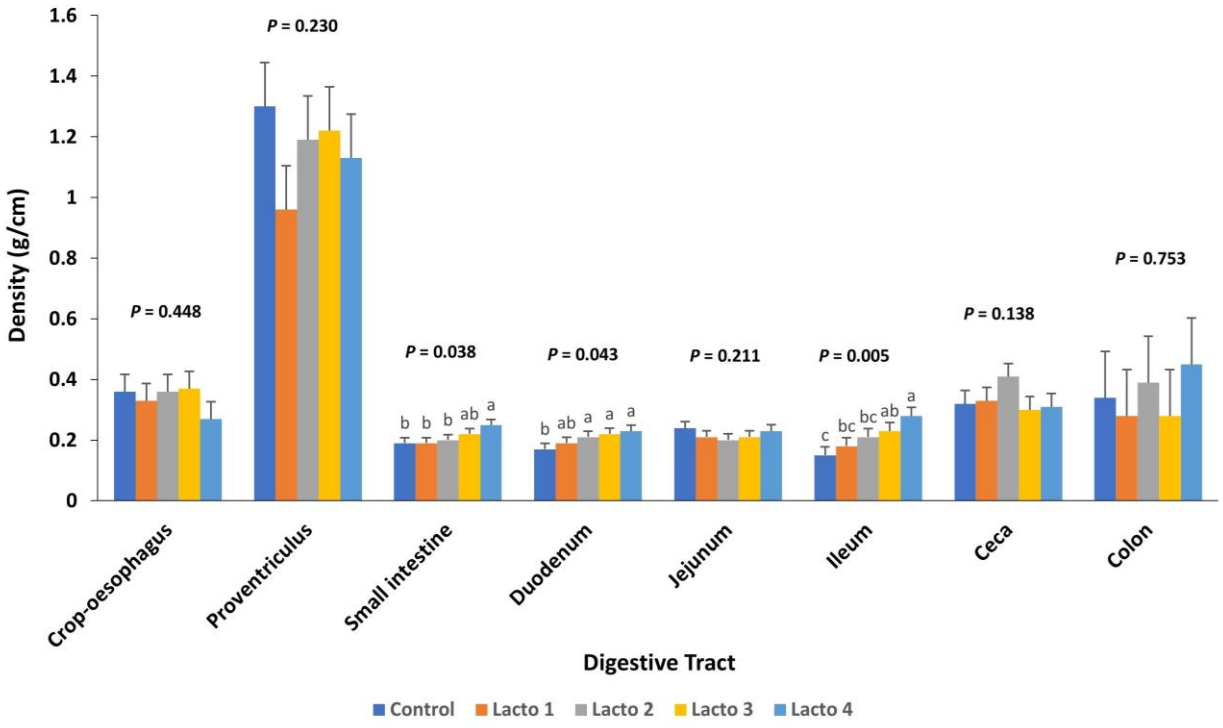


Figure 1. Differences in the digestive tract density of 33-week-old Pegagan laying ducks between the control group and groups supplemented with *Lactobacillus* spp. solutions from ensiled *Hymenachne acutigluma* via drinking water. Control: Group without Lacto solution, Lacto 1, 2, 3, and 4: Groups supplemented with Lacto solution at concentrations of 1×10^6 CFU/mL, 10^7, 10^8, and 10^9, respectively. [a,b, and c] Means without a common superscript letter on the bar graph of each gut segment are significantly different ($p < 0.05$).

Digestibility of feed dry matter, organic matter, and crude fiber

The addition of Lacto solution via drinking water in the Lacto groups significantly affected CFD ($p < 0.05$) and showed a trend toward influencing OMD compared to the control group, while it had no impact on DMD ($p > 0.05$; Figures 2 A-C). The CFD was higher in Lacto 2 Group than in the control and Lacto 1 ($p < 0.05$), but no significant

differences were found among Lacto 2, Lacto 3, and Lacto 4 ($p > 0.05$). Additionally, there was a tendency for OMD to increase in Lacto 4 Group compared to the control group.

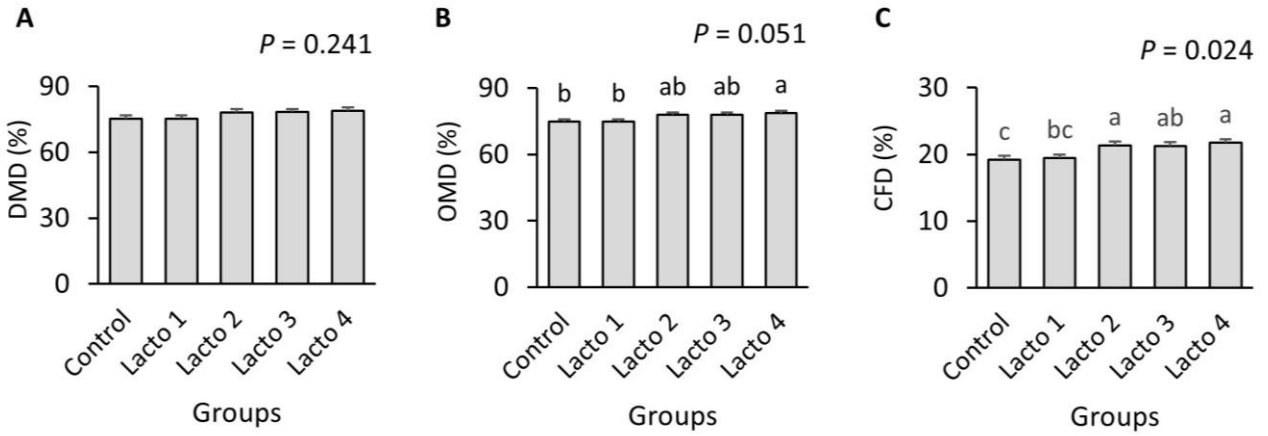


Figure 2. Differences in the dietary digestibility of dry matter, organic matter, and crude fiber in 33-week-old Pegagan laying ducks between the control group and groups supplemented with *Lactobacillus* spp. solutions via drinking water. **A**: Dry matter digestibility (DMD), **B**: Organic matter digestibility (OMD), **C**: Crude fiber digestibility (CFD), Control: Group without Lacto solution, Lacto 1, 2, 3, and 4: Groups supplemented with Lacto solution at concentrations of 1×10^6 CFU/mL, 10^7, 10^8, and 10^9, respectively. [a,b, and c] Means without common superscript letters in the bar graph are significantly different ($p < 0.05$).

Correlation of digestive tract density with nutrient digestibility

According to the Pearson correlation coefficient, the DMD was positively correlated with the density of the proventriculus, duodenum, and total small intestine in 33-week-old Pegagan laying ducks ($p < 0.05$, r = 0.31-0.53; Figure 3a). A positive relationship was found between the CFD and the density of the duodenum, jejunum, and ileum ($p < 0.05$, r = 0.41-0.47; Figure 3b). Additionally, the density of the proventriculus, duodenum, ileum, and total small intestine showed a positive correlation with the OMD ($p < 0.05$, r = 0.32-0.63; Figure 3c).

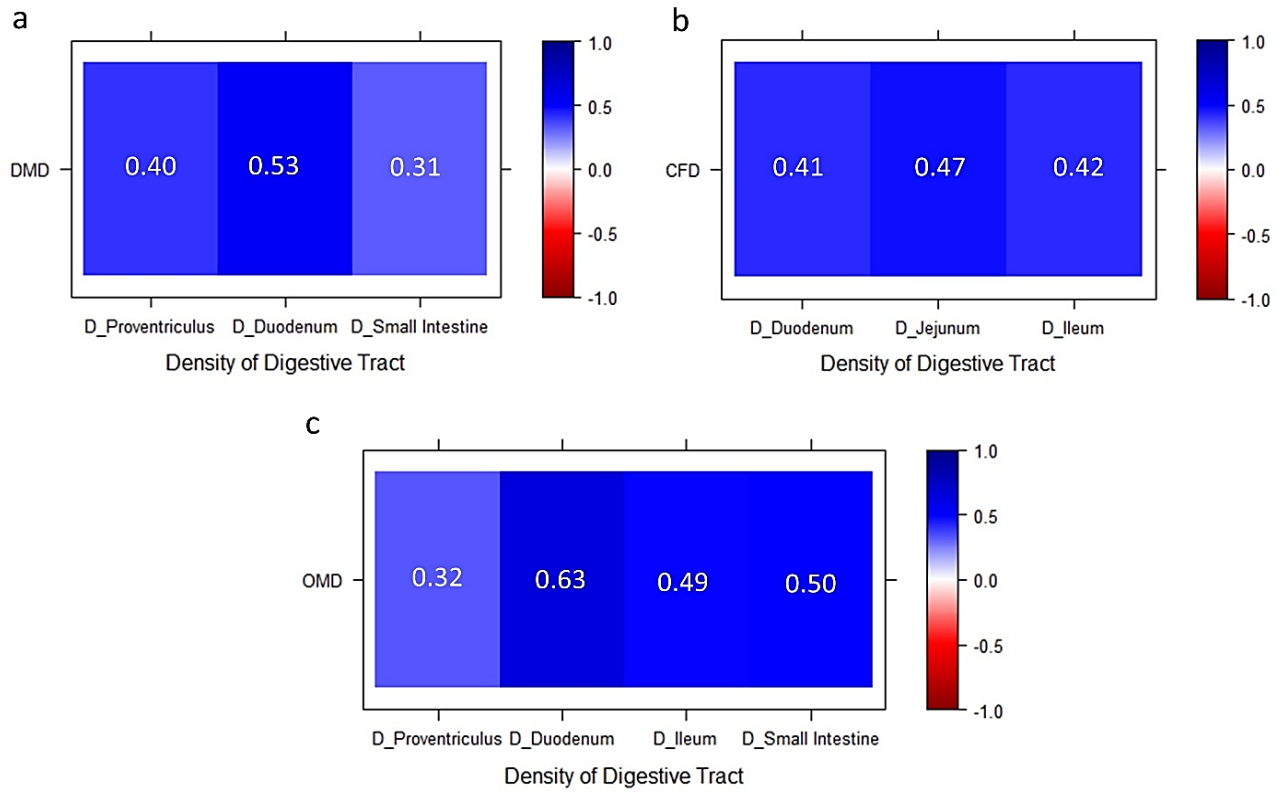


Figure 3. The significant associations of proventriculus and small intestine density with dry matter digestibility, crude fiber digestibility, and organic matter digestibility in 33-week-old Pegagan laying ducks. **a**: Dry matter digestibility (DMD), **b**: Crude fiber digestibility (CFD), **c**: Organic matter digestibility (OMD).

DISCUSSION

The development of the digestive tract of poultry is very important to improve the digestibility of nutrients, which can be supported by providing probiotics as a feed additive (Halder et al., 2024). In the current study, adding Lacto solutions derived from ensiled swamp forage and administered via drinking water appeared to influence the relative weight of the gastrointestinal tract and alter the density of the small intestine, particularly in the duodenum and ileum, in 33-week-old ducks. It was confirmed that the administration of Lacto solution through drinking water was effective in increasing CFD, with less effect on OMD.

In the present study, adding Lacto solutions to drinking water increased the relative weights of the proventriculus and small intestine. These findings aligned with those of Pedroso et al. (2003), who found that probiotics containing *Lactobacillus* spp. (*L. johnsonii* and *L. reuteri*) in drinking water significantly improved broiler intestinal weight at three weeks. The same results for digestive tract improvement were found when *Lactobacillus* was administered in the chicken diet. Olnood et al. (2015) discovered that the addition of four strains of *Lactobacillus*, including *L. salivarius*, *L. crispatus*, *L. johnsonii*, and an unidentified *Lactobacillus* sp., in the diet improved the relative jejunal and ileal weights of 3 and 6-week-old chickens compared to the control, which indicated that both probiotic routes (Diet and drinking water) showed similar responses to changes in small intestinal weight. Overall, the effect of probiotics on the weights of organs in animals remains uncertain, and the underlying mechanism is unknown. It has been suggested that probiotics in the diet or drinking water may modify the intestinal surface by increasing or decreasing its length or the height of the villi. Changing the size of the villi will result in modifications in the surface area for digestion and absorption of feed (Olnood et al., 2015). Additionally, Awad et al. (2009) suggested that an increase in the small intestine relative to body weight when using probiotics may indicate histological changes. The increased villus height, resulting from a larger absorptive surface area, improved nutrient transport mechanisms, and greater expression of brush border enzymes, is believed to coincide with enhanced digestive and absorptive functions of the intestine (Ravindran and Abdollah, 2021). The increased villus height is proposed to be an indicator of stimulated intestinal villus activity (Wang et al., 2025). Furthermore, the higher density of the small intestine, duodenum, and ileum in the present study reinforced the notion that the intestinal villi were more developed after supplementing Lacto solutions. It is assumed that higher density indicates more villi per unit area (Garic et al., 2025). Based on the current results, it is conceivable that the villus development and function might be activated after supplementation of Lacto solutions in drinking water. Nevertheless, histomorphological examination of intestinal villi is essential to validate improved intestinal density and development.

The increased OMD in the Lacto supplementation groups indicated that more nutrients, such as carbohydrates, protein, fat, minerals, and vitamins from the feed, can be absorbed in the intestinal tract of ducks compared to the control group. This suggestion is supported by the results of Chichlowski et al. (2007), which indicated that broiler chickens receiving a multi-probiotic with *Lactobacillus* showed enhanced passive nutrient absorption, particularly for glucose and proline. Additionally, the Lacto supplementation probiotics were found to improve lipid digestibility and the levels of several minerals, such as calcium, phosphorus, and nitrogen, in broiler chickens (Apata, 2008; Wu et al., 2019). The production of extracellular enzymes could play a role in enhancing digestion and nutrient uptake (Jha et al., 2020). A study by Jin et al. (2000) showed that supplementation with *L. acidophilus* and a mixture of *L. fermentum*, *L. acidophilus*, *L. brevis*, and *L. crispatus* was able to increase amylase levels in the small intestine and reduce intestinal glucuronidase activity in broiler chickens after 40 days of feeding. It is suggested that *Lactobacillus* probiotics selectively influence the utilization of major nutrients (Jha et al., 2020). In addition, CFD improved by the administration of Lacto solutions through drinking water. *Lactobacillus* may contribute to an increase in the number of beneficial bacteria in the digestive tract, which can assist in digesting crude fiber and increase the absorption of fermentation-derived metabolites, such as short-chain fatty acids, that serve to improve gut function and health (Silva et al., 2020).

The DMD and OMD were found to be positively linked to the density of the proventriculus. It appeared that an increase in the density of the proventriculus correlated with a rise in the DMD or OMD in the diet. The density of the proventriculus indicated the number of glands or cells per unit area (Langlois, 2003), which means that increasing the density of the proventriculus could increase the number of columnar epithelial cells and glands in the proventriculus that function to secrete mucus and digestive enzymes, especially pepsinogen, to digest protein (Zhu, 2015). Similarly, the density of the small intestine, which includes the duodenum, jejunum, and ileum, exhibited a positive correlation with DMD, OMD, and CFD, indicating that higher small intestine density leads to increased digestibility of these components. A higher density in the intestine indicates more villi, increasing the surface area for absorption (Kai, 2021), which boosts the small intestine's capacity to digest and absorb nutrients, improving overall digestive efficiency. Likewise, the density of the small intestine, including the duodenum, jejunum, and ileum, was positively correlated with DMD, OMD, and CFD, indicating that higher small intestine density is associated with greater digestibility of these

components. High intestinal density indicates more villi, thereby increasing the absorption surface area (Kai, 2021). This results in more nutrients being digested and absorbed by the small intestine, enhancing digestion efficiency.

CONCLUSION

Supplying *Lactobacillus* spp. solutions derived from swamp grass silage at concentrations of up to 1×10^9 CFU/mL via drinking water may enhance the development of the proventriculus, small intestine, and ceca, indicated by increased relative weight and density, along with improved digestibility of nutrients and crude fiber. The correlation findings highlighted the importance of proventriculus and small intestine density in improving feed nutrient digestibility, which is essential for the growth performance of ducks. For future studies, a more comprehensive understanding requires histomorphological examination of the small intestine and ceca in Pegagan laying ducks.

DECLARATIONS

Acknowledgments

The authors gratefully acknowledge the Laboratory of Animal Feed and Nutrition and the Poultry Research Farm, Department of Animal Science, University of Sriwijaya, Indonesia.

Authors' contributions

Fitra Yosi conceptualized the study, collected and analyzed the samples, processed and interpreted the data, and wrote the initial manuscript. Sofia Sandi and Nuni Gofar participated in the design of the study and interpreted the data. Eli Sahara and Meisji Liana Sari participated in data analysis and interpretation. Aryantini Safitri and Farin Farandhita performed animal experiments, collected and analyzed samples. All authors reviewed, revised, and approved the final edition of the manuscript.

Funding

The present study was funded by the Institute for Research and Community Service, University of Sriwijaya, Indonesia, through a Professional Grant under the contract number 0109.26/UN9/SB3.LP2M.PT/2018.

Competing interests

The authors declared no conflict of interest.

Ethical considerations

The present study has not been published elsewhere. The authors have checked the plagiarism index and confirmed that the article is original based on their scientific results.

Availability of data and materials

The data to support the present study are available upon reasonable request from the corresponding author.

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2025, Scienceline Publication
World's Veterinary Journal
World Vet J, 15(3): 743-748.
ISSN 2322-4568

DOI: https://dx.doi.org/10.54203/scil.2025.wvj75
PII: S232245682500075-15

The Pharmacological Study on Therapeutic Efficacy of the Herbal Preparation of Hypericum

Rakhmatullin Emil Kasymovich[1], Rakhmatullov Ruslan Fagimovich[2]*, Kadikov Ilnur Ravilevich[1], Makaeva Alsu Rinatovna[1], Mayorova Ekaterina Nikolaevna[1], Korchemkin Andrey Aleksandrovich[1], Sagdeev Danil Rustamovich[1], and Kurshakova Ekaterina Ivanovna[1]

[1]*Laboratory of Exogenous Ecotoxicants, Department of Toxicology, Federal Center for Toxicological, Radiation and Biological Safety, Kazan, 420075, Russia*
[2]*Penza State University, Federal State Budgetary Educational Institution of Higher Professional Education of the Ministry of Education of the Russian Federation, Penza, Russia*

*Corresponding author's Email: capitalofgreat@icloud.com

ORIGINAL ARTICLE
Received: June 23, 2025
Revised: July 28, 2025
Accepted: August 27, 2025
Published: September 30, 2025

ABSTRACT

Dyspepsia is a condition associated with digestive disorders, including diarrhea, weakness, dehydration, and intoxication. The investigation and application of therapeutic and prophylactic agents for managing gastrointestinal diseases in young cattle, including dyspepsia in newborn calves, is concerning. The present study aimed to investigate the biochemical mechanisms of action of Hypericum in the treatment of dyspepsia in newborn calves. Two groups of black and white breed bulls (five animals per group), aged 8-10 days, were assigned to assess the effects of Hypericum on biochemical parameters, including total protein, albumin, glucose, bilirubin, urea, creatinine, aspartate aminotransferase, alanine aminotransferase, and lactate dehydrogenase. Calves in the first group (n = 5), exhibiting pronounced signs of dyspepsia, were administered the treatment. This treatment consisted of 250 mL of Hypericum, combined with 500-700 mL of warm water, instead of milk, during the following two feedings. Then, milk was added to each serving of this mixture until the total volume reached 1.25-1.50 liters, and it was given to calves until clinical signs of recovery appeared. Calves of the second group (n = 5) were clinically healthy and received boiled water instead of the Hypericum. The current findings demonstrated that the biochemical parameters of the experimental calves improved following the administration of Hypericum, reaching levels comparable to those of clinically healthy calves. After prophylactic administration of Hypericum at a dose of 6.5 mL/kg during the first six feedings, the general condition of the experimental calves was satisfactory. In the experimental group of calves administered Hypericum at a dosage of 6.5 mL/kg mixed with milk over a period of two days, only one out of fifteen calves exhibited signs of dyspepsia, including liquid feces and lethargic behavior within the initial four days, representing a prevalence of 6.7%. Hypericum could prevent the occurrence of dyspepsia by 93%. The utilization of Hypericum had a therapeutic influence and positively impacted the clinical condition, behavior, and appetite of the calves.

Keywords: Albumin, Biochemical indicator, Calf, Dyspepsia, Hypericum, Total protein

INTRODUCTION

In the context of intensive animal husbandry development, acute gastrointestinal diseases in young animals pose a significant problem. The incidence of dyspepsia in young cattle is up to 80-95%, and the mortality rate in severe toxic forms of dyspepsia reached 70% (Klein-Jöbstl et al., 2014; Kurazeeva et al., 2015). The most common causes of diarrhea in newborn calves are rotavirus, coronavirus, and cryptosporidium. Malabsorption is the main mechanism by which these and pathogenic microorganisms cause diarrhea (Naylor, 2009). Dyspepsia is a polyetiological disease accompanied by digestive disorders, diarrhea, weakness, dehydration, and intoxication (Norboyev and Fayziyeva, 2024). As a result, milk production in sick animals decreases by 15-18%, and their fertility is reduced (Zhirkov et al., 2000). Therefore, the prevention and treatment of neonatal gastrointestinal diseases in young animals are key factors for increasing meat and milk production. In recent years, therapeutic and prophylactic agents for treating gastrointestinal diseases in young cattle, including dyspepsia in newborn calves, have been identified (Gerceva et al., 2023). Treatment of calf dyspepsia should be based on the etiopathogenetic principle and should be aimed at normalizing the digestive process, eliminating dysbacteriosis, dehydration, and intoxication (Norboyev and Fayziyeva, 2024). Despite a highly diverse etiology and pathophysiology of gastrointestinal diseases, treatment with antimicrobials is often considered the first-line therapy. Uncontrolled use of antibiotics leads to the development of dysbiosis in newborn calves and the

To cite this paper: Rakhmatullin EK, Rakhmatullov RF, Kadikov IR, Makaeva AR, Mayorova EN, Korchemkin AA, Sagdeev DR, and Kurshakova EI. (2025). The Pharmacological Study on Therapeutic Efficacy of the Herbal Preparation of Hypericum. *World Vet. J.*, 15(3): 743-748. DOI: https://dx.doi.org/10.54203/scil.2025.wvj75

emergence of resistant bacterial strains to these medications (Dobrzanska et al., 2020; Du et al., 2023). Pursuing novel pharmaceuticals and investigating the biochemical mechanisms of their influence in the prevention and treatment of dyspepsia in newborn calves is an urgent priority issue. Medicinal plants offer potential as alternative or complementary treatments. Medicinal plants, due to their multifaceted effects resulting from plant-specific, multi-component compositions, can potentially function as multi-target medicines. Based on European ethnoveterinary studies, a total of 223 medicinal plant species have been identified for treating gastrointestinal and respiratory diseases. Based on these studies, it was found that the most promising candidates for gastrointestinal diseases are *Allium sativum L., Mentha x piperita L.*, and *Salvia officinalis L*. (Ayrle et al., 2016). Additionally, a complex phytomineral preparation was created from opoka from the Taskala deposit and a decoction of camelthorn (*Alhagi maurorum*), and investigated for the treatment of diarrhea in calves. The findings suggested a high therapeutic effect of this preparation (Montayeva et al., 2024). The present study aimed to identify potential medicinal plant species for preventing and treating gastrointestinal and respiratory diseases in calves and piglets, and to investigate the biochemical mechanisms of the herbal drug, Hypericum, in treating dyspepsia in newborn calves.

MATERIALS AND METHODS

Ethical approval

The Animal Ethics Committee of the Federal Center for Toxicological, Radiation and Biological Safety, Kazan, Russian Federation, approved all animal handling protocols used in the current study (no 1 dated 12.02.2023).

Study design

The herbal preparation of Hypericum, based on St. John's Wort, was used for the present study. 300 g of St. John's Wort herb was placed in gauze, poured with 6-7 liters of boiling water, and left until the temperature dropped to 70°C. Then, 250 g of glucose, 50 g of sodium chloride, 10 g of calcium lactate, 5 g of ascorbic acid, and 220 g of sodium carboxymethylcellulose (Blanose) were added to the infusion. After dissolving the added components, enough water at 70°C was added to bring the total volume to ten liters. The resulting solution was left for 12-15 hours at room temperature, and periodically stirred several times. Then, the gauze with the remnants of St. John's Wort was squeezed and discarded. Before use, the preparation was heated to 37-38°C and thoroughly shaken into a homogeneous mass (Rakhmatullin and Sklyarov, 2020). The present study was designed to investigate the dynamics of biochemical blood parameters in calves with dyspepsia before and after the use of Hypericum and to determine the prophylactic effectiveness of Hypericum. The current study was conducted at the Federal State Budgetary Institution, Federal Center for Toxicological, Radiation, and Biological Safety, Kazan, Russia.

To examine the impacts of the pharmaceutical compound of Hypericum on the biochemical parameters of blood, two groups of black-and-white calves, aged 8 to 10 days, were assembled. The first group, consisting of five calves exhibiting pronounced signs of diarrhea, was administered Hypericum orally. The administration of Hypericum continued for five days. For therapeutic purposes, sick calves received a mixture of 250 ml of Hypericum with 500-700 ml of warm water instead of milk during the subsequent two drinking sessions. Then, this mixture was added to each subsequent portion of milk, increasing the total portion volume to 1.25-1.5 liters. The treatment was given orally for five days until the animal had clinically recovered. The calves of the second group, consisting of five calves, were clinically healthy and received 750-950 mL of boiled water instead of the treatment. The calves in the experiment underwent a clinical examination, and their physiological development, response to external influences, reception of cow's milk, and digestive and excretory systems were determined. The frequency of defecation, the consistency, and the color of feces were noted. Blood (5-6 mL) was collected from the jugular vein of calves, both before treatment administration and on the fifth day after the last treatment. The content of total protein and albumin, bilirubin, creatinine, urea, glucose, the activity of aspartate (AST) and alanine aminotransferases (ALT) as indicators of liver damage syndrome, lactate dehydrogenase, and amylase enzymes were studied before treatment administration and on the fifth day after the last administration of the treatment.

To assess the prophylactic effect of Hypericum, two groups of black-and-white calves aged 8-10 days were designed. For the prevention of gastrointestinal diseases in calves of the first group (15 heads), within two days after birth, all calves were given 6.5 mL/kg of the Hypericum medicinal solution once with colostrum of the first six feedings. According to the results of preliminary studies and the findings of Garmaev and Garmaev (2019), it has been established that the most optimal condition is a six-fold preventive administration of the treatment. Considering the composition of the treatment, it had a beneficial influence on the immune system of calves. Calves in the second group (15 heads) were given boiled water within two days after birth and were fed using a nipple drinker. The rates of morbidity and mortality among the calves were used to determine the prophylactic effects.

Biochemical examination

During the experimental studies, the total protein was assessed using the biuret reaction (Lubran, 1978; Tietz et al., 1995; Kondrakhin et al., 2004). The glucose content was determined by the unified glucose oxidase method of orthotolidine oxidation (McMillin, 1990; Tietz, 1995). Activities of ALT and AST were determined using the unified dinitrophenylhydrazine method (Lala et al., 2024). Serum lactate dehydrogenase (LDH) activity was measured by the 2,4-dinitrophenylhydrazine reaction. L-Lactate, in the presence of serum enzyme and NAD, was oxidized to pyruvate, which was then detected through a color reaction with 2,4-dinitrophenylhydrazine (Li et al., 1980; Kondrakhin et al., 2004; Farhana et al., 2023).

Statistical analysis

The results of the experimental studies were processed using the method of variation statistics. therefore, the STATISTICA 13 application software was used. Statistical comparisons between different groups were conducted using one-way analysis of variance (ANOVA). The Least Significant Difference (LSD) test was used to compare variances among means at a significance level of $p < 0.05$. Mean data were expressed with standard deviation.

RESULTS AND DISCUSSION

The occurrence of acute digestive disorders and liver pathology in young animals in the early postnatal period of development indicates the presence of severe metabolic disorders in the breeding stock (Hussain et al., 2014; Urbutis et al., 2023). Additionally, the metabolism of growing animals is characterized by elevated intensity, making them highly sensitive to changes in maintenance and feeding conditions (Batrakov et al., 2021).

The mechanism of action of the medicine was related to its complex mode of action. The treatment contains St. John's wort, which, according to Klemow et al. (2011), exhibited anti-inflammatory, antimicrobial, antiviral, and antidepressant effects.

At the beginning of the experiments, clinical signs such as general weakness, decreased movement, diarrhea, and a decreased sucking reflex were observed in the calves with dyspepsia in the experimental group. After administering Hypericum water mix, the severity of clinical signs, such as diarrhea and depression, in the calves of the experimental group decreased within 24 hours. Additionally, Hypericum had a noticeable therapeutic effect on the clinical condition, behavior, and appetite of calves, and no adverse reactions were detected. On the third day of treatment administration, the calves in the experimental groups were active, with a pronounced appetite, natural reflexes, and accelerated sucking reflexes. Clinical signs of diarrhea in the treatment group entirely resolved by the fourth day of treatment administration. Corresponding biochemical blood parameters, measured before treatment and five days after the last treatment administration, are presented in Table 1.

Table 1. Dynamics of biochemical parameters of blood in black and white breed bulls aged 8-10 days

Measurement results / Indicators	Before treatment		After treatment	
	Experimental group	Control group	Experimental group	Control group
Total protein (g/l)	45.2 ± 3.14*	58.7 ± 3.96	62.6 ± 3.42	62.5 ± 4.21
Albumin (g/l)	24.05 ± 1.9*	35.32 ± 2.8	32.9 ± 4.9	32.1 ± 5.7
Glucose (mmol/l)	1.27 ± 0.21*	2.92 ± 0.47	5.2 ± 0.78	4.3 ± 0.69
Bilirubin (µmol/l)	2.63 ± 0.44	1.92 ± 0.54	2.01 ± 0.75	1.43 ± 0.15
Urea (mmol/l)	5.69 ± 0.64*	2.92 ± 0.38	5.78 ± 2.38*	2.02 ± 0.4
Creatinine (mmol/l)	62.7 ± 7.93	70.3 ± 3.54	84.2 ± 16.5	68.5 ± 2.63
Alanine aminotransferases (U/l)	19.0 ± 2.86	14.1 ± 1.18	20.3 ± 3.91	11.8 ± 1.64
Aspartate aminotransferases (U/l)	32.3 ± 4.56*	56.8 ± 2.69	38.8 ± 5.4	47.2 ± 3.1
AST/ALT ratio	1.99 ± 0.47	2.92 ± 0.33	2.63 ± 0.76	3.82 ± 0.59
Lactate dehydrogenase (U/l)	1623.2 ± 31.96	1735.8 ± 48.1	1629.8 ± 262.7	1812.1 ± 29.1

* $p < 0.05$ in comparison with the results of the control group

According to the present results, it should be noted that in unhealthy calves before the use of Hypericum, the amount of total protein, albumin, glucose, and AST was significantly lower ($p < 0.05$) than in clinically healthy animals in the control group, by 23%, 27%, 56.5% and 43.12%, respectively. Albumin is the primary protein component of blood, primarily produced in the liver, and it plays a crucial role in transporting water and metabolites from tissues. Hepatic

albumin synthesis is not a high priority, and it occurs when the body is adequately nourished. A poor nutritional state, inflammation, exposure to hepatotoxins, and exposure to high colloid osmotic pressure inhibit synthesis (Moman et al., 2022). A decrease in albumin levels is a clear indicator of a liver detoxification system disorder in calves with dyspepsia. Decreased production of albumin is a rare cause of hypoalbuminemia. Significant and severe chronic hepatic impairment is necessary before a noticeable decrease in plasma albumin levels is observed. Most commonly, inadequate synthesis of albumin in the presence of increased catabolism due to significant systemic illness contributes to an overall hypoalbuminemia (Gounden et al., 2021).

The total urea amount in the experimental group before treatment was significantly higher ($p < 0.05$) by 51.3% compared to the control group measurements. Protein degradation was elevated in the bodies of unhealthy calves; consequently, the urea concentration in the blood of all experimental calves was significantly higher than in the control group ($p < 0.05$). The amount of bilirubin in the experimental group before treatment was insignificantly higher ($p > 0.05$) by 37%. The current results indicated that the occurrence of metabolic and functional disorders in the body systems caused an endogenous intoxication. The predominance of catabolic processes over anabolic processes resulted in the decompensation of the body's regulatory systems and the accumulation of proteolytic enzymes, kinins, and other biologically active products of protein degradation, as well as inflammatory mediators, in toxic concentrations. The accumulation of toxic substances in the body subsequently causes liver dysfunction (Velikanov, 2017).

Calves with dyspepsia exhibited significantly lower concentrations of total protein ($p < 0.05$). A reduction in total protein, albumin, and glucose levels signified the dominance of catabolic processes in the bodies of calves suffering from diarrhea.

Administration of Hypericum resulted in the resolution of clinical signs, such as liquid feces and depressed behavior, in three days. Administering treatment to the calves for five days did not cause toxicosis in the experimental animals. Complete recovery in overall health in calves occurred on the tenth day. There was no significant difference in the biochemical parameters of the blood of calves after treatment in the experimental group compared to healthy calves ($p > 0.05$), except for the urea level. The present results indicated that administering Hypericum led to improvements in the biochemical parameters of the experimental calves, which improved to the level of clinically healthy calves. The current findings demonstrated that Hypericum quickly entered the regulatory system of calves after administration and exhibited therapeutic effects. According to Fayzieva et al. (2024), in the calves of the experimental group treated with the probiotic MAXLAC/DW, blood glucose at the beginning of treatment averaged 2.64 ± 0.28 mmol/L and decreased significantly by the fifth day of treatment to 2.48 ± 0.18 mmol/L. Total protein increased significantly from 54.8 ± 1.70 to 67.4 ± 1.82 g/l by the fifth day of treatment. It was observed that the total bilirubin level in the blood of the experimental group, which was injected with Vetom 1.2 probiotic (Bacillus subtilis VKPM I-10641), averaged 4.2 ± 0.05 and 4.4 ± 0.06 μmol/l. The activities of AST and ALT enzymes decreased to 0.58 ± 0.06 (9.67 ± 1.00) and 0.42 ± 0.06 (7.00 ± 1.00) mmol/s/l (U/l), respectively, by the end of the experiments (Norboyev and Fayziyeva, 2024). Montayeva et al. (2024) reported that the created Kazakh complex phytomineral preparation for treating diarrhoea in newborn calves reduced the activity of ALT by 22.4% and AST by 28.7%. Additionally, the total protein level in the calves of the experimental group reached its maximum value, significantly surpassing the comparable metrics of the calves in the control group by 6.1%.

The prophylactic administration of Hypericum at a dose of 6.5 mL/kg during the first six feedings improved the overall health of the experimental calves, with no signs of toxicosis (Table 2). It should be noted that in the calves of the experimental group, which were given Hypericum at a dose of 6.5 mL/kg added to milk for two days, only one calf out of 15 fell ill within the first four days, representing a 6.7% incidence. The Hypericum prevented dyspepsia by 93%. Over a 30-day observation period, no diarrhea was observed in the calves of the experimental group. In contrast, all calves in the control group developed dyspepsia after calving, resulting in a 100% incidence (Shcherbakov et al., 2022).

In the control group, four calves died, representing 26.7% of the total. Explaining the mechanism of the preventive action of Hypericum, it is worth noting that the primary cause of dyspepsia in calves is metabolic disorders in cows during pregnancy, resulting from an inadequate diet and physical inactivity. Metabolic disorders in cows during pregnancy have adverse effects on the embryonic development of calves, resulting in calves born weaker, with reduced natural resistance and susceptibility to diseases (Mischenko et al., 2021).

Table 2. Preventive action of Hypericum in black and white breed bulls aged 8-10 days for five days

Group	Number of calves	Percentage of dyspepsia morbidity	Percentage of cattle deaths
Experimental	15	6.3	0
Control	15	100	26.7

CONCLUSION

The oral administration of Hypericum at a dosage of 6.5 mL/kg for five days effectively restored impaired protein and carbohydrate metabolism, as well as liver function, in calves suffering from dyspepsia, bringing these parameters to levels comparable to those of healthy calves. The administration of Hypericum at a dosage of 6.5 mL/kg demonstrated both therapeutic and prophylactic efficacy in managing calf dyspepsia. Further studies are required to elucidate the impact of Hypericum on the immune system of calves.

DECLARATIONS

Acknowledgments

The authors wish to express their sincere gratitude to the Acting Director of the Federal State Budgetary Institution, Federal Center for Toxicological, Radiation, and Biological Safety, in Kazan, Russia, for their support of the present study.

Funding

The authors declared no financial support from any organization or individual for this study.

Authors' contributions

Emil Rakhmatullin, Ilnur Kadikov, and Ruslan Rakhmatullov developed the study design and substantiated its methodology. Ekaterina Mayorova organized and conducted experimental studies, and Ekaterina Kurshakova and Danil Sagdeev took part in the selection of blood from calves. Andrey Korchemkin conducted the statistical analysis of the results. Emil Rakhmatullin, Ilnur Kadikov, and Ruslan Rakhmatullov analyzed the obtained data and provided a draft of the manuscript. Alsu Makaeva edited and reviewed the manuscript. All authors participated in discussing the results, reviewing the analyzed data, writing the article, and agreeing on the final edition of the manuscript for submission.

Competing interests

The authors declared no conflict of interest.

Ethical considerations

The study was originally conducted, and all the analyzed data were prepared based on the experiment results.

Availability of data and materials

All data of this study are available upon reasonable requests from the authors.

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2025, Scienceline Publication
World's Veterinary Journal
World Vet J, 15(3): 749-755.
ISSN 2322-4568

DOI: https://dx.doi.org/10.54203/scil.2025.wvj76
PII: S232245682500076-15

Effects of Ginger, Turmeric, Teak Leaf Extracts Combined with a Probiotic on Broiler Chicken Performance

Silvi Annas Tasya[1], Veronica Margareta Ani Nurgiartiningsih[2], Fajar Shodiq Permata[2], Muhammad Sasmito Djati[3], Yuli Frita Nuningtyas[1], Adelina Ari Hamiyanti[1], and Muhammad Halim Natsir[1]*

[1]*Department of Animal Nutrition, Faculty of Animal Science, University of Brawijaya, Malang, Indonesia*
[2]*Department of Veterinary Histology, Faculty of Veterinary Medicine, University of Brawijaya, Malang, Indonesia*
[3]*Department of Biology, Faculty of Mathematics and Natural Science, University of Brawijaya, Malang, Indonesia*

*Corresponding author's Email: emhanatsir@ub.ac.id

ORIGINAL ARTICLE
Received: July 04, 2025
Revised: August 07, 2025
Accepted: September 05, 2025
Published: September 30, 2025

ABSTRACT

In poultry production, the transition to antibiotic-free systems has resulted in an increased demand for effective natural growth promoters. The present study aimed to evaluate the effectiveness of a mash-form feed additive made from microwave-extracted ginger, turmeric, and teak leaf phytobiotics, combined with a multi-strain bacterium as a probiotic, administered at graded inclusion levels in broiler chickens. A 35-day feeding experiment was conducted with 300 broiler chickens, arranged in a completely randomized design with five dietary treatments and five replicates per treatment. In the first group, chickens received only the basal diet and served as the control (T0). The treatment groups received the basal feed top-dressed with the phytobiotic and probiotic additive at inclusion levels of 0.2% (T1), 0.4% (T2), 0.6% (T3), and 0.8% (T4). Measurements of performance, including body weight gain (BWG), feed conversion ratio (FCR), performance index, and income over feed cost (IOFC), were collected and analyzed. High-performance liquid chromatography confirmed the presence of key bioactive compounds, such as curcumin and 6-gingerol. The inclusion of 0.2% bacterium as a probiotic additive resulted in optimal BWG, FCR, and the highest IOFC, indicating that this level was biologically effective and economically efficient. In contrast, higher inclusion levels of bacteria as probiotics led to reduced performance, likely due to polyphenol-induced mineral binding and issues with feed palatability. The co-administration of probiotics may enhance the bioavailability of curcumin and promote a healthier gut environment. The present findings highlighted the potential of phytobiotic-probiotic combinations as alternatives to antibiotic growth promoters when used at optimal dose levels.

Keywords: Antibiotic-free, Ginger, Performance, Probiotic, Teak leaf, Turmeric

INTRODUCTION

The global poultry industry, particularly in Asia, has experienced consistent growth, driven by increasing consumer demand, urbanization, and a shift towards protein-rich diets. Broiler meat has become a key protein source in countries such as China, Indonesia, Bangladesh, and India, significantly contributing to food security in developing economies (Haque et al., 2020). However, AGPs have raised concerns over food safety, antimicrobial resistance, and sustainability, leading to the implementation of bans on AGPs, including legislative actions such as Law No. 18/2009 and Law No. 41/2014 in Indonesia.

Phytobiotics, plant-based compounds such as flavonoids and essential oils, have emerged as promising alternatives to AGPs due to their antimicrobial and immunomodulatory properties. Botanicals, such as ginger and turmeric, enhance gut health and carcass quality, aligning with consumer preferences for antibiotic-free meat (Griggs and Jacob, 2021). Advanced techniques, such as microwave-assisted extraction (MAE) and nano-emulsions, can enhance bioavailability, although practical limitations exist (Agustina and Setyaningsih, 2024).

Ginger, turmeric, and teak leaves offer synergistic bioactivities, including antioxidants, anti-inflammatory properties, and antibacterial effects, which enhance broiler health and performance (Hermanto et al., 2022). Combining these herbs with probiotics, such as *Lactobacillus* and *Bacillus*, may further promote the production of short-chain fatty acids and gut integrity (Zhang et al., 2025). Dietary components, such as hemicellulose, divalent minerals, and feeds high in viscosity and protein content, may negatively impact the bio-accessibility of polyphenols. In contrast, digestible carbohydrates, dietary fats, particularly in the case of hydrophobic polyphenols such as curcumin, and the presence of additional antioxidants can help improve the bioavailability of these compounds (Bohn, 2014). The physiological

To cite this paper: Tasya SA, Nurgiartiningsih VMA, Permata FS, Djati MS, Nuningtyas YF, Hamiyanti AA, and Natsir MH (2025). Effects of Ginger, Turmeric, Teak Leaf Extracts Combined with a Probiotic on Broiler Chicken Performance. *World Vet. J.*, 15(3): 749-755. DOI: https://dx.doi.org/10.54203/scil.2025.wvj76

response to polyphenol intake often follows a bell-shaped dose-response curve, highlighting the importance of well-powered trials with sufficient replication (Alhefny et al., 2022).

The present study aimed to evaluate the efficacy of a mash-form feed additive made from microwave-extracted ginger, turmeric, and teak leaf phytobiotics, combined with a multi-strain bacterium as a probiotic, on broiler chickens, providing insights into the minimal effective dose that optimizes performance and economic viability, supporting sustainable antibiotic-free poultry production.

MATERIALS AND METHODS

Ethical approval

All experimental procedures were conducted in accordance with the institutional animal-care guidelines of Brawijaya University No. 91/EC/KEPK/04/2025. Chickens were handled by trained personnel, and humane endpoints were strictly observed. Housing, feeding, and health-management practices complied with national welfare regulations for poultry research.

Maceration

Dried ginger rhizome, turmeric rhizome, and teak leaves were milled to pass a 60-mesh screen. Each botanical flour was macerated separately in ethanol 70% at a 1:5 (w/v) ratio for 24 hours in sealed glass tubes, following the protocol of Kambuno et al. (2024). The use of ethanol 70% was selected due to its proven efficacy in extracting a wide range of polyphenols and other bioactive compounds, as it offered an optimal balance between polarity and solubility. To prevent oxidative degradation, the macerates were protected from light using aluminum foil.

Microwave-assisted extraction

The combined filtrates, which were the pooled maceration extracts of ginger rhizome, turmeric rhizome, and teak leaves, with a total volume of 200 mL, were concentrated using MAE. Maceration liquor was transferred to a Kjeldahl flask to half capacity and irradiated using a pulsed microwave at a 450 W power output mode under reflux condensation at 50-60°C for 15 minutes. The flask was then cooled to ambient temperature, filtered, and re-irradiated for a further 10 minutes. The filtrates obtained from the first and second irradiation steps were combined and pooled to produce an extract with a final solvent-to-solid ratio of 5:1, as described by Pinelo et al. (2005).

Ultrasonic homogenization

To enhance dispersion and reduce particle size, the crude extract was subjected to probe sonication in a 100 mL borosilicate glass vessel using an ultrasonic homogenizer sonicator (UCD-250, 250 W, 220 V, 50 Hz, BIOBASE, China) equipped with a 13 mm titanium alloy probe operating at 20 kHz. Sonication was conducted for a total of 30 minutes at 50-60°C, with 10-minute sonication cycles followed by 5-minute passive cooling intervals at room temperature to prevent overheating (Pinelo et al., 2005). A water bath was used to stabilize the temperature during sonication, and real-time monitoring was performed using a digital thermocouple inserted into the vessel. This ultrasonic homogenization protocol, adapted from Rad et al. (2023), yielded a stable nano-suspension suitable for dry-mixing with carriers. Particle size analysis performed with scanning electron microscopy.

Mash formulation

One hundred mL volumes of ginger, turmeric, and teak extracts were blended at 2200 rpm until visually homogeneous; the mixture was then combined in a 1:1 (w/w) ratio with sun-dried cassava pulp as a carrier. After drying the cassava pulp, the final product exhibited a moisture content of 10.4% (wet basis), determined by oven-drying at 105°C for four hours. The slurry was spread 1-2 cm thick on aluminum trays and oven-dried at 60°C for 24 hours. Subsequently, the sheet was ground into a powder to produce a phytobiotic product suitable for inclusion in poultry feed. The final product was stored in opaque, airtight containers until it was utilized in feed formulation. A commercial multistrain probiotic (Lactoped®, containing viable Lactobacillus spp. and Bacillus spp.) was selected as the microbial component. Preliminary enumeration confirmed a viable count of 1×10^8 CFU/g at the time of mixing, with more than an 85% survival rate in the feed throughout the trial period.

Experimental design and housing

The *in vivo* trial was carried out for 35 consecutive days in Kambingan village, Tumpang district, Malang, East Java, Indonesia. A total of 300 Lohmann-strain day-old chickens with an initial body weight of 42.62 ± 3.52 g (coefficient of variation 8.25%) were obtained from a single commercial hatchery (PT Japfa Comfeed, production code MB 202) to

reduce genetic variation. The chickens were brooded for seven days in floor pens with constant lighting, then moved to raised slatted cages measuring 1.0 m × 0.8 m × 0.5 m, equipped with nipple drinkers and tube feeders. The stocking density was maintained at twelve chickens per cage, providing 0.067 m² per chicken. The ambient temperature was maintained at 34 ± 1°C during the brooding phase and gradually reduced to 29 ± 1°C by day 14, thereafter following the daily room temperature. Feed and water were supplied *ad libitum* throughout the starter (0-14 days) and the finisher (15-35 days) phases.

Dietary treatments and experimental design

A completely randomised design was adopted with five dietary treatments and five replicates per treatment. Each replicate corresponded to one cage of twelve chickens, resulting in a total of 25 experimental units. All chickens received a nutritionally adequate commercial basal diet formulated for fast-growing broilers. The basal diet was divided into three phases, including pre-starter (days 0-7), starter (days 8-21), and finisher (days 22-35), with nutrient specifications detailed in Table 1. In the first group, chickens received only the basal diet and served as the control (T0). The treatment groups received the basal feed top-dressed with the phytobiotic and probiotic additive at inclusion levels of 0.2% (T1), 0.4% (T2), 0.6% (T3), and 0.8% (T4). The additive was incorporated into the basal feed manually using the scoop-and-pour method in clean plastic tubs. Each batch (5 kg) was mixed for 10 minutes using circular and folding motions to ensure uniform distribution. Homogeneity was assured by visual consistency of color and texture across the batch, and mixing was repeated in small quantities to minimize variation.

Table 1. Nutrient content of broiler chickens in the present study

Nutrient content	Pre starter (1-7 days)	Starter (8-21 days)	Finisher (22-35 days)
Water content (%)	12.8	11.5	11.3
Crude protein (%)	22.2	21.6	19.4
Ether extract (%)	5.2	5.5	5.6
Crude fiber (%)	4.7	5.2	5.4
Ash (%)	7.1	7.3	7.2
Calcium (%)	1.1	1.1	1.1
Phosphor (%)	0.5	0.5	0.4
ME (Kcal/kg)	2.900	3.000	3.100
Aflatoxin (µg/Kg)	40.8	50.2	50.1

Note: Proximate analysis data from the Animal Nutrition Laboratory at the Brawijaya University, Malang, Indonesia. ME: Metabolizable energy

Performance measurements

Feed intake was recorded daily as the difference between feed offered and orts. Broiler chickens were weighed collectively at placement and every seven days thereafter; body weight gain (BWG) for each replicate was calculated by subtracting the initial weight from the subsequent weight. Feed conversion ratio (FCR) was expressed as cumulative feed intake divided by cumulative BWG. Mortality was monitored twice daily, and the mass of deceased chickens was used to adjust performance data. The European production efficiency factor, designated locally as performance index (PI), is calculated in accordance with Gonzales et al. (2023).

PI = (final live weight [g] × survival [%]) ÷ (age × FCR) × 100

Economic performance was evaluated solely based on feed-related profitability, calculated as income over feed cost (IOFC). This metric was calculated by subtracting the total feed cost per chicken from the revenue generated by its live body weight at the current farm gate price. The selling price of live chickens was set at 17,500 IDR per kilogram. Feed costs were determined based on the phase-specific prices. Pre-starter and starter feeds were priced at 9,340 IDR per kilogram, while finisher feed was priced at 9,000 IDR per kilogram. The data was calculated using Microsoft Excel.

Statistical analysis

All variables were tabulated in SPSS Statistics and subjected to one-way analysis of variance (ANOVA) using the general linear model procedure. The statistical model, $Y_{ij} = \mu + T_i + \varepsilon_{ij}$, included the fixed effect of dietary treatment (T_i) and the residual error term (ε_{ij}). The significant results were separated with Duncan's multiple-range test to characterize dose-response patterns ($p < 0.05$). Before analysis, data were checked for homogeneity of variance and normality; arcsine transformation was applied to survival percentages where necessary. Statistical software assumptions were met in all cases, and transformed data were returned to the original scale for presentation.

RESULT AND DISCUSSION

The present results of the experimental treatments included the quantified concentrations of key active phytochemicals, measured broiler growth performance indicators, economic efficiency based on income over feed cost, and an interpretation of biological trends with additive dosage. The present data highlighted measurable effects of the dietary treatments on broiler productivity and profitability, with particular emphasis on identifying optimal inclusion levels for practical application.

Feed intake

The current findings indicated that supplementing broiler diets with a phytobiotic-probiotic mix consisting of ginger, turmeric, teak leaf extracts, and probiotics had no statistically significant effects on feed intake ($p > 0.05$). The average cumulative feed intake per chicken ranged from 3,334.98 g to 3,362.72 g across the treatment groups. The highest intake was recorded in Group T3 (3362.72 ± 64.58 g/head), while the control group consumed slightly less (3334.98 ± 0.41 g/head). These narrow variations indicated that the observed BWG improvements were not due to increased intake, but rather to improved nutrient utilization. The present result was consistent with the findings of Ardiansyah et al. (2024), who observed that incorporating fermented feed additives composed of ginger, turmeric, and teak leaf extracts in nanoparticle form did not significantly influence feed intake in broilers. The absence of significant differences in feed intake among treatment groups in the present study aligned with the findings of Ardiansyah et al. (2024), who reported that phytobiotic feed additives composed of ginger, turmeric, and teak leaf nanoparticles did not adversely affect broiler consumption traits. However, direct comparison was limited as feed intake was not quantitatively assessed in that study.

Similarly, Sunmola et al. (2024) indicated that the effects of using 0.3% of ginger and turmeric did not differ significantly in terms of feed consumption, indicating that the addition of these herbs up to a level of 0.30% did not negatively impact palatability or feed acceptance. The present data demonstrated that variables, including the form of administration (drinking water or feed), levels, and fermentation methods, significantly influence the efficacy of feed additives on feed consumption. In contrast with the results of the current study, Al-Mahdawi and Al-Hassani (2023) reported that supplementing broiler chicken feed with 0.5% or 1% of ginger powder significantly reduces feed consumption and weight gain compared to the control group at 35 days of age. This finding suggested that the type of probiotic and the method of administering feed additives play an important role in determining the consumption response of broiler chickens.

Body weight gain

As shown in Table 2, chickens in Group T1 achieved the highest mean BWG (2163.43 ± 31.18 g/head), followed by Group T2 (2133.70 ± 100.55 g/head). In groups T3 and T4, BWG declined to 2076.19 ± 108.79 g/head and 2030.58 ± 112.54 g/head, respectively. Although the differences were not statistically significant ($p > 0.05$), the numerical trend exhibited optimal performance within the inclusion range of 0.2% (T1) to 0.4% (T2). The current findings align with prior evidence that curcumin has dose-dependent effects, with moderate levels promoting microbial balance and nutrient absorption, while high doses may lead to metabolic stress or decrease palatability in laying hens (Nawab et al., 2019). Nuningtyas et al. (2024) reported that the addition of feed additives in the form of liquid nano extracts of ginger, turmeric, teak leaves, and probiotics at levels of 0.4 - 0.8% can increase the percentage of carcass and physical quality of broiler chicken meat, although it does not directly increase BWG significantly. Supplementation of probiotic fermentation with a mixture of turmeric in broiler drinking water can significantly increase ration consumption, BWG, and PI at certain doses. Aderemi and Alabi (2023) highlighted that the inclusion of probiotics did not increase BWG in broiler chickens, emphasizing that the effectiveness of probiotics can be species-dependent and potentially affected by other dietary factor components. The combination of probiotics and herbs in commercial feed additives such as Promix^{TM} had synergistic effects and beneficial roles in improving the growth performance of broiler chickens, including increasing BWG, body weight, and decreasing FCR value; therefore, it can replace AGP as a feed additive in broiler chickens (Nahak et al., 2021). The present findings demonstrated that supplementation with a combination of curcumin, gingerol, and teak leaf extract with probiotics did not directly increase BWG ($p > 0.05$), which was in line with the findings of Martinez et al. (2022), who performed a commercial meta-analysis on phytogenic feed additives and reported improvements in feed conversion efficiency but observed no significant effect on the final body weight of broiler chickens.

Feed conversion ratio

The present findings indicated that supplementing broiler diets with a combination of ginger, turmeric, teak leaf extracts, and probiotics had no statistically significant impact on FCR ($p > 0.05$). The trend in FCR presented the pattern

observed in BWG. The highest feed efficiency, as indicated by the lowest FCR, was observed in Group T1 (1.55), followed by Group T2 (1.57), whereas the control group recorded an FCR of 1.61. Feed conversion ratio values increased at higher inclusion levels, reaching 1.62 and 1.65 for groups T3 and T4, respectively. The current results were consistent with meta-analytical evidence indicating that ginger-based additives can improve feed conversion efficiency, particularly when administered at optimal dosages (Khalil et al., 2022).

The mechanisms behind these improvements are probably multifaceted, involving proper digestion, reduced pathogen levels, and enhanced gut structure effects previously linked to the antimicrobial and pro-digestive properties of gingerol and curcumin (Ferdous et al., 2022). The present results aligned with a study conducted by Windisch et al. (2008), which evaluated the use of red ginger flour as a phytobiotic supplement in broiler chicken feed. The findings revealed that supplementing broiler diets up to 0.4% of red ginger flour did not significantly alter feed conversion ratios in broiler chickens compared to the control group ($p > 0.05$). However, improvements were observed in BWG alongside a reduction in feed intake. These outcomes suggested that herbal additives, such as red ginger, may enhance broiler growth performance without significantly affecting feed conversion efficiency.

Table 2. Growth performance of Lohmann broiler chickens receiving diets supplemented with different levels of phytobiotic and probiotic additives over 35 days

Variables \ Treatment	T0	T1	T2	T3	T4	P-value
Feed intake (g/chicken)	3334.98 ± 0.41[a]	3345.46 ± 26.40[a]	3350.46 ± 26.41[a]	3362.72 ± 64.58[a]	3335.27 ± 0.22[a]	0.67
BWG (g/chicken)	2077.42 ± 124.46[a]	2163.43 ± 31.18[a]	2133.70 ± 100.55[a]	2076.19 ± 108.79[a]	2030.58 ± 112.54[a]	0.84
FCR	1.61 ± 0.1[a]	1.55 ± 0.02[a]	1.57 ± 0.07[a]	1.62 ± 0.07[a]	1.65 ± 0.09[a]	0.26
PI	353.21 ± 49.41[a]	371.66 ± 68.02[a]	369.05 ± 27.64[a]	325.55 ±75.40[a]	354.09 ± 39.03[a]	0.80
IOFC (Rp/chicken)	13884.31 ± 2177.8[a]	15397.13 ± 544.2[a]	14922.89 ± 1758.9[a]	13723.16 ± 1703.5[a]	13053.81 ± 1968.7[a]	0.24

Note: Data are presented as mean ± Standard deviation. T0: Basal diet, T1: Phytobiotic plus probiotic 0.2%, T2: Phytobiotic plus probiotic 0.4%, T3: Phytobiotic plus probiotic 0.6%, T4: Phytobiotic plus probiotic 0.8%. BWG: Body weight gain, FCR: Feed conversion ratio, PI: Performance index, IOFC: Income over feed cost. $P > 0.05$ indicates that no significant differences were observed among the treatment groups.

Performance index

The PI is a comprehensive indicator that integrates growth rate, feed efficiency, and livability to evaluate overall broiler production efficiency. Although numerical differences were observed among treatments during the present study, statistical analysis indicated that the differences in PI among groups were not significant ($p > 0.05$). The highest numerical PI value was recorded in Group T1 (371.66 ± 68.02), followed by Group T2 (369.05 ± 27.64), Group T4 (354.09 ± 39.03), and the control group (353.21 ± 49.41), with the lowest in Group T3 (325.55 ± 75.40). Although the results were not statistically significant, Group T1 exhibited the most favorable overall trend. The enhanced PI in T2 corresponds with the improvement of BWG and the relatively efficient FCR observed at this stage level. The present findings were supported by the study of Urban et al. (2023), which indicated that phytogenic additives, particularly those rich in curcumin and gingerols, can improve the growth performance of broiler chickens and feed conversion by modulating gut microbiota and enhancing nutrient absorption.

The synergistic use of probiotics may contribute to improved intestinal health, immune modulation, and a reduced pathogenic load, all of which are crucial for maximizing performance outcomes (Gadde et al., 2017). The current result aligns with the findings of Amad et al. (2011), who suggested that excessive inclusion of plant-derived bioactives can impair nutrient utilization and palatability. The enhanced PI in broiler chickens at moderate levels, compared to higher levels, supported the present results, which indicated a negative response at high doses.

Income over feed cost

Income over feed cost is a critical economic indicator used to assess poultry production profitability, calculated by subtracting total feed expenses from the income generated per chicken (Pradana and Zuprizal, 2025). The highest IOFC was recorded in Group T1 (15,397.13 ± 544.2 IDR), followed by Group T2 (14,922.89 ± 1,758.9 IDR) and the control group (13,884.31 ± 2,177.8 IDR). Treatments T3 and T4 yielded lower IOFC values, indicating reduced economic returns despite similar feed intake. However, statistical analysis demonstrated no significant differences in IOFC among treatment groups ($p > 0.05$). The numerically superior IOFC in Group T1 suggested that the 0.2% inclusion level of the phytobiotic-probiotic mix may provide an economically efficient balance between feed cost and weight gain. This trend aligns with the findings of Aderemi and Alabi (2023), who reported that moderate supplementation with phytogenic additives can improve growth and feed efficiency in broiler chickens without markedly increasing production costs,

thereby enhancing net income. Although Group T2 resulted in the highest PI, its slightly lower IOFC compared to Group T1 suggested that biological efficiency does not always correlate directly with economic efficiency, particularly as feed additive costs rise with inclusion level. Baurhoo et al. (2007) emphasized the importance of evaluating performance and cost parameters to make informed decisions about feed formulation, particularly in commercial production systems where profit margins are narrow. Interestingly, during the present study, high inclusion levels (0.6% and 0.8%) were associated with declining IOFC values, likely due to diminishing returns in BWG and exacerbating FCR. This trend supported the dose-response relationship described by Amad et al. (2011), where excessive inclusion of phytobiotic compounds may lead to anti-nutritional effects and reduced economic efficiency in broiler chickens.

CONCLUSION

The present study demonstrated that supplementing broiler diets with a feed additive containing ginger, turmeric, and teak leaf extracts combined with a multi-strain *Bacillus*/*Lactobacillus* probiotic offered beneficial effects at an optimal inclusion level of 0.2%. This dose consistently improved body weight gain, feed conversion ratio, performance index, and economic returns. The observed bell-shaped dose-response trend emphasized the importance of dose optimization, as higher inclusion levels (0.6%-0.8%) indicated decreasing returns, possibly due to excessive polyphenolic compounds having anti-nutritional effects. The present findings supported the potential of natural feed additives as effective alternatives to antibiotic growth promoters, particularly in tropical production environments. Future studies should focus on optimizing dosing strategies, evaluating the long-term health effects, and assessing the economic feasibility, including additional costs, to enhance the practical application of phytobiotic-probiotic combinations in broiler production.

DECLARATIONS

Funding

The present study was funded by the Directorate General of Higher Education (DIKTI), Ministry of Education, Culture, Research, and Technology of the Republic of Indonesia, under the grant number 00309.80/UN10.A0501/B/PT.01.03.2/2024.

Acknowledgments

The authors sincerely appreciate the Faculty of Animal Science at Brawijaya University, Malang, Indonesia, for providing the essential facilities and assistance to conduct this study.

Authors' contributions

Silvi Annas Tasya conducted the laboratory and field research. Muhammad Halim Natsir served as the principal investigator and supervised the overall study framework. Adelina Ari Hamiyanti was responsible for the laboratory procedures and ensured methodological accuracy. Veronica Margareta Ani Nurgiartiningsih was responsible for formulating and extracting phytobiotics. Fajar Shodiq Permata contributed to data analysis and statistical interpretation. Muhammad Sasmito Djati supported scientific validation and data synthesis. Yuli Frita Nuningtyas coordinated field activities and contributed to manuscript review. All authors have read and approved the final edition of the manuscript.

Competing interests

The authors confirmed that there are no conflicts of interest associated with this publication.

Ethical considerations

All authors were actively involved in the development of this original manuscript. The authors reviewed the final version of the completed paper, assessed any necessary revisions and updates, and verified the article's similarity index.

Availability of data and materials

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

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2025, Scienceline Publication
World's Veterinary Journal
World Vet J, 15(3): 756-763.
ISSN 2322-4568

DOI: https://dx.doi.org/10.54203/scil.2025.wvj77
PII: S232245682500077-15

Effects of Bitter Melon Fruit Fraction with Fourier Transform Infra-Red (FTIR) Spectroscopy on Histopathological Changes of Koi Fish (*Cyprinus carpio*) Liver Infected with *Aeromonas salmonicida*

Sri Andayani[1]*, Mohamad Fadjar[1], Yenni Risjani[1], Muhamad Sulaiman Dadiono[2], Vina Nur Nadiro[3], and Nadiah Nurandi[4]

[1]*Department of Aquaculture, Faculty of Fisheries and Marine Science, University of Brawijaya, Malang, East Java, Indonesia*
[2]*Aquaculture Study Program, Faculty of Fisheries and Marine Sciences, Jenderal Soedirman University, Purwokerto, Indonesia*
[3]*PSDKU Aquaculture University of Brawijaya, Kediri, East Java, Indonesia*
[4]*Master student of the Department of Aquaculture, Faculty of Fisheries and Marine Science, University of Brawijaya, Malang, East Java, Indonesia*

*Corresponding author's Email: yanik@ub.ac.id

ORIGINAL ARTICLE
Received: June 14, 2025
Revised: July 20, 2025
Accepted: August 25, 2025
Published: September 30, 2025

ABSTRACT

Bitter melon is widely recognized for its medicinal properties, including its potential health benefits in various species. The present study investigated the impact of the bitter melon fraction on liver histological changes of koi fish infected with *Aeromonas salmonicida (A. salmonicida)*. Maceration of bitter melon fruit was carried out for 4 days, then filtered and evaporated to obtain a thick extract. Phytochemical testing included column chromatography for the separation of active compounds and UV-Vis spectrophotometry for the analysis of the absorbance spectra of the fractions. The *A. salmonicida* bacterial infection was performed five days prior to the fish being soaked in a bitter melon fraction, at a bacterial density of 10^7 cells/ml for 48 hours. A completely randomized design was employed, featuring five treatments, including no bitter melon fraction with no infection (K -), other groups infected with *A. salmonicida* and supplemented with 75 ppm of bitter melon (A), 95 ppm of bitter melon (B), 115 ppm of bitter melon (C), and 5 ppm chloramphenicol (antibiotic, K +). Each treatment group consisted of 15 fish (average length and weight of Koi fish were 8 cm and 12 grams) with three replications, which were immersed in their respective solutions for 48 hours. The current study adopted the maceration method with 98% ethanol for the extraction of bitter melon fruit. To determine liver histological damage in Koi fish, the authors employed the scoring method. The findings indicated slight liver damage in histology parameters with an average score of 1.2-1.4 for treatments A and B, respectively; however, treatment C and the positive control exhibited moderate damage with an average necrosis score of 2. According to the obtained data, the bitter melon fruit fraction at a concentration of 95 ppm had the highest preventive effects on the liver of the Koi fish infected with *A. salmonicida*.

Keywords: Bitter melon fruit, Fraction, Koi fish, Liver

INTRODUCTION

The intensive carp production system provides numerous business opportunities, such as seeding, nursery operations, and the expansion of fish farming. In East Java, the aquaculture production value peaked in 2018 (KKP, 2021). However, intensive carp farming increases the risk of disease outbreaks (Kusrini, 2015). One of the primary pathogens causing disease in carp is *Aeromonas salmonicida (A. salmonicida*). The ability of this bacterium to infect fish is closely linked to its capacity to generate toxins. Semwal et al. (2023) classified *A. salmonicida* as a highly virulent pathogenic bacterium. Buller (2004) indicated that the virulence of *A. salmonicida* is influenced by its capacity to generate particular enzymes and toxins that aid in the invasion and infection of fish.

In the management of diseases, especially bacterial infections, a range of antibiotics has been employed, such as chloramphenicol, oxytetracycline, and erythromycin. A significant number of these antibiotics contribute to the development of resistance against new strains in the treatment of the disease (Sumampouw, 2018). Addressing the disease with natural ingredients is essential, emphasizing eco-friendly alternatives such as bitter melon fruit, instead of depending exclusively on saponins and rotenones. Bitter melon is rich in several secondary metabolites that exhibit antimicrobial properties, including alkaloids, flavonoids, tannins, terpenoids, and saponins (Andayani et al., 2023). Alkaloids and flavonoids in bitter melons are known to damage microbial cell membranes, while tannins and saponins inhibit the growth of microorganisms by disrupting their metabolic processes and cell walls. In addition, the phenolic compounds in bitter melon act as antioxidant agents that protect the body from bacterial and fungal infections. The antimicrobial properties of bitter melon allow it to serve as a natural component and a substitute treatment for fish (Hasanah, 2018). According to Ghosh et al. (2018), some alkaloids can increase immunity and vitality in fish. A study by Andayani et al. (2023) demonstrated the capability of bitter melon leukocytes to improve differentials, positioning it as a potential immunostimulant that can enhance the immune response in fish. Additionally, bitter melon fruit is rich in

To cite this paper: Andayani S, Fadjar M, Risjani Y, Dadiono MS, Nadiro VN, and Nurandi N (2025). Effects of Bitter Melon Fruit Fraction with Fourier Transform Infra-Red (FTIR) Spectroscopy on Histopathological Changes of Koi Fish (*Cyprinus carpio*) Liver Infected with *Aeromonas salmonicida. World Vet. J.*, 15(3): 756-763. DOI: https://dx.doi.org/10.54203/scil.2025.wvj77

polyphenols, which are recognized for their antioxidant and anti-inflammatory effects. These substances can mitigate oxidative stress and bolster immune function by stimulating leukocyte activity. An increase in leukocyte differentials, including lymphocytes and neutrophils, would enhance the capacity to combat infections and facilitate disease recovery in fish. Consequently, bitter melon emerges as a promising natural immunostimulant for the enhancement of fish health and resilience (Andayani et al., 2023).

While bitter melon is known for its antimicrobial and anti-inflammatory properties, there is a lack of data about its impact on infected fish with *A. salmonicida*, specifically at the histological level. The present study investigated and contributed new insights into the potential of bitter melon as a natural treatment for bacterial infections in aquaculture, particularly in promoting liver health and mitigating damage caused by bacterial infection. Consequently, valuable information was obtained regarding the use of medicinal plants as alternatives for treating bacterial infections in fish, with a focus on their effects on vital organs such as the liver.

MATERIALS AND METHODS

Ethical approval

All experimental procedures involving animals were conducted in accordance with the ethical standards of the University of Brawijaya, Malang, East Java, Indonesia, and followed national and international guidelines for the care and use of laboratory animals. Every effort was made to minimize the number of animals used and to reduce their suffering during the experimental procedures.

Animals

In the present study, Koi fish were used as a sample, a domesticated variety of the common carp (*Cyprinus carpio*) sourced from a single broodstock at the Blitar Cultivation Center in Blitar City, Indonesia. To maintain the well-being of the fish, it is essential to ensure appropriate water quality, prevent overcrowding, observe for indications of illness, and conduct routine physical assessments. These assessments should include behavioral observation as well as thorough inspections of the skin, fins, gills, and eyes. The housing density of 15 fish per 15 liters, with an average initial length of about 8 cm and an average initial weight of around 12 grams, effectively mitigated stress and inhibited the transmission of infections. Disease prevention strategies, including regular water changes, proper filtration, and maintaining optimal water conditions, such as temperature, pH, and oxygen levels, were regularly controlled. The fish were acclimated over a two-week period, during which 10-20% of the water was replaced weekly and were fed with commercial feed three times daily, offered *ad libitum*.

Bitter melon fruit fractionation

Bitter melon fruit was fractioned in the Brawijaya University laboratory, Indonesia, using the maceration method, where the peeled fruit was blended into a mash and mixed with methanol PA in a 1:1 ratio. This fractionation was repeated three times with 500 ml of fruit and 500 ml of methanol each time, resulting in different fraction weights for yield calculation (Valencia, 2012).

Application of bitter melon fraction

The bitter melon fraction was tested through immersion (Laili, 2016). The immersion setup consisted of three different treatment groups, including treatment A (75 ppm), treatment B (95 ppm), and treatment C (115 ppm). Each container held 8 liters of water. Subsequently, 15 fish were immersed in each treatment container for 48 hours. The *A. salmonicida* bacteria were obtained from the laboratory of FPIK Universitas Brawijaya, Malang, Indonesia. Infection with *A. salmonicida* derived from pure culture was performed 5 days after immersion in the bitter melon fraction. A bacterial density of 10^7 cells/ml was used, then a pure culture was introduced into an appropriate medium, and incubated at a temperature range of 30-37°C for a duration of 24-48 hours. The density was confirmed with plate count or spectrophotometry.

Experimental design

After pre-research, the Lethal Dose 50 (LD50) and Lethal Concentration 50 (LC50) results were obtained. The assessment of toxicity to ascertain the dose or concentration of a substance that results in mortality for 50% of the exposed population of organisms is referred to as LD50 and LC50 (Rand and Petrocelli, 1985). The LD50 measures the dose of a substance given, while the LC50 measures the concentration of a substance in the air that is deadly to 50% of organisms. In the present study, the results of LD50 and LC50 were used to design the randomized experiments, and 3 replications were taken to ensure the accuracy and reliability of the results and to test the toxic effects of substances on

fish under controlled conditions. Fish were exposed to various concentrations of the substance, and their responses were observed under controlled conditions. This approach facilitated understanding the potential risks associated with chemical exposures to aquatic life. The experiment was carried out under a completely randomized design with three replications per treatment. It means that all experimental units were randomly assigned to different treatments without any blocking or grouping. Each treatment was applied to three separate units (replications) to ensure the reliability of the obtained results and to reduce the effect of random variation. The treatment (A) with 75 ppm bitter melon fraction with *A. salmonicida* infection, treatment (B) with 95 ppm bitter melon fraction with *A. salmonicida* infection, treatment (C) with 115 ppm bitter melon fraction with *A. salmonicida* infection, K(-); negative control, neither infected with *A. salmonicida* nor administered bitter melon fraction, serving as the baseline control group, and K(+); positive control group, infected with *A. salmonicida* and treated with 5 ppm chloramphenicol, served as a reference for antibiotic treatment efficacy (Laili, 2016).

Sampling and histopathological examination

The process of removing fish liver started with ethically euthanizing the fish. This was done by using a scalpel or another sharp instrument to quickly and effectively insert it into the brain area, just behind the eyes, ensuring instant death (Sneddon et al., 2014). After the fish was euthanized, it was placed on its back, and an incision was made in the abdominal cavity, extending from below the gills to the stomach using either a knife or scissors. The internal organs, including the liver, were then carefully separated using tweezers or small scissors. Three fish from each treatment A, B, C, K (+), and K (-) were sampled for liver tissue. Then, the samples were put into a microtube containing Davidson preservative solution. The histopathological preparations were made through fixation, dehydration, clearing, impregnation, embedding, staining, and mounting. Histological examination of the Koi fish liver was carried out after 5 days of administration of bitter melon extract. Histological observations were conducted to examine the liver tissue structure of Tilapia subjected to different stocking densities, followed by scoring the extent of tissue damage. According to Izzah (2019), each parameter was evaluated to determine the level of assessment of the network by measuring the extent of damage and calculating the percentage. The reading scoring histology of liver Koi fish starts from the left edge (according to the position of the tail of the preparation) towards the head, then goes down and shifts towards the tail again (zig-zag motion). Each field of view was observed for the level of tissue damage with the criteria of congestion and necrosis, then the percentage of scoring liver fish was assigned a score from 1 to 4 for microscopical evaluation. According to Maftuch et al. (2015), the scoring value for the percentage of damage histology of the liver of the Koi fish is presented in Table 1.

Table 1. Scoring value according to damage percentage in the liver of Koi fish

Scoring value	Damage percentage	Information
0	0	Not broken
1	1-25	Slight
2	26-50	Moderate
3	51-75	Lots
4	76-100	Huge

Remarks: "Huge" can be interpreted as more severe than "lots", because "huge" emphasizes the magnitude of the impact or scale, not just the amount.

Electron microscope examination method

Scanning electron microscopy (SEM) is a high-resolution imaging technique that utilizes a focused beam of high-energy electrons to scan the surface of a specimen, producing detailed topographic and compositional images. In this study, SEM was used to observe the liver tissue of Koi fish. The process began with sample collection and preparation, in which fish livers were aseptically isolated and fixed in a 2.5% glutaraldehyde solution in phosphate-buffered saline (PBS). After fixation, the samples were washed with PBS and subjected to a graded ethanol dehydration series ranging from 30% to 100%. The samples underwent critical point drying to prevent structural deformation. To enhance conductivity, samples were then coated with a thin layer of gold or platinum using a sputter coater. Imaging and analyzing were conducted under high vacuum conditions using the SEM. The monochromatic images obtained, as a result of the electron beams' non-visible wavelength, were examined to evaluate hepatocyte morphology, the integrity of the cell membrane, and indications of tissue stress or damage. Two primary types of images were produced by SEM, including surface topography and compositional maps. Both types were derived from electron-sample interactions that generate signals carrying information about surface structure and material properties. These signals were then detected and processed to form high-detail images for further examination (Masta, 2020).

Fourier transform infrared spectroscopy

Fourier transform infrared spectroscopy (FTIR) spectrophotometry is a spectroscopic technique that utilizes infrared light and incorporates a Fourier transform for spectrum analysis. The FTIR analysis in this case determined the functional groups that are characteristic of a compound and are found in a fraction (Smith, 2011). Testing of Koi fish liver samples using the FTIR method began with taking fish livers, which were then frozen and dried using a freeze-dryer to remove air content. The dried sample was crushed into a fine powder, directly analyzed, or mixed with Potassium Bromide and pressed into a transparent tablet. The sample was then placed on an FTIR spectrometer and scanned at a distance of 4000-400 cm^{-1} to detect functional groups based on the infrared spectrum produced. The spectrum peaks were described with standard references to identify the biochemical composition in Koi fish liver, such as proteins, lipids, and carbohydrates.

RESULTS AND DISCUSSION

Fourier transform infrared spectroscopy of bitter melon fruit

Figure 1 illustrates the wavelength absorption results obtained from the FTIR spectrophotometric analysis of the bitter melon fruit fraction (*Momordica charantia*), along with the absorption band data derived from the same analysis. As depicted in Figure 1, the FTIR spectrum displays several absorption peaks that signify the presence of functional groups within the sample. A prominent peak near 3000 cm^{-1} suggests the existence of -OH or -CH groups, whereas the absorption observed around 1700 cm^{-1} points to the presence of carbonyl (C=O) groups. The range of 1500-500 cm^{-1} serves as a distinctive molecular fingerprint region for each compound. The identification of functional groups within the bitter melon fruit fraction was conducted using FTIR. The spectral data acquired displayed a range of absorption bands that are associated with various chemical bonds and functional groups. A summary of this data is presented in Table 2. The results from the infrared spectrometry identification suggest that the compound under analysis possesses multiple functional groups, each of which contributes to an absorption band located in a specific region. The detection of a C-O band implies the potential presence of functional groups such as alcohol, ether, or carboxylic acid. Additionally, a peak absorption band is noted at a wavelength of 1180-1360 cm^{-1}, indicating the presence of a C-N functional group, specifically amine/amide. Furthermore, the presence of the C-H stretching vibration, which is characteristic of alkanes, is confirmed by the absorption peak observed in the range of 2850-2970 cm^{-1}. The wavelength of 1300-1370 cm^{-1} was the absorption of the NO_2 in the fungus group. The C=O group, which was an aldehyde/ketone / carboxylic acid/ester group, is located at the peak absorption wavelength of 1690-1760 cm^{-1} (Hercos et al., 2021). The C=O group at a wavelength of 1637 cm^{-1} indicated a phenol compound in the sample. The presence of hydroxyl groups (OH) and aromatic hydrocarbon groups in the sample indicated the presence of phenyl propanoic compounds (Sariningsih et al., 2015). A previous study by Coates (2000) announced that the presence of OH (hydroxyl), C=O, aromatic C=C, and aliphatic CH functional groups indicates the presence of flavonoid compounds. The detection of hydroxyl (OH) and aromatic hydrocarbon groups in the sample suggests the presence of phenylpropanoid compounds (Khowas, 2021). Accordingly, it can be found that the dominant content in the third fraction of bitter melon is phenol and its derivatives, namely phenylpropanoids.

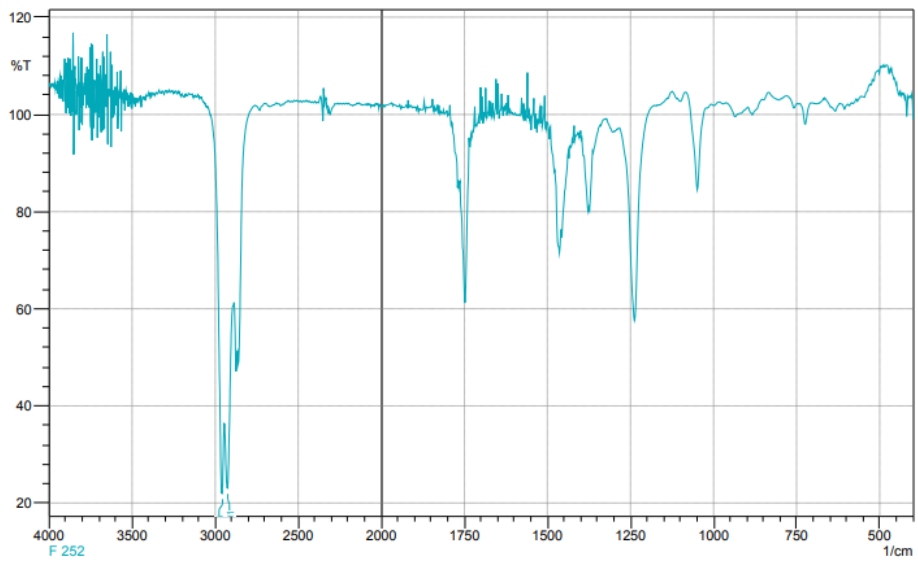


Figure 1. Fourier transform infrared spectroscopy spectral analysis results of bitter melon fruit

Table 2. The Fourier transform infrared spectroscopy spectral data for bitter melon fruit

No	Frequency of absorption band (cm^{-1})	Functional groups (bonds)	Group	Literature
1	1050 - 1300	C-O	Alcohols and Aliphatic Ethers	(Prata and da Costa, 2024)
2	1180 - 1360	C-N	Alkane	(Kristiningrum, 2016)
3	2850 - 2970	C-H	Alkane	(Kristiningrum, 2016)
4	1300 - 1370	NO_2	Aromatic Hydrocarbon	Saputra et al. (2016)
5	1690 - 1760	C=O	Carbonyl	Saputra et al. (2016)

Electron microscopy analysis of the liver

The SEM test provides detailed structural insights into the liver tissue of Koi fish. By analyzing the surface morphology at a microscopic level, SEM assists in identifying cellular integrity, tissue organization, and potential abnormalities (Goldstein et al., 2017). The SEM examination results are presented in Figure 2, highlighting key structural features of the Koi fish liver. The results of the SEM examination of the liver of the control group indicate a visible and complete part composition. These findings included the nucleus, sinusoids, and hepatocytes. The liver damage caused by the bacterial infection treatment showed organ necrosis and discoloration of the tissue, indicating congestion and bleeding.

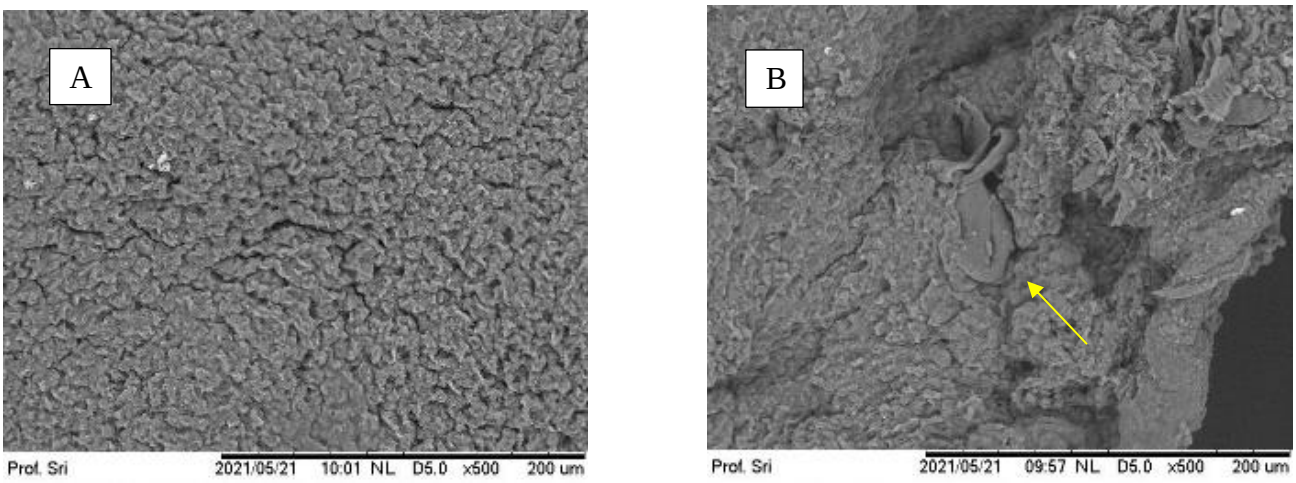


Figure 2. Scanning electron microscopy test of the Koi fish liver. Normal liver (**A**) and damaged liver (**B**, yellow arrow), Koi fish infected with *A. salmonicida* (**B**); 500x.

The results of the histopathological examination of the liver

The present study investigates the histopathological changes in the liver of Koi fish in response to experimental conditions. The results of the histological examination are demonstrated in Figure 3. The study results indicated that treating Koi fish with bitter melon fruit fraction significantly improves the negative impact on the liver of fish infected with *A. salmonicidae* bacteria (Figure 3). The fish appeared more active after being given the bitter melon fruit fraction. The Koi fish treated with bitter melon fruit exhibited minimal histological damage compared to those treated with antibiotics (without the bitter melon fruit fraction). The immune-stimulating effect of bitter melon fraction is linked to its natural components, such as polyphenols, alkaloids, and saponins, which enhance the body's resistance.

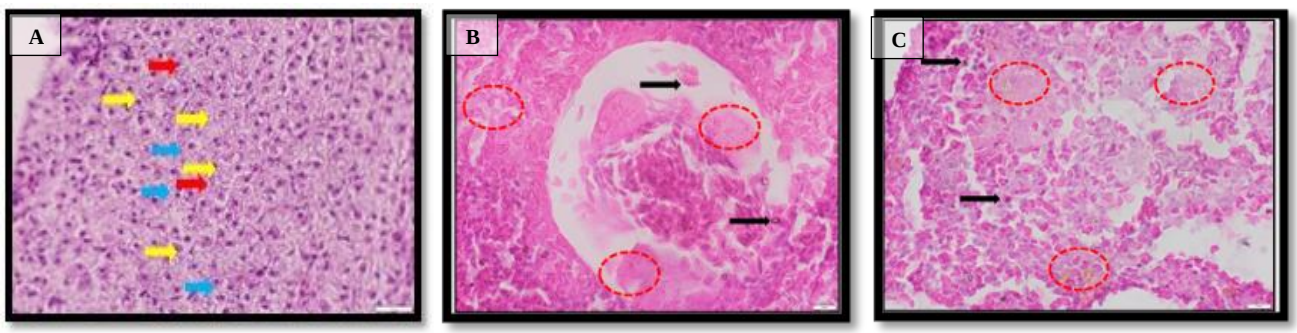


Figure 3. The histopathological examination of the Liver of Koi fish (400x). Normal liver (**A**), Livers with antibiotic treatment (**B**, without bitter melon fruit fraction treatment), Livers with bitter melon fruit fraction treatment (**C**). Yellow arrows: Core cells. Red arrows: Hepatocytes. Blue arrows: Sinusoids. Black arrows: Congestion. Dotted circles: Necrosis.

The immune-boosting effects of bitter melon fraction were considered by Nurandi et al. (2024), who indicated that Alkaloids contribute to detoxification and reduce toxicity in fish infected by bacteria. Saponins act as antibacterial and antiviral agents, contributing to improved immunity and overall health (Syihan, 2024). Moreover, Boshtam et al. (2016) and Maryani et al. (2018) highlighted that saponins boost the immune system by forming hydrogen bonds with cell membranes. This interaction leads to the production of complex compounds that compromise the integrity of bacterial cell membranes, resulting in bacterial cell death and enhanced immune function. Saponins enhance immune function by stimulating immune cell activity, increasing cytokine production, and acting as adjuvants in the immune response. In addition, saponins disrupt the integrity of bacterial cell membranes by forming hydrogen bonds, causing increased permeability and eventual lysis of bacterial cells, thus helping to effectively fight infection. Additionally, the polyphenols and phenolic derivatives found in the bitter melon fraction possess antioxidant properties. These polyphenols function as scavengers of free radicals and antioxidants, and they can also enhance the body's immune response by stimulating cytokine production, activating immune cells, and protecting cells from oxidative stress (Brandão et al., 2013; Sadowska et al., 2020).

In the control group, the liver lobule structure looks normal, characterized by the presence of clear cell nuclei, neatly arranged hepatocyte cells, and sinusoids that appear without any damage or pathological changes. The liver organ observed in fish treated with *A. salmonicidae* bacteria and bitter melon was found to have tissue damage in the form of congestion and necrosis. Congestion refers to the accumulation of excess blood within the small blood vessels or sinusoids of the liver, leading to impaired circulation and potential tissue damage. This condition occurs when blood flow is obstructed or slowed, causing dilation of the sinusoids and an increase in pressure within the hepatic vasculature. As a result, red blood cells may accumulate, leading to the formation of blood clots and reduced oxygen supply to liver tissues. Prolonged congestion can contribute to cellular stress, inflammation, and in severe cases, liver dysfunction (Sartijo et al., 2014). Additionally, necrosis is a condition characterized by the death of cells or tissues, which occurs after the loss of their normal function. It typically results from severe damage, often due to bacterial invasion, which irreparably harms the structure and function of the affected cells (Nurandi et al., 2024).

In the current study, necrosis was caused by infection with the bacteria *A. salmonicida*, which attacked the liver of Koi fish. Necrosis in liver cells is caused by the cytolysis or phagocytosis activity of lymphocytes or histocytes, which causes shrinkage/reduction in the size of the nucleus as a whole (sporadic, Bernet et al., 1999). Necrosis is often seen in cases of severe infections, where the liver tissue changes and looks damaged. This condition causes a decline in overall physiological function and death if it is not treated immediately. The results of scoring histological observations of the liver after treatment with bitter melon fruit fraction are presented in Tables 3 and 4.

Table 3. The score of histological observations of the liver after treatment with bitter melon fruit fraction in Koi fish for 48 hours (congestion)

Treatment	Congestion	Description
A	2	Moderate
B	1.6	Slightly damaged
C	1.8	Slightly damaged
K (+)	2	Moderate
K (-)	0.6	Not broken

Table 4. The score of histological observations of the liver after treatment with bitter melon fruit fraction in Koi fish for 48 hours (necrosis)

Treatment	Congestion	Description
A	1.4	Slightly damaged
B	1.2	Slightly damaged
C	1.8	Slightly damaged
K (+)	2	Moderate
K (-)	0.2	Not broken

The results of the histological scoring test of the liver of the Koi fish showed minor damage in group B. It is indicated that the organ remained largely intact with minimal structural alterations. The limited damage is attributed to the presence of polyphenols in bitter melon, which exhibit strong antibacterial and antioxidant properties. Polyphenols reduced oxidative stress by neutralizing free radicals, thereby preventing lipid peroxidation and cellular damage in liver tissues. Furthermore, these bioactive compounds played a crucial role in modulating the immune response, enhancing the fish's ability to combat bacterial infections, and promoting overall liver health (Andayani et al., 2023). By improving cellular defense mechanisms, polyphenols contribute to maintaining liver integrity and function, ensuring better resilience against environmental stressors and potential pathogens. Additionally, polyphenols helped modulate the immune response, enhancing the fish's ability to combat bacterial infections and maintain overall liver health.

Furthermore, these bioactive compounds played a crucial role in modulating the immune response, enhancing the fish's ability to combat bacterial infections and maintain overall liver health. By improving cellular defense mechanisms, polyphenols contributed to preserving liver integrity and function, promoting resilience against environmental stressors and potential pathogens (Andayani et al., 2023).

CONCLUSION

The bitter melon fruit fraction effectively reduced pathogenic bacterial infestation and improved liver tissue integrity in Koi fish. The optimal result was achieved at a dose of 95 ppm (Treatment B), which indicates minimal liver congestion, scoring 1.6. The present findings suggest the potential of bitter melon fruit as a natural antibacterial and hepatoprotective agent for sustainable aquaculture. Further studies in other fish breeds are needed to indicate the impact of bitter melon fruit fraction on other internal organs.

DECLARATIONS

Funding

This study was funded by Rector's Decree under the grant number No. 6531 of 2024, the Internal Grant of Brawijaya University, Indonesia.

Availability of data and materials

All data produced in this study are relevant and have been incorporated into the published article. Additional details or inquiries will be available through reasonable requests from the corresponding author.

Ethical considerations

All authors have thoroughly examined the manuscript for any ethical concerns, including plagiarism, research misconduct, data fabrication or falsification, and redundant publication.

Acknowledgments

The authors would like to warmly thank the project grant funders, professors, and laboratory staff, namely Mrs. Titin, and postgraduate students Ramanda and Sri Astuti.

Authors' contributions

Sri Andayani was responsible for data collection and analysis, as well as writing the original manuscript. Mohamad Fadjar, Yenni Risjani, Muhamad Sulaiman Dadiono, Vina Nur Nadiro, and Nadiah Nurandi contributed to data analysis, manuscript preparation, and revisions. All authors reviewed and approved the final version of the manuscript.

Competing interests

The authors did not declare any conflict of interest.

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2025, Scienceline Publication
World's Veterinary Journal
World Vet J, 15(3): 764-773.
ISSN 2322-4568

DOI: https://dx.doi.org/10.54203/scil.2025.wvj78
PII: S232245682500078-15

Prevalence of Coccidiosis in Commercial Chickens of Bangladesh: Species Identification with Age and Seasonal Associations

Md. Sahidul Islam[1], Dilruba Akter Mir[1*], Syidul Islam[2], Md. Anwar Jahid[3], Nasrin Akter Sumona[3], Dabobrata Kumar Swar[3], Nasrin Akter[3], Sharmin Zaman[1], Mosammat Mahamuda Khatun[1], Md. Ashraful Islam[2], Md. Asaduzzaman Lovelu[1], and Md. Emran Nazir[4]

[1]*Faculty of Veterinary, Animal and Biomedical Sciences, Khulna Agricultural University, Khulna-9100, Bangladesh*
[2]*Farming System Research Division, Bangladesh Livestock Research Institute, Savar, Dhaka-1341, Bangladesh*
[3]*Faculty of Animal Science and Veterinary Medicine, Patuakhali Science and Technology University, Patuakhali-8602, Bangladesh*
[4]*Faculty of Nutrition and Food Science, Patuakhali Science and Technology University, Patuakhali-8602, Bangladesh*

*Corresponding author's Email: mirdilrubaakter@gmail.com

ORIGINAL ARTICLE
Received: June 22, 2025
Revised: July 26, 2025
Accepted: August 27, 2025
Published: September 30, 2025

ABSTRACT

Coccidiosis is one of the most economically significant diseases in the poultry industry, resulting in substantial economic losses through treatment costs, reduced growth rates, and decreased body weight gain. The present study aimed to determine the prevalence of different *Eimeria* (*E.*) species in commercial chickens and to assess age-specific and seasonal patterns of coccidiosis. A total of 595 chicken fecal samples were randomly collected from three different chicken types, namely broiler breeds (n = 227), layer breeds (n = 205), and Sonali (a dual-purpose crossbred, n = 163) chickens between April 2022 and March 2023, across 27 different farms in the Pirojpur district of Bangladesh. The farms were selected for sampling based on the presence of chickens having clinical signs such as diarrhea, decreased feed consumption, impaired weight gain, lameness, and increased mortality. After sacrificing the suspected chickens, the intestine was carefully inspected and examined for gross pathological lesions. Mucosal scrapings from different intestinal segments and fecal samples were analyzed microscopically for the presence of oocysts using standard parasitological methods. The identification of different *Eimeria* species was conducted based on the morphological and morphometric characteristics of the oocysts. The result revealed that the overall prevalence of coccidiosis was 13.78%. Among the chicken types, Sonali demonstrated the significantly highest prevalence (21.47%), followed by layers (13.66%) and broilers (8.37%). Five different *Eimeria* species were identified, with *E. tenella* (45.12%) being the most prevalent, followed by *E. acervulina* (18.29%), *E. maxima* (15.85%), *E. brunetti* (10.98%), and *E. necatrix* (9.76%). No significant influence of age or season on coccidiosis prevalence was observed in broiler and layer chickens. In Sonali chickens, the prevalence of coccidiosis increased significantly with age, while no significant effects of season were observed. It has been concluded that the total prevalence of coccidiosis was 13.78%, with Sonali chickens exhibiting the highest prevalence. Five distinct species of *Eimeria* were identified, with *E. tenella* being the most common species. Furthermore, no effects of season and age on prevalence of *Eimeria* species were observed, except for Sonali chickens, where the prevalence increased with age.

Keywords: Coccidiosis, *Eimeria*, Morphometry, Poultry, Prevalence, Sonali chicken

INTRODUCTION

The poultry industry plays a vital role in supplying humans with animal protein, particularly through meat and eggs, while making a significant contribution to the national economy in Bangladesh (Hamid et al., 2016). However, several diseases, including coccidiosis, colibacillosis, and chronic respiratory disease, pose significant challenges to the growth and development of this sector. Despite these challenges, the industry continues to employ a large portion of the population, including rural and marginal farmers (Gupta et al., 2019; Yadav et al., 2020). Among these diseases, coccidiosis is considered the most significant parasitic disease affecting the global poultry industry, resulting in substantial economic losses due to both decreased productivity and the need for control measures (Munoz-Gomez et al., 2024). Coccidiosis imposes a substantial economic burden due to decreased productivity, poor feed conversion efficiency, growth retardation, increased mortality, and the associated expenses of treatment and prevention. The disease is caused by *Eimeria* (*E.*), an apicomplexan protozoan parasite that poses a serious threat to the poultry industry (Peek and Landman, 2011). Among the different types of poultry raised in Bangladesh, Sonali chickens, derived from a cross between Rhode Island Red (RIR) cocks and Fayoumi hens, have become a significant part of poultry production. Due to their phenotypic similarity to the native chickens, these crossbred chickens (*Gallus gallus domesticus*) have gained popularity and wider acceptance among farmers as a favorable alternative to broiler chickens (Uddin et al., 2015).

To cite this paper: Islam MS, Mir DA, Islam S, Jahid MA, Sumona NA, Swar DK, Akter N, Zaman S, Khatun MM, Islam MA, Lovelu MA, and Nazir ME (2025). Prevalence of Coccidiosis in Commercial Chickens of Bangladesh: Species Identification with Age and Seasonal Associations. *World Vet. J.*, 15(3): 764-773. DOI: https://dx.doi.org/10.54203/scil.2025.wvj78

Chickens are frequently infected with different *Eimeria* species, which can result in varying degrees of gastrointestinal disorders (Olufemi et al., 2020). There were nine *Eimeria* species among those; *E. tenella*, *E. maxima*, *E. brunette*, and *E. necatrix* were the most pathogenic, followed by *E. mitis*, *E. acervulina*, and *E. mivati*, *which* were moderately pathogenic. On the other hand, *E. hagani* and *E. praecox* were least pathogenic (Nematollahi et al., 2009). Of these, *E. tenella* and *E. necatrix* are especially detrimental, causing bloody lesions, significant morbidity, and mortality in young chickens (Iacob and Duma, 2009). The severity of infection depends on several factors, including the *Eimeria* species involved, the age and immune status of the host, and the number of ingested oocysts (Attree et al., 2021). Poor sanitary conditions in poultry sheds, combined with improper litter management, further increase the risk of infection. Additionally, young chickens are most frequently affected during the wet seasons (Kaboudi et al., 2016). Coccidiosis is still considered the most economically significant parasitic condition affecting poultry production worldwide, which decreases poultry production by increasing feed intake, reducing weight gain, and increasing treatment costs annually to the tune of two billion euros (Peek et al., 2011). Identification of different species based on the morphology of oocysts was a common phenomenon when using the fecal flotation technique under a microscope. The presence of oocysts in fecal samples is primarily examined using the flotation method, which involves a saturated salt solution and a modified McMaster technique (Sharma et al., 2015). The morphology and/or morphometry of the sporocyst and oocyst, their developmental histories, and the location and magnitude of pathological lesions in the chicken's intestine have all been used to identify *Eimeria* species (Aarthi et al., 2010). Every *Eimeria* species has a distinct location within the intestine, which highlights its particular parasitic behavior (Mesa-Pineda et al., 2021). Microscopic analysis of intestinal lesions and oocyst morphology, distinct to each species, can also be used to identify and differentiate *Eimeria* species (Adam et al., 2022). Seven *Eimeria* species were identified by using morphological methods, including *E. tenella* (50.84%), *E. acervulina* (25.42%), *E. maxima* (20.33%), *E. mitis* (8.47%), *E. necatrix* (3.38%), *E. mivati* (3.38%), and *E. praecox* (1.69%; Nasiri et al., 2024).

The prevalence of the disease may vary depending on several factors, including population density, farm management practices, and geoclimatic conditions (Asfaw et al., 2021). Promoting commercial poultry farming in this region remains challenging without fundamental knowledge of the prevalence and pathophysiology of chicken coccidiosis, which has not yet been determined in these areas. The prevalence of coccidiosis was 34.48% in the overall broiler population of Gazipur district, Bangladesh (Rony et al., 2021), and 82.29% in Sylhet district, Bangladesh (Datta et al., 2025). The studies conducted in the Swabi district of Pakistan indicated the overall prevalence of poultry coccidiosis was 44.4% (Begum et al., 2024), while it was 30.12% in the Meghalaya state of India (Das, 2021) and 42.2% in the Gondar town in Ethiopia (Wondimu et al., 2019).

Although coccidiosis is a common disease in the Pirojpur district, Bangladesh, there is a lack of sufficient published information on its prevalence and distribution. Limited studies have been conducted on the topographical distribution of coccidia species in broiler chickens, and the magnitude of the economic loss associated with specific *Eimeria* species in Bangladesh remains largely unknown. The identification of species based on geographic location is the most crucial step for the effective control and prevention of avian coccidiosis. Therefore, the present study aimed to identify the different coccidian species infecting commercial chickens and to determine the age-specific and seasonal prevalence of coccidiosis in the Pirojpur district of Bangladesh.

MATERIALS AND METHODS

Ethical approval

In accordance with the Bangladesh Veterinary Council Act 2019 (Act No. 13 of 2019), all chickens in the present study were treated following the ethical standards established by the Birds Care Instructions and Use Regulations of the Government of the People's Republic of Bangladesh.

Study area and duration

The investigation was carried out from April 2022 to March 2023 across different small-scale commercial poultry farms in the Pirojpur district of Bangladesh (Figure 1). Pirojpur is situated in Southwestern Bangladesh between 22°09’ and 22°52’ North latitude and 89°52’ and 90°13’ East longitude. The soil is fertile, and the majority of the area consists of low-lying plain land, with elevations ranging from 10 to 19 feet above sea level. The average temperature of this district ranged from 25.6 to 33.4 °C, with humidity levels of 63-84%, and the average annual rainfall in the region was approximately 2181.6 mm (Weather2Visit, 2025). The present study was conducted at the Department of Parasitology, Faculty of Veterinary Animal and Biomedical Sciences, Khulna Agricultural University, Khulna-9100, Khulna, Bangladesh.

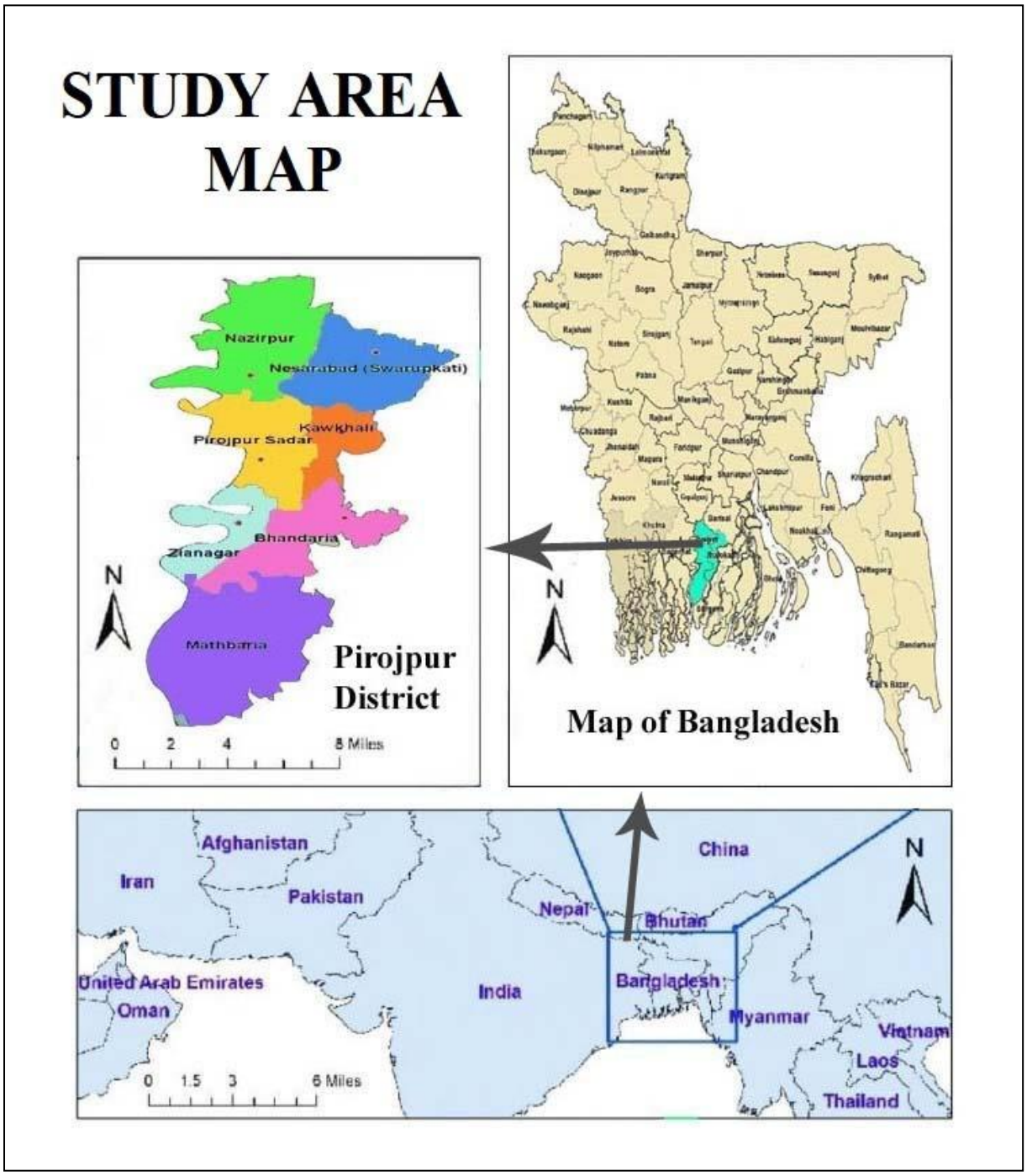


Figure 1. Map of the study area, Pirojpur district of Bangladesh, for investigating coccidiosis in commercial chickens from April 2022 to March 2023

Sample collection

A total of 595 freshly passed fecal samples were randomly collected from several breeds of broiler chickens (n = 227), including different commercial broiler breeds. The Cobb 500 and Hubbard Classic were the most common broiler chickens. The layer chickens (n = 205) comprised different commercial layer breeds, with the most common breeds being Novogen White/Brown and Shaver Brown/White, and the Sonali chickens (n = 163) were found across 27 different farms in the Pirojpur district of Bangladesh. All chickens were reared in floor conditions. The overall number of chickens on each farm varied from 300 to 1500, with ages ranging from 2 to 40 weeks. All farm chickens had no history of vaccination against coccidiosis. Samples were collected from different farms at the farmer's request for the treatment of chickens exhibiting clinical signs, such as diarrhea, decreased feed consumption, impaired weight gain, lameness, and increased mortality. The chickens were taken for necropsy, and fecal samples were collected using a separate spatula for each chicken. Each fecal sample was put into a 50 mL Falcon tube that was previously labeled with the necessary information and transported to the laboratory for parasitological analysis. Upon arrival at the laboratory, these samples

were immediately stored at 4°C until further processing (Wondimu et al., 2019). Information on farm location, flock size, age of the chickens, type of chickens, seasons, owner complaints, clinical history, observed clinical signs (diarrhea, reduced feed and water intake, poor body weight, ruffled feathers, lameness, and drooping wings), mortality rates, and both therapeutic and preventive measures were collected directly from the farmers through a face-to-face interview.

Postmortem examination

In the laboratory, collected chickens were sacrificed by cervical dislocation before necropsy. All visceral organs were initially examined for gross pathological changes. Following a systematic evaluation of all organs, the intestines of each chicken were carefully dissected, opened longitudinally, and inspected for lesions such as whitish spots, petechial hemorrhage, ballooning, reddening, thickening, and caecal cores.

Microscopic examination

The fecal samples were examined for the presence of *Eimeria* oocysts in the parasitology laboratory through standard flotation techniques using concentrated sucrose solution (specific gravity: 1.27) in accordance with the guidelines outlined by Hendrix (1998). A compound microscope (TM450, Meiji Techno Company Limited, Japan) with 10× objectives was used to examine the slides. A sample was considered negative if three separate slides from the same specimen exhibited no oocysts. Positive fecal samples were then sporulated in a wet chamber at 24-26°C in a 2.5% solution of potassium dichromate ($K_2Cr_2O_7$) as described by Al-Quraishy et al. (2009). Mucosal scrapings from different intestinal segments (small intestine and caeca) were collected and analyzed microscopically for the presence and identification of oocysts. Prevalence of coccidiosis and species identification were done based on the morphological traits of sporulated oocysts and sporocysts, including shape, color, micropyle, micropyle cap, presence or absence of polar, residual bodies, as well as the time of sporulation (Soulsby, 1982; Cheru et al., 2023). The morphometric traits, such as length, width, length-to-width ratio, and form index, were evaluated based on the diagnostic guides for identifying sporulated *Eimeria* oocysts outlined by Conway and McKenzie (2007). The results of postmortem and microscopic investigations were compiled and examined comparatively for interpretation.

Statistical analysis

All data collected were entered into a Microsoft Excel 2010 spreadsheet program, and statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) version 26 (SPSS Inc., USA) to determine the prevalence of coccidiosis. The difference among variables was determined by using the chi-square ($\chi 2$) test. A p-value of less than 0.05 was considered statistically significant.

RESULTS

The overall prevalence of coccidiosis was 13.78% (n = 82/595) in commercial chickens in the Pirojpur district of Bangladesh (Figure 2). A significantly higher prevalence was recorded in Sonali chickens at 21.47% (n = 25/163), followed by 13.65% (n = 28/205) in layer and 8.37% (n = 19/227) in broiler chickens ($p < 0.05$, Table 1).

Furthermore, Table 2 displays the prevalence of five identified species, including *E. acervulina*, *E. maxima*, *E. necatrix*, *E. tenella, and E. brunetti.* Among these, *E. tenella* was found to be the most prevalent, with a rate of 45.12%. The prevalence of *E. acervulina*, *E. maxima, E. brunetti, and E. necatrix* was 18.29%, 15.85%, 10.98%, and 9.76% respectively, in commercial chickens.

Based on the findings in Table 3, the highest prevalence of coccidiosis infection (10.09%) was observed in broiler chickens aged between 15 and 35 days, although the difference was not statistically significant ($p > 0.05$). No significant seasonal variation in the prevalence of coccidiosis was observed ($p > 0.05$); however, the rainy seasons demonstrated the highest prevalence (11.63%), followed by winter (6.76%) and summer (5.97%).

The highest prevalence of coccidiosis infection (18.60%) was detected in chickens aged less than eight weeks, with no statistically significant difference in layer chickens ($p > 0.05$). The highest prevalence occurred during the summer (16.67%), followed by the rainy (12.28%) and winter (8.70%) seasons, despite there being no significant association between prevalence and season ($p > 0.05$; Table 4).

The prevalence of coccidiosis in Sonali chickens differed significantly among age groups ($p < 0.05$). The highest prevalence was observed in chickens aged 21-50 days (32.88%), which was significantly greater than in the chickens aged over 51 days (14.55%) and those under 20 days (8.57%) of age. According to the present investigation, Sonali chickens were affected by coccidiosis throughout the year, with only slight seasonal variations. Although the seasonal variations were not statistically significant ($p > 0.05$), a higher prevalence was recorded during the rainy seasons (27.54%), followed by the winter (24.39%) and the summer (11.32%) seasons ($p > 0.05$, Table 5).

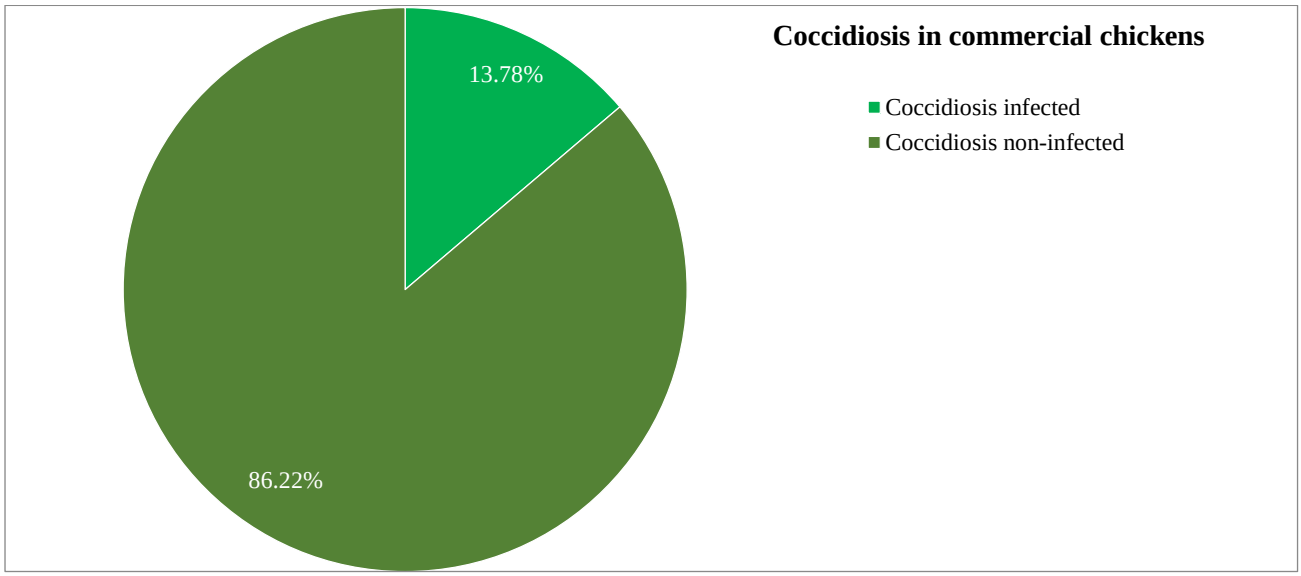


Figure 2. Overall prevalence of coccidiosis in commercial chickens from April 2022 to March 2023 in the Pirojpur district of Bangladesh

Table 1. Prevalence of coccidiosis in commercial chickens in relation to the type of chickens from April 2022 to March 2023 in the Pirojpur district of Bangladesh

Type of chicken	Tested samples		Positive samples		Prevalence	$\chi 2$	p-value
	Total number = 595	(%)	N = 82	(%)	(%)		
Broiler breeds	227	38.15	19	23.17	8.37		
Layer breeds	205	34.45	28	34.15	13.66	13.71	0.001
Sonali chickens	163	27.39	35	42.68	21.47		

N: Number of positive samples, $\chi 2$: Chi-square value, p-value less than 0.05 is considered statistically significant ($p < 0.05$). Note: Sonali is a dual-purpose crossbred (Rhode Island Red ♂ × Fayoumi ♀) chicken type in Bangladesh.

Table 2. Distribution of different *Eimeria* species infecting the commercial chickens from April 2022 to March 2023 in the Pirojpur district of Bangladesh

Species	Broiler chicken		Layer chicken		Sonali chicken		Total	
	n_1	(%)	n_2	(%)	n_3	(%)	n	(%)
Eimeria tenella	6	31.58	13	46.43	18	51.43	37	45.12
Eimeria acervulina	5	26.32	4	14.29	6	17.14	15	18.29
Eimeria maxima	3	15.79	6	21.43	4	11.43	13	15.85
Eimeria brunetti	2	10.53	2	7.14	5	14.29	9	10.98
Eimeria necatrix	3	15.79	3	10.71	2	5.71	8	9.76
Total	19	23.17	28	34.15	35	42.68	82	100

n_1: Number of positive samples in broiler chickens, n_2: Number of positive samples in layer chickens, n_3: Number of positive samples in Sonali chickens, n: Total number of positive samples.

Table 3. Coccidiosis prevalence in broiler chickens in relation to ages and seasons from April 2022 to March 2023 in the Pirojpur district of Bangladesh

Factors	Categories	Tested samples		Positive samples		Prevalence	$\chi 2$	p-value
		N = 227	(%)	n = 19	(%)	(%)		
Age group	≤ 14 days	35	15.42	2	10.53	5.71	0.884	0.643
	15-35 days	109	48.02	11	57.89	10.09		
	≥ 36 days	83	36.56	6	31.58	7.23		
Season	Summer	67	29.52	4	21.05	5.97	1.944	0.378
	Winter	74	32.60	5	26.32	6.76		
	Rainy	86	37.89	10	52.63	11.63		

N: Number of tested samples, n: Number of positive samples, $\chi 2$: Chi-square value, p-value less than 0.05 is considered statistically significant.

Table 4. Age and season distribution of coccidiosis in layer chickens from April 2022 to March 2023 in the Pirojpur district of Bangladesh

Factors	Categories	Tested samples		Positive samples		Prevalence	χ2	p-value
		N = 205	(%)	n = 28	(%)	(%)		
Age group	≤ 8 weeks	86	41.95	16	57.14	18.60	3.236	0.198
	9-20 weeks	72	35.12	8	28.57	11.11		
	≥ 21 weeks	47	22.93	4	14.29	8.51		
Season	Summer	102	49.76	17	60.71	16.67	1.835	0.399
	Winter	46	22.44	4	14.29	8.70		
	Rainy	57	27.80	7	25.00	12.28		

N: Number of tested samples, n: Number of positive samples, χ2: Chi-square value, p-value less than 0.05 is considered statistically significant.

Table 5. Prevalence of coccidiosis in Sonali chickens by age and seasonal changes from April 2022 to March 2023 in the Pirojpur district of Bangladesh

Factors	Categories	Tested samples		Positive samples		Prevalence	χ2	p-value
		N = 163	(%)	n = 35	(%)	(%)		
Age group	≤ 20 days	35	21.47	3	8.57	8.57	10.650	0.005
	21-50 days	73	44.79	24	68.57	32.88		
	≥ 51 days	55	33.74	8	22.86	14.55		
Season	Summer	53	32.52	6	17.14	11.32	4.951	0.084
	Winter	41	25.15	10	28.57	24.39		
	Rainy	69	42.33	19	54.29	27.54		

N: Number of tested samples, n: Number of positive samples, χ2: Chi-square value, p-value less than 0.05 is considered statistically significant.

DISCUSSION

With an overall prevalence of 13.78%, the current study demonstrated that coccidiosis remains a significant health issue for chickens in the Pirojpur district of Bangladesh. The current finding was lower than the 15.38% reported by Islam et al. (2020a) in Bogura district, Bangladesh. On the contrary, the present results were higher than those reported by Islam et al. (2013) and Rahman et al. (2017), who observed 7.53% and 5.8% of coccidiosis, respectively, at different parts of Bangladesh. Numerous factors, including sampling time, sample size, geographic location, population density, and local climate, may contribute to the differences in the results.

The present investigation supported the results of Islam et al. (2023), who reported that coccidiosis is more prevalent in Sonali chickens (18.31%), followed by layers (7.45%) and broiler chickens (2.62%). Similarly, Al Mamun et al. (2019) documented the prevalence of 10.70%, 9.16%, and 6.93% in Sonali, layer, and broiler chickens, respectively. Poor litter management and unsanitary poultry housing contribute to the increased risk of coccidiosis infection. However, Islam et al. (2014) and Islam et al. (2020a) reported that broiler chickens had the highest incidence (15.39% and 20.6% respectively), followed by Sonali (5.56% and 10.47%) and layer (7.5% and 10.25%) chickens. Higher stocking densities may be a contributing factor to the comparatively higher prevalence of coccidiosis in Sonali chickens, as indicated by the sample collection. Overcrowding led to the rapid accumulation of fecal materials and moisture in the litter, creating a suitable environment for *Eimeria* oocysts to sporulate. This significantly increases the risk of ingestion and reinfection across the flocks.

The current results supported the findings of Begum et al. (2024), who reported that *E. tenella* was responsible for the highest prevalence of coccidiosis (42.5%), followed by *E. acervulina* (26.3%), *E. maxima* (20%), and *E. mitis* (11.3%). Similarly, Jan et al. (2022) observed that *E. tenella* was the most prevalent species (20.12%), followed by *E. acervulina* (11.92%), *E. maxima* (10.25%), *E. necatrix* (8.84%), and *E. mitis* (2.69%). However, Bereket and Abdu (2015) reported different findings, with *E. brunetti* (17.8%) demonstrating the highest prevalence of single infection, while lower prevalences of 12.2%, 9.7%, 7.8%, and 6.7% were observed in *E. tenella*, *E. necatrix*, *E. acervulina*, and *E. maxima*, respectively. Additionally, Agishi et al. (2016) identified *E. necatrix* (22.6%) as the most prevalent species, with decreasing prevalence in *E. maxima* (20.4%), *E. tenella* (19.4%), *E. mitis* (15%), *E. acervulina* (10.4%), *E. praecox* (6.9%), and *E. brunetti* (5.3%), which was inconsistent with the present results. In broiler chickens, Meteab et al. (2021) recorded that the highest prevalence of coccidiosis was caused by *E. tenella* (47.05%), followed by *E. acervulina* (23.52%), *E. maxima* (17.64%), mixed infections of *E. maxima* with *E. tenella* (8.23%), and *E. necatrix* (3.52%), consistent with the present results. Conversely, Liao et al. (2024) indicated that *E. acervulina* (36.55%) was the most prevalent species, followed by *E. mitis* (35.28%), *E. tenella* (34.01%), and *E. necatrix* (30.96%) in broiler chickens,

respectively. Additionally, Meteab et al. (2021) recorded that in layer chickens, *E. tenella* (35%) had the highest prevalence, followed by *E. maxima* (25%), *E. acervulina* (15%), mixed infections of *E. tenella* with *E. maxima* (15%), and *E. necatrix* (10%). These variations in findings could be attributed to differences in poultry breeds, management techniques, environmental factors, and study populations.

Broiler chickens aged between 15 and 30 days indicated the highest prevalence of coccidiosis infection. However, there was no significant association between prevalence and age groups. This finding is consistent with the findings reported by Mohamed et al. (2021), who found no infection in broiler chickens aged between 0 and 15 days and above 45 days, while the 15-30-day age groups exhibited the highest percentage of coccidiosis (54.3%), followed by a lower infection rate (45.7%) in the 30-45-day age group. The current results are partially similar to those of Jan et al. (2022) and Amin et al. (2014) who reported that coccidiosis was higher in broiler chicken having 3-4 weeks age (67.23% and 74.90%) followed by those aged 4-6 weeks (23.54% and 17.94%), and lower in chickens less than 3 weeks of age (9.21% and 7.14%). Coccidiosis may be more common in the 15-30-day age group because chickens in this age have not yet developed sufficient acquired immunity against *Eimeria* species and are exposed to a high load of oocysts in the litter. In contrast, chickens aged 0-14 days were protected by maternal immunity. However, some studies have observed a high prevalence of coccidiosis in broiler chickens aged 22-42 days (Nematollahi et al., 2009; Kumar et al., 2014).

According to the present study, the season had no significant influence on the prevalence of coccidiosis in broiler chickens. Nevertheless, the rainy seasons indicated a greater prevalence (11.63%) compared to the winter (6.76%) and summer (5.97%). The disease was detected in broiler chickens throughout most of the year; however, it was most prevalent during the rainy seasons (10.91%), followed by summer (7.31%), and least prevalent in winter (2.37%), as reported by Al Mamun et al. (2019), which is consistent with the present findings. The higher prevalence of coccidiosis in the rainy seasons is attributed to warm and humid conditions that enhance the sporulation, survival, and transmission of *Eimeria* oocysts.

The highest prevalence of coccidiosis infection in layer chickens (18.60%) was detected in the less than 8-week age groups, with no significant difference observed. The highest prevalence (6.3%) of coccidiosis in layer chickens was recorded by Islam et al. (2020b) in the 0-8-week age group, while 4.9% was recorded in the 9-20-week age group, and none was recorded in the over 20-week age group, in the Gazipur district of Bangladesh. In contrast, Semaka et al. (2022) reported different results, with the highest prevalence of coccidiosis at 83.3% in the 25-30-week age group. The prevalence then decreased to 65%, 64.3%, 58.3%, 53.8%, and 40%, respectively, in the over 30-week, 1-6-week, 7-12-week, 19-24-week, and 13-18-week age groups in layer chickens. The crowding factor may be responsible for the variations in results across different age groups of layer chickens.

The present investigation revealed that the highest prevalence of coccidiosis in layer chickens (16.67%) was in the summer, followed by the rainy (12.28%) and winter (8.70%) seasons, with no significant difference. According to Adhikari et al. (2008), the summer and spring seasons had the highest prevalence of coccidiosis (33%), followed by the winter season (23%), while the autumn season had the lowest frequency (14%) in layer chickens. These findings are consistent with the present study. However, Al Mamun et al. (2019) reported coccidiosis prevalence in layer chickens as 11.48% during the rainy seasons, 8% in summer, and 7.78% in winter. Due to the hot and humid conditions in summer, coccidiosis is more prevalent during this season.

In Sonali chickens, the incidence of coccidiosis was found to be significantly associated with age, with chickens aged 21-50 days being mostly affected. These results are consistent with those of Islam et al. (2020c), who demonstrated that coccidiosis was significantly more common in chickens aged 5-9 weeks (20.0%), and less common in chickens aged 1-4 weeks (5.3%) in the Sirajgonj district of Bangladesh. Similarly, Belal (2017) reported that the prevalence of coccidiosis was higher (47.50%) in grower chickens, aged 5-16 weeks, as compared to 23.75% in younger chickens, aged 1-4 weeks, and with a minimum prevalence (10%) in adult chickens over 16 weeks of age. These investigations also supported the present results. It has been noted that coccidiosis primarily affects young chickens during the rainy seasons (Kaboudi et al., 2016).

Seasons did not significantly affect the prevalence of coccidiosis in Sonali chickens. However, the rainy seasons were found to be the most prevalent. Coccidiosis was more prevalent in the rainy seasons (29.17%) than in winter (18.42%) and summer (16.41%; Tipu et al., 2021), which is consistent with the present investigations. In contrast, Al Mamun et al. (2019) reported that the summer season had the highest prevalence (16.85%) in Sonali chickens compared to the rainy (12.50%) and winter (3.92%) seasons.

CONCLUSION

The present study emphasized coccidiosis as a persistent and economically significant disease affecting the poultry sector in the Pirojpur district of Bangladesh. The highest prevalence was found in Sonali chickens among the commercial

breeds, with an overall prevalence rate of 13.78%. *Eimeria tenella* was the most common species, followed by *E. acervulina*, *E. maxima*, *E. brunette*, and *E. necatrix*. Additionally, it was demonstrated that the seasons and ages were associated with the incidence of chicken coccidiosis. Targeted strategies in poultry farming are crucial for long-term success, given the importance of specific *Eimeria* species, their prevalence, and the associated economic and health consequences. It is recommended to conduct molecular-based studies on the epidemiology and risk factors associated with the incidence of chicken coccidiosis in similar areas.

DECLARATIONS

Competing interests

The study reported in this manuscript is free from any conflicting financial interests or personal relationships, as declared by all authors.

Ethical considerations

The authors affirmed that all ethical considerations, including plagiarism, publication permissions, research misconduct, and duplicate submission or publication, have been thoroughly addressed in accordance with the Journal's standards.

Acknowledgments

The poultry farmers' willingness to participate in the present study, their kind assistance, and collaboration were greatly appreciated by the authors, enabling the successful completion of this study.

Funding

The present study did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Author's contributions

This study was carried out in collaboration among all authors. Md. Sahidul Islam, Dilruba Akter Mir, Syidul Islam, Md. Anwar Jahid and Nasrin Akter Sumona designed the study, conducted the laboratory analysis, performed the statistical analysis, and wrote the protocol. Dabobrata Kumar Swar, Nasrin Akter, Sharmin Zaman, and Mosammat Mahamuda Khatun contributed to sample collection and initial draft writing of the manuscript. Md. Ashraful Islam, Md. Asaduzzaman Lovelu, and Md. Emran Nazir managed the literature searches, interpreted the results, and conducted the critical review. All authors read and approved the final edition of the manuscript.

Availability of data and materials

The data are available upon reasonable request from the corresponding author.

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2025, Scienceline Publication
World's Veterinary Journal
World Vet J, 15(3): 774-781.
ISSN 2322-4568

DOI: https://dx.doi.org/10.54203/scil.2025.wvj79
PII: S232245682500079-15

Effects of Black Garlic (*Allium sativum*) Supplementation on Oxidative Stress and Leptin Level in Male Obese Rats

Ibrahim[1,2], Eti Yerizel[3]*, Endrinaldi[4], and Gusti Revilla[5]

[1]*Program Doctoral of Biomedical Science, Faculty of Medicine, Andalas University, Limau Manis, Padang, Sumatra Barat 25175, Indonesia*
[2]*Departement of Medical Laboratory Technology, Syedza Saintika University, Jl. Prof. Dr. Hamka No.228, Air Tawar Tim., Padang, Sumatera Barat 25132, Indonesia*
[3]*Departement of Biochemistry, Faculty of Medicine, Andalas University, Limau Manis, Padang, Sumatra Barat 25175, Indonesia*
[4]*Departement of Biomedical, Faculty of Medicine, Andalas University, Limau Manis, Padang, Sumatra Barat 25175, Indonesia*
[5]*Departement of Anatomy, Faculty of Medicine, Andalas University, Limau Manis, Padang, Sumatra Barat 25175, Indonesia*

*Corresponding author's Email: etiyerizel@yahoo.com

ORIGINAL ARTICLE
Received: June 24, 2025
Revised: July 28, 2025
Accepted: August 27, 2025
Published: September 30, 2025

ABSTRACT

Obesity is characterized by excessive fat accumulation in tissues, leading to leptin resistance and chronic inflammation. Black garlic is a well-documented natural remedy capable of improving leptin resistance and oxidative stress through its antioxidant compounds, such as S-Allyl cysteine and polyphenol flavonoids. The present study aimed to evaluate the effects of black garlic on oxidative stress and leptin levels in obese male rats. Twenty-five adult male Wistar rats, aged 10 to 12 weeks and weighing approximately 200 g, were divided into five experimental groups, each comprising five rats. These groups included a negative control group fed a standard diet (K1), a positive control group given a high-fat diet (K2), Treatment group 1 receiving a high-fat diet plus 200 mg of black garlic (P1), Treatment group 2 receiving a high-fat diet plus 400 mg of black garlic (P2), and Treatment group 3 receiving a high-fat diet plus 800 mg of black garlic (P3). The rats received black garlic orally for 28 days. The blood samples were analyzed for markers of oxidative stress, specifically malondialdehyde, as well as leptin levels. The present findings indicated that black garlic significantly decreased malondialdehyde and leptin concentrations in groups P1, P2, and P3 compared to the positive control group. Black garlic, administered at doses of 200, 400, and 800 mg, demonstrated the capacity to diminish oxidative stress and leptin levels in obese male rats.

Keywords: Antioxidant, Black garlic, Leptin, Malondialdehyde, Obesity, Oxidative stress, Rat

INTRODUCTION

Obesity leads to lipid accumulation in adipose tissue (Aziz et al., 2020). It is a multifactorial disease caused by genetic, cultural, hormonal, psychological, and social factors (Lin and Li, 2021; Panuganti et al., 2023). Obesity is influenced by hormones that regulate food intake, including leptin, ghrelin, adiponectin, and cholecystokinin, among other mediators (Sumadewi et al., 2016). In obesity, leptin resistance often develops, leading to increased hunger and food consumption (Subarjati and Nuryanto, 2015; Lin and Li, 2021). Leptin is a hormone synthesized by adipose cells that reduces food intake, increases energy expenditure through specific signals in the hypothalamus, and maintains body weight homeostasis (Amos et al., 2017; Xu et al., 2018). Excessive lipid accumulation associated with obesity can lead to an elevated production of reactive oxygen species (ROS) and free radicals, both in circulating blood and adipocyte cells (Fajrani et al., 2021). Oxidative stress is characterized by increased lipid peroxides, as indicated by elevated levels of malondialdehyde (MDA; Jovie et al., 2022).

Management of free radicals and oxidative stress has been studied using different medications, including both chemical medicines from the statin class and natural remedies such as *Camellia sinensis*, *Allium sativum*, *Syzygium polyanthum*, *Uncaria*, and botanicals rich in antioxidant compounds, including polyphenols, flavonoids, phenolic acids, and vitamins. One of the traditional plants used as herbal medicine is black garlic (Sembiring and Iskandar, 2019). The active organosulfur compounds in black garlic have a higher antioxidant concentration than regular garlic (Ahmed and Wang, 2021). Black garlic is produced from regular garlic and is popular due to its low cost and minimal side effects. In both *in vitro* and *in vivo* antioxidant tests, black garlic extract exhibited dose-dependent free radical scavenging activity. Black garlic significantly extended the lifespan of *Drosophila melanogaster*, increased the activities of superoxide dismutase (SOD), including CuZn-SOD and Mn-SOD, and catalase (CAT) as its concentration increased, and lowered MDA levels. These findings suggested that black garlic extract may prolong the lifespan of *Drosophila melanogaster* by activating SOD and CAT and reducing MDA production (Lei et al., 2014).

Black garlic comprises slightly different compounds compared to white garlic. Fermentation conducted at temperatures between 60-90°C induces the conversion of γ-glutamyl-S-allylcysteine to S-allyl cysteine, resulting in a 4-8 times increase compared to fresh garlic (Chae et al., 2023). Black garlic contains 106.1 mg/kg of S-allyl cysteine, which

To cite this paper: Ibrahim, Yerizel E, Endrinaldi, and Revilla G (2025). Effects of Black Garlic (*Allium sativum*) Supplementation on Oxidative Stress and Leptin Level in Male Obese Rats. *World Vet. J.*, 15(3): 774-781. DOI: https://dx.doi.org/10.54203/scil.2025.wvj79

is five times higher than the S-allyl cysteine content of white garlic, measured at 21.0 mg/kg (Lee et al., 2009; Zhao et al., 2021). This fermentation process triggers numerous chemical reactions in garlic, including enzymatic browning and Maillard reactions, which cause the color to change from white and yellow to dark brown (Lei et al., 2014; Ha and Kim, 2017). Studies on black garlic and its components against different diseases have been extensively conducted in recent years. However, investigations to establish the therapeutic effects of black garlic remain substantially limited. Accordingly, the present study aimed to evaluate the impacts of black garlic supplementation on oxidative stress markers and leptin levels in male Wistar rats with obesity.

MATERIALS AND METHODS

Ethical approval

The present study was conducted with the approval of the Ethics Committee of the Faculty of Medicine, Andalas University, Indonesia, and in accordance with ethical standards in medical research (ethics permit number: 520/UN.16.2/KEP-FK/2024, issued on October 4, 2024).

Animals

The present study was conducted in the animal house at the Biomedical Laboratory, Faculty of Medicine, Andalas University, Indonesia. Thirty-five Wistar rats, aged 10-12 weeks and weighing 200 g, were obtained from the Biomedical Laboratory of Andalas University, Indonesia. The rats were kept in a controlled environment with a 12-hour light-dark cycle, a room temperature of 22 ± 4°C, and a humidity level of 5 ± 10%. Each rat was allocated 125 cm^2 of floor space (approximately 49.2 in^2) within a cage measuring 14 cm (5.5 inches) in height. During a seven-day adaptation period, the rats were given unlimited access to water and commercial pellet feed. The commercial pellet feed contained a metabolizable energy of 3.4 kcal/g, comprising 60% carbohydrate, 20% protein, 10% fat, and 6% fiber.

Plant collection and identification

Black garlic is a local Indonesian variety indigenous to Bogor Regency, West Java, and its surrounding regions. The black garlic is produced by fermenting *Allium sativum* garlic for 21 days at a temperature of 70°C and a humidity of 80%. The black garlic utilized in the present study was a product of Serambi Botani IPB, MD 655628, and was weighed according to the respective doses for each rat, namely 200 mg, 400 mg, and 800 mg. The black garlic was ground into a smooth paste and mixed with distilled water to facilitate administration to the rats.

Experimental design

Creating an obesity model in rats

Male Wistar rats weighing 200 g were fed a high-fat diet (HFD; 2 mL goat oil and 1 mL egg yolk) for 12 weeks, administered via a tube every morning between 9:00 and 10:00 a.m. After 16 weeks, the body weight and body length of the rats were measured to determine the Lee obesity index in rats, which is similar to BMI in humans. Rats with a Lee index of 0.300 or higher were considered obese (Fajrani et al., 2021).

Experiment procedure

Based on the sample calculation utilizing Federer's (1991) formula, the minimum sample size required was five for each treatment group. Twenty-five rats were divided into five groups, each consisting of five rats, including the negative control group (K1), which received a regular diet with distilled water, the positive control group (K2), which received an HFD with distilled water, Treatment group 1 (P1), which received an HFD and a black garlic at a dose of 200 mg/rat, Treatment group 2 (P2), which received an HFD and a black garlic at a dose of 400 mg/rat, and Treatment group 3 (P3), which received an HFD and a black garlic at a dose of 800 mg/rat. These treatments were administered orally for 28 days. Body weight, body length, and activity levels of rats were monitored weekly. The body weight of male rats was assessed utilizing a precision digital scale (Sartorius, Germany), while the body length was determined with an animal measuring board. Body length was measured from the nose to the anus using a tape measure after administering chloroform anesthesia to the rats (Fajrani et al., 2021). To measure activity, the rats were observed for aggressive behavior and sedentary behavior.

Malondialdehyde and leptin analysis

The MDA and leptin parameters were measured on the final day of the experiment. Blood samples were collected from the orbital sinuses of the rats and then centrifuged to separate plasma and serum. The serum was utilized to determine the MDA and leptin levels. The MDA levels were analyzed using enzymatic samples (UV-Vis Spectrophotometry, Italy) at a wavelength of 532 nm, and the results were calculated based on a curve equation, yielding

MDA levels expressed in nmol/mL (Fajrani et al., 2021). Additionally, leptin hormone levels were assessed using serum samples via the enzyme-linked immunosorbent assay (ELISA). The ELISA leptin assay kit, specifically the Sandwich-ELISA rat leptin (Elabscience®, Catalogue number: E-EL-R0582, England), was employed, and the results indicated leptin levels as expressed in ng/mL.

Statistical analysis

The present data are presented as mean ± standard deviation (SD). Since the data for MDA and leptin were not normally distributed, the Kruskal-Wallis test was used to find significant differences ($p < 0.05$) between the control and treatment groups. Statistical analysis was done with IBM SPSS Statistics version 22.0.

RESULTS

Table 1 displays that there was a significant difference in body weight in Wistar rats fed HFD for 16 weeks ($p < 0.05$). The body weight was the highest in P3 (326.80 ± 3.768) and the lowest in the negative control group (240.20 ± 12.194). In addition, the body weights were 319.20 ± 4.658, 318.20 ± 4.919, and 325.00 ± 5.244 in the positive control group, P1, and P2, respectively. After 28 days of feeding an HFD supplemented with black garlic, the body weight in Group P3 was lower (300.20 ± 14.923) than that of the positive control group (327.80 ± 6.686), Group P1 (307.20 ± 7.225), and Group P2 (310.00 ± 9.935). However, the concentration was still higher than the negative control group (254.80 ± 12.677). A significant difference was observed among the negative control group, the positive control group, and groups P1, P2, and P3 ($p < 0.05$; Table 2).

Table 3 illustrates the levels of leptin hormone following the administration of an HFD supplemented with black garlic over a period of 28 days in Wistar rats. In Group P3, the leptin level was observed to be lower (1.12560 ± 0.160120) in comparison to the negative control group (1.51440 ± 0.241909), the positive control group (3.69020 ± 2.372940), Group P1 (1.41720 ± 0.238614), and Group P2 (1.54500 ± 0.195533). A statistically significant difference was identified among the negative control group, the positive control group, and groups P1, P2, and P3 ($p < 0.05$).

The Effects of black garlic on MDA levels in Wistar rats at doses of 200 mg/rat, 400 mg/rat, and 800 mg/rat given for 28 days are illustrated in Table 4. The MDA levels observed after administering an HFD enriched with black garlic for 28 days indicated that Group P3 exhibited reduced MDA levels (2.2320 ± 0.24793) in comparison to the positive control group (3.9760 ± 0.38927), Group P1 (2.5280 ± 0.16514), and Group P2 (2.4280 ± 0.12337). Nonetheless, these levels remained higher than those observed in the negative control group (1.9300 ± 0.06364). Significant differences were observed among the negative control group, the positive control group, and groups P1, P2, and P3 ($p < 0.05$).

Table 1. Effects of feeding a high-fat diet for 16 weeks on body weight in Wistar rats

Group	Rats (Number)	Minimum body weight (g)	Maximum body weight (g)	Mean ± SD
				Body weight of rats (g)
Negative control	5	221	253	240.20 ± 12.194
Positive control	5	314	326	319.20 ± 4.658
Treatment 1	5	312	324	318.20 ± 4.919
Treatment 2	5	319	332	325.00 ± 5.244
Treatment 3	5	321	331	326.80 ± 3.768
Total	25	221	332	305.88 ± 34.264
Significant				0.002

Data are expressed as minimum, maximum, mean ± Standard deviation (SD). Negative control: Rats that received regular diet with distilled water, positive control: Rats that received an HFD with distilled water, Treatment 1: An HFD and a black garlic at a dose of 200 mg/rat, Treatment 2: An HFD and a black garlic at a dose of 400 mg/rat, Treatment 3: An HFD and a black garlic at a dose of 800 mg/rat.

Table 2. Effects of black garlic on body weight in Wistar rats at doses of 200 mg/rat, 400 mg/rat, and 800 mg/rat given for 28 days

Group	Rats (Number)	Minimum body weight (g)	Maximum body weight (g)	Mean ± SD
				Body weight of rats (g)
Negative control	5	235	266	254.80 ± 12.677
Positive control	5	320	336	327.80 ± 6.686
Treatment 1	5	297	315	307.20 ± 7.225
Treatment 2	5	298	321	310.00 ± 9.935
Treatment 3	5	279	316	300.20 ± 14.923
Total	25	235	336	300.04 ± 26.751
Significant				0.001

Data are expressed as minimum, maximum, mean ± Standard deviation (SD). Negative control: Rats that received regular diet with distilled water, positive control: Rats that received an HFD with distilled water, Treatment 1: An HFD and a black garlic at a dose of 200 mg/rat, Treatment 2: An HFD and a black garlic at a dose of 400 mg/rat, Treatment 3: An HFD and a black garlic at a dose of 800 mg/rat.

Table 3. Effects of black garlic on leptin levels in Wistar rats at doses of 200 mg/rat, 400 mg/rat, and 800 mg/rat given for 28 days

Group	Rats (Number)	Minimum level of leptin	Maximum level of leptin	Mean ± SD
				Leptin hormone (ng/mL)
Negative control	5	1.139	1.763	1.51440 ± 0.241909
Positive control	5	1.964	7.859	3.69020 ± 2.372940
Treatment 1	5	1.134	1.790	1.41720 ± 0.238614
Treatment 2	5	1.294	1.728	1.54500 ± 0.195533
Treatment 3	5	0.955	1.383	1.12560 ± 0.160120
Total	25	0.955	7.859	1.85848 ± 1.365638
Significant				0.008

Data are expressed as minimum, maximum, and Mean ± standard deviation (SD). Negative control: Rats that received regular diet with distilled water, positive control: Rats that received an HFD with distilled water, Treatment 1: An HFD and a black garlic at a dose of 200 mg/rat, Treatment 2: An HFD and a black garlic at a dose of 400 mg/rat, Treatment 3: An HFD and a black garlic at a dose of 800 mg/rat.

Table 4. Effects of black garlic on malondialdehyde levels in Wistar rats at doses of 200 mg/rat, 400 mg/rat, and 800 mg/rat given for 28 days

Group	Rats (Number)	Minimum level of MDA	Maximum level of MDA	Mean ± SD
				MDA (nmol/mL)
Negative control	5	1.85	2.02	1.9300 ± 0.06364
Positive control	5	3.59	4.58	3.9760 ± 0.38927
Treatment 1	5	2.34	2.72	2.5280 ± 0.16514
Treatment 2	5	2.29	2.61	2.4280 ± 0.12337
Treatment 3	5	1.85	2.45	2.2320 ± 0.24793
Total	25	1.85	4.58	2.6188 ± 0.75254
Significant				0.001

Data are expressed as minimum, maximum, and Mean ± Standard deviation (SD). MDA: Malondialdehyde, Negative control: Rats that received regular diet with distilled water, Positive control: Rats that received an HFD with distilled water, Treatment 1: An HFD and a black garlic at a dose of 200 mg/rat, Treatment 2: An HFD and a black garlic at a dose of 400 mg/rat, Treatment 3: An HFD and a black garlic at a dose of 800 mg/rat.

DISCUSSION

An HFD is a dietary regimen in which the majority of daily caloric intake is derived from fat, typically contributing around 40-60% of total energy over a period of 8-16 weeks. Obesity is defined as the excessive accumulation of adipose tissue (Panuganti et al., 2023). In numerous cases involving obesity, leptin levels are elevated concurrently with the development of leptin resistance (Sharebiani et al., 2023). Additionally, obesity is associated with heightened systemic oxidative stress, which results from an imbalance between free radicals and antioxidants, often marked by increased MDA levels (Ashar, 2023). In the study conducted by Chang et al. (2017), rats administered an HFD comprising 2 mL of goat fat per 200 g of body weight and 1 mL of egg yolk per 200 g of body weight over a period of 16 weeks via oral gavage exhibited an approximate 62.9% increase in body weight. Similar findings have been reported in a study conducted by Kim et al. (2017), which demonstrated that a diet comprising 60% fat, administered over six weeks, induced obesity in rats. Additionally, an HFD consisting of 45% kcal over ten weeks resulted in notable weight gain. Likewise, Chae et al. (2023) induced obesity in animals using an HFD at 60% over 16 weeks. Jahan et al. (2024) reported weight gain in rats fed a combination of 90% standard pellets and 10% goat fat oil. Sukmawati and Asgap (2017) developed a hyperlipidemia and obesity model using an HFD comprising 50% goat fat mixed with butter, coconut oil, and cholesterol. Furthermore, Maigoda et al. (2016) confirmed the efficacy of goat fat mixtures in inducing obesity. Additionally, Yu et al. (2019) demonstrated that prolonged consumption of egg yolk resulted in increased adiposity in rats.

A study by Nasution (2023) revealed that Sprague-Dawley rats aged 20-24 weeks and weighing in the range of 250 to 300 g, when subjected to a diet comprising a mixture of beef fat, egg yolk, and palm oil for a duration of 30 days, demonstrated a considerable increase in weight gain in comparison to the control groups. Additionally, these subjects exhibited elevated levels of total cholesterol, triglycerides, and low-density lipoprotein (LDL), alongside a reduction in high-density lipoprotein. Aguila et al. (2003) observed that rats fed egg yolks experienced an average weight gain of 32.6 ± 1.3 g over four weeks, surpassing the gains observed in rats fed oats (28.6 g) or wheat bran (25.7 g). Similarly, Omole and Ighodaro (2013) reported that mice subjected to an HFD gained approximately 40% more body weight, along with an increase in total cholesterol by 30%, triglycerides by 11.9%, and LDL from 56.9 to 81 mg/dL. Yu et al. (2019) investigated the effects of a diet incorporating egg yolk powder combined with vanadium, where the HFD (containing 301.2 g per kg of ether extract derived from egg yolk) over five weeks remarkably elevated body weight and markers of

oxidative stress. Feeding rats utilizing an HFD was linked to the development of obesity, caused by excessive energy intake and disrupted regulation of satiety and hunger signals, which subsequently led to leptin resistance and chronic inflammation.

Black garlic is a fermented form of garlic characterized by a soft, savory texture, mild aroma, slightly sweet or tangy flavor, and a chewy or jelly-like consistency (Choi et al., 2014; Sukrianto et al., 2022). According to Sailah (2021), raw garlic can be processed through fermentation or prolonged heating. The fermentation process is most effective at a temperature of 70-80°C with a controlled humidity of 70-90% for 21 days, without adding any extra ingredients (Lei et al., 2014). This process leads to an increase in total acid content, amino nitrogen, polyphenols, flavonoids, and certain bioactive compounds such as lactic acid, gly-pro-glu, sorbase, and 3,4-dihydro-6-hydroxy-2,5,7,8-tetramethyl-2H-1-benzopyran-2-propanoate (CEHC), while reducing 5-hydroxymethyl furfural levels and enhancing grassy, floral, and fruity aromas (Si et al., 2019). During the present study, rats fed an HFD and supplemented with black garlic for 28 days exhibited different results across the different treatment groups. The rats that received 800 mg of black garlic per rat demonstrated a lower body weight compared to the positive control group and the group receiving 400 mg per rat. The present results support the findings of other studies that highlighted the anti-obesity properties of black garlic. Chang et al. (2017) reported that black garlic supplementation improved HFD-induced obesity, while Chae et al. (2023) demonstrated that aged black garlic mitigates obesity and prevents muscle atrophy. Xu et al. (2018) found that aged garlic extract improved inflammatory and immune responses in obese adults, and Lei et al. (2014) confirmed its role in reducing oxidative stress. Similarly, Kimura et al. (2017) analyzed saccharides and antioxidant activity in black garlic, linking them to weight reduction. In contrast, Sembiring and Iskandar (2019) emphasized the antioxidant and anti-inflammatory effects of black garlic in decreasing both weight and oxidative stress. Other studies, by Nasr and Saleh (2014) and Si et al. (2019), confirmed its pharmacological activity as an antioxidant and anti-obesity agent. Additionally, Ahmed and Wang (2021) demonstrated that black garlic supplementation reduced body weight by 18% and fat mass by 44-63% in obese mice. Also, Chang et al. (2017) exhibited that Wistar rats fed an HFD supplemented with 0.2%, 0.6%, or 1.2% black garlic for six weeks demonstrated significant weight reductions of 4.8-8.9% compared to unsupplemented HFD rats. The beneficial effects of black garlic are attributed to its high antioxidant content, including S-allylcysteine, flavonoids, and polyphenols, which lower oxidative stress and elevate adiponectin levels, a protective hormone secreted by adipose tissue (Setiawan et al., 2021). Mechanistically, black garlic modulates fat metabolism by downregulating the expression of fatty acid synthase (FAS) and inflammatory cytokines, such as interleukin-6 (IL-6; Xu et al., 2018), while increasing the activity of lipolytic enzymes, including ATGL, CPT-1, and HSL, in adipose tissue and the liver. Furthermore, black garlic inhibits lipogenesis by downregulating genes such as SREBP-1c, ACC, FAS, G6PDH, HMG-CoA reductase, and ACAT, while concurrently upregulating CPT-1 expression, thereby promoting fatty acid oxidation (Ha et al., 2015; Lestari and Santika, 2023).

Leptin is a protein hormone composed of 167 amino acids, primarily secreted by adipose tissue (Amos et al., 2017; Nasution, 2023), acting on the central nervous system (CNS) to suppress appetite and increase energy expenditure (Amor et al., 2019; Kim and Kim, 2023). In the present study, supplementation with 800 mg/rat of black garlic resulted in the most significant reduction in leptin levels in rats fed an HFD. These findings are consistent with those of Chae et al. (2023), who reported that black garlic supplementation reduced obesity in HFD-fed mice and lowered leptin levels. Similarly, Chang et al. (2017) indicated that black garlic improved leptin regulation in obese mice, while Xu et al. (2018) demonstrated that aged garlic extract reduced inflammation and modulated leptin and other adipokines in obese individuals. Additionally, Amor et al. (2019) confirmed that HFD-fed mice supplemented with aged black garlic exhibited a marked decrease in plasma leptin concentrations from 19.4 to 9.4 ng/dL.

Moreover, black garlic enhances leptin sensitivity, primarily due to its high Sulfur-containing compounds such as S-allylcysteine and other antioxidant compounds, which reduce oxidative stress and inflammation in the hypothalamus (Amor et al., 2019; Halim and Suzan, 2020). Given that hypothalamic inflammation contributes to leptin resistance, its reduction improved leptin responsiveness and signaling efficiency. Additionally, black garlic has demonstrated effects in modulating the gut microbiota by reducing lipopolysaccharide (LPS), a principal inducer of systemic inflammation. The reduction of LPS levels combined with an increase in short-chain fatty acids contributes to enhanced leptin sensitivity and a decrease in leptin resistance (Ha et al., 2015; Amor et al., 2019; Kim and Kim, 2023; Morawati et al., 2024). Numerous individuals with obesity exhibited high leptin levels without the anticipated decrease in fat mass, which signifies leptin resistance. This condition occurs when increased leptin, caused by greater adiposity, fails to stimulate adequate satiety or energy expenditure. Leptin resistance is frequently linked to chronic inflammation in adipose tissue and the brain. Common signs of leptin resistance include lower levels of soluble leptin receptors, decreased protein-bound leptin, and higher levels of circulating free leptin (Subarjati and Nuryanto, 2015; Halim and Suzan, 2020; Amudi et al., 2021). Black garlic possesses antioxidant and anti-inflammatory properties that aid in leptin resistance by inhibiting the release of pro-inflammatory cytokines such as TNF-α and IL-6, which are recognized for their role in

impairing leptin signaling (Salsabila and Busman, 2021). Overall, leptin is crucial in regulating body weight and lipid metabolism. Black garlic directly enhances leptin sensitivity by reducing oxidative stress, inflammation, and regulating fat metabolism, helping to alleviate leptin resistance and support metabolic homeostasis (Amor et al., 2019; Nasution, 2023).

Malondialdehyde, with the chemical formula $CH_2(CHO)_2$, is a reactive dialdehyde widely recognized as a reliable marker of oxidative stress. It is formed as a terminal product of lipid peroxidation, a process that damages cell membranes. Given that MDA is chemically more stable than numerous other peroxidation byproducts, it is frequently employed in experiments as a marker of oxidative damage (Mulianto, 2020; Huang et al., 2023; Widhiani et al., 2023). An HFD facilitates the production of ROS, thereby accelerating lipid peroxidation and elevating MDA levels, which serve as indicators of oxidative stress. Black garlic, rich in antioxidant and anti-inflammatory compounds, can neutralize ROS, decrease oxidative stress, reduce MDA concentrations, and promote cellular health repair. In the present study, rats given the highest dose (800 mg/rat) exhibited significantly lower MDA values compared to the positive control group and other treatment groups. These findings are consistent with the study conducted by Saravanan and Ponmurugan (2010), which demonstrated that S-allyl cysteine, a key component of garlic and black garlic, decreased MDA and oxidative stress in diabetic rats. Si et al. (2019) highlighted the strong antioxidant potential of black garlic in lowering MDA, while Chae et al. (2023) confirmed its ability to mitigate oxidative stress biomarkers. Chang et al. (2017) demonstrated that black garlic supplementation reduced hepatic oxidative stress in obese rats, and Ha and Kim (2017) reported that supplementation up to 1.5% significantly inhibited lipid peroxidation. Furthermore, Ahmed and Wang (2021) found that garlic fermentation reduced MDA levels, supporting its role as a potent antioxidant intervention. Obesity in the United States has been linked to elevated oxidative stress. According to a study by Lee et al. (2009), MDA levels in the control group were reported as 2.33 ± 0.36. In contrast, rats receiving a diet supplemented with 5% black garlic exhibited notably lower levels at 1.32 ± 0.26, representing an approximate reduction of 43.3%. The reduction in MDA with black garlic supplementation is attributed to its S-allylcysteine, flavonoids, polyphenols, and other organosulfur constituents, which act as scavengers of reactive oxygen and nitrogen species (ROS and RNS). By neutralizing these radicals, black garlic suppresses lipid peroxidation, thereby lowering MDA concentrations (Lee et al., 2009; Dampati and Veronica, 2020; Sukrianto et al., 2022). Black garlic enhances the function of superoxide dismutase, which converts superoxide into hydrogen peroxide (H_2O_2), and CAT and glutathione peroxidase, which break down H_2O_2 into water. This enzymatic defense prevents the formation of hydroxyl radicals, one of the most damaging reactive species responsible for lipid peroxidation. Furthermore, black garlic reduces inflammation and inhibits NF-κB signaling (Xu et al., 2018; Salsabila and Busman, 2021). Chronic inflammation contributes to increased ROS through macrophage activation and NF-κB pathway stimulation. By suppressing pro-inflammatory cytokines such as IL-6 and TNF-α, black garlic lowers ROS generation from inflammatory processes, protecting lipids from oxidative damage, and reducing MDA formation (Lei et al., 2014; Kusriani et al., 2022; Morawati et al., 2024).

CONCLUSION

In the present study, leptin levels were assessed after 28 days of administering an HFD supplemented with black garlic at doses of 200 mg, 400 mg, and 800 mg per rat. The present results suggested that black garlic effectively contributes to weight reduction, lowers leptin concentrations, and decreases MDA levels as an indicator of oxidative stress. The 800 mg/rat dose resulted in a significantly greater reduction compared to the positive control group that received only an HFD. A larger sample size is required to enhance the statistical power and generalizability of the findings. Utilizing animals of different sexes or species could yield a more comprehensive understanding of the effects of black garlic. Furthermore, testing different dosages and determining the optimal dose of administering black garlic are essential to establish the boundaries of efficacy and safety.

DECLARATIONS

Acknowledgments

The authors express their gratitude to the Department of Biomedical Laboratory and the Biochemistry Laboratory, Faculty of Medicine, Andalas University, Indonesia, for their assistance with sample analysis.

Authors' contributions

Ibrahim, Eti Yerizel, Endrinaldi, and Gusti Revilla were responsible for conceptualizing the article. Ibrahim collected the data and the manuscript, while Eti Yerizel reviewed it. All authors considered and agreed on the final edition of the manuscript for publication.

Funding

The present study was privately funded by the authors.

Availability of data and materials
The data are available upon reasonable request from the corresponding author.

Competing interests
The authors declared no conflicts of interest.

Ethical considerations
Ethical issues, including plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy, have been checked by all the authors.

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2025, Scienceline Publication
World's Veterinary Journal
World Vet J, 15(3): 782-789.
ISSN 2322-4568

DOI: https://dx.doi.org/10.54203/scil.2025.wvj80
PII: S232245682500080-15

Determinants Influencing Calving to Service Interval in Danish Holstein Cows

Bui Van Dung[1], Le Thi Le Thuong[2], and Nguyen Hoai Nam[1]*

[1]*Department of Animal Surgery and Theriogenology, Faculty of Veterinary Medicine, Vietnam National University of Agriculture, Ha Noi, Vietnam*
[2]*Undergraduate student, Faculty of Veterinary Medicine, Vietnam National University of Agriculture, Ha Noi, Vietnam*

*Corresponding author's Email: nguyenhoainam@vnua.edu.vn

ORIGINAL ARTICLE
Received: July 05, 2025
Revised: August 09, 2025
Accepted: September 10, 2025
Published: September 30, 2025

ABSTRACT

The calving-to-first-service interval is crucial for dairy herd productivity. However, factors influencing this reproductive parameter remain understudied in modern dairy systems. The present study aimed to investigate the determinants of the calving-to-first-service interval in Danish Holstein cows, focusing on parity, age at first insemination, age at first calving, gestation length, gender of the born calf, and calving season. Data from 699 cows, managed on a single farm in Southwest Denmark, were collected and analyzed to identify risk factors associated with the calving-to-first-service interval. The mean calving-to-the-first-service interval was 87.6 ± 23.7 days. The present results indicated that parity, age at first calving, and calving season are key modifiable factors that affected the calving-to-first-service interval of the investigated Danish Holstein cows. The final multivariate linear regression model, which explained 13.7% of the variation in the calving-to-first-service interval, identified parity, age at first calving, and the gender of the born calf as significant risk factors for the interval. Parity and age at first calving were negatively related to the calving-to-first-service interval. Male calves were linked to an increased calving-to-first-service interval. Gestation length and age at the first artificial insemination indicated no association with the calving-to-first-service interval. Focusing on key factors such as parity, age at first calving, and gender of the born calf can improve reproductive success and profitability in dairy herds.

Keywords: Age, Calving, First calving, Gender, Parity, Service interval

INTRODUCTION

The reproductive efficiency of dairy cows is a key factor influencing the profitability and sustainability of dairy farms (De Vries and Marcondes, 2020). Among different reproductive performance parameters, the calving-to-service interval (CSI) serves as a crucial indicator of a cow's readiness for rebreeding post-parturition (Recce et al., 2021; Temesgen et al., 2022). The CSI can significantly impact the calving interval, influencing milk production cycles and herd productivity (Hossain et al., 2021). A long CSI can delay subsequent pregnancies, reduce the number of calves produced over a cow's lifetime, and potentially lower milk production (Bonato and dos Santos, 2012; Hossain et al., 2021; Temesgen et al., 2022). Conversely, a short CSI can help in maintaining a consistent milk supply, thereby supporting the economic viability of the dairy operation (Hossain et al., 2021). Improving reproductive efficiency is crucial in dairy cows because delays in returning to reproductive activity not only reduce lifetime calf production but also disrupt lactation cycles, causing economic losses (Wiltbank et al., 2011; Dung et al., 2025). Understanding the factors that influence CSI provides valuable insights for developing management strategies that enhance reproductive performance and sustain long-term herd productivity (Dung et al., 2025).

In dairy herds, the CSI can be affected by a range of factors, including cow's parity (Kim et al., 2009; Ali et al., 2013; Hossain et al., 2021), age at first artificial insemination (AFAI; Ali et al., 2013), age at first calving (AFC; Watanabe et al., 2017, Softic et al., 2020), gestation length (Motlagh et al., 2013; Atashi et al., 2019; Senbeta et al., 2024), and environmental factors such as season of calving (Kim et al., 2009). Younger cows or cows in their first parity may display different CSI patterns compared to older, multiparous cows (Softic et al., 2020). Furthermore, the interaction between the age at AFAI and calving season may influence the CSI through their combined effects on ovarian activity, estrous expression, and the effectiveness of reproductive management strategies (Lima et al., 2010; Temesgen et al., 2022). Understanding these relationships is crucial for developing effective reproductive management strategies that optimize herd fertility and increase milk production yield.

The present study aimed to identify the influencing factors of the CSI in Danish Holstein cows, considering parity, AFC, gestation length, gender of the born calf, and calving season, using a multivariate approach.

To cite this paper: Dung BV, Thuong LTL, and Nam NH (2025). Determinants Influencing Calving to Service Interval in Danish Holstein Cows. *World Vet. J.*, 15(3): 782-789. DOI: https://dx.doi.org/10.54203/scil.2025.wvj80

MATERIALS AND METHODS

Ethical approval

The present study was conducted utilizing farm records of Danish Holstein cows on Holdan Argo farm, located at Tyrholmvej 7, 6230 Rødekro, Denmark. It did not incorporate any experimental manipulation or direct intervention with live animals. All data collection and analysis were performed in strict accordance with established ethical standards governing the use of animal production records in research at the Vietnam National University of Agriculture.

Study population

The present study utilized data from 699 Danish Holstein cows, which had previously been used to investigate the factors associated with conception rate (Nam et al., 2025). The investigated cows ranged from first to fourth parity. All cows were born from January 2017 to September 2021. The animals were milked twice daily, with the first milking session starting at 3:00 a.m. and concluding at 11:00 a.m., and the second milking session starting at 3:00 p.m. and ending at 10:30 p.m. The documented calving interval was 394 days. On average, each cow produced about 36 kg of milk per day. The cows were housed indoors and fed alfalfa hay throughout the year. Estrus detection was carried out using pedometers, and insemination with frozen-thawed semen was performed once per estrus cycle. Vaccinations against *Escherichia coli*, Coronavirus, and Rotavirus (Bovigen® Scour, Virbac, Ireland) were given before the cows were dried off.

Data collection

Data of the investigated cows were collected from the dairy management system (Denmark), which was kept on the farm's computer. The essential sample size was calculated using GPower 3.1.9.7 (Germany), based on a linear multiple regression model (fixed model, R^2 deviation from zero; Faul et al., 2009). An effect size of 0.053 was estimated based on a priori $R^2 = 0.05$. Based on a power analysis ($\alpha = 0.05$, power = 0.95) for a model with six predictors, a sample size of 400 was determined to be sufficient. The collected information included birth date, AFAI (days), AFC (days), as well as the insemination date leading to the previous pregnancy, dates of the most recent calving, first service after that calving, and the gender of the born calf at the most recent calving. Based on this information, the collected data were used to calculate the following parameters, including AFAI, AFC, gestation length of the most recent calving (days), and the most recent CSI (days). Data were collected from August to December 2023.

Based on parity number, cows were divided into four categories, including Parity 1, 2, 3, and 4, as these categories naturally represented the distribution of the dataset. The length of the most recent gestation was classified into ≤ 275 days, 276-280 days, and 281-292 days. The AFAI was grouped into three categories, including 365-400 days, 400-430 days, and over 430 days. The AFC was categorized into five groups, including 646-670 days, 671-700 days, 701-730 days, 731-760 days, and over 760 days. The classification of gestation length into three categories, namely short, regular, and long gestation lengths, is based on the mean value of 277 days. The grouping of AFAI and AFC was conducted using monthly intervals to investigate whether variations of approximately one month in these reproductive events exerted an influence on CSI. The calving months were divided into four seasons, including winter (December-March), spring (April-May), summer (June-August), and autumn (September-November). Roughly, the average monthly temperature spanned from 1.96 to 4.75°C in winter, 8.99 to 13.16°C in spring, 16.39 to 18.45°C in summer, and 6.56 to 14.99°C in autumn.

Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics, Version 22.0. The association between the CSI and different independent variables, including parity, gender of the born calf, gestation length, AFAI, AFC, and calving season, was assessed using the Kruskal-Wallis and Mann-Whitney U tests. For significant results, post hoc analyses were conducted using Dunn's test, with the Bonferroni correction applied. Subsequently, a linear regression analysis was employed to construct the most explanatory model for the CSI, which served as the dependent variable. The independent variables included parity, gender of the born calf, AFAI, AFC, recent gestation length, and calving season. The modeling process involved initial univariate analyses to evaluate the individual influence of each factor, followed by multivariate analyses using forward stepwise selection to build the optimal model. The variance inflation factor (VIF) was calculated to check for multicollinearity (Kim, 2019). For all analyses, including one-way ANOVA and Student's t-test, a p-value of less than 0.05 was considered statistically significant.

RESULTS

Calving to service interval in different cow groups

Parity

The CSI decreased significantly from Parity 1 to Parity 3 ($p < 0.05$; Table 1). The mean CSI in the first parity cows was 96.3 ± 19.4 days, and that in the parities 2 and 3 was 86.2 ± 21.8 and 72.2 ± 25.1 days, respectively. Cows in their Parity 4 exhibited a CSI of 96.9 ± 43.9 days, which did not differ significantly from that observed in cows of parities 1, 2, and 3 ($p > 0.05$). The findings related to Parity 4 should be interpreted carefully because of the small sample size.

Gestation length

The gestation length did not significantly impact CSI ($p > 0.05$; Table 1). Cows with gestation lengths ≤ 275 days had a CSI of 85.7 ± 23.1 days, while those with gestation lengths between 276 and 280 days had a CSI of 89.4 ± 24.4 days. For cows with gestation lengths of 281 and 292 days, the CSI was 86.7 ± 24.9 days.

Gender of the born calf

There was no significant difference in the CSI based on the gender of the born calf ($p > 0.05$; Table 1). Cows that gave birth to heifers had a CSI of 87.7 ± 22.0 days, while those that delivered bull calves had a CSI of 87.5 ± 26.8 days.

Age at first artificial insemination

The AFAI did not influence CSI ($p > 0.05$; Table 1). Cows inseminated initially between 365 and 400 days exhibited a CSI of 86.7 ± 23.6 days. Those inseminated within the 400 to 430-day period had a CSI of 87.0 ± 24.2 days, which was not statistically different from the cows inseminated after 430 days (CSI: 92.2 ± 21.2 days; $p > 0.05$).

Age at first calving

Cows with AFC over 760 days exhibited a shorter CSI (81.3 ± 28.6 days) in comparison to cows with AFC ranging from 671 to 700 days (87.7 ± 21.6; $p < 0.05$) and cows with AFC ranging from 701 to 730 days (89.6 ± 23.5; $p < 0.05$). Pairwise comparisons among the other groups revealed no statistically significant differences ($p > 0.05$; Table 1).

Calving season

The calving season influenced CSI. Cows calving in winter had a significantly longer CSI (89.1 ± 24.9) than those calving in spring, summer, and autumn (87.3 ± 22.9, 84.4 ± 17.5, and 86.5 ± 27.8 days, respectively; $p < 0.05$).

Table 1. Calving to service interval of different cow categories in Danish Holstein cows, Holdan Argo farm, Rødekro, Denmark in 2023

Investigated parameters	Calving to service interval (day)
Parity	
1 (n = 284)	96.3 ± 19.4[a]
2 (n = 265)	86.2 ± 21.8[b]
3 (n = 142)	72.2 ± 25.1[c]
4 (n = 8)	95.9 ± 43.9[abc]
Gestation length (day)	
≤ 275 days (n = 216)	85.7 ± 23.1
276-280 days (n = 316)	89.4 ± 23.4
281-292 days (n = 167)	86.7 ± 24.9
Gender of the calf	
Heifer (n = 466)	87.7 ± 22.0
Bull (n = 233)	87.5 ± 26.8
Age at first artificial insemination (day)	
365-400 days (n = 219)	86.7 ± 23.6
401-430 days (n = 387)	87.0 ± 24.2
> 430 days (n = 93)	92.2 ± 21.2
Age at first calving	
646 -670 days (n = 66)	88.8 ± 21.7[a]
671-700 days (n = 270)	87.7 ± 21.6[a]
701-730 days (n = 194)	89.6 ± 23.5[a]
731-760 days (n = 85)	88.1 ± 26.1[ab]
> 760 days (n = 84)	81.3 ± 28.6[b]
Season of calving	
Winter (n = 377)	89.1 ± 24.9[a]
Spring (n = 104)	87.3 ± 22.9[b]
Summer (n = 139)	84.4 ± 17.5[b]
Autumn (n = 79)	86.5 ± 27.8[b]

Data is presented as mean and standard deviation. [a,b, and c] Means within a column with different superscript letters differ significantly ($p < 0.05$). For post-hoc comparisons, the P-values of all the comparisons were: Parity 1 compared to Parity 2,3,4 ($p < 0.001$; < 0.001, and 0.247), Parity 2 compared to Parity 3, 4 ($p < 0.001$ and 0.880), Parity 3 compared to Parity 4 ($p = 0.521$), Gestation length (GL) ≤ 275 days compared to GL= 276-280 and 281-292 days ($p = 0.105$ and 1.000), GL= 276-280 days compared to GL= 281-292 days ($p = 0.771$), Male calf compared to Female calf ($p = 0.679$), Age at first artificial insemination (AFAI) = 365-400 days compared to AFAI = 401-430 and > 430 days ($p = 1.000$ and 0.243), AFAI = 401-430 days compared to AFAI > 430 days ($p = 0.147$), Age at first calving (AFC) = 646-670 days compared to AFC = 671-700, 701-730, 731-760, and >760 days ($p = 1.000$, 1.000, 1.000, and 0.007), AFC = 671-700 days compared to AFC = 701-730, 731-760, and > 760 days ($p = 1.000$, 1.000, and 0.007), AFC = 701-730 days compared to AFC = 731-760, and > 760 days ($p = 1.000$ and 0.002), AFC = 731-760 days compared to AFC >760 days ($p = 0.099$), Winter compared to Spring, Summer and Autumn ($p < 0.001$, < 0.001, < 0.001), Spring compared to Summer and Autumn ($p = 1.000$ and 1.000), Summer compared to Autumn ($p = 1.000$).

Associated factor with calving to service interval in Danish Holstein cows

The multivariate analysis identified the most illustrative model, comprising parity, AFC, and gender of the born calf (Table 2). This model accounted for 13.7% of the variance in the CSI among the investigated Danish Holstein cows. Parity was negatively correlated with CSI (coefficient = -11.7, 95% CI = -13.9 to -9.5; $p < 0.05$). Additionally, AFC demonstrated a significant negative association with the CSI (coefficient = -2.1, 95% CI = -3.6 to -0.7; $p < 0.05$). In the multivariate analysis, the gender of the born calf exhibited a significant relationship with CSI (coefficient = 5.4, 95% CI = 1.8 to 9.0; $p < 0.05$), with a longer CSI observed in cows calving a bull. The VIF ranged from 1.012 to 1.103, indicating the absence of multicollinearity among the model variables.

Table 2. Multivariate analysis of factors associated with calving to service interval in 699 Danish Holstein cows, Holdan Argo farm, Rødekro, Denmark in 2023

Variables	Coefficients/95% confidence interval	P-value	VIF
Constant	113.1 (107.0-119.1)	<0.001	
Parity	-11.7 (-13.9 - [-9.5])	<0.001	1.103
Age at first calving	-2.1 (-3.6 - [-0.7])	0.003	1.012
Gender of the calf	5.4 (1.8-9.0)	0.004	1.090

VIF: Variance inflation factor. The final model explained 13.7% of the variation in calving to first service interval in the investigated animals.

DISCUSSION

The current results revealed that parity, AFC, and gender of the born calf were the most crucial risk factors for CSI in Danish Holstein cows.

Parity effects

Multiparous cows typically exhibit a more stable endocrine-metabolic adaptation around calving compared to primiparous cows, which often encounter greater metabolic imbalance and extended periods of anestrus (Briano et al., 2024). Kim et al. (2009) noted that calving intervals shortened with increasing parity, with the highest reproductive efficiency observed in second-parity cows. This trend aligned with findings of Baek et al. (1998), who documented the shortest calving intervals in second-parity cows, indicating a peak in reproductive performance at this stage. Moreover, Briano et al. (2024) identified prolonged postpartum anestrus in primiparous cows, contributing to their extended calving intervals. These findings collectively highlighted the impact of parity on reproductive efficiency, emphasizing the extended intervals necessary for primiparous cows to resume cyclicity and complete subsequent reproductive processes after calving. This trend may be partially attributable to a reduced dystocia rate in older cows, which facilitates uterine recovery and reduced calving intervals (Gaafar et al., 2011). Nevertheless, older cows face an increased risk of conditions such as dystocia, retained placenta, metritis, and metabolic disorders (Rohmah et al., 2023), which may extend the CSI in higher-parity cows (Motlagh et al., 2013). Several studies indicated that the prevalence of these complications tends to increase remarkably only after the fourth parity, which could explain the observed rise in CSI after this point (Gabr et al., 2005). Inflammatory conditions such as subclinical mastitis can delay postpartum recovery and prolong the resumption of reproductive activity (Bacha and Regassa, 2010). Ghasemian and Asri-Rezaei (2024) reported elevated inflammatory markers such as serum amyloid A, interleukins IL-6 and IL-8 in cows with mastitis caused by *Escherichia coli* and *Staphylococcus aureus*, underscoring the role of systemic inflammation in reproductive inefficiency. The current findings suggest that postpartum multiparous cows generally return to estrus more rapidly than primiparous cows, likely attributable to physiological adaptations and improved reproductive efficiency acquired through successive calvings. Moreover, negative energy balance remains an important challenge in primiparous cows, particularly during their second lactation, as indicated by lower plasma glucose and higher β-hydroxybutyrate and urea levels (Cattaneo et al., 2023). These metabolic markers reflected increased body reserve mobilization and metabolic adaptation, which may contribute to prolonged postpartum anestrus and delayed reproductive recovery in younger cows.

Age at first calving

The negative association between AFC and CSI aligns with the findings of Mufti et al. (2010) and Khan et al. (2015). Cows that calved at a younger age (below 700 days or 23 months) displayed more substantial variation in lifespan compared to those calving between 671 and 823 days (22 to 27 months). The negative association between AFC and CSI highlighted the benefit of calving at a more mature age to support optimal body condition and reproductive health throughout a cow's life. Heifer's calving are more skeletally and metabolically developed, which lowers dystocia

risk, decreases postpartum disease, and eases negative energy balance. These benefits help accelerate uterine involution and restore ovarian cycles more quickly, resulting in a shorter CSI. On the other hand, early calving has been linked to reduced longevity (Haworth et al., 2008). In addition, Nilforooshan and Edriss (2004) found a small positive phenotypic correlation between AFC and lifetime in Iranian Holsteins, implying that delaying calving may help improve reproductive maturity and overall health, potentially extending productive lifespan.

Gender effects

Numerous studies have shown that the gestation period in dairy cows tends to be longer for pregnancies resulting in male calves compared to those resulting in female calves (Silva et al., 1992; Vieira-Neto et al., 2017). This discrepancy may be attributed to fetal weight, as male calves generally exhibit a higher birth weight than female calves, which could potentially result in an extended gestation period (Atashi and Asaadi, 2019). Extended gestation may influence maternal health by elevating the risk of complications and prolonging recovery, thereby extending the interval from calving to the first breeding (Mwangi et al., 2025). Cows that give birth to female calves generally recover their uterine function faster and return to estrus sooner than those birthing male calves (Atashi and Asaadi, 2019). This difference may be due to the larger size of male calves, which results in a longer gestation period and impacts uterine recovery after calving (Atashi and Asaadi, 2019). Additionally, serum estrogen levels are remarkably higher in cows giving birth to female calves than in those birthing male calves (Ibrahim et al., 2017). Estrogen is essential for postpartum uterine recovery, as it stimulates uterine muscle and endometrial activity (Sheldon and Dobson, 2004). Studies conducted by Gad et al. (2017) and Rezende et al. (2020) confirmed that the gender of the born calf substantially influences uterine characteristics, including lumen diameter, curvature, and involution rate. Specifically, cows giving birth to female calves indicated a shorter uterine involution period, while those with male calves experienced a slower return to estrus and delayed uterine involution (Atashi and Asaadi, 2019). Additionally, the higher rate of dystocia linked to male calves, mainly due to their larger birth weight and fetopelvic disproportion, further prolongs the CSI by exacerbating postpartum complications and delaying uterine recovery (De Amicis et al., 2018; Tsaousioti et al., 2024). Therefore, calf gender is a crucial factor influencing the CSI, with cows giving birth to male calves having longer CSIs.

Seasonal effects

The prolonged CSI observed in cows that calve during winter may be associated with increased metabolic demands during colder periods, potentially hindering postpartum recovery and delaying the estrous cycles. Exposure to low temperatures increases energy requirements (Roland et al., 2016; Fu et al., 2022; Wang et al., 2023), reduces feed utilization efficiency (Mota-Rojas et al., 2021), and disrupts hormonal balance (Fu et al., 2022; Lezama-García et al., 2022; Wang et al., 2023), thereby potentially extending CSI. Findings concerning beef cattle indicated that the calving season has considerable effects on the duration of postpartum anestrus. Specifically, cows calving during winter months (November to April) remained in anestrus for an average of 70.8 days, which was nearly twice as long as those calving during the summer months (May to October), with an average of 35.9 days (Peters and Riley, 1982). These findings were consistent with previous studies, which have demonstrated that cows calving in summer generally exhibit shorter CSIs compared to their counterparts calving in winter or spring (Santos et al., 2009). Echevarria et al. (2001) further emphasized the impact of calving season on CSI, with notable differences across different parity groups and seasonal calving cohorts. The seasonal fluctuations in CSI highlighted the importance of implementing targeted management practices to address reproductive challenges that occur during specific periods. Providing enhanced nutritional and reproductive support to winter-calving cows may reduce the duration of CSIs, thereby fostering improved reproductive outcomes and overall herd productivity.

Although the present study identified parity, AFC, and calf gender as the most critical determinants of CSI, other potentially influential factors, such as nutritional status, postpartum diseases, and management practices, were not assessed. Nevertheless, the present findings provided valuable insights into the key factors associated with CSI and contributed to a more comprehensive understanding of reproductive performance in dairy cattle.

CONCLUSION

The present study highlighted the significant effects of parity, AFC, and gender of the born calf at the CSI in Danish Holstein cows. The current findings indicated a significant reduction in CSI as parity increases to the third parity. However, CSI increased in cows with the fourth parity, indicating an age-related reversal in CSI trends beyond the third parity. These results suggested that reproductive management practices in Danish Holstein cows may be optimized by prioritizing an appropriate AFC and carefully monitoring multiparous cows to enhance reproductive outcomes. The present findings provided valuable insights into enhancing breeding and postpartum management strategies, which could

ultimately boost herd fertility and productivity in Holstein dairy systems. Future studies should incorporate nutritional status, body condition during the transition, common postpartum diseases such as metritis and mastitis, as well as metabolic disorders, along with management practices including heat detection efficiency, insemination timing, and housing systems, to provide a more comprehensive understanding of CSI determinants.

DECLARATIONS

Acknowledgments

The authors express their gratitude to Mr. Jacobus A.M. Broeders for his consent to the collection and publication of the herd's data.

Authors' contributions

Bui Van Dung prepared the initial draft of the manuscript. Le Thi Le Thuong collected data. Nguyen Hoai Nam analyzed data and designed the study. All authors reviewed and approved the final edition of the manuscript.

Funding

The present study received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Competing interests

The authors declared no conflicts of interest.

Availability of data and materials

All data and materials supporting the present study are available from the corresponding author upon reasonable request.

Ethical considerations

The authors declare that this manuscript is original and not under consideration for publication elsewhere. All ethical considerations, including consent to publish, research misconduct, data fabrication, and redundancy, have been thoroughly reviewed and approved by all authors.

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2025, Scienceline Publication
World's Veterinary Journal
World Vet J, 15(3): 790-801.
ISSN 2322-4568

DOI: https://dx.doi.org/10.54203/scil.2025.wvj81
PII: S232245682500081-15

Effects of Season and Hygienic Factors on Milk Microbiological and Physicochemical Characteristics in Livestock Communities of Ayacucho, Peru

Gloria Betti Adrianzen-Facundo[1]*, Julio Alberto Ruiz-Maquen[1], Arturo Rodríguez[2], Wilmer Moncada[3], Mardeli Meneses[1], and Krizzya Chávez[1]

[1]*Laboratorio Microbiología e Inmunología Veterinaria,Universidad Nacional de San Cristóbal de Huamanga, Ayacucho, Peru*
[2]*Laboratorio Reproducción y Biotecnología Animal, Universidad Nacional de San Cristóbal de Huamanga, Ayacucho, Peru*
[3]*Remote Sensing and Renewable Energy Laboratory, Universidad Nacional de San Cristóbal de Huamanga, Ayacucho 05000, Peru*

*Corresponding author's Email: gloria.adrianzen@unsch.edu.pe

ORIGINAL ARTICLE
Received: July 02, 2025
Revised: August 07, 2025
Accepted: September 03, 2025
Published: September 30, 2025

ABSTRACT

Climatic changes can influence milking practices and the dairy production chain, thereby influencing the microbiological safety and physicochemical quality of raw bovine milk (RBM), with potential implications for public health. The present study aimed to evaluate the effects of seasonal variability on the hygienic factors and characteristics of RBM produced in livestock communities in the Socos district, Ayacucho, Peru. For mesophilic aerobic bacteria (MAB) and total coliform bacteria (TCB), the compact dry plate method was used. Sixty random samples were collected during the rainy season (RS) and the dry season (DS). Samples were taken from milkers' hands (six in the RS and six in the DS), rags (six in the RS and six in the DS), buckets (six in the RS and six in the DS), receiving tanks (eight in the RS and eight in the DS), and distribution centers (four in the RS and four in the DS). To evaluate physicochemical characteristics, including density, fat, protein, total solids, lactose, pH, and mineral salts, eight samples of Brown Swiss cattle RBM were collected directly from the tanks (four in the RS and four in the DS) of two distribution point centers. The present results indicated that the MAB count for hygienic factors was lower in the DS than in the RS, except in the milker's hands and distribution centers. For the TCB count related to hygienic factors, the values were lower in the DS than in the RS, except in receiving tanks and distribution centers. In the RS, the physicochemical qualities complied with the Peruvian Technical Standard (PTS), except for protein content and mineral salt levels. In the DS, the physicochemical quality did not comply with the PTS; however, these properties were within acceptable limits in the receiving tanks for MAB and on the milker's hands for TCB. As a result, the bacterial load on inert surfaces and the characteristics of RBM were lower in the DS compared to the RS. Still, contamination during milking and at distribution centers was greater.

Keywords: Coliform, Hygienic safety, Mesophilic, Milk, Physicochemical quality, Season

INTRODUCTION

The livestock communities in the Socos district of the Ayacucho region in Peru are known for their milk production from Brown Swiss cattle. This activity represents a major economic effort that supports local traditions and culture while also contributing to regional food security. However, these raw bovine milk (RBM) production processes face significant limitations, such as the absence of appropriate technologies, limited access to livestock services, and a lack of technical and institutional support from local and regional authorities. These factors have negative effects on the management of livestock activities from milking to marketing, affecting the hygiene of RBM and compromising its microbiological and physicochemical quality (Nyokabi et al., 2021; Valdivia Avila et al., 2021). Likewise, changes in the diet of cattle during the rainy (RS) and dry seasons (DS) influence the nutritional quality of the RBM, since during the RS, access to fresh pasture improves the nutritional composition of RBM, which is reflected in enhanced physicochemical qualities of RBM (Páez et al., 2002; Petróczki et al., 2020). In both climatic stages, there is a variation in the bacterial count and physicochemical qualities of RBM. For production systems located in high-Andean areas of Peru, it was found that increased humidity during the RS is associated with significant increases in total coliform bacteria (TCB) and mesophilic aerobic bacteria (MAB) counts. At the same time, RBM sold in local markets exhibited low fat and lactose content, as well as a high pH during the RS, exceeding recommended limits and raising concerns regarding potential public health risks (Millogo et al., 2010).

Raw bovine milk consumption is a vital source of essential nutrients in the diets of consumers in rural communities, which is why it is necessary to maintain safety and nutritional quality standards from milking to marketing (Contero

To cite this paper: Adrianzen-Facundo GB, Ruiz-Maquen JA, Rodríguez A, and Moncada W (2025). Effects of Season and Hygienic Factors on Milk Microbiological and Physicochemical Characteristics in Livestock Communities of Ayacucho, Peru. *World Vet. J.*, 15(3): 790-801. DOI: https://dx.doi.org/10.54203/scil.2025.wvj81

et al., 2021). However, raw milk is a highly perishable product that undergoes rapid changes, either during transport or when stored improperly, and can cause gastrointestinal diseases in consumers (Perin et al., 2019). These vulnerabilities can be mitigated through appropriate technical assistance (Mendez and Osuna, 2007).

From a regulatory perspective, Legislative Decree 1062, the Food Safety Law in Peru, requires national, regional, and local authorities, as well as consumers and economic agents involved in the food supply chain, to cooperate in ensuring the safety of food for human consumption. It is worth noting that, out of the 2.3 million agricultural units, only 64% are livestock farms in the Peruvian highlands, as indicated in the 2012 Fourth National Agricultural Census conducted by INEI-Peru. Peruvian technical standard (PTS; N° 202.001.2003) established specific microbiological limits for LCB, MAB ≤ 10^6 CFU/mL, and TCB ≤ 10^3 CFU/mL (MINAGRI, 2017), which are necessary for assessing the hygienic safety and nutritional quality of RBM before marketing and industrialization, requiring producers to meet a minimum quality standard (Delgado-Callisaya et al., 2016; Feyisa et al., 2023). Therefore, countries regulated these critical physicochemical parameters for collection, shipment, and analysis, with slight differences in performance limits and available methods to ensure that RBM meets appropriate nutritional quality standards (Perin et al., 2019; Contero et al., 2021).

On the other hand, improper handling and sale of RBM in informal channels without temperature control can affect the proliferation of lactic acid bacteria, which rapidly acidify RBM. This is worsened by poor hygiene and low productivity, as small producers often have minimal resources, resulting in underdeveloped production (Knight-Jones et al., 2016). Therefore, hygienic safety should comply with the requirements of the minimum number of bacteria in RBM, as regulated by PTS (N° 202.001.2003), confirmed by Supreme Decree N° 007-2017-MINAGRI, for consumption by the population. The evaluation was performed by counting MAB and TCB throughout the production and marketing process. Milking processes, equipment cleaning and disinfection, milking facility conditions, environmental management of the barn, transportation, nutritional status of cattle, and health management are determining factors in the hygienic safety (Freitas et al., 2009; Chamchoy et al., 2022).

The evaluation of the physicochemical qualities of the RBM encompasses the measurement of total solids, fat, and protein contents, which are vital for evaluating nutritional value and suitability as a raw material in dairy product manufacturing derivatives. This ensures that RBM is safe for consumption and meets the quality standards required for marketing (Solanki and Rizvi, 2001; Park et al., 2007; Renhe et al., 2019).

Among the factors that influence the hygienic safety of RBM include the milking routine, disinfection of hands and equipment, the designated location for milking, cleaning pens free of cow feces, cleaning and drying teats, and feeding cows fresh forage, especially considering that, in most cases, the cows are underfed and do not yield the expected amount of milk with a balanced daily diet (Flórez, 2001). In extreme cases, some RBM traders manipulate milk by adding water either at the barn or during transportation (Brousett-Minaya et al., 2015; Jurado-Gámez et al., 2020), while others maintain poor animal health management practices (Neave et al., 2024; Mendez and Osuna, 2007), in addition to the climatic factors (Brousett-Minaya et al., 2015). Therefore, the present study aimed to assess the seasonal changes in factors affecting the hygienic safety and nutritional quality of RBM in livestock communities in the Socos district, Ayacucho, Peru.

MATERIALS AND METHODS

Ethical approval

The present study was conducted in accordance with the provisions of the Research Ethics Committee of the National University of San Cristóbal de Huamanga (RCU No. 068-2021-UNSCH-CU) and the Law on Animal Protection and Welfare in Peru (Law 30407) for samples that were collected directly from the milkers' hands, from the utensils, and from the collection tanks at distribution centers.

Study area

The Socos district is located in the Cordillera of the central Andes, in the province of Huamanga, Ayacucho Region, Peru, with an average altitude of 3,400 m above sea level (Figure 1). It covers an area of 81.75 km², which represents 0.18% of the Ayacucho region's total area. The high-Andean zone is characterized by three seasonal stages, including the RS with rainfall between December and February, gradually decreasing in March, the DS between May and August, and the intermediate season in April, September, October, and November. The ambient temperature in the high zone ranges from 2 to 13°C, whereas the low zone experiences temperatures between 18 and 21°C. The district is characterized as a grazing zone, with low to moderate vegetation, as indicated by a normalized difference vegetation index range of 0.2 to 0.6 (Moncada et al., 2022).

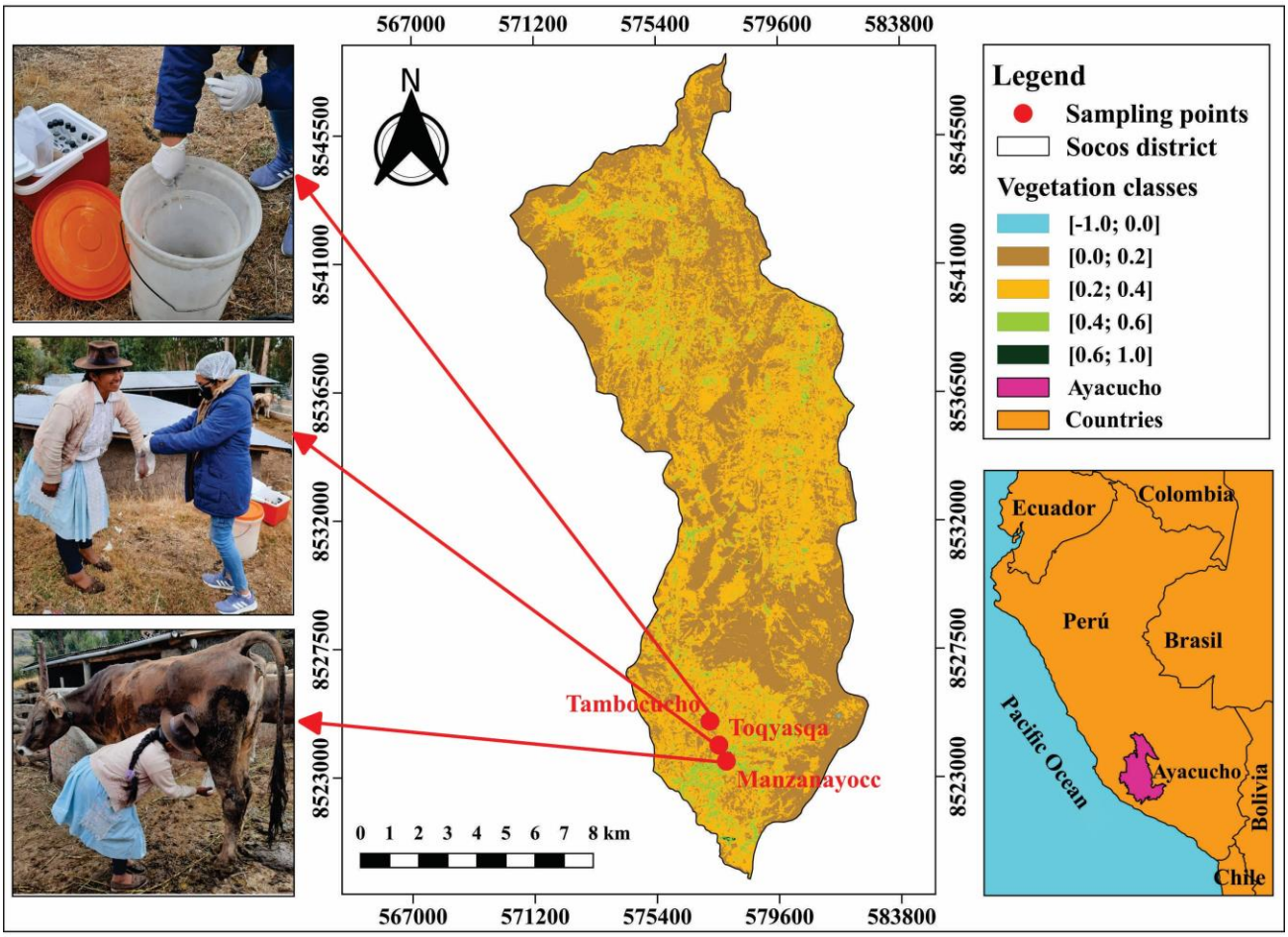


Figure 1. The geographical location of sampling for collecting raw bovine milk data in the towns of Tambocucho, Toqyasqa, and Manzanayocc in the district of Socos, Ayacucho, Peru

Raw bovine milk sampling

The sampling procedure during the RBM production chain, from milking to marketing, and the subsequent evaluation of the hygienic factors and physicochemical characteristics of RBM in the laboratory are illustrated in Figure 2. Initially, 30 RBM samples were collected in the RS (February 2021) and 30 RBM samples in the DS (August 2021) from farms located in the towns of Tambocucho, Toqyasqa, and Manzanayocc, in the district of Socos, Ayacucho, Peru. Samples were collected using the swab method from different sources, including the milker's hands (six in the RS and six in the DS), rags (six in the RS and six in the DS), buckets (six in the RS and six in the DS), receiving tanks (eight in the RS and eight in the DS), and distribution centers (four in the RS and four in the DS), for seasonal hygiene assessment. Additionally, eight RBM samples were obtained from the four teats of the Brown Swiss cow and stored in sterile 500 mL glass jars with lids for the seasonal evaluation of the nutritional quality of RBM.

The sampling method was random, with inclusion criteria for 30 cows in production and exclusion criteria for calves, heifers, dry cows, and cows older than eight years of age. The animals were fed a forage-based diet in extensive farming. The vaccination protocol was supervised by the National Animal Health Service (SENASA, Peru), which was responsible for conducting annual health checks on cattle in the Ayacucho region. Milking was performed manually, given that the average farm size was 50 head of cattle per farm (Gwandu et al., 2018).

The swabbing technique involved utilizing a moistened swab, dipped in 0.1% peptone water, which was systematically passed in a zigzag pattern over the surface of dry cloths arranged on the stones within the animal pen. This method was subsequently employed to sanitize the teats of the cow's udder. The swab was then placed back in the tube containing peptone broth, covered, and stored within an airtight container at 10°C. Additionally, swabs were collected from the inner surfaces of milking buckets and storage tanks at distribution centers (Knight-Jones et al., 2016).

In the lab, the swab inside the test tube was homogenized by rotating it with a vortex mixer. Subsequently, a micropipette was used to extract 1 mL of the homogenized peptone water sample, which was then placed on the compact dry plate for MAB and TCB, respectively. Both samples were incubated at 37°C for 48 hours for MAB and for 24 hours for TCB. Finally, the counts for MAB and TCB were determined utilizing the following formula.

$n \times f$ (CFU) Formula 1

n represents the number of typical colonies, f signifies the dilution factor, and CFU means colony-forming units (Mizuochi and Kodaka, 2000; Wafula et al., 2016).

The method illustrated in Figure 2 enabled the efficient and systematic replication of sampling under different seasonal conditions, facilitating the comparison of hygienic and nutritional quality factors among different samples of RBM collected during the RS and DS. This method was particularly relevant in artisanal LCB production systems, where variations in management and environmental conditions can affect both the hygienic safety and nutritional quality of LCB, from the milking process to its sale in retail outlets (Chiarlone et al., 2025).

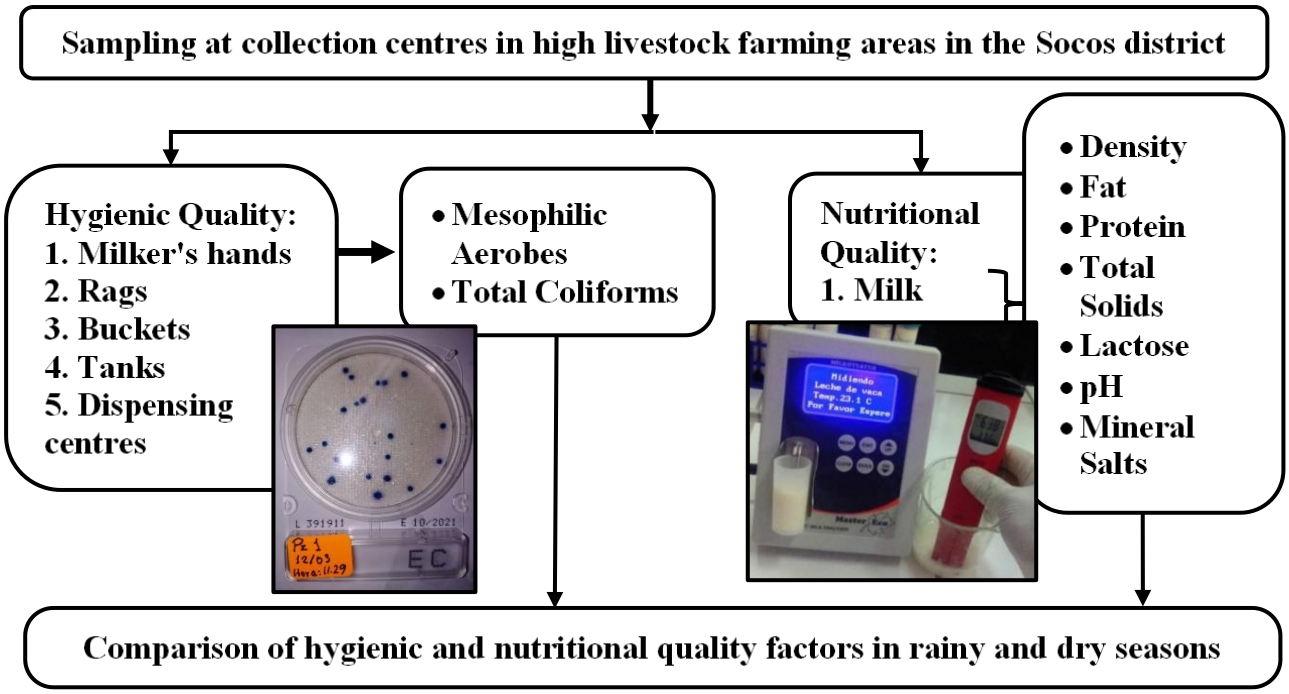


Figure 2. Sampling procedure based on the year and the factors affecting the hygienic safety and nutritional quality of raw bovine milk in the towns of Tambocucho, Toqyasqa, and Manzanayocc, located in Ayacucho, Peru

Hygienic safety

Milk performers were selected through targeted sampling, as the cow owners were responsible for milking their own cows. There was one milker per community, meaning that three milkers repeated or performed the same milking procedure twice in the RS and DS. The milkers resided in their own homes and managed their animals on a nearby farm (Deddefo et al., 2023). The processing of samples for assessing hygiene safety factors was conducted following the guidelines outlined in the technical guide for microbiological analysis of surfaces in contact with food and beverages, approved by resolution of the Ministry of Health (N° 461-2007-MINSA; PTS, 2016). Samples were collected from the hands of the three individuals responsible for milking, twice for each climatic stage, with six samples from the RS and six from the DS. The rinsing liquid was placed into a 150 mL bottle with a lid, then sealed and stored in an airtight container at a temperature not exceeding 10°C for storage, transportation, and laboratory analysis (Shehu et al., 2017).

The cloth rags (10 cm^2) used to clean the cow's udder teats belong to each person responsible for milking in a family group that owns a farm. Inappropriately, exposure of the rags to the environment in the animal pens causes them to become contaminated. After drying, cloth rags were reused to clean the teats of several cows on different occasions (Niboucha et al., 2022). Samples were taken from cloths belonging to different family groups before milking began (six in the RS and six in the DS), using a swab moistened with a diluting solution (0.1% peptone water), which was pressed lightly onto the inner surface of the test tube, pressing lightly with a rotating motion of the swab to remove excess solution. The swab was rubbed four times in a zigzag pattern on the surface of the dry cloth at a 30° angle, each time in the opposite direction to the previous one. The part of the swab containing the sample was deposited in a tube with the diluent solution, removing the part that had been in contact with the fingers. The samples were stored in an airtight container at a temperature not exceeding 10°C for preservation, transport, and analysis in the laboratory (Niboucha et al., 2022).

Plastic buckets, each with a capacity of 50 cm^3 and a volume of 20 liters, were used to collect the RBM during the milking process. These buckets were assigned to each individual responsible for milking a family group that owned a farm. Plastic buckets were thoroughly cleaned with detergent and bleach, rinsed, and subsequently placed on stones

within the animal pens to dry in the sun. Samples were taken from the buckets belonging to different family groups before milking began (six in the RS and six in the DS), in accordance with the procedure indicated in current regulations, using the swab method moistened in peptone water 0.1%, which was pressed lightly against the inner wall of the test tube with a rotating motion to remove excess solution. The swab was rubbed over the edges and internal surfaces of the bucket. The part of the swab containing the sample was placed in a tube with the diluent solution, removing the part that had been in contact with the fingers. The samples were stored in an airtight container at a temperature not exceeding 10°C for preservation, transport, and analysis in the laboratory (Adrianzen Facundo et al., 2024).

Samples were taken from the tanks before depositing the RBM inside them (six in the RS and six in the DS), using a swab moistened with peptone water 0.1 %, which was pressed lightly against the inner wall of the test tube with a rotating motion to remove excess solution. The swab was then rubbed on the inner surface of four tanks, from the base to the top of the neck, with circular movements, focusing more on the irregular surface with indentations. Two samples were taken from each tank for each climatic stage, resulting in a total of eight samples per stage. The part of the swab containing the sample was deposited in a tube with the diluent solution, removing the part that had been in contact with the fingers. The samples were stored in an airtight container at a temperature not exceeding 10°C for preservation, transport, and analysis in the laboratory (Adrianzen Facundo et al., 2024).

During the transportation of the RBM to the distribution centers, the intermediary or responsible individual for marketing the RBM positioned small frozen containers within the tanks to conserve the milk, which was transported approximately two hours after milking. The RBM was stored in 20-liter aluminum tanks, from which four samples from the RS and four from the DS, each measuring 250 mL, were extracted into sterile bottles at two ascertained points of sale supplied by the intermediary. The transfer of these samples to the laboratory took approximately 30 minutes. The bottles were properly labeled and stored within an airtight container containing refrigerant gel, ensuring a temperature not exceeding 10°C (Adrianzen Facundo et al., 2024).

Physicochemical quality of raw bovine milk

The physicochemical qualities of the RBM samples were determined using milk analysis equipment (Master Eco, Milkotester Ltd., Bulgaria). For this analysis, 6 mL of RBM was used to determine density (g/ml), fat (%), protein (%), lactose (%), total solids (%), and mineral salts (%). In addition, the pH of the RBM was determined using a digital pH meter (HI98107 Hanna, Mauritius). The results were evaluated using analysis of variance to identify potential differences between the two climatic periods under study (Geurts et al., 2017; Marini and Walczak, 2020).

Laboratory analysis

The samples were analyzed at the Microbiology Laboratory of the National University of San Cristobal of Huamanga, Peru. In the laboratory, the samples were stored at 2-4°C in a refrigerator for two hours. For processing, a stock solution of 90 mL of peptone broth 0.1% with 10 mL of RBM from each sample was prepared, then a dilution of 10^3 was obtained for the TCB count on compact dry EC plates (24 hours in an incubator at 37°C) and the dilution 10^4 for the MAB count on compact dry TC plates (48 hours in an incubator at 37°C). At the end of incubation, the microorganism colonies were counted using Formula 1. The results were expressed in CFU/ml and compared with the standard values of PTS: 202.001, using the rapid colony counting method with compact dry plates (Adrianzen et al., 2024). The regulation stated that the maximum allowable limits for MAB should not exceed 1 million CFU/mL, and for TCB, it should be 1,000 CFU/mL (PTS: N° 202.001.2003). In the absence of PTS, the Mexican Official Standard (MOS: N° 093-SSA1-1994) was applied to live and inert surfaces in contact with food (Pérez et al., 2016).

Statistical analysis

The measures of central tendency helped determine the mean and range of TCB and MAB counts. One-factor analysis of variance (ANOVA) was used to assess statistical differences between laboratory data and current regulations ($p < 0.05$). A descriptive comparison of changes between climatic periods (RS and DS) was made. All analyses used Excel 2019. Physicochemical results were evaluated using ANOVA ($p < 0.05$) to identify differences between the two climatic periods studied (Marini and Walczak, 2020).

RESULTS AND DISCUSSION

Hygienic safety

The MAB counts on the milker's hands indicated a significant decrease in the RS (20,517 CFU/ml) compared to the DS (1,067,933 CFU/surface; $p < 0.05$; Table 1). However, both values greatly exceeded the limit established by MOS-093 (< 3000 CFU/mL), indicating an apparent failure in milking hygiene. Applying a universal limit of 3000 CFU/mL

without considering the contact surface area might underestimate the actual risk of contamination (Aliyo and Teklemariam, 2022). In rags, the MAB count was higher in the RS (533,333 CFU/mL) than in the DS (6,700 CFU/mL), with values higher than those established by NOM-093 (< 400 CFU/surface; $p < 0.05$). In milking buckets, the count was significantly higher in the RS (640,000 CFU/mL) compared to the DS (3,908 CFU/mL), with values well above those established by MOS-093 (< 400 CFU/mL; $p < 0.05$). The present results indicated that the sanitary conditions in the milking equipment were poor. Since the PTS (RM-461-2007/MINSA) lacked specific standards for food and beverage samples, these MOS guidelines were used as an alternative (Aliyo and Teklemariam, 2022). In contrast, for samples collected from RBM receiving tanks, the MAB count was higher in the RS (409,050 CFU/mL) than in the DS (188,813 CFU/mL); however, these values in the tanks were within the PTS range ($p < 0.05$). Similarly, for RBM samples at distribution centers, the MAB count in the RS (132,500 CFU/mL) complied with the PTS ($p > 0.05$). In contrast, the count in the DS (1,080,050 CFU/mL) significantly exceeded the PTS limit ($p < 0.05$). A significant difference in MAB counts was observed between the RS and DS, potentially attributable to the elevated ambient temperature during the RS ($p < 0.05$). In contrast, the influence of sanitary emergency measures related to the pandemic (SARS-CoV-2, February 2021) may have contributed to strict adherence to cleanliness in the utensils used for selling dairy products during the RS (Adrianzen-Facundo et al., 2024).

Table 1. Mesophilic aerobic bacteria counts and total coliforms in milking equipment and utensils as risk factors for milk safety in two seasons in the district of Socos, Ayacucho, Peru

Sampling zones	Mesophilic aerobic bacteria (MAB)					Total coliform bacteria (TCB)				
	PTS	RS	Complies	DS	Complies	PTS	RS	Complies	DS	Complies
Milker's hands	*< 3000	20,517	No	1,067,933	No	<100	10,375	No	82	Yes
Nipple cleaning cloths	*< 400	533,333	No	6,700	No	<10	12,850	No	1,303	No
Milking buckets	*< 400	640,000	No	3,908	No	<10	1,475	No	393	No
Receiving tank	<1,000,000	409,050	Yes	188,813	Yes	<1,000	5,323	No	7,711	No
Milk dispensing centre	<1,000,000	132,500	Yes	1,080,050	No	<1,000	2,688	No	199,050	No

Note: Six hand rinse samples and swabs from rags and buckets, eight samples from tanks, and four samples of milk were collected from the milk dispensing centres for each climatic season. Complies with regulations: Yes or No. PTS: Peruvian technical standard 202.001.2003 (CFU/ml). RS: Rainy season; DS: Dry season.

Regarding the TCB counts, the sampled zones indicated values above the standard criteria established by the PTS, highlighting that the results during the RS were higher than those of the DS ($p < 0.05$), except for the milker's hands during the DS (82 CFU/mL), which complied with the PTS standards ($p > 0.05$). A significant difference was observed in the majority of the results; the standards established by the PTS were not met ($p < 0.05$). The lack of compliance probably resulted from inadequate staff hygiene practices and exposure to heavily contaminated environments, such as pens soiled with cow feces. Therefore, these data revealed a concerning pattern of surface microbial contamination and related factors throughout the entire process from milking to marketing, posing a significant risk of foodborne illnesses to consumers.

Although the emergency health measures implemented during the SARS-CoV-2 pandemic promoted recommendations for frequent handwashing, evidence indicated that these practices should have improved, considering samples were collected in February 2021. Nonetheless, these practices were not effectively adopted in rural areas, highlighting ongoing deficiencies in hygiene habits at the primary production level (Chiarlone et al., 2025).

The variation in MAB and TCB counts between the DS and the RS is shown in Figure 3. In samples collected from the milker's hands, the yellow bars indicated a notable increase in MAB during the DS (1,047,417 CFU/mL) compared to the RS ($p < 0.05$). A similar pattern was observed for retail outlets, where BAM counts increased significantly (947,550 CFU/mL) during the DS compared to the RS ($p < 0.05$). These results suggested that direct handling of RBM and hygienic conditions at sales outlets were critical factors for bacterial proliferation, consistent with the findings of Lingathurai and Vellathurai (2013), who identified the milker's hands and utensils as primary sources of microbial contamination in RBM.

However, for samples collected from the surfaces of rags used during milking, there was a significant decrease in MAB (-526,633 CFU/mL) in the DS compared to the RS ($p < 0.05$). Similarly, for samples obtained from buckets, there was a notable decrease in MAB (-636,092 CFU/mL) in the DS compared to the RS ($p < 0.05$). The same trend was observed in samples taken from RBM receiving tanks, where the MAB count demonstrated significant reductions (-220,238 CFU/mL) in the DS compared to the RS ($p < 0.05$). This observation may result from frequent replacement of milking utensils and materials during the DS, which could lead to a decrease in MAB, as indicated by Brousett-Minaya et al. (2015) in RBM production systems in Andean regions, Peru. Regarding the variation in TCB counts between the

DS and the RS, there was a significant increase in TCB only in the distribution centers (196,363 CFU/mL) during the DS compared to the RS ($p < 0.05$), as indicated by the light blue bars. This pattern suggested that post-milking handling and exposure to poorly controlled environmental conditions at retail outlets were the major factors contributing to TCB contamination. Similar studies in Peru and Ecuador have demonstrated that the marketing phase was a critical point in the milk safety chain, where significant increases in BCT and *Escherichia coli* have been reported (Albuja Landi et al., 2021). In contrast, significant decreases in TCB counts were observed on the milkers' hands (-10,293 CFU/mL), rags (-11,547 CFU/mL), buckets (-1,082 CFU/mL), and RBM receiving tanks (-2,388 CFU/mL) during the DS compared to the RS ($p < 0.05$). The present results aligned with those reported by Bonfoh et al. (2003), who stated that seasonal variability and hygiene practices during milk handling are key factors influencing the presence of TCB, with lower contamination observed in seasons with reduced relative humidity.

Finally, it is important to note that in high-Andean communities, the hygiene practices of individuals responsible for milking were frequently inadequate, owing to the low temperatures that hinder the frequent washing of hands and utensils. This finding aligned with those of Hassen et al. (2022), who reported that the availability of warm water and environmental conditions serve as limiting factors for adherence to proper hygiene practices in traditional RBM production. These factors, along with a lack of infrastructure and training, contribute to the variability observed in bacterial counts between the DS and the RS.

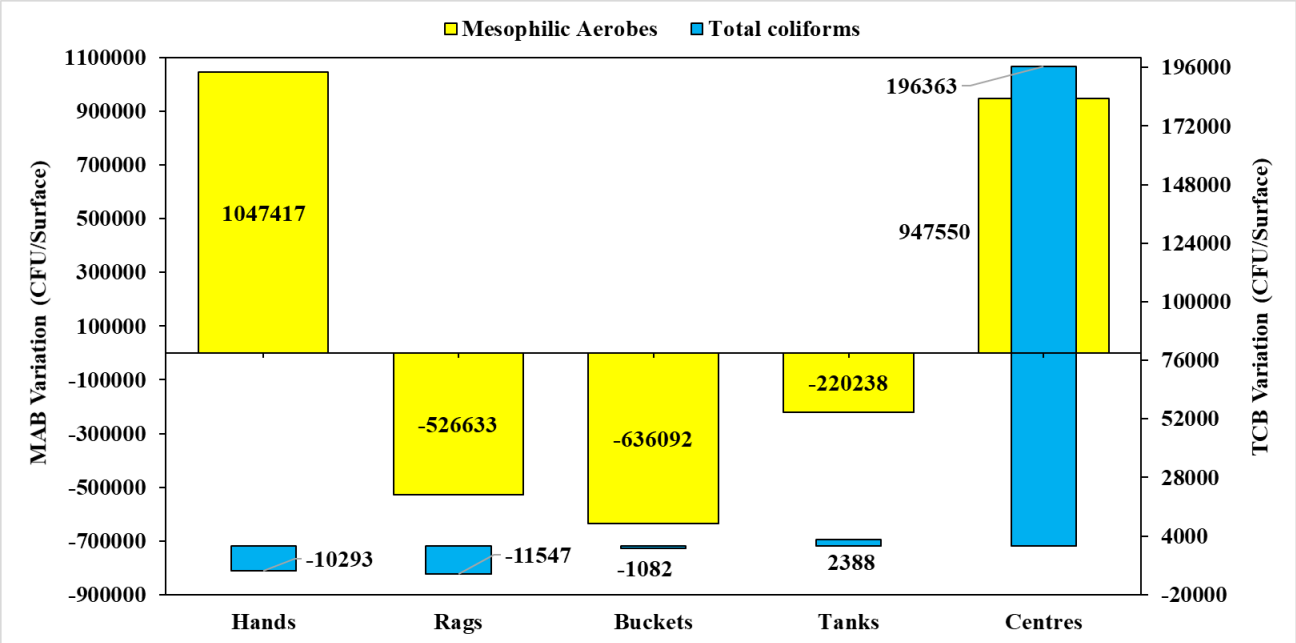


Figure 3. Variation of mesophilic aerobic bacteria and total coliform counts between the rainy season (RS: February 2021) and the dry season (DS: August 2021) in surface samples in the high livestock area of the Socos district, Ayacucho, Peru

Physicochemical characteristics

Table 2 presents a comparison of the mean values of the physicochemical characteristics of RBM (n=120 samples) concerning the parameters outlined by PTS (N° 202-001-2003) as well as additional parameters established in the studies conducted by Zavala (2005), Barbieri (2006), Viera (2013), Zevallos (2023), and Zevallos (2023) in accordance with climatic seasonality.

Regarding density, the mean RBM value during the DS (1.0290 g/mL) was marginally below the minimum threshold specified by the PTS (1.0296 g/mL); nonetheless, no statistically significant differences were observed between the two climatic periods ($p > 0.05$). The current findings are consistent with the values (1.0293 g/mL) reported in the Andean region of Junín, Peru, by Viera (2013). Still, the current results were lower than those documented by Brousett-Minaya et al. (2015) during the DS in Puno, Peru (1.030 g/mL) across eight sampled basins, as well as by Martínez and Díaz (2016) in Colombia (1.031 g/mL).

Regarding the percentage of raw bovine milk fat (RBMF), the average of RBMF in the RS (3.17%) complied with the PTS threshold (≥ 3.2%). In contrast, in the DS (3.03%), RBMF was below ≥ 3.2%, although without a statistically significant difference ($p > 0.05$). This decrease in the DS could potentially influence the technological quality of milk used in producing dairy products that require adequate fat content for optimal texture and flavor. Notably, the percentage of LCB fat in the RS, located in the high-Andean livestock areas of the Socos district, was slightly lower than the

findings of Viera (2013), who recorded 3.73% in Junín, and by De la Sota (2016), who documented 3.72% in Junín. These RBMF results suggested that the reduced fat content may be attributable to a low-fiber pasture diet with limited energy intake, which aligned with the findings of Were et al. (2025) concerning the relationship between forage nutritional quality and milk lipid profile.

Table 2. Comparison of the physicochemical characteristics of the raw bovine milk in the district of Socos, Ayacucho, Peru, during the rainy and dry seasons

Parameters	PTS	Rainy season			Dry Season			p-value (0.05)
		Average	Complies	Limits LL - UL	Average	Complies	Limits LL - UL	
Density[1] (g/ml)	Min. 1.0296 Max. 1.0340	1.0297	Yes	1.0291 - 1.0303	1.0290	No	1.0284 - 1.0297	0.25
Fatty matter[1] (%)	Min. 3.2	3.17	Yes	3.12 - 3.22	3.03	No	3.03 - 3.05	0.27
Protein[2] (%)	3.48	3.46	No	3.45 - 3.47	3.44	No	3.43 - 3.44	0.44
Total solids[1] (%)	Min. 11.4	11.416	Yes	11.411 - 11.421	11.335	No	11.332 - 11.338	0.35
Lactose [2] (%)	5.08	5.09	Yes	5.08 - 5.10	5.04	No	5.03 - 5.05	0.27
pH[3]	6.60	6.60	Yes	6.60 - 6.60	6.56	No	6.56 - 6.56	0.00
Mineral salts[2] (%)	0.60	0.61	No	0.61 - 0.62	0.58	No	0.58 - 0.59	0.00

[1]PTS: Peruvian technical standard 202.001.2003 (CFU/ml, PTS-202.001.2003), [2]Zevallos (2023), [2]Viera (2013), [2]Barbieri (2006), [3]Zevallos (2023). Lower Limit: LL. Upper Limit: UL. Complies with regulations: Yes or No.

Regarding the protein content in RBM, the average values for both climatic seasons (3.44% and 3.46%) were below the reference values reported by Zevallos (2023). Although no statistically significant differences were observed ($p > 0.05$), a marginal advantage was apparent in the RS, likely due to the greater availability of green pastures, consistent with the findings of Evers et al. (2023). Studies in high-Andean regions emphasized that seasonality and nitrogen deficiency in forage are key factors affecting protein synthesis in RBM (Calsamiglia et al., 2010).

The total solids in the RBM indicated compliance with the PTS in the RS (11.416%) but not in the DS (11.335%), which were below these limits. Although there were no statistical differences ($p > 0.05$), a decrease was observed during the dry season, aligning with the findings of Altieri and Nicholls (2008), who linked the reduction in solids to intense frosts, lower precipitation, and low-quality forage. The current results were lower than those reported by Viera (2013), Brousett-Minaya et al. (2015), and De la Sota (2016), indicating a common pattern in high-Andean regions facing climatic stress, such as extreme cold or frost.

Regarding the percentage of lactose in RBM, the content during the RS (5.09%) slightly exceeded the expected value (5.08%) established by Zevallos (2023), but was lower during the DS (5.04%). Although the differences were not significant ($p > 0.05$), the decline in the DS could be related to acidification phenomena during transport and storage, where bacterial fermentation reduces lactose, possibly due to changes in ambient temperature or prolonged exposure to sunlight (O'Connell et al., 2016). The current values were higher than those reported by Viera (2013) in Junín, Peru, who obtained 4.17% during the DS, indicating that RBM may undergo an acidification process during transport and storage. It was estimated that the pH of RBM remained stable during the RS (6.60), which complied with the quality standards outlined by Barbieri (2006) and Zevallos (2023). In contrast, a significant decrease was observed during the DS (6.56; $p < 0.05$). This slight decrease was associated with fermentation processes and increased bacterial proliferation under conditions of lower forage availability and greater heat stress (Barbieri, 2006).

On the other hand, the percentage of mineral salts was significantly higher in the RS (0.61%) than in the DS (0.58%), consistent with the findings reported by Zevallos (2023) and possibly reflecting the influence of the mineralized diet and water availability during the RS. In both cases, significant differences were found between the two climatic periods ($p < 0.05$).

The values of physicochemical characteristics of RBM decreased from RS to DS, possibly due to the lower quality and availability of pasture in the DS (Figure 4; Mamani et al., 2011). Overall, seasonal climate variations in the high-Andean region of Ayacucho, Peru, had different effects on the physicochemical parameters of RBM, with noticeable decreases in fat, total solids, lactose, and pH during the DS. This pattern aligned with reports from other high-Andean ecosystems in Peru and Colombia, where variation in forage nutritional quality mainly explains seasonal changes in RBM (Viera, 2013; Calsamiglia et al., 2020).

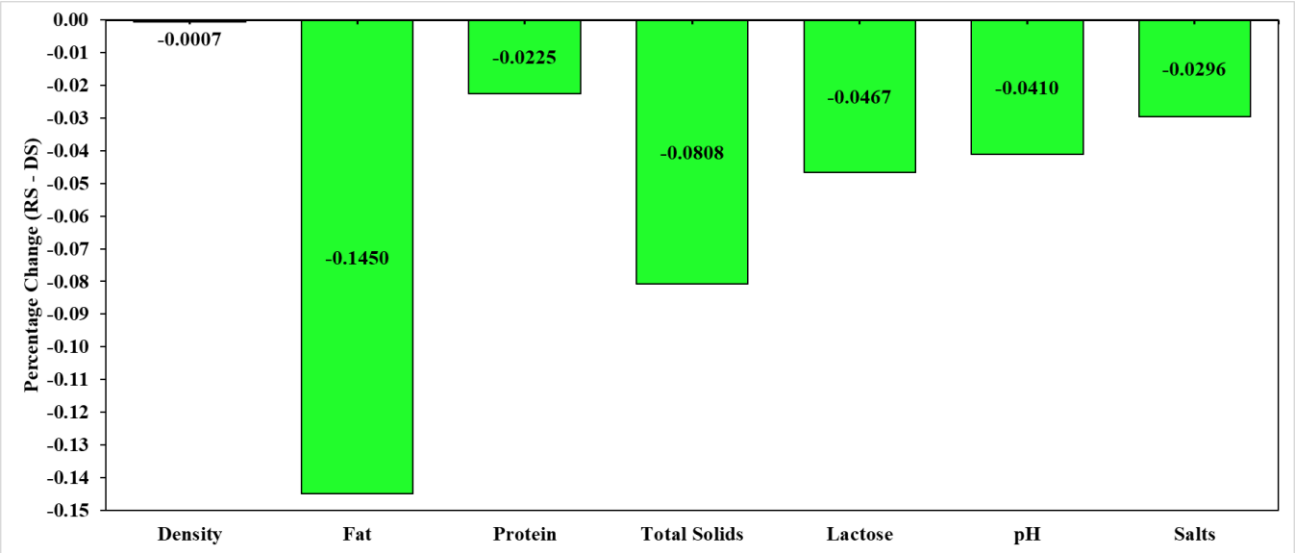


Figure 4. Percentage change of the physicochemical characteristics of raw bovine milk between the rainy (RS: February 2021) and dry (DS: August 2021) seasons in the surface samples in the high livestock area of the Socos district, Ayacucho, Peru

CONCLUSION

The count of mesophilic aerobic bacteria (MAB) on inert surfaces such as rags, buckets, and receiving tanks utilized in high-Andean livestock communities in Socos, Ayacucho, Peru, was significantly lower during the dry season (DS), indicating a possible inhibitory influence of diminished environmental humidity on bacterial growth. Conversely, samples from the milker's hands and raw bovine milk (RBM) at commercial outlets exhibited the highest bacterial counts during the DS, thereby highlighting human handling and post-milking procedures as critical sources of contamination. Regarding the counts of total coliform bacteria (TCB) within the sampled areas, reduced levels were observed during the DS, except in retail outlets. The failure of most results to conform to the thresholds established by the Peruvian Technical Standard (PTS; N° 202.001.2003) or the Mexican Official Standard for surfaces in contact with food (MOS N° 093-SSA1-1994) indicated inadequate hygiene practices during the production process, thereby posing a risk to public health. Concerning nutritional quality, the physicochemical qualities of the RBM were adversely affected during the DS. Key parameters such as density, fat content, total solids, lactose, and pH largely comply with regulatory standards, particularly during the rainy season (RS). Nonetheless, mineral salts exhibited significantly lower values during the DS compared to the RS, failing to meet the reference threshold. This decline in compositional quality was directly attributable to the reduced availability, quality, and nutritional value of forage typical of high-Andean ecosystems during the DS. Therefore, seasonality was a key modulator that interacted with management practices, microbiological risks, and the nutritional value of RBM, with direct implications for food security and the economy of high-Andean livestock communities. It is recommended that future studies use a multi-year (longitudinal) design and include other high-Andean communities. This would help confirm the observed patterns, assess the long-term effects of climate change, and develop predictive models for quality and safety.

DECLARATIONS

Funding

The present study was funded by the Research and Innovation Unit of the National University of San Cristobal de Huamanga (UNSCH), Peru, under the fund No. (RR-1327-2016-UNSCH-R).

Availability of data and materials

Data from the present study is available upon reasonable request.

Acknowledgments

The authors would like to thank the high cattle-raising communities of the Socos district, Ayacucho, for the facilities provided for sampling and data collection. To the Laboratories of Microbiology and Veterinary Immunology, Laboratory of Animal Reproduction and Biotechnology, and Laboratory of Remote Sensing and Renewable Energies of the UNSCH, Ayacucho, Peru.

Competing interests

The authors declared no conflict of interest.

Authors' contributions

Gloria Adrianzén and Julio Ruiz planned the experiment and conducted the fieldwork for sample collection. Wilmer Moncada and Arturo Rodríguez interpreted and statistically analyzed the data and results for the manuscript. Mardeli Meneses and Krizzya Chávez contributed to the field sampling and laboratory analysis. All authors of the revised articles read and approved the final edition of the manuscript.

Ethical considerations

All authors have thoroughly checked and confirmed ethical concerns regarding the originality of the data collected and analyzed before submitting it to the journal. The original article, after checking for plagiarism, was submitted to the journal.

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2025, Scienceline Publication
World's Veterinary Journal
World Vet J, 15(3): 802-810.
ISSN 2322-4568

DOI: https://dx.doi.org/10.54203/scil.2025.wvj82
PII: S232245682500082-15

Detection and Genetic Analysis of Feline Coronavirus in Clinically Suspected Cats

Fia Amalia[1], Madarina Wasissa[2], Siti Isrina Oktavia Salasia[2]*, and Fajar Budi Lestari[3]

[1]*Laboratory of Surgery and Radiology, Faculty of Veterinary Medicine, University of Brawijaya, Malang, Indonesia*
[2]*Department of Clinical Pathology, Faculty of Veterinary Medicine, University of Gadjah Mada, Yogyakarta, Indonesia*
[3]*Department of Bioresources Technology and Veterinary, University of Gadjah Mada, Yogyakarta, Indonesia*

*Corresponding author's Email: isrinasalasia@ugm.ac.id

ORIGINAL ARTICLE
Received: July 08, 2025
Revised: August 10, 2025
Accepted: September 07, 2025
Published: September 30, 2025

ABSTRACT

Feline coronavirus (FCoV) is a common infection in cats, producing outcomes that differ from mild intestinal disease to the fatal form known as feline infectious peritonitis (FIP). Accurate antemortem diagnosis is challenging, and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) targeting the *nucleocapsid* (*N*) gene offers high sensitivity and specificity, while phylogenetic analysis reveals viral variation. The present study aimed to detect FCoV in clinically suspected cats using RT-qPCR and to analyze the phylogenetic relationships of local isolates from Yogyakarta, Indonesia. A total of 45 clinical samples, consisting of 14 ascitic fluid and 31 blood samples, were collected from cats of different breeds (mixed, domestic shorthair, and Persian), aged 0.5-4 years, including 22 males and 23 females, that were presented to seven veterinary clinics in Yogyakarta with clinical signs such as vomiting, diarrhea, abdominal effusion, uveitis, and ataxia. The RT-qPCR revealed 62.2% (28/45) positive cases, with detection rates of 100% (14/14) in ascitic fluid and 45.2% (14/31) in blood. The most frequent clinical findings among FCoV-positive cats were abdominal distension (50%), uveitis (32.1%), ataxia (21.4%), and rhinitis (17.9%). Most infected cats (71.4%) were under two years of age, with a slightly higher prevalence in males (57.1%). Phylogenetic analysis of three isolates from ascitic fluid demonstrated a close genetic relationship with strains from Italy, the United Kingdom, and China, clustering into two distinct clades. The present study highlighted the utility of RT-qPCR targeting the *N* gene as a reliable diagnostic tool for clinical cases, while providing new clinicopathological and molecular data on naturally occurring FCoV infection in Indonesia. These findings contribute to the global understanding of FCoV molecular epidemiology and support future surveillance and control strategies for coronavirus infections in domestic cats.

Keywords: Cat, Feline coronavirus, Feline infectious peritonitis, Phylogenetic analysis

INTRODUCTION

Feline coronavirus (FCoV) belongs to the genus Alphacoronavirus in the family Coronaviridae, order Nidovirales (Theerawatanasirikul et al., 2020), and represents an enveloped, positive-sense, single-stranded RNA virus with a genome size of approximately 27 to 32 kb. The viral genome encodes major structural proteins, including *S*, *M*, *E*, and *nucleocapsid* (*N*), a gene that is highly conserved across strains and abundantly expressed during infection (de Barros et al., 2022; Gao et al., 2023; Shi et al., 2024). This virus is classified into two serotypes, including serotype 1, which predominates in naturally infected cats, and serotype 2, a recombinant form originating from FCoV type 1 and canine coronavirus (Tasker, 2018). Serotyping is primarily based on variations in the *spike* (*S*) gene, which plays a key role in viral entry into host cells. Within each serotype, FCoV occurs in two biotypes, including feline enteric coronavirus (FECV), which usually causes mild self-limiting enteritis, and feline infectious peritonitis virus (FIPV), a virulent variant arising from mutations that allow monocyte and macrophage infection and lead to systemic disease (Pedersen, 2009; Felten and Hartmann, 2019). Approximately 10-12% of FCoV infections can progress to feline infectious peritonitis (FIP), a fatal immune-mediated condition (Tasker et al., 2023).

Feline coronavirus infection is often asymptomatic or presents with mild gastrointestinal signs such as diarrhea and vomiting (Felten et al., 2022). In contrast, FIP occurs in two clinical presentations, including the effusive (wet) form and the non-effusive (dry) form (Tasker et al., 2022). The effusive form is characterized by protein-rich fluid accumulation in the abdominal or thoracic cavities, whereas the non-effusive form is associated with granulomatous inflammatory lesions affecting different organs, particularly the eyes, central nervous system, and abdominal organs (Tasker, 2018; Tasker et al., 2022). Clinical signs of the dry form may include uveitis, ataxia, nystagmus, and chronic rhinitis (Andre et al., 2020; Tasker et al., 2022).

To cite this paper: Amalia F, Wasissa M, Salasia SIO, and Lestari FB (2025). Detection and Genetic Analysis of Feline Coronavirus in Clinically Suspected Cats. *World Vet. J.*, 15(3): 802-810. DOI: https://dx.doi.org/10.54203/scil.2025.wvj82

Antemortem diagnosis of FCoV-associated conditions, particularly FIP, remains challenging because clinical signs often overlap with those of other feline diseases, and conventional diagnostic tools have limited sensitivity and specificity (Tasker et al., 2022). Although immunohistochemistry for detecting viral antigens in tissues is considered the gold standard, it is invasive and unsuitable for routine clinical use (Felten and Hartmann, 2019). Molecular diagnostics such as reverse transcription quantitative polymerase chain reaction (RT-qPCR) are now widely applied due to the high sensitivity and specificity for FCoV detection (Barker and Tasker, 2020). Several RT-qPCR assays have been developed that target different regions of the viral genome, including the *spike* (*S*) gene, the 3′ untranslated region (3′UTR), and the *N* gene (de Barros et al., 2022; Gao et al., 2023; Shi et al., 2024). Among these, the *N* gene is considered a reliable target because it is highly conserved across different FCoV strains and is abundantly transcribed during viral replication, thereby increasing assay sensitivity (Guan et al., 2020; Gao et al., 2023; Shi et al., 2024). Recent studies, including those from Guangxi, China, and northern Vietnam, have successfully employed RT-qPCR assays that include the N gene among conserved targets, demonstrating its stability and diagnostic performance across diverse geographical isolates (Dong et al., 2024; Shi et al., 2024).

Beyond molecular detection, phylogenetic analysis plays a pivotal role in understanding the genetic diversity and evolutionary relationships of circulating strains, thereby supporting epidemiological surveillance and informing preventive measures (Decaro and Lorusso, 2020). In Indonesia, reports on the molecular epidemiology of FCoV remain limited. Wasissa et al. (2021) detected FCoV in clinically suspected FIP cases and identified two genetic clusters of local isolates. Aksono et al. (2023) analyzed FCoV isolates in Surabaya, Indonesia, and demonstrated genetic similarities with strains from Europe and North America. More recently, Fitrawati et al. (2025) reported the detection of FCoV in imported cats, highlighting the risk of cross-border introduction.

Despite significant advances, comprehensive molecular characterization of naturally occurring FCoV cases in high-density pet populations, such as those in Yogyakarta, Indonesia, remains rare. Addressing this knowledge gap is essential not only for regional veterinary diagnostics but also for advancing the global comprehension of FCoV diversity and evolution. Based on the most recent available information, this investigation is the first study to combine clinical observations with RT-qPCR detection and phylogenetic analysis of naturally occurring FCoV infections in domestic cat in Indonesia. Therefore, the present study aimed to detect FCoV in clinically suspected cats in Yogyakarta, Indonesia, using RT-qPCR targeting the *N* gene and to perform phylogenetic analysis of local viral isolates.

MATERIALS AND METHODS

Ethical approval

All experimental procedures were approved by the Research Ethics Committee of the Faculty of Veterinary Medicine, Universitas Gadjah Mada, Indonesia (00113/EC-FKH/Int./2021). Informed consent was obtained from both veterinarians and cat owners before sample collection.

Study duration and location

The present study was conducted from June 2021 to September 2022 at the Clinical Pathology Laboratory, Faculty of Veterinary Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia.

Sample collection

A total of 45 clinical samples (14 ascitic fluid, 31 blood) were obtained from cats of different breeds (mixed, domestic, Persian), aged 0.5 to 4 years (22 males, 23 females), with signs of FCoV-associated disease (vomiting, diarrhea, abdominal effusion, uveitis, and ataxia). Samples were collected from 7 veterinary clinics in Yogyakarta, Indonesia. With the consent of owners, cats underwent clinical examinations, anamnesis, and sample collection conducted by veterinarians. Blood samples (2 mL) were drawn into tubes containing EDTA, while effusion fluids were collected in sterile tubes. All samples were subsequently processed and analyzed at the Clinical Pathology Laboratory, Faculty of Veterinary Medicine, University of Gadjah Mada, Yogyakarta, Indonesia.

Reverse transcription-quantitative polymerase chain reaction for feline coronavirus detection

Viral RNA was extracted using the Quick-RNA Viral Kit (Zymo Research, California, USA) according to the manufacturer's protocol. All RNA samples were stored at -40°C before analysis. Feline coronavirus detection was conducted using RT-qPCR targeting the *N* gene, with the following specific primers including forward 5′-TGCTTCGGCTAACTTTGGTG-3′ and reverse 5′-CAATCATCTCAACCTGTGTGTCAT-3′ (Guan et al., 2020).

The RT-qPCR reaction was performed in a total volume of 20 µL, consisting of 10 µL One Step SYBR Green RT-qPCR buffer 2X, 0.8 µL ABScript II One Step Enzyme Mix, 0.8 µL of forward and reverse primers, 0.4 µL ROX Dye

50X, 6 µL RNAse-free ddH_2O, and 2 µL of template RNA. Amplification was performed using a Bio-Rad CFX96 PCR machine (Bio-Rad, California, USA) as follows. Reverse transcription was carried out at 50°C for 5 minutes, followed by initial denaturation at 95°C for a minute. The reaction then proceeded through 40 cycles of denaturation at 95°C for 15 seconds and annealing at 60°C for 31 seconds, concluding with a melting curve analysis. Samples were considered positive for FCoV when the cycle threshold (Ct) was <35 and the melting curve peaked at 80°C (± 5°C; Sun et al., 2021). Each RT-qPCR and RT-PCR run included positive and negative controls to ensure assay reliability. The positive control (K+) consisted of RNA from a 2-year-old female cat with ascites previously confirmed as FCoV-positive, which consistently amplified the *N* gene and 5'UTR. The negative control (K−) consisted of nuclease-free water to monitor for potential contamination. A negative biological control using RNA from Specific Pathogen Free cats was not included in this study.

Phylogenetic analysis

To investigate the genetic characteristics of local FCoV circulating in Yogyakarta, five RT-qPCR-positive samples were selected for DNA sequencing. Selection criteria included high viral load (lower Ct values) and diversity in clinical presentation and sample type, including blood and ascitic fluid, to ensure representative coverage of positive cases. Conventional RT-PCR was then performed on these samples using the same primer pair. Reactions were carried out with the MyTaq One-Step RT-PCR Kit (Bioline, London, UK) according to the manufacturer's instructions, in a total volume of 50 µL containing 25 µL MyTaq One-Step Mix 2X, 2 µL of each primer, 0.5 µL reverse transcriptase, 1 µL RNase inhibitor, 15.5 µL DEPC-treated water, and 4 µL template RNA.

Cycling included reverse transcription at 55°C for 30 minutes, initial denaturation at 95°C for a minute, and 35 cycles of 94°C for 15 seconds, 60°C for 15 seconds, and 72°C for 10 seconds, followed by melting curve analysis. Polymerase chain reaction products were visualized on 2% agarose gel stained with Safe Red (ABclonal, Wuhan, China), excised at the expected size (386 bp), purified, and submitted for Sanger sequencing. Conventional RT-PCR was used solely to generate DNA amplicons for Sanger sequencing and phylogenetic analysis, and not to confirm RT-qPCR results.

Nucleotide sequences obtained were aligned using ClustalW in MEGA 11 with default parameters and manually inspected as needed. Nucleotide substitution models were evaluated using the Model Selection tool in MEGA 11 based on the Bayesian Information Criterion (BIC), and the model with the lowest BIC value was selected for constructing the Maximum Likelihood phylogenetic tree. Phylogenetic trees were constructed using the Maximum Likelihood method with 1000 bootstrap replicates, and sequences were compared to global FCoV reference strains from GenBank to assess evolutionary relationships and potential geographic origins.

Statistically analysis

Associations between sample origin, clinical signs, and viral burden (*N* gene RT-qPCR Ct values) were analyzed using the Mann–Whitney U test, while differences in sex and age groups were assessed with Fisher's exact test; p-values <0.05 were considered statistically significant.

RESULTS

A total of 45 clinical samples were collected from cats suspected of FIP, including 31 blood samples and 14 ascitic fluid samples. RT-qPCR targeting the *N* gene detected FCoV in 28 of 45 samples (62.2%). Notably, all ascitic fluid samples (100%) tested positive, whereas only 14 of 31 blood samples (45.2%) were positive. The detection rate in ascitic fluid was significantly higher than in blood samples ($p < 0.05$). Data regarding sample codes, controls, clinical origin, sex, breed, age, sample type, and *N* gene cycle threshold (Ct) values are presented in Table 1.

Positive ascitic fluid samples exhibited lower Ct values (mean 22.26 ± 2.19) compared to blood samples (mean 24.10 ± 2.92), indicating higher viral loads, although this difference did not reach conventional statistical significance ($p = 0.069$, Mann–Whitney U test), and the results are presented in Table 2. Polymerase chain reaction amplification of the *N* gene is shown in Figure 1. Clinical records indicated that the most common presenting sign in FCoV-positive cats was abdominal distension (50.0%), followed by uveitis (32.1%) and ataxia (21.4%). Cats with abdominal distension had the lowest mean Ct values (22.26), consistent with the highest viral load, whereas uveitis cases exhibited the highest mean Ct values (24.68), suggesting lower viral burden. Of the 28 FCoV-positive cats, 20 (71.4%) were under 2 years of age, showing a significantly higher positivity rate than older cats ($p = 0.008$, Fisher's exact test). In terms of sex distribution, 57.1% (16/28) of the infected cats were male, and 42.9% (12/28) were female, with no statistically significant difference in infection rate between sexes ($p > 0.05$).

Partial *N* gene sequences (386 bp) from five selected FCoV-positive samples shown in Figure 2 were successfully amplified and analyzed. Phylogenetic analysis revealed that local Indonesian isolates clustered closely with strains from Italy, suggesting a shared evolutionary origin and possible global transmission. All sequences have been submitted to GenBank (Accession Numbers: PV741167 to PV741171). The phylogenetic tree is shown in Figure 2. The five Indonesian sequences (PV741167 to PV741171) formed a well-supported monophyletic clade (100% bootstrap) with very low pairwise genetic divergence (0.0027-0.0304). This clade grouped with strong support (1000 bootstrap replicates) alongside reference strains from Italy (GU017104.1) and the United Kingdom (KP143512.1), indicating a close evolutionary relationship.

Table 1. The RT-qPCR detection of feline coronavirus and confirmation by *N* gene amplification in 45 clinically suspected cats in Yogyakarta during 2021-2022

Sample code	Sex	Breed	Age (year)	Sample type	Sample source	Cycle threshold (*N* gene)	Cycle threshold (5'UTR)	Result
K+*	Female	D	2	Ascites	KP	21.17	21.41	Positive
K-	-	-	-	PCR Water	-	N/A	N/A	Negative
K-01	Female	M	1	Ascites	SWK	20.84	28.18	Positive
K-02	Female	M	1	Ascites	SWK	22.05	26.7	Positive
K-03	Female	M	1	Ascites	SWK	28.17	27.16	Positive
K-04	Female	M	2	Ascites	SWK	24.54	26.91	Positive
K-05	Female	P	1	Blood	NP	22.34	28.14	Positive
K-06	Female	M	3	Blood	NP	N/A	N/A	Negative
K-07	Male	D	2	Blood	NP	25.4	29.7	Positive
K-08	Male	M	2	Blood	DPC	30.55	28.23	Positive
K-09	Male	D	3	Blood	DPC	N/A	N/A	Negative
K-10	Male	D	1	Blood	DPC	N/A	N/A	Negative
K-11	Male	M	1	Blood	DPC	25.24	29.25	Positive
K-12	Male	M	1	Blood	DPC	26	29.07	Positive
K-13	Male	M	1	Blood	DPC	21.83	28.65	Positive
K-14	Female	D	3	Blood	DPC	N/A	N/A	Negative
K-15	Female	P	1	Blood	DPC	N/A	N/A	Negative
K-16	Female	M	1	Blood	DPC	22.11	29.53	Positive
K-17	Male	M	0.5	Blood	DPC	20.14	29.06	Positive
K-18	Male	D	1	Blood	DPC	21.46	23.4	Positive
K-19	Male	P	3	Blood	DPC	N/A	N/A	Negative
K-20	Female	M	1	Blood	DPC	N/A	N/A	Negative
K-21	Male	D	0.5	Blood	DPC	N/A	N/A	Negative
K-22	Female	P	3	Blood	DPC	N/A	N/A	Negative
K-23	Female	M	1	Blood	DPC	N/A	N/A	Negative
K-24	Female	M	2	Blood	DPC	N/A	N/A	Negative
K-25	Female	D	4	Blood	DPC	N/A	N/A	Negative
K-26	Female	D	3	Ascites	SWK	20.9	20.37	Positive
K-27	Male	D	2	Ascites	SWK	21.87	26.95	Positive
K-28	Male	P	2	Ascites	SWK	22.36	28.66	Positive
K-29	Female	P	0.5	Blood	SWK	28.35	30.79	Positive
K-30	Female	D	2	Ascites	SWK	20.28	23.9	Positive
K-31	Female	M	1	Ascites	SWK	20.28	23.6	Positive
K-32	Male	M	1	Ascites	KNG	21.53	24.16	Positive
K-33	Male	D	1	Blood	NP	21.05	25.2	Positive
K-34	Male	P	1	Ascites	PPC	20.17	25.51	Positive
K-35	Male	P	2	Ascites	PPC	20.67	13.73	Positive
K-36	Male	M	2	Blood	KHJ	N/A	N/A	Negative
K-37	Male	M	1	Blood	KHJ	24.64	21.17	Positive
K-38	Male	D	3	Blood	KHJ	N/A	N/A	Negative
K-39	Female	P	2	Blood	KHJ	N/A	N/A	Negative
K-40	Female	P	1	Blood	KHJ	N/A	N/A	Negative
K-41	Female	M	1	Ascites	KP	24.9	25.58	Positive
K-42	Female	P	4	Blood	KP	23.1	25.71	Positive
K-43	Male	D	1	Blood	NP	24.06	24.35	Positive
K-44	Female	D	1	Blood	NP	24.21	28.39	Positive
K-45	Male	P	1	Ascites	KNG	23.1	29.06	Positive

K+*: Tested positive in PCR and RT-qPCR assays targeting the *N* gene and 5'UTR. RT-qPCR: Reverse transcription-quantitative polymerase chain reaction, PCR: Polymerase chain reaction, FCoV: Feline coronavirus, N/A: Not available, D: Domestic, M: Mixed breed, P: Persian, SWK: Satwakita Vet Clinic, NP: Naroo Pet Vet Clinic, DPC: Dji'o Pet Care Clinic, KNG: Kuningan Vet Clinic, PPC: Panda Pet Care Clinic, KHJ: Jogja Vet Clinic, KP: Prosper Vet Clinic.

Table 2. Statistical analysis of feline coronavirus positivity and cycle threshold

Comparison	Test	P-value	Interpretation
Ascitic fluid compared to blood (positivity)	Fisher's exact test	0.00025	Highly significant difference
CT values (ascitic fluid vs blood)	Mann–Whitney U	0.0695	Not significant (trend toward lower Ct in fluid)
Age group and FCoV positivity	Fisher's exact test	0.0081	Significant difference (higher risk in young cats)
Sex and FCoV positivity	Fisher's exact test	0.5420	Not significant

FCoV: Feline coronavirus, Ct: Cycle threshold

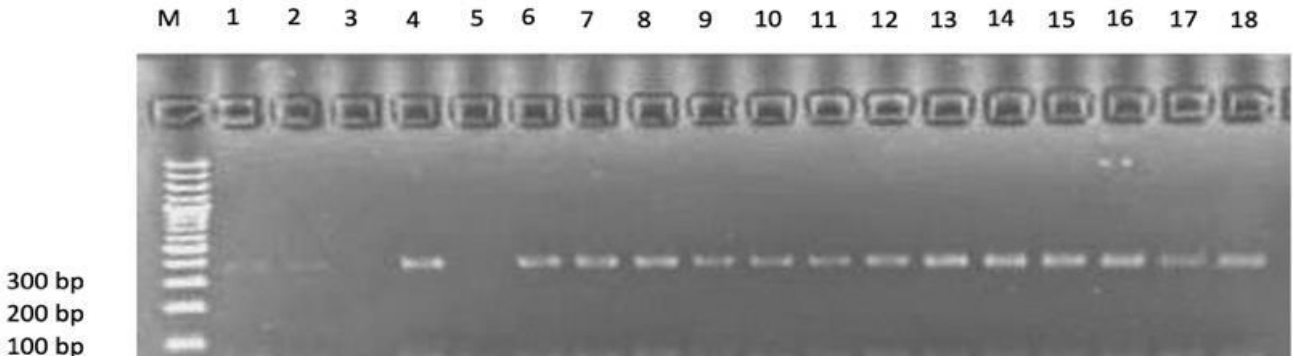


Figure 1. The feline coronavirus-positive samples from cats with different breeds (mixed breed, domestic, and Persian) in Yogyakarta, Indonesia, during 2021-2022. Lanes 4, 6-18: Showing an expected amplicon size of 386 bp for the *N* gene, Lanes 1-3, 5: Negative samples. M: Marker (100 bp).

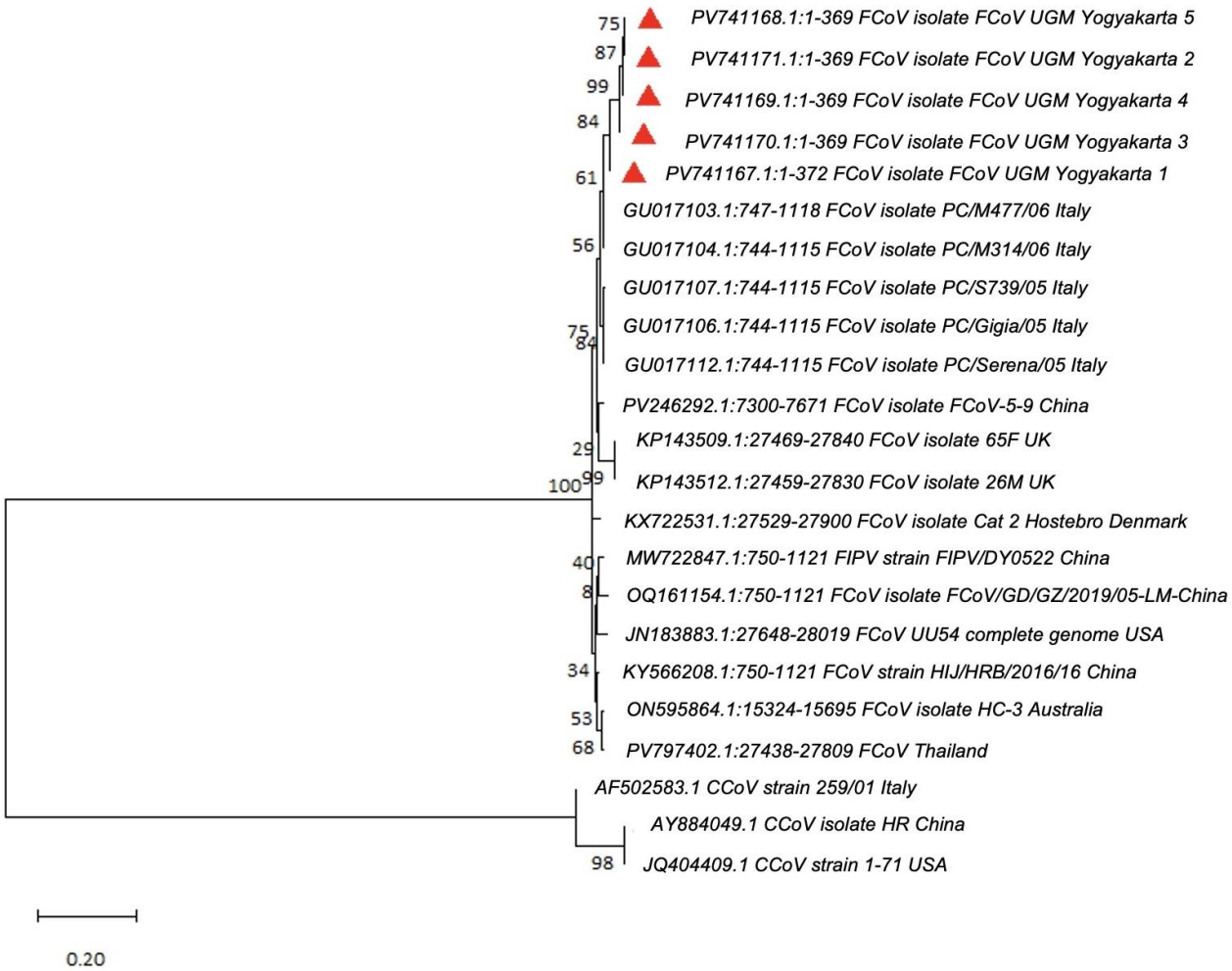


Figure 2. Feline coronavirus isolates (▲) phylogenetic analysis based on the *N* gene compared to reference sequences published in GenBank in Yogyakarta during 2021-2022. The analysis was conducted using MEGA 12 using the Maximum Likelihood model with 1000 bootstrap replicates.

DISCUSSION

The results of the present study demonstrated the effectiveness of RT-qPCR targeting the *N* gene in detecting FCoV infections. With an overall detection rate of 62.22% (28/45), these findings highlighted the utility of RT-qPCR in identifying FCoV-positive cases with greater sensitivity (He et al., 2024). Notably, all effusion fluid samples (14/14) tested positive, whereas 45.16% (14/31) of blood samples were positive, indicating that the viral load in effusion fluid was significantly higher, supporting its role as a primary diagnostic sample. These findings reinforce the importance of using effusion fluid for diagnosing FCoV, particularly in cases suspected of FIP, the severe manifestation of FCoV infection.

Clinical signs observed in infected cats further validate the RT-qPCR results. Abdominal effusion was noted in 50% (14/28) of positive cases, aligning with the classic presentation of wet FIP, which is characterized by exudate accumulation due to vasculitis (Carossino et al., 2022). This observation is consistent with Jayanti et al. (2020), who reported that 80% of FIP cases in domestic shorthair were the wet form. The underlying mechanism involves virus-laden monocytes triggering an inflammatory response that damages blood vessels, leading to plasma leakage into body cavities (Cerna et al., 2022).

Neurological involvement, particularly ataxia, was detected in 21.42% (6/28) of cases, corroborating Doenges et al. (2016), who reported that approximately 10% of FIP cases develop neurological signs, with ataxia being among the most frequent manifestations. The presence of FCoV in the central nervous system suggested that infected monocytes can cross the blood-brain barrier, leading to neuroinflammation (Elmakaty et al., 2022). Uveitis was observed in 32.14% (9/28) of cases, indicating the occurrence of dry FIP. Carossino et al. (2022) have previously identified FIP as a common cause of feline uveitis, with histopathological evidence of severe vasculitis in ocular tissues.

The immunological mechanisms involve immune complex deposition, cytokine-mediated inflammation, and infiltration of virus-infected macrophages, which together disrupt the blood-ocular barrier and drive ocular pathology (Andrew, 2000). Rhinitis was present in 17.85% (5/28) of cases, supporting Andre et al. (2020), who described upper respiratory tract involvement in FIP cases, although FCoV is not typically regarded as a primary respiratory pathogen (Pedersen, 2009).

The present study examined epidemiological factors influencing FCoV infection. Males accounted for 57.14% (16/28) of infected cases, while females constituted 42.85% (12/28). This distribution is consistent with Riemer et al. (2016), who reported that approximately 60-65% of FIP cases occurred in males, and Tasker (2018), who similarly noted that males are more frequently affected than females. Feline infectious peritonitis appears slightly less common in female cats, which may be related to the immunomodulatory effects of estrogen, enhancing antiviral responses (Addie et al., 1995; Pedersen, 2009). However, age, neuter status, breed, and environmental factors also influence susceptibility, indicating that sex differences are likely multifactorial (Worthing et al., 2012). Age also played a critical role, with 71.43% (20/28) of infected cats being under two years old, in line with a study by Guan et al. (2020) and Yin et al. (2021), who reported that younger cats are more susceptible due to an immature immune system and increased exposure to environmental stressors.

Phylogenetic analysis in the present study indicated that the FCoV isolates from Yogyakarta, Indonesia, clustered closely with the Italian isolate (GU017103.1, GU017104.1, GU017107.1, GU017106.1, GU017112.1), supported by phylogenetic clustering and 1000 bootstrap. A similar clustering pattern, as observed with European reference strains, was also reported in isolates from Surabaya (Aksono et al., 2023) and in imported cat cases in Indonesia (Fitrawati et al., 2025). Furthermore, the presence of FCoV in clinically suspected FIP cats in Yogyakarta (Wasissa et al., 2021) indicated that related strains have been circulating locally for several years. These findings suggested that Indonesian FCoV isolates may share a common ancestry with certain European strains, possibly reflecting historical or regional linkage, likely facilitated by international cat travel or trade. A considerable finding of the present study was the close phylogenetic affiliation of Indonesian isolates with European strains from Italy and the UK, rather than with geographically closer Asian strains. This suggested a potential introduction of a European-like strain into Indonesia, likely facilitated by international cat travel or trade, followed by local spread. These findings underscored the global nature of FCoV transmission and highlighted the need for robust genomic surveillance.

The use of RT-qPCR targeting the *N* gene demonstrated reliable sensitivity for FCoV detection in clinical samples, particularly effusion fluid, while partial sequencing of the *N* gene provided valuable insights into the genetic characteristics and phylogenetic relationships of local FCoV strains. Although the present study was conducted in Yogyakarta, Indonesia, the phylogenetic analysis revealed that these isolates shared close genetic proximity to strains originating from Europe and East Asia. This finding underscored the potential of shared transmission pathways, possible cross-border viral introductions, or common evolutionary origins of FCoV strains.

The genetic clustering of Indonesian isolates with international reference strains highlighted the need for continuous, integrated, global surveillance programs to monitor FCoV genetic variation and emergence. The present study represented the first integrated approach in Indonesia that combines clinical observations, RT-qPCR detection, and phylogenetic analysis of naturally occurring FCoV cases in domestic cats, thereby providing novel insights into the molecular epidemiology of FCoV in this region. In a broader context, these results contribute important molecular data from Southeast Asia, a region underrepresented in global FCoV studies, thereby enriching the global coronavirus sequence database and enhancing preparedness for future interspecies transmission events and emerging feline viral diseases. However, the present study was limited by a small sample size for sequencing and only using partial *N* gene fragments, which constrained broader epidemiological analysis conclusions.

CONCLUSION

The current study confirmed that RT-qPCR targeting the *N* gene is a reliable diagnostic tool for detecting FCoV in clinical samples, especially effusion fluids. Younger cats and effusive cases exhibited higher infection rates, supporting the clinical and epidemiological associations with molecular findings. Phylogenetic analysis indicated close relationships between local isolates and strains from Europe and Asia, suggesting possible transboundary transmission or shared evolutionary origins. These findings not only provided essential data for feline health management in Indonesia but also contributed to the global molecular epidemiology of FCoV, reinforcing the importance of international surveillance and control strategies. Future studies should expand the sample size, include full-genome sequencing, and investigate host immune responses to better understand viral evolution, pathogenesis, and potential risk factors for FIP development.

DECLARATIONS

Acknowledgements

The authors express their gratitude to Gadjah Mada University, Yogyakarta, Indonesia, for providing financial support for the current study through RTA No. 5722/UN1.P.III/Dit-Lit/PT.01.05/2022. Additionally, they acknowledge the contributions of all veterinarians, cat owners, and laboratory technicians who supported this study.

Authors' contributions

Amalia Fia, Wasissa Madarina, Lestari Fajar Budi, and Salasia Siti Isrina Oktavia participated in Conceptualization. Data curation was conducted by Amalia Fia, Wasissa Madarina, Amalia Fia, and Wasissa Madarina. Amalia Fia and Wasissa Madarina conducted the investigation. Amalia Fia and Wasissa Madarina provided the methodology. Project administration was done by Salasia Siti Isrina Oktavia. Amalia Fia and Wasissa Madarina prepared the resources. Amalia Fia and Wasissa Madarina conducted work with software. Salasio Siti Isrina Oktavia supervised the study. Amalia Fia and Wasissa Madarina validated the study. Amalia Fia and Wasissa Madarina participated in the visualization. The original draft was written by Amalia Fia. Salasia Siti Isrina Oktavia conducted the writing, reviewing, and editing. All authors have read and approved the final edition of the manuscript before publication in the present journal.

Funding

This study is funded by the University of Gadjah Mada, Yogyakarta, Indonesia, through the RTA research contract No. 5722/UN1.P.III/Dit-Lit/PT.01.05/2022.

Competing interests

The authors declared no conflict of interest.

Ethical considerations

This manuscript is original, conducted solely by the authors, and it has not been published elsewhere.

Availability of data and materials

All data supporting the findings of the present study are presented within the published manuscript. Any additional details can be obtained from the corresponding author upon reasonable request.

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2025, Scienceline Publication
World's Veterinary Journal
World Vet J, 15(3): 811-823.
ISSN 2322-4568

DOI: https://dx.doi.org/10.54203/scil.2025.wvj83
PII: S232245682500083-15

Antibacterial and Sensory Impact of β-carotene and Riboflavin on *Pseudomonas* Biofilm in Raw Milk

Heba E. El-Shora[1]*, Doaa M. Sharaf[1], Eman K. Fathalla[2], Radwa A. Lela[2], Momen M. Aladl[1], and Amal F. Elbanna[2]

[1]*Bacteriology Department, Animal Health Research Institute (AHRI), Tanta branch, Agricultural Research Center (ARC), Giza, Egypt.*
[2]*Food hygiene Department, Animal Health Research Institute (AHRI), Tanta branch, Agricultural Research Center (ARC), Giza, Egypt.*

*Corresponding author's Email: Heba_elsaid@yahoo.com

ORIGINAL ARTICLE
Received: June 24, 2025
Revised: July 29, 2025
Accepted: August 29, 2025
Published: September 30, 2025

ABSTRACT

Milk is a highly nutritious yet perishable food that is susceptible to microbial contamination and spoilage, particularly by *Pseudomonas* species. The present study aimed to assess the antimicrobial and sensory effects of β-carotene and riboflavin as natural additives for preserving raw milk, with a specific emphasis on controlling biofilm-forming *Pseudomonas* strains. A total of 100 raw milk samples were collected from different markets in Gharbia, Egypt, and tested for *Pseudomonas* spp. Biofilm formation was assessed in isolates, followed by the detection of the quorum-sensing transcriptional regulator *lasR*, pellicle formation protein A (polysaccharide biosynthesis protein A) *pelA*, and polysaccharide synthesis locus protein A *pslA* genes. An *in vitro* trial was performed on eight groups of raw milk to evaluate the effects of β-carotene and riboflavin at concentrations of 1 µg/ml and 4 µg/ml, respectively, on *Pseudomonas* counts and sensory properties. The groups include positive control (G1), raw milk inoculated with *Pseudomonas* and fortified with β-carotene (G2), raw milk inoculated with *Pseudomonas* and fortified with riboflavin (G3), raw milk inoculated with *Pseudomonas* and fortified with a combination of β-carotene and riboflavin (G4), negative control **(**G5), milk fortified with β-carotene only (G6), milk fortified with riboflavin only (G7) and milk fortified with both β-carotene and riboflavin (G8). Reverse transcription polymerase chain reaction (RT-PCR) was performed to assess the impact of these additives on *Pseudomonas*, specifically on *16S rRNA* and *pslA* gene expression. *Pseudomonas* spp. were found in 20% of samples, with 75% forming biofilms. The *pslA* gene was detected in 83.33% of these samples. β-carotene and riboflavin indicated dose-dependent antibacterial effects, with minimum inhibitory concentration values, and *minimum bactericidal concentration* values. Milk fortified with these compounds, particularly in G4, demonstrated the most significant reduction in *Pseudomonas* during a 6-day storage period. Additionally, β-carotene maintained a preferable sensory quality compared to other groups. RT-PCR confirmed the highest *pslA* gene suppression with the combined treatment. The current findings indicated that β-carotene at a concentration of 1 µg/ml, whether used independently or in combination with riboflavin, can serve as an effective natural preservative in dairy products.

Keywords: Beta-Carotene, Biofilm, *Pseudomonas*, *PslA* Expression, Riboflavin

INTRODUCTION

Milk is considered one of nature's most nutrient-rich foods, owing to its high levels of bioavailable proteins, lactose, lipids, calcium, and a wide range of bioactive constituents, including peptides, enzymes, immunological agents, growth factors, and hormones (Li et al., 2018). Nevertheless, this nutritional abundance creates a favorable environment for microbial proliferation, potentially resulting in rapid spoilage, decline in quality, shorter shelf life, and a higher risk of foodborne diseases spreading (Dash et al., 2022).

Pseudomonas, an important Gram-negative opportunistic pathogen**,** is considered among the most common psychotropic bacteria associated with milk spoilage (Zhang et al., 2019). These bacteria can grow at refrigeration temperatures (7°C or lower), resulting in putrefaction, fermentation, and the emergence of off-flavors such as fruity or bitter notes due to their capacity to metabolize fats and proteins (Atia et al., 2022). Although psychrotrophic bacteria are not typically part of the native microbiota of the bovine udder (Porcellato et al., 2020), their presence in chilled raw milk is primarily attributed to post-harvest contamination, particularly during or after milking. Milking equipment is frequently identified as a major source of this contamination (Oliveira et al., 2015). These spoilage organisms pose a considerable challenge to the dairy industry (Dogan and Boor, 2003), as they are capable of growing at refrigeration temperatures and producing heat-resistant proteolytic enzymes. Proteolytic enzymes can remain active even after pasteurization, thereby accelerating milk spoilage (Dogan and Boor, 2003; Xin et al., 2017). In addition, *Pseudomonas* spp. possess multiple virulence factors such as biofilm formation, exopolysaccharides, and quorum sensing that enhance their survival under adverse conditions (Zhao et al., 2019). Furthermore, *Pseudomonas* spp. can form biofilms and increase their resistance to disinfectants and antibiotics, making them difficult to eliminate (Drenkard, 2003).

To cite this paper: El-Shora HE, Sharaf DM, Fathalla EK, Lela RA, Aladl MM, and Elbanna AF (2025). Antibacterial and Sensory Impact of β-carotene and Riboflavin on *Pseudomonas* Biofilm in Raw Milk. *World Vet. J.,* 15(3): 811-823. DOI: https://dx.doi.org/10.54203/scil.2025.wvj83

Biofilms formed by *Pseudomonas* spp. pose a significant challenge in dairy processing, as these bacteria can colonize surfaces such as bulk milk tanks and processing equipment, using their polar flagella to aid in motility and surface attachment (Jurado-Marein et al., 2021). These biofilms are composed of structured microbial communities that are embedded in a matrix of extracellular polymeric substances (EPS), which is made up of polysaccharides, proteins, extracellular DNA, and vesicles (Fulaz et al., 2019). This matrix acts as a protective barrier, shielding the bacteria from environmental stresses and antimicrobial agents, thereby promoting contamination and spoilage through the production of degradative enzymes. Thus, controlling biofilm formation is essential to maintaining milk safety and quality (Yuan et al., 2017). Two critical genes involved in biofilm development in *Pseudomonas* are *pslA* and *lasR*. The *pslA* gene encodes a glycosyltransferase responsible for the synthesis of *Psl* polysaccharide, which facilitates initial surface attachment and cellular aggregation. Meanwhile, *lasR* encodes a quorum-sensing transcriptional regulator that governs the expression of genes involved in bacterial communication and EPS production (Abootaleb et al., 2020).

Growing concern about the health hazards and quality deterioration linked to chemical preservatives and thermal processing has generated heightened interest in natural, environmentally sustainable antimicrobial alternatives. Traditional additives such as nitrites and hydrogen peroxide may pose safety risks, while high-temperature treatments can negatively affect the nutritional value and sensory qualities of food (Garcia-Fuentes et al., 2015). In contrast, phytochemicals have demonstrated effective antimicrobial properties against foodborne pathogens, highlighting their potential as viable options for preserving dairy products (Elafify et al., 2020).

Among these compounds, riboflavin (Vitamin B2) and carotenoids serve dual functional roles as natural additives (Farah et al., 2022; Naisi et al., 2023). Carotenoids, such as β-carotene, are naturally occurring pigments found in food sources. β-carotene possesses appropriate antioxidant and anti-inflammatory properties (Fiedor and Burda, 2014) and contributes to improving milk quality by preventing protein oxidation and enhancing flavor stability (Fiedor and Burda, 2014; Yabuzaki, 2017). Additionally, β-carotene exhibits selective antimicrobial activity, targeting harmful pathogens while preserving beneficial lactic acid bacteria, which is advantageous in dairy processing (Tao NengGuo et al., 2010).

Riboflavin plays a vital role in human metabolism as a cofactor in redox reactions (Farah et al., 2022). Furthermore, it exhibits potent antimicrobial activity upon photoactivation, where light exposure generates reactive oxygen species (ROS) that induce lethal oxidative stress in microbial cells (Farah et al., 2022). This property makes riboflavin an effective natural photosensitizer, with proven activity against *Pseudomonas aeruginosa (P. aeruginosa)* (Najari et al., 2024). Riboflavin supports immune function by enhancing pathogen clearance and reducing inflammation (Farah et al., 2022).

The present study aimed to evaluate the antimicrobial and sensory effects of β-carotene and riboflavin, both individually and in combination, as natural preservatives in raw milk, focusing on their influence on *Pseudomonas* spp. survival, inhibition zones, *pslA* gene expression, and sensory attributes during cold storage.

MATERIALS AND METHODS

Ethical approval

The present study was approved by the Research Committee of the Animal Health Research Institute and was authorized by the Institutional Animal Care and Use Committee (IACUC) of the Agricultural Research Center (ARC) under approval number ARC-AHRI 444429.1/24.

Sample collection and preparation

A total of 100 random milk samples were collected from different markets in Gharbia Governorate, Egypt. The collected samples were immediately transported to the Microbiology Department at the Animal Health Research Institute (Tanta Branch) in a chilled container, maintaining strict aseptic conditions, to be prepared and analyzed promptly for detecting *Pseudomonas*.

Isolation and identification of *Pseudomonas*

One mL of the milk sample was inoculated into 9 mL of nutrient broth and incubated at 37°C for 24 hours. Subsequently, a loopful of the enriched culture was streaked onto *Pseudomonas* cetrimide selective agar supplemented with King's B agar, uniformly spread, and incubated at 37°C for 18-24 hours. Colonies exhibiting characteristic blue-green or green-yellow pigmentation with a distinct sweet, grape-like odor were presumptively identified as *Pseudomonas* and selected for further analysis. Biochemical characterization was performed according to ISO (2004) guidelines, including oxidase and catalase tests, gelatin liquefaction and hydrolysis, sugar fermentation assays, and pigment production. Isolates showing biochemical profiles consistent with *Pseudomonas* spp. were subjected to further examination by polymerase chain reaction (PCR) for biofilm-related virulence genes detection (*lasR, pelA, and pslA*).

Detection and evaluation of biofilm production

Tube method

The tube method (TM) is a qualitative method for detecting biofilm formation (Christensen et al., 1985). Bacteria were grown in Trypticase soy broth for 24 hours at 37°C, then the tubes were washed with phosphate-buffered saline (pH 7.2), air dried, stained with 1% crystal violet for 10 minutes, and washed with deionized water. Biofilm formation was assessed utilizing the control strains. The presence of biofilm was identified by a visible film on the walls and base of the tube. The formation of a ring at the liquid interface was indicative of a non-biofilm producer. The results were evaluated visually and categorized as negative (1), weak positive (2), moderate positive (3), and strong positive (4; Figure 1).

Tissue culture plate method

The tissue culture plate (TCP) method was used to assess biofilm formation by *Pseudomonas* isolates (Vasudevan et al., 2003). Biofilm formation was assessed using the microtiter plate method. Bacterial cultures (10^6 CFU/mL) were grown in tryptic soy broth (TSB) supplemented with 1% glucose and incubated at 37°C for 24 hours. The cultures were then diluted 1:100 in fresh TSB, and 200 µL of the diluted suspension was added to each well of a 96-well microtiter plate. After 24 hours of incubation at 37°C, the wells were gently washed three times with phosphate-buffered saline (PBS), air-dried, and stained with 50 µL of crystal violet 1% for 15 minutes. Excess stain was removed by washing the wells three times with 200 µL of sterile distilled water, followed by air drying at 45°C for 20 minutes. To quantify biofilm, the stained biofilm was solubilized with 200 µL of ethanol 95% per well for 30 minutes. The optical density (OD) was measured at 630 nm using an ELISA microplate reader (MR-96, CLINDIAG, China) at the Microanalysis Unit, Faculty of Science, Tanta University, Tanta, Egypt.

Absorbance

Each strain was tested three times, and the average OD readings were calculated. The final OD value for each strain's biofilm activity was obtained by subtracting the cut-off value (ODc) from the average OD. Biofilm production was then classified based on the criteria including $OD \leq ODc$ (Non-biofilm producer), $ODc < OD \leq 2 \times ODc$ (Weak biofilm producer), $2 \times ODc < OD \leq 4 \times ODc$ (Moderate biofilm producer), and $OD > 4 \times ODc$ (Strong biofilm producer), as described by Stepanović et al. (2007). The positive reference strain for biofilm formation was *Pseudomonas aeruginosa* ATCC 27853.

Detection of biofilm virulence genes in *Pseudomonas* using polymerase chain reaction

Isolates exhibiting strong biofilm-forming ability were selected for PCR analysis to detect virulence genes associated with biofilm production (*lasR*, *pelA*, and *pslA*) using uniplex PCR. Genomic DNA was extracted from *Pseudomonas* isolates using the QIAamp DNA Mini Kit (Qiagen, Germany), with slight protocol modifications. Oligonucleotide primers and related analyses were provided by Metabion (Germany), as detailed in Table 1.

Table 1. The primers used for the detection of *Pseudomonas* and biofilm virulence genes

Gene	Sequence	Amplified product (bp)	Reference
Pseudomonas 16S rDNA	GACGGGTGAGTAATGCCTA	618	(Spilker et al., 2004)
	CACTGGTGTTCCTTCCTATA		
pelA	CATACCTTCAGCCATCCGTTCTTC	786	(Ghadaksaz et al., 2015)
	CGCATTCGCCGCACTCAG		
pslA	TCCCTACCTCAGCAGCAAGC	656	
	TGTTGTAGCCGTAGCGTTTCTG		
lasR	CTGTGGATGCTCAAGGACTAC	133	(Saleh et al., 2019)
	AACTGGTCTTGCCGATGG		

Preparation of β-carotene and riboflavin stock solution

A 1 mg/mL riboflavin solution (Sigma-Aldrich) was prepared under dark conditions by dissolving 1 mg of riboflavin in 1 mL of distilled water. Similarly, a 1 mg/mL β-carotene solution (Sigma-Aldrich) was prepared under light-protected conditions by dissolving 1 mg of β-carotene powder in 1 mL of dimethyl sulfoxide (DMSO), the concentration of which did not exceed 1% (v/v). Both solutions were stored in light-protective tubes and used within 30 minutes of preparation to prevent degradation.

***In vitro* evaluation for antibacterial activity of β-carotene and riboflavin**

The well diffusion method was employed to estimate the antibacterial activity of β-carotene and riboflavin against biofilm-forming *Pseudomonas*. After culturing the target bacteria in broth and adjusting the concentration to 10^6 CFU/mL, 100 µL of the bacterial suspension was spread evenly onto agar plates under aerobic conditions. Wells were

aseptically punched into the agar plate using a sterile cork borer with a diameter of 6-8 mm. Freshly prepared solutions of β-carotene, which was dissolved in DMSO, and riboflavin (dissolved in PBS) at concentrations of 0.5, 0.25, and 0.125 mg/mL were dispensed into the wells (20 µL per well). Each compound was tested on separate plates to avoid cross-interference. The plates were then incubated at 37°C for 24 hours to observe zones of inhibition (CLSI, 2020). Positive controls included 10 µg of imipenem, whereas negative controls utilized DMSO for β-carotene and distilled water for riboflavin. Antimicrobial activity was assessed by measuring the diameter of the inhibition zones (mm) around the wells. The inhibition zones were categorized as greater than 15 mm, indicating pronounced antimicrobial activity, 13-15 mm, representing moderate activity, and less than 12 mm, signifying weak or no antimicrobial activity. These criteria were derived from the studies conducted by Ahgilan et al. (2016) on riboflavin and Muntean et al. (2021) on β-carotene.

Determination of minimum inhibitory concentration and minimum bactericidal concentration of β-carotene and riboflavin

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of β-carotene and riboflavin against *Pseudomonas* were determined using a 96-well microtiter plate (CLSI, 2020). Briefly, one *Pseudomonas* strain identified as a strong biofilm producer was selected for the assay. To each well of a microtiter plate, 100 µL of freshly prepared Mueller-Hinton broth was added, followed by 100 µL of bacterial suspension standardized to 0.5 McFarland turbidity. Subsequently, 100 µL of freshly prepared β-carotene and riboflavin solution at a concentration of 0.5 mg/mL was added to the first well and thoroughly mixed. Two-fold serial dilution was done to estimate the MIC of β-carotene and riboflavin. The positive control well contained only bacterial culture without β-carotene and riboflavin, while the negative control well included DMSO and sterile distilled water for β-carotene and riboflavin, respectively.

The microtiter plates were incubated for an additional 24 hours at 37°C. Bacterial growth was assessed by observing changes in turbidity. The MIC was defined as the lowest concentration of β-carotene and riboflavin at which no visible bacterial growth occurred. For determining the MBC, samples from wells with no visible growth were streaked onto antibiotic-free agar plates and then incubated for 24 hours. The MBC was identified as the lowest concentration of β-carotene and riboflavin at which no bacterial colonies were detected.

Evaluation of the addition of β-carotene and riboflavin to milk

Preparation of bacterial cultures

A *Pseudomonas* strain, which was confirmed as a biofilm producer by PCR, was selected for the present study. The bacterial culture was prepared following the United States Food and Drug Administration guidelines (FDA, 2001). The bacterial suspension was then standardized to a final concentration of 10^6 CFU/mL for use in the following process.

Milk sample collection and grouping

The milk samples were divided into eight groups, each consisting of 100 mL, classified according to two main objectives including bacterial proliferation assessment and sensory analysis. The negative control group was employed in both procedure assessments.

Bacterial growth analysis

Five groups were designed to evaluate the antimicrobial effect of β-carotene and riboflavin including positive control (G1), raw milk inoculated with *Pseudomonas* and fortified with β-carotene (G2), raw milk inoculated with *Pseudomonas* and fortified with riboflavin (G3), raw milk inoculated with *Pseudomonas* and fortified with a combination of β-carotene and riboflavin (G4), and the negative control group (G5). The concentrations of riboflavin (4 µg/ml) and β-carotene (1 µg/ml) applied to the milk were derived from their pre-determined MICs. Treated milk samples were stored at 4 ± 1°C, and *Pseudomonas* counts were monitored over 6 days in accordance with the APHA (2004) protocol.

Sensory evaluation

To assess the impact of β-carotene and riboflavin on the sensory properties of milk, the following groups were evaluated. Negative control (Untreated raw milk, G5), milk fortified with β-carotene only (G6), milk fortified with riboflavin only (G7), and milk fortified with both β-carotene and riboflavin (G8). Sensory evaluation included the assessment of appearance, odor, flavor, and overall acceptability, following the method outlined by Clark et al. (2009), which was performed by a trained panel consisting of scientists and students. To monitor temporal changes in milk quality, sensory assessments were carried out daily during the six-day refrigerated storage period. Each attribute was evaluated on a 10-point hedonic scale using the classifications including excellent (9-10), good (8-9), fair to good (7-8), and poor (6). This evaluation aimed to determine the impact of natural additive fortification on sensory characteristics and to estimate shelf life based on consumer acceptability.

Molecular identification

Expression of 16S rDNA and pslA

Milk samples from the different experimental groups were analyzed using reverse transcription quantitative polymerase chain reaction (RT-qPCR) to determine whether the presence of β-carotene and riboflavin in co-culture with *Pseudomonas* influenced the expression of the *16S rDNA* and *pslA* genes. The primers used in RT-qPCR are presented in Table 2. The cycle threshold (Ct) values of each sample were compared to those of the control group using the delta delta Ct (ΔΔCt) method to quantify the relative changes in gene expression among the samples (Spilker et al., 2004; Ghadaksaz et al., 2015).

Table 2. Primers, sequences, and target genes for SYBR green reverse transcription quantitative polymerase chain reaction

Target gene	Primers sequences	Reference
Pseudomonas 16S rDNA	F-GACGGGTGAGTAATGCCTA	(Spilker et al., 2004)
	R-CACTGGTGTTCCTTCCTATA	
PslA	F-TCCCTACCTCAGCAGCAAGC	(Ghadaksaz et al., 2015)
	R- TGTTGTAGCCGTAGCGTTTCTG	

Statistical analysis

The data are presented as the mean ± standard error (SE). The mean values of the different groups were compared using Duncan's post hoc test in a one-way analysis of variance (ANOVA) at a significance level of $p \leq 0.05$. The method cited by Petrie and Watson (1999) was employed to conduct the statistical analysis, which was then computerized using SPSS version 20 (2011).

RESULTS

Incidence of *Pseudomonas* isolated from milk samples

Based on microbiological and biochemical characterization, *Pseudomonas* was isolated from the total collected raw milk samples with an isolation rate of 20% (20/100).

Evaluation of biofilm production

The isolated *Pseudomonas* strains were tested for biofilm production. The current findings revealed that 15 out of 20 isolates (75%) were characterized as biofilm producers with diverse capabilities utilizing the TCP method. Specifically, eight isolates (40%) exhibited strong biofilm-forming capacity, five isolates (25%) demonstrated moderate capacity, and two isolates (10%) were classified as weak. The TM identified 12 isolates (60%) as biofilm producers, with six (30%) classified as strong, four (20%) as moderate, and two (10%) as weak producers (Table 3, Figure 1).

Detection of biofilm-related virulence genes in *Pseudomonas* using polymerase chain reaction

A total of six *Pseudomonas* isolates were confirmed positive for *16S rDNA*. All isolates tested negative for the *pelA* and *lasR* genes, while 83.33% were positive for the *pslA* gene (Figures 2 and 3).

Diameter of the inhibition zone of β-carotene and riboflavin against *Pseudomonas*

The combination of β-carotene and riboflavin exhibited notable antibacterial activity against *Pseudomonas*, as evidenced by the inhibition zone diameters. The diameters demonstrated a proportional increase relative to the concentrations employed (0.5, 1, and 2 mg/ml; Figure 4). Specifically, the inhibition zone diameter for β-carotene exhibited a significant increase ($p < 0.05$), rising from 16 ± 0.5 to 18.5 ± 0.29. Similarly, riboflavin increased from 15.30 ± 0.17 to 16 ± 0.57. Furthermore, the combination of β-carotene and riboflavin illustrated a substantial increase, rising from 17 ± 0.57 to 19.93 ± 0.06 ($p < 0.05$).

Determination of minimum inhibitory concentration and minimum bactericidal concentration of β-carotene and riboflavin

The current results revealed that the MIC of β-carotene and riboflavin was 1 µg/mL and 4 µg/mL, respectively. The MBC was determined to be 2 µg/mL for β-carotene and 8 µg/mL for riboflavin.

Impact of β-Carotene and riboflavin on *Pseudomonas* survival in refrigerated raw milk

The current findings demonstrated that *Pseudomonas* counts progressively increased in the positive control group (G1) over the six-day storage period (Table 4). While milk samples treated with β-carotene (G2), riboflavin (G3), or both combinations (G4) indicated significantly reduced bacterial growth ($p < 0.05$), G4 demonstrated the greatest antimicrobial effect during refrigeration storage at 4 ± 1°c.

Sensory evaluation of raw milk during refrigeration storage

Table 5 illustrates the evaluation of the sensory characteristics of raw milk over six days of refrigerated storage. The current results indicated that the β-carotene group (G6) maintained the highest sensory scores throughout storage, followed by groups fortified with riboflavin (G7) and a mix of β-carotene and riboflavin(G8), while the negative control group (G5) resulted in lower sensory scores.

Effect of β-Carotene and riboflavin on *pslA* gene expression

The current findings demonstrated that the expression of the *pslA* gene in G4 was decreased to a 0.07-fold change compared to the control group (G1). Additionally, groups G2 and G3 exhibited reduced expression levels of the *pslA* gene, with fold changes of 0.31 and 0.54, respectively. These findings suggested a decrease in *pslA* gene expression under the influence of β-carotene and riboflavin (Figures 5 and 6).

Table 3. Degree of biofilm-forming ability of *Pseudomona*s strains isolated from the examined samples

Tested organism	Biofilm producer						Total biofilm producer		Non-biofilm producer	
	Strong positive		Moderate positive		Weak positive					
Pseudomonas	**TCP**	**TM**	**TCP**	**TM**	**TCP**	**TM**	**TCP**	**TM**	**TCP**	**TM**
	8(40%)	6(30%)	5(25%)	4(20%)	2(10%)	2(10%)	15(75%)	12(60%)	5(25%)	8(40%)

Percentage (%) is calculated in relation to the total number of isolated strains. TM: Tube method, TCP: Tissue culture plate method.

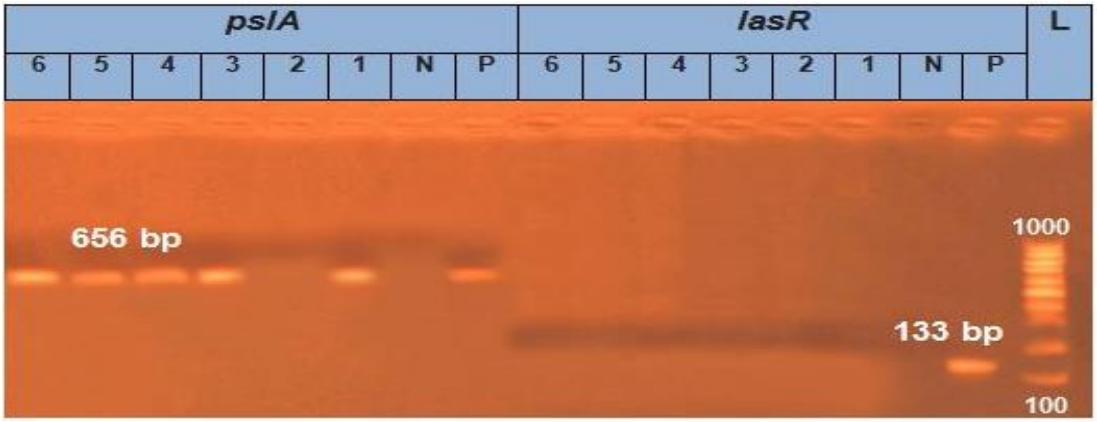


Figure 1. Biofilm formation by the tube method. **A**: Strong biofilm producer, **B**: Moderate biofilm producer, **C**: Weak biofilm producer, **D**: Non-biofilm producer.



Figure 2. Detection of *pslA* and *lasR* genes in 6 *Pseudomonas* isolates at 656 bp and 133 bp, respectively, using agarose gel electrophoresis. L (Ladder): DNA ladder (100-1000bp). Lan (1,3,4,5,6) Positive amplification of the *pslA* gene at 656 bp. Lan (1,2,3,4,5,6) Negative amplification of the *lasR* gene at 133 bp P: Positive control; N: Negative control

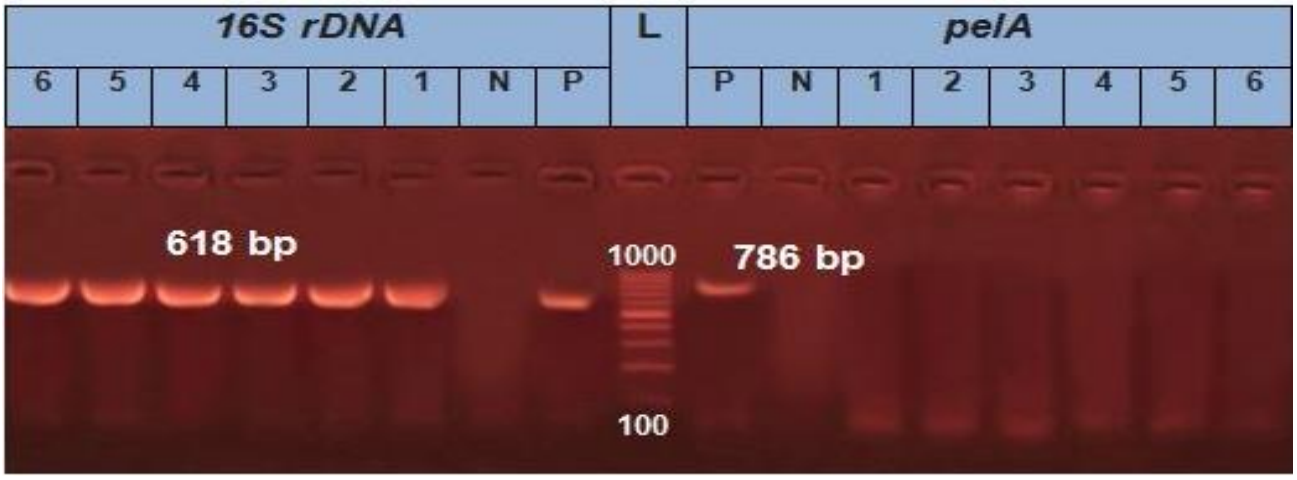


Figure 3. Detection of *16S rDNA* and pel*A* genes in 6 *Pseudomonas* isolates at 618 bp and 786 bp, respectively, using agarose gel electrophoresis. L (Ladder): DNA ladder (100-1000bp). Lan (1,2,3,4,5,6) Positive amplification of the *16S rDNA* gene at 618 bp. Lan (1,2,3,4,5,6) Negative amplification of pel*A* gene at 786 bp P: Positive control; N: Negative control.

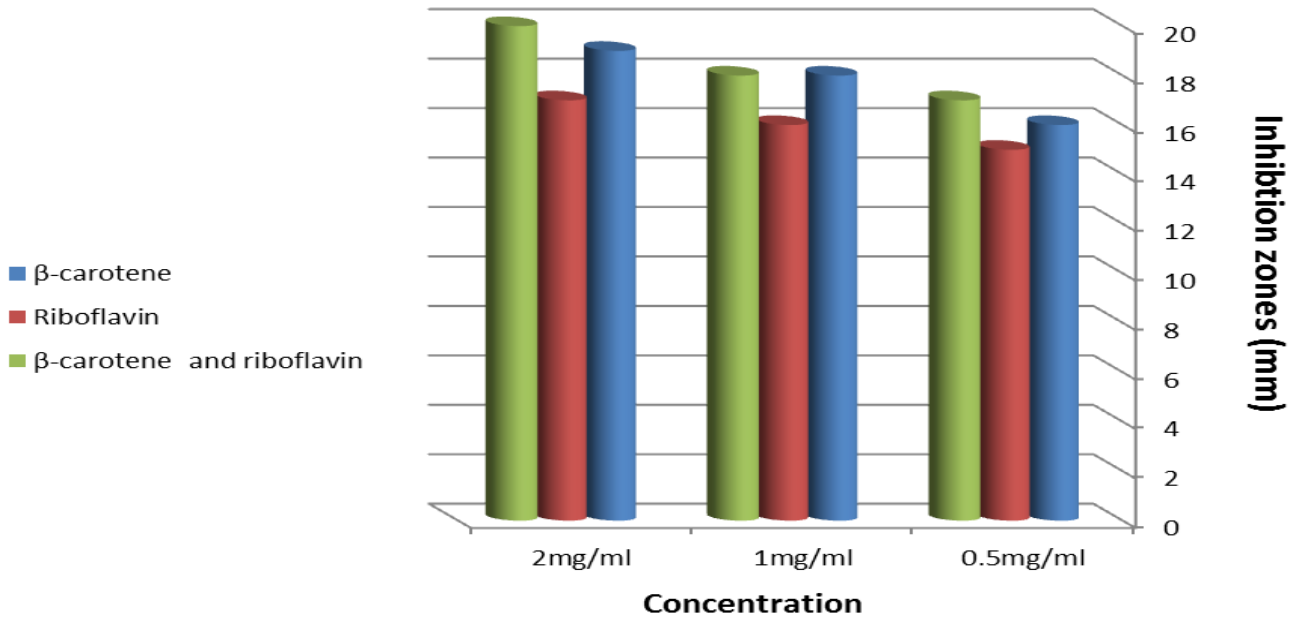


Figure 4. Inhibition zones of β-carotene and riboflavin by agar well diffusion method increased proportionally with the concentrations used.

Table 4. Impact of β-carotene and riboflavin on the survival rate of *Pseudomonas* as log cfu/g in raw milk during refrigeration storage

Groups / Days	G1	G2	G3	G4
Day 0	6.43 ± 0.01^{a}	6.40 ± 0.01^{a}	6.41 ± 0.01^{a}	6.39 ± 0.01^{a}
Day 1	6.92 ± 0.01^{a}	6.25 ± 0.02^{c}	6.39 ± 0.01^{b}	5.92 ± 0.02^{d}
Day 2	7.13 ± 0.01^{a}	5.90 ± 0.02^{c}	5.98± 0.01^{b}	5.34 ± 0.01^{d}
Day 3	7.59 ± 0.06^{a}	5.39 ± 0.01^{c}	5.68 ± 0.02^{b}	4.84 ± 0.01^{d}
Day 4	7.61 ± 0.02^{a}	5.10 ± 0.04^{b}	4.99 ± 0.01^{b}	4.55 ± 0.03^{c}
Day 5	7.81 ± 0.02^{a}	4.83 ± 0.04^{b}	4.98 ± 0.01^{b}	4.35 ± 0.03^{c}
Day 6	7.97 ± 0.02^{a}	4.56 ± 0.06^{c}	4.81 ± 0.01^{b}	3.92 ± 0.01^{d}

G1: Negative control, raw milk without *Pseudomonas*, G2: Raw milk fortified with beta-carotene, G3: Raw milk fortified with riboflavin, G4: Raw milk fortified with a mix of beta-carotene and riboflavin. The values represent as mean ± Standard error. ^{a,b,c,d} Means within a row followed by different superscript letters are significantly different ($p < 0.05$).

Table 5. Sensory evaluation of raw milk during refrigeration storage

Storage period	G5	G6	G7	G8
Appearance scores (10-point scale)				
Day 0	9.85 ± 0.03^{a}	9.80 ± 0.02^{a}	9.70 ± 0.04^{ab}	9.65 ± 0.03^{ab}
Day 1	9.30 ± 0.04^{b}	9.70 ± 0.02^{a}	9.50 ± 0.03^{ab}	9.45 ± 0.05^{ab}
Day 2	8.70 ± 0.05^{b}	9.50 ± 0.02^{a}	9.10 ± 0.04^{ab}	9.05 ± 0.04^{ab}
Day 3	7.90 ± 0.06^{bc}	9.20 ± 0.03^{a}	8.70 ± 0.05^{ab}	8.60 ± 0.05^{ab}
Day 4	7.10 ± 0.07^{c}	8.90 ± 0.04^{a}	8.20 ± 0.04^{ab}	8.10 ± 0.05^{ab}
Day 5	6.40 ± 0.08^{c}	8.60 ± 0.04^{a}	7.60 ± 0.05^{ab}	7.50 ± 0.05^{ab}
Day 6	6.00 ± 0.08^{c}	8.50 ± 0.04^{a}	7.10 ± 0.05^{ab}	7.00 ± 0.05^{ab}
Flavor scores (10-point scale)				
Day 0	9.80 ± 0.03^{a}	9.65 ± 0.02^{a}	9.50 ± 0.04^{ab}	9.45 ± 0.03^{ab}
Day 1	9.00 ± 0.04^{ab}	9.60 ± 0.02^{a}	9.20 ± 0.03^{ab}	9.10 ±0.05^{ab}
Day 2	8.30 ± 0.05^{b}	9.40 ± 0.02^{a}	8.80 ± 0.04^{ab}	8.75 ± 0.04^{ab}
Day 3	7.50 ± 0.06^{bc}	9.10 ± 0.03^{a}	8.40 ± 0.05^{ab}	8.30 ± 0.05^{ab}
Day 4	6.80 ± 0.07^{c}	8.80 ± 0.04^{a}	8.00 ± 0.04^{ab}	7.95 ± 0.05^{ab}
Day 5	6.10 ± 0.08^{c}	8.50 ± 0.04^{a}	7.40 ± 0.05^{ab}	7.30 ± 0.05^{ab}
Day 6	5.70 ± 0.08^{c}	8.00 ± 0.04^{a}	7.20 ± 0.05^{ab}	7.10 ± 0.05^{ab}
Odor scores (10-point scale)				
Day 0	9.80 ± 0.03^{a}	9.82 ± 0.02^{a}	9.75 ± 0.04^{ab}	9.71 ± 0.03^{ab}
Day 1	9.00 ± 0.04^{ab}	9.50 ± 0.02^{a}	9.10 ± 0.03^{ab}	9.05 ± 0.05^{ab}
Day 2	8.40 ± 0.05^{b}	9.30 ± 0.02^{a}	8.70 ± 0.04^{ab}	8.65 ± 0.04^{ab}
Day 3	7.40 ± 0.06^{bc}	9.00 ± 0.03^{a}	8.30 ± 0.05^{ab}	8.25 ± 0.05^{ab}
Day 4	6.60 ± 0.07^{c}	8.70 ± 0.04^{a}	7.90 ± 0.04^{ab}	7.85 ± 0.05^{ab}
Day 5	5.90 ± 0.08^{c}	8.40 ± 0.04^{a}	7.30 ± 0.05^{ab}	7.20 ± 0.05^{ab}
Day 6	6.00 ± 0.02^{c}	7.99 ± 0.03^{a}	7.10 ± 0.02^{ab}	7.00 ± 0.03^{ab}
Overall acceptability scores (10-point scale)				
Day 0	9.81 ± 0.03^{a}	9.75 ± 0.02^{a}	9.60 ± 0.04^{ab}	9.55 ± 0.03^{ab}
Day 1	9.00 ± 0.04^{b}	9.70 ± 0.02^{a}	9.30 ± 0.03^{ab}	9.25 ± 0.05^{ab}
Day 2	8.40 ± 0.05^{b}	9.50 ± 0.02^{a}	8.90 ± 0.04^{ab}	8.85 ± 0.04^{ab}
Day 3	7.60 ± 0.06^{bc}	9.20 ± 0.03^{a}	8.50 ± 0.05^{ab}	8.45 ± 0.05^{ab}
Day 4	6.90 ± 0.07^{c}	8.90 ± 0.04^{a}	8.10 ± 0.04^{ab}	8.05 ± 0.05^{ab}
Day 5	6.20 ± 0.08^{c}	8.60 ± 0.04^{a}	7.50 ± 0.05^{ab}	7.45 ± 0.05^{ab}
Day 6	5.90 ± 0.03^{c}	8.30 ± 0.04^{a}	7.15 ± 0.05^{ab}	7.05 ± 0.05^{ab}

G5: Negative control, raw milk, G6: Raw milk fortified with β-carotene, G7: Raw milk fortified with riboflavin, G8: Raw milk fortified with a mix of β-carotene and riboflavin. The values represent as mean ± Standard error. ^{a,b,c,d} Means within a row followed by different superscript letters are significantly different ($p < 0.05$).

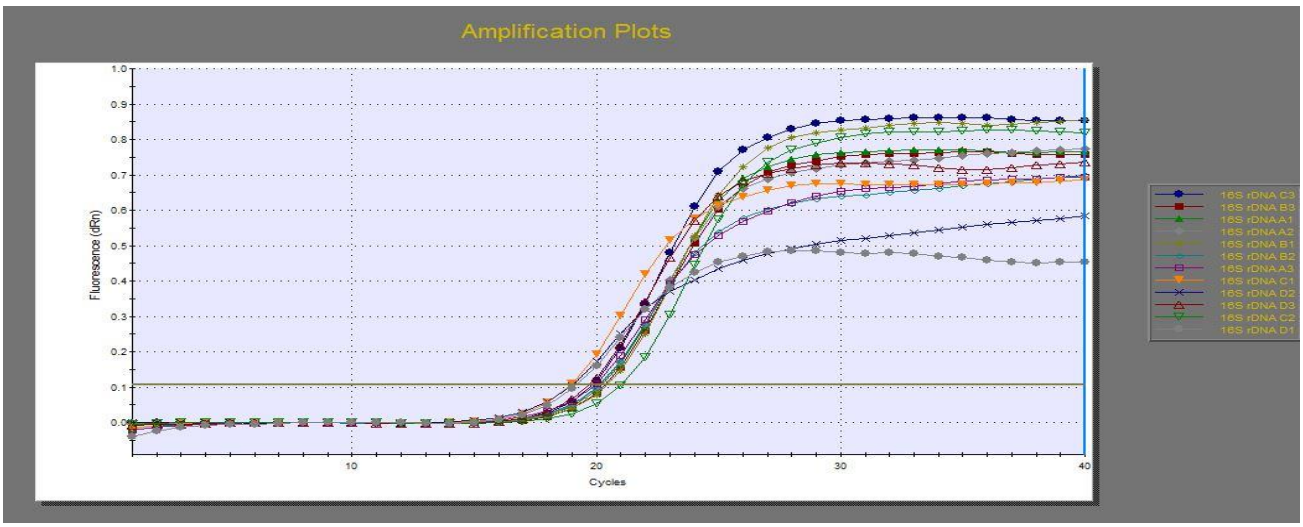


Figure 5. Down-regulation of the *16S rDNA* gene in the *Pseudomonas*-treated groups compared to the non-treated control group

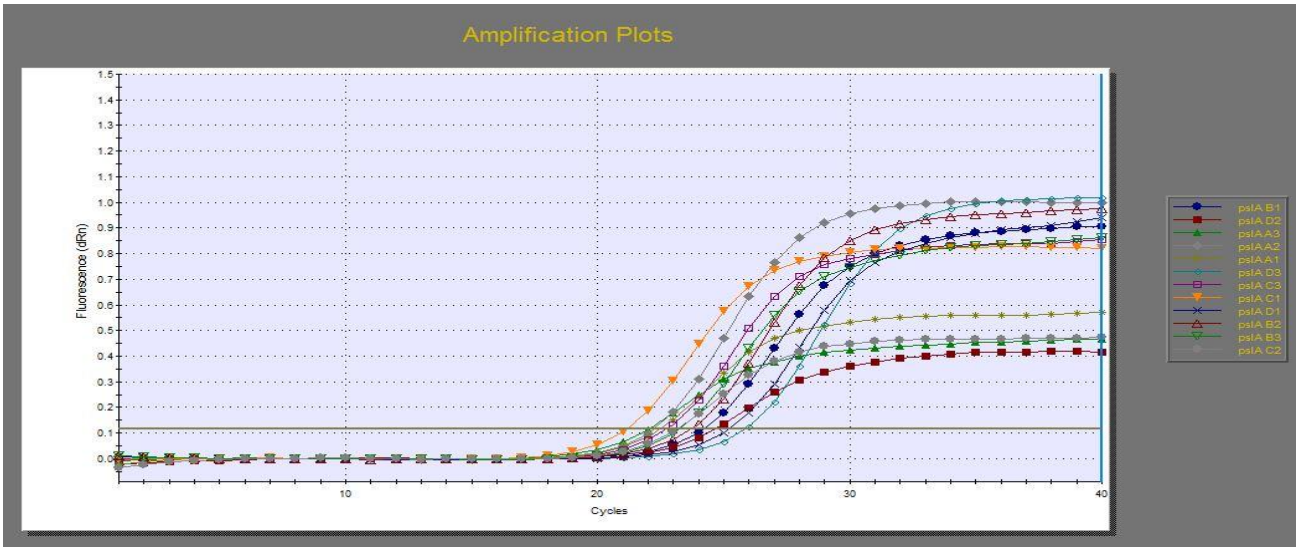


Figure 6. Down-regulation of the *pslA* gene in the *Pseudomonas*-treated groups compared to the non-treated control group

DISCUSSION

In the current study, *Pseudomonas* was isolated from 20% of the samples (20/100). Likewise, Garedew et al. (2012) documented an isolation rate of 18.5% in Gondar town, Ethiopia. Conversely, Atia et al. (2022) reported a higher prevalence of 56% from raw milk samples collected in Shebin El-Kom city, Egypt, whereas Deiab et al. (2023) identified a prevalence of 36% in bulk tank milk from Menofia Governorate, Egypt. The variation in the isolation rates observed in the present study may be attributable to a variety of factors, including hygiene practices, geographic location, disease prevention measures, management strategies, and the efficacy of the immune system.

Bacterial species capable of producing biofilm frequently demonstrate heightened virulence, thereby facilitating their attachment to solid surfaces and the formation of a protective matrix around the cells. This matrix protects bacteria against antibiotics and disinfectants designed to eradicate them (Wilson et al., 2017). During the present study, 75% of isolates were identified as biofilm producers by TCP, while only 60% were identified as biofilm producers by TM, which displayed different levels of biofilm-forming ability. The present findings were lower than those reported by Ngo et al. (2021), who observed biofilm formation in 100% of *Pseudomonas* isolates. Similarly, Deiab et al. (2023) reported a higher prevalence, with 78% of isolates forming biofilms, comprising 48.7% classified as strong, 23.07% as moderate, and 28.2% as weak producers. Conversely, lower rates were observed by Milivojević et al. (2018) and Banda et al. (2020), with biofilm production identified in only 16.3% and 15% of *Pseudomonas* isolates, respectively.

Molecular methods are increasingly preferred for pathogen detection, supplementing traditional culture-based plate enumeration techniques (Zhang et al., 2019). In the present study, 83.33% of the *Pseudomonas* isolates were found to carry the *pslA* gene, which was consistent with the findings of Al-Sheikhly et al. (2020), who observed a 100% prevalence of the *pslA* gene in isolates obtained from the Department of Biology, College of Science, University of Anbar, Iraq. Unlike Abdelraheem et al. (2020), who reported detection rates of 66.7% for *pelA* and 74.1% for *lasR* at Minia, Egypt, neither of these genes was detected in any of the isolates analyzed in the current study. Similarly, Al-Sheikhly et al. (2020) observed the presence of the *pelA* gene in all the isolates. Differences in the prevalence of biofilm-associated virulence genes (*pslA*, *pelA*, and *lasR*) across studies may be attributed to genetic diversity among isolates, differences in sample sources and environmental conditions, as well as different methods. β-carotene, widely recognized as a precursor of vitamin A and a powerful antioxidant, has recently garnered attention for its potential antibacterial properties. Although the precise mechanisms remain incompletely understood, several prospective modes of action have been identified. These mechanisms included disruption of bacterial cell membranes, as β-carotene is a lipophilic compound capable of integrating into and disturbing membrane structure (Hagaggi and Abdul-Raouf, 2023), and the generation of ROS, where β-carotene may act as a pro-oxidant under certain conditions, leading to increased ROS production within bacterial cells (Bae et al., 2021). Similarly, riboflavin (Vitamin B2), an essential nutrient present in both plants and animals, plays a crucial role in human health and has demonstrated antibacterial, antifungal, and anticancer activities (Farah et al., 2022).

The present study assessed the antibacterial activity of β-carotene and riboflavin against *Pseudomonas* spp., which demonstrated notable antibacterial effects, as indicated by clear inhibition zones. These results align with the findings of

Muntean et al. (2021), who reported inhibition zones for β-carotene ranging from 15.33 ± 0.58 mm to 16.0 ± 0.0 mm, and with those of Ahgilan et al. (2016), who observed a 15.7 ± 0.6 mm inhibition zone for riboflavin.

The present study demonstrated that the MIC of β-carotene was 1 µg/mL, and that of riboflavin was 4 µg/mL. The MBC was determined to be 2 µg/mL for β-carotene and 8 µg/mL for riboflavin. Comparatively, Muntean et al. (2021) reported a MIC of 6.25 µg/mL and an MBC of 25 µg/mL for β-carotene, suggesting that during the present study, β-carotene exhibited more potent antibacterial activity at significantly lower concentrations. Importantly, the antibacterial efficacy of β-carotene and riboflavin can be influenced by different factors, including the specific bacterial strain and experimental methodology (Tao NengGuo et al., 2010). Therefore, MIC values can vary across different studies and conditions.

The current results indicate that *Pseudomonas* survival increased over time in the control group, while all treated groups, β-carotene, riboflavin, and their combination, demonstrated antimicrobial effects. β-carotene demonstrated a more substantial reduction compared to riboflavin alone, and the combination of both had the most pronounced inhibitory effect on *Pseudomonas* growth during refrigerated storage of raw milk.

Recent studies suggested that riboflavin can suppress or inactivate a wide range of microorganisms, including bacteria, viruses, fungi, and parasites (Akompong et al., 2000; Tonnetti et al., 2013; Ahgilan et al., 2016). However, studies specifically focused on its antimicrobial properties remain limited. Some evidence indicated that riboflavin alone can inhibit the growth of several pathogenic microbes, such as *Staphylococcus aureus (S. aureus)*, *Enterococcus faecalis (E. faecalis)*, *Salmonella typhi (S. typhi)*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa (P. aeruginosa)*, *Candida albicans*, and *Plasmodium falciparum* (Kashiwabuchi et al., 2013; Ahgilan et al., 2016). Ahgilan et al. (2016) reported that riboflavin, at a concentration of 50 µL, was effective in inhibiting the growth of Gram-positive bacteria, including *S. aureus*, *E. faecalis*, *S. typhi*, and *P. aeruginosa*. During the present study, the group treated with β-carotene demonstrated a significant reduction in comparison to the control group, thereby supporting the hypothesis that β-carotene exhibits antibacterial properties. One proposed mechanism involves the disruption of bacterial cell membranes, where lipophilic characteristics of β-carotene enable it to integrate into the lipid bilayer, thereby increasing membrane permeability, causing leakage of intracellular contents, and ultimately decreasing bacterial viability (Hagaggi and Abdul-Raouf, 2023). Additionally, under specific conditions, β-carotene may act as a pro-oxidant, inducing the generation of ROS. These ROS can damage essential cellular components such as DNA, proteins, and lipids, resulting in bacterial cell death (Sinha et al., 2023).

The sensory evaluation results revealed a significant effect of β-carotene and riboflavin on the sensory attributes of raw milk during cold storage. Among all treatment groups, the milk samples fortified with β-carotene consistently received the highest scores for appearance, flavor, odor, and overall acceptability throughout the 6-day storage period, with values scored 8.50, 8.00, 7.99, and 8.30, respectively, on day 6. The current findings indicated that β-carotene not only enhances the visual appeal of the milk but may also contribute to preserving its organoleptic properties over time. This finding is attributed to the significant antioxidant role of β-carotene that reduces protein and lipid oxidation, stabilizes flavor, and thereby improves overall milk stability (Yabuzaki, 2017).

Numerous studies have indicated that β-carotene enhances sensory stability and shelf life in dairy products. Cakmakci et al. (2014) incorporated 15% carrot juice into set-type yoghurt and observed the highest sensory scores throughout a 21-day cold storage period. Similarly, Senarathne and Wickramasinghe (2019) developed a β-carotene-rich drinkable yoghurt containing 10% carrot pulp, which remained microbiologically stable and sensorially acceptable over 35 days. Overall, the incorporation of β-carotene has demonstrated potential as an effective natural strategy to improve the shelf life and sensory quality of raw milk, thereby offering promising applications in dairy preservation and functional food development.

The current results demonstrated that β-carotene and riboflavin can significantly reduce *Pseudomonas* virulence by targeting and downregulating the *pslA* gene, a key gene involved in biofilm formation. Treatment with β-carotene and riboflavin resulted in a significant decrease in *pslA* gene expression levels across all treated groups in comparison to the untreated control group (G1). Notably, the gene expression in Group G4 was reduced to 0.07-fold, indicating a dramatic suppression of *pslA* gene expression. Additionally, this effect was observed in groups G2 and G3, with fold changes of 0.31 and 0.54, respectively. These findings suggested that both β-carotene and riboflavin can interfere with the regulatory pathways involved in biofilm formation, possibly through their antimicrobial properties or through modulation of gene expression related to exopolysaccharide production. The significant reduction in *pslA* expression corresponds with a concurrent decline in biofilm formation, thereby corroborating the hypothesis that the suppression of this gene directly influences *Pseudomonas* pathogenicity. β-carotene and riboflavin exhibited antibacterial properties at minimal MIC and MBC values; their influence on cell viability may indirectly result in reduced expression of virulence-associated genes, including *pslA*. Alternatively, these compounds might act as signaling modulators, interfering with quorum sensing or other regulatory systems essential for biofilm development. The current results aligned with the

findings of Abdulhadi et al. (2023), who reported that β-carotene pigment exhibited the strongest antimicrobial activity at a concentration of 100 mg/mL, as well as with Ahgilan et al. (2016), who demonstrated the inhibitory effects of riboflavin against several bacterial and fungal species. These findings presented opportunities for developing new anti-virulence strategies that specifically target biofilm-associated mechanisms and pathways.

CONCLUSION

Pseudomonas spp. was detected in 20% of samples and 60% of the isolates were biofilm producers, with 83.33% carrying the *pslA* gene. β-carotene and riboflavin demonstrated vigorous antibacterial activity, low MIC/MBC values, and significantly reduced *Pseudomonas* counts during cold storage, especially when combined. The RT-qPCR revealed significant suppression of the *pslA* gene expression, thereby confirming its anti-biofilm activity effects. Sensory evaluation preferred milk treated with β-carotene, followed by milk with riboflavin, and the combinationβ-carotene and riboflavin demonstrated potential as natural additives to improve safety, prolong shelf life, and preserve milk quality. Future studies should focus on clarifying the molecular mechanisms by which β-carotene and riboflavin suppress biofilm-related genes, verifying their preservative efficacy under real milk processing and storage conditions, and confirming their impact on sensory attributes and nutritional quality to ensure both safety and consumer acceptance

DECLARATIONS

Ethical considerations

This manuscript is the authors' original study and has not been submitted or published elsewhere. The authors have verified the content for plagiarism and confirm that the article is based on their own original scientific findings.

Funding

The present study did not receive any funding or financial support.

Acknowledgments

The authors would like to acknowledge all members of the Food Hygiene and Bacteriology Departments at the Animal Health Research Institute (AHRI), Tanta Laboratory, Agricultural Research Center (ARC), Egypt, for their valuable support and contributions.

Availability of the data and materials

All data supporting the findings of the present study are available in the manuscript.

Author contributions

Heba E. El-Shora and Eman K. Fathalla conceived and supervised the overall study design and execution. Radwa A. Lela and Amal F. Elbanna were responsible for sample collection, data analysis, and interpretation of the results. Doaa M. Sharaf and Momen M. Aladl contributed to the experimental design and critically reviewed the manuscript. All authors contributed to manuscript writing and approved the final edition for submission and publication.

Competing interests

The authors declared no conflict of interest.

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2025, Scienceline Publication
World's Veterinary Journal
World Vet J, 15(3): 824-830.
ISSN 2322-4568

DOI: https://dx.doi.org/10.54203/scil.2025.wvj84
PII: S232245682500084-15

Microbiological and Hematological Diagnostic Profiles of Strawberry Footrot in Sheep in Karawang, Indonesia

Jasmine Yasyfa' Sukmayani[1], Fadeli Bermani[2]*, and Dwi Nawang Wicaksana[3]

[1]*Veterinary Profession Program, Faculty of Medicine, Padjadjaran University, Indonesia*
[2]*Departement of Basic Medical Sciences, Faculty of Medicine, Padjadjaran University, Indonesia*
[3]*Subang Veterinary* Center, *Indonesia*

*Corresponding author's Email: Fadeli@unpad.ac.id

CASE REPORT ARTICLE
Received: June 19, 2025
Revised: July 27, 2025
Accepted: August 29, 2025
Published: September 30, 2025

ABSTRACT

Dermatophilus congolensis (*D. congolensis*) is a gram-positive, coccus-shaped pathogenic bacterium that develops a filamentous structure during the infectious phase. The *D. congolensis* can cause scab lesions on sheep's feet, which may eventually develop into bloody tissue resembling crushed strawberries. The present study aimed to provide diagnostic, microbiological, and hematological profiles of strawberry footrot in sheep in Karawang Regency, Indonesia. Six sheep (*Ovis aries*), with an average age of two years and exhibiting similar signs of strawberry footrot, were sampled from community farms in the region. Blood samples and exudate swabs from scab lesions on the feet and udder were collected in August 2024 for analysis. Hematological testing and bacterial isolation using blood agar, gram staining, fermentation, and biochemical tests were performed. The hematologic results indicated thrombocytosis, macrocytic normochromic anemia, and leukocytosis in the sheep. All samples exhibited beta hemolysis zones on blood agar and were gram-positive, coccus-shaped bacteria with a railroad appearance. Biochemical tests were positive for catalase, negative for indole, negative for Voges-Proskauer (VP), and showed the ability to ferment glucose and maltose. Based on clinical signs, hematological findings, and bacterial isolation, *D. congolensis* was confirmed as the causative agent of strawberry footrot in sheep in Karawang Regency, Indonesia.

Keywords: *Dermatophilus congolensis*, Hematological profile, Microbiology, Strawberry footrot

INTRODUCTION

Strawberry footrot is a disease caused by *Dermatophilus congolensis* (*D. congolensis*) and is often associated with contagious ecthyma caused by *Parapoxvirus* (Blick et al., 2019). Strawberry footrot presents as scabs with defined edges affecting small ruminants, including sheep and goats. These scab lesions can develop into openings on the foot, leading to bleeding tissue that resembles crushed strawberries (Tellam et al., 2021). In cases of secondary infection by pyogenic bacteria, purulent discharge may occur. The occurrence of strawberry footrot is sporadic in tropical regions such as West Africa, East Africa, and the Caribbean and tends to increase during the rainy season or humid conditions (Dirkeswan, 2014). In sheep, morbidity can range from 0.77% to 31%, with mortality reaching 10%, often linked to factors such as poor nutrition and ectoparasites (Faccini et al., 2022). Clinical signs of strawberry footrot include dryness, fistulas, and lesions. Exudative lesions may develop when lesions are exposed to prolonged moisture (Chitra et al., 2017). Lesions can resolve spontaneously within six to eight weeks (Aitken, 2007).

In 1993, an outbreak of skin disease occurred in Ongole crossbred cattle in Kulon Progo Regency, Yogyakarta, Indonesia. An investigation was conducted to assess parasite infestation, and treatment was administered using ivermectin; however, it did not yield a healing response. Microscopic analysis using direct smears and bacterial isolation of the scabs revealed the infection with *D. congolensis*, characterized by long filaments and coccoid bacteria (Dirkeswan, 2014). Until now, comprehensive investigations and reports regarding diagnostics and the prevalence of *D. congolensis* infection in Indonesia remain limited. The present study aimed to provide diagnostic, microbiological, and hematological profiles of strawberry footrot in sheep (*Ovis aries*) in Karawang, Indonesia.

MATERIALS AND METHODS

Ethical approval

The present case report was conducted at Balai Veteriner Subang, a government veterinary diagnostic and research institution operating under the Ministry of Agriculture, Republic of Indonesia. The procedures were performed in accordance with the institution's standardized diagnostic protocols and operational guidelines. Blood samples were obtained by a licensed veterinarian in a calm setting to minimize stress. Farmers and owners received information about the study's purpose and procedures before participation.

Study area

To cite this paper: Yasyfa' Sukmayani J, Bermani F, and Wicaksana DN (2025). Microbiological and Hematological Diagnostic Profiles of Strawberry Footrot in Sheep in Karawang, Indonesia. *World Vet. J.,* 15(3): 824-830. DOI: https://dx.doi.org/10.54203/scil.2025.wvj84

Six male sheep (*Ovis aries*) were obtained from community farms in Indonesia and were approximately two years old. The sheep showed clinical signs suggestive of *D. congolensis* infection, such as fever, facial swelling, swollen knees, and scab lesions on their lower legs. Blood samples and exudate swabs from the scab lesions on the digits and udder were collected from three farms in the Karawang Regency, West Java, Indonesia, on August 8, 2024. Hematological examination and bacterial isolation were performed at the Subang Veterinary Center, West Java, Indonesia.

Hematological analysis

Blood samples were collected from six sheep (*Ovis aries*) displaying clinical signs of suspected *D. congolensis* infection, including fever, scab lesions, facial swelling, and swollen knees, using a three mL syringe with a 23G needle. A total of three mL of blood was taken via jugular vein and placed into the EDTA tube, then analyzed using a hematology analyzer (Rayto RT 7600, China). Hematological testing was performed in cases of suspected *D. congolensis* infection to assess the systemic physiological response of the host to the bacterial infection, with total leukocyte (10^3 /µL), erythrocyte (10^6 /µL), and platelet count (PLT, 10^3 /µL) as parameters (Simsek et al., 2022).

Isolation and identification of *Dermatophilus congolensis*

Swab samples of exudate from the scab lesions of the digit and dry scab of the udder in the sheep were taken for bacterial isolation and identification. Each swab of scab exudate sample was directly inoculated onto blood agar media (Oxoid, UK) using the streaking method with a sterile loop and incubated 37°C for 24 hours (Khan et al., 2024). Gray and beta hemolytic colonies were analyzed by Gram staining and catalase test, followed by biochemical tests such as indole, Voges-Proskauer (VP), and sugar fermentation tests (Glucose, Maltose, Salicin, Mannitol, and Sorbitol). Gram staining was performed to identify the presence of branching, filamentous Gram-positive bacteria with a tram-track appearance to confirm *D. congolensis* as the etiological agent (Habte et al., 2025). The biochemical characteristics of the gray and beta hemolytic colonies on blood agar were compared with previously published profiles of *D. congolensis* to support species-level identification (Samuel et al., 1998; Amor et al., 2011; Walter et al., 2017).

Statistical analysis

The collected data were compiled, processed, and analyzed using descriptive statistical methods. Hematological parameters were evaluated by calculating the mean and standard deviation values to identify relevant patterns and trends.

RESULTS AND DISCUSSIONS

Clinical findings

Clinical examinations were conducted to evaluate the type, distribution, and severity of skin lesions, including an assessment of lesion structure, consistency, location, and exudate. Infected sheep demonstrated a variety of dermatological abnormalities strongly suggestive of *D. congolensis* infection. Sheep exhibiting signs such as fever, lethargy, and swelling in the face, knees, and lower legs were observed for different changes (Moriello, 2019). Confidence in diagnosing *D. congolensis* infection was based on the key clinical signs in affected sheep, which exhibited fever with body temperatures ranging from 41°C to 42°C. These sheep developed scab lesions on the digit, and dry scabs were observed on the udder (Figure 1). Additionally, some sheep showed signs of alopecia and pustular lesions on the head (Figure 2). The observed sheep aligned with findings by Marsella (2014), which indicated that this bacterial infection caused local alopecia and primary lesions such as papules that developed into pustules, potentially leading to matted hair and thick crust formation in some cases. Purulent exudate was readily visible in active lesions.

Motile zoospores of *D. congolensis* infect the epidermis and hair follicles, leading to scab exudate lesions in dermatophilosis. These zoospores germinate and develop into filamentous hyphae that invade and multiply within the live epidermis and hair follicles, areas where the skin's protective barrier is weak (Moriello, 2019). The acute inflammatory response features infiltration of neutrophils and other immune cells, resulting in serous exudation on the skin surface. Scabs composed of crust exudate and necrotic epithelial debris form due to hyperkeratinization and keratinization of the affected epidermal tissue (Tellam et al., 2021). The infection cycle is extended in the presence of moisture because it encourages zoospore growth and release from the hyphae inside these scabs (Tellam et al., 2021). Dry scab lesions on the sheep udder occur from repeated infections in the epidermis, leading to a thickened cornification layer. Eventually, the lesions continue to grow and thicken until they detach and form scab exudate lesions (Aitken, 2007).



Figure 1. Pathological lesions in a 2-year-old male sheep (*Ovis aries*). **A**: Exudate scab lesion on the digit, **B**: Exudate scab lesion on the fourth digit of the sheep, **C**: Dry scab on the udder of the sheep, arrow indicates the wet scab lesions on the udder. Source: Authors of the present study



Figure 2. Pathological lesions in a 2-year-old male sheep head (*Ovis aries*). **A**: Alopecia of the sheep's head, **B**: Pustular lesion of the nose. Source: Authors of the present study

Clinical examination of the affected sheep in Karawang Regency, Indonesia, showed characteristic lesions on different body parts, including the mouth, udder, forelimbs, hindlimbs, and areas of alopecia on the face and body. These clinical signs were suspected to be manifestations of infection with *D. congolensis*, the causative agent of strawberry footrot (Dermatophilosis), and the *parapoxvirus* that causes contagious pustular dermatitis (CPD). According to Kumar et al. (2015), CPD typically presents as lesions located around the lips, oral mucosa, gingiva, and inner mouth, with possible spread to the udder and inguinal region. Lesions on the udder frequently occur along with lesions on the peri-labial area. In some cases, CPD-associated lesions extend to the limbs, particularly the interdigital space and the coronary band, leading to pedal degeneration. The lesions often display a proliferative or papillomatous morphology resembling cauliflower-like growths. The incubation period of CPD ranges from 2 to 3 weeks, and initial clinical signs include fever, lethargy, hypersalivation, gingival and inguinal swelling, followed by the emergence of pustular or papillomatous lesions (Kumar et al., 2015). In clinical cases observed on sheep in Karawang regency, Indonesia, lesions were present in the digit region but were notably absent in the coronary band area. In the present study, lesions observed in sheep were localized to the digits, with no involvement of the coronary band, which is commonly affected in CPD (Mansoor et al., 2025). The presence of exudative, crusted lesions and localized hair loss pointed more toward dermatophilosis, suggesting *D. congolensis* as the primary pathogen rather than a *parapoxvirus* infection.

Hematological findings

The blood sample results of the six sheep can be seen in Table 1.

Table 1. Hematology test results of sheep (*Ovis aries)* with clinical signs of suspected *D. congolensis*

Parameter	Result (Mean ± SD)	Normal range***
HB (g/dL)	9.18 ± 0.2	9-15
HCT (%)	28.1 ± 1.6	27-45
MCH (pG)	14.6 ± 3.6*	8-12
MCHC (pG)	32.12 ± 0.9	31-34
MCV (fL)	45.35 ± 10.8*	28-40
PDW (%)	13.78 ± 3.24	29-96
PLT (10^3/µL)	2.258 ± 1.376*	800-11.000
RBC (10^6/µL)	6.48 ± 1.6**	9-15
RDW (%)	1.6 ± 1.9	16- 22
WBC (10^3/µL)	13.58 ± 8.5*	4-8

* Indicated above normal value, ** Indicated below normal value, ***Source of control data. Source: (Merck, 2024), HB: Hemoglobin, HCT: Hematocrit, MCH: Mean corpuscular hemoglobin, MCHC: Mean corpuscular hemoglobin concentration, MCV: Mean corpuscular volume, PDW: Platelet distribution width, PLT: Platelet count, RBC: Red blood cell count, RDW: Red cell distribution width, and WBC: White blood cell count.

The average red blood cell count (RBC) in the six sheep was below the normal reference range, indicating anemia. According to Nayyef (2020), anemia results from a deficiency of RBC and hemoglobin (HB), leading to inadequate oxygen delivery to meet physiological demands. Similarly, Turner et al. (2023) noted that anemia is defined by reduced erythrocyte counts, HB levels, and hematocrit (HCT) values. This condition compromises physiological efficiency, weakens antioxidant activity, and may progress to hypovolemic shock (Sousa et al., 2022).

Blood analysis of the six sheep revealed macrocytic-normochromic anemia, marked by elevated mean corpuscular volume and mean corpuscular hemoglobin, along with enlarged erythrocytes due to impaired maturation (Killeen and Adil, 2025). Additionally, the hematological analysis revealed leukocytosis, characterized by elevated white blood cell counts, which typically occurs in response to infection, inflammation, malignancy, or genetic disorders (Mank et al., 2024). This finding correlated with the observed clinical signs, as tissue injuries would naturally trigger a leukocytic response to infection. In addition, thrombocytosis (Increased PLT) was detected, a condition often associated with infectious processes, inflammatory states, or hemorrhage, consistent with the open lesions that were inducing both inflammation and bleeding in affected animals (Rokkam et al., 2024). As previously reported by Hamid and Musa (2009) and Simsek et al. (2022), *D. congolensis* infection led to decreased RBC counts, Hb levels, and HCT values, mirroring the current results.

Microbiological findings

Blood agar isolation revealed two colony types, including milky white and grayish, both convex, smooth, and beta hemolytic (Figure 3a). On blood agar, the colonies demonstrated morphological characteristics consistent with *D. congolensis*, appearing as smooth, grayish-white colonies with distinct beta-hemolysis (Burd et al., 2007; Oladunni et al., 2016). A catalase test was performed on bacterial colonies with beta hemolysis. After receiving H_2O_2, bacterial colonies in the catalase test generated foam (Figure 3b). The catalase test yielded a positive result, as evidenced by the immediate formation of oxygen bubbles upon the application of hydrogen peroxide, indicating the presence of catalase enzyme activity. The catalase enzyme is present in *D. congolensis* and can break down H_2O_2 into O_2 and H_2O (Tresamol and Saseendranath, 2015). The bubbles produced from the production of oxygen gas demonstrated positive catalase results. The beta-hemolytic colonies observed in blood agar were subjected to Gram staining, which revealed Gram-positive coccus-shaped bacteria with a characteristic tram track pattern indicated by the blue coloration observed in the staining results (Figure 3c).

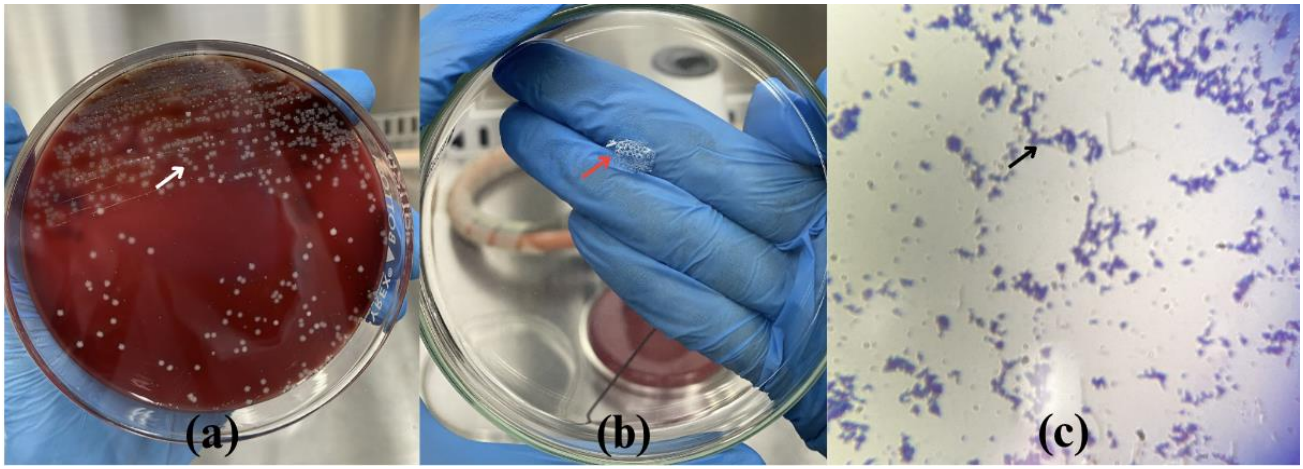


Figure 3. The results of bacterial isolation from the exudate of the scab lesions of the digit and the dry scab of the udder in the sheep (*Ovis aries*) on blood agar media. **A**: White arrow shows grayish bacterial colonies that hemolyze the media, **B**: Red arrow shows catalase positive, and **C**: Black arrow shows gram-positive coccus-shaped and tram track-like appearance. Source: Authors of the present study.

The *D. congolensis* was included in the Gram-positive and has elongated filaments consisting of cocci in rows of two or four cocci and looked like railroad tracks (Tram track-like appearance; Habte et al., 2025). The *D. congolensis* possesses a well-defined life cycle comprising two primary morphological forms, including filamentous hyphae and motile zoospores. The hyphal structures are composed of branching filaments that undergo transverse and longitudinal septation, eventually fragmenting into coccoid cells. These coccoid cells then develop into flagellated zoospores, which constitute the infective stage of the organism. The zoospores are capable of penetrating compromised skin, particularly under conditions of high humidity or mechanical skin damage, initiating infection in a susceptible host (Marsella, 2014).

Fermentation testing was conducted to determine the ability of sugar fermentation in colonies suspected of *D. congolensis*. The ability of *D. congolensis* to ferment sugars was revealed on test agar by a yellow color transition, reflecting acid production and subsequent pH decrease in the culture medium (Mannan, 2009). Based on the fermentation test, the bacteria fermented glucose and maltose (Figure 4; Walter et al., 2017). Isolated crust samples taken from animals with skin lesions indicated that *D. congolensis* can ferment glucose and maltose and cannot ferment mannitol, sorbitol, or salicin. Indole testing performed in the present study yielded negative results, consistent with the findings of Amor et al. (2011) that *D. congolensis* lacks indole-producing enzymes. The negative reaction was confirmed by the absence of a characteristic ring formation upon addition of Kovac's reagent to the culture medium. The VP test is a biochemical test that detects the ability of bacteria to metabolize pyruvate into a neutral intermediate product called acetylmethylcarbinol or acetonin (Shanmugaraj et al., 2021). All isolates of the present study were VP-negative, which was aligned with the findings reported by Samuel et al. (1998) that *D. congolensis* was negative in VP testing. All of the results, including clinical signs, hematological profile, and bacterial isolation, were consistent with *D. congolensis* infection as observed in sheep (*Ovis aries*) from Karawang Regency, Indonesia. The affected sheep exhibited characteristic clinical signs such as crusted skin lesions and alopecia, hematological changes including leukocytosis, thrombocytosis, and macrocytic-normochromic anemia, and the isolation of branching Gram-positive bacteria and a tram track-like appearance.

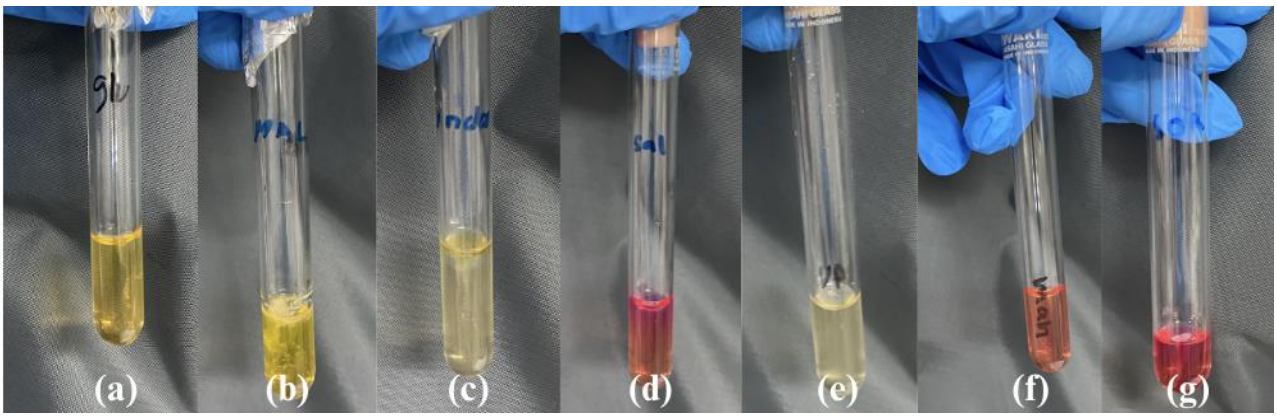


Figure 4. Biochemical and fermentation test results of a colony of beta-hemolytic suspected to *D. congolensis*. **A**: Positive glucose test, **B**: Positive maltose test, **C**: Negative indole test, **D**: Negative salicin test, **E**: Negative Voges-Proskauer test, **F**: Negative mannitol test, **G**: Negative sorbitol test. Source: Authors of the present study

CONCLUSION

The *D. congolensis* causes scab lesions on sheep's digits and udder areas. Hematology examination results illustrated a blood profile of leukocytosis, thrombocytosis, and macrocytic-normochromic anemia. Microbiological analysis performed on swab samples of scab exudate on the digit area indicated that this bacterium is a Gram-positive, coccus-shaped bacterium with a tram track-like appearance, capable of beta hemolysis on blood agar media, and can ferment glucose and mannitol. Further studies should focus on the molecular characterization and genotyping of *D. congolensis* isolates and investigate larger sheep populations to provide epidemiology, risk factors, and distribution patterns of *D. congolensis*.

DECLARATIONS

Availability of data and materials

All data obtained or analyzed in the course of the present study are presented within the manuscript. Additional information or datasets can be provided by the corresponding author upon reasonable request.

Funding

The present study was performed without any external financial assistance from governmental agencies, commercial enterprises, or non-profit organizations. The authors independently funded and managed the study.

Acknowledgments

Gratitude to Subang Veterinary Center and Padjadjaran University, Indonesia, which have helped through the present study. Gratitude is also expressed to all parties who have been involved and helped in the present study.

Authors' contributions

Jasmine Yasyfa' Sukmayani was responsible for directly contributing to sample processing, data processing, and drafting the manuscript. Fadeli Bermani was responsible for supervising the draft and revising the draft manuscript. Dwi Nawang Wicaksana conceptualized and designed the idea for the present study. Each author had an important role in the preparation of the manuscript. All authors were responsible for reviewing the manuscript and contributing to the refinement of the final version of the manuscript. All authors read and approved the final edition of the manuscript for publication.

Competing interests

The authors declare no conflict of interest.

Ethical considerations

All authors have been checked the manuscript for plagiarism, dual publication and/or submission, falsification and/or fabrication, and redundancy.

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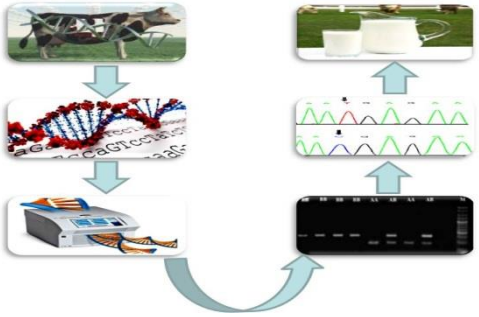
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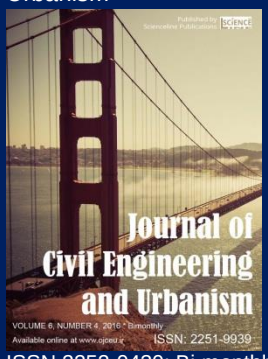
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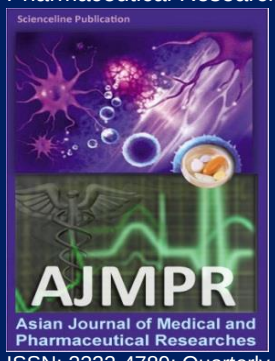
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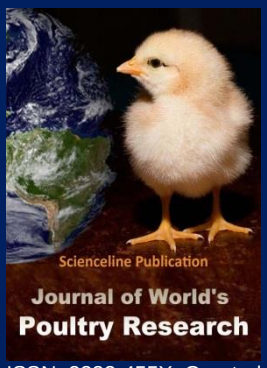
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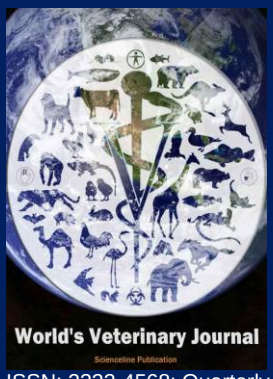
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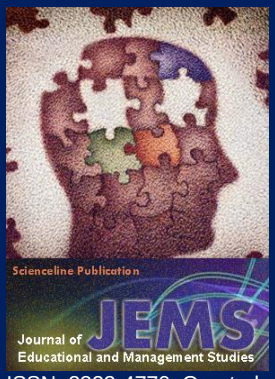
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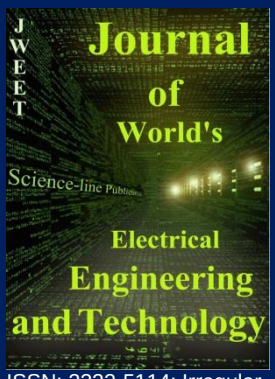
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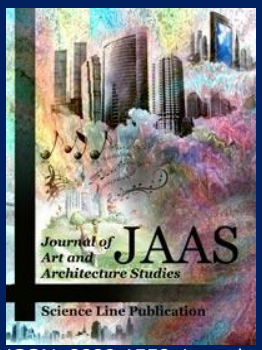
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