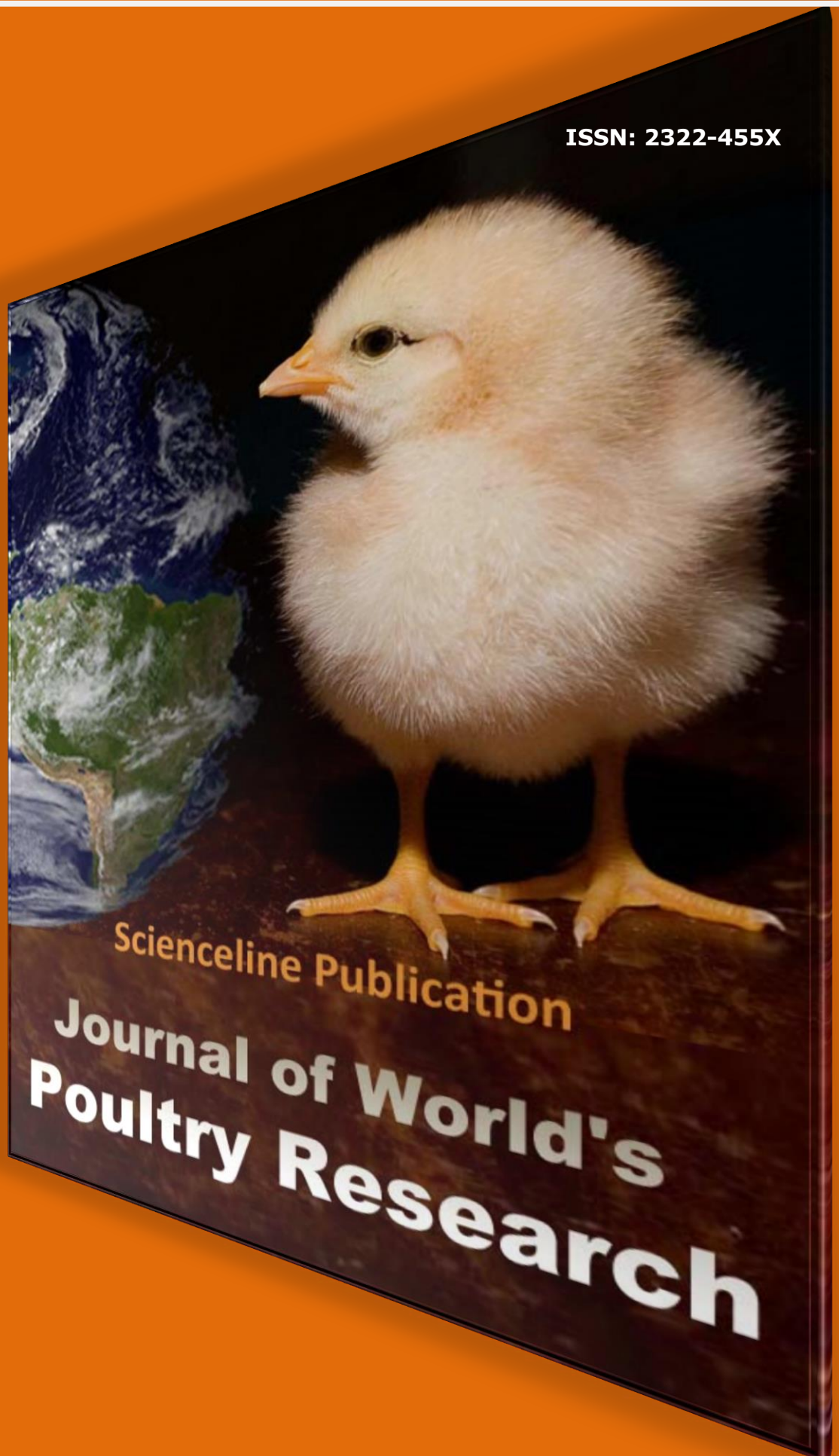




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Volume 16 (3); September 2026

Research Paper

Effects of Dietary Nettle (*Urtica dioica*) Leaf Powder on Performance, Welfare, Hematological Profile, and Oxidative Status in Broiler Turkeys

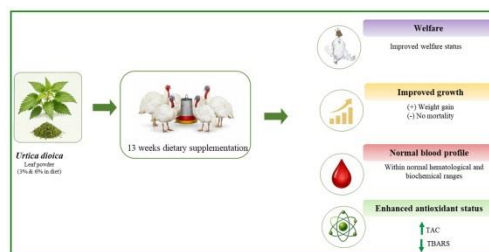
Sadoudi A, Saidj D, Bellik Y, Touazi L, Yahi K, Aberkane B, Iguer-Ouada M, and Moula N.

J. World Poult. Res. 16(3): 243-255, 2026; pii: S2322455X2600022-16

DOI: <https://dx.doi.org/10.36380/jwpr.2026.22>

ABSTRACT: Medicinal plants, such as nettle (*Urtica dioica*), are increasingly popular as sustainable alternatives to synthetic growth promoters and antioxidants in poultry production. The present study aimed to evaluate the effects of dietary nettle leaves on growth performance, welfare indicators, hematological and biochemical parameters, and oxidative status of broiler turkeys (*Meleagris gallopavo*) reared under intensive conditions. Seventy-two turkeys were randomly assigned to one of three dietary treatments from 3 to 15 weeks of age, including a control diet (CN), a diet with 3% nettle leaf powder (N3), and a diet with 6% nettle leaf powder (N6). Growth performance was monitored until week 13, while blood, carcass, and organ parameters were measured at the end of the trial. Mortality occurred only in the control group (3.33%), while there was no mortality in groups N3 or N6. Growth performance was significantly influenced by diet and sex. Male turkeys fed the N3 diet achieved higher final body weight compared to those receiving the CN or N6 diets. Conversely, no growth-promoting effect was observed in female turkeys. Female turkeys receiving the N3 diet exhibited lower final body weight and a higher feed conversion ratio than those in the CN group, indicating reduced feed efficiency. Nettle supplementation had no significant effect on carcass yield, organ development, or welfare indicators, including feather cleanliness and tarsal and plantar lesions. Hematological parameters, notably mean corpuscular volume, mean corpuscular hemoglobin, and platelet count, were significantly affected by nettle supplementation diets, whereas biochemical parameters remained within physiological ranges, indicating preserved systemic homeostasis. Dietary nettle supplementation enhanced antioxidant status, with both supplemented groups exhibiting significantly increased total antioxidant capacity, as measured by 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid; ABTS) radical scavenging activity, and a decrease in lipid peroxidation, assessed by thiobarbituric acid-reactive substances (TBARS), compared to the control group. Nettle leaf supplementation enhanced antioxidant defenses without compromising turkeys' welfare. A sex-dependent pattern was observed in growth performance parameters, with male turkeys exhibiting a greater response than females at the 3% nettle leaf inclusion level. However, both sexes exhibited consistent increases in antioxidant levels, highlighting the potential of nettle as a natural supplement in turkey production.

Keywords: Broiler turkey, Growth performance, Nettle leaf, Oxidative status, Phytogetic feed additive



Sadoudi A, Saidj D, Bellik Y, Touazi L, Yahi K, Aberkane B, Iguer-Ouada M, and Moula N (2026). Effects of Dietary Nettle (*Urtica dioica*) Leaf Powder on Performance, Welfare, Hematological Profile, and Oxidative Status in Broiler Turkeys. *J. World Poult. Res.* 16(3): 243-255. DOI: <https://dx.doi.org/10.36380/jwpr.2026.22>

[Full text-PDF] [[Crossref Metadata](#)] [Scopus] [Export from ePrints]

Research Paper

Effects of *Saccharomyces boulardii* on Productivity, Egg Quality, Excreta Microbiology, and Ammonia Emissions in Indonesian Local Ducks

Ayuningtyas G, Rayani TF, Pantaya D, Syaikhullah G, Subagja H, Prastujati AU, Priyadi DA, and Lestari FEP.

J. World Poult. Res. 16(3): 256-267, 2026; pii:

S2322455X2600023-16

DOI: <https://dx.doi.org/10.36380/jwpr.2026.23>

ABSTRACT: The Cihateup duck is an Indonesian waterfowl with potential as an egg producer. However, the productivity remains suboptimal. Dietary supplementation with the yeast probiotic *Saccharomyces boulardii* (*S. boulardii*) has been reported to improve poultry

Effects of *Saccharomyces boulardii* on Productivity, Egg Quality, Excreta Microbiology, and Ammonia Emissions in Indonesian Local Ducks



Ayuningtyas G, Rayani TF, Pantaya D, Syaikhullah G, Subagja H, Prastujati AU, Priyadi DA, and Lestari FEP (2026). Effects of *Saccharomyces boulardii* on Productivity, Egg Quality, Excreta Microbiology, and Ammonia Emissions in Indonesian Local Ducks. *J. World Poult. Res.* 16(3): 256-267. DOI: <https://dx.doi.org/10.36380/jwpr.2026.23>

productivity. The current study aimed to evaluate the effects of *S. boulardii* yeast probiotic supplementation in Cihateup duck feed on production performance, egg quality, the microbial population in excreta, and excreta ammonia level. A total of 60 female Cihateup ducks aged 71 weeks were randomly assigned to four treatment groups (three replicates per treatment, five ducks per replicate). The treatment consisted of a control (T0), which was a basal diet without *Saccharomyces boulardii*; basal diet plus 0.5 g/kg feed *S. boulardii* (T1), basal diet plus 1.0 g/kg feed *S. boulardii* (T2), and basal diet plus 1.5 g/kg feed *S. boulardii* (T3). The feed treatment lasted for 8 weeks. Production performance, including duck day production, feed conversion ratio (FCR), and total and average egg weight, was evaluated weekly. At the end of the experiment, the physical quality of eggs, fat content, cholesterol, and fatty acid profile (saturated and unsaturated fatty acids) of egg yolks were analyzed. The analysis of excreta microbes and excreta ammonia levels was conducted in the eighth week. *Saccharomyces boulardii* supplementation significantly improved duck day production, total egg weight, and FCR in the T3 group, although it did not significantly affect egg physical quality across all treatments. The egg yolk cholesterol was significantly lower in the T1 and T3 groups than in the other groups. The present study also found that supplementation with *S. boulardii* increased the egg yolk omega-6 fatty acids (linoleic acid, γ -linolenic acid, and arachidonic acid), especially in T2 and T3 treatments. Ammonia in duck excreta decreased significantly in the probiotic groups compared with the control group, with the lowest level in T3. The findings of the present study demonstrated that *S. boulardii* has the potential as a probiotic for enhancing laying performance, improving the functional quality of eggs, and reducing excreta ammonia levels of Cihateup ducks.

Keywords: Cihateup duck, Egg quality, Performance, Probiotic, *Saccharomyces boulardii*

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Research Paper

Effects of *Bacillus amyloliquefaciens* and Organic Acids on Growth Performance, Carcass Traits, Blood Parameters, and Cecal Microbiota in Cockerels

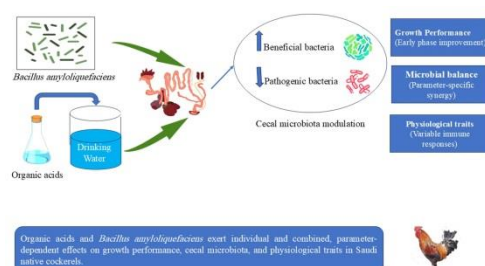
Babiker M, Alhomaidan S, Alhumud A, Alsaleem F, Elmithn S, Alhumimidi A, Alobaydaa A, Aloudah S, and Al-Eid S.

J. World Poult. Res. 16(3): 268-283, 2026; pii: S2322455X2600024-16

DOI: <https://dx.doi.org/10.36380/jwpr.2026.24>

ABSTRACT: The increasing restrictions on antibiotic growth promoters have drawn attention to natural feed additives that enhance gut health and contribute to sustainable poultry production. The present study aimed to investigate the individual and combined effects of organic acids and *Bacillus amyloliquefaciens* (*B. amyloliquefaciens*) on growth performance, organ development, carcass characteristics, blood biochemical and hematological parameters, and cecal microbiota in cockerels. A total of 200 cockerels, weighing 68.5 ± 4.44 grams, were randomly assigned to four treatment groups. Four treatments consisted of a control group (A0B0), organic acids alone (A1B0), *B. amyloliquefaciens* alone (A0B1), and their combination (A1B1), with five replicates of ten cockerels each over 42 days. Two factors were employed, with organic acids supplemented in the drinking water at two levels (0% [A0] and 0.08% [A1]) and *B. amyloliquefaciens* incorporated into the diet at two levels (0% [B0] and 0.01% [B1]) at a concentration of 1×10^9 CFU/g feed. Organic acids enhanced body weight gain during the starter phase (599.6 versus 546.1 g), whereas *B. amyloliquefaciens* reduced cumulative feed intake over the entire period (3044.9 versus 3330.9 g). Group A1B1 did not enhance growth beyond that of the individual additives, indicating no synergistic effect. Organic acids decreased liver and spleen weights but increased proventriculus weight, while *B. amyloliquefaciens* increased spleen weight and decreased gizzard and proventriculus weights. A significant interaction was detected for spleen weight. Most blood biochemical and hematological parameters were unaffected by the treatment. However, organic acids reduced serum total protein and globulin, while hepatic enzyme activities remained unchanged, indicating no adverse effects on liver function. Cecal microbiota profiling demonstrated that organic acids were more effective than the combined treatment in reducing *Escherichia coli* and coliform populations. In contrast, Group A1B1 resulted in the highest *Bacillus* count. Both additives modulated cecal microbial populations compared to the control group. Organic acids improved early growth and reduced pathogenic bacteria, whereas *B. amyloliquefaciens* reduced feed intake and increased *Bacillus* abundance. The combined supplementation of organic acids and *B. amyloliquefaciens* did not provide synergistic benefits, indicating a parameter-dependent response. Neither additive adversely affected hematological indices or liver function.

Keywords: Antibiotic, *Bacillus amyloliquefaciens*, Cecal microbiota, Cockerel, Growth performance, Organic acid



[Full text-[PDF](#)] [[Crossref Metadata](#)] [[Scopus](#)] [[Export from ePrints](#)]

Research Paper

Biotechnological Feed Additives: Alternatives to Antibiotics as Growth Promoters in Broiler Chicken Farming

Matiukha I, Vishchur O, Krasovska O, Broda N, Mudrak D, Masiuk M, Tomchuk V, and Voltornisty A.

DOI: <https://dx.doi.org/10.36380/jwpr.2026.25>

ABSTRACT: The restriction of antibiotic growth promoters in poultry production has grown due to concerns about antimicrobial resistance and food safety. Phytogetic and probiotic feed additives are considered promising candidates for modulating gut health, immune responses, and growth performance in broiler chickens. The present study aimed to assess the effects of dietary supplementation with the probiotic *Kluyveromyces marxianus* strain (KluActive), either alone or in combination with encapsulated hydrolyzable tannins, on innate immune responses and growth performance in antibiotic-free-fed broiler chickens. Sixty broiler chickens, each 15 days old at the start, were used in a 27-day experiment. Broiler chickens were randomly assigned to four dietary treatments, including a control group fed a standard antibiotic-free diet (G1), a group that was fed the standard diet with KluActive at 0.04% (G2), and two groups fed a combination of KluActive with encapsulated tannins at 0.04% (G3) and 0.06% (G4). Phagocytic activity, phagocytic index, phagocytic number, serum bactericidal activity, and serum lysozyme activity were assessed on days 28 and 42. Growth performance parameters, including average daily gain and average body weight gain, were recorded over 42 days. Supplementation with the probiotic and tannins enhanced selected serum innate immune parameters in broiler chickens. In particular, serum bactericidal activity was significantly increased at the higher inclusion level (0.6%), by 12.5% on day 28 and 16.7% on day 42 compared with the control group. Phagocytic activity parameters were consistently elevated in broiler chickens across all experimental groups. The best improvement in feed conversion ratio was observed in the G3 group. The current findings indicated that probiotic-tannin combinations were a promising nutritional alternative to enhance immune function and performance in antibiotic-free broiler chicken production.

Keywords: Growth performance, Immune response, Probiotic, Tannin, Yeast



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Research Paper

Effects of Fungal Bioconversion of Azolla-Enriched Diets on Nutritional Quality and Economic Efficiency in ZamPen Native Chicken

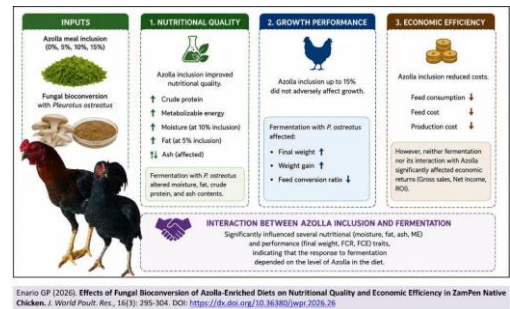
Enario GP.

J. World Poult. Res. 16(3): 295-304, 2026; pii: S2322455X2600026-16

DOI: <https://dx.doi.org/10.36380/jwpr.2026.26>

ABSTRACT: Rising feed prices have driven interest in local feed alternatives, even though these typically offer lower nutritional quality than standard commercial feeds. The present study aimed to evaluate the effects of an Azolla-enriched diet fermented with and without *Pleurotus (P.) ostreatus* on nutrient composition, growth performance, and economic efficiency in ZamPen native chickens. A 4 × 2 factorial experiment under a completely randomized design with 8 treatments and three replications was conducted using 168 two-month-old straight-run ZamPen chickens with an average weight of 665 g. Treatments consisted of 8 diets containing 0%, 5%, 10%, and 15% Azolla, with and without *P. ostreatus* fermentation. Results of nutrient analysis indicated that the fermented Azolla-enriched diet influenced crude protein, ash, and metabolizable energy compared to the non-fermented Azolla-enriched diet. Growth performance, including final body weight, weight gain, and feed conversion efficiency, was not significantly affected by the Azolla-enriched diet. However, feed consumption decreased significantly with increasing Azolla levels (10% and 15%), with animals consuming less feed than those fed 0% Azolla. Fermentation affected weight gain and feed conversion ratio, with non-fermented diets resulting in greater weight gain and better feed conversion than fermented diets. Significant interactions between Azolla and fermentation were observed for final body weight, feed conversion ratio, and feed conversion efficiency. Economic analysis revealed significant reductions in feed cost and total production cost at 10% and 15% Azolla, while gross sales, net income, and return on investment were not significantly affected compared to 0% Azolla. Fermentation with or without *P. ostreatus* did not affect economic performance, and no interaction of Azolla and fermentation was observed. These results suggested that Azolla can be incorporated up to 15% in native chicken diets as a cost-effective alternative feed ingredient without compromising performance. In addition, fermentation with *P. ostreatus* increased the moisture and fat contents of the diets and improved final body weight, weight gain, and feed conversion ratio, but reduced crude protein and ash contents.

Keywords: Alternative feed, Azolla meal, Feed conversion ratio, Fermentation, *Pleurotus ostreatus*



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Effects of Fermented Pineapple Peel and *Indigofera zollingeriana* Leaves on Intestinal Bacterial Population, Performance, and Carcass Traits in Broiler Chickens

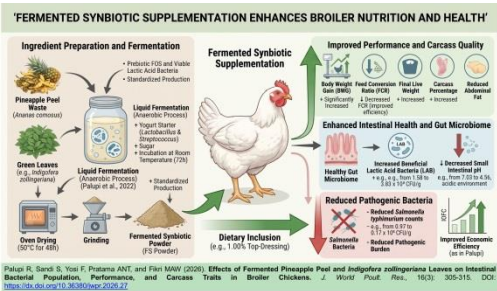
Palupi R, Sandi S, Yosi F, Pratama ANT, and Fikri MAW.

J. World Poult. Res. 16(3): 305-315, 2026; pii: S2322455X2600027-16

DOI: <https://dx.doi.org/10.36380/jwpr.2026.27>

ABSTRACT: The prohibition of antibiotic growth promoters has necessitated the exploration of alternative functional feed additives from agricultural waste to maintain poultry health and productivity. The current study aimed to evaluate the effects of dietary supplementation with fermented synbiotics derived from pineapple peel (*Ananas comosus*) and *Indigofera zollingeriana* leaves on growth performance, carcass traits, intestinal bacterial populations, and internal organ weights in broiler chickens. A total of 100 one-day-old broiler chickens with an average initial body weight of 42 ± 2 g were randomly allocated to five treatments with four replications in a completely randomized design. The dietary treatments initiated on day 1 continued for 28 days and consisted of a basal diet supplemented with fermented synbiotic (FS) at levels of 0% (control), 0.25%, 0.50%, 0.75%, and 1.00%. The FS powder was mixed directly into the basal diet as a top-dressing and provided daily for the entire 28-day experimental period. Feed intake and body weight were recorded weekly. Parameters measured included feed consumption, body weight gain, feed conversion ratio, live weight, carcass percentage, abdominal fat percentage, income over feed cost, relative organ weights (liver, heart, gizzard, pancreas), small intestinal pH, and populations of lactic acid bacteria and *Salmonella typhimurium*. Results demonstrated that supplementation at 1.00% significantly improved body weight gain, feed conversion ratio, live weight, carcass percentage, and income over feed cost compared to the control group. Small intestinal pH decreased from 7.03 (control) to 4.56 (1.00% FS). Lactic acid bacteria populations increased significantly from 1.58 (control) to 3.83×10^6 CFU/g (1.00% FS), while *Salmonella typhimurium* counts were reduced from 0.97 (control) to 0.17×10^6 CFU/g (1.00% FS). No significant effects were observed on relative organ weights across all treatments compared to the control group. In conclusion, fermented synbiotics from pineapple peel and *Indigofera zollingeriana* leaves at 1.00% optimally enhanced growth performance, carcass quality, and intestinal health while improving economic returns in broiler chickens, offering a viable and sustainable alternative to antibiotic growth promoters.

Keywords: Amino acid, Broiler chicken, Fermented synbiotic, Growth performance, *Indigofera zollingeriana*, Pineapple peel



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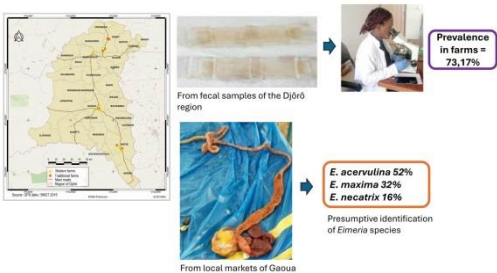
Prevalence of Coccidiosis in Broiler Chickens in Djôrô Region, Southwestern Burkina Faso

Vitouley SH, Hien SIP, Pagabeleguem S, Somda MB, Gniniwéwo Carèle B, and Pousga SS.

J. World Poult. Res. 16(3): 316-326, 2026; pii: S2322455X2600028-16

DOI: <https://dx.doi.org/10.36380/jwpr.2026.28>

ABSTRACT: In Burkina Faso, the poultry industry faces significant health challenges, notably coccidiosis, an intestinal parasitic disease that results in substantial economic losses. This cross-sectional clustered study aimed to determine the prevalence and oocyst load of *Eimeria* (*E.*) spp. in litter-derived fecal samples from broiler chickens in Batié, Dano, Diébougou, and Gaoua in the Djôrô region of Burkina Faso. The study was conducted from June to September 2024. A total of 600 fresh fecal samples were collected from broiler chickens aged 35 to 45 days, originating from 30 traditional and 30 modern farms across the Djôrô region. The droppings were collected from litter, placed in airtight plastic bags, and stored in cooling boxes at 4°C, then transferred to the Laboratory of Animal Biology and Physiology at the Gaoua University Center, Burkina Faso, for subsequent parasitological analysis. Samples were examined using a modified McMaster flotation procedure with saturated sodium chloride, and oocyst counts were determined. For postmortem examination, 25 broiler chickens were randomly selected from a local poultry market in Gaoua, Burkina Faso, then slaughtered and plucked on-site the same day. The plucked, unviscerated carcasses were then stored in cooling boxes at 4°C, and the intestinal tracts were later analyzed at the laboratory for macroscopic lesion identification and site localization. Out of the 600 samples analyzed, 439 (73.17%) tested positive for *Eimeria* spp. oocysts. Positivity rates



Vitouley SH, Hien SIP, Pagabeleguem S, Somda MB, Gniniwéwo Carèle B, and Pousga SS (2026). Prevalence of Coccidiosis in Broiler Chickens in Djôrô Region, Southwestern Burkina Faso. J. World Poult. Res., 16(3): 316-326. DOI: <https://dx.doi.org/10.36380/jwpr.2026.28>

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ranged from 62.7% in Dano city to 92.0% in Diébougou city, with Batié and Gaoua at 72.7% and 65.3%, respectively. Diébougou city had the highest mean oocyst count ($40,310.67 \pm 2,863.40$ OPG). Descriptively, traditional farms had higher oocysts per gram of feces (OPG) values than modern farms. Postmortem examination identified *E. acervulina* (13/25), *E. maxima* (8/25), and *E. necatrix* (4/25) in the intestinal tracts of chickens obtained from the Gaoua slaughter market. Nevertheless, postmortem findings were not generalized to the Djôrô region. The current results demonstrated the substantial *Eimeria* contamination on broiler farms in the Djôrô region, Burkina Faso, and supported the need for improved litter management, biosecurity, and farmer training on coccidiosis prevention.

Keywords: Broiler chicken, Coccidiosis, *Eimeria* spp., Oocyst, Prevalence

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Research Paper

Determining the Ideal Crude Protein Level in Plant-Based Diets for Late-Phase Laying Hens in Tropical Environments

Al Wahid Z, Ratriyanto A, Pradista LA, and Ni'am MN.

J. World Poult. Res. 16(3): 327-334, 2026; pii: S2322455X2600029-16

DOI: <https://dx.doi.org/10.36380/jwpr.2026.29>

ABSTRACT: Reducing dietary crude protein (CP) in laying hens is a strategic approach to improve nitrogen efficiency, reduce feed costs, and enhance sustainability in tropical production systems. The present study aimed to determine the optimal dietary CP concentration required to sustain laying performance in plant-protein-based diets fed to late-phase hens. A total of 3,600 laying hens aged 60 weeks were allocated to three dietary treatments in a completely randomized design with four replicates of 300 hens each. The treatments included a control diet with 18.50% CP (P0), a diet replacing all animal ingredients with corn, rice bran, soybean meal, and corn gluten meal to supply 16.70% CP (P1), and an equivalent plant-protein diet with 16.20% CP (P2). All diets eliminated animal-derived protein sources and were formulated to meet essential amino acid requirements. Feed intake, hen-day production (HDP), egg weight, egg mass, feed conversion ratio (FCR), and income over feed cost (IOFC) were recorded daily for 90 days under tropical conditions (28-34°C) and analyzed over the complete 90-day period. Hens in Group P1 achieved the highest HDP (95.31%), the greatest egg mass (58.58 g/hen/day), and the lowest FCR (2.00), all significantly superior to groups P0 and P2 ($p < 0.05$). Further reduction to 16.20% CP (P2) lowered the HDP to 91.04% and raised the FCR to 2.08, compared with Group P1. In Group P2, egg weight increased to 63.42 g and exceeded that of groups P1 and P0. Group P2 had significantly higher feed intake (119.53 g) than P1 (117.21 g) and P0 (117.48 g). Reducing dietary CP in laying hens improved nitrogen utilization, reduced nitrogen excretion and feed costs, and enhanced the sustainability of egg production under tropical conditions. Diet P1 returned the highest IOFC (726.26 IDR or 0.0446 USD per hen per day) and the lowest feed cost per kilogram of egg (12,606 IDR or 0.773 USD). The results demonstrated that reducing dietary CP from 18.50% to 16.70% in a plant-protein-based formulation optimized laying performance and economic efficiency in late-phase laying hens under tropical conditions. The 16.70% CP level represented a practical threshold that balanced sustainability, nutrient efficiency, and profitability.

Keywords: Crude protein, Egg, Income over feed cost, Laying hen, Tropical condition

16.70% CP plant-protein diet: best laying performance



Al Wahid Z, Ratriyanto A, Pradista LA, and Ni'am MN (2026). Determining the Ideal Crude Protein Level in Plant-Based Diets for Late-Phase Laying Hens in Tropical Environments. *J. World Poult. Res.* 16(3): 327-334. DOI: <https://dx.doi.org/10.36380/jwpr.2026.29>

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Research Paper

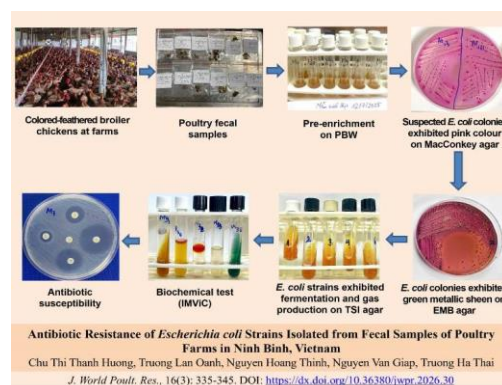
Antibiotic Resistance of *Escherichia coli* Strains Isolated from Fecal Samples of Poultry Farms in Ninh Binh, Vietnam

Huong CTT, Oanh TL, Thinh NH, Giap NV, and Thai TH.

J. World Poult. Res. 16(3): 335-345, 2026; pii: S2322455X2600030-16

DOI: <https://dx.doi.org/10.36380/jwpr.2026.30>

ABSTRACT: Antibiotic resistance is a major global health threat, and antibiotic use in livestock farming contributes to this issue. The present study aimed to assess the prevalence and antibiotic susceptibility of *Escherichia coli* (*E. coli*) isolated from poultry fecal samples in Ninh Binh province, Vietnam. Between May and October 2025, 170 dropping fecal samples were randomly collected from 45 poultry farms in Ninh Binh province, Vietnam, including 24 broiler chicken farms (aged 2.5-3.0 months old) and 21 meat-oriented duck farms (aged 1.5-2.0 months old). Fecal samples were transferred to the laboratory and cultured in



peptone buffer and on selective media, including MacConkey agar, eosin methylene blue agar, and triple sugar iron agar. Antibiotic susceptibility was assessed using the disk diffusion method on Mueller-Hinton agar. Suspected *E. coli* colonies were confirmed using Gram staining and biochemical tests such as citrate utilization, indole production, the methyl red test, and the Voges-Proskauer reaction. *Escherichia coli* was isolated from 134 (78.8%) fecal samples, including 69/85 (81.2%) chicken and 65/85 (76.5%) duck samples. The susceptibility of *E. coli* strains to 10 antibiotics was evaluated using the disk diffusion method. *Escherichia coli* strains isolated from chickens and ducks demonstrated the highest resistance to ampicillin (82.8%), followed by sulfonamides (79.9%), trimethoprim/sulfamethoxazole (74.6%), tetracycline (60.5%), and gentamicin (52.2%). Resistance to the remaining antibiotics, including norfloxacin, doxycycline, nalidixic acid, and cefotaxime, ranged from 14.6% to 38.1%; none of the isolates were resistant to meropenem. Among the isolates, 97 (72.4%) were multidrug-resistant; the proportion of multidrug-resistant isolates from chickens (60 strains, 87.0%) was higher than that from ducks (37 strains, 56.9%). *Escherichia coli* strains isolated from broiler chicken and duck farms in Ninh Binh, Vietnam, exhibited diverse resistance patterns to several antibiotics. Specifically, 34 and 36 resistance patterns were identified in chicken and duck isolates, respectively, with most isolates having multidrug resistance characteristics.

Keywords: Antibiotic resistance, Chicken, Duck, *Escherichia coli*, Poultry farm

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Research Paper

The Effect of Onion Skin Meal on Ileum Histology in Broiler Chickens

Maharani SF, Sudjarwo E, and Sjoftjan O.

J. World Poult. Res. 16(1): 346-352, 2026; pii: S2322455X2600031-16

DOI: <https://dx.doi.org/10.36380/jwpr.2026.31>

ABSTRACT: For many years, plant-based feed additives have served as alternatives to antibiotics, improving feed efficiency and ensuring livestock safety. The present study aimed to investigate the effects of garlic skin, shallot skin, and onion skin on the ileum in broiler chickens. A total of 189 broiler chickens were used in the present study and randomly assigned to nine treatment groups with three replicates, each consisting of seven chickens. The chickens were fed a basal diet supplemented with no onion skin (T0), 0.1% bacitracin (T1), 0.5% garlic skin (T2), 0.5% shallot skin (T3), 0.5% onion skin (T4), a basal diet supplemented with 0.25% garlic and 0.25% shallot skins (T5), a basal diet supplemented with 0.25% garlic and 0.25% onion skins (T6), a basal diet supplemented with 0.25% shallot and 0.25% onion skins (T7), and a basal diet supplemented with 0.167% garlic, shallot, and onion skins each (T8) for 35 days. The current results indicated that adding garlic skin meal, shallot skin meal, and onion skin meal did not affect ileal histology, villi number, villi length, villi width, villi surface area, and crypt depth in broiler chickens. The absence of adverse histological alterations indicated that onion skin meal is safe for utilization as a feed additive.

Keywords: Broiler chicken, Garlic skin, Ileum, Onion skin, Shallot skin



Maharani SF, Sudjarwo E, and Sjoftjan O (2026). The Effect of Onion Skin Meal on Ileum Histology in Broiler Chickens. J. World Poult. Res., 16(1): 346-352. DOI: <https://dx.doi.org/10.36380/jwpr.2026.31>

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Case Report

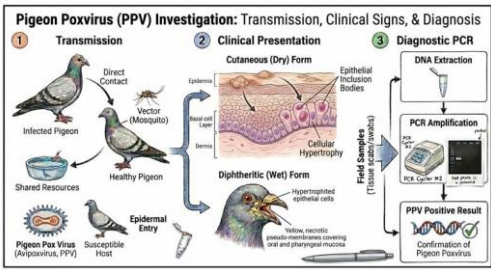
Pigeon Pox Virus Associated with Skin Lesions Isolated from Pigeons in Greece

Aslanidis A, Mavraki Z, Chochtoulas L, Minos E, Varga-Kugler R, Koutoulis K, and Prentza Z.

J. World Poult. Res. 16(3): 353-357, 2026; pii: S2322455X2600032-16

DOI: <https://dx.doi.org/10.36380/jwpr.2026.32>

ABSTRACT: Pigeon breeders in Central and Northern Greece often report pox-like skin lesions in their flocks. However, these reports have not been scientifically validated, and no investigation has determined the presence or prevalence of pigeon pox virus in Greek avian populations. The present study aimed to report the detection of pigeon pox in a flock of pigeons in Karditsa, Greece, confirmed by polymerase chain reaction (PCR) analysis. Following a comprehensive review of the existing scientific literature, the present study provided the first documented confirmation of pigeon pox virus in Greece. In September 2025, a breeder from Karditsa, Greece, reported cutaneous and respiratory signs in 40 of 250 juvenile pigeons in a loft of 450 pigeons. The flock was vaccinated only against paramyxovirus, not against pigeon pox. Lesions on the skin, beak, and legs persisted for 4-8 weeks, with no mortality. The breeder administered doxycycline, enrofloxacin, and local treatment for trichomoniasis and treatment with ganciclovir; however, the treatment was



Aslanidis A, Mavraki Z, Chochtoulas L, Minos E, Varga-Kugler R, Koutoulis K, and Prentza Z (2026). Pigeon Pox Virus Associated with Skin Lesions Isolated from Pigeons in Greece. J. World Poult. Res., 16(3): 353-357. DOI: <https://dx.doi.org/10.36380/jwpr.2026.32>

unsuccessful. Wart-like lesions from the beak, skin, legs, and eyelids were collected for molecular analysis by PCR targeting Avipoxvirus-specific genomic regions, confirming the diagnosis of pigeon pox virus infection. The clinical history, physical examination, and necropsy findings, along with the PCR results, confirmed the poxvirus infection. The present study confirmed the presence of pigeon pox virus in Greece and emphasized the importance of raising awareness and conducting additional epidemiological surveillance of Avipoxvirus infections in domestic pigeons.

Keywords: Avipoxvirus, Lesion, PCR, Pigeon pox, Skin, Virus

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Review

The Role of Dietary Fibre in Modulating Gastrointestinal Development, Nutrient Utilization, and Growth Performance in Broiler Chickens: A Review

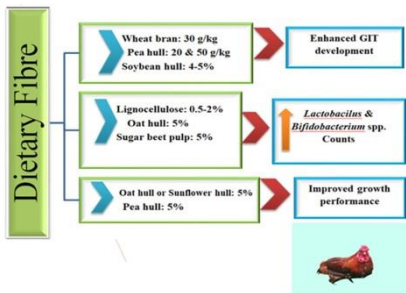
Fekade M.

J. World Poult. Res. 16(3): 358-369, 2026; pii: S2322455X2600033-16

DOI: <https://dx.doi.org/10.36380/jwpr.2026.33>

ABSTRACT: Dietary fibre, known as a diluent in poultry feed, has recently attracted considerable interest owing to its potential functional benefits beyond energy provision. The present study aimed to obtain findings related to the effects of different sources of dietary fibre on gastrointestinal tract (GIT) development, nutrient utilization, gut microbiota, and growth performance in broiler chickens. Dietary fibre is a major constituent of plant feed resources. Traditionally, dietary fibre was regarded as an antinutritional factor. The consideration of dietary fibre as an antinutritional factor was due to its negative effects on nutrient utilization, as it impairs feed intake and digestibility in broiler chickens. However, recent studies have indicated that the inclusion of dietary fibre at 2 to 3% in the diet of broiler chickens served multiple functions. Dietary fibre induces GIT development, gizzard activity and functionality, and digestive physiology. Fibre enhances enzyme secretion, digestion, fermentation, and absorption of nutrients. Dietary fibre keeps homeostasis through balancing the host and gut microbiota, increasing the composition and population of commensal bacteria, and reducing the pH of the GIT. The low pH creates an unfavorable environment for pathogenic bacterial proliferation. Thus, fibre helps maintain immunity, which further enhances the production performance in broiler chickens. In Ethiopia, where financial constraints and limited infrastructure hindered access to conventional feed ingredients, studies have primarily focused on replacing costly concentrates with more accessible and affordable alternatives, including dietary fibre sources. The present study revealed that including dietary fibre in the diet of broiler chickens resulted in different responses, with 2-3% inclusion generally beneficial, while exceeding 30 g/kg may decrease broiler chickens' growth performance; therefore, determining precise levels of dietary fibre inclusion is essential.

Keywords: Broiler chicken, Fibre, Gastrointestinal tract, Growth performance



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Review

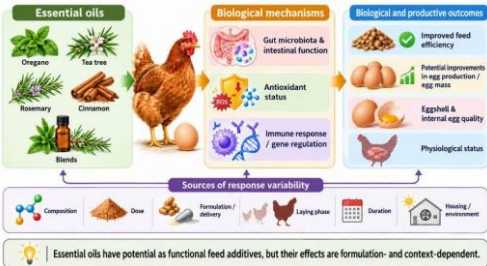
Mechanisms and Biological Outcomes of Essential Oils as Functional Feed Additives in Laying Hens: A Systematic Review

Amalta L, Sjoifjan O, Widodo E, Tistiana H, Nursita IW, Gunawati DN, and Damayanti CA.

J. World Poult. Res. 16(3): 370-379, 2026; pii: S2322455X2600034-16

DOI: <https://dx.doi.org/10.36380/jwpr.2026.34>

ABSTRACT: Essential oils are being investigated as phytogetic alternatives to antibiotic growth promoters in laying-hen nutrition because selected formulations contain compounds with antimicrobial, antioxidant, and immunomodulatory activities. The present study aimed to evaluate the mechanisms and biological outcomes of essential oils as functional feed additives in laying hens' diets, along with their effects on production performance and egg quality. The present systematic review followed the PRISMA 2020 framework. Peer-reviewed English-language studies published from 2020 to 2026 were identified through Scopus and Google Scholar databases. This period highlighted recent findings on the rising use of phytogetic feed additives in poultry nutrition. Twelve studies met the eligibility criteria and were included in the qualitative synthesis. Several studies reported changes in gut microbiota and intestinal morphology, improved antioxidant indicators, or modulation of immune-related variables. Selected studies also reported improvements in feed efficiency, egg



production or egg mass, eggshell characteristics, and internal egg quality, whereas other outcomes were unchanged. The magnitude and consistency of responses varied by essential-oil composition, inclusion level, formulation or delivery method, age or laying phase, experimental duration, housing, and other study conditions. Overall, essential oils have potential as functional feed additives in laying hens, but the evidence does not support a uniform effect across all products. Their practical value depends on the specific formulation, dose, and application strategy.

Keywords: Antioxidant activity, Egg production, Essential oil, Gut microbiota, Immune response, Laying hen, Phytogenic feed additive

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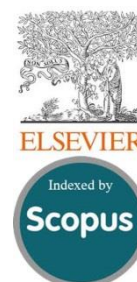
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Effects of Dietary Nettle (*Urtica dioica*) Leaf Powder on Performance, Welfare, Hematological Profile, and Oxidative Status in Broiler Turkeys

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ABSTRACT

Medicinal plants, such as nettle (*Urtica dioica*), are increasingly popular as sustainable alternatives to synthetic growth promoters and antioxidants in poultry production. The present study aimed to evaluate the effects of dietary nettle leaves on growth performance, welfare indicators, hematological and biochemical parameters, and oxidative status of broiler turkeys (*Meleagris gallopavo*) reared under intensive conditions. Seventy-two turkeys were randomly assigned to one of three dietary treatments from 3 to 15 weeks of age, including a control diet (CN), a diet with 3% nettle leaf powder (N3), and a diet with 6% nettle leaf powder (N6). Growth performance was monitored until week 13, while blood, carcass, and organ parameters were measured at the end of the trial. Mortality occurred only in the control group (3.33%), while there was no mortality in groups N3 or N6. Growth performance was significantly influenced by diet and sex. Male turkeys fed the N3 diet achieved higher final body weight compared to those receiving the CN or N6 diets. Conversely, no growth-promoting effect was observed in female turkeys. Female turkeys receiving the N3 diet exhibited lower final body weight and a higher feed conversion ratio than those in the CN group, indicating reduced feed efficiency. Nettle supplementation had no significant effect on carcass yield, organ development, or welfare indicators, including feather cleanliness and tarsal and plantar lesions. Hematological parameters, notably mean corpuscular volume, mean corpuscular hemoglobin, and platelet count, were significantly affected by nettle supplementation diets, whereas biochemical parameters remained within physiological ranges, indicating preserved systemic homeostasis. Dietary nettle supplementation enhanced antioxidant status, with both supplemented groups exhibiting significantly increased total antioxidant capacity, as measured by 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid; ABTS) radical scavenging activity, and a decrease in lipid peroxidation, assessed by thiobarbituric acid-reactive substances (TBARS), compared to the control group. Nettle leaf supplementation enhanced antioxidant defenses without compromising turkeys' welfare. A sex-dependent pattern was observed in growth performance parameters, with male turkeys exhibiting a greater response than females at the 3% nettle leaf inclusion level. However, both sexes exhibited consistent increases in antioxidant levels, highlighting the potential of nettle as a natural supplement in turkey production.

Keywords: Broiler turkey, Growth performance, Nettle leaf, Oxidative status, Phytogetic feed additive

INTRODUCTION

Oxidative stress is a major concern in basic biology and animal production sciences, as it plays a crucial role in

initiating metabolic disturbances, reducing productive performance, and affecting animal welfare (Ponnampalam et al., 2022). An imbalance between pro-oxidant processes

and antioxidant defense mechanisms is characterized by the excessive generation of reactive oxygen species (ROS). When endogenous antioxidant systems fail to adequately neutralize ROS, oxidative damage accumulates at the molecular and cellular levels, disrupts redox homeostasis, and consequently results in altered physiological functions, accelerated cellular aging, and ultimately cell death (Vágási et al., 2019; Yang et al., 2024). This condition is recognized as a critical factor in poultry health and productivity (Surai et al., 2019; Grela et al., 2023).

In modern poultry production systems, particularly in fast-growing species such as broiler turkeys (*Meleagris gallopavo*), oxidative stress is increased by elevated metabolic rates, intensive rearing conditions, environmental challenges, and nutritional imbalances (Aryal et al., 2025). In poultry, oxidative stress damages the intestinal mucosa and impairs digestion and subsequent nutrient absorption (Mishra and Jha, 2019; Li et al., 2024). Moreover, oxidative stress interferes with normal metabolic processes and elevates plasma corticosterone levels, leading to decreased feed intake, reduced body weight gain, and impaired growth performance (Desbruslais and Wealleans, 2022; Oke et al., 2024). Oxidative stress can negatively affect meat quality by increasing the risk and extent of lipid oxidation, thereby reducing shelf life and sensory quality (Choi et al., 2023). Additionally, oxidative stress could potentially compromise immune competence and welfare indicators, thereby reducing overall productivity and economic sustainability (Ghanima et al., 2020). Consequently, nutritional strategies to strengthen antioxidant defenses have become a priority in contemporary poultry nutrition (Surai et al., 2019; Oke et al., 2024).

Turkey production has gained global attention due to the high nutritional value of turkey meat, characterized by high-quality protein and a favorable lipid profile. Compared with broiler chickens, turkeys exhibit longer growth cycles and greater sensitivity to dietary composition and environmental stressors, requiring optimized feeding strategies to ensure adequate growth performance, welfare, and product quality throughout the production period (Surai and Fisinin, 2016; Ncho et al., 2025). In Algeria, turkey production is currently constrained by different structural challenges, including high feed costs, heavy reliance on imported feed products, and extended rearing periods, which collectively limit production efficiency and profitability (Feliachi, 2003; Sadoudi et al., 2024). According to estimates from the Food and Agriculture Organization (FAO), approximately

70,000 turkeys were recorded in 2009, primarily raised in backyard systems in Eastern regions of Algeria, such as Oum El Bouaghi, Batna, and Constantine (FAO, 2019). Over the last two decades, turkey production in Algeria has undergone significant development, driven by the adoption of intensive production systems and improved genetic strains. Recent Food and Agriculture Organization of the United Nations (FAOSTAT) data indicated that turkey meat production reached approximately 34,971 tons in 2024, highlighting the growing importance of turkey farming within the Algerian poultry sector (FAO, 2024). Despite this development, the Algerian turkey industry continues to face significant challenges, including elevated feed costs, reliance on imported raw products, competition with heavy broiler chicken farming, and prolonged rearing periods that limit production turnover and profitability (Sadoudi et al., 2024).

Among phytogetic feed additives, nettle (*Urtica dioica*) has attracted increasing attention due to its broad availability, economical cost, and long history of use as both a nutritional source and medicinal plant in Europe and other temperate regions (Devkota et al., 2022). Nettle leaves are characterized by their nutritional and phytochemical compounds, with reported contents of crude protein, minerals, vitamins, and bioactive compounds such as phenolic compounds and flavonoids. The nutritional properties and antioxidant-related constituents support the potential application of nettle as a natural dietary additive in livestock production (Adhikari et al., 2015; Moula et al., 2019). Different studies have demonstrated the strong antioxidant potential of nettle, largely attributed to its high content of polyphenols and flavonoids, which enhance free-radical scavenging capacity and support endogenous antioxidant defense systems (Gülçin et al., 2004; Jaiswal and Lee, 2022; Wójciak et al., 2024). In animal nutrition, dietary inclusion of nettle powder or extracts has been associated with improvements in growth performance, immune response, and oxidative stability in broiler chickens and other livestock species under experimental and farm conditions (Loetscher et al., 2013; Kregiel et al., 2018). Despite the recognized antioxidant and biological properties of nettle, scientific data regarding its use in turkey nutrition remain limited. Previous studies in broiler chickens have demonstrated that dietary nettle supplementation can modulate antioxidant gene expression, improve physiological responses, and enhance productive performance (Ahmadipour and Khajali, 2019; Pedraza et al., 2025). However, in broiler turkeys, the combined effects of nettle leaf inclusion on growth performance,

welfare indicators, hematological parameters, and oxidative status have not yet been clearly established. The present study aimed to evaluate the effects of dietary nettle leaf (*Urtica dioica*) inclusion on growth performance, welfare indicators, blood parameters, and oxidative status of broiler turkeys reared under intensive conditions, to assess its potential as a functional feed ingredient in sustainable turkey production systems.

MATERIALS AND METHODS

Ethical approval

All study procedures and experimental protocols involving animals were conducted in accordance with institutional ethical standards and were approved by the Animal Welfare, Experimentation, and Zootechnical Group of the University of Blida, Algeria (78 CBEEA-Blida; No. 28/SD/20190331). The present study adhered to national and international guidelines for the care and use of animals in research.

Study location and duration

The current study was conducted in 2021 over 15 weeks at a privately operated apiary owned by Aberkane Saïd, located in Aïn Turk, Bouïra Province, northern Algeria. The location had a Mediterranean climate.

Plant powder preparation

Aerial parts of *Urtica dioica*, including stems and leaves, were gathered from the Azazga district (Tizi Ouzou province), approximately 130 km east of Algiers,

during the period from November 2019 to January 2020. The harvested stems and leaves were air-dried in the shade within a well-ventilated area at ambient temperature for approximately two weeks until a constant weight was achieved, as determined by the absence of further mass loss between two consecutive measurements taken 24 hours apart (difference < 1%). During the drying period, the recorded temperature ranged from 22 to 40°C. Drying was carried out according to the traditional method commonly practiced by local producers in the study area, with a mean of 24°C and a median of 25°C. Variation reflected natural environmental fluctuations during shade drying under field and semi-controlled room conditions rather than a fixed experimental temperature. Shade drying was selected instead of oven drying to minimize potential thermal degradation of heat-sensitive bioactive compounds. The drying process was conducted without direct sunlight exposure or artificial heating to preserve the plant material's phytochemical properties. The primary criterion for confirming drying completion was the stabilization of weight, indicating that the moisture level had reached equilibrium despite temperature fluctuations. The dried plant material was then mechanically ground using a laboratory mill and sieved to obtain a homogeneous fine powder. The resulting powder was stored in paper bags at room temperature until incorporation into the experimental diets. No solvent extraction was applied. The analytical composition of the nettle powder used in the present study is presented in Table 1.

Table 1. Chemical composition of nettle powder used for turkeys' experimental diet

Items	Mean	Standard deviation	Median	Minimum	Maximum	Coefficient of variation (%)
Dry matter (%)	89.02	1.78	88.10	87.28	91.36	2.14
Organic matter (%)	84.93	3.40	84.34	80.62	90.22	4.37
Crude protein (%)	25.08	1.25	24.87	23.36	27.92	5.28
Crude fat (%)	2.69	0.19	2.67	2.08	2.99	6.91
Crude fiber (%)	12.07	0.85	11.65	10.41	13.61	7.24
Gross energy (kcal/kg)	3450	1.73	3337	3268	3612	5.12
Crude ash (%)	13.99	1.68	13.66	8.39	17.50	12.35

Values were obtained from five independent nettle powder samples (n = 5). Data are presented as mean, median, standard deviation (SD), coefficient of variation (CV), minimum, and maximum values.

Animals and management

The experimental trial included two levels of nettle powder (3% and 6%) incorporated into grower and finisher diets manufactured by Sadoudi Frères (Fréha,

Algeria). The trial started at three weeks of age, following a standard adaptation period, during which turkeys were maintained under identical management conditions. Turkeys were housed in floor pens under controlled

environmental conditions, with ventilation and temperature adjusted by age, and a uniform stocking density applied across all treatments. Feed and water were provided *ad libitum* throughout the experimental period. All turkeys were clinically healthy at the start of the trial and received routine preventive veterinary care according to standard farm management practices.

A total of 72 turkeys (36 males and 36 females) were allocated to nine pens (8 turkeys per pen), corresponding to three dietary treatments, each with three replicates. Each pen contained equal numbers of males and females (4 each). Sex was included as a fixed factor in the statistical analysis of performance data. At the beginning of the trial (week 3), turkeys exhibited homogeneous live body weights across dietary treatments, averaging 565.5 g in the control group, 584.8 g in Group N3, and 564.4 g in Group N6, indicating comparable baseline conditions prior to dietary allocation. From 3 weeks of age, all turkeys were randomly assigned to nine floor pens of 15 m² each, with eight turkeys per pen (4 males and 4 females), corresponding to a stocking density of approximately 1.9 m² per turkey. In the present experiment, the litter consisted of chopped straw applied at a rate of 7 kg/m² in all experimental pens. A 16-hour light/8-hour dark lighting program was implemented from the second week of age onwards. Light intensity was maintained at approximately 40 lux, measured at turkey level using a lux meter, to ensure uniform illumination across all pens. The management conditions were applied consistently across all treatments and followed the recommendations of the breeder's management guide ([Aviagen Turkeys, 2024](#)).

Experimental diet

The experimental groups consisted of a control group receiving a basal commercial diet and two supplemented groups receiving the same basal diet enriched with nettle leaf powder at 3% (N3) and 6% (N6). Nettle powder was thoroughly mixed with the commercial feed at each feed preparation to ensure homogeneous distribution. The diets were prepared by incorporating 300 g of nettle powder per 9.7 kg of commercial feed for Group N3, and 600 g per 9.4 kg of commercial feed for Group N6. Feed was prepared in batches and offered daily.

The diet composition and nutritional characteristics of the experimental feeds used during the two rearing phases (grower and finisher) are presented in Tables 2 and 3. The diets were formulated to meet the nutritional requirements of growing turkeys in accordance with the National Research Council ([NRC, 1994](#)) recommendations for poultry.

Table 2. Composition of feed with different levels of nettle leaf powder for turkeys during the grower phase (4-6 weeks)

Diet ingredients	CN	N3%	N6%
Corn	41.4	39.3	38.3
Wheat	3.20	1.74	0.01
Soybean meal	47.6	48.2	48.0
Vegetable oil	2.86	3.14	3.10
Dicalcium phosphate	2.91	2.81	2.79
Salt	0.27	0.27	0.27
Methionine	0.21	0.21	0.21
Lysine	0.25	0.24	0.26
Mineral-vitamin premix ¹	0.45	0.45	0.45
Stinging nettle powder (SNP)	0.45	3.00	6.00
Carbonate de calcium	0.81	0.69	0.63
Nutrient composition			
Crude protein (%)	25.5	25.5	25.2
Calcium (%)	1.01	1.01	1.04
Lysine (%)	1.50	1.51	1.51
Phosphorus (%)	0.54	0.52	0.51
Methionine (%)	0.46	0.46	0.46
Metabolizable energy (kcal/kg)	2840	2840	2831

CN: Control group, N3%: Diets supplemented with nettle leaf powder at 3%, N6%: Diets supplemented with nettle leaf powder at 6%.

Table 3. Composition of feed with different levels of nettle leaf powder for turkeys during the grower phase (7-15 weeks)

Diet ingredients	CN	N3%	N6%
Corn	48.0	49.3	49.3
Wheat	3.00	0.10	0.01
Soybean meal	40.9	39.4	36.6
Vegetable oil	3.68	3.91	3.93
Dicalciumphosphate	2.51	2.56	2.52
Salt	0.45	0.45	0.45
Methionine	0.20	0.19	0.20
Lysine	0.21	0.21	0.22
Mineral-vitamin premix ¹	0.27	0.27	0.27
Nettle leaves	0	3.00	6.00
Carbonate de calcium	0.80	0.63	0.54
Calculated nutrient composition			
Crude protein (%)	21.7	21.7	21.4
Calcium (%)	0.89	0.89	0.91
Lysine (%)	1.32	1.33	1.33
Phosphorus (%)	0.45	0.43	0.44
Methionine (%)	0.43	0.41	0.42
Metabolizable energy (kcal/kg)	2940	2939	2924

CN: Control group, N3%: Diets supplemented with nettle leaf powder at 3%, N6%: Diets supplemented with nettle leaf powder at 6%.

Growth performance

The individual body weights of all turkeys were documented biweekly (weeks 3, 5, 7, 9, 11, and 13), starting in week 3, using a calibrated digital scale. Feed consumption was monitored per pen during these intervals by quantifying the feed supplied and subtracting the remaining feed gathered before each weighing session. Body weight gain was calculated for each 14-day interval (weeks 3-5, 5-7, 7-9, 9-11, and 11-13) as well as cumulatively over the entire experimental period (week 3

to week 13). The feed conversion ratio (FCR) was determined for each period and for the overall study duration as the ratio of total feed intake to body weight gain per pen. Mortality was recorded daily throughout the experimental period, from week 3 to week 15 of age, and cumulative mortality rates were calculated for each treatment group.

Slaughter procedures and carcass traits

At the end of the experimental period, 27 female turkeys (nine turkeys per treatment group) were randomly selected, removed from their pens, and transferred to individual cages. Turkeys were fasted for 12 hours prior to slaughter, with free access to water. Carcass traits were evaluated exclusively in females in order to minimize variability related to sexual dimorphism, which can significantly affect carcass composition and fat deposition in turkeys. Carcass evaluation was performed only on female turkeys to ensure more homogeneous and reliable comparisons between dietary treatment groups. Furthermore, the current study was designed as an exploratory trial, and resource limitations restricted the inclusion of both sexes in the slaughter analysis. Immediately before slaughter, live body weight was documented. After slaughter and plucking, the carcass weight was measured following evisceration. The individual weights of internal organs, namely the liver, heart, proventriculus, gizzard, and spleen, as well as abdominal fat, were measured separately for each turkey. Carcass yield was then computed according to the following equation (Sadoudi et al., 2024).

$$\text{Carcass yield (\%)} = \frac{\text{Eviscerated carcass weight} \times 100}{\text{Live body weight}}$$

Welfare assessment indicators

At the end of the trial, animal welfare was assessed for each turkey using external physical indicators, including feather cleanliness, tarsal joint lesions, and plantar footpad dermatitis (Figure 1). Each parameter was independently evaluated by three trained observers using the *Welfare quality*® (2009) protocol. Scores ranged from 0 to 4, where score 0 indicated the absence of visible alterations, score 1 indicated mild alterations, score 2 indicated moderate lesions without functional impairment, score 3 indicated marked lesions associated with impaired condition, and score 4 indicated severe lesions or extensive tissue damage. Final scores were determined by agreements among observers to minimize subjectivity and inter-observer variability.

Determination of antioxidant activity

Total antioxidant capacity

At the end of the experiment, blood serum samples were collected from 18 turkeys (six per group), and serum was separated. Total antioxidant capacity was carried out using the 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid; ABTS) assay, following the method described by Re et al. (1999). In brief, ABTS radical cation (ABTS⁺) was generated by mixing 7 mM of ABTS with 2.45 mM of potassium persulfate, which was then incubated at room temperature in the dark for 12-16 hours. Before conducting the assay, the ABTS⁺ solution was diluted in phosphate buffer (pH 7.4) to obtain an absorbance of 0.70 ± 0.02 at 734 nm. For the assay, 1 mL of the diluted ABTS⁺ solution was mixed with 10 µL of serum sample, and the decrease in absorbance was recorded at 734 nm exactly six minutes after mixing. Antioxidant activity was expressed as the percentage inhibition of the ABTS radical. All measurements were conducted in triplicate.

Lipid peroxidation

Lipid peroxidation was evaluated by measuring thiobarbituric acid-reactive substances (TBARS) in serum samples obtained at the end of the experiment, following the method described by Devasagayam et al. (2003). Serum samples (1 mL) were deproteinized with 0.5 mL of 30% trichloroacetic acid (TCA) and incubated at 0°C for 120 minutes, then centrifuged at 3000 rpm for 10 minutes at 4°C. One milliliter of the resulting supernatant was mixed with 0.25 mL of 1% thiobarbituric acid (TBA) prepared in 0.05 M NaOH and 0.075 mL of EDTA (0.1 mol/L). The mixture was heated at 95°C for 15 minutes, cooled, and the absorbance was measured at 535 nm. Each sample was analyzed in triplicate. The TBARS levels were used as an index of lipid peroxidation.

Hematological and biochemical analyses

The procedures employed for hematological and biochemical analyses were adapted from the study of Sadoudi et al. (2024).

Blood sampling

Approximately 5 mL of blood was collected from the wing vein of 27 female turkeys (nine per group) at 6, 10, and 14 weeks of age. For hematological assessments, blood was drawn into ethylenediaminetetraacetic acid tubes (EDTA), whereas samples intended for biochemical analysis were collected in lithium heparin tubes. All samples were kept at a cool temperature, then centrifuged at 3000 rpm for 10 minutes, and plasma was analyzed on the same day.

Hematological parameters

Hematological measurements were performed using an automated analyzer (Mindray BC-30s), as described by Post et al. (2003). The evaluated parameters included white blood cell count (WBC), red blood cell count (RBC), hemoglobin concentration (Hb), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), and platelet count (PLT).

Biochemical parameters

Biochemical analyses were performed using a BioSystems BA200 analyzer (BioSystems S.A., Spain). The variables measured included glucose (Gly), triglycerides (TG), total cholesterol (Chol), total protein (Prot T), and albumin (Alb).

Statistical analysis

Data on growth performance, hematological parameters, and biochemical parameters were analyzed using a two-way analysis of variance (ANOVA) according to the following equation.

$$Y_{ijkl} = \mu + A_i + B_j + (AB)_{ij} + e_{ijkl}$$

where Y_{ijkl} represented the observed variables, μ was the overall mean, A_i was the effect of dietary treatment, B_j was the effect of age, $(AB)_{ij}$ was the interaction between diet and age, and e_{ijkl} was the residual error. Mortality rates among groups were compared using Fisher's exact test. Carcass traits and organ weights were analyzed using the PROC ANOVA procedure in SAS software (2012), with statistical significance set at p-value less than 5% ($p < 0.05$).

RESULTS

Growth performance and welfare indicators

Overall mortality recorded throughout the experimental period was 0% in the supplemented groups (N3 and N6), whereas a mortality rate of 3.33% was observed in the control group, indicating enhanced overall flock viability, particularly in the groups receiving nettle supplementation.

At the end of the trial, final body weight differed significantly among the experimental groups (Table 4). Female turkeys in the control group had a significantly higher body weight compared to those in the groups N3 and N6 ($p < 0.05$). Male turkeys fed the N3 diet demonstrated a higher final body weight compared to the control group and Group N6 ($p < 0.05$), while Group N6

did not differ significantly from the control group. In male turkeys, Group N3 exhibited the highest final body weight, significantly exceeding the values recorded in the control group and Group N6 ($p < 0.05$). Additionally, the control group had significantly higher body weight than Group N6 ($p < 0.05$).

The present results indicated that live body weight was influenced by sex and age ($p < 0.05$), whereas the effect of dietary treatment was not statistically significant ($p < 0.05$). The current results demonstrated that growth performance parameters were primarily associated with physiological differences related to sex and age, with a tendency for dietary influences.

The FCR was significantly influenced by dietary treatment (Table 5). Group N3 exhibited a higher FCR compared to the control group and Group N6 ($p < 0.05$), indicating reduced feed efficiency at this inclusion level.

Slaughter traits and organ weights of female turkeys are presented in Table 6. A numerical increase was observed in Group N3 for live weight at slaughter, post-bleeding weight, and empty carcass weight compared to the control group and Group N6. No significant differences were observed among treatments in carcass yield or in internal organ weights, including liver, gizzard, proventriculus, and heart ($p > 0.05$), indicating that dietary nettle supplementation did not adversely affect organ development.

The welfare indicator findings are presented in Figure 2. The present results indicated that neither observer effect nor dietary treatment had a significant impact on lesion scores for any of the evaluated welfare parameters ($p > 0.05$), confirming the reliability and consistency of the scoring procedure.

Regarding feather cleanliness, a score of 0 for clean plumage was observed in the majority of turkeys across all three groups, specifically, 83.4% in Group N3, 80.6% in Group N6, and 86.1% in the control group. Similarly, no significant differences were observed among dietary treatments for tarsal lesions ($p > 0.05$). Most turkeys demonstrated no evidence of tarsal lesions, corresponding to a score of 0 in 91.7% of turkeys in Group N3, 97.2% in Group N6, and 88.8% in the control group. A comparable trend was observed for plantar lesions, with the majority of turkeys across all experimental groups receiving a score of 0, indicating optimal foot health and overall welfare.

Hematological and biochemical parameters

The effects of dietary nettle leaf inclusion on hematological and biochemical parameters are presented in Table 7. Significant differences were observed for

selected hematological indices among the experimental groups. Turkeys in Group N3 exhibited a significantly lower MCV (153.7 fL) compared to those in the control group (161.6 fL) and Group N6 (159.9 fL; $p < 0.05$). In addition, PLT count was significantly higher in Group N3 ($8.78 \times 10^3 \mu\text{L}^{-1}$) than in Group N6 ($4.44 \times 10^3 \mu\text{L}^{-1}$) and the control group ($4.89 \times 10^3 \mu\text{L}^{-1}$; $p < 0.05$).

The MCH was significantly lower in Group N3 (68.8 pg) compared to the control group (71.4 pg; $p < 0.05$), whereas no significant difference was observed between Group N6 and the other treatment groups ($p > 0.05$). No significant differences were found among dietary treatments in the remaining hematological indices or measured biochemical parameters ($p > 0.05$).

Oxidative status

Findings related to the oxidative status of the turkeys are summarized in Table 8. Total antioxidant status (TAS), assessed using the ABTS radical-scavenging assay, demonstrated a significantly higher percentage of inhibition in the nettle-supplemented groups compared to the control group. The ABTS inhibition was 86.3% in Group N3 and 88.0% in Group N6, significantly higher than the 80.2% observed in the control group ($p < 0.05$). Lipid peroxidation, assessed by TBARS determination, was significantly reduced in the nettle-supplemented groups ($p < 0.05$). The lowest TBARS levels were recorded in groups N3 and N6 at 0.204 and 0.195, respectively, compared with 0.271 in the control group ($p < 0.05$).

Table 4. Live weight of turkeys fed diets containing different levels of nettle leaf at 3, 7, and 13 weeks of age

Age (week)	Sex	CN	N3%	N6%	SE	Sex group age	R ²
3	Female	565.5	584.8	564.4	90.0	p < 0.001	0.95
	Male	654.5	718.8	644.4	102.9		
7	Female	1186	1158	1151	90.0		
	Male	1385	1466	1408	102.9		
13	Female	6386 ^a	6098 ^b	5938 ^b	90.0		
	Male	7807 ^b	7908 ^a	7482 ^b	102.9		
p < 0.001	p < 0.001	Diet P < 0.001					

^{a,b} Means values with different superscript letters on the same row indicate significant differences ($p < 0.05$), SE: Standard error, R²: Coefficient of determination, CN: Control group, N3%: Diets supplemented with nettle leaf powder at 3%, N6%: Diets supplemented with nettle leaf powder at 6%.

Table 5. Feed conversion ratio of turkeys fed diets containing different levels of nettle leaf between 3 and 13 weeks of age

Groups					P-value			R ²
Age (week)	CN	N3%	N6%	SE	Groupe	Age	Groupe*Age	
4 - 6	1.89 ^b	1.99 ^a	1.83 ^b	0.03	$P < 0.001$	$P < 0.001$	$P < 0.001$	0.63
7 - 13	2.61 ^b	2.78 ^a	2.58 ^b	0.04				

^{a,b,c} Means with different superscript letters on the same row indicate significant differences ($p < 0.05$). SE: Standard error, R²: Coefficient of determination, CN: Control group, N3%: Diets supplemented with nettle leaf powder at 3%, N6%: Diets supplemented with nettle leaf powder at 6%.

Table 6. Live weight, carcass yield, and weight of different organs of turkeys fed diets containing different levels of nettle leaf at 15 weeks of age (slaughter period)

Parameters	CN	N3%	N6%	SE	P-value	R ²
Live weight (g)	6005	6554	5994	191.9	0.08	0.19
Weight after bleeding (g)	5580	6097	5566	183.7	0.08	0.19
Empty weight (g)	4824	5227	4770	172.3	0.06	0.21
Liver (g)	84.3	104.4	100.2	7.53	0.18	0.13
Rate (g)	7.38	6.75	6.98	0.43	0.61	0.04
Heart (g)	18.9	20.4	19.1	1.04	0.51	0.06
Proventricule (g)	11.4	12.0	11.7	0.43	0.57	0.04
Gizzard (g)	108.5	107.0	103.8	6.21	0.86	0.02
Abdominal fat (g)	21.7	21.6	24.5			
Carcass yield (%)	80.3	79.8	79.6	0.98	0.78	0.04

SE: Standard error, R²: Coefficient of determination, CN: Control group, N3%: Diets supplemented with nettle leaf powder at 3%, N6%: Diets supplemented with nettle leaf powder at 6%.

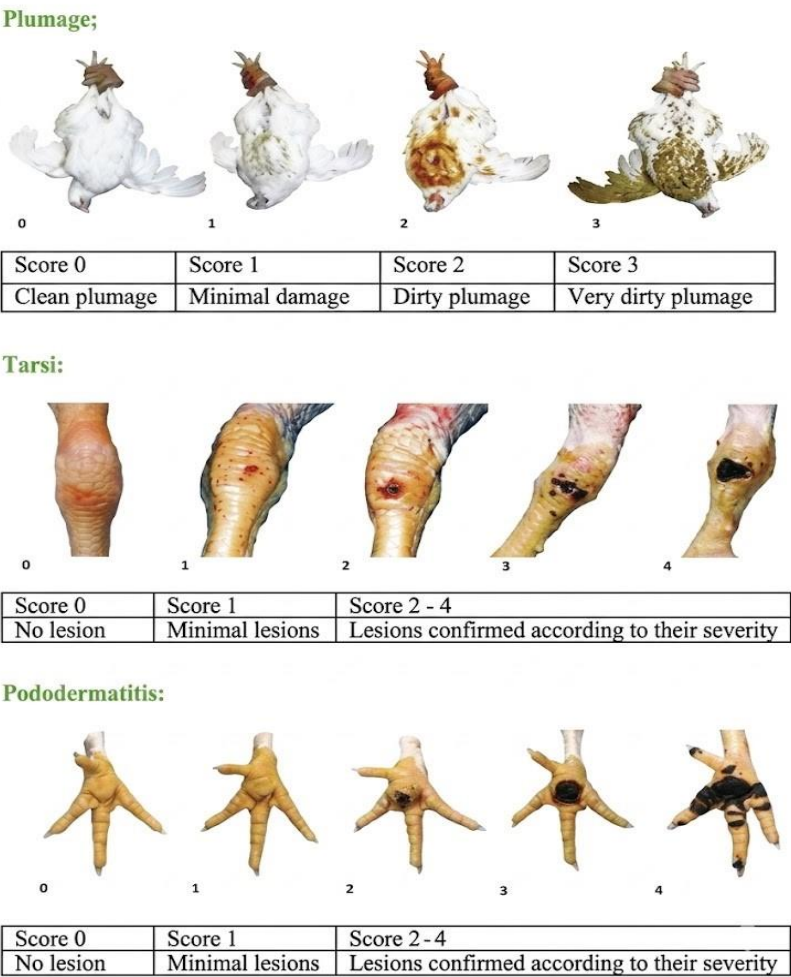


Figure 1. Welfare quality indicators of turkeys in the present experiment

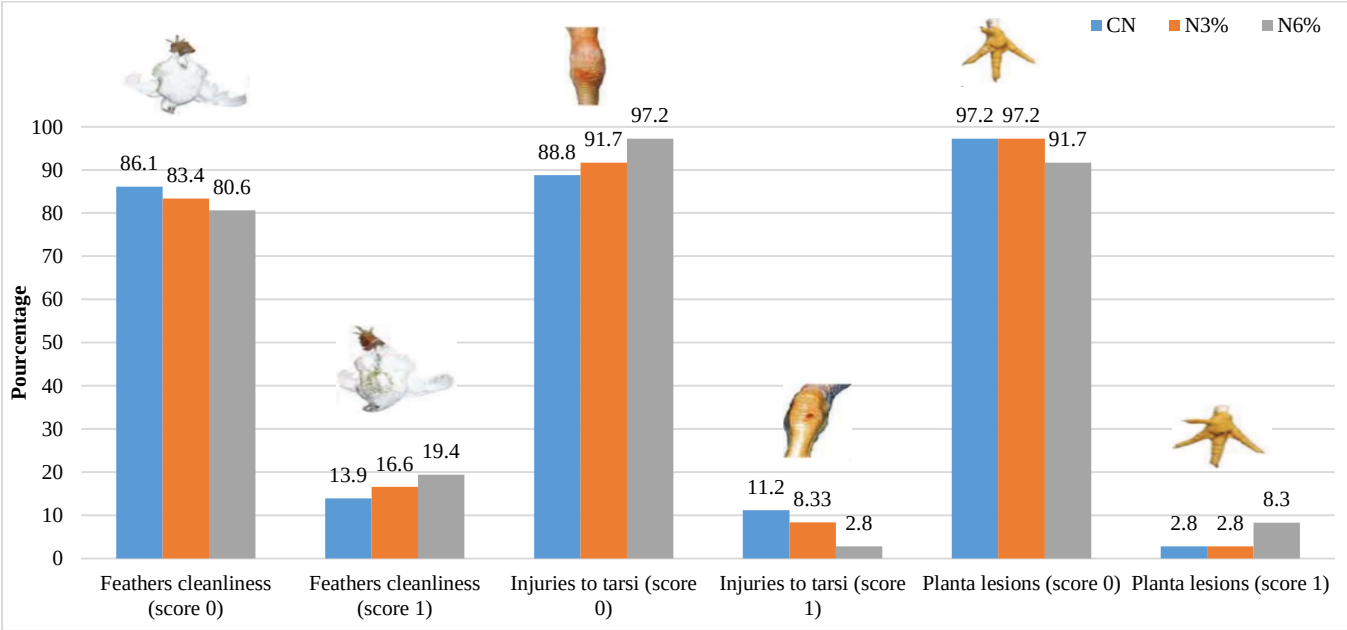


Figure 2. Feather cleanliness scores, shank lesions, and foot lesions of turkeys fed different levels of nettle. CN: Control group, N3%: Diets supplemented with nettle leaf powder at 3%, N6%: Diets supplemented with nettle leaf powder at 6%.

Table 7. Blood and biochemical parameters of turkeys that received different levels of nettle at 14 weeks of age

Parameters	Treatment groups			SE	P-value	R ²
	CN	N3%	N6%			
WB (103 /ml)	106.8	118.0	123.0	7.66	0.31	0.10
RB (106 /ml)	2.09	2.08	2.18	0.06	0.48	0.06
HB (g/dl)	14.9	14.3	15.2	0.44	0.34	0.09
Hct (%)	33.7	32.0	34.8	1.06	0.18	0.13
VGM (fl)	161.6 ^a	153.7 ^b	159.9 ^a	2.03	0.02	0.26
TCMH (pg)	71.4 ^a	68.8 ^b	69.9 ^{ab}	0.55	0.009	0.32
CCMH (g/dl)	44.2	44.8	43.7	0.36	0.14	0.15
Plt (103 /ml)	4.89 ^b	8.78 ^a	4.44 ^b	1.36	0.046	0.21
Gly (g/l)	3.07	2.91	3.09	0.64	0.11	0.16
TG (g/l)	1.03	0.62	0.92	0.29	0.59	0.04
Chol (g/l)	1.07	1.15	1.14	0.07	0.66	0.04
Prot T (g/l)	39.2	41.8	43.0	4.32	0.82	0.02
Alb (g/l)	14.9	16.0	15.7	0.55	0.36	0.08
RDW (g/l)	11.3	12.1	11.7	0.39	0.14	0.25

^{a,b,c} Means values with different superscript letters on the same row indicated significant differences ($p < 0.05$), SE: Standard error, R²: Coefficient of determination, CN: Control group, N3%: Diets supplemented with nettle leaf powder at 3%, N6%: Diets supplemented with nettle leaf powder at 6%.

Table 8. The oxidative status of turkeys that received different levels of nettle at 14 weeks of age

Parameters	Feed Groups			P-value	R ²
	CN	N3%	N6%		
TAS (%)	80.2 ± 0.84 ^a	86.3 ± 0.78 ^b	88.0 ± 0.99 ^b	P < 0.001	0.72

^{a,b,c} Means values with different superscript letters on the same row indicate significant differences ($p < 0.05$), SE: Standard error, R²: Coefficient of determination, CN: Control group, N3%: Diets supplemented with nettle leaf powder at 3%, N6%: Diets supplemented with nettle leaf powder at 6%.

DISCUSSION

Mortality

Mortality remained low across all experimental groups, exhibiting no signs of treatment-related adverse effects. The observed mortality rates were consistent with previous studies reporting that dietary nettle supplementation at inclusion levels between 1% and 6% did not adversely affect survival in poultry species, including broiler chickens and Japanese quail (Loetscher et al., 2013; Safamehr et al., 2013; Moula et al., 2019).

Growth performance

Male turkeys receiving 3% nettle supplementation achieved a higher final body weight (7908 g) than control males (7807 g), whereas female turkeys exhibited lower final body weight and higher FCR values in the present study. The FCR recorded in turkeys receiving 3% nettle supplementation (N3 group; 2.78) was higher than that of the control group (2.61), indicating reduced overall feed efficiency despite the improvement in body weight

observed in male turkeys. Male turkeys exhibited a more favorable response to 3% nettle supplementation than females, as reflected by their higher final body weight. Sex-related differences in growth performance may be associated with differences in growth potential, endocrine regulation, nutrient utilization, and metabolic requirements between male and female turkeys (Lilburn and Nestor, 1991; Havenstein et al., 2003). Male turkeys typically grow faster and accumulate more lean tissue than females, resulting from pronounced sexual dimorphism and variations in nutrient distribution (NRC, 1994; Havenstein et al., 2003). The physiological differences in growth rate, nutrient metabolism, and feed efficiency may enable male turkeys to obtain more nutrients and bioactive compounds from nettle. Similar sex-dependent responses to dietary interventions have been reported by Zuidhof et al. (2014) in poultry, in which males and females exhibited different feed utilization efficiency, growth patterns, and metabolic adaptation to nutritional strategies. The reduced growth efficiency observed in female turkeys receiving 3% nettle supplementation was likely attributable to variations in

their metabolic adaptation to the supplement, rather than a direct adverse effect of the nettle. Consistent with the present study, [Bölükbaşı et al. \(2008\)](#) reported that dietary supplementation with *Nigella sativa* oil improved growth performance and feed efficiency in broiler chickens. More recently, [Sadoudi et al. \(2024\)](#) demonstrated that phytogenic supplementation improved growth performance and antioxidant status in poultry under intensive rearing conditions. In the present study, the response to nettle supplementation was sex-dependent. Such differences among studies might be related to the type of phytogenic additive used, inclusion level, animal species, sex, age, and experimental conditions.

Organ weights

No notable differences were observed among dietary treatments for carcass yield or internal organ weights (liver, spleen, heart, gizzard, and abdominal fat). Consistent with the present results, [Safamehr et al. \(2013\)](#) reported no notable effect of low to moderate dietary nettle inclusion on the development of internal organs in poultry. The absence of remarkable effects on liver and spleen weights observed in the present study was consistent with the findings of [Loetscher et al. \(2013\)](#), who supplemented broiler diets with 25 g/kg (2.5%) dried nettle and reported no major adverse effects on organ development. Although the inclusion levels used in the present study (3% and 6%) were higher than those evaluated by [Loetscher et al. \(2013\)](#), no substantial changes in internal organ weights were detected, suggesting that nettle supplementation at these levels did not adversely affect organ development in turkeys.

Blood biochemical parameters

There were no notable differences in serum biochemical parameters among treatment groups, suggesting that dietary nettle inclusion did not alter the turkeys' metabolic status under the experimental conditions. The absence of notable changes in blood parameters in the present study was consistent with previous studies reporting no alterations in lipid or protein profiles following nettle supplementation ([Khosravi et al., 2008](#); [Keshavarz et al., 2014](#)). Although [Mansoub \(2011\)](#) and [Safamehr et al. \(2012\)](#) documented reductions in cholesterol and triglycerides following nettle supplementation, these reductions were not observed in the current study, possibly due to differences in inclusion levels, dietary formulations, or animal species.

Welfare indicators

Welfare parameters, including feather cleanliness and lesion scores, were not notably affected by nettle supplementation. The predominance of score 0 across all groups indicated an overall favorable welfare status. The observed responses to nettle supplementation could be linked to its strong antioxidant and liver-protective properties, though the precise underlying pathways remain unclear. The findings on welfare parameters were consistent with the results of [Mirsaiedi Farahani and Hosseinian \(2022\)](#), who suggested that antioxidant-rich plant constituents, including vitamin C derived from nettle, might help modulate stress in poultry. Maintaining proper litter conditions is known to decrease footpad lesions ([Eichner et al., 2007](#); [Kaukonen et al., 2016](#)).

Oxidative status

Dietary nettle supplementation improved oxidative status in broiler turkeys, as evidenced by increased antioxidant activity and reduced lipid peroxidation. The Oxidative status findings are consistent with those of [Golshan et al. \(2015\)](#), who reported that nettle supplementation enhanced antioxidant capacity in broiler chickens and improved oxidative stress markers in other animal models, including mice with polycystic ovary syndrome ([Bandariyan et al., 2021](#)). Different studies have demonstrated the strong antioxidant potential of methanolic and ethanolic nettle leaf extracts ([Gülçin et al., 2004](#); [Kataki et al., 2012](#); [Khare et al., 2012](#)). [Gülçin et al. \(2004\)](#) notably reported that aqueous nettle extract at doses of 50, 100, and 250 µg inhibited linoleic acid peroxidation by 39%, 66%, and 98%, respectively. Similarly, [Ghaima et al. \(2013\)](#) observed a 76% inhibition of lipid peroxidation attributable to nettle extract, surpassing the 65% inhibition observed with α -tocopherol. However, [Keshavarz et al. \(2014\)](#) and [Loetscher et al. \(2011\)](#) found no remarkable improvement in TBARS levels in broiler chickens fed diets supplemented with 10 or 25 g nettle powder/kg feed. In addition, [Kukrić et al. \(2012\)](#) reported lower antioxidant activity in ethanolic nettle extracts than in reference antioxidants such as vitamin C and butylated hydroxyanisole.

CONCLUSION

The present study demonstrated that dietary inclusion of nettle leaves (*Urtica dioica*) at 3% was safe and beneficial for broiler turkeys reared under intensive conditions. Nettle supplementation at 3% improved growth performance and carcass traits without negatively

affecting welfare indicators, internal organ development, or hematological and biochemical profiles. In addition, nettle supplementation enhanced systemic antioxidant capacity and reduced lipid peroxidation, suggesting an improved oxidative status in supplemented turkeys. The relationship between nettle supplementation and welfare was a complex issue that could not be directly determined in the present study, as no physiological stress indicators, such as corticosterone levels, were measured. Nevertheless, the current study possessed some limitations. The composition of the gut microbiota, the activity of digestive enzymes, and stress-related hormonal markers were not assessed, thereby limiting a comprehensive understanding of the underlying mechanisms. Future studies should encompass these parameters, as well as dose-response relationships and long-term effects in commercial production settings.

DECLARATIONS

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Authors' contributions

Ahmed Sadoudi contributed to the investigation, experimental trial, conceptualization, methodology, data curation, and preparation of the original draft. Dahia Saidj contributed to the review and editing of the manuscript. Yuva Bellik contributed to supervision, conceptualization, methodology, validation, quality control, and manuscript review and editing. Leghel Touazi participated in the investigation, experimental trial, conceptualization, methodology, laboratory analyses, and manuscript review and editing. Krimou Yahia contributed to data curation, data management, and the experimental trial. Boubekeur Aberkane provided resources and technical support and contributed to the experimental work and

conceptualization. Mokrane Iguer-Ouada contributed to supervision, conceptualization, methodology, validation, quality control, and manuscript review and editing. Nassim Moula contributed to the original draft preparation, conceptualization, methodology, data interpretation, manuscript review and editing, and overall coordination of the study. All authors have read and approved the last edition of the manuscript.

Availability of data and materials

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

Competing interests

The authors declared no conflict of interest.

Ethical considerations

The authors declared that this study is original and has not been submitted for publication elsewhere. They also affirmed that the information contained within this manuscript is free from plagiarism and misconduct and has not been manipulated. Additionally, the authors confirmed that no artificial intelligence tools were utilized in the design, conduct, analysis, or authorship of the present study. The authors accept full responsibility for the originality of this manuscript.

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Effects of *Saccharomyces boulardii* on Productivity, Egg Quality, Excreta Microbiology, and Ammonia Emissions in Indonesian Local Ducks

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ABSTRACT

The Cihateup duck is an Indonesian waterfowl with potential as an egg producer. However, the productivity remains suboptimal. Dietary supplementation with the yeast probiotic *Saccharomyces boulardii* (*S. boulardii*) has been reported to improve poultry productivity. The current study aimed to evaluate the effects of *S. boulardii* yeast probiotic supplementation in Cihateup duck feed on production performance, egg quality, the microbial population in excreta, and excreta ammonia level. A total of 60 female Cihateup ducks aged 71 weeks were randomly assigned to four treatment groups (three replicates per treatment, five ducks per replicate). The treatment consisted of a control (T0), which was a basal diet without *Saccharomyces boulardii*; basal diet plus 0.5 g/kg feed *S. boulardii* (T1), basal diet plus 1.0 g/kg feed *S. boulardii* (T2), and basal diet plus 1.5 g/kg feed *S. boulardii* (T3). The feed treatment lasted for 8 weeks. Production performance, including duck day production, feed conversion ratio (FCR), and total and average egg weight, was evaluated weekly. At the end of the experiment, the physical quality of eggs, fat content, cholesterol, and fatty acid profile (saturated and unsaturated fatty acids) of egg yolks were analyzed. The analysis of excreta microbes and excreta ammonia levels was conducted in the eighth week. *Saccharomyces boulardii* supplementation significantly improved duck day production, total egg weight, and FCR in the T3 group, although it did not significantly affect egg physical quality across all treatments. The egg yolk cholesterol was significantly lower in the T1 and T3 groups than in the other groups. The present study also found that supplementation with *S. boulardii* increased the egg yolk omega-6 fatty acids (linoleic acid, γ -linolenic acid, and arachidonic acid), especially in T2 and T3 treatments. Ammonia in duck excreta decreased significantly in the probiotic groups compared with the control group, with the lowest level in T3. The findings of the present study demonstrated that *S. boulardii* has the potential as a probiotic for enhancing laying performance, improving the functional quality of eggs, and reducing excreta ammonia levels of Cihateup ducks.

Keywords: Cihateup duck, Egg quality, Performance, Probiotic, *Saccharomyces boulardii*

INTRODUCTION

Optimising poultry productivity is a challenge and essential to address the increasing demand for poultry products on a global scale, including meat and eggs. One strategic approach that has been developed is the intervention in nutrient absorption in the digestive tract through the use of feed additives (Bidura et al., 2019).

Feed additives are widely recognised as specific nutritive and non-nutritive ingredients added to poultry feed (Perera and Ravindran, 2025). Feed additives have several benefits, including improving the quality and efficiency of feed utilisation and increasing the quantity and quality of poultry products (Özsoy et al., 2018). Probiotics are a type of feed additive that has been extensively studied,

particularly after several countries announced a ban on antibiotic growth promoters (AGPs; Qiu et al., 2024). Exploration of natural alternatives such as probiotics has gained interest due to their ability to support livestock productivity and health (Wickramasuriya et al., 2022). Probiotics may contain one or more strains of microorganisms and are administered to poultry via feed or drinking water, either as bacteria, yeast, or a consortium of both (Abd El-Hack et al., 2020). *Saccharomyces* spp. yeast has a rich biological content, including proteins, amino acids, vitamin B complex, mannan-oligosaccharides, important trace minerals, and several other growth-promoting factors (Ahiwe et al., 2021).

Yeast-based probiotics, *Saccharomyces cerevisiae* (*S. cerevisiae*) have attracted widespread attention in poultry nutrition science due to their ability to improve gut microbiota balance and immune modulation (Abd El-Hack et al., 2020; Pratama et al., 2026). Similarly, *S. cerevisiae* var. *boulardii* has been investigated for its role as a probiotic in broiler chickens (Qin et al., 2018; Nari et al., 2020; Vu et al., 2025) and laying hens (Miraç and Cannan, 2025). *Saccharomyces boulardii* (*S. boulardii*) is known to improve gut health, trigger innate immunity, and collaborate with gut-dwelling microorganisms to protect the intestinal mucosa when consumed orally. *Saccharomyces boulardii*, with its anti-inflammatory properties, is enriched with nutrients and modulates the host's immune response (Zoghi and Darani, 2025). According to Manafi et al. (2018), *S. boulardii* supplementation in broiler chicken feed has been reported to reduce feed conversion rates and decrease the population of pathogenic bacteria in the caecum. Cihateup duck is one of West Java's leading duck breeds, originating from Tasikmalaya, West Java. Like other local Indonesian ducks, the Cihateup duck is a descendant of the Indian Runner duck, known as a laying duck. Male Cihateup ducks are generally used for meat production, while female ducks are used for egg production. However, the productivity of the local duck as an egg-laying type has not yet reached its optimal level (Ismoyowati et al., 2022).

A strategy to improve production performance and egg quality can be carried out by manipulating the gastrointestinal microbiota through probiotic supplementation (Bidura et al., 2019). A probiotic approach is expected to improve nutrient absorption, suppress colonization of pathogenic microorganisms, and ultimately increase productivity. The current study aimed to evaluate the effects of *S. boulardii* supplementation on production performance, egg quality, excreta microbial

population, and excreta ammonia level in Cihateup duck feed.

MATERIALS AND METHODS

Ethical approval

All protocols in the experimental activities comply with standard operating procedures and were approved by Politeknik Negeri Jember, Indonesia, the animals' Ethics Committee, with registration number (106/PL17.4/PG/2024).

Animals, feed, and study design

A total of 60 Cihateup female ducks aged 71 weeks, with an average body weight of $1,550 \pm 65$ g. Cihateup ducks were kept in open pens using an intensive rearing system. The pen dimensions were 1.5×1.2 m (length $\times$ width). The pen temperature ranged from 23 to 32°C during the experimental period. The pen floor system consisted of 5 cm of rice husk litter, and the lighting duration was set to a minimum of 16 hours. Feed was delivered using a point feeding system at 120 grams per duck per day, and drinking water was provided *ad libitum*. The Cihateup ducks were randomly assigned to four treatment groups, with each treatment group consisting of three replicates. The treatment consisted of a control (T0), which was basal diet without *S. boulardii*, basal diet plus 0.5 g/kg feed *S. boulardii* (T1), basal diet plus 1.0 g/kg feed *S. boulardii* (T2), and basal diet plus 1.5 g/kg feed *S. boulardii* (T3). Dietary supplementation was implemented over 8 weeks, and the composition of the experimental feed is shown in Table 1. Nutrient requirements refer to the standard requirements for laying ducks (Leeson and Summers, 2005). The mixed concentrate duck feed, with a minimum crude protein of 38%, was produced by PT New Hope Indonesia. The *S. boulardii* population used was 10^8 CFU/g.

Table 1. Ingredients and chemical analysis of basal diet over 8 weeks of the study

Ingredient (percentage of dry matter)	Percentage
*Mixed concentrate duck feed	85
Rice bran	10
Azolla meal	5
Total	100
Composition	Content
Dry matter (%)	90.11
Metabolizable energy (Kcal/Kg)	2,800
Crude protein (%)	18.32
Ether extract (%)	4.74
Crude fiber (%)	5.32
Total ash (%)	12.85
Calcium (%)	4.11
Total phosphorus (%)	0.48

Leeson and Summers (2005) analyzed in the Laboratory of the Feed Science and Technology Division, Faculty of Animal Science, IPB University, Indonesia. *Mix concentrate duck feed contains: Soybean meal, fish meal, meat and bone meal, corn protein meal, micro-minerals, and a premix (vitamins, amino acids, and trace minerals).

Data and sample collection

The Cihateup ducks were observed for 8 weeks. Eggs were collected daily, and egg production was calculated daily and weekly. The egg mass was obtained by weighing the total daily eggs in each treatment repetition and averaging to obtain the weight per egg. Feed consumption was recorded daily and accumulated weekly. The feed conversion ratio (FCR) was calculated weekly in the form of w/w, that is, feed consumed (kilograms) per egg mass (kilograms). The production performance data were displayed and evaluated weekly until week 8. Physical quality tests of eggs, analysis of fat content, cholesterol, and fatty acid profile of egg yolks, analysis of excreta microbes, and analysis of excreta ammonia levels were performed in week 8.

Analysis of egg physical quality

The evaluation of egg physical quality includes both the exterior and interior parts of the egg. A total of 24 eggs for each treatment (eight eggs per replicate) were collected over four consecutive days during the eighth week of the study. Measurements and weighing were performed 24 hours after egg collection. Each egg was weighed using an Ohaus CL compact digital scale (CL-501T, OHAUS, USA). The eggs were broken on a glass table, and the height of the albumen and yolk was measured using a tripod micrometre (Mitutoya, No. 2050-08, 0.01-20 mm; Kawasaki, Japan). The length and width of the albumen and the diameter of the yolk were measured using digital callipers. The albumen and yolk indices, Haugh Unit (HU), for each replicate were calculated using the following formulas (Formula 1-3).

$$\text{Albumin index} = (\text{albumen height/average of albumen length and albumen width}) \times 100 \quad (\text{Formula 1})$$

$$\text{Yolk index} = (\text{yolk height/yolk diameter}) \times 100 \quad (\text{Formula 2})$$

$$\text{Haugh units} = [100 \times \log(H + 7.57 - 1.7W^{0.37})],$$

where H is albumen height, and W is egg weight
(Formula 3)

The eggshells were cleaned of any remaining albumin, separated from the egg membrane, and weighed. The thickness of the shell was measured at three areas of the egg (blunt end, pointed end, and middle) using a Mitutoyo Thickness Gauge 7301A 10/0.01 mm, Japan. The egg albumin and yolk are separated using an egg

plastic separator, and the surface of the yolk is cleaned of any remaining albumin and chalaza. The yolk was weighed directly, and the egg white weight was calculated from the difference. The weight of the egg components is presented as a percentage. The yolk color was measured using the Roche yolk color fan scores (RYCF; F. Hoffmann-La Roche, Switzerland), which assigned ratings based on 15 distinct color samples, with 1 representing the lightest shade and 15 the darkest.

Fat content, fatty acids, and cholesterol of egg yolk

Six egg yolk samples per replicate were prepared and homogenized at low temperatures (10°C). Fat content was analysed using AOAC Official Method 989.05, modified with the Mojonnier ether extraction method (1992), whilst the fatty acid profile was analysed in accordance with AOAC Official Method 969.33 (1997). The cholesterol content of egg yolk was determined using the principle of KOH saponification with alcohol, n-hexane extraction, and derivatization with trimethylchlorosilane (TMCS). The results were analyzed using gas chromatography–flame ionization detection (GC–FID) in accordance with method 18-6-5/MU/SMM-SIG (GC–FID) of PT SIG (Saraswanti Indo Genetech). Fat content and fatty acid profile are expressed as a percentage w/w, and cholesterol in mg/grams of egg yolk.

Excreta microbial population

Ten grams of excreta from each replicate were collected in sterile tubes for microbial population enumeration. One gram per sample was diluted into 9 mL of sterile phosphate-buffered saline (PBS) and diluted in a series from 10^{-1} to 10^{-7} . Subsequently, 0.1 mL of the last three dilution suspensions was inoculated onto the appropriate selective medium using the spread plate method. Lactic acid bacteria (*Lactobacilli*) were counted using dilutions of 10^{-5} to 10^{-7} , while *Salmonella* and *Escherichia coli* were counted using dilutions of 10^{-3} to 10^{-5} . *Lactobacilli* were cultured on MRS (de Man, Rogosa, and Sharpe) agar medium (Merck, Darmstadt, Germany) supplemented with 0.5% CaCO_3 (Telmadarre et al., 2020). Mac Conkey Agar media (Merck, Darmstadt, Germany) for *Salmonella* bacteria and Eosin Methylene Blue Agar (EMBA) medium for *Escherichia coli* (*E.coli*). The entire plate was incubated for 24-48 hours at 37°C. The number of colonies was counted with criteria of 25- 250 colonies per plate, and the result was multiplied by the dilution factor. Population data were expressed as logarithms (log colony-forming units per gram or log CFU per gram).

NH₃ (ammonia) excreta

The ammonia concentration in the excreta was determined using the Conway diffusion method, as described by [Adjis et al. \(2022\)](#). The test began by coating the rim of the Conway dish with Vaseline before use; subsequently, 1 mL of boric acid (H₃BO₃) with 2 drops of methyl red indicator was placed in the centre of the Conway dish. A total of 2.5 g of fresh duck excreta was mixed with 25 mL of distilled water, then homogenised, filtered, and centrifuged at 100 rpm for 3 minutes. Subsequently, 1 mL of the supernatant was collected and transferred to the left side of the dish, while 1 mL of saturated sodium hydroxide (NaOH) was placed on the right side. The Conway dish was then tightly sealed and gently shaken until the supernatant and NaOH were mixed. The dish was then left to stand at room temperature for 24 hours to allow NH₃ to bind to boric acid. The presence of bound ammonia is indicated by a color change in the boric acid indicator to green. After 24 hours, the boric acid indicator was titrated with 0.0057 N sulfuric acid (H₂SO₄) until the color changed from green to red. The concentration of NH₃ was calculated using the Formula 4 published by [Adjis et al. \(2022\)](#).

$$\text{NH}_3 (\text{mMol}) = \frac{\text{Volume H}_2\text{SO}_4 \times \text{N H}_2\text{SO}_4 \times 1000}{\text{Sample volume (mL)}} \quad (\text{Formula 4})$$

Statistical analysis

Data were analyzed using a one-way analysis of variance (ANOVA) within a completely randomized design, with dietary yeast dose as the treatment variable. The statistical model employed was $Y_{ij} = \mu + T_i + \epsilon_{ij}$, where Y_{ij} denotes the observed value, μ denotes the overall mean, T_i denotes the treatment effect, and ϵ_{ij} denotes the experimental error. All statistical analyses were conducted using Minitab software (version 19). The normality of residuals and the homogeneity of variances were assessed using the Shapiro–Wilk and Levene's tests, respectively. If a significant treatment effect was found ($p < 0.05$), Tukey's honestly significant difference (HSD) test was used for pairwise comparisons of means.

RESULTS

Production performance

The effect of supplementing Cihateup duck feed with the yeast probiotic *S. boulardii* over an 8-week experimental period on production performance was evaluated and presented as averages over the experimental period (Table 2). Feed intake values showed no significant

difference among replicates, as feed was provided under a controlled point feeding system with a fixed daily allowance of 120 g/duck/day, which was fully consumed at every feeding ($p > 0.05$). Duck day production (DDP), total egg weight, and feed conversion ratio (FCR) were significantly ($p < 0.05$) influenced by *S. boulardii* supplementation. The best performance was demonstrated by the T3 treatment group (1.5 g per kg feed), which showed the highest DDP percentage and total egg weight, as well as the lowest feed conversion ratio, compared to the control, T1, and T2 groups. The increases in DDP and total egg weight in the T3 group were 16.5% and 19.5%, respectively, compared to the control (T0). Meanwhile, the FCR (g/g) for T3 decreased by 17.45% compared to the control. Overall, treatment T3 consistently yielded the best results for most production performance variables (duck day, number of eggs, total egg weight, and FCR), while the average egg weight was not affected by the treatment. Treatments T0, T1, and T2 generally showed no significant differences ($p > 0.05$) among them.

Egg quality

Supplementation with *S. boulardii* for 8 weeks had no significant effect on average egg weight, yolk index, albumin index, yolk color, shell thickness, or the proportion of egg component weights ($p > 0.05$; Table 3). Haugh units, which indicate egg freshness, also showed no significant difference ($p > 0.05$) among all treatment groups. The present results indicated that the treatment did not alter the external and internal morphological structure of the eggs, nor did it affect the integrity of the shell or the pigmentation of the yolk in Cihateup ducks.

Fat content, fatty acids, and cholesterol of egg yolk

No significant differences were observed in the total fat content of Cihateup duck egg yolk across all groups ($p > 0.05$; Table 4). However, the mean fat content for group T3 was the highest among the treatments. The cholesterol content of egg yolk was significantly lower in treatments T1 (1,324.24 mg/100 g) and T3 (1,240.65 mg/100 g) compared with the control group (1,459.29 mg/100 g; $p < 0.05$). In terms of fatty acid profile, the percentage of oleic acid was the highest compared to other fatty acids in all treatment groups, followed by palmitic acid, stearic acid, and linoleic acid. The percentage of the saturated fatty acid stearic acid was significantly higher in T2 than in the control group. The percentage of the monounsaturated fatty acid oleic acid significantly differed ($p < 0.05$) between the T2 and T3 groups compared to the T0 and T1

groups. The highest percentage of oleic acid was reached by the T3 group, as the dose of *S. boulardii* increased. Similarly, the percentage of polyunsaturated fatty acids, which also belong to the omega-6 fatty acid group, namely linoleic acid, γ -linolenic acid, and arachidonic acid was significantly higher in T2 and T3 compared to the control ($p < 0.05$). In contrast to omega-6 fatty acids, the percentage of omega-3 fatty acid linolenic acid did not differ significantly ($p > 0.05$) across treatments, ranging from 0.23% to 0.41%. The ratios of PUFAs to SFAs and of ALA to LA in duck egg yolks were not statistically significant ($p > 0.05$) in all supplemented groups compared to the control. However, the mean values indicated a tendency towards an increase in these ratios in treatments T2 and T3. Overall, based on the study results, the percentage of total fatty acids in Cihateup duck egg yolks increased with increasing yeast probiotic supplementation dose.

Excreta microbial population

Microbiological analysis of duck excreta (Table 5) showed that the supplementation of *S. boulardii* probiotics

in the diet did not affect total lactic acid bacteria, *Salmonella*, and *E. coli* in the excreta of ducks ($p > 0.05$) among the control and treatment groups. Similarly, the ratios of LAB/*E. coli* and LAB/*Salmonella* did not differ significantly ($p > 0.05$), although there was a tendency for both LAB/*E. coli* and LAB/*Salmonella* ratios increased in the yeast-supplemented groups (T1, T2, and T3) compared to the control.

NH₃ (ammonia) excreta

Feed supplementation with the probiotic *S. boulardii* had a significant effect on the ammonia content of excreta ($p < 0.05$; Table 6). Ammonia in duck excreta decreased significantly ($p < 0.05$) across all experimental groups, as the dose of *S. boulardii* probiotic supplementation increased. The ammonia level in the T3 treatment group was the lowest among the treatments (122.22 mM/g). The percentage reductions in excreta ammonia from T1 to T3 relative to the control were 3.5%, 7.6%, and 16.8%, respectively.

Table 2. Production performance of laying ducks (71-79 weeks of age) during the 8-weeks of the study

Variables	T0	T1	T2	T3
Feed intake (g/wee)	840 $\pm$ 0	840 $\pm$ 0	840 $\pm$ 0	840 $\pm$ 0
Duck day (%)	67.48 $\pm$ 4.03 ^a	68.44 $\pm$ 6.14 ^a	63.26 $\pm$ 2.83 ^a	78.64 $\pm$ 2.95 ^b
Number of eggs (Eggs/week)	23.62 $\pm$ 1.41 ^a	23.95 $\pm$ 2.15 ^a	21.67 $\pm$ 1.72 ^a	27.52 $\pm$ 1.03 ^b
Total egg weight (g/week)	1,562.76 $\pm$ 133.58 ^a	1,611.36 $\pm$ 179.34 ^a	1,472.21 $\pm$ 108.34 ^a	1,867.33 $\pm$ 78.74 ^b
Average egg weight (g/egg)	66.14 $\pm$ 1.64	67.25 $\pm$ 3.48	67.88 $\pm$ 2.63	67.94 $\pm$ 0.62
FCR (g/egg)	181.65 $\pm$ 13.31 ^b	179.61 $\pm$ 16.70 ^b	201.04 $\pm$ 12.29 ^b	154.24 $\pm$ 6.47 ^a
FCR (g/g)	2.75 $\pm$ 0.27 ^b	2.68 $\pm$ 0.30 ^{ab}	2.97 $\pm$ 0.20 ^b	2.27 $\pm$ 0.11 ^a

^{a,b} Means within a row with different superscripts differ significantly ($p < 0.05$). T0: Basal feed (containing 5% Azzola) without probiotics, T1: Basal feed plus probiotics 0.5 g/Kg, T2: Basal feed plus probiotics 1 g/Kg, T3: Basal feed plus probiotics 1.5 g/Kg, FCR: Feed conversion ratio

Table 3. Physical quality of laying ducks' eggs (79 weeks of age) after 8 weeks of the study

Variables	T0	T1	T2	T3
Egg weight (g)	68.15 $\pm$ 3.15	64.05 $\pm$ 3.48	65.48 $\pm$ 3.73	66.58 $\pm$ 3.54
Yolk weight percentage (%)	32.32 $\pm$ 3.27	32.13 $\pm$ 2.25	31.93 $\pm$ 3.61	32.13 $\pm$ 2.34
Albumen weight percentage (%)	56.77 $\pm$ 3.47	56.57 $\pm$ 2.84	57.00 $\pm$ 3.34	56.08 $\pm$ 2.23
Shell weight percentage (%)	11.00 $\pm$ 0.53	11.30 $\pm$ 0.77	11.07 $\pm$ 0.36	11.78 $\pm$ 0.90
Yolk index	43.81 $\pm$ 4.25	45.22 $\pm$ 2.61	43.21 $\pm$ 2.05	43.29 $\pm$ 7.50
Albumin index	12.48 $\pm$ 2.09	13.41 $\pm$ 1.90	13.44 $\pm$ 1.43	13.11 $\pm$ 1.55
Haugh unit	89.31 $\pm$ 3.71	91.74 $\pm$ 3.48	92.53 $\pm$ 2.72	93.08 $\pm$ 3.87
Shell thickness (mm)	0.33 $\pm$ 0.02	0.32 $\pm$ 0.02	0.32 $\pm$ 0.03	0.34 $\pm$ 0.02
Yolk color	9.00 $\pm$ 0.89	8.83 $\pm$ 1.33	9.00 $\pm$ 2.00	8.33 $\pm$ 0.52

No significant differences were observed ($p > 0.05$). T0: Basal feed (containing 5% Azzola) without probiotics, T1: Basal feed plus probiotics 0.5 g/Kg, T2: Basal feed plus probiotics 1 g/Kg, T3: Basal feed plus probiotics 1.5 g/Kg

Table 4. Fat, cholesterol, and fatty acid profile of egg yolks in laying ducks (79 weeks of age) after 8 weeks of the study

Variables	T0	T1	T2	T3
Total fat (%)	4.96 ± 2.83	3.62 ± 1.45	7.54 ± 3.06	9.26 ± 3.06
Cholesterol (mg/g)	14.59 ± 0.85 ^b	13.25 ± 0.43 ^a	15.58 ± 0.51 ^b	12.41 ± 1.8 ^a
Saturated fatty acid (SFA)				
Butyric acid (%)	0.08 ± 0.02	0.07 ± 0.01	0.09 ± 0.03	0.12 ± 0.05
Myristic acid (%)	0.24 ± 0.05	0.34 ± 0.11	0.32 ± 0.02	0.35 ± 0.07
Palmitic acid (%)	21.05 ± 1.90	24.35 ± 2.69	26.90 ± 1.74	27.73 ± 0.75
Heptadecanoic acid (%)	0.15 ± 0.01	0.12 ± 0.05	0.13 ± 0.01	0.15 ± 0.06
Stearic acid (%)	4.15 ± 0.22 ^a	4.27 ± 0.59 ^a	5.28 ± 0.12 ^b	5.10 ± 0.28 ^b
Mono unsaturated fatty acid (MUFA)				
Palmitoleic acid (%)	1.54 ± 0.18 ^a	1.70 ± 0.28 ^{ab}	2.02 ± 0.21 ^{bc}	2.17 ± 0.23 ^c
Cis-10-heptadecanoic acid (%)	0.07 ± 0.03	0.07 ± 0.01	0.08 ± 0.02	0.09 ± 0.00
Elaidic acid (%)	0.19 ± 0.05	0.24 ± 0.12	0.20 ± 0.03	0.22 ± 0.04
Oleic acid (%)	35.87 ± 2.07 ^a	36.00 ± 4.51 ^a	44.15 ± 1.42 ^b	44.55 ± 1.95 ^b
Poly unsaturated fatty acid (PUFA)				
Linoleic acid (%)	3.47 ± 0.92 ^a	2.74 ± 0.20 ^a	6.48 ± 0.86 ^b	6.79 ± 0.98 ^b
γ-Linolenic acid (%)	0.07 ± 0.03 ^{bc}	0.04 ± 0.01 ^a	0.12 ± 0.05 ^c	0.08 ± 0.02 ^{bc}
Linolenic acid (%)	0.41 ± 0.32	0.23 ± 0.05	0.39 ± 0.09	0.36 ± 0.14
Arachidonic acid (%)	0.55 ± 0.17 ^a	0.33 ± 0.06 ^a	1.88 ± 0.61 ^b	1.50 ± 0.52 ^b
Total SFA identified (%)	25.67 ± 1.99	29.15 ± 3.28	32.73 ± 1.63	33.46 ± 1.10
Total UFA identified (%)	42.17 ± 2.95	41.34 ± 4.58	55.33 ± 2.69	55.76 ± 3.46
Total MUFA identified (%)	37.67 ± 2.22	38.00 ± 4.67	46.45 ± 1.65	47.03 ± 2.13
Total PUFA identified (%)	4.50 ± 0.74	3.34 ± 0.21	8.87 ± 1.56	8.73 ± 1.45
PUFA:SFA	0.17 ± 0.02	0.12 ± 0.02	0.27 ± 0.05	0.26 ± 0.04
ALA: LA	18.07 ± 0.51	14.21 ± 4.60	18.08 ± 1.94	20.38 ± 5.95
Total fatty acids identified (%)	67.86 ± 4.95	70.49 ± 7.83	88.06 ± 3.77	89.23 ± 4.17

^{a,b,c} Means within a row with different superscripts differ significantly ($p < 0.05$). T0: Basal feed (containing 5% Azzola) without probiotics, T1: Basal feed plus probiotics 0.5 g/Kg, T2: Basal feed plus probiotics 1 g/Kg, T3: Basal feed plus probiotics 1.5 g/Kg; ALA: α-linolenic acid, LA: Linoleic acid

Table 5. Bacterial populations (log₁₀ CFU/g) of the laying duck (79 weeks of age) excreta

Treatment	<i>Escherichia coli</i>	<i>Salmonella</i>	LAB	Ratio LAB/ <i>Escherichia coli</i>	Ratio LAB/ <i>Salmonella</i>
T0	5.06 ± 0.87	4.88 ± 0.62	6.23 ± 0.25	1.29 ± 0.21	1.34 ± 0.2
T1	4.10 ± 0.43	4.41 ± 0.86	6.55 ± 0.10	1.52 ± 0.97	1.41 ± 0.33
T2	4.93 ± 0.26	4.62 ± 0.71	6.7 ± 0.06	1.36 ± 0.68	1.45 ± 0.22
T3	4.77 ± 0.57	4.35 ± 0.077	6.74 ± 0.21	1.41 ± 0.22	1.55 ± 0.06

No significant differences were observed ($p > 0.05$). T0: Basal feed (containing 5% Azzola) without probiotics, T1: Basal feed plus probiotics 0.5 g/Kg, T2: Basal feed plus probiotics 1 g/Kg, T3: Basal feed plus probiotics 1.5 g/Kg. LAB: Lactic acid bacteria

Table 6. Ammonia (NH₃) content of the laying duck (79 weeks of age) excreta

Treatment	NH ₃ (mM)
T0	146.93 ± 1.52 ^d
T1	141.77 ± 1.71 ^c
T2	135.79 ± 0.70 ^b
T3	122.22 ± 1.11 ^a

^{a,b,c,d} Means within a column with different superscripts differ significantly ($p < 0.05$). T0: Basal feed (containing 5% Azzola) without probiotics, T1: Basal feed plus probiotics 0.5 g/Kg, T2: Basal feed plus probiotics 1 g/Kg, T3: Basal feed plus probiotics 1.5 g/Kg

DISCUSSION

The supplementation of feed with yeast probiotics in laying ducks has not yet been extensively studied.

However, the role of yeast-based probiotics has been extensively studied in other laying poultry, such as laying hens (Sarwar et al., 2022; Wahyuni et al., 2023; Qiu et al., 2024) and laying quails (Yousif and Kloor, 2023).

According to Abd El-Ghany (2025), the yeast strains *Saccharomyces cerevisiae* (*S. cerevisiae*) and *S. boulardii* are the most frequently used probiotic cultures in poultry feed formulations. The suggested doses of yeast to be supplemented in poultry feed range from 10^8 to 10^{10} CFU, as noted by Maksimović et al. (2022). In the present study, the type and dose of yeast used were appropriate for acting as a probiotic and supporting optimal poultry productivity. The results of the current study indicated that the probiotic *S. boulardii* supplementation influenced egg production (number of eggs and duck-day production), total egg weight, and FCR. No cases of disease or mortality were observed in the ducks during the study. Qui et al. (2024) reported consistent findings that yeast-based probiotics (2 g per kg feed) can increase egg production and weight as well as reduce the feed conversion ratio in laying hens. Hassanein and Soliman (2010) noted that supplementing laying hens with 0.4% and 0.8% yeast culture for 9 weeks during the late phase (weeks 70-79) could improve egg production, feed conversion, and egg mass through optimised nutrient absorption, modulation of the gut microbiota, increased levels of Lactobacilli, and a reduction in pathogenic bacteria. Similarly, the effect of supplementing laying quails with 2.5% probiotic yeast for 14 weeks also showed an increase in quail day percentage and egg weight, as well as a reduction in FCR (Yousif and Kloor, 2023).

Yeast culture is abundant in peptides, amino acids, vitamins, oligosaccharides, organic acids, minerals, and other advantageous components that enhance poultry production by facilitating the digestion and absorption of nutrients, thus improving poultry production performance (Liu et al., 2021; Qiu et al., 2024; Hossain et al., 2025). Furthermore, the increase in egg production resulting from yeast-based probiotic supplementation is strongly associated with improvements in gut health, the poultry immune system, and increased production and activity of digestive enzymes (Zhang et al., 2020). The mechanism related to yeast probiotics as a feed additive was also reported by Sun et al. (2019), who noted that mixed yeast supplementation (*Saccharomyces cerevisiae* and *Kluyveromyces fragilis*) has been associated with improved nutrient (dry matter) digestibility. The presence of yeast as a probiotic in the digestive tract leads to competition with pathogens for attachment sites on the intestinal wall and for nutrients, thereby suppressing pathogenic bacteria and promoting the development of beneficial gut flora (Ogbuewu et al., 2019). The processes underlying yeast's mechanism of action, as described in the paragraph, are consistent with our findings, which

demonstrate enhanced productivity (egg production and FCR) in ducks.

No significant differences were found in the weight of eggs, albumin, yolk, or eggshell percentage, as well as albumin and yolk weight index, which aligned with those reported in other studies (Özsoy et al., 2018; Sarwar et al., 2022; Yousif and Kloor, 2023). In the present study, the dietary inclusion of *S. boulardii* also did not affect eggshell thickness, Haugh units, or yolk colour score. In contrast to previous studies, such as that by Hassanabadi et al. (2025), supplementation with 2 g of yeast extract per kg of feed significantly increased eggshell thickness in laying hens compared to the control (0.387 versus 0.362 mm). Findings by Park et al. (2020) also showed that the addition of 3% brewer's yeast hydrolysate per kg of layer hens' feed improved albumen height, Haugh unit, yolk colour, and eggshell thickness. Significant differences in the effects of yeast on layer poultry feed may be due to variations in the type of yeast used, dosage, duration of administration, age, and type of layer poultry used, as well as husbandry management conditions. Based on the Haugh unit calculations in the present study, the physical quality of duck eggs in all treatments can be categorised as 'excellent quality or AA grade' (over 72) according to United States Department of Agriculture (USDA) egg quality standards.

The fat content of duck eggs was not affected by supplementation of *S. boulardii* yeast, although the highest average fat content (9.26 ± 3.06) was found in the T3 treatment group (1.5 g per kg of feed). The results of the current study are consistent with the study by Qiu et al. (2024), which found that the addition of 2 g/kg of yeast culture increased the fat content of chicken egg yolks ($8.49 \pm 0.52\%$). In contrast, the cholesterol content of Cihateup duck egg yolks in groups T1 and T3 decreased significantly compared with the control group. The lowest cholesterol content was found in group T3 (12.41 mg/g). Ismoyowati et al. (2022) reported that dietary supplementation in Tegal duck (Indonesian local duck) with a commercial probiotic (2% in feed) showed a yolk cholesterol content of 13.99%, whilst the cholesterol content of the yolks of local Indonesian duck eggs reached 21.94 mg/g with 2% phenolic herbal supplementation in feed (Anggraeni et al., 2023). A reduction in egg cholesterol level following yeast probiotic supplementation was also reported by Yalçın et al. (2010), who reported that the addition of 0-4 g/kg of yeast to the diet of laying hens significantly reduced yolk cholesterol after 16 weeks of treatment. Similarly, Yalçın et al. (2014) reported that supplementation of yeast cell wall over a 26-

week study period significantly reduced yolk cholesterol, with a 13.35% reduction compared to the control. The finding of low cholesterol content in egg yolks (T3) in the current study is thought to be related to the β -glucan content in yeast, which is capable of binding cholesterol in the intestinal lumen, reducing lipoprotein absorption, and enhancing lipid metabolism in the liver (Aoe et al., 2020; Yu et al., 2021).

Another possible factor contributing to the reduction in yolk cholesterol levels is reduced absorption, synthesis, or both of cholesterol in the gastrointestinal tract, as explained in Xiao et al. (2012). Mannan-oligosaccharides (MOS) in the yeast cell wall play an important role in the cholesterol reduction effect. Mannan-oligosaccharides and β -glucans fermented by gut bacteria produce short-chain fatty acids (SCFAs), which acidify the large intestine and serve as a substrate for enterocyte energy production (Liu et al., 2021), thereby enhancing intestinal absorption and indirectly supporting lipid metabolism and reduced cholesterol deposition in the yolk (Bortoluzzi et al., 2018; Perricone et al., 2022). However, the effect of yeast probiotic supplementation on yolk cholesterol has not always shown the same effects across studies. In a study by Xiang et al. (2018), the combination of *S. boulardii* and *Pediococcus acidilactici* as probiotics in laying hens did not have a significant effect on reducing yolk cholesterol. The insignificant reduction in yolk cholesterol may be due to the relatively short duration of administration 5 weeks and the lower dose of 0.05 g/kg of feed. Studies involving bacterial probiotic supplementation over a longer period showed a trend of decreasing yolk cholesterol as the hens aged. The observed findings suggested that the cholesterol-lowering effect may become more pronounced with longer probiotic administration (Ramasamy et al., 2008).

The content of stearic acid, an SFA, was significantly higher in T2 and T3 than in the other treatments. In the current study, the dietary inclusion of *S. boulardii* was able to increase the content of several important fatty acids in the MUFA group, namely oleic acid (C18:1n9c), and PUFAs, namely linoleic acid (C18:2n6c), γ -linolenic acid (C18:3n6), and arachidonic acid (C20:4n6), which is an omega-6 fatty acid. The findings indicated that probiotic yeast modulates lipid metabolism in a beneficial, functional manner. An increase in linoleic acid (C18:2n6c) in chicken egg yolk was also observed as a significant effect of *Saccharomyces cerevisiae* supplementation at dosages of 0.05% and 0.1% of the diet (Özsoy et al., 2018). Yalçın et al. (2012) reported similar findings, noting that supplementation with 2 g of *Saccharomyces*

cerevisiae per kg of feed increased oleic acid (C18:1n9c) levels in chicken egg yolk. The response of other (commercial) probiotic types to the increase in C18:2n6c and C18:3n3 fatty acids in duck eggs was also reported by Ismoyowati et al. (2022). However, in the present study, the increase in C18:2n6c and C18:3n3 was not accompanied by a significant change in the LA: ALA ratio across all treatments. The LA: ALA ratio (14.21-20.38) was higher than that reported by Özsoy et al. (2018), which ranged from 12.50 to 13.16. Meanwhile, in the same type of poultry, namely ducks, the LA: ALA ratio in our study was lower than that reported by Ismoyowati et al. (2022). The result indicates that probiotic supplementation in feed can elicit different fatty acid responses, which depend on the probiotic and the laying poultry used.

Yeast dietary supplements for chickens and ducks exert a modulatory effect on the intestinal microbiota, thereby enhancing colonization resistance against enteric pathogens (Pang et al., 2022). Furthermore, the role of *S. boulardii* in poultry is primarily reflected in enhancing the immune recognition and immune regulation functions of poultry, reducing the colonization of intestinal pathogens, and reducing the number of pathogenic bacteria in feces (Rajput et al., 2013). In the current study, the total counts of lactic acid bacteria, *Salmonella*, and *E. coli* in the excreta did not differ significantly among all treatments compared to the control. The number of *E. coli* bacteria (log10) tended to decrease in group T1, and the LAB/*E. coli* ratio tended to increase compared with the control. The LAB/*Salmonella* ratio was relatively higher in the T3 group. Wahyuni et al. (2023) reported that the addition of a combination of *Saccharomyces cerevisiae* (0.2%) and *Spirulina platensis* algae (0.3%) significantly increased lactic acid bacteria colonies and reduced coliform bacteria in the caecum of laying hens. A study by Pantaya et al. (2026) found that *Saccharomyces cerevisiae* markedly reduced intestinal populations of pathogenic bacteria, including *Salmonella*, *Shigella*, and *E. coli*, at a dose of 1.5 g per kg of feed. The ability of yeast to suppress pathogen populations is linked to several theories, including the notion that yeast cell wall β -glucans promote the growth of beneficial gut bacteria, such as *Lactobacillus* spp., thereby contributing to intestinal health (Fathima et al., 2023). Mannan-oligosaccharides, a component of yeast cell walls, can bind pathogenic bacteria, thereby preventing their adhesion to intestinal epithelial cells (Lee et al., 2021). An increase in the population of lactic acid bacteria, resulting from the presence of yeast in the digestive tract, indirectly initiates a mechanism of

competitive exclusion between gut pathogens, lactic acid bacteria, and yeast (Yeu et al., 2024).

The ammonia content in excreta decreased significantly as the dose of *S. boulardii* supplementation increased. The results are consistent with those reported by Wahyuni et al. (2023), who found that a 0.2% yeast dose reduced excreta ammonia by 52.82%. In the current study, T3 levels decreased by 16.8% compared to the control. Similar results were reported by Pantaya et al. (2026), who found that real-time ammonia gas emissions in Pekin ducks fed the probiotic *S. cerevisiae* decreased significantly. Rezaee et al. (2019) stated that probiotics can inhibit the activity of urease and other proteolytic enzymes that catalyse the deamination of amino acids and the hydrolysis of urea in the large intestine, thereby reducing the production of ammonia as a metabolic end-product excreted from the poultry's body. The reduction in ammonia excretion is also associated with increased digestibility of crude protein in the feed and nitrogen retention (Park et al., 2016). The more protein that is digested, the less nitrogen is lost in the excreta. The level of ammonia in excreta is typically determined by the amount of nitrogen in the excreta (Jeong and Kim, 2014).

The findings in the present study demonstrated that *S. boulardii* has the potential to serve as a probiotic for laying ducks. The protein, vitamin, and mineral content of yeast is a key factor in its safe use in the poultry sector (Liu et al., 2021). Moreover, yeast has functional mechanisms to modulate the immune system, histamine release, toxin binding, and interactions with pathogenic cells and gut constituents, thereby improving poultry performance (Hatoum et al., 2012). The presence of *S. boulardii* in the digestive tract of ducks in the present study allows for antagonistic activity of microorganisms, such as competition for nutrients, pH changes in the medium as a result of growth-coupled ion exchange or organic acid production, and secretion of antibacterial proteins and protease compounds (Pais et al., 2020).

CONCLUSION

The dietary supplementation of *S. boulardii* increased egg production and total egg weight, reduced the feed conversion ratio, and lowered the ammonia content of excreta. The present study also found that yeast probiotic supplementation decreased yolk cholesterol and increased the omega-6 fatty acid content (linoleic acid, γ -linolenic acid, and arachidonic acid) in egg yolks. In general, the optimal dosage of *S. boulardii* in the feed of Cihateup ducks was 1.5 g per kg. The findings demonstrated that *S.*

boulardii has the potential to serve as a probiotic, leading to improved laying performance and indicating enhanced quality of Cihateup duck eggs. A limitation of the present study is that an in-depth analysis regarding the biological mechanisms by which probiotics influence gut microbiota composition and lipid metabolism was not directly investigated. Further studies on gut microbiota composition are suggested to substantiate the explanation of the beneficial effects of the probiotic *S. boulardii* on the productivity and lipid metabolism of laying ducks.

DECLARATIONS

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Authors' contributions

Gilang Ayuningtyas contributed to the conceptualization of the study, method development, and its implementation. Validated and processed the data and drafted the original manuscript. Also addressed reviewers' comments, revised the statistical analysis, and updated the manuscript. Dadik Pantaya was involved in the conceptualization of the study, validated the methodology, supervised the study process and data processing, and was responsible for funding acquisition, also addressed reviewers' comments. Tera Fit Rayani, Gayuh Syaikhullah, Fitriani Eka Puji Lestari, Anis Usfah Prastujati, and Dwi Ahmad Priyadi contributed as study collaborators, recorded and curated the data, and participated in the writing of the manuscript. Hariadi Subagja supervised and reviewed the manuscript and was responsible for funding acquisition. All authors have read and agreed to the substance written in the final version of the manuscript.

Availability of the data and materials

Data available upon reasonable request from the corresponding author.

Competing interests

The authors declare no conflict of interest.

Ethical considerations

Ethical issues, including plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy, have been checked by all the authors. All authors also state

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Effects of *Bacillus amyloliquefaciens* and Organic Acids on Growth Performance, Carcass Traits, Blood Parameters, and Cecal Microbiota in Cockerels

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ABSTRACT

The increasing restrictions on antibiotic growth promoters have drawn attention to natural feed additives that enhance gut health and contribute to sustainable poultry production. The present study aimed to investigate the individual and combined effects of organic acids and *Bacillus amyloliquefaciens* (*B. amyloliquefaciens*) on growth performance, organ development, carcass characteristics, blood biochemical and hematological parameters, and cecal microbiota in cockerels. A total of 200 cockerels, weighing 68.5 ± 4.44 grams, were randomly assigned to four treatment groups. Four treatments consisted of a control group (A0B0), organic acids alone (A1B0), *B. amyloliquefaciens* alone (A0B1), and their combination (A1B1), with five replicates of ten cockerels each over 42 days. Two factors were employed, with organic acids supplemented in the drinking water at two levels (0% [A0] and 0.08% [A1]) and *B. amyloliquefaciens* incorporated into the diet at two levels (0% [B0] and 0.01% [B1]) at a concentration of 1×10^9 CFU/g feed. Organic acids enhanced body weight gain during the starter phase (599.6 versus 546.1 g), whereas *B. amyloliquefaciens* reduced cumulative feed intake over the entire period (3044.9 versus 3330.9 g). Group A1B1 did not enhance growth beyond that of the individual additives, indicating no synergistic effect. Organic acids decreased liver and spleen weights but increased proventriculus weight, while *B. amyloliquefaciens* increased spleen weight and decreased gizzard and proventriculus weights. A significant interaction was detected for spleen weight. Most blood biochemical and hematological parameters were unaffected by the treatment. However, organic acids reduced serum total protein and globulin, while hepatic enzyme activities remained unchanged, indicating no adverse effects on liver function. Cecal microbiota profiling demonstrated that organic acids were more effective than the combined treatment in reducing *Escherichia coli* and coliform populations. In contrast, Group A1B1 resulted in the highest *Bacillus* count. Both additives modulated cecal microbial populations compared to the control group. Organic acids improved early growth and reduced pathogenic bacteria, whereas *B. amyloliquefaciens* reduced feed intake and increased *Bacillus* abundance. The combined supplementation of organic acids and *B. amyloliquefaciens* did not provide synergistic benefits, indicating a parameter-dependent response. Neither additive adversely affected hematological indices or liver function.

Keywords: Antibiotic, *Bacillus amyloliquefaciens*, Cecal microbiota, Cockerel, Growth performance, Organic acid

INTRODUCTION

Poultry production is recognized as the most significant supplier among different food supply sectors (FAO, 2023). The increasing global consumption of poultry products is driven by population growth and ongoing urbanization (Mottet and Tempio, 2017). Maintaining high-quality, safe nutrients is crucial for the poultry industry to meet

consumer expectations and regulatory standards. In the poultry industry, the use of dietary antibiotics has historically been widespread (Abreu et al., 2023). For many decades, antibiotics were routinely used as a key management practice, considered essential for maintaining production efficiency and protecting birds from infectious diseases (Bao et al., 2022). Nevertheless, the long-term

misuse of antimicrobial agents has contributed to the emergence of bacterial resistance, a major global challenge in livestock production (Haulisah et al., 2021). The worldwide prohibitions on antibiotic growth promoters (AGPs) in poultry production, notably in organic systems, necessitated the development of innovative alternatives (Salim et al., 2018). The suggested non-antibiotic options should aim to boost innate immunity and influence the cecal microbiome to support the bird's health and performance (Paneru et al., 2024). As a result, multiple studies have examined non-antibiotic alternatives such as organic acids (Khan et al., 2022; Abd El-Ghany, 2024) and *Bacillus*-based probiotics (Bao et al., 2022; Bahaddad et al., 2023). Different categories of feed additives have been suggested as alternatives to AGPs, such as organic acids, enzymes, probiotics, prebiotics, herbal extracts, essential oils, and immunostimulants. The suggested feed additives are used to mitigate production losses following the ban on antibiotics (Khan et al., 2022; Abd El-Ghany, 2024). Organic acids and probiotics have generated substantial interest due to their potential not only to enhance poultry growth but also to maintain a well-balanced gut microbiota and stimulate immune activity (Ebeid et al., 2021). Among the alternatives to AGPs, organic acids, particularly propionic, formic, citric, and acetic acids, have emerged as promising candidates (Khan et al., 2022). Organic acids, classified as short-chain fatty acids (SCFAs), are characterized by their volatility and broad-spectrum antimicrobial activity against enteric pathogens (Abd El-Ghany, 2024). Organic acids enhance gut health through multiple mechanisms, including reducing intestinal pH, improving nutrient digestibility and absorption, and suppressing pathogenic bacterial proliferation (Adil et al., 2020). Consequently, SCFAs, including propionic, acetic, butyric, lactic, and fumaric acids, have been demonstrated to enhance poultry health and performance (Khan et al., 2022). The positive effects of SCFAs include modulating the gut microbiota, enhancing carcass traits (Hamid et al., 2018), inhibiting pathogenic microorganisms (Melaku et al., 2021), enhancing nutrient digestibility (Ghazalah et al., 2011), and improving intestinal morphology (Deng et al., 2023).

Probiotics included in poultry diets are beneficial microorganisms that colonize the gut of homeothermic animals (Khan, 2013). The strains of *Bacillus*, *Bifidobacterium*, and different yeasts are the most commonly used probiotics for broiler chickens and laying hens (Khan, 2013). *Bacillus* species produce different digestive enzymes, including cellulase, α -amylase, and proteases, which optimize nutrient utilization and enhance

nutrient digestibility and absorption (Lee et al., 2008). *Bacillus* species have distinct advantages in feed applications due to their ability to form resilient endospores and inhibit gut-pathogenic bacteria (Bahaddad et al., 2023). Spore formation and antimicrobial activity significantly encourage the viability and utility of *Bacillus* species in commercial feed production (Elisashvili et al., 2019). *Bacillus amyloliquefaciens* (*B. amyloliquefaciens*), in particular, produces antimicrobial substances such as subtilin and barnase, which invariably reduce the abundance of pathogenic microorganisms in the gut (Lisboa et al., 2006). As a microbial feed additive, *B. amyloliquefaciens* has been recognized as a suitable replacement for AGPs in poultry production (Lei et al., 2015), mainly due to its ability to produce extracellular enzymes that promote feed utilization (Lee et al., 2008). Although different studies have investigated the effects of organic acids (Dibner and Buttin, 2002; Adil et al., 2011; Khan et al., 2022) and *B. amyloliquefaciens* (Ahmed et al., 2014; Lei et al., 2015; Bao et al., 2022) individually, few studies have examined their combined effect during the growth phase of cockerels, a period essential for enhancing growth efficiency and immune system development. The cecal microbiota is a major factor determining the metabolic fate of nutrients, immune status, and the health status of chickens (Stanley, 2014). However, the effects of concurrent use of *B. amyloliquefaciens* and organic acids on physiological indicators, including blood biochemical parameters and microbial diversity, remain largely unexplored in cockerels. Previous studies have indicated that each additive offers notable benefits individually (Khan et al., 2022; Abd El-Ghany, 2024). For instance, supplementation with *B. amyloliquefaciens* has been indicated to improve growth performance in broiler chickens (Ahmed et al., 2014), although similar studies in native cockerels remain limited. Additionally, organic acids have been indicated to effectively control enteric pathogens, including *Clostridium perfringens*, *Escherichia coli* (*E. coli*), and *Salmonella* spp. (Islam et al., 2024).

Despite promising findings in individual applications, potential synergistic interactions among these additives, particularly regarding metabolic and microbiological responses and pharmacodynamic interactions within the gastrointestinal tract of native cockerels, remain largely unexamined. It remained uncertain whether their concurrent supplementation could enhance beneficial microbiota, antagonistically suppress pathogens, or lead to unexpected interactions in organ development and metabolic functions. Therefore, the present study aimed to

investigate the individual and combined effects of organic acids and *B. amyloliquefaciens* on growth performance, organ development, blood parameters, and cecal microbiota in Saudi native cockerels.

MATERIALS AND METHODS

Ethical approval

The study protocol was reviewed and approved by the Internal Scientific Committee of the National Organic Agriculture Center, Ministry of Environment, Water and Agriculture, Saudi Arabia (Approval No. NOAC/2023/014, dated 15 March 2023). The experimental chickens were obtained from the Organic Poultry Unit of the National Organic Agriculture Center. All experimental procedures were conducted in accordance with local regulations (Royal Decree No. M / 59, dated 14/09/1431 H) and internationally accepted guidelines for the care and use of animals in research.

Cockerels and management

The current experiment was conducted at the National Organic Agriculture Center of the Ministry of Environment, Water, and Agriculture in Saudi Arabia. One-day-old Saudi native cockerels were obtained from the Organic Poultry Unit of the National Organic Agriculture Center. The cockerels hatched from eggs laid by an in-house breeding flock of Saudi native chickens raised at the center. Eggs were incubated at 37.5°C and 55-60% humidity in the hatchery center. After hatching, male chickens (cockerels) were individually selected, with an initial body weight of 68.5 ± 4.44 g, and randomly assigned to experimental treatments. All cockerels were housed in 20 floor wire pens ($100 \times 100 \times 100$ cm) within an environmentally controlled house. The floor of each pen was covered with a 5-cm layer of wood shavings as litter. Each pen had a feeder and a drinker to provide *ad libitum* feed and water throughout the experimental period. The temperature was maintained at 35°C for the first week of age, then gradually reduced to 24-27°C at 3°C per week, with relative humidity ranging from 50 to 70%. Continuous lighting was kept throughout the experiment. The same lighting program was applied to all treatment groups to ensure uniform management conditions and to avoid potential confounding effects of lighting on the evaluation of the dietary treatments.

Experimental design and treatments

A total of 200 cockerels were randomly allocated to four treatments in a completely randomized design,

employing a 2×2 factorial experiment with five replications of ten cockerels each. The two factors included the use of organic acids and *B. amyloliquefaciens*. The organic acid was used at two levels in the drinking water; 0% (drinking water without organic acids; A0) and 0.08% (A1). The two levels of *B. amyloliquefaciens* were 0% (basal diet without supplementation; B0) and 0.01% in the diet, at a concentration of 1×10^9 CFU/g of feed (B1). Consequently, four treatments included a control group without supplementation of either additives (A0B0), a group with only organic acids at 0.08% (A1B0), a group supplemented with *B. amyloliquefaciens* at 0.01% (1×10^9 CFU/g feed; A0B1), and a combined group receiving both organic acids at 0.08% and *B. amyloliquefaciens* at 0.01% (1×10^9 CFU/g of feed; A1B1). For organic acid supplementation, a commercial organic acid blend (Agrocid Super, CID LINES N. V., Belgium) containing formic acid, propionic acid, lactic acid, citric acid, and sorbic acid was added to the drinking water at a final concentration of 0.08%, which was within the safe range and below toxicity-associated levels (Adil et al., 2011). Based on the manufacturer's specifications, the addition of 0.08% organic acid blend (Agrocid Super) reduced the pH of drinking water from approximately 7.2 (plain water) to approximately 4.0-4.5. A fresh organic acid solution was prepared daily and freely accessible to chickens during the 42-day experimental period. For probiotic supplementation, *B. amyloliquefaciens* CECT 5940 (Evonik España y Portugal S. A. U., Spain) was incorporated into the feed at 0.01% (w/w), yielding a final concentration of 1×10^9 CFU/g of feed. The probiotic was mixed thoroughly with the basal diet. Treated feed was prepared weekly and stored in sealed bags at room temperature until further use. The stability of *B. amyloliquefaciens* CECT 5940 in feed during the 7-day storage period was verified by laboratory testing using the spread plate method on nutrient agar after heat treatment at 80°C for 10 minutes. Feed samples were collected on days 0 and 7, and viable *Bacillus* counts were determined using standard serial dilution and plating methods. Colony counts were calculated from plates containing 30-300 colonies. The current results confirmed that the probiotic remained stable throughout storage, with viability exceeding 95% of the initial count on day 7. Basal starter (0-21 days) and finisher (22-42 days) diets were formulated to meet or exceed the nutrient requirements recommended by the National Research Council (NRC, 1994) for poultry. All experimental diets were designed to be isoenergetic with equal metabolizable energy (ME) and iso-nitrogenous, containing the same crude protein

(CP) to ensure that any observed effects resulted from the additives themselves rather than from differences in energy or

protein levels. The composition of the experimental diets is detailed in Table 1.

Table 1. Ingredients and chemical composition of the experimental diets

Ingredients (%)	Starter diet with <i>Bacillus</i> <i>amyloliquefaciens</i>	Starter diet without <i>Bacillus</i> <i>amyloliquefaciens</i>	Finisher diet with <i>Bacillus</i> <i>amyloliquefaciens</i>	Finisher diet without <i>Bacillus</i> <i>amyloliquefaciens</i>
Corn	56.9	57.65	57.6	57.20
Soybean meal (40% CP)	32.55	32.5	32.8	32.80
Corn germ cake	0.59	0.59	0.00	0.00
Wheat bran	2.40	2.26	1.49	1.90
Salt	0.20	0.20	0.20	0.20
Vegetable oil	3.90	3.50	4.10	4.10
Dicalcium phosphate	1.75	1.75	1.80	1.80
Limestone	1.20	1.20	1.30	1.30
DL-Methionine	0.10	0.10	0.20	0.20
Lysine	0.20	0.05	0.30	0.30
Vitamin-mineral premix ¹	0.20	0.20	0.20	0.20
<i>Bacillus amyloliquefaciens</i>	0.01	0.00	0.01	0.00
Calculated analysis				
ME (kcal/kg)	3198	3196	3201	3202
Crude Protein	20.06	20.09	20.01	20.03
Calcium	0.98	0.98	1.01	1.01
Available phosphorus	0.36	0.36	0.35	0.35
Lysine	1.45	1.45	1.43	1.43
Methionine	0.54	0.54	0.53	0.53

¹Vitamin-mineral premix provided the following per kilogram of diet. Vitamin A (retinyle acetate): 10,000 IU, Vitamin D₃ (cholecalciferol): 2,500 IU, Vitamin E (α -tocopheryl acetate): 60 mg, Vitamin K₃ (menadione sodium bisulfite complex): 15 mg, Vitamin B₁ (thiamine hydrochloride): 2 mg, Vitamin B₂ (riboflavin): 8 mg, Vitamin B₆ (pyridoxine hydrochloride): 4 mg, Vitamin B₁₂ (cyanocobalamin): 0.04 mg, Pantothenic acid: 15 mg, Nicotinic acid: 40 mg, Folic acid: 1.5 mg, Biotin: 0.2 mg, Choline chloride: 200 mg, Iron: 50 mg, Manganese: 50 mg, Copper: 10 mg, Zinc: 50 mg, Calcium: 352 mg, Iodine: 1.46 mg, Cobalt: 0.5 mg, Selenium: 0.2 mg. ME: Metabolizable energy (Kcal/Kg), CP: Crude protein, DL-Methionine: A racemic mixture of the D- and L- isomers of methionine (synthetic methionine). Experimental diets were formulated according to [NRC \(1994\)](#).

Growth performance

Feed consumption and body weight gain were recorded weekly for each replicate. The collected data were used to calculate the feed conversion ratio (FCR). Growth performance parameters were evaluated across three periods, including starter (0-21 days), finisher (22-42 days), and the entire experimental period (0-42 days).

Slaughter performance

At the end of the trial, two cockerels from each pen were selected based on their proximity in weight to the average weight of the cockerels within their respective pen (experimental unit). The selected cockerels were subsequently slaughtered to determine the dressing percentage, as well as the relative weights of the proventriculus, gizzard, liver, pancreas, and spleen, along with the measurements of the intestine and ceca lengths. After slaughter, the abdominal cavity was opened, and the internal organs were carefully excised. The proventriculus and gizzard were opened, and their contents were completely removed by gentle scraping and rinsing with saline solution. All organs, including the liver, spleen,

proventriculus, gizzard, and pancreas, were gently dried with filter paper to remove surface blood and moisture before weighing. The relative weights of the organs were calculated as a percentage of live body weight. The lengths of the intestine and ceca were measured following gentle expression of their contents. The cockerel's carcass sections, such as breast, thigh, and drumstick, were measured relative to their live body weights.

Blood sample collection

On day 42, two cockerels were selected randomly from each pen (10 cockerels per treatment) to collect the blood samples. The cockerels were fasted for 4 to 6 hours prior to blood collection, with feed removed, and water, free of organic acids, was available *ad libitum*. Two blood samples were collected via wing veins from each selected cockerel before slaughter to avoid post-mortem changes. The first sample was collected into a tube containing anticoagulant (EDTA) for hematological parameters using an automated hematology analyzer (HumaCount, 30TS, Germany). The hematological parameters analyzed were red blood cells (RBCs), packed cell volume (PCV),

hemoglobin (Hb), white blood cells (WBCs), differential leukocyte counts, and mean corpuscular hemoglobin concentration (MCHC). The second blood sample was placed in a plain test tube, centrifuged (DLAB, DM0412, China) at $6,000 \times g$ for 10 minutes to separate the serum, and stored at -20°C until further biochemical analysis. Blood biochemical parameters were measured spectrophotometrically using a HumaLyzer 4000 (HUMAN, Germany) according to the manufacturer's instructions. Commercial diagnostic kits (HUMAN, Germany) were used to determine glucose, total protein, albumin, cholesterol, calcium, phosphorus, alanine aminotransferase (ALT), aspartate aminotransferase (AST), triglyceride (TG), and high-density lipoprotein (HDL). The globulin concentration was calculated as the difference between the total protein and albumin (Bushner, 1990). Low-density lipoprotein (LDL) and Very-low-density lipoprotein (VLDL) concentrations were calculated using the Friedewald formula (Friedewald et al., 1972).

Cecal microbial count

Cecal samples (1 g each) were collected aseptically from two cockerels per replicate (10 samples per treatment) for microbial analysis. All dissecting tools were autoclaved at 121°C for 15 minutes and flamed with 70% ethanol between samples. Cecal contents were collected directly into pre-sterilized tubes using sterile forceps and scalpels. All procedures were performed on a sterile surface near a Bunsen burner flame. Plating was conducted in a laminar flow cabinet. Gut microbial analysis involved quantifying total aerobic bacteria, *E. coli*, and *Bacillus* spp., using established methods (McDonald et al., 1983; van Horn et al., 1996; Hossain et al., 2015). Serial decimal dilutions of the homogenized digesta were prepared with sterile buffered peptone water (Liofilchem, Italy; Cat. No. 611014) for microbial counting. Selective agar media were employed for each target organism. Nutrient agar (Mast Group, Germany; Cat. No. DM179D) for total aerobic bacteria, chromatic coliform agar (Liofilchem, Italy; Cat. No. 610610) for coliforms, chromatic *E. coli* O157 agar (Liofilchem, Italy; Cat. No. 610614) for *E. coli*, and polymyxin egg yolk mannitol bromothymol blue agar (PEMBA; Neogen, USA; Cat. No. LAB193) for *Bacillus cereus* were used. All plates were aerobically incubated at 37°C . Bacterial counts were reported as log₁₀ colony-forming units (CFU) per gram of digesta based on colony morphology and distinctive growth characteristics.

Statistical analysis

All data were analyzed using SPSS, version 21. Two-way analysis of variance (ANOVA) was used to assess the effects of two independent variables, namely organic acids and *B. amyloliquefaciens*, on performance, blood parameters, carcass characteristics, and cecal microbiota of cockerels. The model analyzed the primary effects of each factor along with their interactions. Differences between mean values of the treatments were determined using Duncan's multiple-range test (Statistical significance was defined at $p \leq 0.05$). The results were expressed as the means and standard error of the mean (SEM).

RESULTS

Growth performance

The effects of water supplemented with organic acids and the dietary incorporation of *B. amyloliquefaciens* on the growth performance of the cockerels during the starter (0-21 days), finisher (22-42 days), and entire experimental periods are presented in Table 2. Organic acid supplementation did not significantly affect feed consumption during the starter period (0-21 days), finisher period (22-42 days), or the entire experimental period (0-42 days; $p > 0.05$). In contrast, the dietary inclusion of *B. amyloliquefaciens* significantly reduced the cumulative feed intake (3044.9 g) in comparison to B0 (3330.9 g) throughout the entire experimental period (0-42 days; $p < 0.05$). However, no significant effects were observed during the starter or finisher phases when considered separately ($p > 0.05$). Supplementing with organic acids significantly increased body weight gain in Group A1, reaching 599.6 g compared to 546.1 g in Group A0 during the starter period (0-21 days; $p < 0.05$). No significant effects on weight gain were observed during the finisher period or the entire experimental period ($p > 0.05$). *Bacillus amyloliquefaciens* supplementation did not significantly affect weight gain during any period (starter, finisher, or entire period; $p > 0.05$). Organic acid supplementation significantly impaired FCR throughout the entire 0-42 day experimental period, with A1 at 3.2 compared to A0 at 2.9 ($p < 0.05$); however, no significant effects were observed during the starter or finisher periods ($p > 0.05$). *Bacillus amyloliquefaciens* supplementation did not significantly affect FCR during any period ($p > 0.05$). No significant interaction effects between organic acids and *B. amyloliquefaciens* were observed for any growth parameters, including feed consumption, weight gain, or FCR, during any period ($p > 0.05$).

Table 2. Effects of organic acids and *Bacillus amyoliquefaciens* on cockerels' growth performance during starter, finisher, and entire experimental periods

Treatments	Weight gain (g)			Feed consumption (g)			Feed conversion ratio (g:g)		
	Starter	Finisher	Entire period	Starter	Finisher	Entire period	Starter	Finisher	Entire period
Control (A0B0)	565.4	409.8	1043.6	1777.4	1445.4	3223	3.00	3.52	2.86
<i>Bacillus amyoliquefaciens</i> (A0B1)	526.8	397	986.6	1660	1375	2998.4	3.16	3.46	2.96
Organic acids (A1B0)	595.2	406.2	1014.4	1824.4	1428.6	3438.8	3.36	3.52	3.38
Combination (A1B1)	604.0	390.6	1001.6	1699	1394.4	3091.4	2.82	3.58	3.10
SEM	24.8	7.35	27.59	143.1	58.52	133.2	0.18	0.127	0.162
Organic acids effects									
A0	546.1 ^b	403.4	1016.6	1718.7	1410.2	3110.7	3.1	3.5	2.9 ^b
A1	599.6 ^a	398.4	1019	1761.7	1411.5	3265.1	3.1	3.6	3.2 ^a
SEM	17.5	5.2	19.5	101.2	41.38	94.19	0.13	0.09	0.12
p-value	0.046	0.506	0.759	0.768	0.983	0.263	0.956	0.644	0.048
<i>Bacillus amyoliquefaciens</i> Effects									
B0	580.3	408	1029	1800.9	1437	3330.9 ^a	3.2	3.5	3.1
B1	565.4	393.8	995.6	1679.9	1384.7	3044.9 ^b	3.0	3.5	3.0
SEM	17.5	5.2	19.5	101.2	41.38	94.2	0.13	0.09	0.12
p-value	0.556	0.071	0.244	0.409	0.385	0.047	0.307	0.981	0.587
Organic acids × <i>Bacillus amyoliquefaciens</i> (p-value)	0.353	0.851	0.466	0.978	0.761	0.657	0.07	0.644	0.259

A0 and A1: Levels of organic acids at 0 and 0.08%, respectively, B0 and B1: Levels of *Bacillus amyoliquefaciens* as probiotics at 0 and 0.01%, respectively, with a concentration of 1×10^9 CFU/g. SEM: Standard error of the mean, starter: From day 0 to 21, finisher: From day 22 to 42, entire period: From day 0 to 42. ^{a,b} Mean different superscript letters in the same column were significantly different at $p \leq 0.05$.

Slaughter performance

The percentages of internal organs in relation to body weight, as well as the lengths of the intestine and ceca of the cockerels affected by organic acids and *B. amyoliquefaciens*, were presented in Table 3. The present results indicated that organic acids significantly decreased the relative weight of the liver and spleen ($p < 0.05$), compared with Group A0. Conversely, the proventriculus weight was significantly increased in cockerels receiving organic acids ($p < 0.05$). No statistically significant differences were observed in the weights of the gizzard and pancreas, nor the lengths of the intestines and ceca between treated and non-treated groups ($p > 0.05$). Similarly, adding *B. amyoliquefaciens* to the cockerels' diet did not significantly affect the weight of the pancreas or the lengths of the intestine and ceca compared to Group B0 ($p > 0.05$). Furthermore, the liver weight was not influenced by the inclusion of *B. amyoliquefaciens* in the diet. In contrast, cockerels receiving diets with *B. amyoliquefaciens* revealed a significant increase in spleen relative weight ($p < 0.05$), while the relative weights of the gizzard and proventriculus were significantly decreased compared to cockerels receiving diets without *B. amyoliquefaciens* ($p < 0.05$). No significant effects of *B. amyoliquefaciens* supplementation were observed on pancreas weight or the lengths of the intestine and ceca (p

> 0.05). Liver weight remained unaffected. A significant interaction between organic acids and *B. amyoliquefaciens* was detected for spleen weight ($p < 0.05$). Group A0B1 significantly increased spleen weight (0.396%) compared to Group A0B0 (0.184%; $p < 0.05$). However, Group A1B1 (0.192%) demonstrated no statistically significant difference compared to Group A1B0 (0.176%; $p > 0.05$). No significant effects were observed in any carcass parameter, including carcass weight, dressing percentage, breast, thigh, and drumstick, for either organic acids (A1 and A0) or *B. amyoliquefaciens* groups (B1 and B0; $p > 0.05$; Table 4).

Serum biochemical parameters and liver enzymes

The effects of organic acids and *B. amyoliquefaciens* on serum biochemical parameters, liver enzymes, and calcium and phosphorus of the cockerels are shown in Tables 5 and 6. Most serum biochemical parameters, including glucose, cholesterol, triglycerides, HDL, LDL, VLDL, albumin, calcium, and phosphorus, were not significantly affected by any treatment ($p > 0.05$). However, Group A1 exhibited significantly lower total protein and globulin levels (3.47 g/dL and 2.23 mg/dL, respectively) in comparison to those observed in Group A0 (3.88 g/dL and 2.61 mg/dL, respectively; $p < 0.05$). There were no significant differences between Group B1

and B0 in terms of reduced total protein and globulin levels ($p > 0.05$). Supplementation with organic acids and *B. amyloliquefaciens* had no significant effect on serum AST or ALT activities ($p > 0.05$).

Hematological parameters

The results of the hematological parameters influenced by organic acids and/or *B. amyloliquefaciens* are summarized in Table 7. Supplementation with organic acids and/or *B. amyloliquefaciens* had no significant effect on any hematological parameter, including RBC, PCV, Hb, WBC, and MCHC ($p > 0.05$). Similarly, supplementation with organic acids or *B. amyloliquefaciens* did not significantly affect the differential leukocyte profile, including heterophils, lymphocytes, monocytes, or the heterophil-to-lymphocyte ratio ($p > 0.05$). Additionally, no significant interactions were observed for any hematological parameters ($p > 0.05$).

Cecal microbiota

The present results indicated that both organic acids and *B. amyloliquefaciens* significantly affected cecal microbiota populations (Table 8). Group A1 had lower coliform, *E. coli*, and total aerobic bacterial counts than Group A0. Conversely, Group B1 exhibited an increase in *Bacillus* spp. while concurrently decreasing the populations of coliform and *E. coli* compared to Group B0. Significant interactions between the two factors were detected for all microbial variables ($p < 0.001$). Group A1B1 had the highest *Bacillus* spp. count ($5.11 \log_{10}$ CFU/g), whereas the lowest coliform ($5.91 \log_{10}$ CFU/g) and *E. coli* ($4.71 \log_{10}$ CFU/g) counts were observed in Group A1B0. In contrast, *B. amyloliquefaciens* increased total aerobic bacterial counts, potentially mitigating the suppressive effect of organic acids and resulting in higher counts in the combined treatment group than in the cockerels receiving organic acids alone.

Table 3. Effects of organic acids and *Bacillus amyloliquefaciens* on the relative weights of internal organs and the lengths of the intestine and ceca of the cockerels at 42 days of age

Treatments	Liver (%)	Spleen (%)	Proventriculus (%)	Gizzard (%)	Pancreas (%)	Intestinal length (cm)	Cecal length (cm)
Control (A0B0)	1.654	0.184	0.412	1.528	0.188	119.0	18.6
<i>Bacillus amyloliquefaciens</i> (A0B1)	1.482	0.396	0.164	1.472	0.194	116.4	14.6
Organic acids (A1B0)	1.404	0.176	0.448	1.708	0.19	119.6	14.0
Combination (A1B1)	1.352	0.192	0.36	1.254	0.152	114.7	14.9
SEM	0.081	0.026	0.05	0.111	0.015	6.358	1.529
Organic acids effects							
A0	1.568 ^a	0.290 ^a	0.288 ^b	1.5	0.191	117.7	16.6
A1	1.378 ^b	0.184 ^b	0.404 ^a	1.48	0.171	117.2	14.5
SEM	0.057	0.019	0.035	0.078	0.011	4.5	1.08
p-value	0.031	0.001	0.034	0.866	0.211	0.932	0.179
<i>Bacillus amyloliquefaciens</i> effects							
B0	1.529	0.180 ^b	0.430 ^a	1.62 ^a	0.189	119.3	16.3
B1	1.417	0.294 ^a	0.262 ^b	1.36 ^b	0.173	115.6	14.8
SEM	0.057	0.019	0.035	0.078	0.011	4.5	1.08
p-value	0.183	0.001	0.004	0.035	0.313	0.564	0.326
Organic acids × <i>Bacillus amyloliquefaciens</i> (p-value)	0.467	0.002	0.129	0.091	0.171	0.859	0.129

A0 and A1: Levels of organic acids at 0 and 0.08%, respectively, B0 and B1: Levels of *Bacillus amyloliquefaciens* as probiotics at 0 and 0.01%, respectively, with a concentration of 1×10^9 CFU/g. SEM: Standard error of the mean. ^{a,b} Mean different superscript letters in the same column were significantly different at $p \leq 0.05$.

Table 4. Effects of organic acids and *Bacillus amyloliquefaciens* on carcass weight, dressing percentage, and cut-up part yields of the cockerels at 42 days of age

Treatments	Carcass weight (g)	Dressing (%)	Breast (%)	Thigh (%)	Drumstick (%)
Control (A0B0)	700.6	68.13	13.78	5.59	5.26
<i>Bacillus amyloliquefaciens</i> (A0B1)	673.6	65.74	11.79	5.84	4.90
Organic acids (A1B0)	657.4	64.46	12.3	5.83	5.38
Combination (A1B1)	697.2	64.76	11.594	5.25	4.80
SEM	38.742	2.617	0.878	0.369	0.292
Organic acids effects					
A0	687.1	66.93	12.78	5.71	5.08
A1	677.3	64.61	11.95	5.54	5.09
SEM	27.4	1.85	0.621	0.261	0.206
p-value	0.804	0.389	0.355	0.646	0.973
<i>Bacillus amyloliquefaciens</i> effects					
B0	679	66.3	13.04	5.71	5.32
B1	685.4	65.25	11.69	5.54	4.85
SEM	27.4	1.85	0.621	0.261	0.206
p-value	0.871	0.694	0.145	0.653	0.128
Organic acids × <i>Bacillus amyloliquefaciens</i> (p-value)	0.401	0.614	0.477	0.273	0.701

A0 and A1: Levels of organic acids at 0 and 0.08%, respectively, B0 and B1: Levels of *Bacillus amyloliquefaciens* as probiotics at 0 and 0.01%, respectively, with a concentration of 1×10^9 CFU/g. SEM: Standard error of the mean.

Table 5. Effects of organic acids and *Bacillus amyloliquefaciens* on cockerels' serum biochemical parameters at 42 days of age

Treatments	Glucose (mg/dL)	Cholesterol (mg/dL)	TG (mg/dL)	HDL (mg/dL)	LDL (mg/dL)	VLDL (mg/dL)	Total protein (g/dL)	Albumin (g/dL)	Globulin (g/dL)
Control (A0B0)	244.0	99.0	60.8	34.46	52.4	12.15	3.93	1.26	2.67
<i>Bacillus amyloliquefaciens</i> (A0B1)	246.4	106.4	63.4	34.06	59.5	12.76	3.84	1.31	2.55
Organic acids (A1B0)	262.4	100.4	45.4	37.14	54.2	9.08	3.48	1.28	2.202
Combination (A1B1)	219.6	103.8	62.8	35.98	55.2	12.55	3.45	1.19	2.26
SEM	17.503	9.281	5.246	3.127	6.978	1.049	0.165	0.076	0.164
Organic acids effects									
A0	245	103	62.0	34.2	56.0	12.5	3.88 ^a	1.29	2.61 ^a
A1	241	102	54.0	36.6	54.7	10.8	3.47 ^b	1.23	2.23 ^b
SEM	12.4	6.6	3.7	2.2	4.9	0.74	0.12	0.05	0.12
p-value	0.81	0.949	0.147	0.473	0.86	0.138	0.022	0.501	0.035
<i>Bacillus amyloliquefaciens</i> effects									
B0	253	100	53.0	35.8	53.3	10.6	3.7	1.27	2.44
B1	233	105	63.0	35.0	57.4	12.7	3.65	1.25	2.41
SEM	12.4	6.6	3.7	2.2	4.9	0.74	0.022	0.05	0.12
p-value	0.265	0.569	0.075	0.806	0.568	0.07	0.73	0.835	0.857
Organic acids × <i>Bacillus amyloliquefaciens</i> (p- value)	0.215	0.832	0.178	0.905	0.67	0.192	0.867	0.335	0.599

A0 and A1: Levels of organic acids at 0 and 0.08%, respectively, B0 and B1: Levels of *Bacillus amyloliquefaciens* as probiotics at 0 and 0.01%, respectively, with a concentration of 1×10^9 CFU/g. TG: Triglycerides, HDL: High-density lipoprotein, LDL: Low-density lipoprotein, VLDL: Very low-density lipoprotein, SEM: Standard error of the mean. ^{a,b} Mean different superscript letters in the same column were significantly different at $p \leq 0.05$.

Table 6. Effects of organic acids and *Bacillus amyloliquefaciens* on cockerels' liver enzymes and serum calcium and phosphorus at 42 days of age

Treatments	ALT (U/L)	AST (U/L)	Calcium (mg/dL)	Phosphorus (mg/dL)
Control (A0B0)	4.43	248.2	7.48	6.22
<i>Bacillus amyloliquefaciens</i> (A0B1)	4.77	249	8.72	5.5
Organic acids (A1B0)	5.58	271.4	7.66	5.02
Combination (A1B1)	5.25	284.8	7.56	5.1
SEM	0.46	23.65	0.446	0.432
Organic acids effects				
A0	4.6	248.6	8.1	5.86
A1	5.42	278.1	7.61	5.06
SEM	0.323	16.72	0.315	0.305
p-value	0.092	0.23	0.288	0.082
<i>Bacillus amyloliquefaciens</i> effects				
B0	5.0	259.8	7.57	5.62
B1	5.01	266.9	8.14	5.3
SEM	0.323	16.72	0.315	0.305
p-value	0.991	0.768	0.315	0.469
Organic acids × <i>Bacillus amyloliquefaciens</i> (p-value)	0.474	0.793	0.152	0.368

A0 and A1: Levels of organic acids at 0 and 0.08%, respectively, B0 and B1: Levels of *Bacillus amyloliquefaciens* as probiotics at 0 and 0.01%, respectively, with a concentration of 1×10^9 CFU/g. ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, SEM: Standard error of the mean. All measurements were performed on serum samples collected at 42 days of age. No significant differences were observed for any parameter ($p > 0.05$).

Table 7. Effects of organic acids and *Bacillus amyloliquefaciens* on cockerels' hematological profile at 42 days of age

Treatments	RBC ($10^6/\mu\text{L}$)	PCV (%)	Hb (g/dL)	WBC ($10^3/\mu\text{L}$)	Heterophils ($10^3/\mu\text{L}$)	Lymphocytes (%)	Monocytes (%)	H/L ratio	MCHC (g/dL)
Control (A0B0)	3.01	30.72	21.23	24.60	7.87	53.46	8.22	0.56	69.40
<i>Bacillus amyloliquefaciens</i> (A0B1)	3.06	31.16	21.65	31.08	9.63	54.86	9.30	0.71	69.62
Organic acids (A1B0)	2.89	30.38	20.80	26.28	7.23	54.80	8.86	0.53	68.42
Combination (A1B1)	2.84	28.72	19.97	21.60	7.67	52.60	8.12	0.57	69.50
SEM	0.120	1.335	0.808	4.14	0.719	0.856	0.609	0.052	1.397
Organic acids effects									
A0	3.03	30.94	21.44	27.84	8.75	54.16	8.76	0.63	69.51
A1	2.87	29.55	20.38	23.94	7.45	53.70	8.49	0.55	68.96
SEM	0.085	0.994	0.572	2.93	0.508	0.605	0.431	0.55	0.988
p-value	0.188	0.313	0.210	0.360	0.089	0.598	0.664	0.137	0.699
<i>Bacillus amyloliquefaciens</i> effects									
B0	2.95	30.55	21.01	25.44	7.55	54.13	8.54	0.55	68.91
B1	2.95	29.94	20.81	26.34	8.65	53.73	8.71	0.54	69.56
SEM	0.085	0.944	0.572	2.93	0.508	0.605	0.431	0.037	0.988
p-value	0.993	0.654	0.803	0.831	0.145	0.647	0.784	0.104	0.648
Organic acids × <i>Bacillus amyloliquefaciens</i> (p-value)	0.677	0.443	0.448	0.196	0.371	0.062	0.155	0.295	0.762

A0 and A1: Levels of organic acids at 0 and 0.08%, respectively, B0 and B1: Levels of *Bacillus amyloliquefaciens* as probiotics at 0 and 0.01%, respectively, with a concentration of 1×10^9 CFU/g. RBC: Red blood cells, PCV: Packed cell volume, Hb: Hemoglobin, WBC: White blood cells, H/L: Heterophils/Lymphocyte's ratio, MCHC: mean corpuscular hemoglobin concentration, SEM: standard error of the mean.

Table 8. Effects of organic acids and *Bacillus amyloliquefaciens* on cockerels' cecal microbial count (log₁₀ CFU/g) at 42 days of age

Treatments	<i>Bacillus</i> count	Coliforms	Aerobic bacteria	<i>Escherichia coli</i>
Control (A0B0)	3.65	10.14	13.045	7.49
<i>Bacillus amyloliquefaciens</i> (A0B1)	4.826	6.85	11.675	5.11
Organic acids (A1B0)	3.554	5.905	9.86	4.705
Combination (A1B1)	5.11	7.912	12.765	5.90
SEM	0.041	0.126	0.132	0.11
Organic acids effects				
A0	4.238 ^b	8.495 ^a	12.360 ^a	6.300 ^a
A1	4.332 ^a	6.908 ^b	11.313 ^b	5.303 ^b
SEM	0.029	0.089	0.093	0.078
p-value	0.035	0.0001	0.0001	0.0001
<i>Bacillus amyloliquefaciens</i> effects				
B0	3.602 ^b	8.022 ^a	11.452 ^b	6.098 ^a
B1	4.968 ^a	7.381 ^b	12.220 ^a	5.505 ^b
SEM	0.029	0.089	0.093	0.078
p-value	0.0002	0.0001	0.0001	0.0002
Organic acids × <i>Bacillus amyloliquefaciens</i> (p-value)	0.0001	0.0002	0.0001	0.0002

A0 and A1: Levels of organic acids at 0 and 0.08%, respectively, B0 and B1: Levels of *Bacillus amyloliquefaciens* as probiotics at 0 and 0.01%, respectively, with a concentration of 1×10^9 CFU/g. SEM: Standard error of the mean. ^{a,b} Mean different superscript letters in the same column are significantly different at $p \leq 0.05$.

Growth performance

The current study revealed that supplementing drinking water with organic acids notably improved body weight gain in cockerels during the starter period (0-21 days) by 9.8% compared to the control group. Improved body weight gain observed during the starter phase in the present study was consistent with findings of Adil et al. (2011) and Dibner and Buttin (2002), who reported that organic acids enhanced early growth by improving nutrient digestibility, reducing intestinal pH, and suppressing pathogenic bacteria. Although early growth improved, supplementing with organic acids considerably increased the FCR throughout the experimental period. The impaired FCR observed during the experimental period may be attributed to microbial adaptation to organic acids over time, changes in gut microbiota dynamics, or a decline in the effectiveness of organic acids as the cockerels' age (Ricke, 2003). The present findings indicated that careful optimization of the timing and duration of organic acid administration was required in cockerel production. Throughout the growth periods, body weight gain and FCR in cockerels were not affected by the dietary inclusion of *B. amyloliquefaciens*. Despite the substantial reduction in cumulative feed intake, no improvement in FCR was observed during the present

study. *Bacillus* strains, including *B. amyloliquefaciens* and *B. subtilis*, have been demonstrated to modulate the gut microbiota, suppress pathogenic bacteria, and produce beneficial enzymes and antimicrobial substances (Jeong and Kim, 2014; Wang et al., 2021). No notable interactions have been recorded between organic acids and *B. amyloliquefaciens* across all investigated growth parameters. The absence of interaction indicated that organic acids and *B. amyloliquefaciens* may exert their effects through distinct physiological pathways that did not overlap sufficiently to generate interactive responses under the conditions examined, which is consistent with previous reports in broilers showing variable responses to their combined use depending on the parameter assessed (Rodjan et al., 2018; Ebeid et al., 2021). The present findings reinforce that combining feed additives does not inherently yield interactive benefits and emphasize the need for parameter-specific evaluation.

Slaughter performance

In the present study, organic acid supplementation was associated with reduced relative weights of the spleen and liver. Reduced spleen and liver weights occurred in the absence of physiological stress indicators, suggesting that the observed changes were not related to pathological

conditions. Nonetheless, caution was necessary when interpreting reductions in lymphoid organ size, since these changes alone did not definitively indicate altered immune function. The antimicrobial properties of organic acids, such as formic, propionic, lactic, citric, and sorbic acids, likely helped modulate the intestinal microbiota and decrease microbial antigen exposure, potentially affecting the development of immune organs (Dibner and Buttin, 2002; Klasing, 2007; Adil et al., 2010).

In contrast, dietary inclusion of *B. amyloliquefaciens* was associated with an increase in spleen relative weight. However, spleen enlargement was interpreted with caution, since organ size alone did not definitively indicate immune activation, especially in the absence of additional immunological data. The lack of changes in the heterophil-to-lymphocyte ratio further supported the absence of physiological stress. Therefore, the biological importance of this finding remained unclear. Zhyla et al. (2022) observed a similar increase in lymphoid organ weights after probiotic supplementation. It is interesting to note that acidification of the upper gastrointestinal tract can influence the gut environment and microbial populations (Van Immerseel et al., 2006), potentially indirectly affecting the development of digestive organs, including the proventriculus. Conversely, the absence of notable alterations in gizzard and pancreas weights, in addition to intestinal and cecal lengths, indicated that organic acids had a limited impact on the structural development of the lower gastrointestinal tract. Gunal et al. (2006) likewise reported limited morphological changes despite functional responses to organic-acid supplementation. The gut-modulatory and antimicrobial properties of organic acids support their potential use as alternatives to antibiotic growth promoters in poultry (Van Immerseel et al., 2003; Ebeid and Al-Homidan, 2022).

The absence of notable changes in pancreatic weight and intestinal and cecal length values following *B. amyloliquefaciens* supplementation suggested that this probiotic strain did not directly alter the structural development of cockerels' digestive and metabolic organs. The current results demonstrated that organic acids or *B. amyloliquefaciens* did not remarkably affect the carcass weight, dressing percentage, or yields of cut-up parts such as breast, thighs, wings, and drumsticks in cockerels. The absence of carcass responses indicates that these additives act primarily through physiological and microbial pathways rather than by altering tissue deposition. The current findings were consistent with those of Cengiz et al. (2015), Rehman et al. (2020), and Ebeid et al. (2021), who reported that such additives may enhance gut function

without necessarily resulting in measurable changes in carcass composition under standard production conditions.

Blood biochemical parameters and liver enzymes

The current findings indicated that supplementation with organic acids and/or *B. amyloliquefaciens* did not remarkably affect the main blood biochemical parameters or liver enzyme activities in cockerels. The stability of glucose and lipid profiles (cholesterol, TG, HDL, LDL, VLDL) was consistent with the findings of Adil et al. (2010), who reported that organic acids did not affect blood lipid metabolites or glucose levels. The lack of notable changes in liver enzymes (AST and ALT) suggested that neither organic acids nor *B. amyloliquefaciens* affected liver function. The present findings were consistent with those of Qiu et al. (2023), who reported that the organic acidifier did not affect AST and ALT activities. However, the reduction in total protein and globulin levels following organic acid supplementation may reflect an immunomodulatory effect, potentially attributed to organic acids' role in modulating the gut microbiota and reducing antigenic stimulation (Adil et al. 2010).

Hematological parameters

Hematological parameters did not change with the addition of organic acids, *B. amyloliquefaciens*, or their combination, indicating that neither additive disrupted hematopoiesis nor systemic physiological homeostasis under the conditions of the present investigation. The absence of changes in differential leukocyte profiles and the heterophil-to-lymphocyte ratio indicated that the treatments did not elicit measurable stress or inflammatory responses. The unchanged hematological indices after probiotic supplementation were consistent with the findings of Nguyen et al. (2018), who similarly reported no changes. The current findings indicated that the organic acids and *B. amyloliquefaciens* additives primarily act at the gastrointestinal level without causing systemic hematological changes, supporting their safe use in poultry diets under similar production conditions.

Cecal microbiota

The current results confirmed that supplementing organic acids, *B. amyloliquefaciens*, or their combination altered the gut microbiota composition, encouraging the growth of beneficial bacteria and reducing pathogenic microorganisms. *Bacillus amyloliquefaciens* contains a variety of bioactive substances, including antimicrobial peptides that inhibit pathogenic bacteria, promote the

proliferation of beneficial gut microorganisms, and help maintain intestinal homeostasis (Sutyak et al., 2008; Luan et al., 2019). Probiotics have been shown to increase *Lactobacillus* and *Bifidobacterium* populations and decrease *Clostridium* and coliform populations in broiler chickens, including Ross 308 (Giannenas et al., 2012), Ross (Al-Fataftah and Abdelqader, 2014), and Arbor Acres (Song et al., 2014) strains. The observed increase in *Bacillus* counts following organic acid supplementation suggested a favorable modulatory effect of acidification on the establishment of spore-forming probiotic strains. The increase in *Bacillus* counts following organic acid supplementation was likely mediated through pH reduction and competitive inhibition of acid-sensitive microbial populations (Rodjan et al., 2018). In contrast, the stronger effect observed with *B. amyloliquefaciens* alone likely resulted from its natural ability to produce antimicrobial compounds and competitively outcompete pathogenic bacteria (Tang et al., 2017).

Organic acids and *B. amyloliquefaciens* demonstrated strong antimicrobial effects, notably reducing *E. coli* and coliform populations. The antimicrobial effects of both additives likely resulted from distinct yet complementary mechanisms, such as pH-dependent microbial inhibition by organic acids and the production of bacteriocins (subtilin, subtilisin) by *Bacillus* spp. (Lisboa et al., 2006; Liu et al., 2021; Rathnayake et al., 2021). However, the combined treatment was less effective than organic acids alone at inhibiting *E. coli*, indicating a parameter-dependent interaction that needs mechanistic investigation, which may reflect differences in the responses of intestinal microbial populations to organic acids and probiotics when administered individually or in combination, as similarly reported in poultry by Rodjan et al. (2018). Rapid acidification may impose transient acid stress on *B. amyloliquefaciens*, potentially affecting its growth and metabolic activity during adaptation to a low-pH environment (Chowdhury et al., 2021). Furthermore, the establishment of *B. amyloliquefaciens* may require an extended adaptation period, especially under low-pH and bile-rich intestinal conditions (Rathnayake et al., 2021). The differing impacts of the two additives on total aerobic bacteria reflected their distinct mechanisms of action. Organic acids notably decreased aerobic counts by broadly inhibiting microbes, a reduction linked to lower pH (Papatsiros et al., 2014). Whereas *B. amyloliquefaciens* increased total aerobic bacterial counts, likely through competitive exclusion and metabolic cross-feeding. Similar increases in aerobic and lactic acid bacteria

following probiotic supplementation have been reported previously by Shargh et al. (2012).

The total aerobic bacterial counts in the combined treatment were more similar to those in the control than to those in the organic acids' treatment alone. The observed similarity in total aerobic counts between the combined treatment and the control may be attributed to the direct contribution of *B. amyloliquefaciens*, an aerobic spore-forming bacterium, to the total aerobic population. Additionally, *B. amyloliquefaciens* can secrete extracellular enzymes, such as proteases, amylases, and cellulases, which facilitate the release of fermentable substrates and promote the proliferation of other aerobic commensals (Lee et al., 2008). The acidifying effect of organic acids may be less pronounced in the cecum compared to the upper gastrointestinal tract, thereby facilitating the survival of acid-tolerant aerobes (Gunal et al., 2006; Van Immerseel et al., 2006). Based on the present findings, the interaction between organic acids and *B. amyloliquefaciens* was not consistently synergistic. In the current study, synergistic effects mainly appeared in the growth of beneficial *Bacillus* strains and overall aerobic bacteria. However, the reduced ability to suppress pathogens indicated some functional interference when applied together. Taken together, these findings indicate a parameter-dependent interaction between organic acids and *B. amyloliquefaciens*, with the combined treatment increasing *Bacillus* counts but providing less suppression of *E. coli* and coliforms than organic acids alone.

CONCLUSION

The present study revealed that organic acids and *B. amyloliquefaciens* had distinct effects on growth, physiological traits, and cecal microbiota in native cockerels. Organic acids improved body weight gain during the starter phase and significantly reduced *E. coli*, coliforms, and total aerobic bacteria. In contrast, *B. amyloliquefaciens* reduced feed intake without adversely affecting growth and increased *Bacillus* counts in the cecum. The combined supplementation did not consistently enhance performance or microbial responses compared to individual treatments, indicating that the interaction between the two additives was parameter-dependent. No significant effects were observed on carcass characteristics, hematological indices, or liver enzyme activities, suggesting that the treatments did not adversely affect physiological status. The present study acknowledged limitations. One notable constraint was that the cecal microbiota was evaluated using culture-

dependent methods, which may not comprehensively capture the microbial diversity. In addition, molecular indicators of immune function were not evaluated. Therefore, future studies should focus on dose-response relationships using advanced microbiome techniques (16S rRNA sequencing) to further clarify the effects of organic acids and probiotic supplementation on poultry nutrition.

DECLARATIONS

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Authors' contributions

All authors contributed equally to the investigation, writing, reviewing, editing, and approval of the final edition of the manuscript. Mutaz Babiker contributed to conceptualization, methodology, data curation, formal analysis, supervision, and drafting the original edition. Saleh Alhomaidan carried out the methodology. Abdulmajeed Alhumud and Fahd Alsaleem contributed to software analysis. Saleh Almithn validated the study. Abdulrahman Alhumimidi, Abdullah Alobaydaa, and Sultan Al-Eid contributed to the validation process, with Sultan supervising resources and overseeing the project. Salman Aloudah was responsible for project management and securing funding. All authors read and approved the final edition of the manuscript.

Availability of the data and materials

The data presented in this study are available on request from the corresponding author. The data are not publicly available due to institutional and ethical restrictions.

Competing interests

The authors declared that they have no competing interests.

Ethical considerations

The study protocol was reviewed and approved by the Internal Scientific Committee of the National Organic Agriculture Center, Ministry of Environment, Water and Agriculture, Saudi Arabia (Approval No. NOAC/2023/014, dated 15 March 2023). All procedures involving birds were conducted in accordance with applicable local regulations (Royal Decree No. M/59, dated 14/09/1431 H) and internationally accepted guidelines for the care and use of animals in research. Animal welfare considerations were observed throughout the experimental period. During the preparation and revision of this manuscript, the authors used ChatGPT (OpenAI) solely as an AI-assisted tool for language refinement and grammatical editing. The tool was not used to generate, analyze, or interpret experimental data, perform statistical analyses, or independently formulate scientific conclusions. The authors confirmed that the original experimental results and data reported in this study were generated independently of AI and were verified by the authors before submission and again before acceptance and publication. The authors take full responsibility for the content, accuracy, originality, and integrity of the manuscript.

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Biotechnological Feed Additives: Alternatives to Antibiotics as Growth Promoters in Broiler Chicken Farming

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ABSTRACT

The restriction of antibiotic growth promoters in poultry production has grown due to concerns about antimicrobial resistance and food safety. Phytogenic and probiotic feed additives are considered promising candidates for modulating gut health, immune responses, and growth performance in broiler chickens. The present study aimed to assess the effects of dietary supplementation with the probiotic *Kluyveromyces marxianus* strain (KluActive), either alone or in combination with encapsulated hydrolyzable tannins, on innate immune responses and growth performance in antibiotic-free-fed broiler chickens. Sixty broiler chickens, each 15 days old at the start, were used in a 27-day experiment. Broiler chickens were randomly assigned to four dietary treatments, including a control group fed a standard antibiotic-free diet (G1), a group that was fed the standard diet with KluActive at 0.04% (G2), and two groups fed a combination of KluActive with encapsulated tannins at 0.04% (G3) and 0.06% (G4). Phagocytic activity, phagocytic index, phagocytic number, serum bactericidal activity, and serum lysozyme activity were assessed on days 28 and 42. Growth performance parameters, including average daily gain and average body weight gain, were recorded over 42 days. Supplementation with the probiotic and tannins enhanced selected serum innate immune parameters in broiler chickens. In particular, serum bactericidal activity was significantly increased at the higher inclusion level (0.6%), by 12.5% on day 28 and 16.7% on day 42 compared with the control group. Phagocytic activity parameters were consistently elevated in broiler chickens across all experimental groups. The best improvement in feed conversion ratio was observed in the G3 group. The current findings indicated that probiotic-tannin combinations were a promising nutritional alternative to enhance immune function and performance in antibiotic-free broiler chicken production.

Keywords: Growth performance, Immune response, Probiotic, Tannin, Yeast

INTRODUCTION

The safe use of veterinary medicines, especially antibiotics in animal husbandry, is crucial for protecting the health of both animals and humans. The worldwide challenge of antibiotic resistance (AMR), driven by the overuse of antibiotics, has led many countries to implement strict legal limits on their use in animal husbandry, poultry farming, and livestock products (Tang et al., 2017).

A significant step toward aligning national regulations with European Union standards has recently been made in

Ukraine. The Verkhovna Rada of Ukraine approved draft law No. 12285-d, intended to align Ukrainian veterinary and animal welfare regulations with EU standards (Verkhovna Rada of Ukraine, 2025). Recent regulatory reforms in Ukraine have imposed strict limits on antibiotic use in animal production, establishing a regulatory framework consistent with European Union standards (Matiukha et al., 2024). The restrictions on antibiotic growth promoters, limits on prophylactic use without clinical necessity, and bans on critically important antimicrobials for human medicine all reflected a

comprehensive effort to address AMR. Beyond regulatory requirements, these restrictions transform production standards towards greater sustainability, enhanced biosecurity, and more responsible use of antimicrobials (Casewell et al., 2003; Abreu et al., 2023). In this changing regulatory environment, relying on antibiotics as performance boosters is unsustainable, prompting the need to develop alternative approaches to maintain productivity and protect animal health. Therefore, there is a growing focus on biotechnological feeds, especially functional additives that can influence gut microbiota, improve metabolic efficiency, and strengthen immune responses (Khan et al., 2025).

Yeast-derived feed additives, including probiotics and postbiotics, have become promising options among new supplements due to their diverse mechanisms of action, such as boosting immunity, acting as antioxidants, and supporting gut barrier health (Zamojska et al., 2021). Their application provided a well-established approach to maintaining animal performance even when antibiotics are limited. Yeast-derived feed additives play an important role in developing next-generation feeding strategies for livestock production systems. Implementing these new methods not only meets legal requirements but also supports sustainable growth in agriculture and industry, with a focus on product safety and consumer trust.

Probiotics are live microorganisms that provide health benefits to the host when given in sufficient quantities (Hill et al., 2014). Probiotics are associated with several health benefits, including improved digestion, enhanced protection against infections, and support for the immune system (Jha et al., 2020). Different effective bacterial probiotics, including *Lactobacillus*, *Bifidobacterium*, *Bacillus subtilis*, and *Enterococcus* spp., are commercially available for poultry production and are widely used to improve gut health, growth performance, and immune function (Yaqoob et al., 2022). Probiotic yeasts are increasingly used in different animal production systems (Pendon et al., 2024). In addition to bacterial probiotics, some nonpathogenic yeasts, such as *Saccharomyces cerevisiae* (*S. cerevisiae*), *Saccharomyces boulardii* (*S. boulardii*), and *Kluyveromyces marxianus* (*K. marxianus*), are used as probiotics. Yeast probiotics typically offer greater stability during feed processing and are more resistant to environmental and gastrointestinal stresses compared to bacterial probiotics. Among yeast probiotics, *K. marxianus* is considered a promising probiotic for applications in the food and feed industries (Jin et al., 2021). Furthermore, *K. marxianus* yeast has been designated as Generally Recognized as Safe (GRAS) by

the Food and Drug Administration (FDA) and as Qualified Presumption of Safety (QPS) by the European Food Safety Authority (EFSA; Diaz-Vergara et al., 2017).

In addition to probiotics, safe alternatives to antibiotics include tannins, polyphenolic compounds that precipitate proteins. These are secondary metabolites found in plants, seeds, bark, wood, leaves, and fruit skins, serving as defense mechanisms against predators. In addition to probiotics, other promising and safe alternatives to antibiotics include phytobiotics, among which tannin-rich plant extracts are of particular interest. A relevant example is chestnut (*Castanea sativa*) extract, known for its high content of hydrolyzable tannins, polyphenolic compounds that can precipitate proteins and are classified as secondary plant metabolites. Tannins are widely distributed in plants, including seeds, bark, wood, leaves, and fruit skins, where they act as natural defenses against herbivores and pathogens (Mueller-Harvey, 2006; Redondo et al., 2014; Fraga-Corral et al., 2021). High levels of tannins are known to have antinutritional effects in monogastric animals, as they can decrease feed intake, nutrient digestibility, and growth performance in chickens (Garcia et al., 2004). In poultry production, tannins have recently attracted significant interest as a substitute for antibiotic growth promoters, owing to their antimicrobial, antioxidant, and anti-inflammatory effects (Daglia, 2012; Brus et al., 2018). However, the effects of tannins on the growth performance and gut ecosystem of chickens remain inconsistent, and their mode of action is unclear. Therefore, a comprehensive understanding of the chemical properties and biological effects of tannins is crucial to optimizing their application as feed supplements in poultry. Additionally, it is important to develop evidence-based guidelines for their safe inclusion levels, both as individual compounds and within feed additive complexes. A combination of biologically active ingredients, including probiotics and phytobiotics such as tannins, can function synergistically in antibiotic-free diets, enhancing each other's beneficial effects and ultimately improving productivity while maintaining high-quality performance (Gadde et al., 2017).

Supplementation of tannins in combination with other bioactive compounds may be more beneficial for poultry than the use of tannins alone. Tannins can form complexes with proteins or polysaccharides, potentially decreasing their ability to bind with endogenous and dietary proteins, as well as metal ions. In addition, different bioactive compounds may exhibit synergistic antimicrobial effects against both Gram-negative and Gram-positive bacteria. Bioactive compounds can influence the gut through

different mechanisms, potentially improving overall gut health and function in a complementary manner (Redondo et al., 2014; Tonda et al., 2018). Finally, using multiple bioactive compounds could lower the risk of antimicrobial resistance because pathogenic bacteria are less able to adapt to diverse, combined modes of action. Prior studies had already established that probiotics in antibiotic-free diets positively affect productivity and immune parameters (Matiukha et al., 2024). The present study aimed to assess whether combining tannins with the yeast-based probiotic (*K. marxianus*) is beneficial in poultry nutrition by evaluating its effects on the immune system and productivity of broiler chickens fed antibiotic-free diets.

MATERIALS AND METHODS

Ethical approval

The present study was conducted in strict accordance with established ethical guidelines for animal research and welfare. The experimental protocol was approved by the Institute of Animal Biology NAAS (approval number 04/01/2025) and adhered to the guidelines established by the World Organisation for Animal Health (OIE) to ensure the welfare of animals used for scientific purposes.

Experimental design and animals

The study involved 60 clinically healthy Ross 308 broiler chickens, from day 15 to day 42 of age, under controlled conditions. Chickens were randomly assigned to four groups (15 chickens each) following the principle of analogs. From day 1 to day 14 of age, broiler chickens were reared under standard commercial conditions on a private poultry farm. On day 15, chickens were transferred to an experimental facility for the trial. During days 15-17, chickens remained on the same diets and feeding schedule as at the initial farm to reduce stress-related effects during the transfer and adaptation period. Starting from day 17 of age, following the adaptation period, the chickens were allocated to different experimental feeding treatments. Broiler chickens were housed within a commercial-type experimental facility under controlled environmental conditions. Chickens were housed in battery cages at a stocking density of five chickens per cage, providing approximately 0.05 m² per chicken. Feed and water were provided *ad libitum* throughout the experimental period. Feed intake was recorded to calculate feed conversion parameters. Environmental conditions, including temperature, ventilation, and lighting program, were maintained in accordance with Ross 308 management guidelines. No antibiotics were used during the

experimental trial. All chickens were clinically healthy and vaccinated in accordance with the standard farm vaccination protocol prior to the experiment.

Location and veterinary supervision

The experiment was conducted at a commercial poultry farm in the Lviv region, Ukraine. All procedures, such as weekly health checks, body weight measurements, and chicken handling, were performed under the supervision of a licensed veterinary specialist to ensure animal welfare and data reliability.

Diets and experimental treatments

The first group, serving as the control (G1), received a standard, nutritionally balanced diet specifically formulated for broiler chickens at each phase; prestarter, starter, grower, and finisher. The experimental groups received the same basal diet supplemented according to the experimental design (Table 1). The nutrient profile of the basal diet remained unchanged across all experimental groups, as the addition of experimental supplements had no impact. The second group was given a standard diet supplemented with 0.04% KluActive (SD + 0.04% KluActive, G2).

Table 1. Nutritional composition of the diets for broiler chickens (as-fed basis)

Nutrient/parameter	Starter phase (0-10 days)	Grower phase (11-29 days)	Finisher phase (30-42 days)
Metabolizable energy (ME, kcal/kg)	2950	3050	3150
Crude protein (%)	22.5	20.0	18.0
Crude fat (%)	5.0	5.5	6.0
Crude fiber (%)	3.5	4.0	4.5
Crude ash (%)	6.5	6.0	5.8
Lysine (%)	1.30	1.10	0.95
Methionine + cystine (%)	0.95	0.85	0.75
Calcium (Ca, %)	1.00	0.90	0.85
Available phosphorus (P) (%)	0.50	0.45	0.42
Sodium (Na, %)	0.18	0.17	0.16

Values are provided according to the feed manufacturer's specifications

KluActive is an encapsulated probiotic yeast (*K. marxianus*) that was isolated, identified, and evaluated for its technological stability and probiotic potential at the R&D center (Biotechnology Company Enzyme Group in Lviv, Ukraine). The third group was provided with a standard diet supplemented with a combination of KluActive and encapsulated tannins (CHT) at a level of 0.04% (SD + KluActive + CHT 0.04%, G3), and the

fourth group received the basal diet supplemented with a combination of KluActive and tannins (SD + KluActive + CHT 0.06%, G4). The phytobiotics component, in the form of encapsulated hydrolyzable tannins, was obtained as a commercially available product from a feed additive supplier.

KluActive production

The encapsulation of the commercial probiotic product was performed at the manufacturer's industrial production facilities (Enzyme Group, Lviv, Ukraine). A specially developed fluidized-bed drying technology enabled the production of biocapsulated probiotic yeast, which exhibited high resistance to technological stresses. The granules were approximately 0.5-2 mm in size and contained a viable yeast cell count exceeding 1.0×10^9 CFU/g, using the strain *K. marxianus*. The technology guaranteed product stability under thermal processing conditions (up to 60°C without loss of biological activity), as well as resistance to acidic pH and viability in the gastrointestinal tract. The technology and formulation protocol were developed by the producer.

Blood sampling and biochemical analysis

Blood samples were collected from 10 chickens in each experimental group on days 28 and 42 of the study. Sampling was conducted in accordance with established avian blood collection protocols, ensuring aseptic conditions and minimizing stress to the birds. Blood was obtained from the brachial (wing) vein using sterile needles (23-25 G) and syringes. The puncture site was disinfected with 70% ethanol prior to venipuncture. Blood samples were collected into plain tubes without anticoagulants and left to clot at room temperature. The serum was then separated by centrifugation at 3000 rpm for 10-15 minutes and stored at -20°C until biochemical analysis in a certified laboratory. Serum bactericidal activity (SBA) was assessed using an established microbiological assay that detects the inhibition of *Escherichia coli* growth, adapted for poultry studies as described by [Vlizlo et al. \(2012\)](#). Serum lysozyme activity (SLA) was measured using a turbidimetric method with *Micrococcus lysodeikticus* as the substrate, based on the technique outlined by [Shugar \(1952\)](#). To assess the phagocytic immune response, leukocyte phagocytic function was evaluated using standard microscopic techniques. To assess the phagocytic immune response, leukocyte phagocytic function was evaluated using standard microscopic techniques. Phagocytic activity (PA, %) indicated the number of neutrophils actively involved

in ingesting test microorganisms. The phagocytic index (PI) measured the average number of microorganisms each neutrophil ingested, while the phagocytic number (PN) demonstrated the total number of microorganisms ingested per 100 neutrophils. These parameters were determined using established hematological methods for leukocyte functional assessment, as described by [Harmon et al. \(1998\)](#). Phagocytosis assays were conducted using heat-inactivated bacterial cultures, following established veterinary immunology protocols for poultry. The selected immune parameters served as indicators of nonspecific (innate) immunity, which was essential for maintaining health and optimal performance in broiler chickens.

Productive performance measures

Productive performance parameters were recorded throughout the experimental period in compliance with established poultry production methodologies. Average daily gain (ADG, g/day), calculated from individual or group body weight values during the experimental period, absolute body weight gain (ABWG, g) indicated the total increase in body weight during the experimental period, feed intake (FI, g/bird) was the total feed consumed per chicken, feed conversion ratio (FCR, kg feed/kg gain) was calculated as the ratio of feed intake to body weight gain, and feed efficiency was measured as body weight gained per unit of feed consumed. All measurements were performed under controlled housing and feeding conditions to minimize environmental variability.

Statistical analysis

Data were analyzed utilizing one-way analysis of variance (ANOVA), followed by Tukey's honestly significant difference (Tukey's HSD) post hoc test to identify pairwise differences between groups. All statistical analyses were conducted using Microsoft Excel (Microsoft Office Professional Plus, Microsoft Corporation, USA) with the Analysis ToolPak add-in. Differences between groups were deemed statistically significant at a p-value less than 5% ($p < 0.05$). The present results were presented as mean $\pm$ standard deviation (SD).

RESULTS

Humoral and cellular innate immunity parameters

The effects of dietary supplementation with KluActive alone or in combination with CHT on phagocytic activity, phagocytic index, and phagocytic number in broiler chickens are presented in Figure 1.

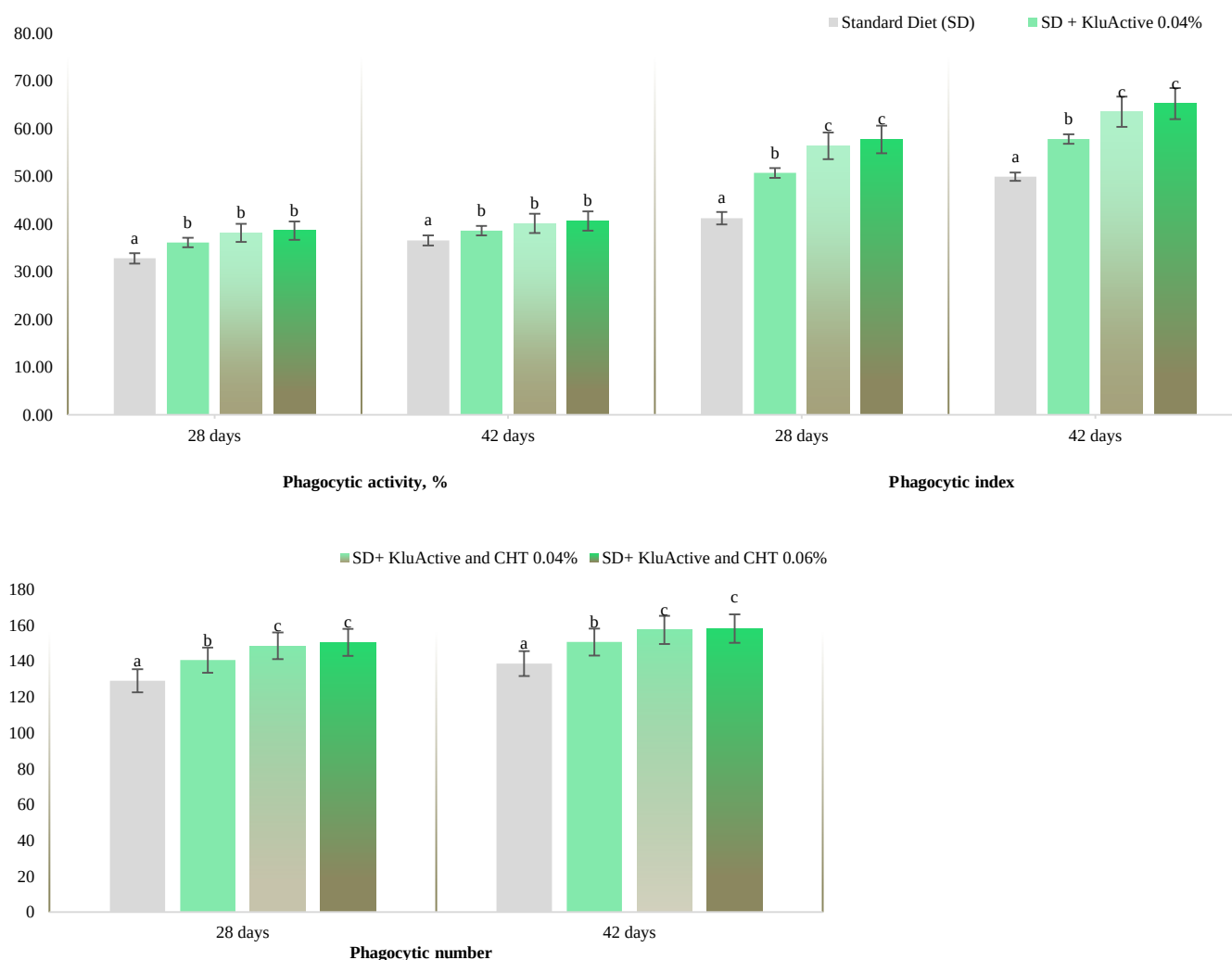


Figure 1. Changes in phagocytic activity, phagocytic index, and phagocytic number of neutrophils in broiler chickens at 28 and 42 days of age. Values are expressed as (mean $\pm$ SD). ^{a,b,c} Means with different superscript letters within the day of study indicate a significant difference ($p < 0.05$).

At 28 days of age, broiler chickens fed the standard diet (G1) demonstrated the lowest phagocytic activity, phagocytic index, and phagocytic number. Broiler chickens in G2 demonstrated significantly higher phagocytic activity than the control group ($p < 0.05$). Broiler chickens in G4 exhibited the highest average phagocytic activity; however, this difference was not statistically significant compared to the G2 and G3 groups ($p > 0.05$). In contrast, phagocytic index and phagocytic number increased progressively across treatment groups. Broiler chickens in G3 and G4 groups exhibited significantly higher levels of these parameters than those in the control and G2 groups ($p < 0.05$). Specifically, the number of phagocytes increased significantly in G2, G3, and G4 groups by 8.9%, 15.1%, and 16.7%, respectively, compared to the control group ($p < 0.05$). Similarly, the phagocytic index increased significantly across all

supplemented groups, from 23.02% to 40.06%, exceeding the control value ($p < 0.05$).

At 42 days of age, a similar pattern was observed. Phagocytic activity in the G2 group remained significantly higher than in the control group ($p < 0.05$), while the inclusion of encapsulated tannins in combination with the probiotic resulted in additional increases. The phagocytic index and phagocytic number demonstrated the same trend, with significantly higher values in G3 and G4 groups than in G1 and G2 groups ($p < 0.05$). Furthermore, chickens in the G4 group demonstrated significantly higher phagocytic responses than those in the G3 group, indicating a dose-dependent effect ($p < 0.05$).

The effects of dietary supplementation with *K. marxianus* alone or in combination with encapsulated tannins on SBA and SLA in broiler chickens are shown in Figure 2.

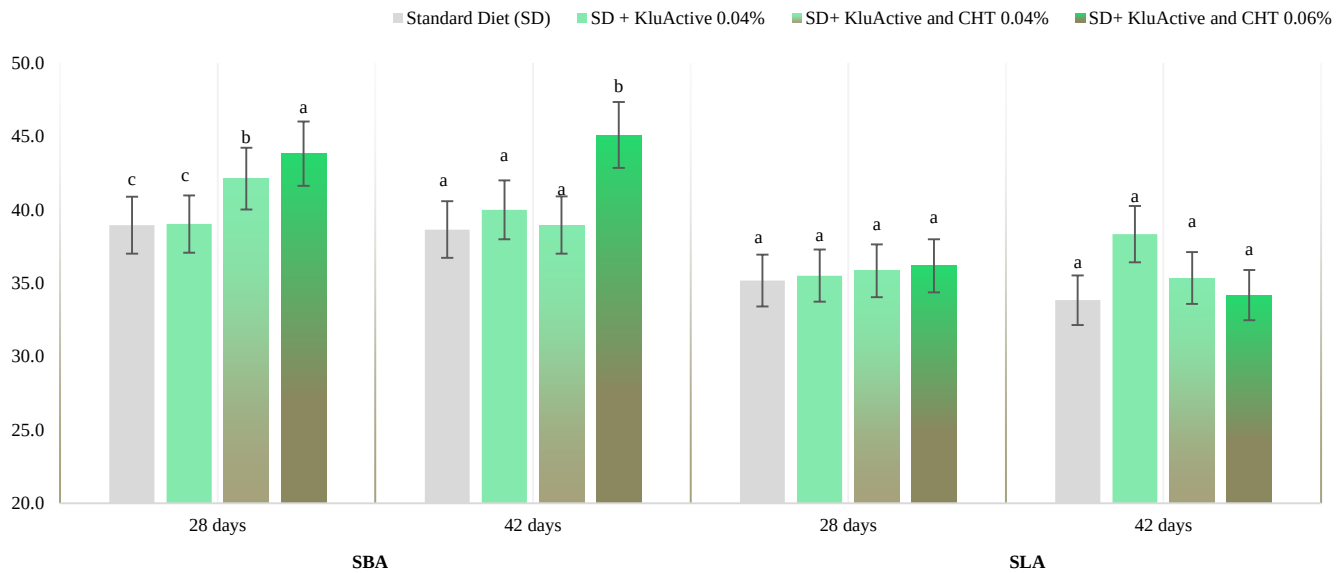


Figure 2. Lysozyme and bactericidal activities of blood serum in broiler chickens at 28 and 42 days of age. Values are expressed as (mean $\pm$ SD). ^{a,b,c} Means different superscript letters within the same day of study indicate a significant difference ($p < 0.05$).

At 28 days of age, SBA was the lowest in broiler chickens fed the standard diet and increased progressively with dietary supplementation. A slight numerical increase in SBA was observed in the G2 group (39.015 ± 0.992) compared with the control group (38.93 ± 0.515); however, this difference was not statistically significant ($p > 0.05$). The highest value was observed in the G4 group compared with the control group, indicating a dose-dependent response ($p < 0.05$). In G4 group, SBA was 12.5% higher than in the control group, and in the G3 group, SBA was 8.2% higher. At day 42, broiler chickens supplemented with KluActive and encapsulated tannins at the higher inclusion level (G4 group) exhibited significantly higher SBA than the control and the other G2 and G3 groups, respectively ($p < 0.05$). Serum bactericidal activity (SBA) was significantly higher in the G4 group, increasing by 12.5% on day 28 and 16.7% on day 42 compared with the corresponding values in the serum of birds from the control group ($p < 0.05$).

Serum lysozyme activity revealed minor differences among treatments at 28 days of age. However, at 42 days, SLA was significantly higher in the G1 group than in the control group ($p < 0.05$). In the blood serum of chickens from G3 and G4 groups, SLA values were lower than those in the G2 group but still exceeded those in the control group. Overall, dietary supplementation with *K. marxianus* was associated with improvements in phagocytic parameters and bactericidal activity in broiler chickens. The combined use of the probiotic and

encapsulated tannins generally resulted in higher values than the control, with a tendency toward greater responses at the higher inclusion level (0.06%). Lysozyme activity increased mainly in the G2 group, while values in the G3 and G4 groups persisted at levels equal to or exceeding those of the control.

Productive performance

Supplementation of the antibiotic-free diet with all three additives improved growth performance, feed utilization, and feed conversion efficiency, although the responses differed by additive type and dosage (Table 2). Dietary supplementation with experimental additives positively affected the growth performance of broiler chickens. Analysis of ADG demonstrated higher values across all experimental groups than in the control group. Broiler chickens in G2 and G3 groups reached ADG values of 98.35 and 100.45 g/day, respectively, while the control group had an ADG of 88.49 g/day. Similarly, the final body weight of broiler chickens increased by 11.14% in the G2 group, 13.52% in the G3 group, and 4.85% in the G4 group compared to the control group. Evaluation of feed efficiency parameters further indicated improved feed utilization in all supplemented groups. The lowest FCR was observed in the G3 group, at 1.5, compared to 1.67 in the control group. These findings indicate that combining probiotic and tannin additives may enhance nutrient utilization efficiency and growth performance in broiler chickens fed antibiotic-free diets.

Table 2. Growth performance of broiler chickens fed experimental diets (days 15-42 of age)

Treatment group	Average daily gain (g/day)	Absolute body weight gain (g)	Change versus control (%)	Feed intake (g/bird/period)	Feed conversion ratio	Feed efficiency (%)
Standard diet (SD)	88.49	2300.67	-	3850	1.67	59.76
SD + 0.04% KluActive	98.35	2557.00	11.14	3960	1.55	64.57
SD + KluActive + 0.04% CHT	100.45	2611.67	13.52	3925	1.50	66.54
SD + KluActive + 0.06% CHT	92.78	2412.33	4.85	3905	1.62	61.78

CHT: Chestnut hydrolyzable tannins

DISCUSSION

The present study demonstrated that supplementation of antibiotic-free broiler diets with *K. marxianus*, either alone or in combination with CHT, improved growth performance and certain innate immune parameters in real-world production settings. Previous studies have separately demonstrated the beneficial effects of probiotics and tannin-based phytogetic additives in broiler chickens, including improvements in growth performance, gut health, and immune responses (Shehata et al., 2022; Ranjan et al., 2023). In the present study, intestinal barrier function, microbial ecology, and systemic immune responses were not directly evaluated. Hydrolyzed tannins, characterized by their lower molecular weight and greater bioavailability than condensed tannins, have been indicated to modulate local immune responses by regulating cytokine expression, enhancing epithelial barrier integrity, and exerting antioxidant and antimicrobial effects. Consequently, these actions help reduce immune pressure associated with enteric pathogens (Guo et al., 2023). Encapsulation technology can further enhance these effects by increasing tannin stability and enabling targeted release throughout the gastrointestinal tract, which reduces potential antinutritional interactions while maintaining biological activity (Choi and Kim, 2020).

The present results demonstrated that probiotic yeast supplementation stimulated the phagocytic component of innate immune defense, as well as SLA and SBA in broiler chickens at the end of the experimental period. A more substantial impact on phagocytic activity was observed following supplementation with the combined additive comprising probiotic and phytobiotic components. In particular, SBA remained elevated throughout the study, with the highest values recorded in broiler chickens receiving the higher inclusion level of the combined additive. These findings generally align with the findings of Akhoundzadeh et al. (2025), who studied the

immunomodulatory effects of probiotics and tannins in poultry and other animals.

Concurrently, *K. marxianus*, a probiotic yeast, demonstrated immunomodulatory potential, including stimulation of humoral immunity, enhancement of immune organ development, and modulation of gut microbiota composition in broiler chickens, similar to conventional *Saccharomyces*-based products (Wang et al., 2017; Rassmidatta et al., 2024). The components of the yeast cell wall and metabolites of *K. marxianus* have the potential to activate innate immune pathways and support the function of gut-associated lymphoid tissue (GALT), thereby enhancing immune competence without inducing excessive inflammatory activation (Maccaferri et al., 2012). The combined use of hydrolyzable tannins and probiotic *K. marxianus* may result in complementary and reinforcing effects. Probiotic yeasts stabilize intestinal morphology and microbial balance, while tannins reduce oxidative and microbial stress, collectively promoting a more balanced and efficient immune response (Romanin et al., 2016; Wang et al., 2017; Akhoundzadeh et al., 2025). The synergistic interactions were particularly relevant in antibiotic-free broiler production systems, where supporting immune resilience and gut health was vital to maintaining performance during commercial rearing (Yuan et al., 2023).

The markers of phagocyte-mediated innate immunity were notably increased by the addition of the experimental feed additives, which is especially important since the diets were entirely antibiotic-free throughout the rearing period. Different studies have investigated the role of yeasts and their derivatives in animal and poultry nutrition (Abd El-Ghany et al., 2015; Shurson, 2018; Perricone et al., 2022). Yeasts and their byproducts are widely used as a partial or complete substitute for excessive antibiotic use in animal production systems. In addition, due to their valuable composition and highly digestible protein content, yeast-derived byproducts are frequently incorporated into poultry diets as an alternative to

conventional protein sources such as soybean meal and fish meal. However, yeast products should not be regarded solely as protein sources, as their primary value lies in their rich content of bioactive compounds, which can positively influence animal health, immunity, and gut function (Bilal et al., 2021; Abd El-Ghany, 2025). While direct evidence on the immunomodulatory activity of *K. marxianus* was limited, findings from *Saccharomyces cerevisiae* may offer relevant mechanistic insights, as both yeasts have similar cell wall compositions rich in mannan oligosaccharides (MOSs) and β -glucans (Gheisari and Kholehipour, 2006). Furthermore, the cell wall of *K. marxianus* was characterized by a substantial content of structural polysaccharides, including mannans and β -glucans, which are known for their immunomodulatory and prebiotic properties (Perpetuini et al., 2019; Tang et al., 2022). Therefore, similar immunological mechanisms might play an important role in the beneficial effects of *K. marxianus* in animal nutrition.

Previous studies demonstrated that dietary supplementation with hydrolyzable tannins (0.15-0.30%) or organic acids (0.20-0.40%) improved FCR and intestinal health in broiler chickens, suggesting that these additives may positively modulate nutrient utilization and gut functionality (Khorasgani et al., 2025). These effects may partially explain the beneficial responses observed following *K. marxianus* supplementation in the present study. Tannin sources and animal species may influence the immunoregulatory effects on gut health in broiler chickens (Guo et al., 2023). In the present study, supplementation of antibiotic-free diets with the probiotic KluActive (G1) improved FCR by 7.19% compared with the control group. Likewise, supplementation with the probiotic-tannin product improved FCR by 10.18% at the lower dose (G2) and by 2.99% at the higher dose (G3), indicating a non-linear dose-response relationship.

The observed improvements in productive performance were consistent with previous studies indicating that probiotic supplements improved broiler nutrition and that phytogenic compounds such as tannins promoted growth (Redondo et al., 2014). Supplementing *K. marxianus* at a high dose (0.25%) proved to be more effective for improving feed efficiency and intestinal health in broiler chickens, whereas innate immunity was optimized at a medium dose (0.1%; Wang et al., 2017). *In vitro* studies using intestinal epithelial cell culture systems have demonstrated that *K. marxianus* may exert immunomodulatory effects and support intestinal barrier integrity, as evidenced by modulation of pro-inflammatory cytokines and preservation of tight junction proteins

(Kourelis et al., 2010; Romanin et al., 2010; Maccaferri et al., 2012). Supplementation with tannins and probiotics enhanced growth performance in broiler chickens compared with the antibiotic and control groups. No remarkable differences were observed in the retention of dry matter, ether extract, or phosphorus among broiler chickens fed diets supplemented with tannins, probiotics, or antibiotics, except for calcium digestibility, as reported by Pramod Ranjan et al. (2023). Present results indicated that adding tannins and probiotics to the diet could improve productive performance traits such as body weight gain, FCR, and performance index, consistent with findings by Ranjan et al. (2023). Similarly, Wang et al. (2017) found that increasing the dietary *K. marxianus* level consistently lowered FCR during the grower phase and overall rearing period in broiler chickens, demonstrating enhanced feed efficiency, especially at higher supplementation levels (0.2% and 0.25%).

CONCLUSION

The yeast probiotic containing *Kluyveromyces marxianus* (KluActive) at a dose of 0.04% demonstrated positive effects on innate immune parameters and enhanced feed conversion ratio by 7.2%. Supplementing the diet with KluActive at 0.04% and chestnut hydrolyzable tannins (CHT) at 0.04% resulted in a feed conversion ratio of 1.50, representing a 10.2% improvement over the control group. Supplementing the diet with KluActive alone was associated with increased serum lysozyme activity and improved phagocytic activity. In the group receiving KluActive with CHT at 0.04%, lysozyme activity demonstrated only a slight increase, remaining close to the control level. Conversely, supplementing the diet of broiler chickens with a higher level of CHT at 0.06% primarily enhanced serum bactericidal activity and phagocytic capacity, while its impact on lysozyme activity was less significant. The current results indicated that KluActive and CHT differentially influenced selected nonspecific immune parameters in broiler chickens. Dietary supplementation with KluActive improved growth performance in broiler chickens, particularly when combined with CHT at a 0.04% inclusion level. Chickens received a combination of KluActive and CHT at 0.04%, resulting in improved productive performance and feed efficiency, along with a reduced feed conversion ratio. Increasing the concentrations of KluActive and CHT to 0.06% slightly reduced the growth-promoting effects, although the results remained generally superior to those of the control group. The present findings indicated that

combined supplementation with KluActive and CHT may improve productive performance and selected immune-related parameters in broiler chickens. Future studies should include a broader assessment of blood biochemical indicators and should validate the results obtained under commercial production conditions. Additionally, large-scale trials on commercial poultry farms are needed to evaluate the real-world effectiveness and cost-efficiency of adding these supplements to broiler diets.

DECLARATIONS

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Authors' contributions

All authors contributed to data analysis and the writing of the manuscript. All authors contributed to the study conception, experimental design, data collection, analysis, and manuscript preparation. Iryna Matiukha was responsible for study organization, data collection during the experiment, result analysis, and manuscript preparation. Olena Krasovska was responsible for yeast strain collection and identification. Oleh Vishchur contributed to the study organization and preparation of the results. Natalia Broda performed blood parameter analysis and statistical processing of the results. Daria Mudrak provided veterinary supervision of the experiment. Maria Masiuk contributed to the analysis of blood parameters and productive performance indicators. The manuscript was reviewed and edited by Victor Tomchuk and Andriy Voltornisty. All authors have reviewed and approved the final edition of the manuscript and have given their permission for this article to be published.

Availability of data and materials

All materials required to complete the current study are available at the Institute of Animal Biology NAAS.

Competing interests

The authors disclosed no conflicts of interest related to the present study.

Ethical considerations

The authors affirm that this study is original and has not been previously submitted for publication elsewhere. The authors also declared that the information contained within this publication is accurate and has not been modified. No AI tools were employed in conducting and preparing this study. The authors have reviewed all

content and take full responsibility for the accuracy, originality, and integrity of the final manuscript, from submission through revision to publication.

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Effects of Fungal Bioconversion of Azolla-Enriched Diets on Nutritional Quality and Economic Efficiency in ZamPen Native Chicken

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ABSTRACT

Rising feed prices have driven interest in local feed alternatives, even though these typically offer lower nutritional quality than standard commercial feeds. The present study aimed to evaluate the effects of an Azolla-enriched diet fermented with and without *Pleurotus (P.) ostreatus* on nutrient composition, growth performance, and economic efficiency in ZamPen native chickens. A 4×2 factorial experiment under a completely randomized design with 8 treatments and three replications was conducted using 168 two-month-old straight-run ZamPen chickens with an average weight of 665 g. Treatments consisted of 8 diets containing 0%, 5%, 10%, and 15% Azolla, with and without *P. ostreatus* fermentation. Results of nutrient analysis indicated that the fermented Azolla-enriched diet influenced crude protein, ash, and metabolizable energy compared to the non-fermented Azolla-enriched diet. Increasing Azolla levels increased crude protein content, moisture, fats, and metabolizable energy at 15%, 10%, and 5%, respectively, compared to 0% Azolla. Growth performance, including final body weight, weight gain, and feed conversion efficiency, was not significantly affected by the Azolla-enriched diet. However, feed consumption decreased significantly with increasing Azolla levels (10% and 15%), with animals consuming less feed than those fed 0% Azolla. Fermentation affected weight gain and feed conversion ratio, with non-fermented diets resulting in greater weight gain and better feed conversion than fermented diets. Significant interactions between Azolla and fermentation were observed for final body weight, feed conversion ratio, and feed conversion efficiency. Economic analysis revealed significant reductions in feed cost and total production cost at 10% and 15% Azolla, while gross sales, net income, and return on investment were not significantly affected compared to 0% Azolla. Fermentation with or without *P. ostreatus* did not affect economic performance, and no interaction of Azolla and fermentation was observed. These results suggested that Azolla can be incorporated up to 15% in native chicken diets as a cost-effective alternative feed ingredient without compromising performance. In addition, fermentation with *P. ostreatus* increased the moisture and fat contents of the diets and improved final body weight, weight gain, and feed conversion ratio, but reduced crude protein and ash contents.

Keywords: Alternative feed, Azolla meal, Feed conversion ratio, Fermentation, *Pleurotus ostreatus*

INTRODUCTION

The poultry industry in the Philippines is an important part of the economy because it supports people's livelihoods and helps ensure food supply (Ortega et al., 2021). It is also one of the fastest-growing areas in agriculture, and includes broiler, layer, and native chickens. In 2023, the total chicken inventory reached 202.82 million, with layer chickens accounting for 22.3%, broilers for 34.7%, and native chickens for 43% (PSA, 2023).

However, the industry faces challenges due to high feed ingredient prices (Shirisha and Mohanty, 2017; Mirafior, 2021; Tan, 2022), with the Philippines still heavily dependent on imports of feed raw materials (Tan, 2021). In 2018, the Philippines imported approximately 4 million metric tons of animal feed ingredients, including corn, soybean meal, and fishmeal (Cruz et al., 2015; OEC, 2022; Statista Research Department, 2024; Tan, 2024). Rising feed prices have put pressure on poultry farmers

and consumers, leading to higher prices of livestock and poultry products (Mirafior, 2021; Reeve, 2021; Eardly, 2022).

The current crisis has encouraged farmers to use alternative feed ingredients for poultry to help lower feed costs (Ito, 2024; Nadgauda, 2024). Feed accounts for about 70% of the total production cost (Oladokun and Johnson, 2012; Mallick et al., 2020), so using alternative feeds can help reduce expenses and improve farmers' income. Locally available feed sources such as corn, azolla, moringa, and copra meal can be used as substitutes by being included in formulated rations. However, several factors must be considered in feed formulation, including nutrient availability of the feed ingredients (PCAARRD-DOST, 2000). One challenge in using alternative feed is that it often contains lower nutrient levels than conventional feed ingredients (BCRC, 2024).

Fungal fermentation is a biological process that uses fungi to break down and transform feed ingredients, improving their nutritional quality and digestibility (Imelda et al., 2008; Sun et al., 2021). During this process, fungi produce enzymes that degrade complex carbohydrates and other organic compounds that are otherwise difficult for poultry to digest (Sun et al., 2023). These complex carbohydrates include non-starch polysaccharides (NSP), such as cellulose, pectins, gums, glucans, mucilages, inulin, and chitin, as well as lignin, which make up a large portion of the dietary fiber component in plant-based feed ingredients (Choct, 2015; Eunjo et al., 2022; Nguyen et al., 2022). These dietary fibers are the least digestible constituents of poultry feed, as birds lack endogenous NSPs and the enzymes required to degrade lignocellulose (such as cellulases, xylanases, glucanases, amylase, and protease) (Devi and Diarra, 2019; Eunjo et al., 2022). Non-starch polysaccharides and lignin can affect gut physiology and function by altering the secretion of water, lipids, electrolytes, and proteins within the digestive tract. These compounds are considered anti-nutritional factors because they can reduce nutrient digestion and absorption in poultry (Nguyen et al., 2021). Through fermentation, however, fungi produce metabolites and enzymes that help break down these complex compounds, thereby improving nutrient availability and utilization in the feed (Sun et al., 2023). As a result, fermentation enhances the nutritional value of the feed and can improve the growth and production performance of poultry birds (Chu et al., 2016; Chuang et al., 2020; Ahmad et al., 2023; Ibrahim et al., 2023).

Pleurotus ostreatus (*P. ostreatus*), known as the oyster mushroom, is a fungal species widely used in the

feed industry as a white-rot fungus for fermentation and for degrading lignin, cellulose, and hemicellulose (Bánfi et al., 2015; Heidari et al., 2022). A study by Nuraini et al. (2022) showed that fermentation using *P. ostreatus* improved the nutrient quality of Azolla. Combining fungal fermentation with the mechanical treatment of canola meal is a suitable strategy to improve its nutritional quality and significantly reduce its antinutrient content (Heidari et al., 2022). Another study showed that *P. ostreatus* improved the nutritive value of corn stover by increasing its energetic values, crude protein, microbial mass, and total volatile fatty acid concentrations (Olagunju et al., 2023).

However, fermentation using *P. ostreatus* in locally available feed ingredients for native chicken diets is scarce. Most previous studies have focused on common commercial animals or controlled laboratory experiments, leaving a gap in studies on its effects on the growth performance of native chickens. Thus, the present study aimed to use *P. ostreatus* to ferment Azolla-incorporated diets and evaluated the nutritive value, economic efficiency, and production performance in ZamPen native chickens.

MATERIALS AND METHODS

Ethical approval

The current study followed the ethical standards outlined in the Philippines' Animal Welfare Act (RA 8485, as amended by RA 10631). Proper care, handling, and housing were ensured to minimize animal stress and discomfort in the chickens.

Study area and experimental design

The study was conducted in Ozamiz City, Misamis Occidental, Philippines, using a 4×2 factorial experiment arranged in a Completely Randomized Design (CRD) with three replications. The experimental factors consisted of fermentation and an Azolla-enriched diet. The fermentation factor included diets prepared either with or without *P. ostreatus* fermentation, while the Azolla factor consisted of four dietary levels, including 0% Azolla (A1), 5% Azolla (A2), 10% Azolla (A3), and 15% Azolla (A4). A total of 168 two-month-old straight-run ZamPen native chickens with an average initial body weight of 665 g per chicken were used in the study. Seven chickens were randomly assigned to each replication, resulting in 21 chickens per treatment combination. The chickens were randomly allocated to the eight treatment combinations and reared for a 30-day experimental period.

Feed formulation

The feed formulation process followed a trial-and-error method (PCAARRD-DOST, 2000). Nutrient composition values for the feed ingredients were obtained from published feed composition tables and relevant literature (PCAARRD-DOST, 2000; Seid, 2023). The proportions of yellow corn, Azolla meal, soybean meal (SBM), rice bran, limestone, dicalcium phosphate (Dicaphos), salt, molasses, coconut oil, and vitamin-mineral premix were adjusted iteratively until the

calculated nutrient levels met the nutrient requirements of growing native chickens. The diets were balanced for metabolizable energy (ME), crude protein (CP), crude fiber (CF), calcium (Ca), and phosphorus (P), while maintaining the desired Azolla inclusion levels (0%, 5%, 10%, and 15%). Microsoft Excel (version 2019) was used to calculate the nutrient contribution of each ingredient and the total nutrient composition of the formulated diets (Table 1).

Table 1. Feed Ingredients and diet chemical composition of ZamPen native chickens for a 30-day experimental period

Feed ingredients	Treatments							
	Fermented with <i>P. ostreatus</i>				Fermented without <i>P. ostreatus</i>			
	T ₁	T ₂	T ₃	T ₄	T ₅	T ₆	T ₇	T ₈
Corn	46.3	47.75	51.2	54.95	46.3	47.75	51.2	54.95
Azolla meal	0	5	10	15	0	5	10	15
Soybean meal	22.1	20	18	16.5	22.1	20	18	16.5
Rice bran D1	21.85	18	11.8	4.8	21.85	18	11.8	4.8
Calcium carbonate	2	1.5	1.5	1.5	2	1.5	1.5	1.5
Dicalcium phosphate	1	1	0.75	0.5	1	1	0.75	0.5
Salt	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Molasses	3	3	3	3	3	3	3	3
Coconut oil	3	3	3	3	3	3	3	3
Vitamin-mineral premix	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Total	100	100	100	100	100	100	100	100
Chemical analysis								
Moisture (%)	9.59	9.04	9.91	9.96	10.56	9.26	13.47	10.79
Fat (%)	2.78	5.87	2.00	2.12	3.19	5.11	2.48	4.04
Crude Protein (%)	17.42	18.18	17.91	18.07	17.05	17.45	17.03	18.30
Ash (%)	9.20	9.48	9.69	10.00	9.36	9.73	8.95	8.68
ME (kcal)	3386	3552	3316	3308	3363	3496	3227	3423

Diet formulation, nutrient composition, and native chicken nutrient recommendations were based on PCAARRD-DOST (2000), while Azolla nutrient composition was obtained from Seid (2023). T₁-T₈: Different treatments with and without *Pleurotus ostreatus* fermentation. Vitamin-mineral premix composition: Vitamin A, D, E, potassium iodide, zinc, copper, manganese, iron, cobalt, sodium chloride, and magnesium oxide according to the recommendation of PCAARRD-DOST (2000).

Fermentation of the formulated azolla-enriched diets

Feed fermentation was carried out using *P. ostreatus* and compared with non-fermented azolla-enriched diets. Prior to fermentation, the formulated feeds were sterilized for 6 hours and allowed to cool to room temperature. The sterilized feeds were then inoculated with *P. ostreatus* tissue at 3% (w/w) and subjected to solid-state fermentation for 15 days at 60% moisture content. Upon completion of the fermentation period, the fermented feeds were heated at 80°C for 2 hours to inactivate the fungal culture and then dried at 60°C for 8 hours. The non-fermented feeds underwent the same processing conditions, except for inoculation with *P. ostreatus* (Nuraini et al., 2022).

Nutrient analysis of formulated and fermented feeds

The nutrient analysis of the formulated and fermented feeds, with and without *P. ostreatus*, was conducted at the Department of Science and Technology–Regional Standards and Testing Laboratory (DOST-RSTL), Region 10, Philippines. Moisture content was determined using the AOAC Official Method 930.15 (Oven Drying Method), ash content was analyzed using AOAC Official Method 942.05 (Gravimetric Furnace Method), crude protein was determined through AOAC Official Method 991.20 using the Automated Kjeldahl Method, and total fat was measured following AOAC Official Method 932.06 through acid hydrolysis and solvent extraction. All analyses were performed in accordance with the Official Methods of Analysis (OMA) of the Association of Official

Analytical Chemists (AOAC), 21st Edition (AOAC, 2023).

Growth parameters

The growth performance was measured using several parameters, including initial weight (IW), final weight (FW), and weight gain (WG). The initial body weight (g) was measured by individually weighing the chickens at the start of the experiment to establish their baseline weight. Final body weight (g) was measured at the end of the study to assess overall growth. Weight gain (g) was subsequently determined by subtracting the initial body weight from the final body weight, as shown in Formula 1. Feed consumption (FC) was measured as the total amount of feed consumed per chicken throughout the study period. FCR and FCE were computed using Formulas 2 and 3 (Shamsudin et al., 2022).

$$\text{Weight gain (g)} = \text{Final Weight (g)} - \text{Initial Weight (g)} \quad (\text{Formula 1})$$

$$\text{Feed conversion ratio} = \text{Feed Consumption (g)} / \text{Weight Gain (g)} \quad (\text{Formula 2})$$

$$\text{Feed conversion efficiency} = \text{Weight Gain (g)} / \text{Feed Consumption (g)} \quad (\text{Formula 3})$$

Economic analysis

The cost-and-return analysis was conducted to evaluate the cost-effectiveness and profitability of feeding strategies for ZamPen chickens. The parameters include calculations of feed cost (FC), total production cost (TPC), gross sales, net income, and return on investment (ROI). Feed cost was determined by summing all feed-related expenses incurred throughout the experiment. Total production cost was calculated by summing all production-related costs, including housing, feed, chicks, healthcare, labor, and other operational needs. Gross sales referred to the total income generated from selling the chickens, calculated at a standard market price of ₱350 (Philippine peso) per kilogram based on carcass weight. Net income was calculated by subtracting total production costs from gross sales, as shown in Formula 4. Return on investment was used to measure profitability by comparing net income to total production cost, indicating the efficiency of the production system, as shown in Formula 5 (PSA, 2022).

$$\text{Net Income} = \text{Gross Sales (₱)} - \text{Total Production Cost (₱)} \quad (\text{Formula 4})$$

$$\text{Return On Investment} = \frac{\text{Net Income (₱)}}{\text{Total Production Cost (₱)}} \times 100\% \quad (\text{Formula 5})$$

Statistical analysis

A two-way ANOVA under a factorial CRD was computed using the IBM SPSS Software version 26 to evaluate the significant effects of varying Azolla levels and fermentation treatments (with and without *P. ostreatus*) on the measured parameters. Where significant differences were observed, Tukey's test was used for multiple comparisons to identify specific differences among treatment means. Statistical significance was declared at the 5% probability level ($p < 0.05$).

RESULTS AND DISCUSSION

Nutrient analysis

The nutrient analysis of Azolla-enriched diets with and without *P. ostreatus* fermentation is presented in Table 2. Moisture content differed significantly among the Azolla-enriched diets, with the highest observed in the 10% Azolla diet (11.69%, $p < 0.05$). The variation in moisture content may be associated with its fibrous structure, which promotes moisture retention (Singh and Kim, 2021), and with Azolla's inherent water-retention capacity (Shamsudin et al., 2022). Fermentation also significantly affected moisture content, with higher values observed in the treatment without *P. ostreatus* ($p < 0.05$). The moisture result may reflect differences in water utilization during fungal growth, as optimal *P. ostreatus* fermentation requires controlled moisture conditions (Wiesnerova et al., 2023). The interaction effect of azolla and fermentation suggests relatively stable moisture at lower Azolla inclusion levels but variation at higher levels (10%), with non-fermented azolla-enriched diets exhibiting greater moisture retention. The observed decrease in moisture content of the inoculated diets at 10% Azolla inclusion may indicate a partial reduction in fiber-associated water-holding capacity following fungal degradation (Yasar et al., 2020; Zhang et al., 2024). Fat content was significantly influenced by Azolla inclusion, with the highest at 5% Azolla (5.49%), while 10% and 15% Azolla showed reduced fat content ($p < 0.05$). The decrease in fat at higher Azolla levels (10% and 15%) may be due to a dilution effect from Azolla's low lipid content. Fermentation resulted in lower fat content, particularly in *P. ostreatus*-treated feeds, likely due to microbial lipolysis and lipid utilization during fermentation (Boukid et al., 2022; Heidari et al., 2024). The significant interaction between Azolla and fermentation indicates that lipid reduction was more pronounced at higher Azolla levels, suggesting substrate-dependent effects.

Table 2. Nutrient analysis of azolla-enriched diets fermented with or without *P. ostreatus* in ZamPen native chickens for a 30-day experimental period

Treatments	Moisture	Fat	Crude protein	Ash	Metabolizable energy
Azolla-enriched diet					
A ₁	10.07 ^b	2.99 ^b	17.24 ^a	9.28	3375.25 ^b
A ₂	9.15 ^a	5.49 ^c	17.81 ^{ab}	9.60	3524.22 ^c
A ₃	11.69 ^c	2.24 ^a	17.47 ^a	9.32	3271.88 ^a
A ₄	10.38 ^b	3.08 ^b	18.18 ^b	9.34	3365.32 ^b
P-value	.001 ^{**}	.001 ^{**}	.005 ^{**}	.389 ^{ns}	.001 ^{**}
Fermentation					
B ₁	9.63	3.19	17.89	9.59	3390.94
B ₂	11.02	3.71	17.46	9.18	3377.39
P-value	.001 ^{**}	.001 ^{**}	.018 [*]	.011 [*]	.127 ^{ns}
A x B	.001 ^{**}	.001 ^{**}	.125 ^{ns}	.003 ^{**}	.001 ^{**}
CV	13.2	39.7	3.3	5.4	3.0

^{a,b,c,d} Different superscript letters within a column showed a significant difference ($p < 0.05$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, NS: Not significant, A₁: 0% Azolla, A₂: 5% Azolla, A₃: 10% Azolla, A₄: 15% Azolla, B₁: with *Pleurotus ostreatus*, B₂: without *Pleurotus ostreatus*. A × B: Interaction of Azolla-enriched diet and fermentation, CV: Coefficient of variation.

Similar reductions in ether extract following fungal fermentation have been reported in different feed substrates, although responses vary with feed composition (Yalchil and Hajieghrari, 2011). Crude protein content was significantly affected by Azolla inclusion and fermentation ($p < 0.05$). Crude protein increased with higher Azolla inclusion levels, reaching 18.18% at 15% inclusion, consistent with Azolla's relatively high protein content (Swain et al., 2022). Fermentation with *P. ostreatus* resulted in a slight increase in CP, which may be attributed to microbial degradation of fiber and the resulting concentration effects following substrate breakdown (Wang et al., 2021; Heidari et al., 2022). However, the observed increase in crude protein was marginal. Ash content was not significantly affected by Azolla inclusion, indicating stable mineral contributions across diets ($p > 0.05$). In contrast, fermentation significantly increased ash content ($p < 0.05$). The increase is possibly due to the release of bound minerals during microbial degradation of complex feed matrices (Yasar and Tosun, 2020). The interaction between Azolla inclusion and fermentation significantly affected ash content ($p < 0.05$). In the absence of *P. ostreatus* fermentation, ash content decreased with increasing Azolla inclusion, suggesting alterations in the mineral composition of the diets. Fermentation may have moderated this response through fungal degradation of organic substrates and subsequent changes in nutrient distribution within the feed matrix

(Heidari et al., 2022). Metabolizable energy was significantly affected by Azolla inclusion level ($p < 0.05$). The diet containing 5% Azolla exhibited the highest ME value (3524.22 kcal/kg), which was significantly higher than those of the 0% (3375.25 kcal/kg), 10% (3271.88 kcal/kg), and 15% Azolla diets (3365.32 kcal/kg, $p < 0.05$). The reduction in metabolizable energy at higher Azolla inclusion levels may be attributed to increased fiber content, which can limit nutrient digestibility and reduce the availability of dietary energy (Mtei et al., 2019; Jha and Mishra, 2021). Fermentation did not significantly influence metabolizable energy, although diets fermented with *P. ostreatus* (3390.94 kcal/kg) showed a numerically higher metabolizable energy than non-fermented diets (3377.39 kcal/kg). Azolla inclusion level and fermentation interacted significantly to influence metabolizable energy ($p < 0.05$). This interaction indicates that different Azolla inclusion levels modified the effect of fermentation on dietary energy utilization. The chemical composition and fiber content of the substrate likely influenced the nutritional effects of fermentation by affecting nutrient availability and energy release during digestion (Olukomaiya et al., 2019).

Growth performance

Growth performance data are presented in Table 3. Results on the initial weight showed no significant differences among treatments, confirming uniformity at

the start of the experiment ($p > 0.05$). The lack of significant difference in initial weight supports the validity of subsequent comparisons, as initial weight is a key determinant of growth response in chickens (Khulel and Sabri, 2020). Final weight ranged from 1125.09 g to 1230.64 g and was not significantly affected by Azolla levels, fermentation, or their interaction ($p < 0.05$). The non-significant results for final weight indicated that Azolla can be incorporated at levels up to 15% without negatively affecting growth performance, consistent with the findings of Subramanian (2021). The lack of a fermentation effect indicates limited improvement under the present conditions, in contrast to reports of enhanced growth following fungal fermentation (Ahmad et al., 2023). Weight gain was not significantly affected by Azolla inclusion levels or by the Azolla-fermentation interaction ($p > 0.05$). Although numerical reductions in weight gain were observed at higher Azolla levels, this trend may be attributed to Azolla's increased dietary fiber content, which can reduce nutrient digestibility and energy utilization (Jha and Mishra, 2021). In contrast, fermentation significantly affected weight gain, indicating that the fermentation treatment influenced growth performance ($p < 0.05$). Feed consumption was significantly affected by Azolla inclusion, with reduced consumption at higher inclusion levels ($p < 0.05$). Fermentation and interaction effects were not significant ($p > 0.05$). The reduction in feed consumption is likely associated with Azolla's high fiber content, which may

reduce palatability and voluntary feed intake (Singh and Kim, 2021; Swain et al., 2022). Feed conversion ratio ranged from 5.09 to 5.89 and showed no significant main effects of Azolla or fermentation ($p > 0.05$). However, a significant interaction effect indicates that fermentation influenced feed efficiency depending on Azolla level ($p < 0.05$). The improved feed conversion ratio observed at moderate Azolla inclusion levels (5-10%) suggests more efficient nutrient utilization under these conditions. However, this advantage was not maintained at higher inclusion levels (15%), likely due to Azolla's increased dietary fiber content. While moderate amounts of fiber may support normal digestive function, excessive fiber can reduce nutrient digestibility, increase digesta passage time, and limit the availability of energy and other nutrients for growth. Consequently, chickens fed higher levels of Azolla may have utilized feed less efficiently than those receiving moderate inclusion levels, resulting in diminished improvements in feed conversion ratio (Kumar, 2018; Katu et al., 2025). Feed conversion efficiency was not significantly affected by any factor ($p > 0.05$). The decrease observed at higher Azolla inclusion levels supports the notion that excessive dietary fiber can impair nutrient utilization efficiency (Joysowal et al., 2018; Kheravii et al., 2018). The limited effects of fermentation on performance parameters suggest that any improvements in digestibility were insufficient to produce substantial changes in overall performance (Zentek and Boroojeni, 2020).

Table 3. Growth performance of ZamPen native chicken fed fermented diets with or without *P. ostreatus* for a 30-day experimental period

Treatments	IW (g)	FW (g)	WG (g)	FC	FCR	FCE
Azolla-enriched diet						
A ₁	662.93	1230.64	567.712	2826.95 ^b	5.09	19.98
A ₂	679.13	1218.23	539.10	2749.74 ^{ab}	5.16	19.52
A ₃	650.93	1136.01	485.08	2625.79 ^a	5.45	18.44
A ₄	670.96	1125.09	454.1233	2601.04 ^a	5.89	17.32
P-value	.434 ^{ns}	.137 ^{ns}	.088 ^{ns}	.036 [*]	.057 ^{ns}	.088 ^{ns}
Fermentation						
B ₁	664.29	1172.04	507.75	2706.88	5.50	18.58
B ₂	667.68	1182.94	515.26	2694.87	5.29	19.04
P-value	.001 ^{**}	.001 ^{**}	.018 [*]	.833 ^{ns}	.011 [*]	.127 ^{ns}
A x B	.001 ^{**}	.001 ^{**}	.125 ^{ns}	.079 ^{ns}	.003 ^{**}	.001 ^{**}
CV	13.2	39.7	3.3	6.3	5.4	3.0

^{a,b,c,d} Different superscript letters within a column showed a significant difference ($p < 0.05$). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; NS: Not significant, A₁: 0% Azolla, A₂: 5% Azolla, A₃: 10% Azolla, A₄: 15% Azolla, B₁: with *Pleurotus ostreatus*, B₂: without *Pleurotus ostreatus*, IW: Initial weight, FW: Final weight, WG: Weight gain, FC: Feed consumption, FCR: Feed conversion ratio, FCE: Feed conversion efficiency, A × B: Interaction of Azolla-enriched diet and fermentation, CV: Coefficient of variation.

Table 4. Economic efficiency of ZamPen native chickens fed fermented diets, with or without *P. ostreatus* for a 30-day experimental period

Treatments	FC	PC	GS	NI	ROI
Azolla-enriched diet					
A ₁	590.09 ^b	2245.18 ^b	2804.43	559.25	24.81
A ₂	548.87 ^b	2203.95 ^b	2775.03	571.09	25.82
A ₃	494.80 ^a	2149.89 ^a	2588.83	438.95	20.37
A ₄	458.64 ^a	2113.73 ^a	2563.11	449.38	21.18
P-value	.001 ^{**}	.001 ^{**}	.137 ^{NS}	.479 ^{NS}	.591 ^{NS}
Fermentation					
B ₁	524.94	2188.36	2670.70	482.35	21.83
B ₂	521.26	2168.01	2695.00	526.99	24.25
P-value	.745 ^{NS}	.085 ^{NS}	.780 ^{NS}	.562 ^{NS}	.474 ^{NS}
A x B	.115 ^{NS}	.115 ^{NS}	.138 ^{NS}	.150 ^{NS}	.133 ^{NS}
CV	11.2	2.7	8.9	39.6	36.9

^{a,b,c,d} Different superscript letters within a column showed a significant difference ($p < 0.05$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, NS: Not significant, A₁: 0% Azolla, A₂: 5% Azolla, A₃: 10% Azolla, A₄: 15% Azolla, B₁: w/ *Pleurotus ostreatus*, B₂: w/out *Pleurotus ostreatus*, FC: Feed cost, PC: Production cost, GS: Gross sales, NI: Net income, ROI: Return on investment, A x B: Interaction of Azolla-enriched diet and fermentation, CV: Coefficient of variation.

Economic performance

Table 4 presents the economic efficiency of ZamPen native chickens fed Azolla-enriched diets with or without *P. ostreatus* fermentation. Increasing Azolla inclusion significantly reduced feed cost, with the lowest cost observed at 15% inclusion, followed by the 10%, 5%, and 0% Azolla diets ($p < 0.05$). The reduction result confirmed the cost-reducing potential of Azolla as a locally available feed ingredient (Kumar et al., 2018; Swain et al., 2022). The reduction in feed cost also significantly reduced production cost, with the 15% Azolla diet recording the lowest production cost, followed by the 10%, 5%, and 0% diets ($p < 0.05$). This finding agrees with Sun et al. (2023), who identified feed as the major component of poultry production costs. *P. ostreatus* fermentation did not significantly influence feed or production costs, suggesting that fermentation neither increased nor reduced overall production expenses. Dai et al. (2020) similarly reported that fermentation treatments did not substantially alter the economic cost of feed production. Likewise, Azolla inclusion, fermentation, and their interaction did not significantly affect gross sales, net income, and return on investment ($p > 0.05$). Although the differences were not significant, the 5% Azolla diet numerically recorded the highest net income and return on investment among the Azolla treatments. Similarly, diets fermented with *P. ostreatus* numerically produced higher net income and return on investment than non-fermented diets. These trends suggest that moderate Azolla inclusion and fermentation may offer economic advantages; however, further studies are needed to confirm these effects.

CONCLUSION

The present study demonstrated that the fungal bioconversion of Azolla-enriched diets using *P. ostreatus* influenced the nutritional quality and affected selected growth performance and economic efficiency of ZamPen native chickens. Azolla inclusion increased crude protein content and metabolizable energy while reducing feed consumption, feed cost, and production cost. Fermentation with *P. ostreatus* altered moisture, fat, crude protein, and ash contents and affected final weight, weight gain, and feed conversion ratio. Furthermore, the interaction between Azolla inclusion and fermentation significantly influenced several nutritional and performance traits, indicating that the response to fermentation depended on the level of Azolla in the diet. However, the combined use of Azolla and *P. ostreatus* fermentation did not significantly affect economic returns. Thus, Azolla can be incorporated into diets for ZamPen native chickens at up to 15%, while a 5% inclusion level provided the most favorable balance among nutritional quality, growth performance, and economic return under the conditions of this study. Future studies should investigate higher Azolla inclusion levels, improved fermentation strategies, and longer feeding periods in native chickens.

DECLARATIONS

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Authors' contributions

Gether Pacatang Enario designed the study; conducted the experiment; performed data collection, statistical analysis, and interpretation; and prepared the original draft. The author also reviewed, edited, and approved the final manuscript.

Availability of data and materials

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

Competing interests

The author declares no competing interests.

Ethical considerations

The author assures that this manuscript complies with ethical standards and that no issues related to plagiarism, consent to publish, research misconduct, data fabrication and/or falsification, double publication and/or submission, or redundancy are present. Grammarly was used solely for English language editing and proofreading. The author reviewed and approved all revisions and accepts full responsibility for the content of the manuscript before submission and before publication.

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Effects of Fermented Pineapple Peel and *Indigofera zollingeriana* Leaves on Intestinal Bacterial Population, Performance, and Carcass Traits in Broiler Chickens

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ABSTRACT

The prohibition of antibiotic growth promoters has necessitated the exploration of alternative functional feed additives from agricultural waste to maintain poultry health and productivity. The current study aimed to evaluate the effects of dietary supplementation with fermented synbiotics derived from pineapple peel (*Ananas comosus*) and *Indigofera zollingeriana* leaves on growth performance, carcass traits, intestinal bacterial populations, and internal organ weights in broiler chickens. A total of 100 one-day-old broiler chickens with an average initial body weight of 42 ± 2 g were randomly allocated to five treatments with four replications in a completely randomized design. The dietary treatments initiated on day 1 continued for 28 days and consisted of a basal diet supplemented with fermented synbiotic (FS) at levels of 0% (control), 0.25%, 0.50%, 0.75%, and 1.00%. The FS powder was mixed directly into the basal diet as a top-dressing and provided daily for the entire 28-day experimental period. Feed intake and body weight were recorded weekly. Parameters measured included feed consumption, body weight gain, feed conversion ratio, live weight, carcass percentage, abdominal fat percentage, income over feed cost, relative organ weights (liver, heart, gizzard, pancreas), small intestinal pH, and populations of lactic acid bacteria and *Salmonella typhimurium*. Results demonstrated that supplementation at 1.00% significantly improved body weight gain, feed conversion ratio, live weight, carcass percentage, and income over feed cost compared to the control group. Small intestinal pH decreased from 7.03 (control) to 4.56 (1.00% FS). Lactic acid bacteria populations increased significantly from 1.58 (control) to 3.83×10^6 CFU/g (1.00% FS), while *Salmonella typhimurium* counts were reduced from 0.97 (control) to 0.17×10^6 CFU/g (1.00% FS). No significant effects were observed on relative organ weights across all treatments compared to the control group. In conclusion, fermented synbiotics from pineapple peel and *Indigofera zollingeriana* leaves at 1.00% optimally enhanced growth performance, carcass quality, and intestinal health while improving economic returns in broiler chickens, offering a viable and sustainable alternative to antibiotic growth promoters.

Keywords: Amino acid, Broiler chicken, Fermented synbiotic, Growth performance, *Indigofera zollingeriana*, Pineapple peel

INTRODUCTION

In the past two decades, the use of functional feed additives in poultry nutrition has developed rapidly alongside increasing attention to digestive health and production efficiency. Synbiotics have gained considerable interest among feed additives. Synbiotics combine probiotics (live beneficial microorganisms) and prebiotics (substrates selectively utilized by host microorganisms) to improve gut microbiota balance,

enhance nutrient absorption, and suppress pathogenic colonization in poultry (Gibson et al., 2017). Prebiotics themselves have been shown to improve growth performance (Rehman et al., 2020), modulate the immune system (Al-Baadani et al., 2025), and enhance the quality of poultry products (Dong et al., 2024). Previous studies have reported that supplementation with conventional prebiotics such as fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS), and inulin can improve the

balance of intestinal microflora, suppress the colonization of pathogenic bacteria, and enhance feed conversion efficiency in broiler chickens (Pourabedin and Zhao, 2015; Ricke, 2018). Synbiotics offer the synergistic advantage of delivering both live beneficial bacteria and their specific substrates, thereby enhancing the survival and activity of probiotics in the gut (Śliżewska et al., 2020).

The use of synbiotics has become increasingly relevant following the ban on Antibiotic Growth Promoters (AGPs) in many countries due to the risks of antibiotic resistance and residues in animal products (Manyi-Loh et al., 2018). The AGP ban has driven the exploration of alternative prebiotic sources that are more economical and sustainable (Cheng et al., 2014). However, the high production costs of commercial synbiotics have encouraged the investigation of alternative sources, particularly from agricultural waste, to improve economic viability and sustainability.

Pineapple peel (*Ananas comosus*) is rich in pectin fiber, the bromelain enzyme, and oligosaccharides such as FOS, which can serve as prebiotic substrates (Hikal et al., 2021; Gómez-García et al., 2022). *Indigofera zollingeriana* leaves contain high crude protein and bioactive compounds but also contain tannins as anti-nutritional compounds that can reduce digestibility (Susanto et al., 2025). The fermentation process combining the two materials using a yogurt starter (containing *Lactobacillus delbrueckii subsp. bulgaricus* and *Streptococcus thermophilus*) produces a fermented synbiotic that contains both viable lactic acid bacteria (probiotics) and prebiotic oligosaccharides from the plant materials (Sah et al., 2016; Lu et al., 2024). Furthermore, fermentation by lactic acid bacteria produces tannase enzymes and creates an acidic environment (pH < 4.5), which hydrolyzes tannin-protein complexes and reduces anti-nutritional effects (Jeyakumar and Lawrence, 2022). The fermentable sugars and oligosaccharides from pineapple peel serve as an energy source that stimulates the growth and metabolic activity of lactic acid bacteria, thereby enhancing the bacterial production of tannase and organic acids (Parra-Matadamas et al., 2015). Consequently, the increased microbial activity facilitates more effective degradation of tannins present in *Indigofera zollingeriana* leaves compared to fermentation of *Indigofera zollingeriana* alone (García Méndez et al., 2021). The combination of pineapple peel and *Indigofera zollingeriana* leaves may yield a broader spectrum of prebiotic oligosaccharides compared to either substrate alone; pineapple peel provides FOS, while *Indigofera zollingeriana* leaves contribute other fermentable

carbohydrates, potentially enhancing selective stimulation of beneficial gut microbiota.

To date, studies on pineapple peel as a feed ingredient in poultry have been reported (Sukri et al., 2023; Abba et al., 2026), while *Indigofera zollingeriana* has been investigated as a feed supplement for chickens (Mohamad Termizi et al., 2024; Tanjung et al., 2025). However, studies on the synergistic effects of the fermented synbiotic combination of both on growth performance, carcass quality, and intestinal microbial populations in broiler chickens remain limited. Therefore, the current study aimed to evaluate the effects of dietary supplementation with a fermented synbiotic from pineapple peel and *Indigofera* leaves on growth, carcass characteristics, intestinal pH, and the populations of beneficial and pathogenic bacteria.

MATERIALS AND METHODS

Ethical approval

An animal feeding experiment was conducted at the experimental station, Department of Animal Science, Faculty of Agriculture, Universitas Sriwijaya, South Sumatra, Indonesia. The animals were cared for according to the Indonesian Institute of Sciences Animal Welfare Guidelines. The approval of the experiment was granted by Universitas Sriwijaya with approval number KPPHP-2021-1.

Experimental design and chickens preparation

The current study employed a completely randomized design (CRD) with five treatments and four replications. The treatments consisted of a basal diet (control) supplemented with fermented synbiotic from pineapple peel and *Indigofera* leaves (FS) at the following proportions, including 100% control diet (FS0), 0.25% FS ratio (FS25), 0.50% FS ratio (FS50), 0.75% FS ratio (FS75), and 1% FS ratio (FS100). A total of 100 one-day-old broiler chickens (DOC) of the Lohman MB-202 strain with an average initial body weight of 42 ± 2 g were randomly allocated into 20 litter-floor pens ($100 \times 100 \times 60$ cm), resulting in 5 broiler chickens per pen. Initial body weight was measured for all broiler chickens before the start of the experiment, and randomization was performed without any weight-based selection to avoid bias.

Upon arrival, all chickens received drinking water mixed with 5% sugar for the first four hours as a standard practice to aid recovery from transport stress by providing an immediate energy source. The same protocol was applied uniformly to all treatment groups. After four hours, the sugar water was replaced with plain drinking

water. A few hours later, the DOC was provided with the treatment diets sprinkled on paper for five days to familiarize them with the feed. Feed and drinking water were provided twice daily (morning and afternoon). The observation period lasted 28 days (4 weeks), with parameters including chicken body weight and treatment feed consumption measured weekly.

Diet and the synbiotic production process

The synbiotic powder used in the present study was produced through a liquid fermentation process combining pineapple peel (*Ananas comosus*) and *Indigofera zollingeriana* leaves, following the method described by Palupi et al. (2020) with modifications to ensure standardization.

Pineapple peel waste was collected from local farmers in Indralaya, South Sumatra, Indonesia, and *Indigofera* leaves were harvested from the experimental farm of the Department of Animal Science, University of Sriwijaya, Indralaya, Indonesia.

The yogurt starter used in the present study was a commercial product (Cimory, Indonesia). According to the product label, the yogurt contains a consortium of lactic acid bacteria, including *Lactobacillus delbrueckii* subsp. *Bulgaricus*, *Streptococcus thermophilus*, *Lactobacillus acidophilus*, and *Bifidobacterium* spp. The starter contained a high density of viable cells, typically expected in commercial yogurt products, which is sufficient to drive fermentation. To ensure standardization, all fermentation batches used the same product batch number, and the starter was added at a consistent volume of 300 mL per 12 L of fermentation medium.

The fermentation ingredients consisted of 5,640 g of ground pineapple peel, 360 g of ground *Indigofera* leaves, 300 mL of yogurt starter, 90 g of granulated sugar, and 12 L of distilled water. All materials were mixed in a 20-L fermentation jar, sealed, and incubated at room temperature ($\pm 28^{\circ}\text{C}$) for 72 hours, with daily stirring to maintain homogenization. After fermentation, the biomass was separated using a sterile strainer, oven-dried at 50°C for 48 hours, ground to pass a 120-mesh sieve, and stored in airtight containers at 4°C until diet preparation.

For dietary supplementation, the synbiotic powder was mixed into the basal diet as a top-dressing (added on top, not substituted for any basal ingredient such as corn or soybean meal) at levels of 0% (control), 0.25%, 0.50%, 0.75%, and 1.00% (w/w) and provided daily for the 28-day experimental period. The same basal diet was used for all treatments; only the FS supplementation level differed. The basal diets were commercial broiler starter

(days 1-21) and finisher (days 22-28) feeds obtained from PT Japfa Comfeed Indonesia Tbk. According to the manufacturer, the diets are formulated to meet or exceed the nutrient requirements for broiler chickens as recommended by the NRC (1994) and are suitable for the Lohman MB-202 strain. The nutrient composition of the basal diets is presented in Table 1.

Table 1. Nutrient composition of the commercial basal diets (as a feed basis) for starter and finisher

Nutrient	Starter feed (1-14 days of age, BR I)*	Finisher feed (15-28 days of age, BR II)*
Water content (%)	12	12
Crude protein (%)	22.00	20.00
Crude fiber (%)	5.00	5.00
Fat (%)	5.00	5.00
Ash (%)	7.00	7.00
Calcium (%)	1.00	0.90
Phosphorus (%)	0.50	0.45
Metabolizable energy (kcal/kg)	3,000	3,100
Aflatoxin ($\mu\text{g/kg}$)	50.00	50.00

*PT Japfa Comfeed Indonesia Tbk. Diets are formulated to meet NRC (1994) recommendations and are suitable for Lohman MB-202 broilers. BR I: Broiler starter feed (1-21 days), BR II: Broiler finisher feed (22-28 days).

Data collection and laboratory measurements

Feed consumption, body weight gain, and feed conversion ratio were measured following the methods described by Quintana-Ospina et al. (2023). Carcass percentage and abdominal fat were determined using the approach outlined by Maynard et al. (2023). The calculation of Income Over Feed Cost (IOFC) referred to the procedures reported by Anggitasari et al. (2016). Internal organ weights (liver, heart, gizzard, and pancreas) were measured according to Henry et al. (2023). The pH of small intestine digesta was determined following the protocols described by Yardimci et al. (2006) and Nkukwana et al. (2015). Intestinal bacterial populations (lactic acid bacteria and *Salmonella typhimurium*) were enumerated using the standard serial dilution and colony-forming unit (CFU) method on selective agar media (Makras et al., 2006; Kowalska et al., 2020).

Slaughter procedure

At the end of the 28-day experimental period, all broiler chickens were fasted for 6 hours before slaughter to ensure empty digestive tracts, with water provided *ad libitum*. Each broiler was manually restrained, and

slaughter was performed by severing the jugular veins and carotid arteries using a sharp knife (exsanguination). Broiler chickens were allowed to bleed for 2-3 minutes until death. The carcasses were then scalded in hot water at 55-60°C for 1-2 minutes, and feathers were removed manually. After defeathering, the carcasses were eviscerated, and the feet, chicken, and neck were removed.

Feed consumption

The investigated parameters included consumption of ration (g/chicken/day), which was measured based on the difference between the ration given (g) and the rest of the ration remaining (g) during a specific period (days; Quintana-Ospina et al., 2023).

Body weight gain

Body weight gain (g/chicken/day) was measured by weighing the difference between body weight at the end of the study (g) and the initial body weight (g), then dividing by the length of growing time (days; Quintana-Ospina et al., 2023).

Feed conversion ratio

Feed conversion ratio was measured based on the ratio between weight gain and feed consumption (Quintana-Ospina et al., 2023).

Live weight

The quality of the broiler carcass included the measurement of live weight, which was measured based on the results of weighing at the end of the study (Quintana-Ospina et al., 2023).

Carcas percentage

The percentage of the carcass was calculated based on the percentage of weight comparison of broilers without blood, feathers, chicken, legs, and digestive organs (g) divided by live weight (g). All 5 broilers per pen were used for carcass measurement (no broilers remained; Maynard et al., 2023).

Abdominal fat

Percentage of abdominal fat (%), which is calculated based on the percentage comparison between the weight of abdominal fat contained in the abdominal cavity and fat attached to the digestive organs (g) and the live weight of broilers (g; Maynard et al., 2023).

Income over feed cost

Income over Feed Cost (IOFC) was calculated based on the difference between the revenue obtained from the

sale of live broilers (IDR/kg live weight) and the feed cost incurred during the rearing period (IDR/kg live weight). The IOFC value represents the economic efficiency of feed utilization in generating profit. A higher IOFC indicates more optimal conversion of feed expenditure into income from broiler production (Anggitasari et al., 2016).

Internal organ weight

The percentage of internal organ weight was measured at the end of the rearing period. Organs assessed included the liver, heart, gizzard, and pancreas. The percentage was calculated by comparing the weight of each organ (g) to the live body weight of the broiler (g), and then multiplying the ratio by 100% (Henry et al., 2023). Relative organ development in relation to overall body growth is indicated by these percentages.

pH of small intestine digesta

The pH of small intestine digesta was measured in broilers at 28 days of age. Broilers were fasted for 6 hours before slaughter (Yardimci et al., 2006). After dissection, the small intestine was removed, and the digesta was collected into a container for pH determination using a pH meter. For measurement, 1 g of digesta was homogenized with 10 mL of distilled water. The electrode was rinsed, dried, and then immersed in the sample solution. Readings were taken once the values stabilized, and the pH was recorded.

Measurement of small intestinal bacteria

Samples for bacterial enumeration were collected from the same broilers used for pH measurement. Approximately 1 g of ileal digesta was serially diluted (10^{-1} to 10^{-6}) in sterile 0.85% NaCl (Reuben et al., 2019). Lactic acid bacteria (LAB) were enumerated by pouring 1 mL from dilutions 10^{-4} - 10^{-6} onto DeMan-Rogosa-Sharpe (MRS) agar (selective medium), followed by aerobic incubation at 37°C for 48 hours (Li et al., 2024). *Salmonella typhimurium* was quantified by spreading 100 µL from dilutions 10^{-1} - 10^{-3} onto Xylose Lysine Deoxycholate (XLD) agar (selective and differential), then incubating aerobically at 37°C for 24 hours (Velasquez et al., 2018). All bacterial counts were transformed to log₁₀ CFU/g before statistical analysis.

Statistical analysis

All data were analyzed using SPSS software (version 20), based on the design used. Data were subjected to one-way analysis of variance (ANOVA). When a significant difference was detected among treatment

means, further comparison was performed using Duncan's multiple range test (Steel and Torrie, 1980). The significance level was set at less than 0.05.

RESULTS AND DISCUSSION

Production performance

Feed consumption showed a gradual increase with the rising level of FS (Table 2). Although the average consumption (76.62-77.11 g/broiler/day) was lower compared to studies using pure prebiotics (Rehman et al., 2020; Xiao et al., 2024), the increasing pattern at levels of 0.75% and 1.00% indicated an improvement in palatability. The primary mechanism involved is the degradation of tannins during the fermentation process. Tannins are known to have astringent properties that reduce palatability by binding to salivary proteins and taste receptors (Wina et al., 2005). Fermentation by lactic acid bacteria (LAB) has been shown to degrade tannins into phenolic compounds with lower molecular weights that are no longer astringent (Mullen et al., 2015). Furthermore, organic acids, particularly lactic acid produced during fermentation, impart an acidic taste associated with a drop in pH, thereby helping to inhibit the growth of pathogenic bacteria in the digestive tract (Gao et al., 2021). Acidic conditions are expected to increase feed intake due to the accelerated rate of degradation.

The significant increase in daily body weight gain (BWG) and decrease in feed conversion ratio (FCR) at the 1.00% FS level indicate an enhanced efficiency of nutrient utilization. The mechanism is explained through two synergistic actions of the components from pineapple peel and *Indigofera* leaves. Pineapple peel contains FOS, which functions as a selective prebiotic, while the viable LAB from the yogurt starter acts as a probiotic, together forming a fermented synbiotic (Gibson et al., 2017; Yang et al., 2025). Fructo-oligosaccharides are not digested in the small intestine but are fermented by the colonic microbiota, primarily *Bifidobacterium* and *Lactobacillus* (Ricke, 2018). Fermentation of FOS produces short-chain fatty acids (SCFAs) that lower the intestinal pH, thereby inhibiting the proliferation of pathogenic bacteria such as *Salmonella* and *Escherichia coli*, while simultaneously stimulating the proliferation and differentiation of intestinal villous epithelial cells (Wu et al., 2022). An increased ratio of villus height to crypt depth significantly expands the surface area for nutrient absorption (Awad et al., 2008).

Additionally, *Indigofera* leaves are rich in protein, but their tannin content constitutes a major anti-nutritional

factor (Choi and Kim, 2020). Tannins can form insoluble complexes with feed proteins and digestive enzymes, such as trypsin and pepsin, thereby reducing the digestibility of protein and amino acids (Wina et al., 2005). The fermentation process with a consortium of LAB breaks down tannin-protein bonds through the activity of tannase enzymes and the acidic conditions (Jiménez et al., 2014; Jeyakumar and Lawrence, 2022). This degradation liberates the bound protein and amino acids, increasing their availability for absorption in the small intestine (Mullen et al., 2015). Thus, fermentation transforms *Indigofera* leaves from a protein source with limited availability into a more readily digestible protein source. The synergy between the prebiotic action of FOS from pineapple peel and the enhanced nutrient availability from *Indigofera* leaves explains the improvement in growth performance and feed efficiency at higher FS levels. This synergistic action is supported by studies demonstrating that synbiotic supplementation (combining a prebiotic such as FOS with a probiotic, such as *Lactobacillus plantarum*) results in superior outcomes. For instance, Song et al. (2022) reported that a synbiotic composed of *Lactobacillus plantarum* and FOS significantly improved average daily gain and the digestibility of calcium and phosphorus in broilers, while Soumeh et al. (2019) found that adding MOS to fermented soybean meal had synergistic effects, leading to greater body weight gain and enhanced intestinal morphology. The increased lactic acid bacteria populations, decreased intestinal pH, and improved nutrient digestibility observed in the present study collectively support the proposed dual mechanism of action.

The increase in final live weight and carcass percentage at the high supplementation levels (0.75-1.00%) reflected the efficiency of nutrient utilization. The synergy between the enhanced intestinal absorption capacity through FOS and the increased availability of protein substrate from *Indigofera* leaves, liberated from tannin complexes, directs energy and amino acids more efficiently towards the anabolic pathways of muscle protein synthesis, rather than storage as adipose tissue or utilization for basal metabolic requirements. The improved digestibility and nutrient availability, particularly the balance of essential amino acids, optimize the effective energy-to-protein ratio in the diet, thereby reducing excess energy that could potentially be deposited as fat (Leeson and Summers, 2005; Musigwa et al., 2024).

From an economic perspective, the significant increase in Income Over Feed Cost (IOFC) in the FS75 and FS100 treatments indicated that the additional cost

incurred by FS supplementation was offset by the increased revenue from chickens with higher live weights and carcass yields. The significantly higher final body weight and carcass yield in the FS75 and FS100 groups support [Bollido \(2020\)](#), who demonstrated that direct profitability is largely determined by final body weight gain. Thus, despite the increase in feed consumption, the

proportional increase in body weight and carcass quality resulted in a positive net financial return. Overall, FS supplementation at the 1.00% level was not only effective in leveraging the prebiotic FOS and the probiotic LAB to enhance biological performance but also proved to be economically viable within a commercial broiler production system.

Table 2. Production performance of broiler chickens as affected by dietary fermented synbiotic supplementation

Variables	Treatments				
	FS ₀	FS ₂₅	FS ₅₀	FS ₇₅	FS ₁₀₀
Feed consumption (g/chicken/day)	76.62 ± 1.48 ^a	76.70 ± 1.63 ^{ab}	76.94 ± 0.47 ^b	77.05 ± 1.45 ^{bc}	77.11 ± 1.66 ^c
Body weight gain (g/chicken/day)	56.12 ± 1.78 ^a	56.57 ± 0.58 ^a	57.04 ± 1.50 ^a	57.40 ± 0.77 ^a	59.69 ± 0.41 ^b
Feed conversion ratio (FCR)	1.58 ± 0.10 ^b	1.55 ± 0.05 ^b	1.52 ± 0.03 ^{ab}	1.50 ± 0.08 ^{ab}	1.42 ± 0.04 ^a
Live weight (g)	1,625.95 ± 42.50 ^a	1,673.00 ± 51.73 ^{ab}	1,687.10 ± 7.93 ^b	1,699.25 ± 20.90 ^b	1,724.00 ± 39.08 ^b
Carcass percentage (%)	71.99 ± 0.90 ^a	72.72 ± 2.85 ^a	72.21 ± 2.21 ^a	74.35 ± 0.91 ^a	77.76 ± 1.60 ^b
Abdominal fat (%)	1.23 ± 0.22 ^a	1.30 ± 0.25 ^a	1.26 ± 0.27 ^a	1.09 ± 0.17 ^a	1.01 ± 0.18 ^a
Income over feed cost (USD)	0.52 ± 0.05 ^a	0.58 ± 0.06 ^{ab}	0.59 ± 0.01 ^{ab}	0.60 ± 0.02 ^b	0.62 ± 0.05 ^b

FS₀: Control ratio, FS₂₅: 0.25% FS ratio, FS₅₀: 0.50% FS ratio, FS₇₅: 0.75% FS ratio, FS₁₀₀: 1% FS ratio. Different superscripts in the same row indicate significant differences ($p < 0.05$)

Organs and the digestive tract

The results showed that supplementation with fermented prebiotics from pineapple peel and *Indigofera zollingeriana* leaves (FS) did not have a significant effect on the relative weights of internal organs, including the liver, heart, gizzard, and pancreas (Table 3; $p > 0.05$). The finding indicated that the supplementation strategy did not impose a physiological burden or toxic effects on these vital organs. Specifically, the relative liver weight ranged from 2.13-2.34%, which falls within the normal range (2.5-3.5%) for healthy broiler chickens ([Iqbal et al., 2014](#)). The relative weights of the heart (0.37-0.47%), gizzard (1.92-2.09%), and pancreas (0.19-0.25%) were also consistent with standards reported in the previous studies ([Emiola et al., 2007](#); [Hafsan et al., 2021](#)).

The stability of these internal organ weights can be explained by the indirect mechanism of synbiotic action. FS supplementation, particularly at higher levels, increased the population of LAB and decreased the pH of the small intestine (Table 3). A more optimal intestinal environment supports the efficiency of digestion and nutrient absorption, thereby preventing accessory digestive organs such as the liver and pancreas from undergoing hypertrophy due to nutrient deficiencies or the toxic burden from pathogens ([Lan et al., 2022](#); [Ringseis and Eder, 2022](#)). Furthermore, the fermentation process that degrades tannins in *Indigofera* leaves plays a crucial role

in maintaining organ function, as dietary tannins have been shown to induce hepatic inflammation ([Yuan et al., 2023](#)) and reduce the relative weight of vital organs, including the liver, bursa of Fabricius, and spleen ([Choi and Kim, 2020](#); [Hidayat et al., 2021](#)). Undegraded tannins can form complexes with proteins and minerals, potentially inducing oxidative stress and burdening the detoxification function of the liver ([Wina et al., 2005](#)). By reducing tannins through fermentation, the risk of anti-nutritional effects can be minimized, thereby maintaining stable organ function and morphology, as reported in other studies using natural ingredients ([Choi and Kim, 2020](#); [Mahawan et al., 2025](#)).

Compared to the control group, FS supplementation at 0.75% and 1.00% significantly decreased small intestinal pH, and supplementation at 0.50%, 0.75%, and 1.00% significantly increased LAB populations ($p < 0.05$). Small intestinal pH decreased significantly from a neutral condition (7.03 in the control) to a more acidic one (4.56 in FS100), indicating increased fermentative activity by LAB ($p > 0.05$). The pH reduction is driven by two synergistic factors; the FOS substrate from pineapple peel fermented by LAB produces lactic acid as the main product ([Cao et al., 2019](#); [Yang et al., 2025](#)), and the inoculation of active LAB from the fermented product, which used yogurt as a starter, means that viable LAB continue to contribute to organic acid production in the

digestive tract (Ayivi and Ibrahim, 2022; Li et al., 2024). The decrease in pH creates a selective environment that inhibits the growth of pathogens sensitive to acidic conditions, such as *Salmonella*, while simultaneously supporting the proliferation of LAB (Neal-McKinney et al., 2012; El Baaboua et al., 2018). A lower pH condition enhances the activity of endogenous digestive enzymes and improves intestinal mucosal health (Ma et al., 2021; Han et al., 2023).

The LAB population was significantly higher in the FS50, FS75, and FS100 groups compared to FS0 and FS25 ($p < 0.5$), with no significant differences among FS50, FS75, and FS100 ($p > 0.05$). The highest LAB population (3.83×10^6 CFU/g) was observed in FS100. This increase confirmed the effectiveness of FS as a prebiotic substrate, where FOS from pineapple peel serves as a selective "food" for indigenous LAB (Al-Khalaifah, 2018; Kaewarsar et al., 2023). Furthermore, the reduction in tannin content due to fermentation also supports LAB colonization, as tannins are known to possess antimicrobial activity that can inhibit the growth of both beneficial and pathogenic bacteria (Reverón et al., 2017; Jeyakumar and Lawrence, 2022). Reducing tannin content through fermentation removes an inhibitory barrier, thereby allowing LAB to colonize more effectively and

compete for nutrients and adhesion sites (Zhang et al., 2023). The increase in the LAB population creates a biological barrier effect through competitive exclusion of pathogens (Adeyemi and Nahashon, 2026). Conversely, the *Salmonella typhimurium* population decreased drastically with increasing FS levels, from 0.97×10^6 CFU/g (control) to 0.17×10^6 CFU/g (FS100). The suppression of *Salmonella typhimurium* results from a multifaceted assault by LAB, supported by the modified intestinal environment. The decreased pH is bactericidal or bacteriostatic to enteropathogens (Ringseis and Eder, 2022), while LAB produce antimicrobial metabolites such as bacteriocins, hydrogen peroxide, diacetyl, and reuterin that specifically inhibit pathogens (Chen et al., 2025). Additionally, LAB compete with pathogens for nutrients and adhesion sites on the intestinal epithelium (Hammami et al., 2019) and produce SCFAs from FOS fermentation, which serve as an energy source for colon epithelial cells, strengthening tight junction integrity and reducing pathogen translocation (Lan et al., 2022). Thus, FS supplementation not only increases the LAB population and decreases small intestinal pH but also can effectively suppress *Salmonella typhimurium* colonization through comprehensive biological mechanisms.

Table 3. Effect of fermented synbiotic supplementation on digestive organ weights, intestinal pH, and microbial populations in broiler chickens

Variables	Treatments				
	FS ₀	FS ₂₅	FS ₅₀	FS ₇₅	FS ₁₀₀
Liver weight percentage (%)	2.34 ± 0.34 ^a	2.20 ± 0.16 ^a	2.54 ± 0.22 ^a	2.13 ± 0.19 ^a	2.28 ± 0.42 ^a
Heart weight percentage (%)	0.42 ± 0.02 ^a	0.37 ± 0.07 ^a	0.44 ± 0.01 ^a	0.40 ± 0.12 ^a	0.47 ± 0.04 ^a
Gizzard weight percentage (%)	2.04 ± 0.34 ^a	2.09 ± 0.15 ^a	2.08 ± 0.50 ^a	1.92 ± 0.16 ^a	1.99 ± 0.24 ^a
Pancreas weight percentage (%)	0.25 ± 0.05 ^a	0.19 ± 0.06 ^a	0.23 ± 0.02 ^a	0.24 ± 0.02 ^a	0.25 ± 0.03 ^a
Small intestinal pH	7.03 ± 0.38 ^b	6.64 ± 0.88 ^b	6.46 ± 0.18 ^b	5.10 ± 0.33 ^a	4.56 ± 0.36 ^a
Lactic acid bacteria ($\times 10^6$ CFU/g)	1.58 ± 0.49 ^a	2.37 ± 0.23 ^a	3.49 ± 0.34 ^b	3.53 ± 0.20 ^b	3.83 ± 0.56 ^b
<i>Salmonella typhimurium</i> ($\times 10^6$ CFU/g)	0.97 ± 0.24 ^b	0.85 ± 0.43 ^b	0.67 ± 0.20 ^b	0.20 ± 0.10 ^a	0.17 ± 0.06 ^a

FS0: Control ratio, FS25: 0.25% FS ratio, FS50: 0.50% FS ratio, FS75: 0.75% FS ratio, FS100: 1% FS ratio. Different superscripts in the same row indicate significant differences ($p < 0.05$).

CONCLUSION

Supplementation with fermented synbiotics from pineapple peel and *Indigofera zollingeriana* leaves at high levels (1.00%) was shown to improve growth performance, carcass quality, and income over feed cost (IOFC), while also decreasing small intestinal pH, increasing the lactic acid bacteria (LAB) population, and suppressing pathogenic bacterial growth without affecting relative organ weights. Therefore, this supplementation

value is recommended to achieve maximum biological and economic benefits with minimal negative impacts. However, the short duration (28 days) and controlled experimental conditions limit the generalizability of these findings to long-term commercial production systems. Future studies should investigate the underlying molecular mechanisms of the synbiotic's action, including its effects on intestinal gene expression and immune modulation. Long-term feeding trials under commercial conditions are

also recommended to confirm the economic benefits and assess the stability and shelf life of the synbiotics.

DECLARATIONS

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Authors' contributions

Rizki Palupi provided the original text, contributed to the experimental design, carried out the experiments, and conducted the statistical analysis. Sofia Sandi and Fitra Yosi contributed to both the statistical analysis and the design of the experiments. Anggriawan Naidilah Tetra Pratama and M. Akbar Wijaya Fikri assisted in carrying out the experiments and collecting the data. All authors analyzed and reviewed the results and then composed the final manuscript. All authors have reviewed and approved the final edition of the manuscript for publication.

Availability of data and materials

The data obtained in the present study can be obtained from the corresponding author upon reasonable request.

Competing interests

The authors have declared no competing interests.

Ethical considerations

Plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy have been checked by all the authors. During the preparation of this work, the authors used Grammarly solely for language and grammar improvement. No generative artificial intelligence tools were used to generate data, results, or conclusions. The authors take full and sole responsibility for all use of artificial intelligence in the preparation of this manuscript. The authors further affirm that they are solely responsible for the accuracy, originality, and integrity of the final content both before initial submission, during the revision process, and upon final publication. The authors have checked and verified all scientific data, interpretations, and conclusions without reliance on AI for intellectual content.

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Prevalence of Coccidiosis in Broiler Chickens in Djôrô Region, Southwestern Burkina Faso

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ABSTRACT

In Burkina Faso, the poultry industry faces significant health challenges, notably coccidiosis, an intestinal parasitic disease that results in substantial economic losses. This cross-sectional clustered study aimed to determine the prevalence and oocyst load of *Eimeria* (*E.*) spp. in litter-derived fecal samples from broiler chickens in Batié, Dano, Diébougou, and Gaoua in the Djôrô region of Burkina Faso. The study was conducted from June to September 2024. A total of 600 fresh fecal samples were collected from broiler chickens aged 35 to 45 days, originating from 30 traditional and 30 modern farms across the Djôrô region. The droppings were collected from litter, placed in airtight plastic bags, and stored in cooling boxes at 4°C, then transferred to the Laboratory of Animal Biology and Physiology at the Gaoua University Center, Burkina Faso, for subsequent parasitological analysis. Samples were examined using a modified McMaster flotation procedure with saturated sodium chloride, and oocyst counts were determined. For postmortem examination, 25 broiler chickens were randomly selected from a local poultry market in Gaoua, Burkina Faso, then slaughtered and plucked on-site the same day. The plucked, uneviscerated carcasses were then stored in cooling boxes at 4°C, and the intestinal tracts were later analyzed at the laboratory for macroscopic lesion identification and site localization. Out of the 600 samples analyzed, 439 (73.17%) tested positive for *Eimeria* spp. oocysts. Positivity rates ranged from 62.7% in Dano city to 92.0% in Diébougou city, with Batié and Gaoua at 72.7% and 65.3%, respectively. Diébougou city had the highest mean oocyst count (40,310.67 ± 2,863.40 OPG). Descriptively, traditional farms had higher oocysts per gram of feces (OPG) values than modern farms. Postmortem examination identified *E. acervulina* (13/25), *E. maxima* (8/25), and *E. necatrix* (4/25) in the intestinal tracts of chickens obtained from the Gaoua slaughter market. Nevertheless, postmortem findings were not generalized to the Djôrô region. The current results demonstrated the substantial *Eimeria* contamination on broiler farms in the Djôrô region, Burkina Faso, and supported the need for improved litter management, biosecurity, and farmer training on coccidiosis prevention.

Keywords: Broiler chicken, Coccidiosis, *Eimeria* spp., Oocyst, Prevalence

INTRODUCTION

In Burkina Faso, cattle and poultry farming significantly affects livelihoods, contributing between 71 and 115 million USD to the economy and playing a crucial role in food security and poverty alleviation (FAO, 2018). The poultry sector involved 98% of rural households and comprised 46 million chickens in 2021 (MARAH, 2022; Dione et al., 2025). However, diseases such as avian coccidiosis are a major constraint to the development of poultry production. Coccidiosis is an enteric disease caused by an intracellular

protozoan parasite of the genus *Eimeria* (*E.*), family *Eimeriidae* (Taylor et al., 2015). In domestic chickens (*Gallus gallus domesticus*), five *Eimeria* spp. are considered the most pathogenic, including *E. acervulina*, *E. brunetti*, *E. maxima*, *E. necatrix*, and *E. tenella*, which cause significant economic losses (El-Shall et al., 2022). Animals are mainly infected orally through contaminated food, water, or litter containing sporulated oocysts of *Eimeria* species. Following ingestion, sporozoites are released into the host's intestine (Abd El-Ghany, 2021). The life cycle of *Eimeria* spp.

comprises three main phases, including sporogony, which occurs in the external environment, followed by schizogony and gametogony, both of which occur within the epithelial cells lining the host's intestine (López-Osorio et al., 2020; McDougald et al., 2020). Each *Eimeria* spp. has its own intestinal tract localization preference, resulting in different clinical signs in affected chickens (Liao et al., 2024). Clinical signs range from mild subclinical to severe, depending on the *Eimeria* spp., infection level, host age, and immune status (El-Shall et al., 2022). The most common signs of *Eimeria* infection in chickens include huddling and feather ruffling, dehydration, decreased feed consumption, reduced weight gain or egg production, and hemorrhagic diarrhea with fresh or digested blood and mucus (Abd El-Ghany, 2021). Disease control mainly relies on chemoprophylactic compounds such as amprolium (Snyder et al., 2021). However, anticoccidial resistance and toxicity to non-target organisms have been increasingly reported (Mooney et al., 2020; Liao et al., 2024), driving continued investigation into alternatives such as vaccination and herbal remedies (Ghafouri et al., 2023; Bai et al., 2026). Coccidiosis, endemic in tropical and subtropical regions, has impacted the commercial broiler industry worldwide (Alito, 2023). The economic impact is substantial, with annual losses due to coccidiosis estimated at 2.4-3 billion USD (Badri et al., 2024). A recent study in Bobo-Dioulasso, located in the Guiriko region of western Burkina Faso, reported a predominance of *Eimeria* spp. (53.13%) among gastrointestinal parasites in peri-urban local chickens, followed by *Heterakis* spp. (43.13%) and *Ascaris* spp. (0.63%; Zongo et al., 2026). However, data on coccidiosis in broiler chicken farms within the Djôrô region of southwestern Burkina Faso remain scarce. The Djôrô region is one of the wettest regions of Burkina Faso and a vital source of agricultural, forestry, and pastoral potential (Thienon, 2025; ANAM-BF, 2026). Therefore, the present study aimed to determine the prevalence of *Eimeria* oocyst contamination in litter-derived fecal samples from broiler chicken farms across the Djôrô region of Burkina Faso.

MATERIALS AND METHODS

Ethical approval

The present study followed animal welfare guidelines and protocols at the University Center of Gaoua in Gaoua, Burkina Faso. The present study used field-reared broiler and market chickens, with licensed veterinarians supervising all procedures and farm activities. Informed consent was obtained from all poultry owners before sample collection.

Study location and duration

The present study was carried out from June to September 2024 on peri-urban chicken farms in the cities of Diébougou, Dano, Gaoua, and Batié, capitals of the provinces of Bougouriba, Ioba, Poni, and Nounbiel, respectively, within the Djôrô region of Burkina Faso. The Djôrô region is located between isohyets of 1,000 and 1,200 mm and has an annual rainfall of 1,071.09 mm from June to October. The dry season starts from November to April, with temperatures ranging from 17°C to 36°C. These environments, along with the predominance of Savannah, gallery forests, and open woodlands, make the Djôrô region suitable for crop farming and animal husbandry (Thienon, 2025; ANAM-BF, 2026). The Djôrô region ranks among the greenest areas of Burkina Faso and supports a wide diversity of plant species. Cultivated crops include cereals such as sorghum, millet, maize, and rice, cash crops including cotton, cashew, and sesame, and food crops such as yam, cowpea, and sweet potato. Fruit tree cultivation is also significant, with mango, banana, papaya, and citrus widely grown (Bene and Fournie, 2012).

Study design

A cross-sectional, clustered study was conducted across 60 selected poultry farms in the cities and peri-urban areas of Gaoua, Diébougou, Batié, and Dano. The inclusion criterion was farmers' consent to participate after the study objectives were explained. Sampling was conducted on 30 modern and 30 traditional broiler chicken farms, with broiler chickens aged 35 to 45 days. A certified veterinarian examined all farms to assess chicken health status and prevent infectious diseases. Modern poultry farms were characterized by medium- to high-level biosecurity measures, such as vector- and pest-proofing, strict visitor control, routine use of footbaths and farm clothes, and fully enclosed houses with litter floors. In this farming system, chickens received commercially balanced feeds, such as Galdus and The Koudijs Broiler Concentrate (KBC), produced by Koudijs Animal Nutrition (the Netherlands) and customized for different ages and production targets. The ingredient composition of Galdus, as indicated on the product packaging, included maize, cooked soybean meal, wheat, corn gluten, rapeseed meal, oat hulls, wheat screenings, chemically refined soybean oil, physically refined palm kernel fatty acid distillates, feed phosphate, calcium carbonate, fish meal, crude lecithins, sodium chloride, and sodium bicarbonate. According to the product packaging, the Koudijs broiler concentrate consisted of concentrated plant proteins derived from soybean meal, a mineral mixture (phosphate, calcium carbonate, sodium

chloride), a vitamin premix (Vitamins A, D₃, E, B), and trace elements (iron sulfate, zinc oxide, calcium iodate, manganese sulfate, selenium), as well as synthetic amino acids including L-lysine, DL-methionine, and threonine. The Koudijs broiler concentrate was incorporated into diets formulated with locally available cereals and oilseed by-products, including maize, soybean, cottonseed meal, and wheat bran. In addition to the basal diets, the poultry received the nutritional complex Amin'Total (Laprovét, France) and the vitamin supplement Genevits (Granovit AG, Switzerland). In modern poultry farms, anticoccidials (Amprolium 20%, Laprovét, France) were routinely used either in feed or water. In contrast, traditional poultry farms used simple, open-sided shelters with compacted dirt floors, bare earth, or straw or mixed organic waste. This farming system was characterized by free-range scavenging, including insects, seeds, household scraps, supplemented with grains such as maize or sorghum. Depending on farmers' financial capacity, poultry received supplementary inputs such as soybean meal, cottonseed meal, fish meal, and the nutritional complexes Amin'Total (Laprovét, France) and Genevits (Granovit AG, Switzerland). Anticoccidials were rarely or never used on traditional farms (Tapsoba et al., 2024; Dione et al., 2025). Generally, the Marek's disease vaccine was administered at the hatchery using Nobilis Rismavac (MSD Animal Health, The Netherlands) via subcutaneous injection. Subsequently, chickens were vaccinated against Newcastle disease on day 7, with a booster given on day 18, utilizing Nobilis ND (MSD Animal Health, The Netherlands) via drinking water. Gumboro vaccination was administered on day 21 using either Nobilis Gumboro (MSD Animal Health, The Netherlands) via drinking water or Cevac Transmune IBD (Ceva Santé Animale, France) through subcutaneous injection in modern and traditional farms.

Sample size

The estimated sample size was calculated using Formula 1 (Stevenson, 2021).

$$n - \text{clustered} = n - \text{srs} \times \text{DEFF} \quad (\text{Formula 1})$$

where $n - \text{srs}$ was the base sample size using a simple random sample, and DEFF was the design effect.

The Thrusfield formula was used to calculate the simple random sample as follows (Thrusfield, 2005; Pearl, 2006).

$$n - \text{srs} = 1.96^2 \times \text{Pexp} (1 - \text{Pexp}) / d^2 \quad (\text{Formula 2})$$

where n was the required sample size, Pexp was the expected prevalence (0.53; Zongo et al., 2026), d was the

desired absolute precision (0.05), and 1.96 was the constant for a 95% confidence interval.

$$n - \text{srs} = ([1.96]^2 \times 0.53 [1 - 0.53]) / (0.05)^2 = 383$$

The design effect was calculated using Formula 3.

$$\text{DEFF} = 1 + (m - 1) \times \rho \quad (\text{Formula 3})$$

With m as the average cluster size (10) and the intra-cluster correlation coefficient (ICC), ρ , as 0.05.

$$\text{DEFF} = 1 + (10 - 1) \times 0.05 = 1.45$$

Finally, the sample size was estimated using Formula 1.

$$n - \text{clustered} = n - \text{srs} \times \text{DEFF} = 383 \times 1.45 = 555$$

The study set the sample size at 555, while 600 broiler chicken feces were sampled to maximize precision.

Due to limited resources, only 25 randomly selected slaughtered and plucked broiler chickens were collected from the chicken slaughter market in Gaoua, Burkino faso, supplied by nearby poultry farms.

Fecal sample collection

Freshly deposited fecal samples were collected early in the morning from different areas of the litter using clean disposable gloves, ensuring that samples were not intentionally mixed. Each sample was placed in a labeled plastic bag indicating the date, farm code, and city, and kept at 2-4°C during transport. Subsequently, the samples were stored at 4°C until analysis. Fecal appearance was recorded descriptively according to consistency and visible abnormalities, including formed/normal, watery, mucoid, or blood-stained material. The chickens on the farms were aged 35 to 45 days (approximately 5 to 6 weeks). Farmers collected feces only from enclosures housing poultry that had reached sale age, as collecting feces from younger chickens could induce stress.

Fecal sample examination

Eimeria spp. oocysts were detected and quantified using a modified McMaster flotation procedure adapted from the study of Conway (2007). Briefly, 3 g of feces were homogenized with 42 mL of a 30% sodium chloride flotation solution, filtered through gauze-lined fine strainers, and mixed well. The suspension was then transferred into the two chambers of a McMaster slide (1 mL in each) and left for about 15 minutes before microscopic analysis. Afterward, the McMaster slide was examined at 40 and 100x magnification using an optical microscope (Labomed, USA). Oocysts of *Eimeria* spp. were identified by size, shape, presence or absence of a micropyle, and sporulation time, and expressed as oocysts per gram of feces (OPG; Haug et al., 2008; Carvalho et al., 2011).

Postmortem sampling and examination

A total of 25 broiler chickens were randomly selected and purchased from the chicken market in Gaoua, Burkina Faso, supplied by nearby peri-urban traditional and modern poultry farms. These chickens, aged 35 to 45 days, were fed locally available cereals, including maize and millet, and had ad libitum access to water. No therapeutic treatments or vaccinations were administered to these chickens. Gaoua, in the Poni Province of the Djôrô Region of Burkina Faso, was chosen because it was close to the laboratory at the University Center of Gaoua, Burkino faso. Postmortem samples were used only to identify the *Eimeria* spp. present in the slaughtered chickens collected from the market of Gaoua city, because farmers preferred to sell their chickens to professional poultry dealers or processors. The selected chickens were then slaughtered and plucked on-site the

same day. The time between slaughter and collection was approximately 15 minutes. The plucked, non-eviscerated chickens were collected in clean, labeled polythene bags and transported in cooling boxes (2°C-4°C) to the laboratory at the University Center of Gaoua, Burkina Faso, for necropsy. The duodenum (upper small intestine), mid-intestine, distal intestine, and ceca were carefully opened and examined for gross lesions including mucosal thickening, petechiae, necrosis, whitish plaques, cecal cores, and hemorrhage (Nana-Mariam et al., 2023). Lesion severity was graded from 0 to 4 based on the Johnson and Reid scale. Score 0 indicated no visible lesion, 1 indicated very mild, 2 indicated moderate, 3 indicated severe, and 4 indicated extremely severe lesions (Johnson and Reid, 1970).



Figure 1. Study location in the provinces of Poni, Nounbiel, Ioba, and Bougouriba in the Djôrô region, Burkina Faso

Data analysis

All statistical analyses were conducted utilizing R software version 4.5.1 (R Core Team, 2025). Descriptive statistics were employed to determine the means and standard errors of the OPG. The χ^2 test was used to compare qualitative parameters, including breeding system, sampling sites, and feces appearance. The normality of the distributions for quantitative data was assessed via the Shapiro-Wilk test. Comparisons of OPG means were carried out using the nonparametric Kruskal-Wallis test, followed by Dunn's post hoc test with a Bonferroni correction to account for multiple comparisons. The threshold for statistical significance was established at a p-value less than 0.05.

RESULTS AND DISCUSSION

Prevalence in farms

In the Djôrô region of Burkina Faso, 73.17% of fecal samples from broiler chicken litters were infected with *Eimeria* species. Broiler chickens are more likely to have higher *Eimeria* spp. infection rates, and insufficient biosecurity measures can contribute to this high prevalence (Hofmann et al., 2020; Begum et al., 2024). The use of poor-quality veterinary medicines has increased across West Africa (Teko-Agbo et al., 2011; Dembele et al., 2026). A study in Burkina Faso found that 53% of veterinary antiparasitic medicines sold in official and unofficial markets failed to meet quality standards (Teko-Agbo et al., 2011). Farmers' low skill level, combined with the poor quality of veterinary antiparasitic medicines, may have contributed to the high oocyst load observed in farms in the present study. A study on broiler chickens from Pichincha and Santo Domingo de los Tsáchilas provinces in Ecuador has indicated a higher prevalence of coccidiosis (100%) than the present result, using flotation and McMaster techniques (Cevallos-Gordon et al., 2024).

In the present study, chickens were aged 35-45 days old. Several studies have reported the importance of chicken age in susceptibility to natural *Eimeria* spp. infection. An investigation in Medea province, Algeria, found that broiler chickens aged 31 to 40 days (about 4 to 6 weeks) had the highest coccidial prevalence (24.8%) (Debbou-Iouknane et al., 2018). In Swabi district, Pakistan, a study using the fecal flotation technique reported the highest *Eimeria* spp. infection rate (55.5%) in chickens aged 4-6 weeks (Begum et al., 2024). Likewise, in Borno State, Nigeria, the prevalence of coccidiosis was higher in chickens aged less than 8 weeks (70.3%) using the same fecal flotation diagnostic technique (Lawal et al.,

2025). These results were consistent with the present study, indicating that the highest *Eimeria* spp. infection rate was observed in broiler chickens aged between 4 and 8 weeks. A study in Medea province, Algeria, found that younger flocks (10 to 20 days old) had the highest oocyst counts (19,930 OPG) from fecal samples collected on broiler farms (Bachene et al., 2026). This finding indicated that peaks in oocyst shedding occur earlier and differ by *Eimeria* spp. and host age. All reported results reflected peaks in oocyst shedding in broiler chickens, typically occurring between 28 and 35 days after infection (Williams, 2005).

The current study was performed between June and September, which period corresponded to the rainy season in Burkina Faso (ANAM-BF, 2026). Increased rainfall, ambient temperatures (25°C to 30°C), and humidity fostered optimal conditions for sporulation, survival, and sustained transmission of coccidian oocysts in poultry flocks, leading to a high infection rate (Lawal et al., 2025). Consistent with the present study, Lawal et al. (2025) conducted a study in Borno State, North-eastern Nigeria, under the same environmental conditions and using the same diagnostic technique to explore the effects of seasonal differences in coccidiosis in chickens. This seasonal effect can influence the occurrence and spread of avian coccidiosis outbreaks (Parveen et al., 2021). Moreover, several studies that collected fecal samples to evaluate coccidiosis in broiler chickens have reported lower prevalence rates than the present results. Bachene et al. (2026) recorded a prevalence rate of 42.9% in Medea province, Algeria, an area characterized by a Mediterranean climate with both continental and orographic influences. In the Swabi district, Pakistan, with a humid subtropical climate, 53.63% of fecal samples tested positive for *Eimeria* spp. (Begum et al., 2024). Similarly, coccidiosis prevalence among broiler chickens in the Tarai region of Uttarakhand, India, characterized by a tropical monsoon and subtropical climate, was 60% (Pant et al., 2018; Bora et al., 2024). In Gwallameji, Bauchi State, Nigeria, coccidiosis prevalence reached 46.7% in a tropical savannah climate with distinct wet and dry seasons (Yaro et al., 2025). These differences might be attributed to the distinct agro-ecological zones in different countries and regions.

At sampling sites in the Djôrô region of Burkina Faso, farm-level contamination frequency differed significantly ($p < 0.05$), with rates of 92% in Diébougou city, 72.7% in Batié, 65.3% in Gaoua, and 62.7% in Dano region (Table 1). Notably, Diébougou region had a significantly higher average oocyst load ($40,310.67 \pm 2,863.40$ OPG) than

Batié ($15,612.00 \pm 1,578.34$ OPG), Dano ($14,734.67 \pm 1,397.11$ OPG), and Gaoua region ($13,115.33 \pm 1,461.06$

OPG; $p < 0.001$; Table 1; Figure 2).

Table 1. Prevalence and oocyst loads (oocysts per gram) of *Eimeria* spp. in broiler chickens in the Djôrô region of Burkina Faso, 2024

Sampling Site	Number of positive samples and prevalence percentage	OPG (mean $\pm$ standard error)	P-value
Batié	109 (72.7%)	$15,612.00 \pm 1,578.34^a$	< 0.001
Dano	94 (62.7%)	$14,734.67 \pm 1,397.11^a$	
Diébougou	138 (92%)	$40,310.67 \pm 2,863.40^b$	
Gaoua	98 (65.3%)	$13,115.33 \pm 1,461.06^a$	

OPG: Oocysts per gram. ^{a,b} Mean different superscript letters in the same column are statistically different at $p < 0.05$.

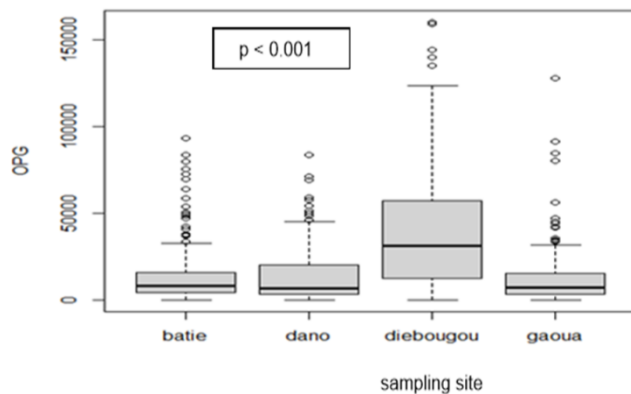


Figure 2. *Eimeria* spp. oocyst loads of broiler chickens (oocysts per gram) in different sampling sites in Burkina Faso, 2024

Diébougou is a strategically positioned city at the crossroads of roads connecting Ivory Coast and Ghana, which underscores its potential role in facilitating animal transportation and commercial exchanges (Werthmann, 2006). The considerable mobility of animals with diverse health conditions, including poultry arriving from diverse locations without quarantine protocols, may be associated with the observed *Eimeria* spp. infection rate in Diébougou region. In addition to this geographical factor, inadequate biosecurity measures, farmers' limited awareness, and limited access to veterinary care could explain the higher *Eimeria* spp. oocyst load observed in the Diébougou region (Hofmann et al., 2020; Saleem et al., 2025). The influence of geographical location on the prevalence of coccidiosis in broiler chickens has been examined worldwide. For instance, a study in Medea province, Algeria, found prevalence ranging from 57.1% to 19% in the districts of Beni Slimane and Oumari, respectively (Bachene et al., 2026). In India, broiler chickens in Uttar Pradesh province were more infected with coccidiosis (88%) than those in Madhya Pradesh province (60%; Bora et al., 2024). A similar pattern was observed in broiler chickens in South Africa, with

prevalence ranging from 9.8% in Scottburgh to 17.1% in Phoenix in KwaZulu-Natal province (Fatoba et al., 2020). Beyond geographical considerations, variations in poultry management practices may explain these differences.

In the present study, traditional poultry farms had significantly higher oocyst loads than modern farms ($p < 0.038$; Figure 3). Traditional farms were characterized by poor housing, low biosecurity, and limited access to veterinary care. A recent study demonstrated that inadequate housing conditions compromised the immune systems of chickens, thereby enhancing their susceptibility to diseases, regardless of geographic location (Hofmann et al., 2020). In the field, inadequate management can easily contaminate feed and water, increasing parasitic pressure on poultry (Mramba, 2023; Cevallos-Gordon et al., 2024; Makouloutou-Nzassi et al., 2024). In the present study, external personnel were often observed on traditional farms without adhering to biosecurity measures. Their clothing and vehicles may serve as mechanical vectors, promoting oocyst transfer between farms. Management practices differ across countries depending on poultry production systems. Poultry production systems are typically categorized into indigenous/traditional, small-scale, and large-scale commercial types, each with distinct differences in housing, feeding, biosecurity, and health management (Abadula et al., 2022). In line with this classification, the farms in the present study were identified as either traditional or modern, primarily based on their housing, biosecurity protocols, feed sources, and use of anticoccidials. In contrast to the present study, the poultry-keeping system did not notably affect coccidiosis occurrence in broiler chicken farms in Mazandaran province, Iran (Shirzad et al., 2011). In addition, a recent study in Borno State, Nigeria, reported higher coccidiosis prevalence in broiler chickens reared in intensive systems (78.5%) than in extensive systems (23%; Lawal et al., 2025). Consistent with the previous finding, the risk of

Eimeria spp. infection in broiler chickens from an intensive rearing system was eight times higher than in an extensive system in Bangladesh (Rahman et al., 2025). These findings indicated that effective management, stocking pressure, litter quality, and biosecurity are essential for controlling coccidiosis in poultry farms.

Fecal appearance demonstrated no significant correlation with *Eimeria* spp. oocyst load in poultry breeding systems ($p = 0.098$; Figure 4), indicating a high prevalence of subclinical coccidiosis forms.

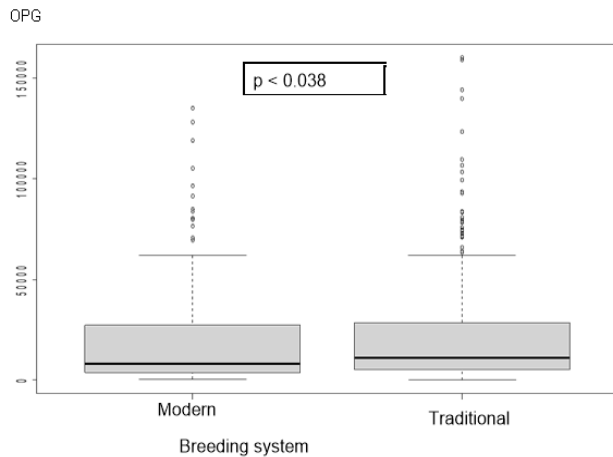


Figure 3. *Eimeria* spp. oocyst loads (oocysts per gram) in broiler chicken farm breeding systems in Burkina Faso, 2024

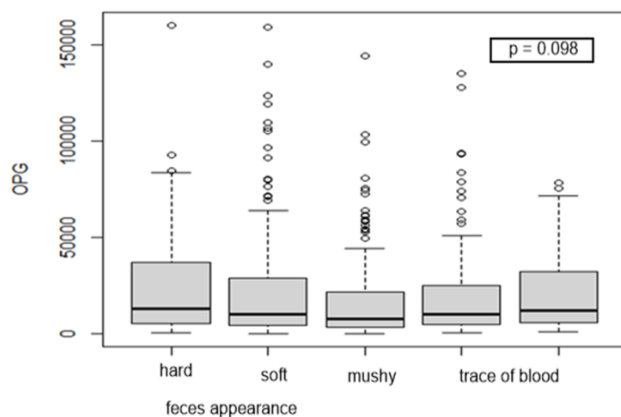


Figure 4. Fecal sample appearance characteristics of chickens infected with *Eimeria* spp., collected from different regions of Djôrô, Burkina Faso, 2024

Postmortem examination of chicken intestinal tracts

The upper intestinal (duodenum) lesion score ranged from 1 to 2, indicating mild to moderate lesions with white transverse streaks, small red spots, a ladder-like pattern,

and thickened mucosa. In the mid-intestine (jejunum), the lesion score was 3, exhibiting severe lesions with thickened mucosa, orange mucus, ballooning, and hemorrhage. In the distal intestine (ileum) and ceca, the lesion score was 4, indicating extremely severe lesions, including cecal cores, hemorrhage, and necrosis. Among the 25 gastrointestinal tracts examined from the Gaoua slaughter market, gross lesion distribution and morphology were presumptively compatible with *E. acervulina* in 13/25 samples (52%), *E. maxima* in 8/25 (32%), and *E. necatrix* in 4/25 (16%). Lesion locations that matched *E. acervulina*, *E. maxima*, and *E. necatrix* were biologically plausible, as these species tend to affect different parts of the intestine. However, lesion scoring alone did not definitively identify the species (Johnson and Reid, 1970). Furthermore, these postmortem findings were only from samples examined at the Gaoua market, Burkino faso, and offered preliminary insights. Therefore, the postmortem results did not reflect the true prevalence of species in the study farms or across the whole Djôrô region in Burkina Faso.

Control of avian coccidiosis in the Djôrô region of Burkina Faso mainly depended on amprolium, a chemical coccidiostat that inhibits parasite development. However, its effect diminishes notably after elimination (Ahmad et al., 2024; Dabire et al., 2025), leading to recurrence of the infection (Avi et al., 2023; Ahmad et al., 2024). Amprolium is used in layer and broiler chickens as a feed additive during one or more growth phases (Attree et al., 2021). Reports of resistance to anticoccidial medicines such as amprolium, toltrazuril, and clodol against *Eimeria* spp. have increased globally (Ojimelukwe et al., 2018; Flores et al., 2022; Bai et al., 2025), alongside growing demand for drug-free poultry meat (Bharti et al., 2025). Therefore, alternative control methods such as vaccines (Fatemi Motlagh et al., 2022; van Eerden et al., 2022; Zhang et al., 2025) and phytochemical compounds are needed. Several herbs, including garlic (*Allium sativum*), turmeric (*Curcuma longa*), and neem (*Azadirachta indica*), are documented for their antioxidant, antimicrobial, and anti-inflammatory effects. These herbs have been reported to enhance poultry immunity and diminish the severity of coccidiosis in poultry farming (Eftekhari et al., 2023).

CONCLUSION

In the Djôrô region, 73.17% of broiler chicken fecal samples collected from litters in farmhouses tested positive for *Eimeria* spp. The highest infection rate was

recorded in Diébougou city, at 92%. Postmortem findings on samples collected from the Gaoua slaughter market in Burkina Faso demonstrated presumptive identifications of *E. acervulina* (52%), *E. maxima* (32%), and *E. necatrix* (16%). These findings supported an immediate focus on litter management, routine cleaning, farm biosecurity training, and parasitological monitoring, along with the rational use of anticoccidials. Key limitations included limited data on farmers' management practices in Djôrô and limited use of postmortem findings because of sampling and analytical constraints. Further studies should include a knowledge, attitude, and practices survey on coccidiosis among poultry farmers in the Djôrô region in Burkina Faso. Moreover, analyzing farm samples using molecular diagnostic techniques can help characterize *Eimeria* spp. circulating in the region.

DECLARATIONS

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Authors' contributions

Séna Hervé Vitouley designed and implemented the study and wrote the manuscript. Sié Isidore Papis Hien contributed to data collection and manuscript writing. Soumaïla Pagabeleguem analyzed the field samples. Martin Bienvenu Somda analyzed the data, Gniniwéwo Carèle Botoni contributed to field sample analysis, and Salimata Sondé Pousga supervised the entire study. All authors read and approved the final edition of the manuscript for publication.

Availability of data and materials

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

Competing interests

The authors declare no conflict of interest.

Ethical considerations

All authors checked the ethical issues, including plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or

submission, and redundancy. The authors confirmed that they did not use any AI tools to prepare or write this article. All authors accept full responsibility for the manuscript's originality before submission and after acceptance, and approve the revised edition.

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Determining the Ideal Crude Protein Level in Plant-Based Diets for Late-Phase Laying Hens in Tropical Environments

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ABSTRACT

Reducing dietary crude protein (CP) in laying hens is a strategic approach to improve nitrogen efficiency, reduce feed costs, and enhance sustainability in tropical production systems. The present study aimed to determine the optimal dietary CP concentration required to sustain laying performance in plant-protein-based diets fed to late-phase hens. A total of 3,600 laying hens aged 60 weeks were allocated to three dietary treatments in a completely randomized design with four replicates of 300 hens each. The treatments included a control diet with 18.50% CP (P0), a diet replacing all animal ingredients with corn, rice bran, soybean meal, and corn gluten meal to supply 16.70% CP (P1), and an equivalent plant-protein diet with 16.20% CP (P2). All diets eliminated animal-derived protein sources and were formulated to meet essential amino acid requirements. Feed intake, hen-day production (HDP), egg weight, egg mass, feed conversion ratio (FCR), and income over feed cost (IOFC) were recorded daily for 90 days under tropical conditions (28-34°C) and analyzed over the complete 90-day period. Hens in Group P1 achieved the highest HDP (95.31%), the greatest egg mass (58.58 g/hen/day), and the lowest FCR (2.00), all significantly superior to groups P0 and P2 ($p < 0.05$). Further reduction to 16.20% CP (P2) lowered the HDP to 91.04% and raised the FCR to 2.08, compared with Group P1. In Group P2, egg weight increased to 63.42 g and exceeded that of groups P1 and P0. Group P2 had significantly higher feed intake (119.53 g) than P1 (117.21 g) and P0 (117.48 g). Reducing dietary CP in laying hens improved nitrogen utilization, reduced nitrogen excretion and feed costs, and enhanced the sustainability of egg production under tropical conditions. Diet P1 returned the highest IOFC (726.26 IDR or 0.0446 USD per hen per day) and the lowest feed cost per kilogram of egg (12,606 IDR or 0.773 USD). The results demonstrated that reducing dietary CP from 18.50% to 16.70% in a plant-protein-based formulation optimized laying performance and economic efficiency in late-phase laying hens under tropical conditions. The 16.70% CP level represented a practical threshold that balanced sustainability, nutrient efficiency, and profitability.

Keywords: Crude protein, Egg, Income over feed cost, Laying hen, Tropical condition

INTRODUCTION

The global poultry industry encounters an increasing challenge of balancing productive efficiency with environmental sustainability and economic viability, especially in tropical regions where heat stress exacerbates nutritional requirements (Oluwabenga and Fraley, 2023). Dietary crude protein (CP) is the most expensive nutritional component in poultry feed formulations after energy (Chathuranga and Heo, 2025), and excessive CP inclusion increases nitrogen excretion, ammonia

emissions, and feed costs (de Rauglaudre et al., 2023; Heo et al., 2023). With rising global soybean meal prices and growing environmental regulations, developing low-protein diets that reduce nitrogen excretion and feed costs while limiting losses in productive performance has become an important focus in poultry studies (Oketch et al., 2026).

Advances in crystalline amino acid (AA) technology have enabled the formulation of reduced-CP diets without compromising essential AA supply, allowing hens to express their genetic potential at lower protein intakes

(Macelline et al., 2021; Ali et al., 2025). Torki et al. (2015) demonstrated that dietary CP in laying hens can be reduced by 2 to 4% below the conventional 17% to 19% CP. However, the threshold at which further CP reduction impairs laying persistence and egg quality remains context-dependent and is influenced by genotype, age, ambient temperature, and the specific dietary protein sources (Hu et al., 2024).

Late-phase laying hens (beyond 60 weeks of age) represent a critical production stage in which laying rate naturally declines, metabolic efficiency decreases, and susceptibility to nutrient imbalances increases (Kleyn and Ciacciariello, 2023). Optimizing CP levels in the late-laying phase is particularly important because an imbalanced energy-to-protein ratio in low-protein diets induces hepatic lipid deposition and disrupts lipid metabolism (Hu et al., 2024). Hepatic lipid accumulation is further exacerbated when low-protein diets are combined with either low or high energy levels, depending on the energy-to-protein ratio (Hu et al., 2024). Furthermore, tropical climatic conditions characterized by chronic heat stress (ambient temperatures of 28–34°C) reduce voluntary feed intake, increase maintenance energy requirements, and alter AA utilization patterns (Torki et al., 2015). Although numerous studies have examined low-CP diets for laying hens, most were conducted under temperate conditions and used traditional protein sources, such as fish meal or meat and bone meal, including animal-based ingredients (Torki et al., 2015; Iqbal et al., 2026). Comparatively few studies have evaluated exclusively plant-protein-based formulations in late-phase hens under actual tropical field conditions with large flock sizes. The present study aimed to identify the optimal dietary CP concentration in plant-protein-based diets for 60-week-old laying hens managed under commercial tropical conditions in Central Java, Indonesia.

MATERIALS AND METHODS

Ethical approval

All experimental procedures involving live hens were reviewed and approved by the Animal Ethics Committee, Universitas Sebelas Maret, Indonesia, under approval number 18/UN27.20/PT01.01/2023. The hens were managed throughout the trial in accordance with the Lohmann Brown-Classic management guide. The authors declared that no procedure that would cause avoidable pain or distress was performed, and that the flock remained under routine commercial husbandry throughout the experimental period.

Study time and location

The study was conducted over 90 days, from 9 September to 10 December 2025, at a commercial layer farm in Central Java, Indonesia. The region has a tropical climate with high ambient temperatures and relative humidity. Ambient temperature ranged from 28°C to

34°C, and relative humidity from 64% to 75% during daylight hours throughout the experimental period. Temperature and relative humidity were recorded with a Lutron Environmental Data Logger (Taiwan).

Experimental animals and housing

A total of 3,600 Lohmann Brown laying hens aged 60 weeks were used in the present study, and the hens were randomly allocated into three dietary treatment groups. Each treatment consisted of 1,200 hens, further divided into 4 replications (300 hens per replication) to ensure statistical reliability. The hens were housed in a conventional battery cage system comprising 600 cages, each measuring 60 × 55 × 40 cm and holding 6 hens, providing 550 cm² per hen. Cages were arranged in 3 tiers and 4 double-sided rows. Husbandry practices followed the guidelines outlined in the Lohmann Brown-Classic management manual (Lohmann Breeders, 2021). Feed was provided at 130 g per chicken, and leftover feed was weighed daily. Chickens had unrestricted access to water. The lighting schedule consisted of 16 hours of illumination followed by 8 hours of darkness, with the average light intensity at bird level maintained at 25 lux throughout the experiment.

The flock received vaccination in line with the farm health program, based on baseline antibody titer monitoring, and was administered before the experiment began, following the vaccine manufacturer's recommendations (PT Vaksindo Satwa Nusantara, Bogor, Indonesia). At 58 weeks of age, hens were vaccinated with an inactivated Newcastle disease and infectious bronchitis vaccine (NDIB killed) via intramuscular injection (0.5 mL/hen). At 60 weeks of age, hens received an inactivated Avian Influenza vaccine (AI killed) by intramuscular injection (0.5 mL/hen). At 64 weeks of age, hens received live Newcastle disease and infectious bronchitis vaccines (NDIB Live) via drinking water. No further vaccinations were given during the remainder of the experimental period. No other supplements or medications were given, except the vitamin-mineral premix and feed additives incorporated into the experimental diets.

Dietary treatments

The experimental diets were formulated to meet the nutrient requirements of late-phase Lohmann Brown layers, as specified in the Nutrient Requirements of Poultry (NRC, 1994) and the Lohmann Brown-Classic management guide (Lohmann Breeders, 2021), with different CP concentrations and protein sources. The first group was the control group and received a conventional mash diet containing 18.50% CP (P0). The second group received a full plant-protein diet containing 16.70% CP (P1), and the third group received a full plant-protein diet containing 16.20% CP (P2). The control diet (P0) was mixed on the farm, and all diets (P0–P2) were prepared from locally sourced ingredients using the BRILL formulation software. Diets P1 and P2 excluded all

animal-derived protein sources and were balanced for the essential AAs lysine, methionine, threonine, tryptophan, valine, and isoleucine on an ideal-protein basis using the standard AA matrix in the BRILL formulation software; thus, the reduction in total protein was compensated. The ingredients and calculated nutrient composition of the experimental diets are presented in Table 1.

Table 1. Ingredient composition and calculated nutrient content of the experimental diets fed to laying hens aged 60 weeks (as-fed basis)

Ingredient (%)	P0	P1	P2
Corn	51.39	56.94	58.78
Rice bran	8.00	7.94	7.00
Soybean meal	25.20	19.50	16.42
Corn gluten meal	-	3.00	4.50
Meat bone meal	4.00	-	-
Limestone	9.50	10.07	10.75
Monocalcium phosphate	0.70	1.40	1.35
Salt and sodium bicarbonate	0.45	0.45	0.45
Additives (choline, betaine, GAA)	0.32	0.32	0.32
Micro-ingredients ¹	0.14	0.14	0.14
Synthetic amino acids ²	0.30	0.24	0.29
Total	100.00	100.00	100.00
Calculated nutrient composition[†]			
Metabolizable energy (kcal/kg)	2,650	2,671	2,696
Crude protein (%)	18.50	16.70	16.20
Crude fiber (%)	3.22	3.37	3.14
Ether extract (%)	3.85	3.55	3.49
Calcium (%)	4.15	4.12	4.37
Total phosphorus (%)	0.72	0.71	0.68
Available phosphorus (%)	0.42	0.41	0.38
Lysine (%)	0.95	0.82	0.78
Methionine + cystine (%)	0.76	0.69	0.71
Threonine (%)	0.68	0.61	0.60

¹Micro-ingredients supplied per kilogram of diet: A vitamin-mineral premix, a toxin binder, vitamin E, and selenium (Se). ²Synthetic amino acids included L-lysine HCl, DL-methionine, L-threonine, L-tryptophan, L-valine, and L-isoleucine. P0: Conventional diet containing 18.50% CP, P1: Plant-protein-based diet containing 16.70% CP, P2: Plant-protein-based diet containing 16.20% CP, CP: Crude protein, GAA: Guanidinoacetic acid. [†]Values were calculated from the tabulated ingredient composition using standard feed-matrix values.

The higher proportion of corn and the lower inclusion of soybean meal in diets P1 and P2 resulted from the least-cost formulation based on locally available feed ingredients (Surakarta, Central Java, Indonesia). Locally sourced cereal ingredients mainly supplied energy, while crystalline AA supplements provided essential AAs instead of adding more intact protein sources (NRC, 1994; Macelline et al., 2021). The crystalline AAs L-lysine HCl, DL-methionine, L-threonine, L-tryptophan, L-valine, and L-isoleucine were obtained from PT. Menjangan Sakti, Jakarta, Indonesia. Monocalcium phosphate was the only supplemental phosphorus source in the experimental diets; the remaining phosphorus was from the plant ingredients. Choline chloride, betaine, and guanidinoacetic acid were obtained from PT. Fenanza Putra Perkasa, Bandung, Indonesia. Guanidinoacetic acid was included because it

supports the productive performance of aged laying hens fed reduced-protein diets at high ambient temperatures (Mahdavi et al., 2026). Betaine was included because it can improve laying-hen performance under tropical conditions (Wahid et al., 2021) and support gut microbiota composition and intestinal health under heat stress, as demonstrated in laying pullets (Wahid et al., 2024) and quail (Pradista et al., 2026).

Data collection

Production performance was monitored continuously over the 90-day experimental period (Days 1 to 90), and data were summarized by replicate for statistical analysis. Feed intake was determined daily (Days 1-90) for each replicate by subtracting the weight of feed refusals from the weight of feed offered, expressed as grams per hen per day. Hen-day production (HDP) was recorded daily (Days 1-90) as the total number of eggs produced divided by the number of live hens present on that day, expressed as a percentage. For egg weight determination, a random sample of 100 eggs per replicate was collected and weighed daily (Days 1-90) using a digital electronic balance (ACIS BC-500, 600×0.01 g, PT. Libramas, Indonesia). Daily egg mass was calculated as the product of daily HDP and daily mean egg weight (g/hen/day). The overall feed conversion ratio (FCR) was calculated at the end of the 90-day period as total cumulative feed intake divided by total cumulative egg mass produced per replicate over the entire experimental period. Production performance parameters were calculated following the general procedures of Oketch et al. (2025), with modifications to the sampling frequency and measurement period.

Economic evaluation

At the end of the 90-day period, economic efficiency was evaluated using the income over feed cost (IOFC) method to determine the most cost-effective feed formulation (North et al., 1990). The calculation used local market prices observed during the experimental period. The feed cost was 6,500 IDR/kg for P0, 6,300 IDR/kg for P1, and 6,240 IDR/kg for P2, corresponding to 0.399 USD/kg, 0.387 USD/kg, and 0.383 USD/kg, respectively. The egg selling price remained stable at 25,000 IDR/kg (1.534 USD/kg) throughout the experimental period. Income over feed cost was determined using Formula 1. The feed cost per kilogram of egg was calculated based on Formula 2. All amounts in Indonesian rupiah were converted to United States dollars at a rate of 1.00 USD = 16,300 IDR in January 2025.

IOFC (IDR/hen/day) = (HDP [%] × egg weight [g] × egg price [IDR/kg]) – (feed intake [g/hen/day] × feed price [IDR/kg]) (Formula 1)

Feed cost (IDR/kg egg) = (feed intake [g/hen/day] × feed price [IDR/kg]) ÷ (HDP [%] × egg weight [g]) (Formula 2)

Statistical analysis

The data were analyzed using a completely randomized design, with the replicate (300 hens) as the experimental unit, yielding four observations per treatment. Normality of residuals and homogeneity of variance were verified with the Shapiro-Wilk test and the Levene test, respectively, before a one-way analysis of variance was performed. Tukey's honestly significant difference test was used to separate differences between treatment means, and differences were considered significant at p -value less than 0.05. The magnitude of the treatment effect was expressed as eta squared (η^2), derived from the F statistic and its degrees of freedom; values of 0.01, 0.06, and 0.14 were interpreted as small, medium, and large effects, respectively. All results were presented as mean $\pm$ standard deviation (SD). All statistical analyses were performed using R Statistical Software, version 4.3.2 (R Core Team, 2025).

RESULTS AND DISCUSSION

Production performance

The effects of dietary CP levels on the production performance of late-phase hens are presented in Table 2. Dietary CP levels significantly influenced all measured production parameters ($p < 0.05$). Feed intake differed significantly ($p = 0.0293$) among the dietary treatment groups ($\eta^2 = 0.54$). Hens in Group P2 consumed significantly more feed (119.53 ± 0.80 g/hen/day) than those in P1 (117.21 ± 0.37 g/hen/day; $p < 0.05$), whereas feed intake did not differ significantly between Group P0 (117.48 ± 1.67 g/hen/day) and Group P1 ($p > 0.05$). The higher feed intake in Group P2 did not improve egg production or feed efficiency, suggesting that the lower CP concentration did not adequately support productive performance (Iqbal et al., 2026).

Feed conversion ratio differed significantly ($p = 0.0124$) among dietary treatments ($\eta^2 = 0.62$). Hens in Group P1 achieved the lowest FCR (2.00 ± 0.00), indicating greater feed utilization efficiency than hens in Group P0 (2.04 ± 0.02) or P2 (2.08 ± 0.01 ; $p < 0.05$). This improvement in FCR at the moderate protein reduction was consistent with the principle, established in broiler low-CP feeding trials, that a precisely balanced low-CP diet containing supplemental crystalline AA can improve nitrogen utilization, because less dietary protein is deaminated and excreted (de Rauglaudre et al., 2023). The current findings were in line with those of Ali et al. (2025), who reported that a 16% CP diet supplemented with essential AA maintains a feed efficiency comparable to that of a 17% CP control in Hy-Line Brown hens. In contrast, the FCR deteriorated at 16.20% CP (Group P2) in the present study. The poorer FCR observed in P2 indicated that reducing dietary CP to 16.20% may have limited the availability of non-essential AAs and other nitrogen-containing compounds required for efficient egg

synthesis (He et al., 2021). In the present study, the superiority of Group P1, with a 16.70% CP, was primarily due to the strategic balance of essential AAs. Supplementation with crystalline lysine, methionine, threonine, tryptophan, valine, and isoleucine ensured that the AA profile of the reduced-CP diets met the requirement for egg protein synthesis despite the lower total nitrogen supply (He et al., 2021; Ali et al., 2025).

Hen-day production was significantly ($p = 0.0001$) affected by dietary treatment ($\eta^2 = 0.87$). Group P1 had the highest HDP ($95.31 \pm 0.18\%$), exceeding the control group ($94.20 \pm 0.28\%$) and Group P2 ($91.04 \pm 0.29\%$). The 4.3% decline in HDP from 16.70% CP (Group P1) to 16.20% CP (Group P2) indicated that 16.20% CP was insufficient to sustain laying persistency in 60-week-old hens, possibly because an energy-to-protein imbalance disrupted hepatic lipid metabolism via the gut-liver axis and limited the availability of non-essential AAs required for egg synthesis (Hu et al., 2024). Similarly, Liu et al. (2024) stated that excessively reducing CP in laying hens should be done cautiously, as the resulting productivity losses might reduce the environmental gains. The high HDP observed in Group P1 under tropical temperatures (28–34°C) was considerable, since heat stress can inhibit ovarian function and reduce laying rates in certain conditions (Oluwagbenga and Fraley, 2023). The present result suggested that the 16.70% CP level with balanced AA provided sufficient nutrients for egg synthesis even under chronic heat exposure, possibly because lower-protein diets generate less metabolic heat during digestion. Similarly, Torki et al. (2015) found that increasing heat did not reduce laying performance in hens fed low-CP diets supplemented with AA under high ambient temperatures.

Egg mass was significantly higher ($p = 0.0004$) in Group P1 (58.58 ± 0.23 g/hen/day) compared to groups P0 (57.34 ± 0.15 g/hen/day) and P2 (57.74 ± 0.23 g/hen/day; $\eta^2 = 0.82$). The superior egg mass in Group P1 was due to high HDP and optimal egg weight, reflecting the most favorable balance between laying rate and egg size. The present egg mass values were consistent with those of Iqbal et al. (2026), who maintained egg mass at 14.50% CP with glycine and serine supplementation. In contrast, Liu et al. (2024) found that a diet with 13.85% CP reduced egg production and egg mass compared with 15.63% CP in 38-week-old hens, suggesting that the threshold in the present study was within the range reported for AA-balanced low-protein diets (Iqbal et al., 2026).

Egg weight increased significantly ($p = 0.0001$) as dietary CP decreased ($\eta^2 = 0.87$). Group P2 produced the heaviest eggs (63.42 ± 0.09 g), followed by Group P1 (61.46 ± 0.16 g) and Group P0 (60.87 ± 0.08 g). The heavier eggs observed in Group P2 may be associated with the lower laying rate in this group. This shift reflected an inverse relationship between egg number and egg size, a well-documented reproductive strategy in avian species. Notably, the increase in egg weight observed in Group P2

was insufficient to offset the decline in HDP, resulting in

lower egg mass and poorer FCR than in Group P1.

Table 2. Effects of dietary crude protein level on production performance in late-phase laying hens

Parameters	Group P0	Group P1	Group P2	p-value	η^2
Feed intake (g/hen/day)	117.48 $\pm$ 1.67 ^{ab}	117.21 $\pm$ 0.37 ^b	119.53 $\pm$ 0.80 ^a	0.0293*	0.54
FCR	2.04 $\pm$ 0.02 ^b	2.00 $\pm$ 0.00 ^c	2.08 $\pm$ 0.01 ^a	0.0124*	0.62
HDP (%)	94.20 $\pm$ 0.28 ^b	95.31 $\pm$ 0.18 ^a	91.04 $\pm$ 0.29 ^c	0.0001**	0.87
Egg mass (g/hen/day)	57.34 $\pm$ 0.15 ^b	58.58 $\pm$ 0.23 ^a	57.74 $\pm$ 0.23 ^b	0.0004**	0.82
Egg weight (g)	60.87 $\pm$ 0.08 ^c	61.46 $\pm$ 0.16 ^b	63.42 $\pm$ 0.09 ^a	0.0001**	0.87

P0: Conventional diet containing 18.50% CP, P1: Plant-protein-based diet containing 16.70% CP, P2: Plant-protein-based diet containing 16.20% CP. CP: Crude protein, FCR: Feed conversion ratio, HDP: Hen-day production. ^{a, b, c} Mean different superscript letters in a row differ significantly ($p < 0.05$). * indicates $p < 0.05$, ** indicates $p < 0.01$, η^2 : Eta squared, an estimate of the proportion of total variance explained by the dietary treatment. Data are presented as mean $\pm$ SD.

Economic parameters

Dietary CP levels significantly affected all economic indicators measured (Table 3; $p < 0.05$). Feed cost differed significantly ($p = 0.0403$) among treatment groups ($\eta^2 = 0.51$). Group P1 recorded the lowest feed cost (738.45 $\pm$ 2.33 IDR/hen/day, equivalent to 0.0453 USD/hen/day), followed by Group P2 (745.85 $\pm$ 5.01 IDR/hen/day, equivalent to 0.0458 USD/hen/day) and Group P0 (IDR 763.65 $\pm$ 10.87/hen/day, equivalent to 0.0469 USD/hen/day). The lower feed cost of the plant-protein-based diets (P1 and P2) reflected lower inclusion of expensive soybean meal and lower total protein content, which reduced formulation cost. The slightly higher feed cost of Group P2 relative to P1, despite its lower CP content, resulted from higher inclusion of corn gluten meal (4.50% versus 3.00%). This higher inclusion counteracted the cost savings achieved by reducing soybean meal. This finding emphasized that feed cost optimization in low-CP diets depends not only on protein level but also on the specific ingredient used. This strategy was consistent with Macelline et al. (2021), who noted that the economic success of low-CP formulations relies heavily on selecting complementary ingredients and crystalline AAs rather than on protein reduction alone (Macelline et al., 2021).

Egg revenue was significantly ($p = 0.0004$) influenced by treatments ($\eta^2 = 0.82$). Group P1 had the highest daily egg revenue (1,464.70 $\pm$ 5.85 IDR/hen/day, equivalent to 0.0898 USD/hen/day), which exceeded the values of groups P0 (1,433.73 $\pm$ 3.92 IDR/hen/day, equivalent to 0.0880 USD/hen/day) and P2 (1,443.74 $\pm$ 5.93 IDR/hen/day, equivalent to 0.0886 USD/hen/day). This superiority of Group P1 primarily resulted from the higher HDP and egg mass in P1 hens, leading directly to increased saleable egg output.

Income over feed cost, the most critical indicator of economic efficiency in commercial egg production, indicated a highly significant difference ($p = 0.0002$) among treatments ($\eta^2 = 0.85$). Group P1 produced the highest IOFC (726.26 $\pm$ 4.41 IDR/hen/day, equivalent to 0.0446 USD/hen/day), which represented a significant

improvement of 8.4% than the control group (670.07 $\pm$ 10.92 IDR/hen/day equivalent to 0.0411 USD/hen/day) and 4.1% improvement than Group P2 (697.89 $\pm$ 9.73 IDR/hen/day, equivalent to 0.0428 USD/hen/day; $p < 0.05$). Group P1 attained a higher IOFC by reducing feed cost and increasing egg revenues. The economic advantage of a moderate CP reduction in the present study contrasts with the findings of Oketch et al. (2026), who found that balanced protein reduction decreased egg income and feed costs but did not affect overall profit margin. In tropical production systems, where feed costs account for 65-75% of total production costs (Sonaiya and Swan, 2004), the 8.4% improvement in IOFC at 16.70% CP was a meaningful gain in farm profitability, as observed in this study.

Feed cost of egg was significantly lower ($p = 0.0015$) in Group P1 (12,606 $\pm$ 34 IDR/kg of egg, equivalent to 0.773 USD/kg of egg) than in groups P2 (12,924 $\pm$ 125 IDR/kg of egg, equivalent to 0.793 USD/kg of egg) and P0 (13,316 $\pm$ 188 IDR/kg of egg, 0.817 USD/kg of egg; $\eta^2 = 0.76$). Because feed cost per kilogram of egg reflects both feed efficiency and feed cost, the lower value observed in Group P1 indicates that the 16.70% CP formulation achieved the most cost-effective conversion of feed into egg output. The 5.3% decrease in feed cost per kilogram of eggs from P0 to P1 resulted in savings of 710 IDR/kg of eggs, or about 0.044 USD/kg of eggs. This saving is noticeable in large-scale commercial operations. These findings were consistent with those of Iqbal et al. (2026), who reduced feed cost per unit of egg output by supplementing glycine and serine in a low-protein diet. By contrast, Liu et al. (2024) reported that excessively lowering CP can reduce egg production and feed-cost savings. In the present study, feed costs were reduced without lowering production, which is consistent with the favorable range reported by Iqbal et al. (2026).

Using plant-protein sources in the experimental diets enhanced the practical relevance of the present findings. Eliminating animal-derived protein ingredients such as fish meal and meat and bone meal simplified supply chain

logistics, reduced the risk of contamination with antibiotic residues or pathogens, and aligned with growing consumer demand for plant-based animal production systems (Attia et al., 2024). The present results demonstrated that a

formulation based on corn, soybean meal, and corn gluten meal, when combined with appropriate crystalline AA supplementation, can fully support laying performance in late-phase hens without reliance on animal protein.

Table 3. Effects of dietary crude protein level on the economic performance of late-phase laying hens

Parameters	Group P0	Group P1	Group P2	p-value	η^2
Feed cost of formulated feed (IDR/hen/day)	763.65 ± 10.87 ^a	738.45 ± 2.33 ^b	745.85 ± 5.01 ^b	0.0403*	0.51
Feed cost of formulated feed (USD/hen/day)	0.0469 ± 0.0007 ^a	0.0453 ± 0.0001 ^b	0.0458 ± 0.0003 ^b	0.0403*	0.51
Egg revenue (IDR/hen/day)	1,433.73 ± 3.92 ^b	1,464.70 ± 5.85 ^a	1,443.74 ± 5.93 ^b	0.0004**	0.82
Egg revenue (USD/hen/day)	0.0880 ± 0.0002 ^b	0.0898 ± 0.0004 ^a	0.0886 ± 0.0004 ^b	0.0004**	0.82
IOFC (IDR/hen/day)	670.07 ± 10.92 ^c	726.26 ± 4.41 ^a	697.89 ± 9.73 ^b	0.0002**	0.85
IOFC (USD/hen/day)	0.0411 ± 0.0007 ^c	0.0446 ± 0.0003 ^a	0.0428 ± 0.0006 ^b	0.0002**	0.85
Feed cost of egg (IDR/kg of egg)	13,316 ± 188 ^a	12,606 ± 34 ^c	12,924 ± 125 ^b	0.0015**	0.76
Feed cost of egg (USD/kg of egg)	0.8169 ± 0.0115 ^a	0.7733 ± 0.0021 ^c	0.7929 ± 0.0077 ^b	0.0015**	0.76

P0: Conventional diet containing 18.50% CP, P1: Plant-protein-based diet containing 16.70% CP, P2: Plant-protein-based diet containing 16.20% CP. CP: Crude protein, IDR: Indonesian rupiah, IOFC: Income over feed cost, USD: United States dollar. Amounts in USD were obtained by converting the corresponding IDR value at USD 1.00 = IDR 16,300; the p-value and η^2 apply to both currencies because the conversion was a constant multiplier. ^{a, b, c} Mean different superscript letters in a row differ significantly ($p < 0.05$). * indicates $p < 0.05$, ** indicates $p < 0.01$, η^2 : Eta squared, an estimate of the proportion of total variance explained by the dietary treatment. Data are presented as mean ± SD.

CONCLUSION

Reducing dietary CP from 18.50% to 16.70% in a plant-protein-based formulation supplemented with crystalline AA significantly improved productive performance and economic efficiency in 60-week-old Lohmann Brown hens under tropical conditions in Central Java, Indonesia. The 16.70% CP diet produced the highest HDP (95.31%), the lowest FCR (2.00), the greatest egg mass (58.58 g/hen/day), and the highest IOFC (726.26 IDR or 0.0446 USD/hen/day). Further reducing CP to 16.20% decreased laying persistency and feed efficiency, indicating that 16.70% CP was the optimal threshold for this production system. The present findings support the practical adoption of moderate CP reduction in tropical layer production to improve sustainability, nutrient efficiency, and farm profitability simultaneously. The present study did not include egg quality traits such as shell thickness, albumen height, and Haugh unit. Additionally, nitrogen balance and ammonia emissions were not measured, so the environmental benefits of reduced-CP diets were not quantified, which are recommended to be investigated in future studies.

DECLARATIONS

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Authors' contributions

Zainudin Al Wahid conceptualized the study, designed the methodology, interpreted the data, prepared the visualizations, and wrote the original draft. Adi Ratriyanto conceptualized the study, interpreted the data, supervised the study, and wrote the original draft. Luthfi Adya Pradista designed the methodology, interpreted the data, and prepared the visualizations. Muhammad Nidhomun Ni'am designed the methodology, interpreted the data, and prepared the visualizations. All authors read and approved the final edition of the manuscript.

Availability of data and materials

The datasets generated and analyzed during the present study are available from the corresponding author upon reasonable request. The raw production and economic records are submitted with the present article.

Competing interests

The authors declared no conflicts of interest.

Ethical considerations

The authors declare that this article is original, has not been published elsewhere in whole or in part, and is not under consideration by any other journal. All authors have avoided plagiarism, data fabrication, data falsification, and

duplicate publication. Regarding the use of artificial intelligence, the authors declare that generative AI tools (Gemini, ChatGPT, and Claude AI) were used solely for language editing to improve readability, and the authors have reviewed and revised the language-edited version and take full responsibility for the final content of the article. The authors accept full responsibility for the originality and the integrity of the content of this article, before submission and after acceptance.

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Antibiotic Resistance of *Escherichia coli* Strains Isolated from Fecal Samples of Poultry Farms in Ninh Binh, Vietnam

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ABSTRACT

Antibiotic resistance is a major global health threat, and antibiotic use in livestock farming contributes to this issue. The present study aimed to assess the prevalence and antibiotic susceptibility of *Escherichia coli* (*E. coli*) isolated from poultry fecal samples in Ninh Binh province, Vietnam. Between May and October 2025, 170 dropping fecal samples were randomly collected from 45 poultry farms in Ninh Binh province, Vietnam, including 24 broiler chicken farms (aged 2.5-3.0 months old) and 21 meat-oriented duck farms (aged 1.5-2.0 months old). Fecal samples were transferred to the laboratory and cultured in peptone buffer and on selective media, including MacConkey agar, eosin methylene blue agar, and triple sugar iron agar. Antibiotic susceptibility was assessed using the disk diffusion method on Mueller-Hinton agar. Suspected *E. coli* colonies were confirmed using Gram staining and biochemical tests such as citrate utilization, indole production, the methyl red test, and the Voges-Proskauer reaction. *Escherichia coli* was isolated from 134 (78.8%) fecal samples, including 69/85 (81.2%) chicken and 65/85 (76.5%) duck samples. The susceptibility of *E. coli* strains to 10 antibiotics was evaluated using the disk diffusion method. *Escherichia coli* strains isolated from chickens and ducks demonstrated the highest resistance to ampicillin (82.8%), followed by sulfonamides (79.9%), trimethoprim/sulfamethoxazole (74.6%), tetracycline (60.5%), and gentamicin (52.2%). Resistance to the remaining antibiotics, including norfloxacin, doxycycline, nalidixic acid, and cefotaxime, ranged from 14.6% to 38.1%; none of the isolates were resistant to meropenem. Among the isolates, 97 (72.4%) were multidrug-resistant; the proportion of multidrug-resistant isolates from chickens (60 strains, 87.0%) was higher than that from ducks (37 strains, 56.9%). *Escherichia coli* strains isolated from broiler chicken and duck farms in Ninh Binh, Vietnam, exhibited diverse resistance patterns to several antibiotics. Specifically, 34 and 36 resistance patterns were identified in chicken and duck isolates, respectively, with most isolates having multidrug resistance characteristics.

Keywords: Antibiotic resistance, Chicken, Duck, *Escherichia coli*, Poultry farm

INTRODUCTION

Antibiotics are widely used worldwide in livestock farming to prevent disease, treat infections, promote growth, and increase production (Pokharel et al., 2020; Rahman et al., 2022). Disease in livestock is a primary driver of antibiotic use (AMU), and global AMU is expected to rise by 8% by 2030 (Mulchandani et al., 2023). Over recent decades, antibiotic overuse in human and veterinary medicine has increased antibiotic resistance (AMR) and accelerated its spread among pathogenic and harmless bacteria (Moffo et

al., 2022; Salam et al., 2023). Additionally, AMU in livestock can foster AMR, jeopardizing the sustainability of the livestock industry by causing treatments to fail in animals (Mulchandani et al., 2023; Stevenson, 2023). AMR has become one of the most critical global health issues, and the World Health Organization (WHO) recognized AMR as one of the top ten public health threats (Bhargav et al., 2022). Antibiotic resistance is frequently referred to as a silent pandemic due to its deadly yet largely invisible impacts (Zhou et al., 2022).

Escherichia coli (*E. coli*) is among the most common microorganisms in the guts of warm-blooded animals, including mammals and chickens (Kaper et al., 2004; Kerek et al., 2025). While most *E. coli* strains are harmless, some are pathogenic, highly transmissible, and can form colonies. Because of their widespread presence and resistance to different antibiotics, these strains are found globally (Paitan, 2018). One important AMR mechanism in *Enterobacteriaceae*, including *E. coli*, is the production of Extended-spectrum β -lactamases (ESBLs) or β -lactamase type C (AmpC; Geser et al., 2012). With more than 600 variants, ESBLs/AmpC not only confer cephalosporin resistance but are also associated with resistance to many other antibiotics, including fluoroquinolones, trimethoprim-sulfamethoxazole, and tetracyclines (Woodford et al., 2004; Geser et al., 2012). Therefore, the WHO has included *E. coli* on the list of 12 priority AMR pathogens (Asokan et al., 2019).

Poultry industry of Vietnam ranks tenth globally in chicken production and second in waterfowl production, with approximately 584.4 million chickens annually, producing about 2.46 million tons of meat and 20.352 billion eggs, including 3.516 billion chicken eggs and 6.913 billion waterfowl eggs (Thanh Tra, 2025). The use of antibiotics in poultry farming for disease prevention, treatment, and growth promotion poses a significant risk of AMR bacteria emerging through genetic or non-genetic mechanisms, making poultry a major global reservoir of AMR *E. coli* (Osman et al., 2018; Liu et al., 2025). In response to AMR, Vietnam's Ministry of Agriculture and Rural Development (MARD) implemented a National Action Plan in 2017 to address AMR in livestock and aquaculture (MARD, 2017). The plan aimed to reduce non-therapeutic AMU, such as growth promotion, while strengthening surveillance and raising awareness. However, implementation of the AMR plan has faced challenges because poultry farms frequently use antibiotics for disease prevention, to boost productivity, and to prevent outbreaks (Carrique-Mas et al., 2015). To date, several studies have examined AMU and bacterial resistance on poultry farms in Vietnam (Carrique-Mas et al., 2015; Vounba et al., 2019; Nohrborg et al., 2024).

Additional studies are needed to fully understand the AMR characteristics of different bacteria, including *E. coli*, to inform AMR prevention strategies. Therefore, the present study aimed to determine the prevalence and antibiotic susceptibility of *E. coli* isolated from poultry fecal samples in Ninh Binh province, Vietnam.

MATERIALS AND METHODS

Ethical approval

The present study was conducted in accordance with the animal welfare and safety guidelines of the Committee on Animal Research and Ethics at the Faculty of Veterinary Medicine, Vietnam National University of Agriculture, Vietnam (Approval No. CARE-2025/06). The authors addressed farmers' ethical concerns and obtained their consent before conducting the study.

Sample size determination

The study sample size was calculated using the prevalence of *E. coli* to achieve a 95% confidence level and a 5% margin of error. For sample size determination, the present study used the following formula:

$$N = Z^2 \times P(1-P) / L^2$$

$$N = (1.96^2 \times 0.7105 \times 0.2895) / (0.05^2)$$

where N was the required sample size, Z was the Z-statistic for a 95% confidence level (1.96 for a two-tailed test), P was the prevalence of *E. coli*, and L was the margin of error (5%). In the present study, P was set at 71.05%, based on findings from Nguyen et al. (2021). Substituting these values into the formula yielded approximately 316 samples. However, due to budget constraints and fewer farms consenting to sampling than expected, the study collected 170 samples.

Sampling

In this cross-sectional study, 170 fresh fecal samples were randomly collected from 24 colored-feather broiler chicken farms and 21 meat-oriented duck farms in Ninh Binh Province, Vietnam, between May and October 2025. These farms were located between 20°23' and 20°44' North latitude and between 105°45' and 106°15' East longitude in Ninh Binh Province, Vietnam. The temperature in the poultry houses was maintained between 20°C and 25°C, humidity between 60% and 70%, and stocking density at 6-8 animals per m². Certified veterinarians selected broiler chicken and meat-oriented duck farms for sampling. These farms operated under a semi-closed husbandry model, specializing in raising a single breed of broiler chicken or meat-oriented duck, with flock sizes typically ranging from 2,000 to 3,000 chickens or ducks per farm. The colored-feather broiler flocks comprised indigenous breeds such as Luong Phuong, Ho, Mia, and Dong Tao (aged 2.5 to 3.0 months), while the meat-oriented duck flocks consisted of Super-meat, Grimaud, Cherry Valley, Bau, and Co breeds (aged 1.5 to 2.0 months). The flocks had been fully vaccinated against key diseases, including Newcastle disease at 5-7, 18-21, and 35-42 days of age, Gumboro disease at 10-14 and 21-25 days of age for chickens; and Duck viral hepatitis at 1-3 days of age, along with Duck plague at 7-10

and 35-40 days for ducks. Vaccines against Newcastle disease and Gumboro disease were typically supplied by companies such as Boehringer Ingelheim (Germany), Ceva Santé Animale (France), MSD Animal Health (USA), Zoetis (USA), Vaccindo (Indonesia), and Biovac (Israel). Vaccines against viral hepatitis in ducks and duck cholera were supplied by companies such as Hanvet, Navetco, and Vetvaco (Vietnam). Certified veterinarians conducted health checkups and clinical examinations to prevent external infectious diseases. All medications to prevent diseases were administered according to the veterinarians' recommendations. All poultry flocks were fed a balanced commercial poultry feed from well-known animal feed manufacturers, with diets tailored to each age stage's nutritional needs. At the time of sampling, the poultry flocks were healthy and demonstrated no clinical signs of disease, as evidenced by normal activity levels and average water and feed intake. Three to five samples of freshly voided poultry feces were collected from each flock in accordance with the [MARD \(2011\)](#) guidelines (QCVN 01-83:2011/BNNPTNT). Approximately 2-3 grams of fresh feces were collected from the pen floor using an autoclaved spoon. Each sample was placed in a separate sterile polyethylene bag, labeled, stored in a cool box with dry ice, and transported to the Department of Veterinary Microbiology and Infectious Diseases, Faculty of Veterinary Medicine, Vietnam National University of Agriculture, for analysis within 24 hours.

Isolation of *Escherichia coli*

Escherichia coli were isolated from fecal samples using the method described by [Deng et al. \(2024\)](#), with some modifications. In the laboratory, approximately 1 g of each fecal sample was homogenized with buffered peptone water (BPW; Merck, Germany) at a 1:9 ratio. A loopful of the homogenate was streaked onto MacConkey agar (Merck, Germany) and incubated at 37°C under aerobic conditions for 24 hours. Pink colonies on MacConkey agar were then cultured onto eosin methylene blue agar (EMB; Merck, Germany) and incubated at 37°C under aerobic conditions for 24 hours. A typical colony exhibiting a green metallic sheen on EMB agar was streaked into a triple sugar iron (TSI; Merck, Germany) agar tube and incubated at 37°C under aerobic conditions for 24 hours. Colonies exhibiting typical TSI characteristics, including glucose and lactose fermentation with gas production and no hydrogen sulfide, were identified as *E. coli* by Gram staining and biochemical tests, including citrate utilization, indole production, the methyl red test, and the Voges-Proskauer reaction, performed in Simmons citrate agar (SCA; Merck, Germany), Tryptone broth (TB; Merck, Germany), and methyl-red

Voges-Proskauer broth (MR-VP; Merck, Germany), respectively. All isolated strains were cultured in brain-heart infusion broth (BHI; Merck, Germany) under aerobic conditions for 16-18 hours. Then, 0.5 mL of the bacterial culture was transferred to an Eppendorf tube containing 0.5 mL of glycerol (Merck, Germany). The mixture was shaken thoroughly to ensure homogeneity, incubated for an additional 2-3 hours at 37°C, and finally stored at -80°C for the following experiment.

Antibiotic susceptibility testing

The agar disk diffusion method was performed on Mueller-Hinton agar (MHA; Merck, Germany) following the methodology of [Bauer et al. \(1966\)](#). Ten antibiotics manufactured by Oxoid, United Kingdom, from seven antibiotic classes were tested, including penicillins (ampicillin, 10 µg), cephalosporins (cefotaxime, 30 µg), carbapenems (meropenem, 10 µg), aminoglycosides (gentamicin, 10 µg), quinolones (nalidixic acid, 30 µg; norfloxacin, 10 µg), tetracyclines (doxycycline, 30 µg; tetracycline, 30 µg), and sulfonamides (sulfonamides, 300 µg; trimethoprim/sulfamethoxazole, 1.25/23.75 µg). Briefly, *E. coli* colonies were suspended in nutrient broth (Merck, Germany), and the suspension's turbidity was adjusted to the 0.5 McFarland standard. A sterile swab was dipped into the standardized bacterial suspension, pressed against the tube wall to remove excess liquid, and then streaked across prepared MHA plates. The inoculated plates were left to stand for 10-15 minutes, after which sterile forceps were used to place five antibiotic discs onto the agar surface. The plates were incubated at 37°C under aerobic conditions for 16-18 hours ([Bauer, 1966](#)). After incubation, the zone of inhibition around each plate was measured to the nearest millimeter with a ruler, recorded, and classified as resistant, intermediate, or susceptible according to the Clinical and Laboratory Standards Institute guidelines ([CLSI, 2024](#)). The *Escherichia coli* ATCC 25922 strain, provided by the Department of Pharmacology, Internal Medicine, and Toxicology, Faculty of Veterinary Medicine, Vietnam National University of Agriculture, was used as a quality control strain. An *E. coli* strain was classified as resistant when it was confirmed resistant to at least one tested antibiotic, and as multidrug-resistant when it exhibited resistance to at least one antibiotic from each of three antibiotic classes ([Magiorakos et al., 2012](#)).

Data analysis

Data on *E. coli* isolation and antimicrobial susceptibility testing were recorded and edited in a Microsoft Excel spreadsheet. A two-tailed Fisher's exact test was used to assess differences in prevalence and antibiotic susceptibility

between *E. coli* isolated from chicken and duck fecal samples. P values less than 0.05 were considered significant.

RESULTS

Of 170 samples collected, 134 (78.8%) tested positive for *E. coli*. Specifically, *E. coli* was detected in 69 of 85 chicken fecal samples (81.2%) and in 65 of 85 duck fecal samples (76.5%; Table 1). Overall, *E. coli* strains isolated from chicken and duck fecal samples were resistant to nine antibiotics tested (Table 2). Among the 134 *E. coli* strains, resistance was highest for ampicillin (82.8%), followed by sulfonamides (79.9%), trimethoprim/sulfamethoxazole (74.6%), tetracycline (60.5%), and gentamicin (52.2%). Resistance rates were lower for norfloxacin (16.4%), doxycycline (20.9%), nalidixic acid (30.6%), and cefotaxime (38.1%); none of the isolates exhibited resistance to meropenem. Resistance rates for ampicillin did not differ significantly ($p = 0.398$) between *E. coli* isolates from chicken and duck samples (85.5% versus 80.0%). Similarly, resistance rates to trimethoprim/sulfamethoxazole was not significantly different ($p = 0.549$) between chicken and duck isolates (76.8% versus 72.3%). Cefotaxime (43.5% versus 32.3%; $p = 0.183$) and nalidixic acid (33.3% versus 27.7%; $p = 0.479$) demonstrated no significant differences. Doxycycline resistance was comparable between *E. coli* isolates from chicken and duck (21.7% versus 20.0%; $p = 0.805$). Norfloxacin resistance did not differ significantly ($p = 0.148$) between *E. coli* isolates from chicken and duck samples (21.7% versus 10.8%). However, Sulfonamide resistance was significantly higher in chicken

isolates ($p = 0.012$; 88.4% versus 70.8%). A similar pattern was observed for tetracycline, with chicken isolates demonstrating a significantly higher ($p = 0.001$) resistance rate than duck isolates (78.3% versus 41.5%). Gentamicin resistance was significantly higher ($p = 0.039$) in isolates from chicken fecal samples than in duck isolates (60.9% versus 43.1%).

Among the isolates, 97 (72.4%) were multidrug-resistant. The proportion of multidrug-resistant isolates from chickens (60 strains, 87.0%) was significantly higher ($p < 0.001$) than that from ducks (37 strains, 56.9%; Figure 1). All 69 *E. coli* strains isolated from chicken fecal samples were resistant to at least one antibiotic and exhibited 34 distinct resistance patterns (Table 3). The most common resistance pattern was ampicillin - gentamicin - tetracycline - trimethoprim/sulfamethoxazole - sulfonamides pattern, observed in 9 strains (13.04%). The second most frequent resistance pattern was ampicillin - cefotaxime - gentamicin - trimethoprim/sulfamethoxazole - sulfonamides - nalidixic acid - norfloxacin, observed in 5 strains (7.25%). Of the 65 *E. coli* strains isolated from ducks, 4 were susceptible to all 10 antibiotics, and the remaining 61 exhibited 36 distinct resistance patterns (Table 4). The most common resistance patterns were ampicillin - trimethoprim/sulfamethoxazole and ampicillin - trimethoprim/sulfamethoxazole - sulfonamides, observed in 6 (9.23%) and 7 (10.77%) strains, respectively. Additionally, the ampicillin alone and ampicillin - gentamicin - trimethoprim/sulfamethoxazole - sulfonamides resistance patterns were each detected in 4 strains (6.15%).

Table 1. Isolation rate of *Escherichia coli* from poultry fecal samples in Ninh Binh, Vietnam

Sources	Sample	Samples (number)	Positive isolates number (%)	95% confidence interval
Chicken		85	69 (81.2)	(71.24 - 88.84)
Duck		85	65 (76.5)	(66.03 - 85.00)
Total		170	134 (78.8)	(71.81 - 84.71)

Table 2. Antibiotic resistance rates among *Escherichia coli* strains isolated from poultry fecal samples in Ninh Binh, Vietnam

Antibiotic classes	Antibiotics	Number and resistance rate (%) of <i>Escherichia coli</i> strains				
		Chicken (n = 69)	Duck (n = 65)	P-value	Total (n = 134)	95% confidence interval
Penicillin	Ampicillin	59 (85.5) ^a	52 (80.0) ^a	0.398	111 (82.8)	(75.4 - 88.8)
Cephalosporin	Cefotaxime	30 (43.5) ^a	21 (32.3) ^a	0.183	51 (38.1)	(29.8 - 49.8)
Carbapenems	Meropenem	0 (0.0) ^a	0 (0.0) ^a	nd	0 (0.0)	(0.0 - 0.2)
Aminoglycosides	Gentamicin	42 (60.9) ^a	28 (43.1) ^b	0.039	70 (52.2)	(43.4 - 60.9)
Quinolones	Nalidixic acid	23 (33.3) ^a	18 (27.7) ^a	0.479	41 (30.6)	(22.9 - 39.1)
	Norfloxacin	15 (21.7) ^a	7 (10.8) ^a	0.148	22 (16.4)	(10.6 - 23.8)
Tetracyclines	Doxycycline	15 (21.7) ^a	13 (20.0) ^a	0.805	28 (20.9)	(14.4 - 28.8)
	Tetracycline	54 (78.3) ^a	27 (41.5) ^b	0.001	81 (60.5)	(51.6 - 68.8)
Sulfonamides	Sulfonamides	61 (88.4) ^a	46 (70.8) ^b	0.012	107 (79.9)	(72.1 - 86.3)
	Trimethoprim/ Sulfamethoxazole	53 (76.8) ^a	47 (72.3) ^a	0.549	100 (74.6)	(66.4 - 81.7)

n: number of samples, nd: Not detected. ^{a,b} Mean different superscript letters on the same row indicate statistically significant differences at p-value less than 0.05.

Table 3. Multidrug-resistance profiles of *Escherichia coli* strains isolated from chicken fecal samples in Ninh Binh, Vietnam

AMR pattern	Number of AMR	Number of classes	Number of strains (%)
AMP	1	1	1 (1.45)
TET	1	1	1 (1.45)
AMP, TET	2	2	1 (1.45)
STX, SUL	2	1	1 (1.45)
AMP, STX, SUL	3	2	3 (4.35)
AMP, TET, SUL	3	3	3 (4.35)
TET, STX, SUL	3	2	1 (1.45)
CTX, DOX, TET	3	2	1 (1.45)
CTX, TET, SUL	3	3	2 (2.90)
AMP, GEN, STX, NAL	4	4	1 (1.45)
AMP, GEN, STX, SUL	4	3	1 (1.45)
AMP, TET, STX, SUL	4	3	3 (4.35)
AMP, CTX, GEN, TET	4	4	1 (1.45)
CTX, DOX, TET, SUL	4	3	1 (1.45)
AMP, GEN, DOX, TET, STX	5	4	1 (1.45)
AMP, DOX, TET, STX, SUL	5	3	2 (2.90)
AMP, CTX, TET, STX, SUL	5	4	2 (2.90)
AMP, GEN, TET, STX, SUL	5	4	9 (13.04)
AMP, TET, STX, SUL, NAL	5	4	3 (4.35)
CTX, GEN, TET, SUL, NOR	5	5	1 (1.45)
AMP, GEN, DOX, TET, STX, SUL	6	4	3 (4.35)
AMP, CTX, GEN, STX, NAL, NOR	6	5	1 (1.45)
AMP, CTX, GEN, TET, STX, SUL	6	5	4 (5.80)
AMP, GEN, TET, STX, SUL, NAL	6	5	2 (2.90)
AMP, CTX, TET, STX, SUL, NAL	6	5	2 (2.90)
AMP, CTX, GEN, SUL, NAL, NOR	6	5	2 (2.90)
GEN, TET, STX, SUL, NAL, NOR	6	4	1 (1.45)
CTX, GEN, DOX, TET, SUL, NOR	6	5	1 (1.45)
AMP, CTX, GEN, DOX, TET, STX, SUL	7	5	3 (4.35)
AMP, GEN, DOX, TET, STX, SUL, NAL	7	5	1 (1.45)
AMP, CTX, GEN, STX, SUL, NAL, NOR	7	5	5 (7.25)
AMP, GEN, DOX, TET, SUL, NAL, NOR	7	5	1 (1.45)
AMP, CTX, GEN, DOX, TET, STX, SUL, NAL	8	6	1 (1.45)
AMP, CTX, GEN, TET, STX, SUL, NAL, NOR	8	6	3 (4.35)

The total number of *Escherichia coli* strains isolated from chicken fecal samples was 69. AMP: Ampicillin, CTX: Cefotaxime, DOX: Doxycycline, GEN: Gentamicin, NAL: Nalidixic acid, NOR: Norfloxacin, SUL: Sulfonamides, TET: Tetracycline, SXT: Trimethoprim/Sulfamethoxazole

Table 4. Multidrug-resistance profiles of *Escherichia coli* strains isolated from duck fecal samples in Ninh Binh, Vietnam

AMR pattern	Number of AMR	Number of classes	Number of strains (%)
Susceptible	0	0	4 (6.15)
AMP	1	1	4 (6.15)
STX	1	1	1 (1.54)
TET	1	1	1 (1.54)
AMP, STX	2	2	6 (9.23)
AMP, TET	2	2	1 (1.54)
STX, SUL	2	1	3 (4.62)
AMP, STX, SUL	3	2	7 (10.77)
AMP, CTX, SUL	3	3	1 (1.54)
AMP, SUL, NAL	3	3	1 (1.54)
AMP, TET, SUL	3	3	1 (1.54)
TET, STX, SUL	3	2	1 (1.54)
TET, SUL, NAL	3	3	1 (1.54)
AMP, GEN, STX, SUL	4	3	4 (6.15)
AMP, GEN, TET, STX	4	4	1 (1.54)
AMP, TET, STX, SUL	4	4	1 (1.54)
AMP, CTX, GEN, STX, SUL	5	4	1 (1.54)
AMP, CTX, GEN, STX, NAL	5	5	1 (1.54)
AMP, CTX, TET, STX, SUL	5	4	1 (1.54)
AMP, GEN, TET, STX, SUL	5	4	2 (3.08)
AMP, GEN, TET, SUL, NAL	5	5	1 (1.54)
AMP, CTX, DOX, TET, STX, SUL	6	4	3 (4.62)
AMP, GEN, DOX, TET, STX, SUL	6	4	1 (1.54)
AMP, CTX, GEN, STX, SUL, NAL	6	5	3 (4.62)
AMP, CTX, GEN, TET, STX, SUL	6	5	1 (1.54)
AMP, GEN, TET, STX, SUL, NAL	6	5	1 (1.54)
AMP, CTX, GEN, DOX, TET, SUL	6	5	1 (1.54)
AMP, GEN, DOX, TET, SUL, NAL	6	5	1 (1.54)
CTX, GEN, STX, SUL, NAL, NOR	6	4	1 (1.54)
AMP, CTX, GEN, DOX, TET, STX, SUL	7	5	1 (1.54)
AMP, CTX, GEN, STX, SUL, NAL, NOR	7	5	1 (1.54)
AMP, CTX, GEN, DOX, TET, SUL, NAL	7	6	1 (1.54)
AMP, CTX, GEN, DOX, TET, STX, SUL, NAL	8	6	1 (1.54)
AMP, GEN, DOX, TET, STX, SUL, NAL, NOR	8	5	1 (1.54)
AMP, CTX, GEN, TET, STX, SUL, NAL, NOR	8	6	1 (1.54)
CTX, GEN, DOX, TET, STX, SUL, NAL, NOR	8	5	1 (1.54)
AMP, CTX, GEN, DOX, TET, STX, SUL, NAL, NOR	9	6	2 (3.08)

The total number of *Escherichia coli* strains isolated from duck fecal samples was 65. AMP: Ampicillin, CTX: Cefotaxime, DOX: Doxycycline, GEN: Gentamicin, NAL: Nalidixic acid, NOR: Norfloxacin, SUL: Sulfonamides, TET: Tetracycline, SXT: Trimethoprim/Sulfamethoxazole

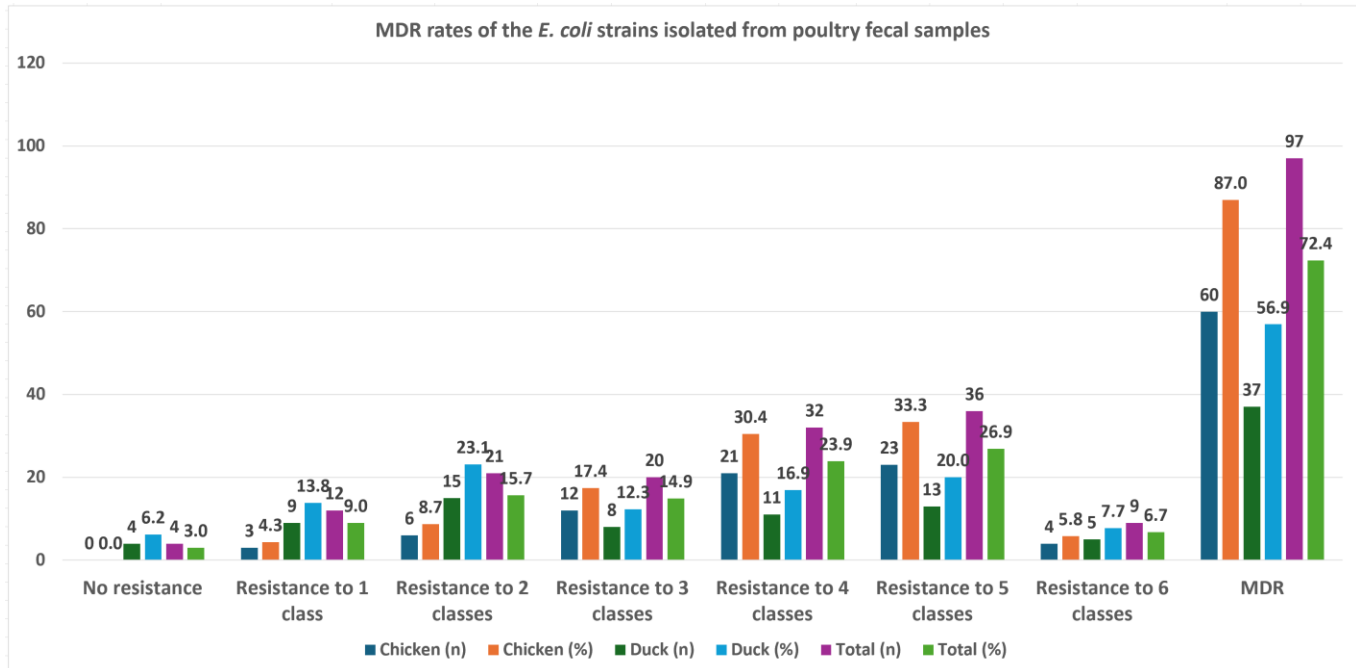


Figure 1. Multidrug resistance pattern of *Escherichia coli* strains isolated from poultry fecal samples in Ninh Binh, Vietnam

DISCUSSION

The *E. coli* isolation rate from poultry fecal samples in the current study was comparable to the 86.39% observed in broiler chickens fecal samples in China (Liu et al., 2025) but higher than the 36.7%-66.7% rate documented in broiler chicken fecal samples from Kenya (Langata et al., 2019), Egypt (Badr et al., 2022), Iraq (Hussein et al., 2025), Nigeria (Ugbo et al., 2025), South Africa (Gumede et al., 2026), and Pakistan (Khan et al., 2026). Notably, previous studies in Nigeria (Agusi et al., 2025), Bangladesh (Ibrahim et al., 2023), and India (Patel et al., 2023) have reported higher *E. coli* isolation rates from broiler chicken fecal samples, ranging from 86.4% to 99.44%. *Escherichia coli* isolation rates varied across studies due to differences in sampling methods, sample sizes, isolation methods, study sites, and farm hygiene conditions (Roy et al., 2025).

The 82.8% ampicillin resistance rate observed in the present study was predictable, given the widespread and long-term use of this antibiotic in human and veterinary medicine in Vietnam (Nga-do et al., 2014; Nohrborg et al., 2024). Similarly, high ampicillin resistance rates in avian-derived *E. coli* strains, ranging from 78.3% to 87.8%, have been reported in studies from India (Patel et al., 2023), Kenya (Deng et al., 2024), and China (Afayibo et al., 2022; Liu et al., 2025). However, the ampicillin resistance rate of *E. coli* strains in the current study was lower than

E. coli strains isolated from broiler chickens in Egypt (Awad et al., 2016; Badr et al., 2022), Algeria (Barour et al., 2022), Poland (Racewicz et al., 2022), Bangladesh (Ibrahim et al., 2023), Nepal (Bhattarai et al., 2024), and South Africa (Gumede et al., 2026), where resistance rates ranged from 92.9% to 100%. Conversely, lower rates of ampicillin resistance, ranging from 45.5% to 75.5%, were observed in broiler chicken-derived *E. coli* strains in Nigeria (Agusi et al., 2024; Adetunji et al., 2025; Ugbo et al., 2025), Tanzania (Mtemisika et al., 2022), and Pakistan (Khan et al., 2026). Notably, chicken- and duck-derived *E. coli* strains from Canada demonstrated lower rates of ampicillin resistance than those in the present study, ranging from 4.17% to 15.92% (Varga et al., 2019).

The cefotaxime resistance rate (38.1%) in the current study was comparable to the 29.27% and 38.3% rates observed in *E. coli* isolates from broiler chickens in Slovakia (Sciberras et al., 2019) and Nigeria (Adetunji et al., 2025). However, some studies have reported higher cefotaxime resistance rates of *E. coli* isolates from broiler chickens, including 59.09% in Bangladesh (Khan et al., 2026) and 52.6-80.12% in China (Afayibo et al., 2022; Liu et al., 2025). In contrast, previous studies from Bangladesh (Ibrahim et al., 2023), Iraq (Hussein et al., 2025), and Spain (Sevilla-Navarro et al., 2022) reported lower cefotaxime resistance rates of 3.0%, 6.6%, and 9.0% among *E. coli* isolates from broiler chickens. Gumede et

al. (2025) observed that all *E. coli* strains from poultry farms in South Africa were susceptible to cefotaxime.

All *E. coli* strains in the present study were susceptible to meropenem, consistent with findings in *E. coli* from chickens and ducks in Canada (Varga et al., 2019), broiler chickens in South Africa (Gumede et al., 2026), and Nigeria (Adetunji et al., 2025). Nonetheless, meropenem resistance was reported at higher rates in *E. coli* from broiler chickens, including 3.2% in Tanzania (Mtemisika et al., 2022) and 9.09% in Pakistan (Khan et al., 2026). These differences may reflect variations in management practices and AMU, which can influence the development of meropenem resistance in livestock isolates.

In the current study, 52.2% of *E. coli* strains were resistant to gentamicin, a widely used antibiotic in livestock and veterinary medicine in Vietnam (Nga-do et al., 2014). Previous studies in China (Liu et al., 2025), Nepal (Shrestha and Banjara, 2025), and Slovenia (Sciberras et al., 2019) reported higher resistance rates of *E. coli* strains from broiler chickens, at 62.2%, 65.38%, and 75.6%, respectively. In contrast, lower resistance rates, ranging from 22.0% to 44.7%, were observed in *E. coli* strains isolated from broiler chickens in Bangladesh (Ibrahim et al., 2023), India (Khan et al., 2026), Tanzania (Mtemisika et al., 2022), Nepal (Bhattarai et al., 2024), China (Afayibo et al., 2022), Poland (Racewicz et al., 2022), and Nigeria (Adetunji et al., 2025). Notably, studies from Kenya and South Africa (Langata et al., 2019; Gumede et al., 2026) did not detect gentamicin-resistant *E. coli* isolates derived from broiler chickens, whereas Varga et al. (2019) reported a low resistance rate of 6.42% in *E. coli* strains from chicken and duck sources in Canada.

The resistance rate to nalidixic acid (30.6%) among isolated *E. coli* strains was lower than that reported in other poultry-related studies, such as 78.7% reported in Nigeria (Adetunji et al., 2025), 90.0% documented in Bangladesh (Ibrahim et al., 2023), 94.44% identified in India (Patel et al., 2023), and 97.96% found in Algeria (Barour et al., 2022). Conversely, poultry-derived *E. coli* strains in Canada (Varga et al., 2019) and Kenya (Langata et al., 2019) exhibited lower nalidixic acid resistance rates, ranging from 2.0 to 4.17%. The present study observed 16.4% resistance to norfloxacin, lower than the 56.76% recorded in *E. coli* strains isolated from chickens and ducks in Canada (Varga et al., 2019), and the 66.1% and 91.7% recorded in *E. coli* strains isolated from broiler chickens in Kenya (Badr et al., 2022) and Iraq (Hussein et al., 2025), respectively. The relatively low resistance levels to nalidixic acid and norfloxacin observed in the

present study may reflect less frequent use of these antibiotics on poultry farms in Vietnam compared with other antibiotics (Carrique-Mas et al., 2015).

The 60.5% tetracycline resistance rate in the present study aligns with the antibiotic's widespread global use. Numerous studies have documented high rates of tetracycline resistance in broiler chicken-derived *E. coli* isolates, such as 97.22% in India (Patel et al., 2023), 78.7–81.92% in Nigeria (Agusi et al., 2024; Adetunji et al., 2025), 81.1–100% in Nepal (Bhattarai et al., 2024; Shrestha and Banjara, 2025), 87.4% in Tanzania (Mtemisika et al., 2022), 84.6–95.2% in China (Afayibo et al., 2022; Liu et al., 2025), 92.68% in Slovakia (Sciberras et al., 2019), 93.1–100% in Egypt (Awad et al., 2016; Badr et al., 2022), 98.0% in Bangladesh (Ibrahim et al., 2023), 100% in Algeria (Barour et al., 2022), and South Africa (Gumede et al., 2026). However, some studies reported lower tetracycline resistance rates than those in the present study, such as 12.0% and 38.0% resistance rates in broiler chicken-derived *E. coli* isolates in Kenya (Langata et al., 2019) and Spain (Sevilla-Navarro et al., 2022), respectively, and 41.67–43.3% resistance rates in broiler chicken- and duck-derived *E. coli* strains reported in Canada (Varga et al., 2019). The doxycycline resistance rate (20.9%) among *E. coli* strains in the current study was comparable to the 27.0% observed in *E. coli* isolates from broiler chickens in Spain (Sevilla-Navarro et al., 2022). Higher rates of doxycycline resistance have been reported in *E. coli* isolates from broiler chickens, such as 39.42% and 76.4% in Nepal (Bhattarai et al., 2024; Shrestha and Banjara, 2025), 62.6% and 93.9% in China (Afayibo et al., 2022; Liu et al., 2025), and 88.0% in Poland (Racewicz et al., 2022).

In the current study, 79.9% of isolated *E. coli* strains were resistant to sulfonamides, comparable to the 84.3% reported in China (Afayibo et al., 2022) but higher than the 53.2% and 66.0% documented in Nigeria (Agusi et al., 2024; Adetunji et al., 2025). Furthermore, 74.6% of isolates were resistant to trimethoprim/sulfamethoxazole, consistent with previous studies reporting high resistance rates of *E. coli* isolates derived from broiler chicken samples in Nepal (53.0%; Shrestha and Banjara, 2025), Egypt (58.6%–82.1%; Badr et al., 2022; Awad et al., 2026), Spain (66.0%; Sevilla-Navarro et al., 2022), Tanzania (69.8%; Mtemisika et al., 2022), and in South Africa (76.0%; Gumede, 2026). However, resistance levels in broiler chicken-derived *E. coli* strains were reported as 84.0% in Poland (Racewicz et al., 2022), 90.74% in India (Patel et al., 2023), and 100% in Algeria (Barour et al., 2022). Conversely, *E. coli* isolates collected

from poultry samples in Canada (Varga et al., 2019) and Kenya (Langat et al., 2019) exhibited much lower resistance to trimethoprim/sulfamethoxazole, at 8.38-16.67% and 26.0%, respectively.

The isolated *E. coli* strains exhibited a broad spectrum of AMR patterns, with 34 in chicken-derived strains and 36 in duck-derived strains. Overall, 72.4% of isolates were multidrug-resistant, representing 87.0% of chicken isolates and 56.9% of duck isolates. The worldwide rise of multidrug-resistant *E. coli* strains poses a noticeable challenge for choosing effective antibiotics. The prevalence of multidrug-resistant *E. coli* strains in this study aligns with rates of 74.0% reported in Nigeria (Adetunji, 2025) 74.0% in South Africa (Gumede et al., 2025), and 81.1% in Poland (Racewicz et al., 2022), but exceeded the multidrug-resistance rates of 12.5-19.55% and 35.3% observed in poultry-origin *E. coli* strains in studies from Canada (Varga et al., 2019) and Nigeria (Ugbo et al., 2025), respectively. Notably, very high rates of multidrug-resistance have been documented in *E. coli* isolates from broiler chickens, including 86.3% in Tanzania (Mtemisika et al., 2022), 90.38% in Nepal (Shrestha and Banjara, 2025), 96.45-100% in China (Afayibo et al., 2022; Liu et al., 2025), 100% in Algeria (Barour et al., 2022), 100% in Spain (Sevilla-Navarro et al., 2022), and 100% in Bangladesh (Ibrahim et al., 2023). In the current study, multidrug-resistant *E. coli* demonstrated co-resistance to some antibiotics such as ampicillin, tetracycline, sulfonamide, trimethoprim/sulfamethoxazole, and gentamicin, as well as to newer antibiotics. These resistance patterns indicated that *E. coli* strains on poultry farms are frequently exposed to different antibiotics because of drug-use practices by farm owners and technical staff during rearing.

CONCLUSION

The present study confirmed the presence of AMR *E. coli* strains in colored-feather broiler chicken and meat-oriented duck farms in Ninh Binh, Vietnam, with most isolates exhibiting multidrug-resistance patterns. The isolated *E. coli* strains demonstrated high resistance to several commonly used antibiotics, including ampicillin, sulfonamides, tetracycline, trimethoprim/sulfamethoxazole, gentamicin, and cefotaxime. However, due to the small sample size and the lack of investigation into poultry drug-use practices in the study area, the study did not establish a link between *E. coli* prevalence and specific poultry breeds or between AMU and the AMR rate of the isolated *E. coli* strains. The

relationship between the phenotypic and genotypic profiles of these strains remains unclear. Therefore, further studies should examine how farm hygiene and medication practices contribute to AMR in poultry.

DECLARATIONS

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Authors' contributions

Chu Thi Thanh Huong and Nguyen Van Giap designed the study and methodology and contributed to the manuscript. Truong Lan Oanh and Nguyen Hoang Thinh conducted the experiments and analyzed the data. Truong Ha Thai supervised the study, provided methodological support, and wrote the manuscript. All authors revised and approved the final edition of the manuscript.

Availability of data and materials

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

Competing interests

The authors have no conflicts of interest.

Ethical considerations

This article was originally written without copying from published articles and books. No AI was used in this study. The authors take full responsibility for the originality of the present study data before submission and after that, during the peer review process.

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The Effect of Onion Skin Meal on Ileum Histology in Broiler Chickens

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ABSTRACT

For many years, plant-based feed additives have served as alternatives to antibiotics, improving feed efficiency and ensuring livestock safety. The present study aimed to investigate the effects of garlic skin, shallot skin, and onion skin on the ileum in broiler chickens. A total of 189 broiler chickens were used in the present study and randomly assigned to nine treatment groups with three replicates, each consisting of seven chickens. The chickens were fed a basal diet supplemented with no onion skin (T0), 0.1% bacitracin (T1), 0.5% garlic skin (T2), 0.5% shallot skin (T3), 0.5% onion skin (T4), a basal diet supplemented with 0.25% garlic and 0.25% shallot skins (T5), a basal diet supplemented with 0.25% garlic and 0.25% onion skins (T6), a basal diet supplemented with 0.25% shallot and 0.25% onion skins (T7), and a basal diet supplemented with 0.167% garlic, shallot, and onion skins each (T8) for 35 days. The current results indicated that adding garlic skin meal, shallot skin meal, and onion skin meal did not affect ileal histology, villi number, villi length, villi width, villi surface area, and crypt depth in broiler chickens. The absence of adverse histological alterations indicated that onion skin meal is safe for utilization as a feed additive.

Keywords: Broiler chicken, Garlic skin, Ileum, Onion skin, Shallot skin

INTRODUCTION

The use of antibiotics as feed additives in broiler nutrition has caused several adverse effects, including drug residues in livestock products and the development of bacterial resistance, which may pose significant human health risks (Mehdi et al., 2018). Several countries have banned antibiotics in livestock feed because of concerns about drug residues and antimicrobial resistance (Castanon, 2007). Since January 1, 2006, the European Union has formally prohibited antibiotic use in livestock feed under Regulation No. 1831/2003 (Castanon, 2007). A similar policy was implemented in Indonesia through Minister of Agriculture Regulation No. 14/2017, which banned the use of antibiotics as feed additives since January 2018 (Azizah et al., 2024). Following the prohibition on using antibiotics as feed additives in livestock production, numerous alternatives, including probiotics, prebiotics, phytobiotics,

and organic acids, have been explored (El-Hack et al., 2022; Anyigor et al., 2026).

One promising alternative is phytobiotics: plant-derived feed additives containing bioactive compounds with diverse biological effects, such as antioxidant, antibacterial, anti-inflammatory, antiviral, antifungal, and immunomodulatory activities (Ivanova et al., 2024). The plant-based feed additives are known to increase productivity, improve feed utilization efficiency, and enhance gut morphofunctional properties (Selionova et al., 2025). Garlic skin, shallot skin, and onion skin are different types of phytobiotic feed additives (Kothari et al., 2019). Garlic skin possesses flavonoids, tannins, phenolics, and anthocyanins (Dos Santos et al., 2022). Shallot skin has phenolic and flavonoid compounds (Moldovan et al., 2024). Onion skin is a food waste that can cause environmental pollution when not properly managed. Onion skin contains bioactive compounds beneficial for poultry health, including flavonoids,

phenolics, anthocyanins, tannins, saponins, phenols, and quercetin (Benitez et al., 2011; Shabir et al., 2022). Bioactive compounds in onion skins have antibacterial, antioxidant, and immunomodulatory properties (Adeyemi et al., 2021) that may enhance gut health and improve poultry productivity (Obianwuna et al., 2024). The antibacterial properties of onion skins can inhibit the growth of pathogenic bacteria in the gut (Kumar et al., 2022). Reducing the pathogenic bacterial population may help maintain intestinal health, thereby supporting optimal gut function. This mechanism can improve villus morphology, which plays an important role in nutrient absorption (Heak et al., 2017). No scientific reports have addressed the impacts of dietary onion skin on poultry intestines. Therefore, the present study aimed to investigate the effects of garlic skin, shallot skin, and onion skin meals on the histological structure of the ileum in broiler chickens.

MATERIALS AND METHODS

Ethical approval

The Animal Care and Use Committee of the University of Brawijaya, Malang, Indonesia, approved all procedures performed in the present study.

Study design

A total of 189 unsexed Lohmann MB 202 broiler chickens with an average body weight of 44.78 g/chicken were randomly divided into nine treatment groups with three replicates, and each replicate consisted of seven chickens. The first group received only the basal feed without any feed additives (T0), and the second group received the basal feed supplemented with 0.1% bacitracin

(T1; Widodo et al., 2022). The third, fourth, and fifth groups received the basal feed supplemented with 0.5% garlic skin (T2), 0.5% shallot skin (T3), and 0.5% onion skin (T4), respectively. The sixth group was fed the basal feed supplemented with 0.25% each of garlic skin and shallot skin (T5), the seventh group received the basal feed supplemented with 0.25% each of garlic skin and onion skin (T6), and the eighth group received the basal feed supplemented with 0.25% each of shallot skin and onion skin (T7). The last group received the basal feed supplemented with 0.167% each of garlic, shallot, and onion skins (T8). Before the chickens arrived, the pens were cleaned and disinfected. The chickens were raised for 35 days in 27 floor pens measuring 100 × 100 × 80 cm, which were lined with rice husks. The ambient temperature was maintained at approximately 32°C during the first three days and gradually reduced to 23°C by the end of the rearing period, while relative humidity was maintained between 50 and 70% (EFSA AHAW Panel et al., 2023). The chickens were fed a basal feed manufactured by PT New Hope, Indonesia, using a mash-form starter feed from 1 to 21 days of age and a crumble-form finisher feed from 22 to 35 days of age, including soybean meal, corn, fish meal, meat and bone meal, corn gluten, palm kernel meal, oil, and calcium phosphate (Table 1). Drinking water was provided *ad libitum*. Chickens were vaccinated with Newcastle disease and infectious bronchitis vaccines (PT Vaksindo Satwa Nusantara, Indonesia) at 1 day of age via eye drops. The Gumboro vaccine (PT Medion Ardhika Bhakti, Indonesia) was administered at 10 days of age via drinking water. Each vaccine was administered once during the rearing period. There were no mortality rates in the present study.

Table 1. Nutrient content of the basal feed for broiler chickens at starter and finisher phases

Nutrient content	Starter (1-21 days)	Finisher (22-35 days)
Water content (%)	Max 14	Max 14
Crude protein (%)	Min 22	Min 20
Crude fat (%)	Min 5	Min 5
Crude fiber (%)	Max 5	Max 5
Ash (%)	Max 8	Max 8
Calcium (%)	0.8-1.1	0.8-1.1
Total phosphorus (with phytase enzyme, %)	Min 0.5	Min 0.5
Total aflatoxin (µg/kg)	Max 50	Max 50
Lysine (%)	Min 1.2	Min 1.2
Methionine (%)	Min 0.45	Min 0.45
Methionine + cystine (%)	Min 0.8	Min 0.8
Tryptophan (%)	Min 0.19	Min 0.19
Threonine (%)	Min 0.75	Min 0.75

Source: PT New Hope East Java, Max: Maximum, Min: Minimum.

Onion skin meal

Garlic skins, shallot skins, and onion skins were obtained from traditional markets in Malang City, Indonesia, and collected on the same day. A total of 1.2 kg of garlic skins, 3 kg of shallot skins, and 1.8 kg of onion skins were collected in dry and intact condition. The skins were washed with clean water, then oven-dried at 50°C for 24 hours. After the drying process, the skins were ground into a 100-mesh meal. The resulting meals were stored in airtight glass jars and kept in a dry place away from direct sunlight. The meals were then added to the basal feed according to treatment doses.

Ileum histology

Ileum samples were obtained at 35 days of age by slaughtering one chicken from each experimental unit, resulting in a total of 27 chickens. The chickens were slaughtered with a sharp knife by cutting the neck to sever the carotid arteries, jugular veins, trachea, and esophagus (Salwani et al., 2016). The small intestine was then removed. Samples were taken by cutting the ileum of the small intestine along 1 cm, then cleaned with distilled water and fixed in 10% neutral buffered formalin. Histological examination was performed by cutting the ileum into 4 µm sections using a microtome, then mounting them on slides for staining with Harris Hematoxylin and Eosin (Tejeda and Kim, 2021).

Examinations were performed using a light microscope (Nikon, Japan) equipped with an Optilab camera connected to a computer, and analyzed using Image Raster software (version 4.0.5). The number of villi (per transverse cut), villi length, villi width, and crypt depth were measured. In addition, the villi surface area was calculated using Formula 1 (Iji et al., 2001).

$$\text{Villi surface area} = (b + c) / c \times a \quad (\text{Formula 1})$$

where a is the villi length, b is the basal width of villi, and c is the apical width of villi.

Statistical analysis

One-way analysis of variance (ANOVA) was used to analyze the data with SPSS software version 27. Significant differences between mean values were tested using Duncan's Multiple-Range Test (DMRT), with the significance level set at P value less than 0.05.

RESULTS AND DISCUSSION

Histological examination of the ileum demonstrated that adding garlic, shallot, and onion skin meal to the feed did not produce statistically significant changes in the measured parameters across all treatment groups ($p > 0.05$; Table 2). Histological findings of the ileum sampled from broiler chickens across different treatment groups are illustrated in Figure 1.

Table 2. Effects of onion skin meal on ileum histology in 35-day-old broiler chickens

Treatment	Villi amount (per transverse cut)	Villi length (µm)	Villi width (µm)	Villi surface area (µm ²)	Crypt depth (µm)
T0	4.80	377.20	41.83	1,054.60	110.70
T1	5.73	374.43	46.78	1,248.10	78.40
T2	7.33	399.87	34.90	1,119.27	100.17
T3	5.27	376.07	37.33	1,135.93	95.20
T4	4.80	336.20	40.60	862.30	81.30
T5	5.53	333.90	37.67	931.23	76.37
T6	5.00	338.43	42.05	893.37	83.47
T7	6.60	445.27	31.70	1,330.13	84.57
T8	5.13	353.43	37.13	961.57	86.83
SEM	0.28	9.42	1.45	53.44	3.38

T0: Basal feed (control), T1: Basal feed + 0.1% bacitracin, T2: Basal feed + 0.5% garlic skin, T3: Basal feed + 0.5% shallot skin, T4: Basal feed + 0.5% onion skin, T5: Basal feed + 0.25% each of garlic skin and shallot skin, T6: Basal feed + 0.25% each of garlic skin and onion skin, T7: Basal feed + 0.25% each of shallot skin and onion skin, T8: Basal feed + 0.167% each of garlic, shallot, and onion skins. SEM: Standard error of the mean

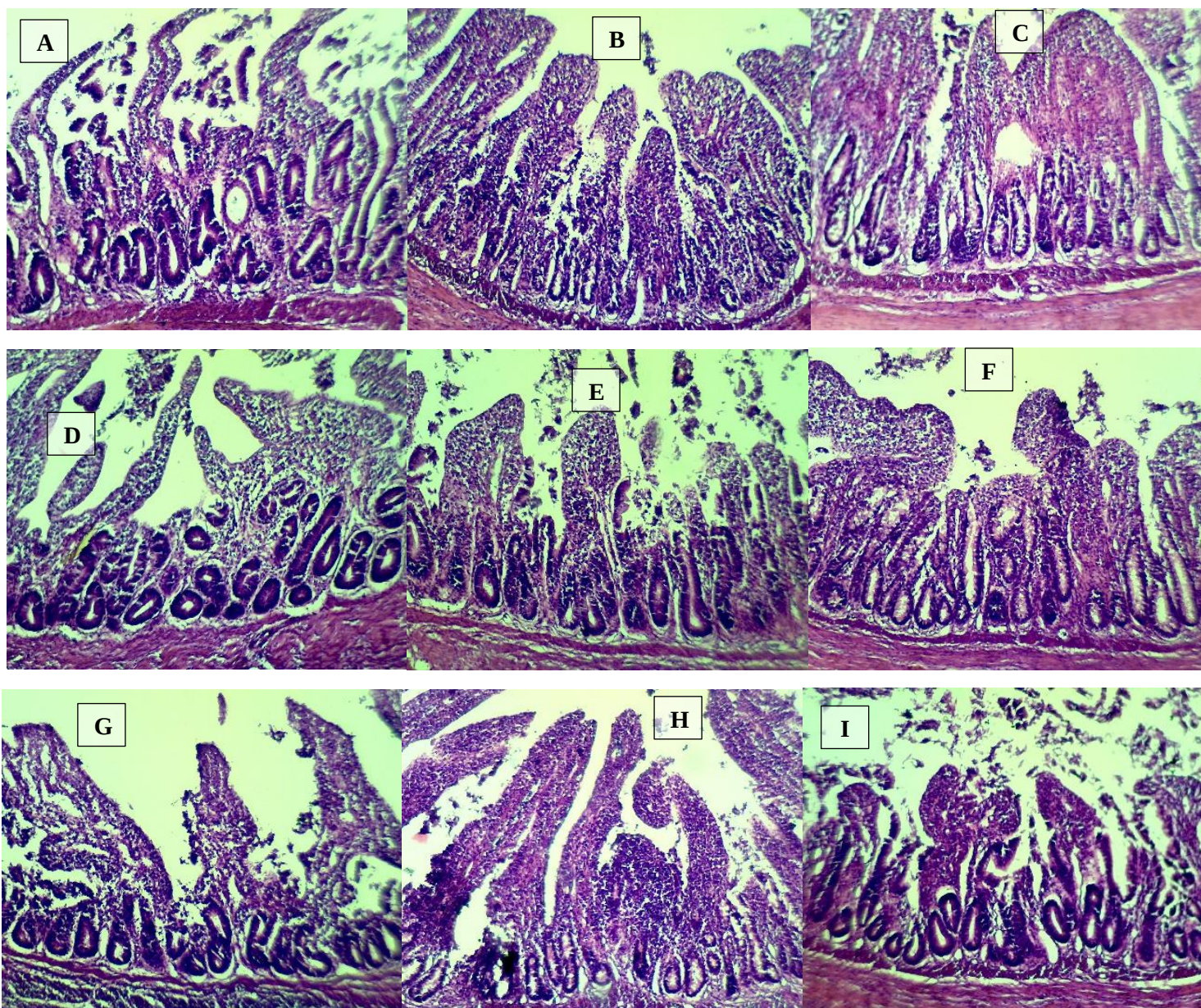


Figure 1. Ileal histology of 35-day-old broiler chickens fed diets supplemented with garlic, shallot, and onion skin meals. **A:** Without onion skin (T0), **B:** 0.1% bacitracin (T1), **C:** 0.5% garlic skin (T2), **D:** 0.5% shallot skin (T3), **E:** 0.5% onion skin (T4), **F:** 0.25% each of garlic skin and shallot skin (T5), **G:** 0.25% each of garlic skin and onion skin (T6), **H:** 0.25% each of shallot skin and onion skin (T7), **I:** 0.167% each of garlic skin, shallot skin, and onion skin (T8).

Garlic skins, shallot skins, and onion skins are agricultural byproducts rich in bioactive compounds and can be used as natural feed additives to support the digestive health of broiler chickens (Dulia et al., 2026). Onion skins contain different bioactive compounds, including flavonoids, alkaloids, terpenoids, tannins, saponins, phenols, anthocyanins, and quercetin, which have been reported to possess antibacterial activity (Shabir et al., 2022). However, the level of onion skins used in the current study was not sufficient to produce significant changes in the ileum of broiler chickens. The absence of significant effects in the present study was likely due to the low level of onion skin meal added. Mozin et al. (2015) reported that adding shallot by-products at levels ranging from 0.5% to 2% had no remarkable effects on small intestinal characteristics in broiler chickens, including villi height and crypt depth. These findings indicated that these levels of bioactive compounds were insufficient to optimally stimulate intestinal villi development.

Intestinal morphology is related to the intestine's capacity to absorb nutrients (Awad et al., 2009). Longer, wider villi provide a larger surface area for nutrient absorption, making the process more efficient (Prakatur et al., 2019). Feed additives can support intestinal development and optimize nutrient absorption (Oni and Oke, 2025). One method for assessing the effects of feed additives on the digestive tract involves histological examination of intestinal morphology (Alsafy et al., 2024). The current results demonstrated that the onion skin meal had no notable effects on the ileum in broiler chickens, indicating that the bioactive compounds in onion skin meal could not enhance villi growth, so the surface area for nutrient absorption did not increase notably (Hossain and Amin, 2026). Onion skins contain bioactive compounds with antibacterial properties, such as flavonoids and phenols (Albandary, 2023). Flavonoids act as antibacterial agents by targeting key bacterial processes such as nucleic acid replication, membrane functionality, and energy production (Shamsudin et al., 2022). Moreover, flavonoids may also reduce adhesion and biofilm formation, porin expression on cell membranes, membrane permeability, and pathogenicity, all of which are critical mechanisms for bacterial growth (Shamsudin et al., 2022). Furthermore, phenol demonstrates its antibacterial efficacy by disrupting cell membranes (Dembinska et al., 2025). Specifically, phenolic compounds interact with the lipid layer, increasing membrane permeability and triggering leakage of intracellular components, eventually resulting in cell lysis (Dembinska et al., 2025). Such bioactive compounds can suppress the population of pathogenic

bacteria in the digestive tract and create a healthier gut environment (Gilani et al., 2021).

According to Li et al. (2024), lactic acid bacteria such as *Lactobacillus* sp. and *Enterococcus* sp. are beneficial microorganisms found in the chicken gut, whereas pathogenic bacteria such as *Escherichia coli* and *Salmonella* sp. can disrupt gut health. Active compounds in onion skins can modulate the composition of the gut microbiota by promoting the growth of beneficial bacteria while suppressing pathogenic bacteria (Adeyemi et al., 2021). However, when onion skins were included in the diet at low levels, the resulting antibacterial activity did not demonstrate sufficient potency to influence the composition of the gut microbiota and, ultimately, the villi morphology.

CONCLUSION

The addition of garlic skin, shallot skin, and onion skin meal did not negatively affect ileal histology in broiler chickens. However, the inclusion levels used in the present study were too low to induce significant histological changes. Therefore, further studies at higher levels of garlic, shallot, and onion skin meal are recommended to achieve significant histological improvements.

DECLARATIONS

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Authors' contributions

Salsabila Fyrda Maharani conducted the research and wrote the manuscript. Edhy Sudjarwo contributed to designing the study and proofreading the manuscript. Osfar Sjojfan participated in designing the study, proofreading the manuscript, and analyzing the data for publication. All authors approved the final edition of the manuscript prior to publication in the present journal.

Availability of data and materials

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

Competing interests

The authors declare no competing interests.

Ethical considerations

All authors have affirmed that this study is free of plagiarism, misconduct, data fabrication and/or

falsification, duplicate publication and submission, and redundancy, and have provided their consent for publication. The authors declare that they did not use any artificial intelligence tools for the composition and preparation of this study. Additionally, the authors state that they are responsible for the accuracy, originality, and integrity of the final content before submission, during the revision process, and upon final publication of the article.

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Pigeon Pox Virus Associated with Skin Lesions Isolated from Pigeons in Greece

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ABSTRACT

Pigeon breeders in Central and Northern Greece often report pox-like skin lesions in their flocks. However, these reports have not been scientifically validated, and no investigation has determined the presence or prevalence of pigeon pox virus in Greek avian populations. The present study aimed to report the detection of pigeon pox in a flock of pigeons in Karditsa, Greece, confirmed by polymerase chain reaction (PCR) analysis. Following a comprehensive review of the existing scientific literature, the present study provided the first documented confirmation of pigeon pox virus in Greece. In September 2025, a breeder from Karditsa, Greece, reported cutaneous and respiratory signs in 40 of 250 juvenile pigeons in a loft of 450 pigeons. The flock was vaccinated only against paramyxovirus, not against pigeon pox. Lesions on the skin, beak, and legs persisted for 4-8 weeks, with no mortality. The breeder administered doxycycline, enrofloxacin, and local treatment for trichomoniasis and treatment with ganciclovir; however, the treatment was unsuccessful. Wart-like lesions from the beak, skin, legs, and eyelids were collected for molecular analysis by PCR targeting *Avipoxvirus*-specific genomic regions, confirming the diagnosis of pigeon pox virus infection. The clinical history, physical examination, and necropsy findings, along with the PCR results, confirmed the poxvirus infection. The present study confirmed the presence of pigeon pox virus in Greece and emphasized the importance of raising awareness and conducting additional epidemiological surveillance of *Avipoxvirus* infections in domestic pigeons.

Keywords: *Avipoxvirus*, Lesion, PCR, Pigeon pox, Skin, Virus

INTRODUCTION

Avian pox is a disease caused by different strains of the genus *Avipoxvirus* (APV; Mohamed et al., 2024). According to the International Committee on Taxonomy of Viruses (ICTV), APV comprises twelve species, including fowlpox, pigeonpox, canarypox, flamingopox, penguinpox, quailpox, and turkeypox (ICTV, 2025). *Avipoxvirus* infects more than 370 species of wild and domesticated birds (Williams et al., 2021) and can be transmitted via water, feed, eggs, and direct contact through skin abrasions (Abbiw et al., 2025). Mosquitoes and other insects may also contribute to the mechanical transmission of the virus (Abbiw et al., 2025). Avian wildlife may serve as reservoirs or vectors for the transmission of APV (Bertelloni et al., 2022). In Europe,

poxvirus has been identified in different bird species since 1850 (Bolte et al., 2010). Additionally, APV infections can cause significant economic losses in commercial poultry (Minhas et al., 2023).

Two common clinical manifestations of pigeon pox are the diphtheritic and cutaneous forms. The cutaneous form of pigeon pox is the most frequently documented form (Abbiw et al., 2025). In affected pigeons, nodular lesions are observed on the skin and feathers, as well as on the beak, legs, and non-feathered regions (Mohamed et al., 2024). Nodules located around the ocular area and beak can impair vision, feeding, and breathing. In addition, viremia may occur; however, gross internal pox lesions are rarely observed in pigeons (Tudor, 1978). Pigeon pox is generally a self-limiting disease; however, it may result in more severe disease in young or immunocompromised

pigeons (Hibl et al., 2019). Disease in pigeons can be mitigated by administering a commercially available live pigeon pox virus (PPV) vaccine via the wing web or by injection.

Recently, APV (including PPV) infection has been documented in Central Italy (Bertelloni et al., 2022), and specific PPV cases have been reported in Croatia and Egypt (Prukner-Radovic et al., 2006; Mohamed et al., 2024; Helal et al., 2025). However, no investigation has confirmed the presence or prevalence of PPV within Greek avian populations, particularly in Central and Northern Greece, which frequently report pox-like skin lesions in their flocks. Therefore, the present study aimed to provide the first molecular confirmation of PPV in Greece through polymerase chain reaction (PCR) analysis of samples from a backyard flock in Karditsa.

CASE PRESENTATION

Ethical approval

Ethical approval under European Commission (2010) EU Directive 2010/63/EU was not required for this study, as the pigeons were euthanized as part of the diagnostic procedure to identify the flock disease and direct treatment for the remaining animals, which constitutes standard veterinary practice in flock health management. Under Article 1, Section 5(a) of EU Directive 2010/63/EU, non-experimental agricultural or clinical veterinary practices are explicitly excluded from the definition of an animal experiment. Informed consent to collect samples from pigeons and to use the results and animal data was obtained from the farmer.

History

On September 26, 2025, a breeder from Karditsa, Thessaly, Greece, presented a loft of 450 racing pigeons (*Columba livia*) to the Department of Poultry Diseases at the Faculty of Veterinary Medicine, University of Thessaly, Greece. The breeder reported skin lesions and respiratory signs in several juvenile pigeons. All sampling procedures were conducted in accordance with the highest principles of veterinary care. Initially, a visit to the pigeon breeding facility was conducted to inspect the loft and obtain history. The loft appeared crowded, housing 30 canaries, 20 goldfinches, 70 backyard chickens, nearby dogs and cats, and an ammonia odor was noticeable. In addition, no signs of pigeon pox were observed in the neighboring avian species. Pigeons were fed a commercial pigeon diet, vaccinated only with Pigeon Paramyxovirus Type 1 (Pharmagal-Bio, Slovakia) at four weeks of age,

according to the company's recommendation, and left unvaccinated for PPV. The breeder reported that 40 out of the 250 juveniles from the summer hatching period exhibited cutaneous lesions accompanied by respiratory signs. The signs persisted for approximately 4 to 8 weeks before resolving, with no mortality reported. Antibiotic treatment with doxycycline and enrofloxacin, as well as treatment for trichomoniasis and local ganciclovir, was administered by the breeder; however, the treatment failed without therapeutic effect. Additionally, similar pathogenicity was reported to have reemerged annually during the same season (summer to early fall) over the past three to four years.

Clinical examination and necropsy

Four pigeons aged 1-2 months, displaying the most noticeable clinical signs, were taken to the Clinic for Poultry at the Faculty of Veterinary Science, University of Thessaly, in Karditsa, Greece, for further evaluation. The observed signs included conjunctivitis, abscesses near the Harderian gland, and wart-like lesions on the beak, skin, legs, and eyelids (Figure 1). Following the clinical examination, the pigeons were euthanized by cervical dislocation (Leary et al., 2020). A postmortem examination was conducted, during which caseous material within the lesions and bile in the ventriculus (muscular stomach) were found, indicating anorexia (Figure 2). Wart-like lesions on the beak, skin, legs, and eyelids were collected for PCR analysis. Lesions from the four pigeons were pooled into a single sample for PCR, as all pigeons had the same lesions (Abbiw et al., 2025).

Swabs from the collected lesions were vigorously vortexed in 1 mL of phosphate-buffered saline (PBS) at room temperature (20-25°C) and then centrifuged at $10,000 \times g$ for five minutes. Viral nucleic acid was extracted from the supernatant using the MagMAX Express 24 Magnetic Particle Processor (Thermo Fisher Scientific, Waltham, MA, USA) with the IndiMag Pathogen kit (cat. no. SP947257, Indical Bioscience, Leipzig, Germany) according to the manufacturer's instructions. Briefly, 200 µL of the sample was mixed with 20 µL Proteinase K solution and 500 µL of lysis buffer containing 1 µL carrier RNA (1 µg/µL), 25 µL magnetic beads, 400 µL buffer ACB, and 100 µL buffer VXL. After lysis, samples were washed twice with 700 µL of each wash buffer 1 and 2, then eluted with 100 µL of elution buffer. *Avipoxvirus* was detected by real-time PCR on a Rotor-Gene Q thermocycler using the KYLT fowlpox virus qPCR kit (SAN Group Biotech Germany GmbH, Germany). Briefly, 10 µL of 2x qPCR-mix and 6 µL of

primer-probe-mix were combined with 4 μ L of extracted DNA, then incubated at 95°C for 10 minutes, followed by 42 cycles of 95°C for 15 seconds and 60°C for 1 minute. Fluorescence was observed during the annealing and extension step at 60°C for 1 minute on the 6-carboxyfluorescein (FAM, green) channel. The qPCR assay yielded a cycle threshold of 20.56, indicating a relatively high viral load in the initial sample.

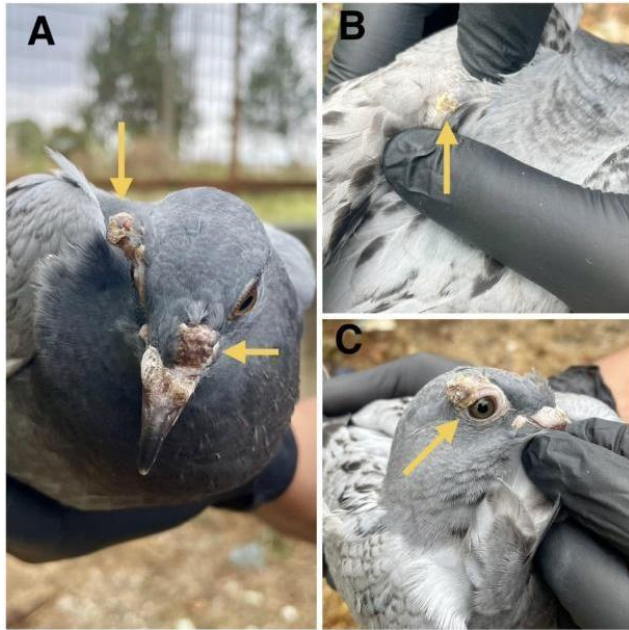


Figure 1. Wart-like lesions in pigeons infected with pigeon pox virus. **A:** Lesions on the beak and upper eyelid, **B:** Lesions on the feather, **C:** Lesions on the upper eyelid.



Figure 2. Postmortem examination of a pigeon infected with pigeon pox virus. **A:** Bile inside the empty ventriculus, **B:** Lesions on the legs, **C:** Conjunctivitis, **D:** Caseous material within the lesions.

Treatment

Topical therapy consisting of tobramycin (0.1% w/v) and dexamethasone (0.3% w/v; Tobradex, Novartis Hellas, Greece) was administered as a drop per eye once daily for 5 days to all 40 affected pigeons. As there is no gold standard treatment for pigeonpox, this is an empirically selected treatment based on the clinical experience. Reducing inflammation was essential for the pigeons' well-being and recovery from anorexia; therefore, dexamethasone was combined with tobramycin to prevent secondary bacterial infections and enhance the host's response to viral infections (Gopinath et al., 2018). Post-treatment evaluation revealed no signs of conjunctivitis or cutaneous lesions, and the appetite was improved.

DISCUSSION

Following a comprehensive review of the published studies, the present study reported the first laboratory-confirmed detection of PPV in Greece, as no prior confirmed cases had been identified. The initial clinical suspicion was based on the cutaneous and respiratory manifestations observed, while the definitive diagnosis was confirmed by PCR analysis. The clinical signs observed in the present study, characterized by prominent wart-like lesions on the beak, eyelids, and legs, conjunctivitis, and severe anorexia, closely aligned with the classical pathology of APV infections (Hibl et al., 2019). The gross pathological findings observed during necropsy were consistent with lesions described in recently confirmed cases of pigeon pox reported in Croatia and Egypt (Prukner-Radovic et al., 2006; Mohamed et al., 2024; Helal et al., 2025). Additionally, the detection of APV in asymptomatic wild birds in Central Italy underscores the virus's potential to persist in the environment (Bertelloni et al., 2022). Mohamed et al. (2024) detected APV in 16 of 50 Egyptian backyard flocks and reported 91% nucleotide similarity between PPV and fowlpox virus, which was consistent with the phylogenetic difference observed in the present study.

The occurrence of APV during the warmer months (summer through fall) might be epidemiologically notable. Elevated temperatures promote the proliferation of insect vectors, particularly mosquitoes, which are identified as mechanical transmitters of APV (Mohamed et al., 2024). Peak insect population densities during summer may increase the likelihood of PPV transmission (Mohamed et

al., 2024). The seasonal pattern of APV transmission was consistent with the current study and those of Mohamed et al. (2024), who reported a high rate of APV infections in pigeons and chickens throughout the summer. Furthermore, management-related factors such as high stocking density and elevated ammonia levels may predispose poultry to skin abrasions, thereby facilitating viral entry through compromised skin barriers, consistent with the present observations in the loft. Although mortality was not observed in the present study, pigeon pox can cause severe disease, particularly in young or immunocompromised pigeons (Abbiw et al., 2025).

The presence of skin lesions can strongly suggest PPV infection; however, definitive confirmation requires molecular diagnostic techniques such as PCR. Although histopathological examination was not performed in the current study, Abbiw et al. (2025) indicated that such analysis could help confirm APV by identifying eosinophilic intracytoplasmic inclusion bodies (Bollinger bodies), which are characteristic features of poxvirus infections.

Currently, there is no specific antiviral treatment for poxvirus infections in pigeons. Supportive therapy was administered to prevent secondary bacterial infections and decrease inflammation. Previous studies suggested that topical administration of aminoglycosides could improve host responses to viral infections (Gopinath et al., 2018). Several studies have indicated that azithromycin, as well as the combination of oral acyclovir, azithromycin, Vitamin A, neem leaves, and turmeric paste, were effective in treating pigeon pox (Sudhakara Reddy and Sivajothi, 2018; Somasundaram et al., 2020). However, these combinations were not used in the present study. In the current study, topical treatment was chosen to attain higher local medicine concentrations, thereby potentially enhancing lesion management and clinical outcomes.

CONCLUSION

The present study reported the first laboratory-confirmed case of PPV infection in a domestic pigeon flock in Karditsa, Greece, characterized by cutaneous and diphtheritic clinical signs of PPV. The clinical signs included wart-like lesions on the beak, eyelids, and legs, as well as conjunctivitis and severe anorexia, closely aligned with the classical pathology of APV infections. Moreover, the absence of pigeon pox vaccination in the affected flock likely contributed to the recurrent outbreaks observed over consecutive summers. The present study

highlighted the need for increased awareness regarding PPV infection among pigeon breeders and veterinarians in Greece. Future studies should emphasize systematic surveillance and epidemiological studies to assess the prevalence and distribution of PPV in Greece.

DECLARATIONS

Authors' contributions

Zoi Prentza and Konstantinos Koutoulis conceptualized the study and supervised the study. Argyrios Aslanidis, Zoi Mavraki, and Loukas Chochtoulas visited the pigeon loft, collected the samples, and conducted the postmortem examination. Renáta Varga-Kugler performed the PCR examination, and Evangelos Minos analyzed the data. All authors contributed to the writing and editing of the manuscript. 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.

Competing interests

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical considerations

This study has been submitted to this journal for the first time and has not been submitted elsewhere. The authors declare that the present study has been checked for plagiarism, data fabrication, and duplication to ensure scientific integrity. No AI tools/apps were used in preparing any part of the present study. All authors take full responsibility for the originality of the data prior to submission and throughout the peer review process.

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The Role of Dietary Fibre in Modulating Gastrointestinal Development, Nutrient Utilization, and Growth Performance in Broiler Chickens: A Review

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ABSTRACT

Dietary fibre, known as a diluent in poultry feed, has recently attracted considerable interest owing to its potential functional benefits beyond energy provision. The present study aimed to obtain findings related to the effects of different sources of dietary fibre on gastrointestinal tract (GIT) development, nutrient utilization, gut microbiota, and growth performance in broiler chickens. Dietary fibre is a major constituent of plant feed resources. Traditionally, dietary fibre was regarded as an antinutritional factor. The consideration of dietary fibre as an antinutritional factor was due to its negative effects on nutrient utilization, as it impairs feed intake and digestibility in broiler chickens. However, recent studies have indicated that the inclusion of dietary fibre at 2 to 3% in the diet of broiler chickens served multiple functions. Dietary fibre induces GIT development, gizzard activity and functionality, and digestive physiology. Fibre enhances enzyme secretion, digestion, fermentation, and absorption of nutrients. Dietary fibre keeps homeostasis through balancing the host and gut microbiota, increasing the composition and population of commensal bacteria, and reducing the pH of the GIT. The low pH creates an unfavorable environment for pathogenic bacterial proliferation. Thus, fibre helps maintain immunity, which further enhances the production performance in broiler chickens. In Ethiopia, where financial constraints and limited infrastructure hindered access to conventional feed ingredients, studies have primarily focused on replacing costly concentrates with more accessible and affordable alternatives, including dietary fibre sources. The present study revealed that including dietary fibre in the diet of broiler chickens resulted in different responses, with 2-3% inclusion generally beneficial, while exceeding 30 g/kg may decrease broiler chickens' growth performance; therefore, determining precise levels of dietary fibre inclusion is essential.

Keywords: Broiler chicken, Fibre, Gastrointestinal tract, Growth performance

INTRODUCTION

The global demand for meat consumption was anticipated to increase by approximately 60 to 70 percent by the year 2050, driven by population growth and economic development (Sumbule et al., 2021). The production of broiler chickens plays a pivotal role in ensuring a rapid and sustainable supply to satisfy the growing demand. According to Oke et al. (2024), broiler chicken production has been increasing worldwide due to population growth, industrialization and urbanization, intensive production systems, and increased use of improved breeds. However, inadequate availability coupled with poor-quality feed

resources are major constraints of the poultry sector, especially in developing countries including Ethiopia (Abera et al., 2024).

Nutrition is the backbone of profitable broiler chicken production. On average, feed accounts for the largest portion of total production costs. Specifically, in modern broiler chicken production, feed is the primary expense, making up approximately 70% of the overall production costs (Sugiharto, 2019). Consequently, several studies have been conducted to investigate the productive performance of broiler chickens using potential alternative feed resources (Sittiya et al., 2020; Tejeda and Kim, 2020;

Zhang et al., 2023). Recently, alternative feed ingredients that enhance the production performance of broiler chickens have been of great concern for scientific communities (El-Deek et al., 2020; Grigore et al., 2025). The need for alternative feed ingredients has led to the use of dietary fibre as a strategy to decrease the cost of feeds (Amerah et al., 2009; Yadav and Jha, 2021). Fibre naturally exists in plant-based feed resources and is a vital constituent in poultry diets (Jha et al., 2019). Dietary fibre refers to a group of heterogeneous compounds that includes oligosaccharides, non-starch polysaccharides, pectin, waxes, and lignin (McDonald et al., 2008). Dietary fibre is highly resistant to enzymatic hydrolysis by alimentary enzymes (Jha and Mishra, 2021). However, an optimal level of dietary fibre is important in poultry nutrition, as it supports improved productive performance (Esmail, 2012; Barszcz et al., 2024).

Previous findings indicated that dietary fibre had adverse effects on feed intake and nutrient digestibility (Jha et al., 2019), while its impact on growth performance was not notable (Sittiya et al., 2020). A recent study demonstrated that dietary fibre at 3% inclusion improved growth performance and digestive physiology in broiler chickens (Rasool et al., 2023). Furthermore, soluble dietary fibre (SDF) is generally recognized to diminish nutrient utilization primarily due to its viscous nature, which decreases the passage rate (Mateos et al., 2012). On the contrary, insoluble dietary fibre increases nutrient utilization by improving gastrointestinal development and enzyme secretion (Donadelli et al., 2019). Additionally, particle size plays a significant role in the use of dietary fibre in broiler nutrition. For instance, large particle size (coarse particles) of dietary fibre increases broiler chicken performance, while fine particle fibres negatively affect growth performance (Xu et al., 2017; Donadelli et al., 2019). Consequently, it has been recommended that a feed particle size of at least 1 mm should be used to enhance gizzard development (Svihus, 2011).

The incorporation of dietary fibre at a level of 2% lignocellulose within the grower and finisher diets for broiler chickens yielded beneficial effects on their performance (Kheravii et al., 2017). Accordingly, Abdollahi et al. (2019) demonstrated that the dietary inclusion of insoluble fibre products such as 10 g/kg lignocellulose improved digestive function and boosted growth performance in broiler chickens. It has been observed that insoluble fibre, made up of 15% coarse hulls with equal parts of oats and barley hulls, promotes the development of the gastrointestinal tract (GIT; Sacranie et al., 2012), improves nutrient digestibility (Jiménez-

Moreno et al., 2013a), and enhances utilization (Donadelli et al., 2019). Nowadays, the inclusion of fibre in broiler chicken diets is more advanced and has been given more attention by animal nutritionists (Bedford et al., 2024). As a result, there are many studies related to the use of fibre in poultry nutrition worldwide (Adibmoradi et al., 2016; Tejeda and Kim, 2021; Nguyen et al., 2022). However, the use of dietary fibre in broiler chicken nutrition, with special reference to the digestive system, nutrient utilization, and performance, has not been extensively reviewed. Therefore, the present study aimed to review the current findings on the role of dietary fibre in broiler chicken nutrition in relation to GIT development, nutrient utilization, gut microbiota, and growth performance.

SEARCH CRITERIA

This study used a conventional literature review methodology. The keywords used to find relevant information on the role of dietary fibre in modulating the GIT, nutrient utilization, and performance were poultry, chicken, broiler, fibre, dietary fibre, dietary fibre sources, dietary fibre level, fibre feeding, dietary fibre-based feeding, fibre and GIT, fibre and growth performance, and fibre and microbiota. The data were retrieved using the Web of Science (WoS), Scopus, and Google Scholar databases. Research articles sourced from reputable scholarly journals, encompassing both reviews and original research papers, were prioritized during the selection process. Additionally, the reference lists of most articles related to the current review were thoroughly examined to identify further pertinent findings. After collecting all the necessary information, the study selected relevant articles or research papers published from 2003 onward from different databases for the current review. Although the total number of articles downloaded from the different searching databases at the beginning was 217, the total number of articles used to write the present study was 83. Old publications, duplicate publications, those published in non-reputable journals, and articles that did not directly relate to the aim of the current study were excluded.

DIETARY FIBRE AND GASTROINTESTINAL TRACT DEVELOPMENT

The avian stomach contains two different compartments, which include the proventriculus and the ventriculus (Hyder et al., 2023). The physiology of the gut, particularly the proventriculus and ventriculus, has been comprehensively discussed (Svihus, 2014). The

proventriculus secretes hydrochloric acid and digestive enzymes such as pepsin, which are added to the feed (Scanes and Pierzchala-Koziec, 2014). However, since the proventriculus has a very limited capacity and the feed is retained there for a short period, most of the hydrolysis of feed materials takes place in the gizzard (Svihus, 2011).

The gizzard controls many physiological functions of the GIT and plays a key role in mechanical breakdown and enzymatic digestion of ingesta (Scanes and Pierzchala-Koziec, 2014). Gizzard development and functionality are positively affected by dietary fibre inclusion (Mousavi et al., 2024). The inclusion of dietary crude fibre from 7 to 9% enhances GIT development and growth performance in broiler chickens aged between 22 and 42 days (Zhang et al., 2023). The benefits of including moderate levels of fibre (2-3%) are more significant when incorporated into low fibre diets (Mateos et al., 2012). Nevertheless, the effect of fibre on GIT development is highly determined by different interacting factors such as fibre sources used, particle size of the fibre, and amount of fibre (Mateos et al., 2012; Jiménez-Moreno et al., 2013b). According to Jiménez-Moreno et al. (2013a), fibre enhances GIT development, but higher fibre levels might negatively affect intestinal mucosal structure in young broiler chickens, since their requirement ranges from 82 to 101 g/kg diet.

Dietary changes have significant effects on GIT development, particularly the gizzard of broiler chickens (Celi et al., 2017). Therefore, the size of the gizzard rapidly enlarges due to the inclusion of structural components such as dietary fibre in the diet of broiler chickens (Jiménez-Moreno et al., 2013b; Kheravii et al., 2017). Shang et al. (2020) reported that wheat bran inclusion at 30 g/kg in the diet of broiler chickens did not affect the relative weight of the proventriculus and small intestine, and the relative length of the small intestine, but resulted in a higher (12.7 g/kg of body weight [BW]) relative weight of the gizzard. In contrast, Jiménez-Moreno et al. (2011) indicated that the inclusion of 20 and 50 g/kg of pea hulls in the diet of broiler chickens improved the relative weight of the GIT. In addition, supplementation of soybean hull at 4% had more remarkable effects on the gizzard (29 g) and small intestine sections, including the jejunum (29 g), duodenum (12 g), and ileum (28 g; Tejeda and Kim, 2020). Sittiya et al. (2020) observed that supplementing broiler chickens' diet with 5% soybean hulls increased the relative length of the small intestine sections, including the duodenum (1.42), the jejunum (3.72), and the ileum (3.9 cm). Furthermore, the data regarding the impact of different sources of fibre inclusion on GIT development in broiler chickens are briefly summarized in Table 1.

Table 1. Effects of different dietary fibre sources on gastrointestinal tract development in broiler chickens

Strain	Organ	Treatment						Duration	Reference	
		Oat hulls (g)			Sugar beet pulp (g)					
Broiler chickens		Control	25	50	75	25	50	75	18 days	
	Liver (g/kg BW)	27.7	27	27.1	26.8	27.8	27	28.9	(p = 0.97)	Jiménez-Moreno et al. (2013b)
	Pancreas (g/kg BW)	3.35	3.13	3.02	3.47	3.28	3.61	3.8	(p = 0.04)	
Broiler chickens		Oat hull (%)								
		0	2	4	6	8	P-value			
	Gizzard plus proventriculus (LW%)	1.64	1.40	1.76	1.32	1.82	0.20	35 days	Mossami and Kalmendal (2011)	
	Duodenum (LW%)	0.90	0.92	0.82	0.92	0.94	0.46			
	Jejunum (LW%)	1.82	2.00	1.64	2.02	2.26	0.04			
	Ileum (LW%)	1.16	1.64	1.14	1.54	1.70	0.004			
	GIT (LW%)	7.32	8.16	7.34	8.58	9.04	0.06			
		Control	BMD	3% OH	FCOH		P-value			
Broiler chickens	Gizzard (g/kg BW)	19.6	19.9	20.0	24.6		< 0.0001	36 days	Adewole et al. (2020)	
		Control	SRH	IRH			P-value			
		Weight of empty digestive organs (% of BW, without digesta)								
	Gizzard	2.16	2.41	1.86			0.07	21 days	Hartini et al. (2018)	
	Small intestine	3.26	3.49	2.80			0.12			
	Ceca	0.35	0.33	0.19			0.001			

Bacitracin, methylene disalicylate, BW: Body weight, 3% OH: Fine oat hulls incorporated in the diet at 3%, FCOH: Free choice oat hulls, SRH: Soluble rice hull, IRH: Insoluble rice hull, LW%: Percent of live weight, GIT: Gastrointestinal tract

DIETARY FIBRE AND GUT FLORA PROFILE

The gut of the broiler chicken comprises different structures, including the crop, proventriculus, gizzard, small intestine, and large intestine, with a dense and diverse microbial community (Yeoman et al., 2012). The gut, extending from the crop to the colon, serves as the site of microbial activity (Pan and Yu, 2013). Microbes are densely populated in the large intestine, particularly in the caecum and colon, at about 10^{11} to 10^{12} colony-forming units/g of luminal content (Wuraola, 2024). The gut microbiome contains large, diverse microbial communities crucial for chicken growth and health (Wei et al., 2013). The diverse microbial communities inhabiting the GIT include bacteria, methanogenic archaea, fungi, and viruses (Yeoman et al., 2012). Among these microbial communities, bacteria have the largest population and diversity (Shang et al., 2018). Paiva and McElroy (2014) noted that antibiotics were used in broiler chickens' diets to maintain GIT health and thus help optimize growth performance. However, repetitive use of antibiotics resulted in their prohibition within the European Union and other countries (Casewell et al., 2003; Cardinal et al., 2019). The complete prohibition of antibiotics increased the risk of bacterial diseases and may have limited the growth performance of broiler chickens (Attia et al., 2025). Therefore, potential alternative feed resources to replace antibiotic growth promoters have been investigated (Gadde et al., 2017). Thus, dietary fibers with prebiotic functions have garnered significant attention due to their specific influences on the composition and activity of gut microbiota (Qiu et al., 2022).

Furthermore, the positive and symbiotic interaction between the GIT and the gut microbiota plays an essential role in nutrient absorption, immune development, and disease resistance (Pan and Yu, 2013). Hence, the disturbance of gut flora may adversely affect growth and health performance in broiler chickens (Kohl, 2012). The interrelationship between GIT and nutrition is indicated in Figure 1. Several factors such as the environment, feed, and the microbiota within the gut affect the overall performance and health of the GIT in broiler chickens (Shang et al., 2018). Among these factors, feed is the major constraint that directly influences the nature of the microbiota in the host, and its effect is extremely significant (Yadav and Jha, 2019). Feed plays a principal role in determining the composition and density of the intestinal microflora in broiler chickens post-hatching (Shang et al., 2018; Yadav and Jha, 2019). According to

Fathima et al. (2022), the bacterial population in the GIT was affected by diet changes. Changes in the bacterial population are attributed to specific bacterial species having specific substrate preferences (Haberecht et al., 2020).

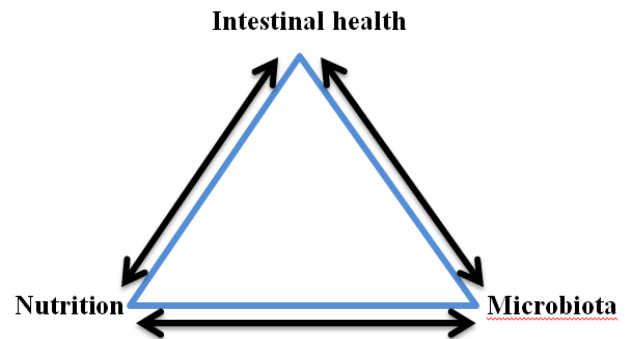


Figure 1. Relationship between intestinal health, nutrition, and microbiota in poultry

Dietary fibre plays a vital role in modulating the microbial population in the GIT (Qiu et al., 2022). Thus, the use of fibre sources such as wheat bran in broiler chicken nutrition is essential to help control the gut microbiome (Vermeulen et al., 2018). Wheat bran, a major end product of flour milling industries, provides fibre for poultry (Bach et al., 2026). Fibre particularly serves as a source of energy for microbes in the GIT via fermentation (Zhang et al., 2023). Dietary fibre may decrease gastrointestinal pH by improving fermentation in the foregut and secretion of HCl in the proventriculus (González-Alvarado et al., 2008). The reduction in GIT pH makes it unfavorable to pathogenic bacteria and enhances the proliferation of beneficial bacteria (Svihus, 2011; Yadav et al., 2016). Bogusławska et al. (2015) reported that dietary lignocellulose supplementation at 0.5% in broiler chickens' diet increased *Lactobacillus* spp. count in the ileum and *Bifidobacterium* spp. count in the ileum and caeca, while decreasing the ileal and caecal *Escherichia coli* and *Clostridium* spp. Additionally, Kheravii et al. (2017) indicated that lignocellulose supplementation at 2% decreased caecal *Clostridium* spp. counts, implying its potential to control *Clostridium* spp. in the GIT. Controlling *Clostridium* species in the GIT suggested that feeds rich in specific types of dietary fibre, such as structural components, could help reduce some disease risks (Guo et al., 2020). Studies on dietary fibre and its effects on gut flora in broiler chickens are summarized in Table 2.

Table 2. Responses of gut microbiota to different sources of dietary fibre in broiler chickens

Strain	Duration (days)	Fibre source	Level (%)	<i>Lactobacillus</i> spp. (log ₁₀ CFU/g)		<i>Bifidobacterium</i> spp. (log ₁₀ CFU/g)		<i>Escherichia coli</i> (log ₁₀ CFU/g)		Reference
Broiler chicken	42	Inulin		Ileum	Caecum	Ileum	Caecum	Ileum	Caecum	Nabizadeh (2012)
	0		7.29	8.18	6.22	7.91	7.06	8.76		
	0.5		7.42	8.48	7.01	8.30	6.64	7.54		
	1		7.54	8.83	6.77	8.74	6.18	6.29		
	SEM		0.071	0.224	0.200	0.100	0.245	0.071		
				<i>Lactobacillus</i> spp. (log ₁₀ CFU/g)	<i>Lactobacillus</i> spp. (log ₁₀ CFU/g)	<i>Clostridium perfringens</i> (log ₁₀ CFU/g)	<i>Enterobacteriaceae</i> (log ₁₀ CFU/g)			
				Crop	Caecum	Caecum	Caecum			
Broiler chicken	36	Oat hull and sugar beet pulp	1.6% CF	7.9	9.8	5.9	8.4		Jiménez-Moreno et al. (2011)	
	5% OH		7.1	8.6	1.2	5.9				
	5% SBP		8.4	10.0	6.2	8.4				
	P-value		p < 0.01	p = 0.08	p < 0.05	p < 0.01				
				<i>Helicobacter pullorum</i> (%)	<i>Megamonas hypermegale</i> (%)	<i>Campylobacter jejuni</i> (%)	<i>Escherichia coli</i> (%)	<i>Firmicutes</i> (%)	<i>Bacteroidetes</i> (%)	
				Caecum	Caecum	Caecum	Caecum	Caecum	Caecum	
Broiler chicken	21	DDGS and WB*	Low fibre **	5.3	2.3	0.05	1.9	36.2	41.2	Walugembe et al. (2015)
			High fibre ***	12.2	12.7	0.7	12.4	54.8	26.4	

CF: Crude fibre, DDGS: Dried distillers' grain with soluble, OH: Oat hull, SBP: Sugar beet pulp, SEM: Standard error of mean, WB: Wheat bran. *Both lower and higher fibre diet were based on corn-soy bean meal, but the higher fibre diet had the addition of 60.0 g/kg of DDGS and 60.0 g/kg of wheat bran, **Lower fibre diet: Based on corn-soy bean meal with no wheat bran and DDGS, ***High fibre diet: Formulated based on corn-soy bean meal-DDGS-wheat bran with the addition of 80.0 g/kg of DDGS and 80.0 g/kg of wheat bran

DIETARY FIBRE AND NUTRIENT UTILIZATION

Dietary crude fibre inclusion at 7 to 9% in the broiler chickens' diet improved their growth performance (Zhang et al., 2023). Under practical feeding conditions, supplementation of dietary fibre sources such as wood shavings at 6% in the diet of broiler chickens enhanced gizzard function (Shirzadegan and Taheri, 2017). Moderate inclusion of insoluble fibers (2 to 3%) in the diet of broiler chickens enhanced gizzard development, which resulted in increased nutrient digestibility and utilization and improved growth performance (Xu et al., 2017; Kheravii et al., 2018; Donadelli et al., 2019). Jiménez-Moreno et al. (2013a) observed that the addition of 2.5% dietary fibre improved the feed conversion ratio of broiler chickens aged between 1 and 18 days. However, according to Walugembe et al. (2015), supplementation with a higher dietary source (60 g/kg dried distillers' grain with solubles and 80 g/kg wheat bran) was reported to affect nutrient metabolism in broiler chickens. In addition, Walugembi et al. (2015) reported that the growth performance of broiler chickens was not affected when supplemented with 60 to 120 g/kg dried distillers' grain with solubles in the diet. Furthermore, supplementing broiler chickens' diet with 30 g/kg wheat bran improved digestibility of dry matter (0.73

versus 0.75), organic matter (0.77 versus 0.79), gross energy (0.76 versus 0.78), and crude protein (0.63 versus 0.67) compared to the control (Shang et al., 2020). It has been indicated that supplementation of dietary fibre improved GIT immunity, physiology, and digestion of nutrients in broiler chickens raised under a concentrate feeding system (González-Alvarado et al., 2008). Gut health can occur due to enhanced proventriculus and gizzard activity and longer retention time in the upper part of the GIT (Svihus, 2011). Gizzard activity enhances fermentation in the crop (Classen et al., 2016) and decreases gut pH (Naderinejad et al., 2016), and improves secretion and functionality of digestive enzymes, such as increased pepsin activity (Svihus, 2011; Husvéth et al., 2015). Moreover, the high gizzard activity provides sufficient time for HCl secretion (Mateos et al., 2012; Qaisrani et al., 2015). However, previous studies indicated that the amount, particle size, and sources of dietary fibre were among the key factors affecting nutrient utilization (Xu et al., 2017; Vermeulen et al., 2018). For instance, nutrient utilization was higher in broiler chickens fed coarse fibre particles compared to those fed fine fibre particles (Donadelli et al., 2019). Figure 2 illustrates the mechanisms through which coarse dietary fibre influences nutrient utilization.

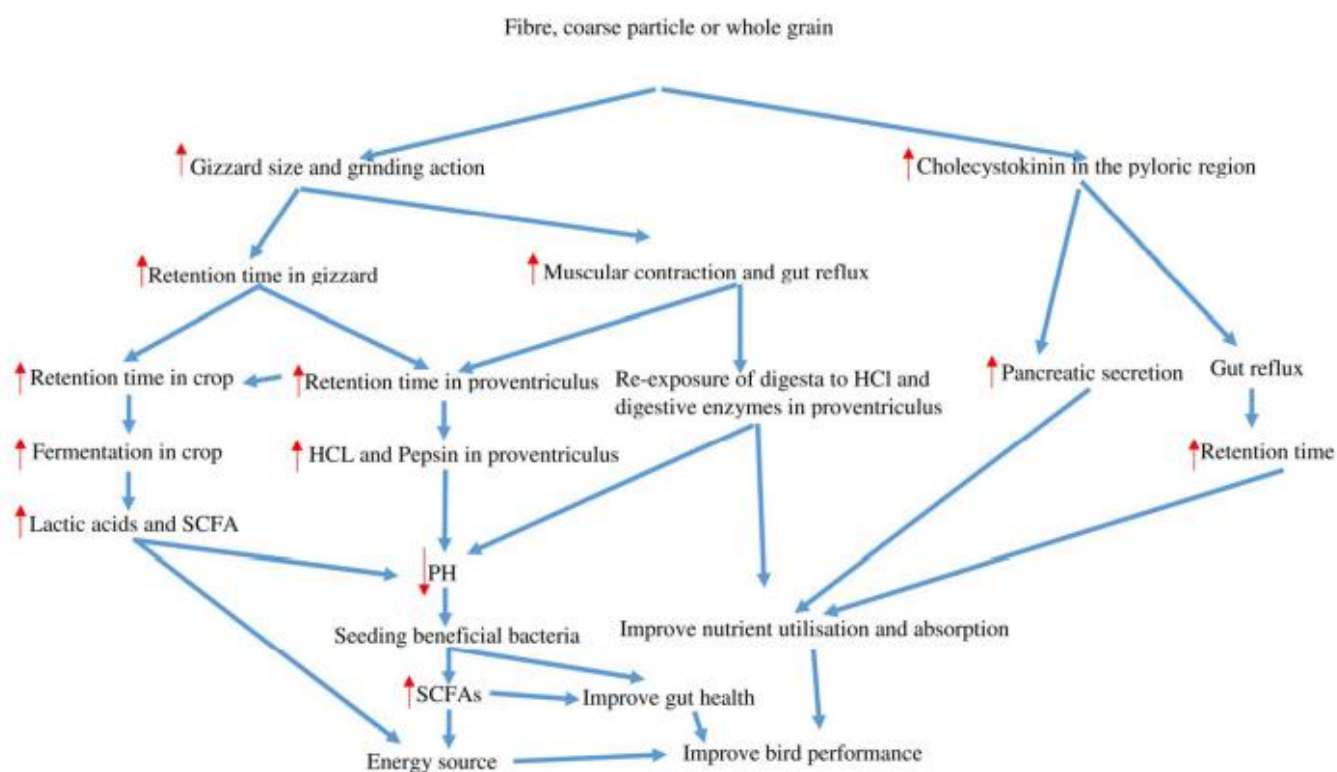


Figure 2. Mechanisms of dietary fibre to improve digestibility, growth performance, and gut health in poultry. Source: Kheravii et al. (2018)

DIETARY FIBRE AND GROWTH PERFORMANCE

Several studies have been conducted regarding the impact of fibre on growth performance in broiler chickens (Walugembe et al., 2015; Donadelli et al., 2019; Sittiya et al., 2020). Nursiam et al. (2021) demonstrated that the inclusion of 1% commercial fibre source improved GIT development and productive performance in broiler chickens. In addition, Mateos et al. (2012) reported that adding oat hulls at 2-3% to the broiler chickens' diet enhanced growth performance. According to Ginindza et al. (2022), the supplementation of 6.4% crude fibre increased feed intake and growth performance in broiler chickens. The challenge of proposing an exact fibre level arises from different interacting factors, such as age and species, fibre sources used, management, and prevailing environmental conditions (Mateos et al., 2012).

Furthermore, Adibmoradi et al. (2016) indicated that inclusion of dietary hull at 0.75 to 1.5% in the broiler chickens' diet resulted in improved growth performance. Kheravii et al. (2017) found that sugarcane bagasse supplementation at an inclusion level of 2% improved body weight gain in broiler chickens aged 0 to 24 and 0 to 35 days. González-Alvarado et al. (2010) indicated that adding oat hulls in the diet of broiler chickens aged between 1 to 42 days improved their growth performance. Conversely, adding 1 or 2% lignocellulose fibre had minimal impact on the growth performance of broiler chickens from 0 to 24 days and 0 to 35 days (Kheravii et al., 2017). Similarly, supplementation of sunflower hulls at a 3% inclusion level had no impact on the growth performance of broiler chickens (Kimiaetalab et al., 2017).

Furthermore, Aziz-Aliabadi et al. (2021) observed that broiler chickens exhibited optimal performance when fed diets devoid of fibre and fat at 7 days of age. However, the lowest feed conversion ratio was recorded at 14 days with a diet comprising 0.98% sugar beet pulp and 1.0% soybean oil. Dos Santos et al. (2019) reported decreased body weight gain at 2.5% soybean hull inclusion in the diet of broiler chickens aged between 1 and 21 days. However, Adewole et al. (2020) found no significant effect on growth performance and carcass yield when oat hulls were supplemented at 3% in the diet of broiler chickens. According to García et al. (2019), 3% oat hull supplementation in broiler chickens' diet did not have any effect on their growth performance.

Although fibre has been reported to have a positive impact on poultry at modest levels (2-3%), inclusion of fibre in the diet at 30 g/kg could have a negative influence on growth performance (Sadeghi et al., 2015). The impact of dietary fibre supplementation depends on its inclusion level. Higher fiber content might negatively affect growth, while lower levels may not provide significant benefits (Kheravii et al., 2018). For instance, Jiménez-Moreno et al. (2011) revealed that the addition of dietary pea hulls at a level of 5% enhanced growth by 15% and feed conversion ratio by 3% in broiler chickens aged between 1 and 18 days.

However, increasing levels of dietary pea hulls to 7.5% affected growth performance. Another study conducted by Jiménez-Moreno et al. (2013a) indicated that increasing the sugar beet pulp from 25 to 75 g/kg of diet decreased the growth performance of broiler chickens aged between 1 and 6 days. Walugembe et al. (2014) observed that higher dietary fibre (60 g/kg of both dried distillers' grains with solubles and wheat bran reduced the average daily gain of broiler chickens by 11.2 g per head. Furthermore, the effect of different sources of dietary fibre on the growth performance of broiler chickens is summarized in Table 3.

CONCLUSION

The inclusion of dietary fibre in broiler chickens' diet plays an important role in enhancing growth performance, GIT development, nutrient utilization, and gut microbiota balance. The effects of dietary fibre on growth performance, GIT development, and nutrient utilization in broiler chickens depend on many factors, including age and species of the broiler chickens, fibre sources used in the diet, particle size, management, and prevailing environmental conditions. Including 2 to 3% dietary fibre in broiler chicken diets enhanced production performance, while excessive inclusion (exceeding 30 g/kg) might decrease overall performance, and lower inclusion might not exert any significant effect. It is recommended to identify different dietary fibre sources and determine their precise levels in broiler chicken nutrition. Furthermore, reviewing the effects of dietary fibre in relation to the histology of broiler chickens is essential.

Table 3. Effects of different sources of dietary fibre on the growth performance in broiler chickens

Strain	Duration (days)	Fibre source	Level (treatment)	ADG (g/head)	FI (g/day)	FCR	Reference
Broiler chicken	1-56	Maize and barley	100% maize	38.81	-	2.67	Ali et al. (2020)
			66.6% maize and 33.3% barley	37.89	-	2.72	
			33.3% maize and 66.6% barley	35.88	-	2.84	
			100% barley	31.18		3.14	
				BWG (g/day)	FI (g/day)	FCR	
Broiler chicken	1-42	No supplemental fibre	No	52.4	93.1	1.77	Saadatmand et al. (2019)
		Sugar beet pulp	30 g/kg SBP	49.3	86.8	1.76	
		Rice hull	30 g/kg RH	49.1	85.6	1.72	
				DWG (g/day)	DFI (g/day)	-	
Broiler chicken	1-42	Sugar beet pulp	No	59.2	112.1	-	Sadeghi et al. (2015)
		Rice hull	30 g/kg SBP	56.1	115.4	-	
			30 g/kg RH	58.3	114.5	-	
			Equal combination of SBP/RH	58.9	112.0	-	
				BWG (g/bird/day)	DFI (g/h/d)	FCR	
Broiler chicken	11-42	Barley and rice hulls	No	72.23	126.53	1.75	Adibmoradi et al. (2016)
			LBH (0.75% barley hull)	74.59	128.52	1.72	
			HBH (1.5%barley hull)	75.48	127.25	1.68	
			LRH (0.75% rice hull)	74.05	131.74	1.79	
			HRH (1.5% rice hull)	76.69	131.69	1.71	

ADG: Average daily gain, BWG: Body weight gain, DFI: Daily feed intake, DWG: daily weight gain, FCR: Feed conversion ratio, FI: Feed intake, HBH: The diets contained high level of barley hulls (1.5%), HRH: The diet contained high level of rice hulls (1.5%), LBH: The diets contained low level of barley hulls (0.75%), LRH: The diet contained low level of rice hulls (0.75%), RH: Rice hull, g/h/d: Gram/head/day, SBP: Sugar beet pulp, -: Not reported

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Authors' contributions

Muluze Fekade was responsible for conceptualization, data collection, writing the initial manuscript draft, reviewing the draft, and submitting the manuscript for publication. The author has read and approved the final edition of the manuscript before publication in the present journal.

Availability of data and materials

All information related to the present study is available upon reasonable request from the author.

Competing interests

The author declares no competing interests related to the present study.

Ethical considerations

The author confirmed that this study has not been submitted for publication elsewhere. Furthermore, no AI tools were employed in the preparation and writing the draft and revisions of the manuscript. The author is solely responsible for the accuracy, originality, and integrity of the final content prior to submission, throughout the revision process, and at the point of final publication.

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Mechanisms and Biological Outcomes of Essential Oils as Functional Feed Additives in Laying Hens: A Systematic Review

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ABSTRACT

Essential oils are being investigated as phytogetic alternatives to antibiotic growth promoters in laying-hen nutrition because selected formulations contain compounds with antimicrobial, antioxidant, and immunomodulatory activities. The present study aimed to evaluate the mechanisms and biological outcomes of essential oils as functional feed additives in laying hens' diets, along with their effects on production performance and egg quality. The present systematic review followed the PRISMA 2020 framework. Peer-reviewed English-language studies published from 2020 to 2026 were identified through Scopus and Google Scholar databases. This period highlighted recent findings on the rising use of phytogetic feed additives in poultry nutrition. Twelve studies met the eligibility criteria and were included in the qualitative synthesis. Several studies reported changes in gut microbiota and intestinal morphology, improved antioxidant indicators, or modulation of immune-related variables. Selected studies also reported improvements in feed efficiency, egg production or egg mass, eggshell characteristics, and internal egg quality, whereas other outcomes were unchanged. The magnitude and consistency of responses varied by essential-oil composition, inclusion level, formulation or delivery method, age or laying phase, experimental duration, housing, and other study conditions. Overall, essential oils have potential as functional feed additives in laying hens, but the evidence does not support a uniform effect across all products. Their practical value depends on the specific formulation, dose, and application strategy.

Keywords: Antioxidant activity, Egg production, Essential oil, Gut microbiota, Immune response, Laying hen, Phytogetic feed additive

INTRODUCTION

Egg production is an important component of the global animal-protein supply, and maintaining laying performance requires attention to both productivity and hen health. In this review, physiological stability refers to the maintenance of oxidative status, immune-related responses, blood biochemical indicators, and intestinal health within ranges compatible with normal laying function. Antibiotic growth promoters can support feed efficiency and control enteric pathogens, but inappropriate or prolonged antibiotic use in poultry production is linked to antimicrobial resistance and residue-related food-safety concerns (Abou-Jaoudeh et al., 2024). Studies of poultry

production similarly identified antimicrobial resistance as a major issue associated with routine antibiotic use and emphasize the need for effective non-antibiotic strategies (Mehdi et al., 2018). Alternative-feed strategies were expected to maintain performance while supporting intestinal and immune function rather than serving as a simple replacement for antimicrobial inputs (Gadde et al., 2017).

Restrictions on routine antibiotic growth promoters have increased interest in alternative feed additives, including probiotics, prebiotics, organic acids, enzymes, phytogetic products, and essential oils (Acharya and Barsila, 2025). Phytogetic feed additives derived from herbs, spices, and other plant materials have been

investigated for antimicrobial, antioxidant, digestive, and immunomodulatory functions in poultry and other food-producing animals (Windisch et al., 2008; Hashemi and Davoodi, 2011). Phytochemicals have been suggested as antibiotic alternatives because of their effects on host defense and intestinal health (Lillehoj et al., 2018). Essential oils contain diverse bioactive constituents, and their biological activity depends on the plant source and chemical profile. Studies on non-ruminant nutrition indicated that essential oil activity depends on composition, dose, and interaction with the gastrointestinal environment (Zeng et al., 2015). In laying hen investigations, carvacrol and thymol from oregano preparations, cinnamaldehyde from cinnamon-based products, and terpinen-4-ol from tea tree oil have gained attention (Amerah and Ouwehand, 2016; Puvača et al., 2020). Feng et al. (2021) demonstrated that an oregano product containing carvacrol and thymol can affect intestinal and microbial responses in laying hens. Studies in post-peak laying hens have evaluated cinnamaldehyde combined with carvacrol or thymol, illustrating that specific combinations can cause different responses in intestinal health and egg quality (Wang et al., 2022).

Published studies in laying hens have reported effects of essential oil supplementation on intestinal morphology and microbiota, antioxidant status, immune-related variables, production traits, and egg quality; however, the findings have been inconsistent. Differences in essential oil type, bioactive composition, inclusion level, formulation, laying phase, and experimental conditions indicated that essential oils should not be regarded as a single intervention. Naim et al. (2024) examined essential oils' effects on egg production; however, the relationship between mechanistic responses and productive outcomes was not fully integrated, particularly given heterogeneity across studies. Therefore, a focused synthesis is needed to link biological mechanisms with production outcomes while distinguishing product-specific and study-specific responses. The present study aimed to evaluate the mechanisms and biological outcomes of essential oils as functional feed additives in laying hens, with emphasis on gut microbiota, intestinal function, antioxidant status, immune response, production performance, egg quality, and factors contributing to variation in responses across studies.

MATERIALS AND METHODS

This study used a systematic literature-review design and followed the Preferred Reporting Items for Systematic

Reviews and Meta-Analyses (PRISMA) 2020 guideline (Page et al., 2021). The purpose was to identify and qualitatively synthesize recent experimental evidence on essential oil interventions in laying hens; however, this study was not designed as a meta-analysis. Scopus and Google Scholar were used as the information sources. Scopus was selected to capture peer-reviewed literature indexed in a major international bibliographic database, while Google Scholar was used for broader scholarly searching to identify additional relevant articles. PubMed and Web of Science were not searched in the present review, and this restricted database coverage is acknowledged as a methodological limitation. Search terms included combinations of essential oil, phytogetic feed additive, laying hen, egg production, egg quality, intestinal health, gut microbiota, immune response, antioxidant status, blood parameters, digestibility, and gene expression. Boolean operators AND and OR were used to combine terms and refine retrieval. The publication window was limited to 2020-2026 to focus on recent evidence generated during increasing interest in phytogetic feed additives as alternatives to antibiotic growth promoters in poultry nutrition (References published before 2020 were used only for background, methodological context, or discussion and were not counted as included studies). Only peer-reviewed articles written in English were considered. The last literature search was conducted on 20 April 2026.

Peer-reviewed articles published in English between 2020 and 2026 that were conducted in laying hens, evaluated an essential oil or an essential-oil-based phytogetic additive, and reported at least one relevant outcome related to production performance, egg quality, gut health or microbiota, antioxidant status, immune response, blood parameters, digestive function, gene expression, or other physiological outcomes were considered eligible. Studies were excluded when they solely involved broiler chickens, quail, or other poultry without data on laying hens, lacked an essential-oil intervention, were reviews, conference abstracts, theses, dissertations, or other non-peer-reviewed sources, were not published in English, reported outcomes unrelated to the review questions, or failed to provide enough information for qualitative analysis. A total of 1,841 records were identified in Scopus (n = 1,341) and Google Scholar (n = 500). After duplicate removal, 1,711 records were screened by title; 1,321 records were excluded, leaving 390 records for title/abstract assessment. A further 345 records were excluded at this stage. Forty-five full-text articles were assessed for eligibility; 33 were

excluded, and 12 studies were included in the final qualitative synthesis. Figure 1 shows the selection pathway.

For each included study, extracted information included author and year, essential oil type, main bioactive compound (when reported), inclusion level or dose, experimental duration, age or laying phase, delivery or formulation method (when reported), parameters observed, and key findings. Age or laying phase was recorded because physiological stage can modify the context in which a response was observed. Study findings were grouped into biological mechanisms, production performance and egg quality, and factors explaining variation in responses. A qualitative synthesis was used

because the included studies differed substantially in essential oil source and composition, dose units, formulations, laying phase, duration, housing or challenge conditions, and outcome definitions. These differences did not provide a sufficiently homogeneous basis for a defensible pooled effect estimate. The reporting of findings without statistical pooling was informed by the Synthesis Without Meta-analysis (SWiM) guidance (Campbell et al., 2020). Table 1 summarizes the characteristics of the included studies, whereas Table 2 presents their key findings and outcome synthesis. References published before 2020 were used only for background, methodological context, or discussion and were not counted as included studies.

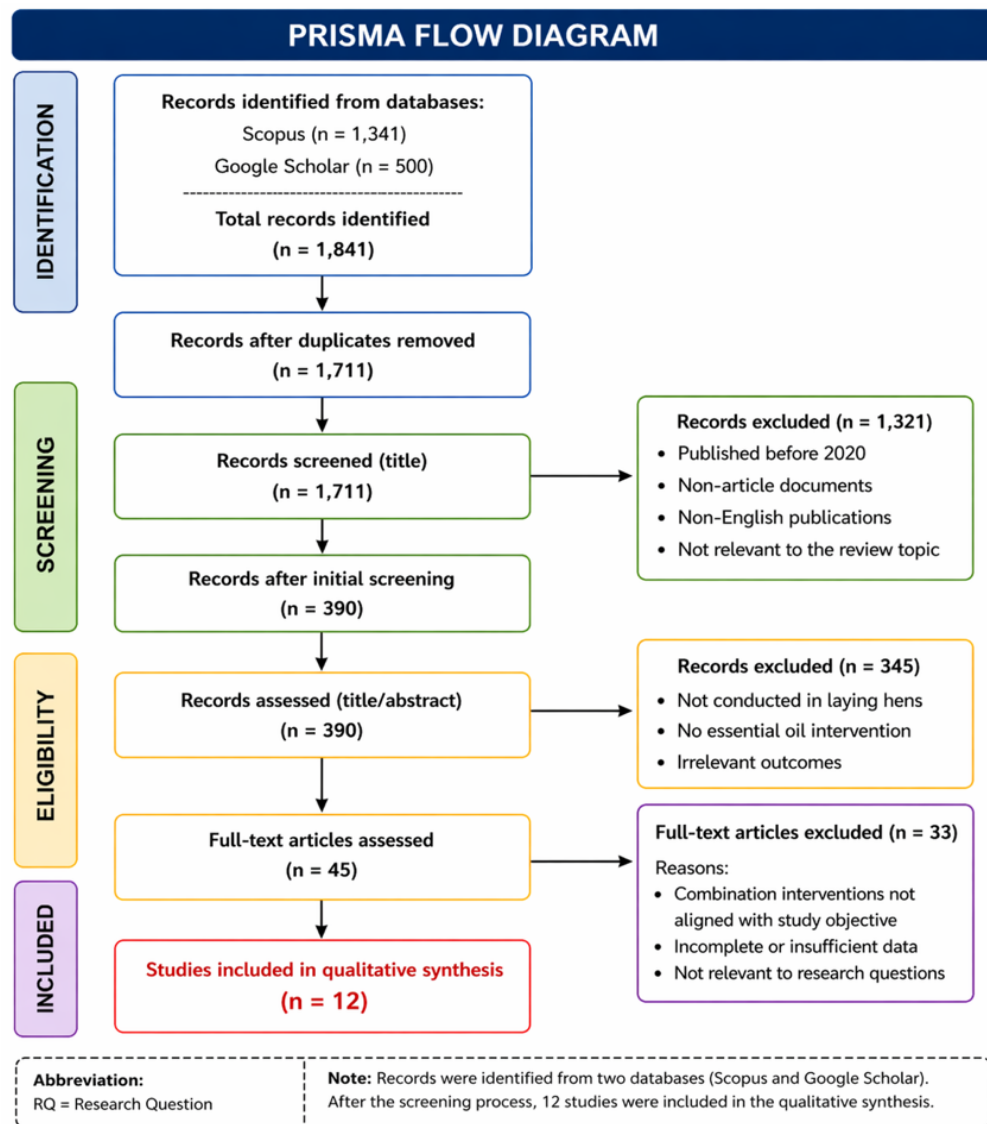


Figure 1. PRISMA flow diagram of the study selection process. The diagram was prepared by the authors based on the [PRISMA \(2020\)](#) guideline and summarizes article identification, screening, eligibility assessment, and final inclusion in the qualitative synthesis. Source: Authors' own illustration based on [PRISMA \(2020\)](#).

Table 1. Characteristics of studies on essential oils as functional feed additives in laying hens included in the present study (2020-2026)

Authors and year	Essential oil type	Main bioactive compound, if reported	Inclusion level or dose	Duration	Age or laying phase	Parameters observed
Marume et al. (2020)	<i>Citrullus lanatus</i> essential oil	Not reported	1 and 2 g/kg feed	8 weeks	18 weeks (point-of-lay)	Egg production, egg quality, yolk fatty acid profile, hematology, body weight, and tibia bone parameters
Feng et al. (2021)	Oregano essential oil (microencapsulated product)	Carvacrol and thymol	100, 200, and 400 mg/kg product	12 weeks	60 weeks (late phase)	Feed efficiency, egg quality, digestive enzymes, intestinal morphology, epithelial barrier, mucosal immune markers, and ileal microbiota
Ramirez et al. (2021)	Oregano essential oil (<i>Lippia organoides</i>)	Not reported	80 and 150 ppm	Study period June-December 2018; treatment duration not explicitly reported	70 weeks	Performance, egg quality, and intestinal morphometry
Xian et al. (2024)	Oregano oil	Not reported	25, 50, 75, and 100 mg/kg	100 days	55 weeks (late phase)	Egg quality, yolk nutrients, intestinal morphology, and cecal microbiota
Johnson et al. (2025)	Oregano essential oil (commercial product)	Not reported	275 mg/kg diet	16 weeks	24-40 weeks (early/peak phase)	Production, blood lipid profile, antioxidant capacity, liver-health indicators, egg quality, and yolk lipid peroxidation
Puvača et al. (2020)	Tea tree essential oil (<i>Melaleuca alternifolia</i>)	Terpinen-4-ol (40.0%), gamma-terpinene (23.0%), and alpha-terpinene (10.4%)	40 and 80 mg/kg	56 days	54 weeks at allocation; 56-day trial	Production performance, egg quality, eggshell traits, and yolk fatty acid profile
Xiao et al. (2022)	Essential oil blend	Carvacrol (2.5%), thyme-derived component (5%), and cinnamaldehyde (2.5%)	300 mg/kg	8 weeks	55-63 weeks (late phase)	Laying performance, egg quality, serum biochemical indices, antioxidant status, and cecal microbiota
Hanif et al. (2024)	Mineral mix-enriched essential oil	Not reported	0.25% in diet	6 weeks	31-37 weeks	Egg production, feed efficiency, antibody titer, physical and chemical egg quality, yolk antioxidant activity, and fatty acid profile
Abo Ghanima et al. (2020)	Rosemary essential oil or cinnamon essential oil	Not reported	300 mg/kg diet	48 weeks	28-76 weeks	Performance, egg quality, hematology, blood chemistry, immunity, antioxidant status, and housing-system effects
Laptev et al. (2021)	Essential oil-based phytobiotic, Intebio® (garlic, lemon, thyme, and eucalyptus oils)	Individual bioactive compounds not reported	90 g/ton feed	21 days before challenge; sampling at 1 and 7 days after inoculation; egg traits recorded for 27 days	346 days at study start; challenge at 367 days	Gene expression, blood biochemical and immunological variables, cecal microbiome, <i>Salmonella</i> challenge, and egg production
Reyer et al. (2026)	Phytogenic feed additive based mainly on thyme and star anise essential oils with quillaja bark	Not reported	150 mg/kg phytogenic supplement	4 weeks	68 weeks	Nutrient digestibility, egg quality, gene expression in pancreas, kidney, uterus, and follicles, and immune-related transcriptomics
Mus et al. (2026)	Oregano essential oil alone or combined with cinnamon or peppermint essential oil	Not reported	250 mg/kg oregano; or 125 mg/kg oregano + 125 mg/kg cinnamon; or 125 mg/kg oregano + 125 mg/kg peppermint	28 days	57 weeks	Intestinal microbiota, egg sensory and texture properties, fatty acid profile, and nutritional indices

Table 2. Key findings and outcome synthesis of included studies

Authors and year	Essential oil type	Inclusion level	Main outcome category	Key findings
Marume et al. (2020)	<i>Citrullus lanatus</i> essential oil	1 and 2 g/kg feed	Production; egg quality; hematology; bone	Egg mass and feed conversion ratio improved, as did albumen height and Haugh unit; selected yolk fatty acids, hematological values, and tibia parameters also changed. Total egg number was not significantly increased.
Feng et al. (2021)	Oregano essential oil	100, 200, and 400 mg/kg product	Gut function/microbiota; immune; production; eggshell	Feed conversion ratio, eggshell thickness, digestive-enzyme activity, intestinal morphology, epithelial-barrier markers, mucosal immune status, and ileal microbiota were affected; several favorable responses were strongest at 200 mg/kg.
Ramirez et al. (2021)	Oregano essential oil (<i>Lippia origanoides</i>)	80 and 150 ppm	Production; egg quality; intestinal morphometry	Both oregano treatments improved feed conversion and intestinal morphometry; 150 ppm produced the clearest improvements in egg production, egg mass, and internal and external egg-quality traits.
Xian et al. (2024)	Oregano oil	25, 50, 75, and 100 mg/kg	Egg quality; intestinal morphology; cecal microbiota	Oregano oil altered cecal microbiota and intestinal morphology and improved selected eggshell and yolk nutrient traits; 25 mg/kg produced the best overall response among the tested levels.
Johnson et al. (2025)	Oregano essential oil	275 mg/kg diet	Production; blood/liver; antioxidant; egg quality	Supplementation was associated with lower feed conversion ratio, changes in blood lipid profile, greater antioxidant capacity, lower lipid peroxidation, improved liver-health indicators, and selected improvements in internal egg quality.
Puvača et al. (2020)	Tea tree essential oil (<i>Melaleuca alternifolia</i>)	40 and 80 mg/kg	Production; eggshell; yolk fatty acids	Egg production and eggshell thickness improved in selected periods. Changes in the yolk fatty acid profile were directionally favorable but were not statistically significant overall.
Xiao et al. (2022)	Essential oil blend	300 mg/kg	Egg quality; serum biochemistry; antioxidant; microbiota	The blend increased shell-breaking strength and antioxidant indicators, altered serum biochemical indices, and shifted cecal microbial composition; production responses were more limited.
Hanif et al. (2024)	Mineral mix-enriched essential oil	0.25% in diet	Production; immunity; egg quality; yolk antioxidant	Egg production, feed efficiency, and yolk antioxidant activity increased. AI and ND antibody titers and yolk fatty acid profile were not significantly changed.
Abo Ghanima et al. (2020)	Rosemary essential oil or cinnamon essential oil	300 mg/kg diet	Production; egg quality; blood; immunity; antioxidant	Rosemary or cinnamon supplementation was associated with favorable changes in laying performance and selected egg-quality, blood, immune, and antioxidant measures. Housing system was evaluated concurrently.
Laptev et al. (2021)	Essential oil-based phytobiotic, Intebio®	90 g/ton feed	Gene expression; blood; microbiome; challenge; production	The phytobiotic modulated immune-related gene expression, blood biochemical and immunological variables, and cecal microbiome profiles during <i>Salmonella Enteritidis</i> challenge, with concurrent changes in egg-production traits.
Reyer et al. (2026)	Thyme/star-anise phytogetic feed additive with quillaja bark	150 mg/kg	Digestibility; transcriptomics; immune-related gene expression	Supplementation increased apparent ileal crude-protein digestibility and altered expression of genes related to antimicrobial activity, biomineralization, nutrient use, and immune responses.
Mus et al. (2026)	Oregano alone or with cinnamon/peppermint essential oils	250 mg/kg oregano; or 125 + 125 mg/kg combinations	Intestinal microbiota; egg composition; sensory/texture; fatty-acid indices	Oregano-based treatments reduced selected undesirable bacterial groups, increased lactic-acid bacteria, improved several fatty-acid-based nutritional indices, and did not produce undesirable sensory or texture changes in cooked eggs.

AI: Avian influenza; ND: Newcastle disease.

BIOLOGICAL MECHANISMS

The included studies identified intestinal function, microbiota, oxidative status, and immune regulation as recurrent biological domains, but the specific response depended on the intervention. [Feng et al. \(2021\)](#) found that a microencapsulated oregano essential oil product at 100-400 mg/kg affected digestive-enzyme activity, villus-to-crypt relationships, epithelial-barrier gene expression, mucosal immune markers, and ileal microbiota in 60-week-old late-phase hens. Several responses were most favorable at 200 mg/kg, suggesting the dose-response relationship is not linear. [Xian et al. \(2024\)](#) similarly reported that oregano oil at 25-100 mg/kg altered cecal microbial composition and intestinal morphology in 55-week-old hens, with 25 mg/kg yielding the most favorable overall response across egg production and intestinal health indicators. [Xiao et al. \(2022\)](#) observed that in late-laying hens, a 300 mg/kg essential oil blend enhanced total antioxidant capacity, boosted superoxide dismutase activity, altered specific serum biochemical indices, and modified the cecal microbiota. These findings support a connection between intestinal and oxidative responses, while also showing that not all microbiota-related and production endpoints changed in the same direction.

[Laptev et al. \(2021\)](#) reported that an essential oil-based phytobiotic containing garlic, lemon, thyme, and eucalyptus oils modulated immune-related gene expression, blood biochemical and immunological variables, and cecal microbiome profiles in hens infected by *Salmonella Enteritidis*. [Reyer et al. \(2026\)](#) found that a

thyme- and star-anise-based phytogetic additive containing quillaja bark enhanced apparent ileal crude-protein digestibility and induced transcriptomic changes in the pancreas, kidney, uterus, and follicles, involving genes related to antimicrobial activity and immune function.

Antioxidant responses were shown to be product-specific. [Johnson et al. \(2025\)](#) reported greater antioxidant capacity and lower lipid peroxidation with oregano essential oil, while [Abo Ghanima et al. \(2020\)](#) observed changes in antioxidant and blood parameters with rosemary or cinnamon essential oils. Reduced lipid peroxidation may indicate enhanced cellular integrity rather than a universal consequence of essential oils, aligning with findings from several studies suggesting that essential oil constituents can modulate oxidative and inflammatory mechanisms ([Bakkali et al., 2008](#); [Miguel, 2010](#)). Additionally, the antimicrobial efficacy of essential oils may be associated with the physicochemical properties of individual constituents and their interactions with microbial cellular structures ([Burt, 2004](#)). In poultry nutrition, proposed mechanisms include modulation of gut microbial ecology, digestive activity, and antioxidant or immune-related pathways ([Brenes and Roura, 2010](#)). These findings indicated that biological effects may include microbiota modulation, intestinal barrier support, oxidative balance, immune signaling, and systemic biochemical changes, each influenced by composition, dose, formulation, laying phase, and experimental conditions. These proposed mechanisms and their reported downstream outcomes are summarized in Figure 2.

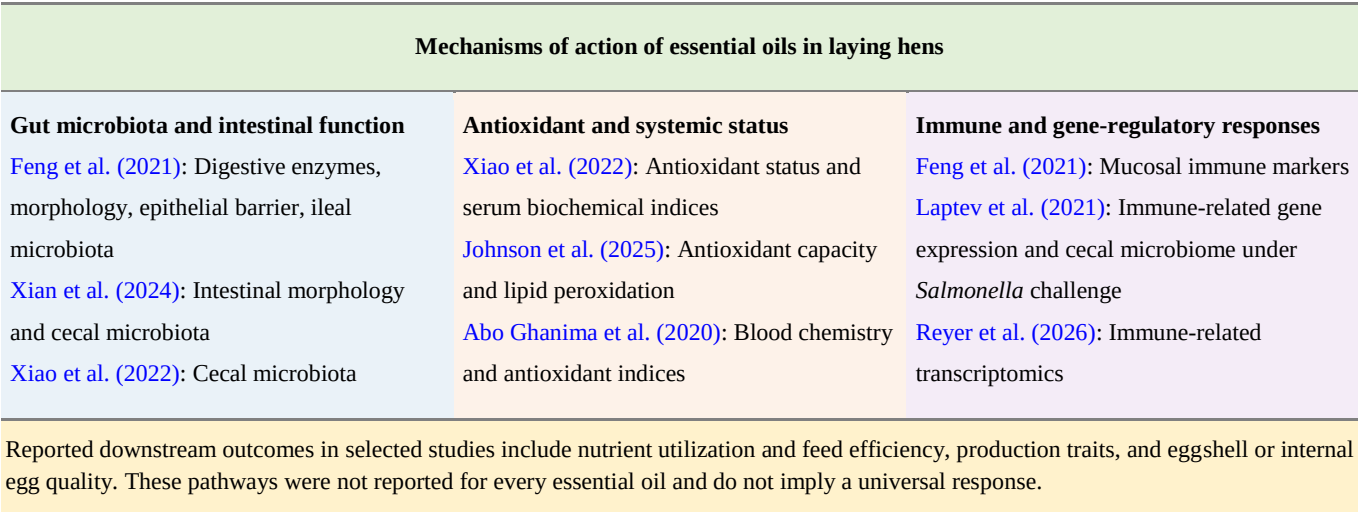


Figure 2. Proposed mechanisms by which essential oils influence biological and productive responses in laying hens. Essential oils may modulate gut microbiota, support intestinal morphology, enhance antioxidant defense, and regulate immune responses. These mechanisms may improve nutrient utilization, physiological stability, egg production, and egg quality, depending on essential oil composition, dose, and experimental conditions. Source: Authors' own illustration based on the synthesis of included studies.

PRODUCTION PERFORMANCE AND EGG QUALITY

Several included studies reported improvements in selected production traits after essential oil supplementation, although the magnitude and consistency of the response differed among products and experiments. [Marume et al. \(2020\)](#) found that administering 1 or 2 g/kg of *Citrullus lanatus* seed oil improved egg mass and feed conversion ratio in 18-week-old laying hens. However, total egg number did not increase significantly. [Ramirez et al. \(2021\)](#) reported improved feed conversion and intestinal morphometry with 80 or 150 ppm of *Lippia origanoides* essential oil. Notably, the 150 ppm treatment produced the most pronounced improvements in egg production, egg mass, and several internal and external egg-quality parameters.

Oregano-based supplementation yielded context-specific responses in other studies. [Feng et al. \(2021\)](#) reported a lower feed conversion ratio and greater eggshell thickness in late-phase hens, with the strongest responses at 200 mg/kg. [Johnson et al. \(2025\)](#) reported that administering 275 mg/kg of oregano essential oil from 24 to 40 weeks of age resulted in a lower feed conversion ratio, along with alterations in blood lipid profile, antioxidant capacity, liver health indicators, and internal egg quality. These findings suggested that production responses can occur alongside digestion, metabolism, or antioxidant activity; however, the mechanism applicable to each oregano formulation differed across studies.

Other essential oil sources produced more selective outcomes. Tea tree essential oil at 40 or 80 mg/kg improved egg production and eggshell thickness, whereas overall yolk fatty-acid changes were not statistically significant ([Puvača et al., 2020](#)). [Hanif et al. \(2024\)](#) discovered that enriching essential oil products with a 0.25% mineral mix enhanced egg production, feed efficiency, and yolk antioxidant activity. However, this product did not significantly affect antibody titers for avian influenza or Newcastle disease, nor did it alter the yolk fatty-acid profile. [Abo Ghanima et al. \(2020\)](#) observed improvements in laying performance, specific egg quality traits, blood parameters, immune response, and antioxidant markers when using rosemary or cinnamon essential oils at 300 mg/kg of diet, and also evaluated floor and cage housing systems.

The effects on egg quality varied by trait. Albumen height and Haugh unit increased with the addition of *Citrullus lanatus* ([Marume et al., 2020](#)), eggshell thickness

or strength improved with several oregano and tea tree oils ([Puvača et al., 2020](#); [Feng et al., 2021](#); [Xiao et al., 2022](#)), and [Xian et al. \(2024\)](#) reported changes in eggshell traits and yolk nutrients with oregano oil. The available evidence supports the conclusion that selected essential-oil formulations can improve specific production or egg-quality endpoints, rather than the broader claim that all essential oils improve all production traits.

VARIATION IN ESSENTIAL OIL RESPONSES

Differences across studies were closely related to chemical composition and formulation. Oregano-based products were the most commonly represented interventions in these 12 studies; however, they were not chemically or technologically identical. [Feng et al. \(2021\)](#) employed a microencapsulated oregano product containing carvacrol and thymol, while other oregano studies utilized different commercial formulations or *Lippia origanoides* oil ([Ramirez et al., 2021](#)). Tea tree oil contains terpinen-4-ol, gamma-terpinene, and alpha-terpinene ([Puvača et al., 2020](#)), while [Xiao et al. \(2022\)](#) used a mixture containing carvacrol and cinnamaldehyde with a thyme-derived component. Variation in chemical composition, stability, and formulation is a recognized source of inconsistent responses when essential oils are used as feed additives ([Dajić Stevanović et al., 2018](#)).

Additionally, dose can influence response variability, but current findings did not indicate a uniform optimal dose for all products. [Feng et al. \(2021\)](#) found that the most pronounced intestinal and production responses occurred at 200 mg/kg compared with 100 mg/kg and 400 mg/kg. In a study by [Xian et al. \(2024\)](#), the lowest tested level, 25 mg/kg, produced the best overall response compared with the higher tested levels of 50, 75, and 100 mg/kg. Thus, lower or intermediate tested doses sometimes performed more consistently than the highest tested dose, but this pattern cannot be generalized across essential oils because dose units, product concentration, and bioactive composition differed among studies.

Formulation and delivery further complicated comparison across the studies. Most included trials administered the essential oil via feed; however, the products included individual oils, oil mixtures, a microencapsulated oregano formulation, a mineral-enriched preparation, and a phytobiotic derived from essential oils. Encapsulation can improve the physical stability of essential oils and alter their release in the gastrointestinal tract; consequently, nominal inclusion

levels are not always directly comparable between encapsulated and unencapsulated formulations (Fontana et al., 2025). Mus et al. (2026) directly compared oregano essential oil alone with oregano-cinnamon and oregano-peppermint combinations in 57-week-old hens and observed treatment-specific changes in intestinal microbiota and egg fatty-acid indices without undesirable sensory effects. Xiao et al. (2022) evaluated the effects of a different essential oil mixture. These studies indicated that combining multiple oils did not automatically mean the mixture was synergistic.

Age and laying phase were another source of heterogeneity among the included studies. The included studies ranged from 18-week-old (Marume et al., 2020) to 70-week-old laying hens (Ramirez et al., 2021), with late-phase chickens evaluated in the studies of Feng et al. (2021), Xiao et al. (2022), and Xian et al. (2024) and 68-week-old laying hens in the study conducted by Reyer et al. (2026). The physiological priorities of early, peak, post-peak, and late laying differ, so age was treated as a study-level modifier rather than ignored during synthesis.

Experimental settings differed across the included studies. Abo Ghanima et al. (2020) explicitly evaluated floor and cage housing with rosemary or cinnamon

essential oils, whereas the remaining studies did not uniformly report housing, ambient conditions, or heat-stress status. Experiment durations ranged from 28 days in the study by Mus et al. (2026) to 48 weeks in the study conducted by Abo Ghanima et al. (2020). The evaluated factors spanned a broad range, including standard performance, egg-quality metrics, microbiota sequencing, blood indices, gene expression, and transcriptomic analyses. Consequently, inconsistent findings may reflect differences in measured factors, sampling times, and physiological or environmental conditions.

Across the included studies, oregano-based essential oils were most frequently associated with positive responses in gut function or microbiota, feed efficiency, antioxidant status, and selected egg-quality traits. This pattern should be interpreted cautiously because oregano-based studies were also the most numerous and differed in inclusion level, duration, laying phase, formulation, and measured outcomes. The qualitative design of the present review does not permit ranking oregano as universally superior to other essential oils. The main product- and study-level factors contributing to response variability are summarized in Figure 3.

Factors influencing variation in essential oil responses in laying hens		
Composition and bioactive profile Oregano preparations, tea tree oil, rosemary/cinnamon oils, single oils, blends, and phytobiotics differed in chemical profile.	Inclusion level Doses varied within and between products, with oregano studies using 25-400 mg/kg and other studies using g/kg, ppm, %, or g/ton units.	Formulation and delivery Most studies used feed inclusion; formulations included microencapsulated oregano, blends, mineral-enriched products, and a phytobiotic.
Age or laying phase Hens ranged from point-of-lay (18 weeks) to late/post-peak phases (55-70 weeks or older), creating different physiological contexts.	Housing and environment Housing, environmental conditions, and heat-stress status were not uniformly reported.	Duration and outcome measurement Experimental duration ranged from 28 days to 48 weeks, and studies measured different biological, production, egg-quality, blood, and omics endpoints.
Interpretation: variability across studies reflects interacting product- and study-level factors; the qualitative synthesis does not establish a universal ranking of essential oils.		

Figure 3. Factors contributing to variability in essential oil responses in laying hens. Differences in chemical composition, inclusion level, delivery method, laying phase, housing system, and environmental condition may influence the consistency and magnitude of biological and production outcomes across studies. Source: Authors' own illustration based on the synthesis of included studies.

CONCLUSION

Twelve studies were included and indicated that specific essential oil formulations can affect gut microbiota and

intestinal health, antioxidant levels, immune responses, systemic biochemical markers, production performance, and egg quality in laying hens. Oregano-based preparations were the most common intervention and were

most often linked to positive effects across outcomes, especially gut function, feed efficiency, antioxidant status, and specific egg-quality traits. The present findings should not be interpreted as definitive evidence that oregano is the most effective essential oil overall, since the review was qualitative and did not include a meta-analysis. Responses to essential oil formulations differed across studies due to variation in chemical composition, dose, formulation or delivery method, age or laying phase, duration, housing, and experimental conditions. The present study was constrained by reliance on Scopus and Google Scholar databases, heterogeneity in experimental design and outcome measurement, and incomplete reporting of bioactive composition or treatment details across studies. Future studies should report essential oil composition and delivery more consistently, use clearly defined laying phases and comparable outcome measures, and broaden database coverage.

DECLARATIONS

Authors' contributions

Luky Amalta contributed to the study conception and design, literature search, screening, data extraction, qualitative synthesis, and manuscript drafting. Osfar Sjoifan, Eko Widodo, Heli Tistiana, Ita Wahju Nursita, Dita Novarina Gunawati, and Cindy Audina Damayanti contributed to methodological review, interpretation of the qualitative synthesis, critical revision, and improvement of intellectual content. All authors read and approved the final edition of the manuscript.

Availability of data and materials

All data analyzed in this review were obtained from previously published articles cited in the manuscript.

Competing interests

The authors declare no competing interests.

Ethical considerations

The authors confirm that this manuscript is original and is not currently under consideration for publication elsewhere. The authors accept full responsibility for the originality of the content, accuracy of the data extraction, and the final edited version of the manuscript. Since this study was a systematic literature review and did not involve human participants or live animals, ethical approval and informed consent were not required. The authors did not use any AI tools to conduct or prepare this study. The authors accept full responsibility for the

originality and the integrity of the present review manuscript, before submission and at publication time.

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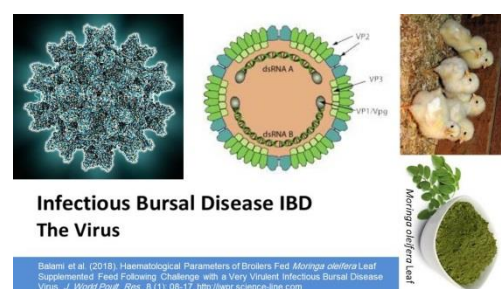
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d) For books:

AOAC (1990). Association of Official Analytical Chemists. Official Methods of Analysis, 15th Edition. Washington D.C. pp. 69-88. Pelczar JR, Harley JP, Klein DA (1993). Microbiology: Concepts and Applications. McGraw-Hill Inc., New York, pp. 591-603.

e) Books, containing sections written by different authors:

Kuney M (1979). Pig Fattening. In: A. Alexiev (Editor), Farm Animal Feeding. Vol. III. Feeding of Different Animal Species, Zemizdat, Sofia, p. 233-243 (Bg).

In referring to a personal communication the two words are followed by the year, e.g. (Brown, J. M., personal communication, 1982). In this case initials are given in the text.

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Decilitre	dl	Kilogram	kg
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Micrometer	mm	Minutes	min
Molar	mol/L	Mililitre	ml
Percent	%		

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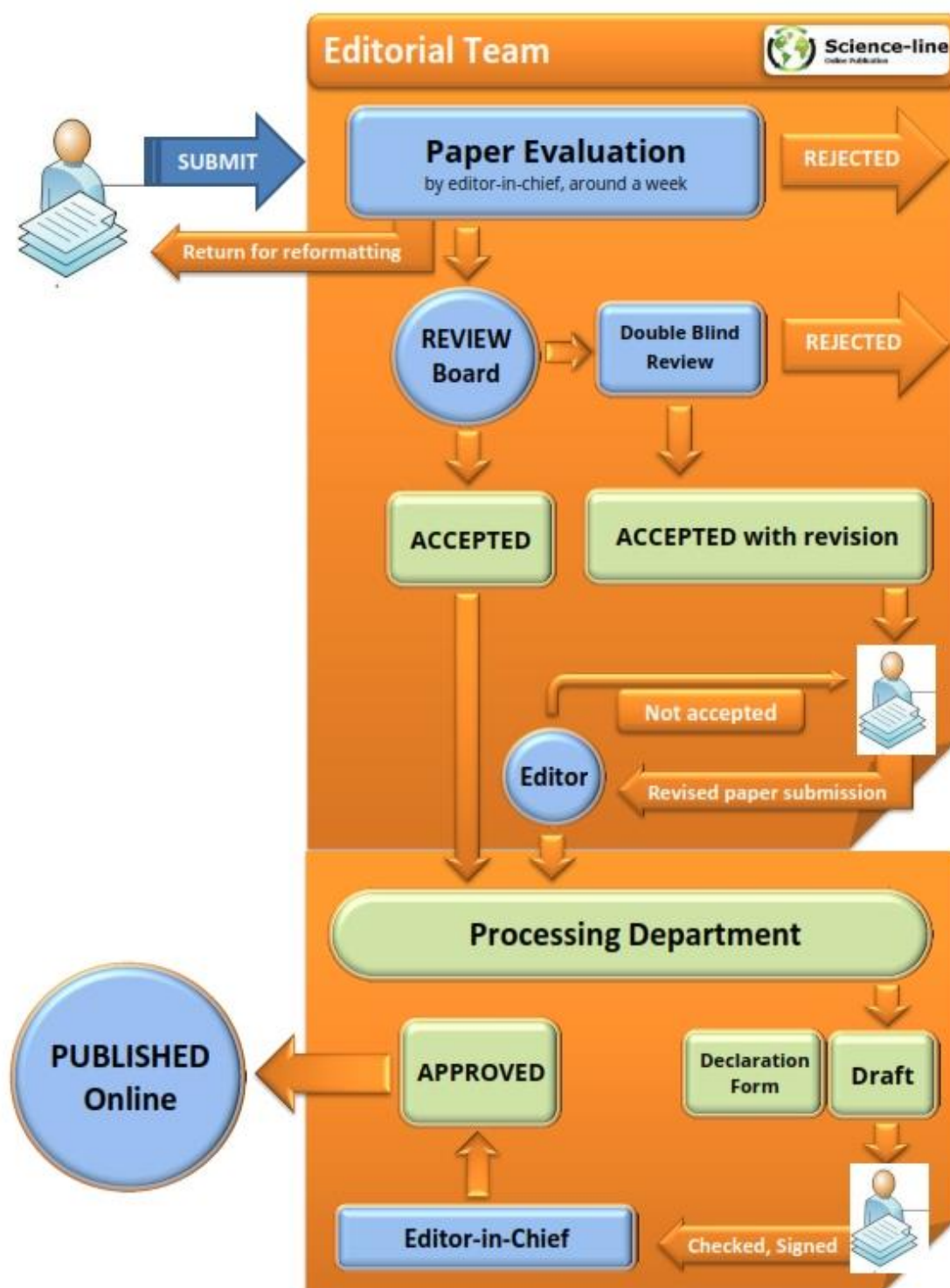
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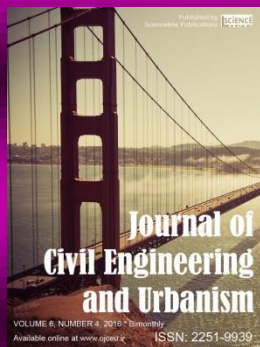
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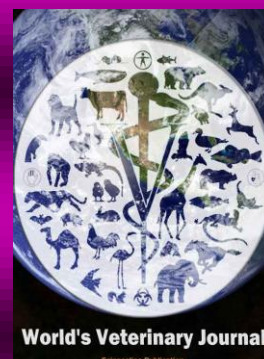
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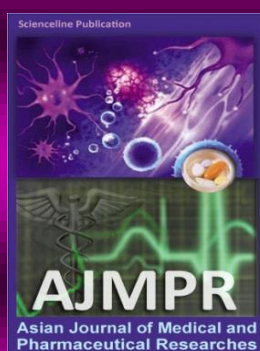
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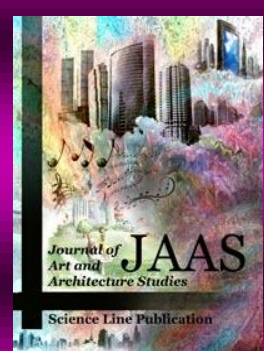
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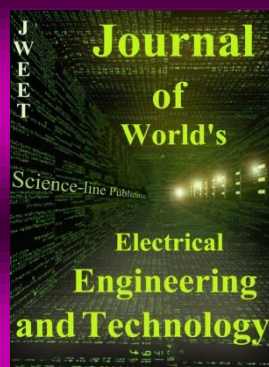
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