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BOOKLET

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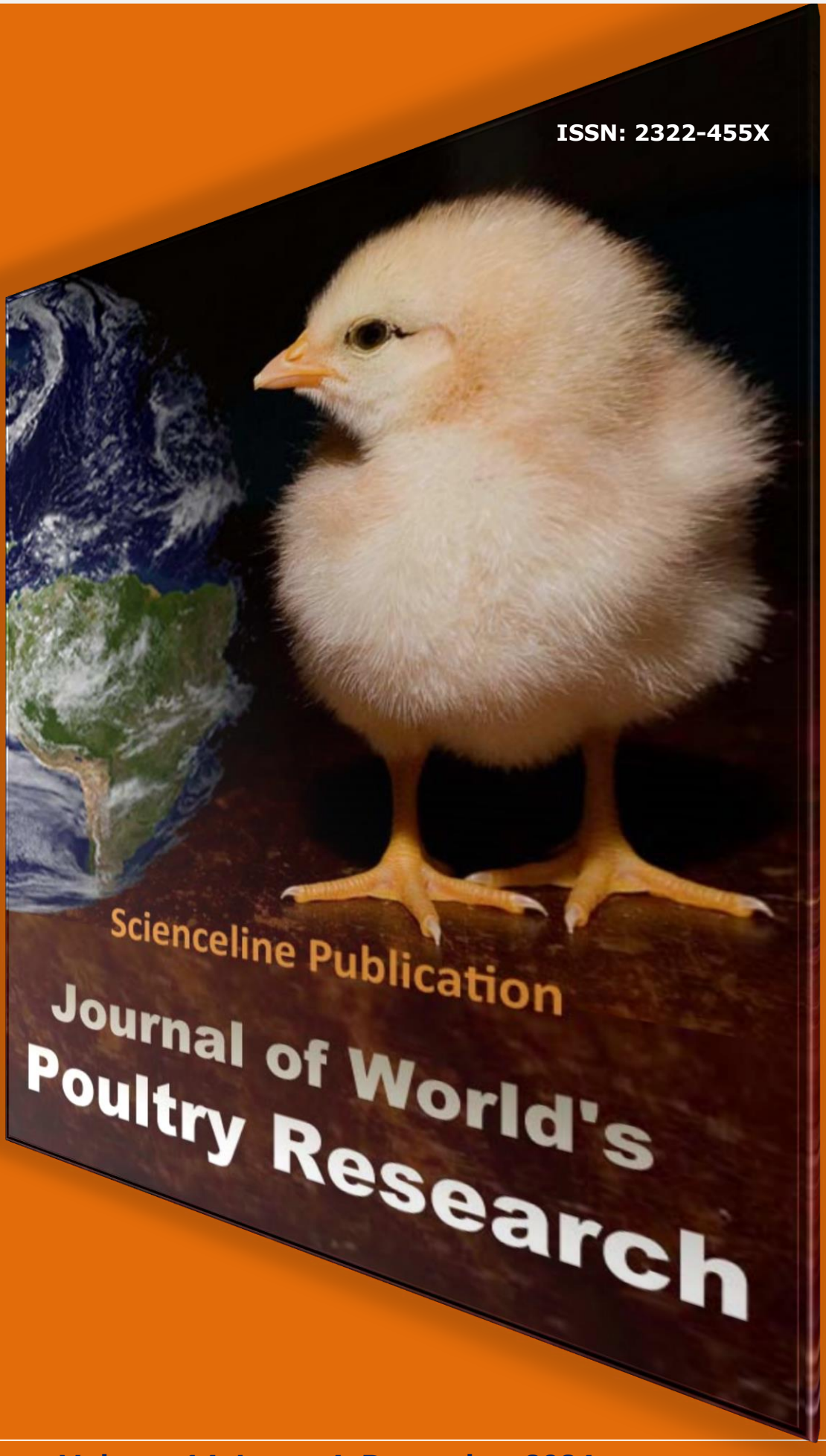

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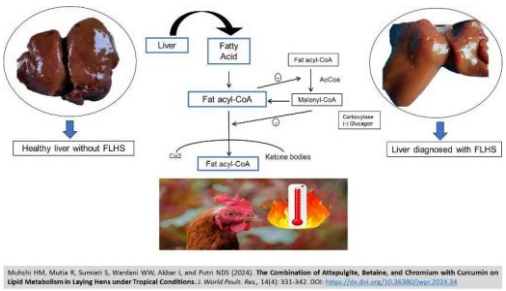
Research Paper

The Combination of Attapulgite, Betaine, and Chromium with Curcumin on Lipid Metabolism in Laying Hens under Tropical Conditions

Muhshi HM, Mutia R, Sumiati S, Wardani WW, Akbar I, and Putri NDS.
J. World Poult. Res. 14(4): 331-342, 2024; pii: S2322455X2400034-14
DOI: https://dx.doi.org/10.36380/jwpr.2024.34

ABSTRACT: Liver health in laying hen is associated with lipid synthesis and metabolism. This study focused on oxidative parameters to maintain liver health and lipid metabolism in laying hens. The efficacy of curcumin as a herbal compound, combined with attapulgite, betaine, and organic chromium in a feed additive called Citrus XL, was evaluated in terms of its impact on lipid metabolism. This study involved 2000 ISA Brown strain laying hens aged 82-87 weeks. The heat stress index was calculated based on a temperature of 26.71 ± 1.11 oC and humidity of 83.21 ± 6.86 % in open-house cages with an 18-hour lighting period. This experiment included four treatments with five replications, including a basal diet with no Citrus XL (Control), a basal diet plus 0.5 kg/ton of Citrus XL, a basal diet plus 1.0 kg/ton of Citrus XL, and a basal diet plus 1.5 kg/ton of Citrus XL. To do so, blood biochemistry, fat content, liver score, malondialdehyde (MDA), and superoxide dismutase (SOD) levels were measured. The results indicated a significant increase in HDL levels as well as a reduction in LDL and MDA levels, liver scores, and egg yolk fat content. In conclusion, the treatment with 1.0 kg/ton of Citrus XL yielded the best results in terms of HDL, liver score, and liver MDA while Citrus XL treatment with 0.5 kg/ton produced the best results for LDL and yolk fat content.
Keywords: Curcumin, Heat stress, Laying hens, Lipid metabolism, Liver



[Full text-PDF]

Research Paper

Effects of in ovo Injection of Soursop (*Annona muricata*) Leaf Extract on Blood Profile, Immune Organs, and Intestinal Morphology of Noiler Chicks

Kuka TT, N'nanle O, Karou S, Tona K, and Bakoma B.
J. World Poult. Res. 14(4): 343-350, 2024; pii: S2322455X2400035-14
DOI: https://dx.doi.org/10.36380/jwpr.2024.35

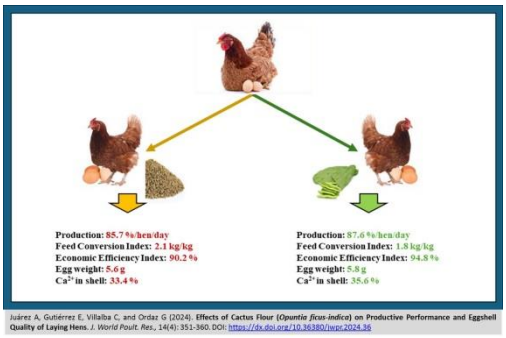
ABSTRACT: Plant extracts in poultry production are widely recognized for their significant benefits in improving productive performance. Specifically, the *in ovo* administration of soursop leaf extract (SLE) shows promise in improving the health and productivity of Noiler chickens. This study aimed to provide valuable insights into the health of the chicks and evaluate the effectiveness and safety of *in ovo* SLE in poultry production by examining the blood profile, internal organs, and intestinal morphology of Noiler chickens. For this experiment, 640 hatching eggs were incubated and randomly divided into four experimental groups, including 0.25 µg SLE, 0.5 µg SLE, 0.75 µg SLE, and a non-injected control group. Three treatment groups received a direct injection of 0.2 ml of the respective SLE concentrations into the air cells of the eggs on the 18th day of incubation. After hatching, chickens from each group were divided into five replicates of 15 chicks each and raised using a completely randomized design. At ten days of age, blood samples were collected from two chicks per replicate for hematology and serum analysis. Two chicks per replicate were sacrificed on day 10 to assess the internal organs and intestinal morphology. The results showed no significant changes in hematological parameters, serum biochemistry, and internal organs in all groups. The soursop leaf extract groups had markedly longer villi, deeper crypts, and thicker muscular walls compared to the control group. In conclusion, the *in ovo* injection of soursop leaf extract at 0.75 µg improved intestinal health by enhancing the intestinal surface structures in Noiler chickens.
Keywords: Hematology, Internal organ, In ovo, Intestinal morphology, Noiler chicken, Soursop Leaf Extract

[Full text-PDF]

Research Paper

Effects of Cactus Flour (*Opuntia ficus-indica*) on Productive Performance and Eggshell Quality of Laying Hens

Juárez A, Gutiérrez E, Villalba C, and Ordaz G.
J. World Poult. Res. 14(4): 351-360, 2024; pii: S2322455X2400036-14
DOI: https://dx.doi.org/10.36380/jwpr.2024.36



Juárez A, Gutiérrez E, Villalba C, and Ordaz G (2024). **Effects of Cactus Flour (*Opuntia ficus-indica*) on Productive Performance and Eggshell Quality of Laying Hens**. *J. World Poult. Res.*, 14(4): 351-360. DOI: https://dx.doi.org/10.36380/jwpr.2024.36

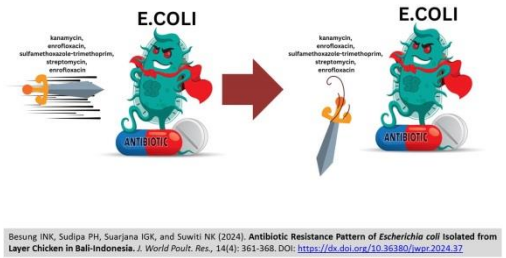
ABSTRACT: The poultry industry plays a crucial role in the production of animal proteins for human consumption and generating sources of employment. Thus, it is essential to explore effective strategies to enhance both the productivity of laying hens and the quality of their products, particularly eggshell quality, due to its significant economic implications for the poultry sector. This study aimed to evaluate the effects of cactus flour (CF; Opuntia ficus-indica) on the productive performance and eggshell quality of laying hens. Twenty-four Rhode Island Red laying hens were randomly divided into two groups (n = 12 experimental units -hens-/group) consisting of a control group and another fed CF (1% of the diet volume). The variables assessed included initial and final weight, weight gain, feed and calcium intake, egg production, egg mass, feed conversion index/kg of egg, economic efficiency index, egg weight, shell weight, shell thickness, shell percentage, and calcium levels in eggshells and excreta (daytime and nighttime). The addition of CF in the diet affected the final weight of hens, with the CF-fed hens (2.1 kg) being heavier than the control (1.9 kg). Egg production was higher in the CF-fed hens than in the control hens. Additionally, the mean egg weight was higher (68.5 g) in the CF-fed hens than in the control (62.2 g). The feed conversion index was lower in the CF-fed hens (2.1 kg/kg) than in the control (1.8 kg/kg). The economic efficiency index was higher in the CF-fed hens (94.8 %) than in the control (90.2 %). Eggshell weight (5.8 g), thickness (0.31 mm), and calcium levels (35.6 %) were significantly higher in the CF-fed hens than in the control (5.1 g, 0.27 mm, and 33.4 % for eggshell weight, thickness, and calcium levels, respectively). In conclusion, the inclusion of CF in the diet of laying hens improved the productive indicators and eggshell quality, thereby enhancing economic efficiency.
Keywords: Egg production, Cactus flour, Calcium, Laying hens, Poultry farming

[Full text-PDF]

Research Paper

Antibiotic Resistance Pattern of *Escherichia coli* Isolated from Layer Chicken in Bali-Indonesia

Besung INK, Sudipa PH, Suarjana IGK, and Suwiti NK.
J. World Poult. Res. 14(4): 361-368, 2024; pii: S2322455X2400037-14
DOI: https://dx.doi.org/10.36380/jwpr.2024.37



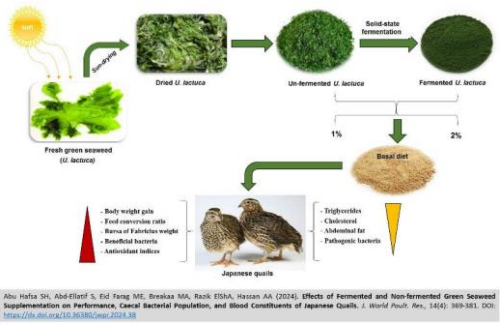
Besung INK, Sudipa PH, Suarjana IGK, and Suwiti NK (2024). **Antibiotic Resistance Pattern of *Escherichia coli* Isolated from Layer Chicken in Bali-Indonesia**. *J. World Poult. Res.*, 14(4): 361-368. DOI: https://dx.doi.org/10.36380/jwpr.2024.37

ABSTRACT: Antibiotics have been used as growth promoters in the poultry industry worldwide, which might lead to the emergence of anti-microbial resistant bacterial strains. Theoretically, older animals should have been exposed to antibiotics and anti-microbial resistant (AMR) strains for longer periods, which may result in the discovery of more resistant strains. The present study aimed to evaluate the antibiotic resistance of *Escherichia coli* isolated from fecal samples of layer chicken that showed signs of watery diarrhea. In the current study, 134 fecal samples were taken from the layer chicken farms in Penebel village, Tabanan District, Bali, Indonesia. The chickens were classified into three groups including Group 1 under 7 days of age, group 2 aged 7-30 days, and Group 3 chickens older than one month. The samples were cultured in Eosin Methylene Blue agar. The suspected colonies were stained, and subjected to biochemical tests. *Escherichia coli*-positive colonies were subjected to a bacteria sensitivity test using multiple antibiotic discs. The result demonstrated multi-drug resistance (MDR) of *Escherichia coli,* while the isolated *Escherichia coli* was resistant to the most common antibiotics in layer farms in the study area including kanamycin, enrofloxacin, sulfamethoxazole-trimethoprim, streptomycin, and enrofloxacin. In addition, the present study confirmed that although all sample groups were sensitive to bacitracin, oxytetracycline, and clindamycin, they were resistant to sulfamethoxazole-trimethoprim, kanamycin, and ampicillin.
Keywords: Antibiotic resistance, Bali, Colibacillosis, *Escherichia coli*, Layer chicken

[Full text-PDF]

Effects of Fermented and Non-fermented Green Seaweed Supplementation on Performance, Caecal Bacterial Population, and Blood Constituents of Japanese Quails

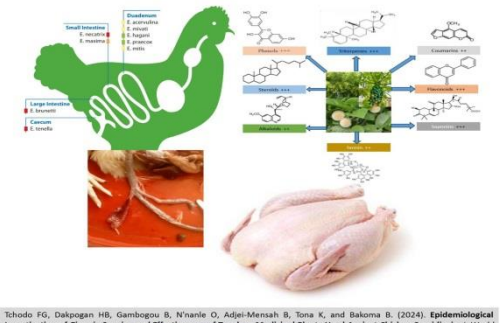
Abu Hafsa SH, Abd-Ellatif S, Eid Farag ME, Breakaa MA, Razik ElShA, and Hassan AA.
J. World Poult. Res. 14(4): 369-381, 2024; pii: S2322455X2400038-14
DOI: https://dx.doi.org/10.36380/jwpr.2024.38



ABSTRACT: Green Seaweeds are a valuable feedstock that can be utilized in poultry feed. Due to their recalcitrant polysaccharide structure, their use is still limited in poultry farming. This structure can be broken by a biotechnological approach such as solid-state fermentation (SSF) such as Trichoderma reesei, which simultaneously increases the nutritional value of the biomass. The current study aimed to investigate the effect of supplementation of fermented and non-fermented green seaweed (Ulva lactuca) on growth performance, nutrient digestibility, carcass characteristics, caecal microbiota, serum biochemistry, and antioxidant status in growing Japanese quails. Japanese quails (n = 375; one day old) were divided into five groups, with three replicates per group (25 quails in each replication). The quails were fed with five experimental diets, namely a control diet (basal diet without supplement), a basal diet supplemented with 1% and 2% green seaweed (GS) as well as 1% and 2% fermented green seaweed (FGS) for 42 days. The results showed that the groups fed FGS had a greater body weight gain and better feed conversion ratio than the other groups. The FGS groups showed the highest digestibility of crude protein and crude fiber, followed by the GS groups. FGS supplementation decreased abdominal fat percentage while increasing the bursa of Fabricius weight. The count of Lactobacillus was significantly increased in quails fed either GS or FGS, while Clostridium perfringens and Escherichia coli were decreased. The green seaweed-fed groups had significantly greater total protein, albumin, and globulin levels than the control group. Total lipids, triglycerides, cholesterol, HDL, and LDL were decreased in quails-fed diets containing 1% and 2% FGS. The quails in FGS diet groups had higher levels of total antioxidant capacity, catalase, and superoxide dismutase than the other groups, but lower levels of MDA. In conclusion, adding up to 2% fermented Ulva lactuca to the basal diet of Japanese quail promotes the growth and health of quails.
Keywords: Antioxidant status, Caecal microbiota, Fermentation, Green seaweed, Performance, Lipid profile, Japanese quail
[Full text-PDF]

Epidemiological Investigation of *Eimeria* Species and Effectiveness of Togolese Medicinal Plants Used Against Chicken Coccidiosis

Tchodo FG, Dakpogan HB, Gambogou B, N'nanle O, Adjei-Mensah B, Tona K, and Bakoma B.
J. World Poult. Res. 14(4): 382-398, 2024; pii: S2322455X2400039-14
DOI: https://dx.doi.org/10.36380/jwpr.2024.39



ABSTRACT: *Eimeria* species cause coccidiosis, a poultry disease that occurs worldwide. Infection is linked to decreased feed efficiency and body weight increase. The present study aimed to assess the prevalence of coccidian species in Togolese poultry farms and evaluate the anticoccidial efficacy of three local medicinal plants. From July to September 2023, two hundred and ninety-five fecal samples were randomly collected using a cross-sectional observational study in the maritime region of Togo, specifically in Vo, Lacs, Zio, and Grand-Lomé districts. Data on risk factors were collected through an interview with the poultry farmers. All fecal samples collected were subjected to *Eimeria* oocyst counting using the standard McMaster technique. The anticoccidial activity of the extract of *Azadirachta indica* leaves *Carica papaya* seeds, and *Sarcocephalus latifolius* roots in a completely randomized design was evaluated on 23-day-old male Isa brown chicks infected with 30.10^4 oocysts. Body weight gain, feed efficiency, lesion score, proportion of bloody droppings, anticoccidial index, and excretion of coccidia oocysts were assessed. The results revealed an overall prevalence of 39.66% (117/295) for coccidiosis, with 75% of positive samples having fewer than 10,000 oocysts/g. The logistic regression test indicated that the interval between two anticoccidial prophylaxis applications, age, management, and breed were significant risk factors associated with coccidial infection, with young chicks (≤ 8 weeks) being 5.66 times more susceptible than those older ones (8 weeks) with 0.86 as an odd ratio. Six *Eimeria* species were identified, with *E. maxima* (54.17%), *E. brunetti* (33.33%), and *E. tenella* (25%) being the most common. The anticoccidial efficacy of *Azadirachta indica* leaves, *Carica papaya* seeds *Sarcocephalus latifolius* roots extract, and amprolium was demonstrated by a reduction in lesion scores, bloody diarrhea, and oocysts per gram in feces (OPG) as well as an improvement in body weight, feed conversion ratio, and production efficiency factor when compared to infected and untreated groups. The anticoccidial index was marked in the chickens treated with *Sarcocephalus latifolius* roots extract (170) and amprolium (176). The findings of this large-scale epidemiological study and anticoccidial efficacy tests revealed that these Togolese medicinal plants can be sustainable and cost-effective strategies for coccidiosis control.

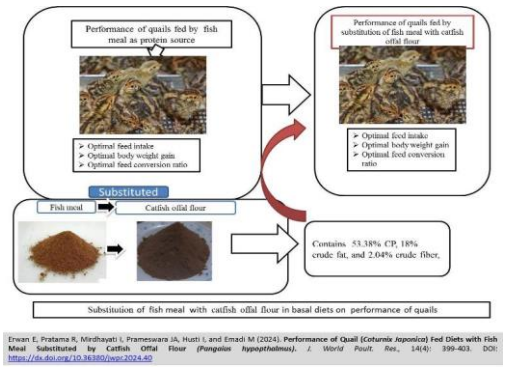
Keywords: Anticoccidial drug sensibility, Coccidiosis, Prevalence, Risk factor, Togo

[Full text-PDF]

Research Paper

Performance of Quail (*Coturnix Japonica*) Fed Diets with Fish Meal Substituted by Catfish Offal Flour (*Pangaius hypopthalmus*)

Erwan E, Pratama R, Mirdhayati I, Prameswara JA, Husti I, and Emadi M.
J. World Poult. Res. 14(4): 399-403, 2024; pii: S2322455X2400040-14
DOI: https://dx.doi.org/10.36380/jwpr.2024.40



ABSTRACT: Catfish offal flour (COF; Pangaius hypopthalmus) has the potential to replace fish meal (FM) due to its high crude protein content. The present study aimed to investigate the effects of substituting FM with COF in basal diets on food intake (FI), body weight gain (BWG), and feed conversion ratio (FCR) in quails. A total of 100 male quails were randomly assigned to five treatment groups, each with four replicates. The treatment groups were fed with basal diet + 0% COF and 100% FM (T0), basal diet + 25% COF and 75% FM (T1), basal diet + 50% COF and 50% FM (T2), basal diet + 75% COF and 25% FM (T3), and basal diet + 100% COF and 0% FM (T4). Feed intake, BWG, and FCR were measured from 0 to 35 days of age. The findings indicated that substituting FM with COF up to 100% did not significantly affect FI, BWG, and FCR. It can be concluded that COF has the potential to replace FM in basal diets while maintaining performance in quails.
Keywords: Catfish offal flour, Body weight gain, Feed intake, Feed conversion ratio, Quail

[Full text-PDF]

Research Paper

Assessing the Productivity of BLRI-Developed Native Ducks at the Community Level Compared to Indigenous Ducks in Conventional Farming Systems

Islam S, Islam MA, Sultana S, Islam R, Rahman MH, and Khatun R.
J. World Poult. Res. 14(4): 404-417, 2024; pii: S2322455X2400041-14
DOI: https://dx.doi.org/10.36380/jwpr.2024.41

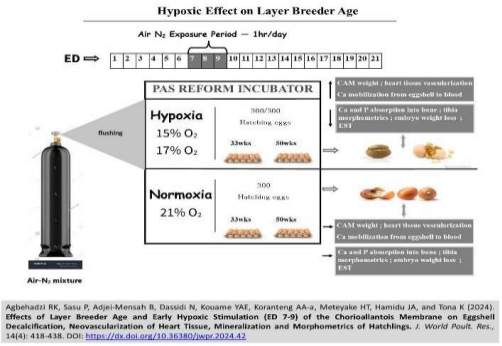
ABSTRACT: Duck farming is a profitable business in low-lying areas of Bangladesh. The present study aimed to disseminate Bangladesh Livestock Research Institute (BLRI) developed native ducks BLRI-1(Rupali) and BLRI-2 (Nageswari) and validate their production ability compared to indigenous ducks under existing farming conditions in Bhanga upazila of Faridpur. An experiment was done at the community level where 45 farmers were selected based on their duck type. Data on the productive potentials of BLRI-developed native ducks were recorded and compared with the local germplasm of ducks. Among 45 duck-rearing farmers, with an average age of 38.58 years and farming experience of 12.38 years. Ducks were raised under scavenging conditions where 82.2% of farmers used separate duck houses and regular house cleaning was practiced by 68.89% of farmers. Ducks were consistently fed paddy, rice, and rice bran whereas 82.2% of farmers provided supplement feed with duckweed, and 15.6% supplied ready-made feed. The highest growth performance was observed for Rupali ducks growing to 1505.62 g by 24 weeks, compared to 1486.07 g for Nagesawri ducks. The highest egg production was 192.00 ± 5.70 eggs in Nageswari ducks followed by 181.33 ± 7.55 eggs for Rupali. Statistically significant differences were observed in adult male and female weights, eggs per clutch, and egg weight among the three breeds. Most of the farmer (84.4%) vaccinate their duck, against Duck Plague and Duck Cholera. The highest incidences of Duck Plague and Duck Cholera were observed in Native duck farms in comparison to BLRI-developed duck farms. Farmers obtained the highest Net income 8149.00 BDT (68.04 USD) and Benefit-Cost Ratio (BCR) of 1.60 in Rupali ducks compared to the Indigenous ducks at 1.30 whereas the overall BCR in duck rearing was 1.49. Major constraints regarding duck farming were disease outbreaks (73.3%) and high feed prices (64.4%). Thus, the study highlighted the significant variations in the performance and economic viability of ducks and emphasized farmers' training and breed-specific management strategies such as improved housing; feeding, and disease management practices to boost the profitability of duck farming.
Keywords: Benefit-cost ratio, BLRI duck, Disease outbreak, Growth performance, Native duck, Profitability

[Full text-PDF]

Research Paper

Effects of Layer Breeder Age and Early Hypoxic Stimulation (ED 7-9) of the Chorioallantois Membrane on Eggshell Decalcification, Neovascularization of Heart Tissue, Mineralization and Morphometrics of Hatchlings

Agbehadzi RK, Sasu P, Adjei-Mensah B, Dassidi N, Kouame YAE, Koranteng AA-a, Meteyake HT, Hamidu JA, and Tona K.
J. World Poult. Res. 14(4): 418-438, 2024; pii: S2322455X2400042-14
DOI: https://dx.doi.org/10.36380/jwpr.2024.42



ABSTRACT: Oxygen concentration (O_2) during incubation is crucial for embryo development, and hypoxic conditions can influence phenotypic plasticity in poultry. Although low O_2 (hypoxia) can be detrimental, it may also promote adaptive responses. Breeder age, a known genetic determinant of egg quality and embryonic development, is likely to interact with O_2 levels during incubation, however, this relationship remains understudied in layer breeders. This study examined how layer breeder age and reduced oxygen (O_2) levels during early embryonic development affect various factors, including eggshell decalcification (Dcal-SHL), chorioallantoic membrane (CAM) weight, heart tissue vascularization, egg weight loss (EWL), eggshell temperature (EST), and calcium (Ca) and phosphorus (P) content in bone and blood and tibia and femur morphometrics. A total of 900 eggs from 33 and 50-week-old ISA brown layer breeders were incubated in a 2x3 factorial design with O_2 levels of 15%, 17% (hypoxic), and 21% (control). Oxygen was reduced for 1hr/day from embryonic days (ED) 7-9 using air-N_2 flushing. Results showed increased CAM weight and heart tissue vascularization under hypoxia, especially in older breeders (50 weeks). Hypoxic conditions (15% and 17% O_2) reduced embryo weight loss and eggshell temperature compared to controls during the post-exposure phase (ED 15-18). There was an interaction between breeder age and O_2 levels on mineral absorption, with reduced oxygen leading to lower Ca and P absorption in bones, higher eggshell P retention, and decreased tibia morphometrics (weight, length, diameter, and seedor index) in hatchlings. Additionally, CAM weight correlated negatively with Dcal-SHL Ca at 15% O_2. The study concluded that reduced oxygen during early embryonic development increases CAM weight, heart neovascularization, and Ca mobilization from eggshell to blood. However, older flocks exhibited reduced Ca transfer to bones, likely due to homeostatic imbalance.

Keywords: Breeder age, Bone mineralization, Eggshell quality, Hypoxia, Oxygen level

[Full text-PDF]

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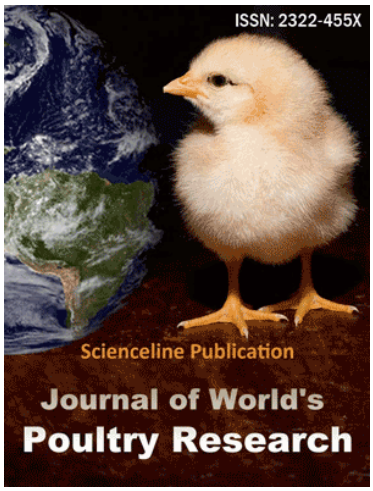

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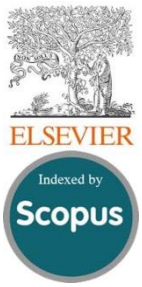

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The Combination of Attapulgite, Betaine, and Chromium with Curcumin on Lipid Metabolism in Laying Hens under Tropical Conditions

Hafidz Muhammad Muhshi[1], Rita Mutia[2]*, Sumiati Sumiati[2], Wira Wisnu Wardani[3], Ilham Akbar[3], and Nofitra Dewi Suparno Putri[3]

[1]*Graduate School of Nutrition and Feed Science, Faculty of Animal Science, IPB University, Dramaga, Bogor 16680, Indonesia*
[2]*Department of Nutrition and Feed Technology, Faculty of Animal Science, IPB University, Dramaga, Bogor 16680, Indonesia*
[3]*Nutricell R&D, Cibis nine 12th Floor Unit G1, Jakarta 12560, Indonesia*

*Corresponding author's E-mail: ritamutia@apps.ipb.ac.id

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ABSTRACT

Liver health in laying hen is associated with lipid synthesis and metabolism. This study focused on oxidative parameters to maintain liver health and lipid metabolism in laying hens. The efficacy of curcumin as a herbal compound, combined with attapulgite, betaine, and organic chromium in a feed additive called Citrus XL, was evaluated in terms of its impact on lipid metabolism. This study involved 2000 ISA Brown strain laying hens aged 82-87 weeks. The heat stress index was calculated based on a temperature of 26.71 ± 1.11 ^{o}C and humidity of 83.21 ± 6.86 % in open-house cages with an 18-hour lighting period. This experiment included four treatments with five replications, including a basal diet with no Citrus XL (Control), a basal diet plus 0.5 kg/ton of Citrus XL, a basal diet plus 1.0 kg/ton of Citrus XL, and a basal diet plus 1.5 kg/ton of Citrus XL. To do so, blood biochemistry, fat content, liver score, malondialdehyde (MDA), and superoxide dismutase (SOD) levels were measured. The results indicated a significant increase in HDL levels as well as a reduction in LDL and MDA levels, liver scores, and egg yolk fat content. In conclusion, the treatment with 1.0 kg/ton of Citrus XL yielded the best results in terms of HDL, liver score, and liver MDA while Citrus XL treatment with 0.5 kg/ton produced the best results for LDL and yolk fat content.

Keywords: Curcumin, Heat stress, Laying hens, Lipid metabolism, Liver

INTRODUCTION

Curcumin is a phytobiotic herbal ingredient known for its essential role in the lipid metabolism rate. It can be used in animal feed, due to its anti-inflammatory, antioxidant, and anti-cancer properties, as well as its ability to prevent liver disease (Perrone et al., 2015). Betaine has potential benefits for laying hens, functioning as an osmolytic agent to mitigate heat stress and positively influencing tonic immobility (Abd El-Ghany and Babazadeh, 2022). Attapulgite is a mineral utilized as a supplement in chicken feed, characterized by its octahedral structure, which enhances viscosity, improves absorption capacity, and binds toxins within the digestive system of chickens (Kanoulas et al., 2023). Chromium, though not required in poultry feed, is naturally present in trace amounts and has been shown to improve the physiological, immunological, growth, and lipid profiles of chickens (Fraz et al., 2023). Curcumin has an efficient therapeutic role and protects against the effects of oxidative disorders in the liver through molecular and cellular mechanisms as hepatoprotective (Farzaei et al., 2018). This mechanism can improve cellular responses to oxidative stress disorders by expressing catalase (CAT), glutathione (GSH), glutathione peroxidase (GPx), and superoxide dismutase (SOD). The indication of oxidative stress can be excessive reactive oxygen species and an imbalance between the oxidants and antioxidants in the body, leading to the degradation of lipids, cell proteins, and DNA (Cichoz-Lach and Michalak, 2014). In layer poultry, the use of curcumin can specifically prevent a liver disease

called fatty liver hemorrhagic syndrome (FLHS), which is caused by a metabolic disorder that causes a very significant decrease in egg production, causing mass mortality.

The curcumin combined with attapulgite, betaine, and chromium is marketed as Citrus XL. As an industrial feed additive product for poultry, it potentially improves the lipid metabolism process, reduces heat stress, protects the liver function from fatty liver disease, and prolongs egg production in aged laying hens. Curcumin has a role in regulating lipid metabolism and inhibits hepatic steatosis (Feng et al., 2019). The mineral type of attapulgite can increase the absorption capacity of feed in the digestive tract and exchange ions. It can also help maintain intestinal health in chickens by preventing diarrhea in laying hens (Zhou et al., 2014; Tzora et al., 2017). The use of organic chromium (Cr) is also beneficial in protecting livestock from metabolic disorders and heat stress, as it helps prevent oxidative stress in cells and tissues (Youssef et al., 2022). The inclusion of betaine in the diet can optimize feed conversion ratios, increase chest muscle circumference, and enhance egg production. The osmolytic property of betaine reduces heat stress and has good potential for tonic immobility, thereby reducing stress in chicks (Abd El-Ghany and Babazadeh, 2022). Information regarding the combination of attapulgite, betaine, and chromium with curcumin has not yet been extensively studied or published.

Lipid metabolism refers to the utilization of fat digested from diet and absorbed from body fat to be used by body tissues. According to Attia et al. (2022), lipid compounds primarily form lipid metabolites involved in egg formation. In addition, the emergence of oxidative stress is caused by free radicals which break down into malondialdehyde (MDA) as an oxidative degradation product. Heat stress is an indicator of disturbances in livestock caused by high temperatures arising from environmental conditions (Thornton et al., 2021). Heat stress, triggered by environmental factors, can disrupt lipid metabolism in laying hens by promoting the formation of free radicals, thereby increasing MDA levels and reducing superoxide dismutase (SOD) activity (Emami et al., 2021). The enzymatic reaction in the layer liver to external stressors can disrupt the lipid metabolism process and the balance of nutritional requirements due to oxidative stress from hot temperatures (Bacou et al., 2021). The effects of heat stress from high body temperature will influence immunological biomarkers, blood chemistry, brain function, and other physical parameters, leading to oxidative damage of membranes and DNA (He et al., 2018). A score indicator is obtained by measuring the Heat Stress Index (HSI), which involves environmental temperature and humidity with various tolerance limits (Esnaola-Gonzalez et al., 2020). In chickens, heat stress can decrease feed intake efficiency (Killic and Simsek, 2013), reduce growth rates (Gholamreza et al., 2019), lower egg production, slow body weight gain, increase feed consumption ratio and raise mortality rates (Wasti et al., 2020). These effects are often influenced by a combination of air temperature, environmental heat, wind speed, and humidity.

The present study aimed to evaluate the effects of Citrus XL supplementation at specific doses on lipid metabolism and liver health in ISA Brown laying hens aged 82-87 weeks.

MATERIALS AND METHODS

Ethical approval

All experimental procedures were approved by the Animal Ethics Committee of IPB University following the guidelines for the use and care of animals in research (No. 053-2023 IPB).

Animal diets and research design

This experiment was carried out at Cisadane Pradana Farm in Semplak, Bogor City, Indonesia. A total of 2000 laying hens were used, housed individually in battery cages with dimensions of 50 cm (length) x 38 cm (width) x 45 cm (height). The feed additive, Citrus XL, produced by PT. Nutricell Pacific (Perseroan Terbatas) was used for the experiment. Citrus XL was a combination of organic chromium, attapulgite, phytobiotic curcumin, and betaine (Table 1).

Table 1. Citrus XL feed additive ingredient on laying hen lipid metabolism under tropical conditions

Material[1]	Amount	Unit
Chromium Organic	100.00	mg
Attapulgite	200.00	g
Phytobiotic	350.00	g
Betaine	200.00	g

[1)] PT: Nutricell Pacific.

The product was developed in collaboration between PT. Nutricell Pacific and IPB University, based on local farming conditions, and was provided by the factory for the trials. The laying hen used were the ISA Brown strain, aged 82-87 weeks. After an adaptation period, the

chickens were randomly assigned to four groups with five replications (n = 20), resulting in 100 hens per replication. The hens were fed complete diets supplemented with Citrus XL at 0, 0.5, 1.0, and 1.5 kg/ton of feed from 82 to 87 weeks of age. The diet used was complete rations provided by PT. Sreeya Sewu Indonesia, Tbk. and consisted of corn, bran, soybean meal, meat, and bone meal, olein, palm oil meal, wheat bran, distillers dried grains with solubles (DDGS), feather meal, amino acids, minerals, vitamins, phytase enzyme, non-starch polysaccharide (NSP) enzyme, antifungals, antioxidants, and organic acids. The complete nutritional components of the feed can be seen in Table 2. The feed was provided through restricted feeding at a rate of 115 g/day based on diet guidance daily feeding requirements (ISA Brown Product Guide, 2022), while water was provided *ad libitum* through nipple drinkers. Lighting was regulated for 18 hours per day, with 6 hours of darkness. Temperature and humidity were recorded to determine the Heat Stress Index (HSI), with an index score of < 150 indicating comfort for hens and >160 indicating heat stress (Pakpahan et al., 2023). The HSI during the study ranged from a minimum of 154 to a maximum of 172, with an average of 163.43 ± 5.01. The HSI results are shown in Figure 1. At the end of the study, five hens from each treatment group (one per replicate) were randomly selected for blood sampling. Approximately 3 ml of blood was drawn from the brachial vein in the left wing using a syringe and collected in vacuum tubes. The samples were transported to the Faculty of Animal Science laboratory at IPB University, where they were prepared under cold conditions and centrifuged at 1500 rpm for 30 minutes. The liver condition was assessed using a Nutricell Eggspert color scan tool to detect the color, which was reviewed with the L a b color coordinates (L = lightness; a = red/green; b = yellow/green). The color data were processed in Microsoft Excel and sent to the laboratory for testing fat, MDA, and SOD levels.

Table 2. Nutrient composition of experimental diet on the lipid metabolism of laying hens in tropical conditions

Nutrient Content[1] \ Treatments[2]	T1	T2	T3	T4
Water (max%)	13.00	13.00	13.00	13.00
Ash (max%)	14.00	14.00	14.00	14.00
Crude protein (min%)	17.00	17.00	17.00	17.00
Crude fat (min%)	3.00	3.00	3.00	3.00
Crude fiber (max%)	7.00	7.00	7.00	7.00
Calcium (%)	3.25-4.25	3.25-4.25	3.25-4.25	3.25-4.25
Phytase ≥ 400 FTU kg	0.45	0.45	0.45	0.45
Aflatoxin totally (max µ kg^{-1})	50	50	50	50
Lysine (min%)	0.84	0.84	0.84	0.84
Methionine (min%)	0.42	0.42	0.42	0.42
Methionine+cysistin (min%)	0.70	0.70	0.70	0.70
Threonine (min%)	0.58	0.58	0.58	0.58
Tryptophan (min%)	0.19	0.19	0.19	0.19

[1]PT. Sreeya Sewu Indonesia Tbk. [2]T1: diet without Citrus XL supplementation (Control), T2: Control diet supplemented with Citrus XL 0.5 kg/ton, T3: Control diet supplemented with Citrus XL 1.0 kg/ton, T4: Control diet supplemented with Citrus XL 1.5 kg/ton.

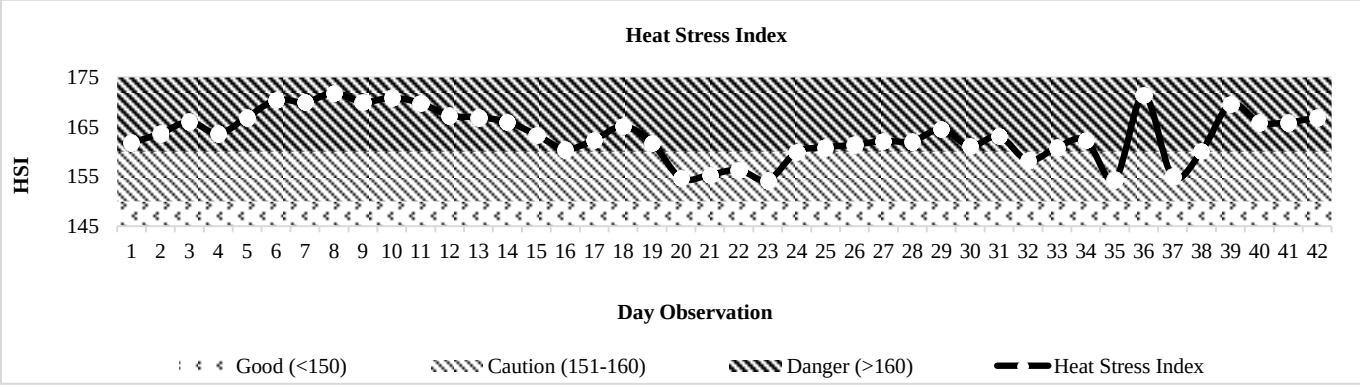

Figure 1. Heat stress index condition during the observation of a feeding Citrus XL in the diet of laying hen aged 82-87 weeks.

Blood cholesterol and triglyceride

The analysis of total blood cholesterol and triglyceride levels was conducted following the method of Sharma et al. (1987). Samples containing 0.5 ml of blood were randomly drawn from each treatment and replication group. Enzyme reagents and standard solutions from Liquidcolor, Human Diagnostics Worldwide (Germany), were prepared for the analysis. The preparation steps included filling the blank tube with 1000 µL of enzyme reagent. The standard tube was filled with 10 µL of standard cholesterol solution (for cholesterol analysis), 1000 µL of enzyme reagent, and 10 µL of standard triglyceride solution (for triglyceride analysis). Then, 1000 µL of enzyme reagent was added to the plasma preparation. The sample tube was filled with 10 µL of blood plasma and 1000 µL of enzyme reagent cholesterol and triglyceride. The mixture was then homogenized with vortex and then incubated at 20°C – 25°C for 20 minutes. Absorbance was measured using a spectrophotometer at 500 nm in wavelength, resulting in the blood cholesterol (mg/dL) and triglyceride (mg/dL) levels.

Blood high-density lipoprotein level

The analysis of blood high-density lipoprotein (HDL) levels was performed using the method of Wieland and Seidel (1983). A 3 µL blood sample was randomly taken for each treatment. Reagent 1 was prepared in a volume of 225 µL, consisting of 4-Aminoantipyrin (1.4 mmol/L), cholesterol esterase (1.6 KU/L), cholesterol oxidase (1 KU/L), and peroxidase (5 KU/L) as the component to detect the concentration of HDL level. Blood serum was homogenized with reagent 1 and incubated for 5 minutes at 37°C. The absorbance at 600nm was measured using a spectrophotometer as A1, representing the standard and initial value of HDL level. Reagent 2, consisting of 75 µL of HEPES buffer (25 mmol/L) and TOOS (N-Ethyl-N-(2-hydroxy-3-sulfopropyl)-3-methylaniline, sodium salt, dihydrate; 1.3 mmol/L), was then prepared. The blood serum was homogenized with reagent 2 and incubated for 5 minutes at 37°C. The absorbance was measured again at 600 nm using a spectrophotometer as A2, focusing on the red and purple coloration of the HDL levels. The change in absorbance (ΔA) was calculated using the following formula to determine the final HDL level (mg/dL).

$\Delta A = A2-A1$ (Formula 1)

Blood low-density lipoprotein level

The analysis of blood low-density lipoprotein (LDL) levels was carried out following the method of Wieland and Seidel (1983) using blood samples taken randomly in 3 µL volumes. Reagent 1, consisting of 225 µL HEPES buffer (23 mmol/L), 4-Aminoantipyrin (1.5 mmol/L), cholesterol esterase (1.6 KU/L), cholesterol oxidase (1 KU/L), and peroxidase (5.5 KU/L), was prepared. The blood serum was homogenized with reagent 1 and incubated for 5 minutes at 37°C, and the absorbance was measured at 600nm using a spectrophotometer as A1. Next, 75 µL of reagent 2 was prepared using HEPES buffer (25 mmol/L) and TOOS (1.3 mmol/L). The blood serum was homogenized with reagent 2, then incubated for 5 minutes at 37°C. The absorbance was read again at 600 nm with a spectrophotometer as A2. The change in absorbance (ΔA) was calculated using the following formula to determine the LDL level (mg/dL).

$\Delta A = A2-A1$ (Formula 2)

Egg yolk and liver fat analysis

The Soxhlet method was used to analyze the fat content of liver and egg yolk (AOAC, 2005). The sample sizes were 10 ml of egg yolk and 7 g of liver. After being dried for 30 minutes at 105^{o}C in the oven, the fat flask was cooled for 15 minutes in a desiccator. Next, the chilled pumpkin was weighed (A). A syringe was used to extract 5 cc of egg yolk (S) and 5 g of liver (S), which were placed on filter paper and tied with fat-free cotton wool. After being placed inside a paper thimble and extracted with hexane for 3-4 hours at 80^{o}C, the filter paper was chilled and weighed (B). The fat content was calculated using the following formula.

$$\text{Fat content (\%)} = \frac{B-A}{S} \times 100\% \quad \text{(Formula 3)}$$

Superoxide dismutase analysis

The analysis of SOD was conducted via the method of Ulhusna et al. (2019). A 0.5 g sample of liver tissue was chopped until fine and homogenized in 2.5 ml of phosphate buffer (PB, pH 7). The homogenate was placed in a tube and centrifuged at 3000 rpm for ten minutes at 4^{o}C. The supernatant was mixed with 8 ml of a chloroform-ethanol (3:5) solution and vortexed before being centrifuged again at 3000 rpm for 10 minutes at 4°C. The supernatant was then measured for absorbance at a wavelength of 480 nm using a spectrophotometer. Absorbance was measured at the first, second, third, and fourth minutes, and the SOD activity in the liver was expressed in U/mL.

Malondialdehyde analysis

The analysis of malondialdehyde (MDA) levels in the liver followed the thiobarbiturate acid reactive substance (TBARS) method as described by Ulhusna et al. (2019). A

0.5 g sample of liver tissue was finely chopped and homogenized in 5 ml of phosphate-buffered saline (PBS), with the remaining liver organs saved for further research. The homogenate was centrifuged at 3500 rpm for 10 minutes at 4°C, and the supernatant was extracted and transferred to an Eppendorf tube carried out in cold conditions. The amount of 1 ml of the supernatant was extracted from the Eppendorf and transferred into a test tube. A 4-milliliter test tube was filled with a cold 0.25 N HCl mixture that contained 0.5% butylated hydroxytoluene (BHT), 15% trichloroacetic acid (TCA), and 0.38% thiobarbituric acid (TBA). The oven was set to 80^{o}C for one hour, heating the test tube. The liquid tube was then allowed to cool for ten minutes in a water-filled bath. The tube was centrifuged for 10 minutes at 40° C at 3500 rpm. The supernatant was read at a wavelength of 532 nm with a UV-Vis spectrophotometer (Thermo Scientific Genesys 10S UV-Vis, USA). A standard curve was prepared using 1,1,3,3-tetraethoxypropane (TEP) at concentrations of 0, 1, 2, 3, 4, 5, 10, 20, 40, 80 µ, where Y is the MDA level (nmol/mg), and the liver MDA levels were calculated using the following formula.

$$Y = aX + b \quad \text{(Formula 4)}$$

Liver color score

The liver color indicator was measured using a colorimeter calibrated with standard white ceramic tiles. Samples were taken from each replicate by comparing the color of the chicken liver and measuring its brightness, identified by the Nutricell Eggspert Digital Colorimeter LS175 (China 2022).The lightness (L), redness (a), and yellowness (b) values of each liver sample were assessed in triplicate, and these values were translated into red (R), green (G), and blue (B). Identification of liver disease was based on the method described by Zhu et al., (2020), which assigns a score of 1-4 by evaluating the color of the liver and the number of hemorrhage points. A score of 1 indicates a normal, dark red liver with no hemorrhage. A score of 2 shows a mild, slightly yellow liver with 1-5 hemorrhages. A score of 3 indicated a moderate, yellow liver with 6-15 hemorrhages. Finally, a score of 4 signifies extreme, showing a brittle, dark yellow liver with 16-25 hemorrhages.

Statistical analysis

Data collected from this study were statistically analyzed using analysis of variance (ANOVA) with SPSS (2017) software version 25. If significant differences were found between treatment groups, Duncan's multiple range test (Duncan, 1955) was used for further analysis. Polynomial contrasts, including Linear, Quadratic, and Cubic models, were also applied. P values less than 0.05 were considered significant, and the mean data were expressed with standard deviations.

RESULTS

Blood biochemistry, liver malondialdehyde, and superoxide dismutase

Citrus XL supplementation at 0.5 to 1.5 kg/ton of feed did not have a significant effect on blood triglyceride levels in all groups. The best result was observed at 1.0 kg/ton, with 1355.4 ± 445.13 mg/dL, compared to the control treatment with 1502.6 ± 300.53 mg/dL ($p = 0.897$, $p > 0.05$). Blood cholesterol levels showed the best result at 0.5 kg/ton of Citrus XL, with 124.8 ± 36.09 mg/dL, compared to 151.6 ± 28.99 mg/dL in the control group ($p = 0.811$, $p > 0.05$). Supplementation of Citrus XL at 1.0 kg/ton had a significant effect (35.4 ± 5.85 mg/dL) on increased blood HDL levels compared to the control group (27.4 ± 4.39 mg/dL) with a p-value of 0.038, and quadratic polynomial contrast results of 0.031 ($p < 0.05$). Supplementation of Citrus XL at 0.5 kg/ton significantly reduced blood LDL levels (58.0 ± 15.24 mg/dL) compared to the control group (85.4 ± 16.50 mg/dL) with a p-value of 0.038 and a quadratic polynomial contrast result of 0.013 ($p < 0.05$). Supplementation of 1.0 kg/ton of Citrus XL had a significantly reduced liver MDA level (0.235 ± 0.013 U/mL) compared to the control group (0.264 ± 0.015 U/mL) with a p-value of 0.038 and a quadratic polynomial contrast result of 0.015 ($p < 0.05$). Although no significant effects on liver SOD levels were observed, polynomial contrast analysis showed a quadratic result with a p-value of 0.047, with the optimal dose identified as 1.0 kg/ton (Tables 3 and 4).

Fat level and liver score

Supplementation of Citrus XL at 0.5 to 1.5 kg/ton did not have any significant effect on the abdominal fat, which ranged between 2.98% and 4.26% of body weight. However, the control group had the lowest percentage, with 2.98 ± 1.19% ($p = 0.172$). Liver fat levels ranged from around 18.93% to 21.25% of liver weight, with the best result observed in the Citrus XL 1.0 kg/ton group, which presented 18.93 ± 9.13% ($p = 0.136$). Citrus XL supplementation at 0.5 kg/ton had a significant effect (47.93 ± 9.78%) in reducing egg yolk fat compared to the control group (63.39 ± 4.72%), with a p-value of 0.004 and a quadratic polynomial contrast of 0.001 ($p < 0.05$). Additionally, supplementation of Citrus XL at 1.0 kg/ton

had a significant effect (2.0 ± 0.70) in reducing the liver score compared to the control group (3.4 ± 0.89) with a p-value of 0.012 and a quadratic polynomial contrast of 0.002 ($p < 0.05$), as shown in Tables 5 and 6.

Table 3. Effects of dietary inclusion of Citrus XL on blood biochemistry, liver malondialdehyde, and superoxide dismutase of laying hen aged 82-87 weeks under tropical conditions

Variables \ Treatments[1]	T1	T2	T3	T4	P value[2]
Triglyceride (mg/dL)	1502.6 ± 300.53	1389.6 ± 455.97	1355.4 ± 445.13	1490.0 ± 216.42	0.897
Cholesterol (mg/dL)	151.6 ± 28.99	124.8 ± 36.09	133.2 ± 65.17	137.2 ± 37.93	0.811
HDL (mg/dL)	27.4 ± 4.39^{b}	35.4 ± 5.85^{a}	37.2 ± 5.80^{a}	34.4 ± 4.03^{a}	0.038
LDL (mg/dL)	85.4 ± 16.50^{b}	58.0 ± 15.24^{a}	62.2 ± 16.21^{a}	70.6 ± 7.36^{ab}	0.038
Liver SOD (U/mL)	83.64 ± 20.72	105.45 ± 15.21	112.73 ± 19.91	101.82 ± 9.95	0.084
Liver MDA (nmol/mg)	0.264 ± 0.015^{b}	0.249 ± 0.018^{ab}	0.235 ± 0.013^{a}	0.255 ± 0.011^{b}	0.038

[1]T1:diet without Citrus XL supplementation (Control), T2: Control diet supplemented with Citrus XL 0.5 kg/ton, T3: Control diet supplemented with Citrus XL 1.0 kg/ton, T4: Control diet supplemented with Citrus XL 1.5 kg/ton. [2]Probability Value. [a-b]Means in the same row with superscripts differ significantly ($p < 0.05$).

Table 4. Effects of dietary inclusion of polynomial contrast of Citrus XL on blood biochemistry, liver malondialdehyde, and superoxide of laying hen aged 82-87 weeks under tropical conditions

Variables	Polynomial Contrast[1]				X-Optimum[3]	R-Square[4]
	Linear	Quadratic	Cubic	Model[2]		R^2
Triglyceride (mg/dL)	0.923	0.463	0.904	Ns	-	-
Cholesterol (mg/dL)	0.699	0.448	0.661	Ns	-	-
HDL (mg/dL)	0.040	0.031	0.877	Qd	0.96	0.997
LDL (mg/dL)	0.180	0.013	0.353	Qd	0.86	0.914
Liver SOD (U/mL)	0.088	0.047	0.916	Qd	0.94	-
Liver MDA (nmol/mg)	0.184	0.015	0.253	Qd	0.87	0.878

[1]If there were significant differences ($p < 0.05$), the most appropriate equation model is selected. [2]Ns: no structure ($p > 0.05$), Ln: Linear; Qd: Quadratic; Cu: Cubic. [3]Maximum value of dose (x) to variable (y). [4]Variable variation rate (0-1).

Table 5. Effects of dietary inclusion of Citrus XL on fat levels and liver health of laying hen aged 82-87 weeks under tropical conditions

Variables \ Treatments[1]	T1	T2	T3	T4	P value[2]
Liver Score	3.4 ± 0.89^{bc}	2.4 ± 1.14^{ab}	2.0 ± 0.70^{a}	3.8± 0.44^{c}	0.012
Red[3]	114.6 ± 8.04	114.0 ± 16.53	114.4 ± 6.42	120.0 ± 7.00	0.770
Green[3]	84.8 ± 10.03	86.0 ± 12.7	81.4 ± 9.28	88.4 ± 2.88	0.703
Blue[3]	62.8 ± 4.86	61.4 ± 12.48	66.6 ± 6.58	62.2 ± 7.12	0.763
Liver Fat (%)	21.25 ± 4.27	19.47 ± 3.90	18.93 ± 9.13	19.56 5.48	0.136
Yolk Fat (%)	63.39 ± 4.72^{c}	47.93 ± 9.78^{a}	51.93 ± 5.63^{ab}	59.79 ± 2.09^{ac}	0.004
Abdominal Fat (%)	2.98 ± 1.19	3.97 ± 0.98	4.26 ± 0.94	4.07 ± 0.41	0.172

[1]T1:diet without Citrus XL supplementation (Control), T2: Control diet supplemented with Citrus XL 0.5 kg/ton, T3: Control diet supplemented with Citrus XL 1.0 kg/ton, T4: Control diet supplemented with Citrus XL 1.5 kg/ton. [2]Probability Value. [3]Liver color was calculated using a Nutricell Eggspert digital colorimeter. [a-c] Means in the same row with different superscript letters significantly ($p < 0.05$).

Table 6. Effects of dietary inclusion of polynomial contrast of Citrus XL on fat levels and liver health of laying hen aged 82-87 weeks of age under tropical conditions

Variables	Polynomial Contrast[1]				X-Optimum[3]	R-Square[4]
	Linear	Quadratic	Cubic	Model[2]		R^2
Liver Score	0.639	0.002	0.353	Qd	0.72	0.939
Red[5]	0.434	0.513	0.842	Ns	-	-
Green[5]	0.747	0.502	0.371	Ns	-	-
Blue[5]	0.840	0.690	0.342	Ns	-	-
Liver Fat (%)	0.650	0.664	0.994	Ns	-	-
Yolk Fat (%)	0.592	0.001	0.227	Qd	0.78	0.919
Abdominal Fat (%)	0.074	0.178	0.903	Ns	-	-

[1]If there were significant differences ($p < 0.05$), the most appropriate equation model is selected. [2]Ns: no structure ($p > 0.05$), Ln: Linear; Qd: Quadratic; Cu: Cubic. [3]Maximum value of dose (x) to variable (y). [4]Variable variation rate (0-1). [5]Liver color was calculated using a Nutricell Eggspert digital colorimeter.

DISCUSSION

The results showed that using Citrus XL as a feed additive at a level of 1.0 kg/ton significantly increased HDL levels in the blood and significantly reduced liver MDA levels. The optimum level of Citrus XL for increasing HDL was estimated to be 0.96 kg/ton, with a quadratic polynomial contrast. The reduction of MDA levels was optimal at an estimated level of 0.87 kg/ton. Curcumin has been shown to positively influence metabolism and inhibit excess fat accumulation (Aderemi and Alabi, 2023). It contains beneficial therapeutic properties due to antioxidants and herbal ingredients that help improve blood circulation throughout the organism (Mughal, 2019). In the study by Deng et al. (2023), curcumin supplementation in feed was found to increase HDL levels in human metabolism. The antioxidant content in curcumin had a positive effect on the health of old chickens by maintaining the stability of excessive fat growth and facilitating lipid metabolism in the blood. This was achieved by modulating HDL activity and regulating the activity and levels of biomarkers such as apolipoprotein A1, cholesterol acyltransferase, paraoxonase -1, and myeloperoxidase (Xie et al., 2012; Saberi-Karimian et al., 2021). A meta-analysis study by Ilyas et al. (2023), highlighted the crucial role of mineral supplementation in the activity of GPx enzymes in the liver, which contributes to increasing blood HDL levels, and reducing LDL and cholesterol levels. Citrus XL supplementation in the diet reduced liver MDA levels compared to the control treatment, where it was known that the liver was a precursor in the formation of egg yolk (Cui et al., 2020). The liver also plays a role in detoxifying toxins in the body as well as metabolic waste substances, contributing to lipid metabolism in poultry (Zaefarian et al., 2019). Low MDA levels indicated a form of antioxidant status in livestock and its response in organs, reflecting the final product of lipid peroxidation as a biomarker of oxidative stress (Respati et al., 2023). The inclusion of betaine in Citrus XL likely protected the liver from oxidative stress due to decreased liver function in old chickens. Previous studies have shown that optimizing betaine enhanced antioxidant activity by increasing liver detoxification, thereby alleviating the development of liver fibrosis and preventing radiation-induced liver damage (Shedid et al., 2018).

Providing Citrus XL in the diet at a level of 0.5 kg/ton had a significant effect on reducing LDL levels in the blood, with the optimum levels estimated at 0.86 kg/ton. Older layers typically have elevated LDL levels, which contribute to higher amounts of bad cholesterol circulating in their bodies. The increased amount of LDL in the blood causes endothelial damage and increased lines of fat formation, leading to coronary artery disease, which attacks medium and large arteries characterized by endothelial cells and lipids in the middle, making it essential to maintain normal LDL levels to preserved cellular function and structure (Rafieian-Kopaei et al., 2014; Bandyopadhyay et al., 2018). The meta-analysis by Qin et al. (2017) indicated that curcumin reduces LDL levels, thereby supporting lipid metabolism and promoting overall health.

Other results regarding the use of Citrus XL at various doses did not show a significant effect on triglyceride levels. However, it was noted that a dose of 1.0 kg/ton resulted in better triglyceride levels than other treatments. This improvement can be attributed to the combination of organic chromium, betaine, and curcumin, which exhibit good antioxidant properties and help maintain cellular integrity in the blood. Additionally, older chickens face complexities in lipid metabolism; as they age, triglyceride levels tend to increase which will, in turn, bring about a decrease in egg production (Liu et al., 2018). Chromium (Cr) is vital for proper insulin action and is necessary for normal protein, fat, and carbohydrate metabolism when included in feed. Its mineral use can help manage heat stress in chickens, maintain a healthy metabolic rate (Chowdhury et al., 2003; Haq et al., 2016), and decrease triglyceride levels in the blood chemistry profile (Wardani et al., 2020a). Han et al. (2020) showed that the use of Cr was beneficial to the health of chickens in limited quantities and significantly reduced triglyceride levels in the blood with various other additions compared to the control group. Citrus XL at 0.5-1.5 kg/ton doses had no significant effect on liver SOD in laying hens aged 82-87 weeks under tropical conditions; however, the highest superoxide dismutase (SOD) levels were observed with the 1.0 kg/ton treatment, which outperformed the control. This indicated that SOD was an antioxidant enzyme which was vital for the body and was produced for the defense against oxidative stress (Thorpe et al., 2013). It was central in scavenging superoxide anions from cell oxygen molecules (Hwang et al., 2020). Higher oxidative stress levels were associated with older chickens; this was demonstrated by the fact that higher stress levels were correlated with lower SOD levels, indicating that this product provided effective protection against lipid peroxidase (Surai, 2016). Oxidative stress in cells was caused by intracellular disruptions in the equilibrium

between the generation of reactive oxygen species (ROS) and antioxidant defenses (Kakkar et al., 2017), with free radicals damaging DNA, protein, and lipid structures (Schieber and Chandel, 2014) as well as SOD (Wardani et al., 2020b; Kemal et al., 2023) and MDA (Rehman et al., 2018). The use of Citrus XL may beneficially affect liver SOD levels in chickens, aiding in the management of heat stress conditions and accelerating the lipid metabolism cycle in the liver. The betaine content improved mitochondrial function and prevented fatty liver disease to maintain cells (Zhang et al., 2019) while Cr enhanced chicken by boosting antioxidant capacity, reducing stress, and enhancing immunity (Xin et al., 2022). There was no significant effect on total cholesterol levels in the blood, with the lowest cholesterol results observed at the 0.5 kg dose, which was better than the other treatments compared to the control. The potential of Citrus XL was partly due to Cr. According to the study by Sarai et al. (2022), the use of Cr can reduce total cholesterol levels in the blood during heat stress in chickens. Chromium can boost egg production while maintaining egg quality, making it beneficial for older laying hens (Chen et al., 2021). Controlling cholesterol in the blood is very beneficial for maintaining health and has the potential to extend the life of egg production in aged laying hens by incorporating components with high nutritional value. The total amount of cholesterol in the blood also provided information that healthy chickens can produce eggs, which is healthy for humans (El-Sabrout et al., 2022; Myers and Ruxton, 2023). Controlling lipid metabolism in blood-circulated tissues by reducing cholesterol can stabilize lipid accumulation and improve the health status of poultry (Tan et al., 2022). Elevated total cholesterol levels were a concern as they were associated with increased age and the risk of cardiovascular disease (Van Vliet et al., 2009). Using Citrus XL at a dose of 1.0 kg/ton showed better cholesterol levels compared to other treatments.

The findings indicated that the use of Citrus XL significantly improved liver condition to prevent fatty liver hemorrhagic syndrome with a dose of 1.0 kg/ton and resulted in a score of 2.0 compared to the control having a score of 3.4. This improvement was accompanied by a reduction in liver fat levels across various treatments, with the lowest fat levels observed at the 1.0 kg/ton dose. In the control treatment, the liver condition was fragile and easily damaged, exhibiting multiple hemorrhagic spots indicative of fatty liver disease. Liver damage in turkeys and broilers can be assessed by measuring the concentration of metabolites as biomarkers in the blood and observing any decrease in protein levels (Beyoğlu and Idle, 2020; Tardieu et al., 2021). According to Heeren and Scheja (2021), a fragile liver is caused by liver lipid metabolism disorders or lipotoxicity problems, which trigger stress and reduce liver function in old chickens (You et al., 2023). A healthy avian liver, characterized by a fresh brownish-red color, suggests optimal liver function in detoxifying toxins and synthesizing fats (Zaefarian et al., 2019). In the present observation, liver color in the treatments was evaluated using a digital colorimeter, assessing scores on Lightness (L), red/green coordinate points (a), and yellow/blue coordinate points (b), which were converted to Red, Green, and Blue. A deep and fresh liver color indicates a healthy liver and can potentially prolong egg production in old age. The use of curcumin to improve liver function in laying hens, complemented by betaine and organic chromium, was a beneficial combination for re-energizing liver function and extending the productive period in laying hens. Furthermore, curcumin aids chicken metabolism in preventing heat stress conditions, particularly during extreme temperatures (Hu et al., 2019; Liu et al., 2020; Wasti et al., 2020). Curcumin has also been optimized as a hepatoprotective agent to protect against injury and the effects of aging (Afrin et al., 2017; Li et al., 2021). Chickens exposed to heat stress can experience cell damage and disrupt egg production, necessitating proper management to prevent declines in egg yield, which could lead to financial losses (Li et al., 2020; Ahmad et al., 2022). A yellowish liver color indicated a health problem in the body. Anatomically, hepatocytes are microscopic cells, if they become filled with pigment or accumulate fat deposits, their color will change.

The results of this study did not show a significant effect on abdominal fat; however, there was a notable impact on reducing yolk fat. There was a lower fat content in the egg yolk in each treatment but the lowest fat content was observed at 0.5 kg/ton. The optimum levels of Citrus XL in reducing the fat content of the yolk was estimated at 0.78 kg/ton. High fat levels in the liver can inhibit the egg formation process, and if left uncontrolled, may lead to fatty liver disease, consequently affecting the fat concentration in egg yolk. Given that egg yolks contain high levels of fat, it was essential to limit their content to maintain overall health by managing fat intake. The age of the chickens also affected laying performance, egg yolk fat content, and their health status (Keum et al., 2018). The decrease in egg yolk fat content observed with various treatments of Citrus XL shows its potential to mitigate heat stress at the metabolic level. It improved internal egg quality due to optimal fat mobilization in adipose tissue

(Chen et al., 2023), which was an important consideration for consumers seeking low-fat eggs. Based on research from Yusuf et al. (2023), egg quality can be improved for consumer's health by including vitamin D3 supplements in their diet. However, the results of this study indicated that the abdominal fat in aged laying hens during 82 to 87 weeks of age, when using various doses, could not reduce the abdominal fat content of laying hens in hot environmental conditions. An indicator of heat stress exposure was improper food absorption, leading to fat deposits in the chicken's stomach (Brugaletta et al., 2022). The accumulation of fat in poultry, especially in broilers and laying hens, often results from their early life exposure to environmental stressors, including both heat and cold stress. In addition, older chickens tend to accumulate more abdominal fat, which restricts nutrient absorption, necessitating the implementation of effective feeding strategies (Fouad and El-Senousey, 2014).

CONCLUSION

This study indicated that supplementation at a level of 1.0 kg/ton of Citrus XL provided the best results in treating heat stress and preventing FLHS in chickens aged 82-87 weeks. It significantly improved lipid metabolism and liver health scores, increased blood HDL levels, and reduced egg yolk fat and MDA in ISA Brown laying hens. Future research should be carried out to evaluate Citrus XL during the first laying period until peak egg production.

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Author's contributions

Hafidz Muhammad Muhshi contributed to the conception design of the research, data analysis, writing, review, and drafting of the manuscript. Rita Mutia, Sumiati Sumiati, and Wira Wisnu Wardani were involved in each step of the research and manuscript revision. Ilham Akbar and Nofitra Dewi Suparno Putri were involved in data collection, critical review, and research analysis. All authors confirmed the last edition of the manuscript for submission and publication.

Competing interests

The authors declare no competing interest in the publication of this study.

Ethical considerations

All the authors had checked and confirmed the article through ethical issues such as plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy before the submission.

Availability of data and materials

The availability of data and supplemental materials contained the original contributions that were presented in the study. To inquire, kindly get in touch with the corresponding author.

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Effects of *in ovo* Injection of Soursop (*Annona muricata*) Leaf Extract on Blood Profile, Immune Organs, and Intestinal Morphology of Noiler Chicks

Timothy T. Kuka[1]*, Oumbortime N'nanle[1], Simplice Karou[2], Kokou Tona[1], and Batomayena Bakoma[3]

[1]*Regional Center of Excellence in Poultry Science, University of Lome, BSP 1515, Lome, Togo*
[2]*Laboratory of Microbiology and Quality Control of Foodstuffs (LAMICODA), Higher School of Biological and Food Techniques (ESTBA), University of Lome Togo*
[3]*Pharmaceutical Sciences Research Laboratory, University of Lome, BSP 1515, Lome, Togo*

*Corresponding author's E-mail: kukatar6@gmail.com

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ABSTRACT

Plant extracts in poultry production are widely recognized for their significant benefits in improving productive performance. Specifically, the *in ovo* administration of soursop leaf extract (SLE) shows promise in improving the health and productivity of Noiler chickens. This study aimed to provide valuable insights into the health of the chicks and evaluate the effectiveness and safety of *in ovo* SLE in poultry production by examining the blood profile, internal organs, and intestinal morphology of Noiler chickens. For this experiment, 640 hatching eggs were incubated and randomly divided into four experimental groups, including 0.25 µg SLE, 0.5 µg SLE, 0.75 µg SLE, and a non-injected control group. Three treatment groups received a direct injection of 0.2 ml of the respective SLE concentrations into the air cells of the eggs on the 18th day of incubation. After hatching, chickens from each group were divided into five replicates of 15 chicks each and raised using a completely randomized design. At ten days of age, blood samples were collected from two chicks per replicate for hematology and serum analysis. Two chicks per replicate were sacrificed on day 10 to assess the internal organs and intestinal morphology. The results showed no significant changes in hematological parameters, serum biochemistry, and internal organs in all groups. The soursop leaf extract groups had markedly longer villi, deeper crypts, and thicker muscular walls compared to the control group. In conclusion, the *in ovo* injection of soursop leaf extract at 0.75 µg improved intestinal health by enhancing the intestinal surface structures in Noiler chickens.

Keywords: Hematology, Internal organ, *In ovo*, Intestinal morphology, Noiler chicken, Soursop Leaf Extract

INTRODUCTION

The success of a poultry enterprise is often quickly judged by the growth performance forgetting the health status of the flock which plays such an important role that with time even the growth will be affected if no attention is given. Despite genetic improvement leading to several fast-growing breeds of chicken (Mancinelli et al., 2023), none of them can reach their full genetic potential without proper health management. For this reason, antibiotic growth promoters became an important component of poultry production for an average farmer (Mehdi et al., 2018). However, the restriction on antibiotic growth promoters in poultry production has led to exploring various alternatives, with a preference for natural substances with little to no residual risks. (Bean-Hodgins and Kiarie, 2021).

Among the alternatives to antibiotics growth promoters, phytogenics or botanicals have experienced a

wider exploration due to their tract record in human health management and broad-spectrum scope (Kikusato, 2021; Ndomou and Mube, 2023). Different application methods of phytobiotics have been reported in poultry research with varying results on performance parameters. *In ovo* administration of bioactive substances influences the developing embryos by improving the development and function of the intestine, immune system, and hatching parameters (Ayalew et al., 2023; Kpodo and Proszkowiec-Weglarz, 2023). Substances like carbohydrates, amino acids, vitamins, minerals, prebiotics, probiotics, synbiotics, and phytobiotics have been experimented with and reported to have influenced embryo and chick physiology (Babazadeh and Asasi, 2021; Kpodo and Proszkowiec-Weglarz, 2023). *In ovo* injection of some plant extracts (Moringa and soursop) have also been reported to influence the hatching process by reducing the incubation period which is of economic advantage to the hatchery managers (Bilalissi et al., 2019; Kuka et al., 2024)

Although numerous benefits of phytobiotics have been reported in poultry production, different plants have different chemical compositions which may negatively impact the health of animals. Additionally, the dosage, mode of administration, and duration of usage can lead to toxic effects, leading to inconsistent results (Oladokun et al., 2023). Therefore, the use of every plant material must be carefully evaluated in all possible ways to ascertain the safety of the material used.

Soursop leaf extract has been reported to contain bio-active components such as flavonoids, alkaloids, tannins, saponins, glycosides, ascorbic acid, beta-carotene, and minerals that exhibit antibacterial, antioxidant, anti-inflammatory, and immune-boosting activities leading to improved health and performance of poultry (Oluwayinka et al., 2017; Kuka et al., 2022). *In ovo* injection of soursop leaf extract enhanced chick weight, overall chick quality, survivability, and growth performance of chicks (Kuka et al., 2023).

However, there is currently a lack of research on the impact of *in ovo* soursop leaf extract on the health of Noiler chicks. It is crucial, therefore, to evaluate the potentially toxic effects of bioactive substances on the blood, intestine, and internal organs of Noiler chickens because any harmful effects of a substance consumed by chickens may not be immediately apparent in their growth but can be detected earlier in their tissues.

Hence, the present study aimed to evaluate the effect of *in ovo* administration of soursop leaf extract on the blood profile, intestinal morphology, and internal organs of Noiler chicks.

MATERIALS AND METHODS

Ethical approval

The experiment was conducted at the experimental unit of the Regional Center of Excellence in Poultry Science (CERSA) following the CCAC (2009) guidelines and the ethical approval (008/2021/BC-BPA/FDS-UL) from the University of Lomé. All experimental animals were handled with care to minimize any potential suffering

Soursop leaf extract preparation

Fresh soursop leaves were harvested within Lome, Togo, air-dried at 20^{o}C then milled to powder. The extraction followed the procedure of Kuka et al. (2023). The soursop leaf powder was soaked in ethanol (80%) in a ratio of 1:1, and the mixture was set on a shaking machine for 72 hours of regular shaking. The content was filtered twice, first through cotton wool fitted in a filtration funnel and finally through a coffee filter bag (# 6) in a filtration funnel. The filtrate was concentrated at 40°C in a rotary evaporator equipped with a chiller and vacuum pump to produce the leaf extract that was then refrigerated for later use. In preparation for injection, a stock solution (1000 µg) was created by reconstituting ten (10) milligrams of the extract with ten milliliters of saline solution (0.9% NaCl). Another 10µg working solution was created by reconstituting one milliliter of the stock solution with 100 milliliters of saline solution. To obtain the 0.25, 0.5, and 0.75 µg concentrations needed for the injection, 0.25 ml, 0.5 ml, and 0.75 ml of the working solution were finally reconstituted with 10 ml of saline solution (Kuka et al., 2023).

Experimental design and *in ovo* injection

A completely randomized design was used for the experiment, 756 Noiler chicken eggs were purchased from a breeder farm in Lome, Togo. These eggs were weighed and assigned unique codes for easy identification and monitoring during the experiment. Subsequently, the eggs were incubated at 37.7^{o}C and a relative humidity of 60% using a Royal Pas Reform (SmartPro TM) combi incubator from the Netherlands. Throughout the incubation period, which lasted until day 18, the eggs were regularly turned at a 45^{o} angle, once every hour. On day 18, the eggs were examined under intense light (candling) to identify and remove any infertile ones. The remaining

eggs, which displayed evidence of developing embryos, were then randomly divided into four groups, each containing 160 eggs.

The broad ends of the eggs were perforated using 21 g needles, and the extract was injected into the air cell through the holes using an automatic syringe with 22 gauge (13mm), after that, the holes were sealed with adhesive tape. The control group comprised normal (non-injected) eggs; groups one, two, and three were injected with 0.25 µg/ml, 0.5 µg/ml, and 0.75 µg/ml of soursop leaf extract, respectively at day 18 of incubation. All the eggs were then transferred into the hatching baskets in three replicates and allowed to hatch at 37.7^{o}C and 70% relative humidity.

Since there was no discernible difference between the saline-injected and non-injected eggs in earlier works (Karamik and Kop-Bozbay, 2020; Atan and Kop-Bozbay, 2021), no group of eggs received saline injection in this experiment. A volume of 0.2 milliliters was administered to each egg within the air cell. Hatched chicks were raised for ten days in five replicates of fifteen (15) each, based on the groupings. The chicks had 24 hours of light and *ad libitum* feeding. The chick mash contained 20% crude protein and 2800 Kcal/kg ME (Table 1) as recommended by Amo Sieberer Hatchery LTD. According to the manufacturer's instructions, the chicks received multivitamins (Introvit A + Oral, Netherlands) in their water and were vaccinated following the schedule for Noiler chickens.

Table 1. The starter diet of Noiler chicken administered *in ovo* soursop leaf extract

Ingredients	Amount (kg)
Maize	53.00
Soya bean meal (46%)	33.00
Wheat bran	7.00
Concentrate (5% JLC)*	5.00
Oyster shells	2.00
Total	100.00
Nutrients composition	
Crude protein (%)	20.00
Metabolizable Energy (kcal)	2800.00
Crude fibre (%)	4.01
Calcium (%)	1.04
Phosphorus (%)	0.45
Methionine (%)	0.50
Lysine (%)	1.00

*Jubaili layer concentrate: Crude protein, 30%; Crude fats, 2%, Calcium, 4%; Phosphorus, 4.1%; Lysine, 2.0%; Methionine, 3.0%; Methionine + cystine, 3.5%; Sodium, 2.0%, Met. Energy, 2280 Kcal/kg; Vitamins; Minerals, enzymes. A diet formulated according to Amo Farm Sieberer Hatchery Ltd.

Sample collection

Two chicks per replicate were sampled for blood on day 10 in the morning, and one ml of blood was collected from the jugular vein with the aid of a needle and syringe into anticoagulant (Ethylenediaminetetracetic acid) bottles for haematological analysis. Another 2 ml of blood was collected into plane tubes and allowed to clot. Blood samples were transported to the Regional Center of Excellence in Poultry Science (CERSA) laboratory at the University of Lome, Togo, using ice box to maintain cold conditions. An automatic hematology analyzer (DH36, Dymind Biotechnology Co. Ltd Shenzhen, China) was used to analyze the following parameters: hematocrit (HCT %), red blood cell (RBC 10^{12}/L) count, hemoglobin (Hb g/L) level, white blood cell (WBC 10^{9}/L) count, lymphocytes, granulocytes, mean corpuscular volume (MCV fL), mean corpuscular hemoglobin (MCH pg), mean corpuscular hemoglobin concentration (MCHC g/L), and platelet count (PLT 10^{9}/L). The analyzer uses impedance for WBC, RBC, and PLT analysis, and cyanide-free colorimetry for the Hb test (Dymind Technologies). Coagulated samples were centrifuged at 3000 rpm for 15 minutes to extract serum for further analysis. Levels of total protein, albumin, globulin (g/dL), alanine aminotransferase (ALT U/L), aspartate aminotransferase (AST U/L), and triglycerides (g/dL) in the serum were determined using specific reagents (Cypress diagnostic, Belgium) and the Gen5™ Microplate Reader and Imager Software (BioTek Instruments USA), following the manufacturer's instructions.

The heart, liver, spleen, thymus, and bursa of Fabricius from two chicks per replicate were weighed with a sensitive balance, and the weights were expressed to the percentage of the body weight using the formula below to obtain the organ's relative weight.

$$Relative\ weight = \frac{Organ\ weight\ (g)}{Chick\ weight\ (g)} \times 100$$

(Formula 1)

Two chicks from each replicate were sacrificed by cervical dislocation and dissected on day 10. One-inch mid-section of the ileum was cut, flushed with distilled water, and then fixed in a 10% formalin solution. The samples were processed using standard histological procedures, including dehydration, clearing, and embedding in paraffin wax. Six (6 mm) thick sections were sliced from each sample, placed on glass slides, and stained with Hematoxylin-Eosin (H & E) stain. Microscopic observation and examination were conducted using a light microscope (Olympus, Japan). Villus height,

crypt depth, thickness of muscularis, and mucosae were determined**.** Photomicrography of the H & E slides was done at X40 magnification with a Digital Microscope camera, Amscope® 319CU CMOS Camera. The morphometric analyses were performed using TS View CX Image® Software, version 6.2.4.3, and Motic Images Plus 2.0 ML (China).

Statistical analysis

Data collected was computed using one-way analysis of variance (ANOVA) in SPSS Software (Version 23, 2018). Differences between treatment means were compared using Duncan's multiple range test and significant differences were considered at $p \leq 0.05$.

RESULTS AND DISCUSSION

Table 2, presents the effect of *in ovo* injection of soursop leaf extract on the hematology of Noiler chicks. No significant differences ($p > 0.05$) were observed among the parameters analyzed in all groups. The finding indicated that the chickens did not suffer any impairment due to the treatment effect in this study. Furthermore, it is possible that the doses of soursop leaf extract used in the present study were not high enough to induce a significant change in the parameters. In contrast to these findings, Asa et al. (2022) found a significant increase in red blood cell (RBC) and hemoglobin (Hb) levels in broiler chickens that received a combination of carbohydrates and antioxidants through *in ovo* administration. The discrepancy between the present study and other reports may be attributed to differences in the timing of sample collection, as the chickens in the present experiment were sampled at ten days old. Likewise, Oke et al. (2022) observed a significant difference in RBC levels of broiler chicks at hatch but could not find any variation in blood parameters at the finisher stage when black cumin was administered through *in ovo* injection. The higher percentage of granulocytes in the injected groups could imply the mobilization of defense cells in response to infection or abnormal conditions (Ünal et al., 2022).

Serum biochemical parameters revealed no significant differences, except for ALT (Table 3). *In ovo* feeding of soursop leaf extract at 0.25 µg, had a significantly higher level of ALT ($p < 0.037$) compared to the other treatment groups. This result indicated the possibility of a negative effect which that concentration could not amend. However, it was worth noting that the values reported in this study fell within the range of values reported for Noiler chickens (Idowu et al., 2021). No significant differences ($p > 0.05$) were observed in the immune and vital organs of the chicks among the treatment groups (Table 4). This result indicated that the extract did not cause any possible inflammation in the assessed organs. Visual observation also confirmed that the organs were not inflamed. This report corresponds to those of other researchers who used plant extracts, indicating the potential safety of *in ovo* phytobiotics. Saki and Salary (2015) who administered *in ovo* thyme and savory extracts in broiler chicken reported similarities in organ weights. Likewise, Bakyaraj et al. (2011) observed that *in ovo* administration of amino acids, trace minerals, fatty acids and vitamins (Vit. A 200 IU, vit. D3 20 IU, vit. E1 IU, thiamine 72 µg, riboflavin 144 µg, pyridoxine 140 µg, pantothenic acid 400 µg, niacin 140 µg, vit. C 8 mg, linoleic acid 20 mg) did not influence the spleen and bursa weights but influenced the thymus weight of the broiler chicks.

On the contrary, El-Kholy et al. (2021) reported that *in ovo* administration of cinnamon, thyme, and clove extract had a significant difference in bursa weight without a difference in thymus and spleen weights. The size of the thymus, spleen, and bursa of Fabricius are used alongside other indicators to determine the health status of chickens (Lutful Kabir, 2009; Sikandar et al., 2017).

The effect of *in ovo* injection of soursop leaf extract on the intestinal morphology of Noiler chicks was presented in Table 5 and Figure 1. Villus height, crypt depth, muscular, and mucosae thickness were significantly different ($p < 0.05$) among the treatment groups. *In ovo* injection of soursop leaf extract presented higher values of villus height, crypt depth, and muscular thickness than the un-injected group. Ilea villi height was longer in the 0.75 µg group while the crypt was deeper in the 0.25 µg group ($p < 0.05$). The improvement in the intestinal surface structures in the present study could be inferred from the influence of the antibiotic and anti-inflammatory properties of soursop leaf extract. Researchers have established that *in ovo* injection of nutrients improves intestinal development leading to longer intestinal villi (Kadam et al., 2013; Siwek et al., 2018). Furthermore, *in ovo* administration of bioactive compounds such as probiotics and plant extracts can create the right environment for colonizing the embryonic gut by beneficial microbe that promotes intestinal health (Yadav et al., 2016; Shehata et al., 2022).

The outcome of this study agrees with Cheled-Shoval et al. (2011) who reported greater villus height and intestinal muscle thickness in chickens caused by *in ovo* injection of mannan oligosaccharide. Similar results were

obtained by other researchers who injected *in ovo Baccilus subtilis* and raffinose in broiler chickens (Oladokun et al., 2021; Shehata et al., 2022). Furthermore, oral administration of soursop leaf extract in broiler chicken was reported to have improved villus height, crypt depth, and feed conversion ratio (Kuka et al., 2022).

Intestinal structures and their well-being were of utmost significance because they provide the surfaces for nutrient absorption. Changes in these structures affect the optimum functioning of the intestine thereby affecting the growth and general performance of the animal. Increased absorptive surface of the intestine and nutrient absorption were associated with longer villi of the intestine, the outcome was an improved feed conversion ratio and growth performance (Prakatur et al., 2019**)**. The improved intestinal morphology in the present study relates to the improved feed conversion ratio and growth performance of Noiler chicks reported by Kuka et al. (2023). The present study and other studies have shown that soursop leaf extract has the potential to promote intestinal health and development in chickens (Kuka et al., 2022; Artawiguna et al., 2023).

Table 2. Effect of *in ovo* injection of soursop leaf extract on hematology of Noiler chickens at 10 days of age

Parameters	Treatment (Soursop leaf extract)				p-value
	Control	0.25 (µg/ml)	0.50 (µg/ml)	0.75 (µg/ml)	
HCT (%)	25.30 ± 0.09	29.40 ± 0.04	28.40 ± 0.09	31.20 ± 0.12	0.656
RBC (10^{12}/L)	1.91 ± 0.65	2.14 ± 0.30	2.12 ± 0.58	2.33 ± 0.85	0.666
Hgb (g/L)	107.43 ± 37.45	119.00 ± 15.98	116.86 ± 36.61	127.00 ± 47.47	0.793
MCV (fL)	131.39 ± 7.01	137.10 ± 5.43	133.20 ± 5.76	133.24 ± 4.52	0.322
MCH (pg)	55.97 ± 2.59	55.53 ± 2.38	54.81 ± 2.20	54.33 ± 1.29	0.510
MCHC (g/L)	416.29 ± 15.70	405.00 ± 11.46	411.71 ± 5.68	407.71 ± 7.95	0.062
PLT (10^9/L)	1.71 ± 1.38	1.29 ± 0.48	1.71 ± 2.05	1.00 ± 1.29	0.736
WBC (10^9/L)	51.82 ± 21.29	61.03 ± 5.36	61.67 ± 8.45	59.39 ± 19.34	0.608
Lymph (%)	0.88 ± 0.04	0.89 ± 0.02	0.89 ± 0.03	0.91 ± 0.02	0.182
Gran (%)	0.04 ± 0.02^a	0.02 ± 0.01^b	0.03 ± 0.01^a	0.02 ± 0.01^b	0.050

HCT: Hematocrit, RBC: Red blood cell, Hgb: Hemoglobin, MCV: Mean corpuscular volume, Lymp: Lymphocytes, MCH: Mean corpuscular hemoglobin, MCHC: Mean corpuscular hemoglobin concentration, PLT: Platelets, Gran: Granulocytes. ^{a,b}: means within a row with different superscripts are significantly different

Table 3. Effect of *in ovo* injection of soursop leaf extract on serum biochemistry of Noiler chickens at 10 days of age

Parameters	Treatment (Soursop leaf extract)				p-value
	Control	0.25 (µg/ml)	0.50 (µg/ml)	0.75 (µg/ml)	
Total Protein (g/dl)	4.71 ± 0.49	4.25 ± 0.47	4.12 ± 0.49	4.14 ± 0.44	0.061
Albumin (g/dl)	2.98 ± 0.21	2.91 ± 0.15	2.66 ± 0.42	2.63 ± 0.49	0.140
Globulin (g/dl)	1.83 ± 0.43	1.34 ± 0.53	1.46 ± 0.65	1.50 ± 0.75	0.419
ALT (U/L)	20.18 ± 5.11^a	15.23 ± 3.66^b	15.10 ± 2.87^b	14.59 ± 4.51^b	0.037
AST (U/L)	32.64 ± 16.77	21.83 ± 9.89	23.65 ± 10.07	25.64 ± 13.11	0.367
Triglycerides (g/dl)	197.68 ± 46.07	172.69 ± 27.85	165.42 ± 30.23	167.82 ± 25.88	0.218

ALT: Alanine aminotransferase, AST: Aspartate aminotransferase. ^{a,b} Means with different superscripts within a row are significantly different.

Table 4. Effect of *in ovo* injection of soursop leaf extract on relative organs' weight of Noiler chickens at 10 days of age

Organs	Treatment (Soursop leaf extract)				p-value
	Control	0.25 (µg/ml)	0.50 (µg/ml)	0.75 (µg/ml)	
Liver (%)	3.68 ± 1.10	3.52 ± 0.64	3.24 ± 0.32	3.57 ± 0.38	0.609
Heart (%)	0.74 ± 0.17	0.75 ± 0.12	0.75 ± 0.08	0.84 ± 0.09	0.282
Spleen (%)	0.14 ± 0.05	0.19 ± 0.27	0.09 ± 0.02	0.10 ± 0.05	0.450
Bursa (%)	0.29 ± 0.08	0.26 ± 0.05	0.27 ± 0.19	0.24 ± 0.08	0.801
Thymus (%)	0.16 ± 0.05	0.15 ± 0.04	0.22 ±0.07	0.24 ± 0.12	0.118

N = 10

Table 5. Effect of *in ovo* injection of soursop leaf extract on intestinal morphology of Noiler chickens at 10 days of age

Parameters	Treatment (Soursop leaf extract)				p-value
	Control	0.25 (µg)	0.50 (µg)	0.75 (µg)	
Villus height (µm)	307.42 ± 91.26[b]	343.50 ± 77.51[ab]	315.66 ± 82.99[b]	394.52 ± 94.45[a]	0.009
Crypt depth (µm)	133.92 ± 34.06[b]	167.48 ± 41.43[a]	140.87 ± 31.51[b]	159.71 ± 50.29[ab]	0.032
Villus crypt ratio	2.44 ± 0.99[ab]	2.14 ± 0.55[b]	2.33 ± 0.17[ab]	2.76 ± 1.16[a]	0.014
Muscular thickness (µm)	374.61 ± 56.47[c]	496.82 ± 99.33[ab]	453.11 ± 89.75[b]	540.99 ± 65.71[a]	< 0.001
Mucosae thickness (µm)	140.38 ± 50.15[a]	112.26 ± 33.42[ab]	103.99 ± 23.67[b]	136.11 ± 48.08[ab]	0.012

[a,b] Means with different superscripts within a row are significantly different. n = 40

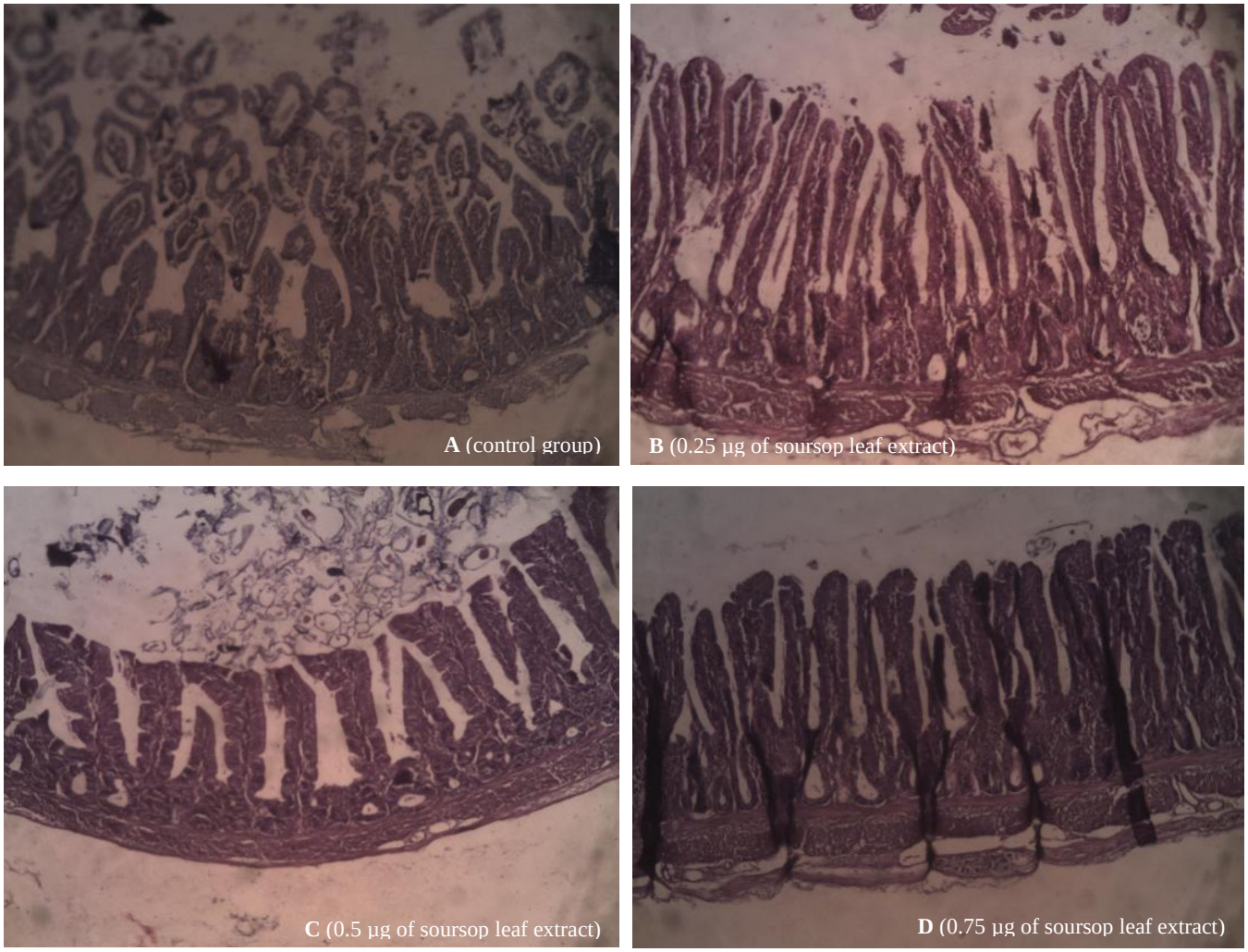

Figure 1. Effects of *in ovo* injection of sour sop leaf extract on intestinal structures of Noiler chickens at 10 days of of age. Viewed at X40 magnification. 2024.

CONCLUSION

In ovo administration of soursop leaf extract in Noiler chickens positively influenced intestinal villi, crypt depth, and muscular wall thickness. These effects can improve nutrient absorption leading to enhanced growth of the chickens. Furthermore, *in ovo* injection of soursop leaf extract did not cause a significant change in the blood profile and internal organs of the chickens which showed their normal state of health. We recommend that higher concentrations of soursop leaf extract should be experimented with to ascertain the benefits and safety of chicken production. Also, an expanded immune response

of chicken administered *in ovo* soursop leaf extract should be studied.

DECLARATIONS

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Authors' contributions

Kokou Tona, Simplice Karou, and Batomayena Bakoma validated the protocol, provided access to facilities, and supervised the experiment, Timothy Kuka conducted the experiment and wrote the manuscript; Oumbortime N'nanle assisted with validation and laboratory analysis. All authors contributed to the experiment and the write-up of the manuscript and approved the last edition of the article before submission to the journal.

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Competing interests

The authors declare that there is no personal or professional conflict of interest with the present study.

Ethical considerations

The authors have avoided plagiarism, misconduct, data falsification, and double submission/publication and have given consent to publish this article.

Availability of data and materials

The original contributions presented in the study are included in the article/supplementary material. For inquiries, please contact the corresponding author.

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Effects of Cactus Flour (*Opuntia ficus-indica*) on Productive Performance and Eggshell Quality of Laying Hens

Aureliano Juárez[1], Ernestina Gutiérrez[1], Carlos Villalba[1], and Gerardo Ordaz[2]*

[1]*Instituto de Investigaciones Agropecuarias y Forestales, Universidad Michoacana de San Nicolás de Hidalgo, México*
[2]*Centro Nacional de Investigación Disciplinaria en Fisiología y Mejoramiento Animal, INIFAP, México*

*Corresponding author's E-mail: ordaz.gerardo@inifap.gob.mx

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ABSTRACT

The poultry industry plays a crucial role in the production of animal proteins for human consumption and generating sources of employment. Thus, it is essential to explore effective strategies to enhance both the productivity of laying hens and the quality of their products, particularly eggshell quality, due to its significant economic implications for the poultry sector. This study aimed to evaluate the effects of cactus flour (CF; *Opuntia ficus-indica*) on the productive performance and eggshell quality of laying hens. Twenty-four Rhode Island Red laying hens were randomly divided into two groups (n = 12 experimental units -hens-/group) consisting of a control group and another fed CF (1% of the diet volume). The variables assessed included initial and final weight, weight gain, feed and calcium intake, egg production, egg mass, feed conversion index/kg of egg, economic efficiency index, egg weight, shell weight, shell thickness, shell percentage, and calcium levels in eggshells and excreta (daytime and nighttime). The addition of CF in the diet affected the final weight of hens, with the CF-fed hens (2.1 kg) being heavier than the control (1.9 kg). Egg production was higher in the CF-fed hens than in the control hens. Additionally, the mean egg weight was higher (68.5 g) in the CF-fed hens than in the control (62.2 g). The feed conversion index was lower in the CF-fed hens (2.1 kg/kg) than in the control (1.8 kg/kg). The economic efficiency index was higher in the CF-fed hens (94.8 %) than in the control (90.2 %). Eggshell weight (5.8 g), thickness (0.31 mm), and calcium levels (35.6 %) were significantly higher in the CF-fed hens than in the control (5.1 g, 0.27 mm, and 33.4 % for eggshell weight, thickness, and calcium levels, respectively). In conclusion, the inclusion of CF in the diet of laying hens improved the productive indicators and eggshell quality, thereby enhancing economic efficiency.

Keywords: Egg production, Cactus flour, Calcium, Laying hens, Poultry farming

INTRODUCTION

Genetic improvements have increased the productivity of laying hens and caused physiological modifications, which can lead to productive inefficiency in these animals (Li et al., 2017). Therefore, further research is important to establish efficient strategies to improve productivity indicators in hens (Geraldo et al., 2006). Specifically, eggshell quality is the most important indicator associated with the improved productive performance of laying hens. It has important economic repercussions on egg production, accounting for 6-10% of the total decrease in egg production (Lichovnikova, 2007; Świątkiewicz et al., 2015; An et al., 2016).

Since calcium carbonate makes up 95% of the eggshell's chemical composition, calcium supply is the most important nutritional factor for eggshell quality and productive longevity as hens (Li et al., 2017). With increased longevity, hens produce eggs with weak shells and compromised skeletal structure due to the decreased efficiency of the intestinal absorption of calcium (Diana et al., 2021), leading to greater dependence on bone-derived calcium, thus increasing the risk of fractures (de Juan et al., 2023). The National Research Council (NRC, 1994)

prescribed the mineral requirements for poultry almost 30 years ago. Since then, the genetic makeup, housing, management, and diet of laying hens have changed (Li et al., 2017).

Many studies have reported discrepancies in the calcium requirements for laying hens established by the NRC (1994). Previous studies have demonstrated that the NRC-specified calcium levels are adequate for optimal shell formation and that an additional increase in the dietary calcium level (> 3.6%) does not affect the eggshell quality (Valkonen et al., 2010; Pastore et al., 2012). However, variations in eggshell quality have also been reported using the calcium levels outlined by NRC (1994). This may be due to the variable absorption of calcium. Therefore, source and bioavailability of minerals, serum levels of calcium, phosphorus, vitamin D3, parathyroid hormone, gastrointestinal pH, and mineral content in the diet should be evaluated as indicators of dietary calcium usage (Koreleski and Swiakiewicz, 2004; Lichovnikova, 2007; Li et al., 2017; de Juan et al., 2023).

Determining the optimal calcium requirements for laying hens is a challenge for nutritionists and poultry producers (Pastore et al., 2012; An et al., 2016). Continuous genetic improvements in commercial hens, without considering the modifications of their nutritional requirements, along with the increasing incorporation of novel ingredients in animal diets to reduce reliance on conventional raw materials, have expanded research on alternative ingredients for animal feed (Andhale, 2024). In women, the consumption of cactus flour (CF) improves osteoporosis by counteracting the reduction in bone mineral density due to menopause; this effect is attributed to the amount (35.3 mg/g) and bioavailability (0.12 molar ratio of oxalate; Calcium) of calcium in CF (Aguilera-Barreiro et al., 2013; Rojas-Molina et al., 2015).

The consumption of CF by breeding sows (Ordaz et al., 2020) and rats (Kang et al., 2012) increases the concentration of osteocalcin, a marker of bone formation associated with improved bone mineral density, possibly due to the high calcium content of CF (Hernandez-Becerra et al., 2020). According to these reports, adding CF to the diet could improve the eggshell quality-related indicators in laying hens. It should be noted that the results of the use of CF in laying hens have been limited to the use of CF from cacti older than two years (Sousa et al., 2024). These are characterized by being metabolically unviable for monogastric, including humans, due to their high lignin content (Castellano et al., 2021). Therefore, the current study aimed to assess the impact of CF (*O. ficus-indica*) on the productivity and eggshell quality of laying hens.

MATERIALS AND METHODS

Ethical approval

The study adhered to the Official Mexican Standard 062-ZOO (NOM-062-ZOO, 1999) for the production care and use of laboratory animals, and the technical specifications of the International Guiding Principles for Biomedical Research with Animals for the Council for International Organizations of Medical Sciences (CIOMS, 1985) in the handling of all the hens.

Experimental conditions

The study was performed during May and June 2023 at the poultry facilities of the Faculty of Veterinary Medicine and Zootechnics (FVMZ) of the Universidad Michoacana de San Nicolás de Hidalgo (UMSNH), Mexico. The FVMZ-UMSNH is located at km 9.5 of the Morelia Zinapécuaro highway, municipality of Tarímbaro, Michoacán, between the coordinates 19º47'11″ north latitude and 101º10'35″ west longitude, at an altitude of 1,864 meters above sea level. The climate is temperate subhumid with summer rains, with annual rainfall ranging from 600 to 800 mm and temperatures between 2.5 and 25.1ºC (INEGI, 2017).

Animals, diet, and housing

In the present study, twenty-four Rhode Island Red hens, aged 75 weeks, with 53 weeks of laying experience, were employed. Each hen was considered an experimental unit. Every hen was allocated at random to a single, standard battery-style cage measuring 46 × 40 × 43 cm in length, width, and height. The cages were equipped with automatic waterers and galvanized iron feeders that covered the entire front region of the cage. The hens were randomly divided into two groups (12 hens - experimental units-/group) that included hens that did not consume CF (control) and animals that consumed CF 1% of the volume of the diet ration (Experimental; Table 1). The diets were balanced according to the recommended requirements of the National Research Council (NRC, 1994) for laying hens. The hens were administered the diets for 10 weeks, followed by two weeks of adaptation and 8 weeks of evaluation. Notably, 120 g/hen/day feed was supplied to both groups. Free access to water was provided.

Obtention and production of cactus pear meal

The cladodes used to produce the flour weighed 500 ± 50 g (approximately 125 days old). For dehydration, the cladodes were cut into 10 cm long and 1 cm wide fragments and dried in the sun on metal trays for a week.

They were subsequently ground using a 0.5-mm mesh in a laboratory mill (Arthur H. Thomas Co. Philadelphia, PA).

Table 1. Composition and nutritional value of the diet with cactus flour for laying hens

Ingredients (%)	Control diet	Cactus flour diet
Corn	69.00	68.00
Soybean meal	18.88	18.88
Limestone	8.55	8.55
Cactus flour	--	1.00
Dicalcium phosphate	1.35	1.35
Kaolin	0.59	0.59
Soy oil	0.53	0.53
Minerals + vitamins premix[&]	0.50	0.5
Salt (NaCl)	0.43	0.43
DL-methionoine-98%	0.12	0.12
L-lysine-78%	0.03	0.03
Butylhydroxytoluene	0.02	0.02
Total	100.00	100.00
Estimated analysis		
Metabolizable energy (kcal/kg)	2800	2800
Crude protein (%)	15.00	15.37
Crude fiber (%)	4.00	4.10
Total calcium (%)	3.60	4.80
Total phosphorus (%)	0.40	0.41
Digestible arginine (%)	0.84	0.84
Digestible lysine (%)	0.64	0.64
Digestible methionine (%)	0.34	0.34
Digestible Met + Cis (%)	0.56	0.56

[&]Levels per Kg of diet: Vit. A – 8000 IU; Vit. D3 – 2000 IU; Vit. E – 50 mg; Vit. K – 3 mg; Vit. B1 – 1.5 mg; Vit. B2 – 4 mg; Vit. B6 – 0.12 mg; Vit. B12 – 15 mg; Ac. Folic – 0.6 mg; Ac. Pantothenic – 10 mg; Niacin – 30 mg; Biotin – 0.1 mg; Choline – 300 mg; Iron – 50 mg; Copper – 10 mg; Zinc – 70 mg; Manganese – 100 mg; Iodine – 1 mg; Selenium – 0.3 mg; Antioxidants 50 mg.

Laboratory analysis

The chemical composition of CF (Table 2) used in the diet was analyzed by the FVMZ-UMSNH Nutrition and Food Analysis Laboratory. An adiabatic bomb calorimeter (Model 1281; Parr, Moline, IL) was used to measure the energy content of raw materials, experimental diets, and CF. The dry matter and crude protein contents were determined following the AOAC (2000) guidelines 934.01 and 976.05, respectively. The Mexican Standard-Y-021-SCFI (NMX-Y-021-SCFI, 2003) was used to measure the calcium levels in CF and experimental diets.

Table 2. Chemical composition of cactus flour (*O. ficus indica*)

Indicator	Amount
Humidity (%)	7.33
Protein (%)	12.87
Fat (g)	2.53
Soluble fiber (g)	14.91
Insoluble fiber (g)	41.65
Ash (%)	21.17
Calcium (%)	4.33
Phosphorus (%)	0.29
Calcium oxalates (mg/g)	5.73
Calcium/Phosphorus Ratio	14.93

Experimental procedure

The body weight of each hen was recorded at the beginning and end of the experimental period using a digital scale (Dibatec®; 0.005–40 kg). Feed intake was assessed based on the difference with the supply (120 g) using a digital scale (Noval NBE-CF®; 0.01-2 kg).

Egg production was determined by the Formula 1.

$$\text{Egg production (\%/hen/day)} = \left(\frac{number\ of\ eggs\ laid}{number of\ hens}\right) * 100 \quad \text{(Formula 1)}$$

Egg weight (g), which was calculated using a digital scale (OHAUS Scoutv®; 120 g x 0.001 g).

Egg mass was determined by the Formula 2.

$$\text{Egg mass (g/hen/day)} = egg\ weight * egg\ production \quad \text{(Formula 2)}$$

The feed conversion ratio was determined by the Formula 3.

$$\text{Feed conversion index (FCI) (kg/kg)} = \left(\frac{feed\ intake}{egg\ mass}\right) \quad \text{(Formula 3)}$$

Calcium intake (g/hen/day), which was estimated according to the feed intake and calcium that the diets contained, shell weight (g), which was calculated using a digital scale (OHAUS Scout®; 120 g x 0.001 g), shell thickness (mm), which was estimated using a digital micrometer (Baxlo®; 0–10 mm),

Shell percentage was determined by the Formula 4.

$$\text{Shell percentage} = \left(\frac{shell\ weight}{egg\ weight}\right) * 100 \quad \text{(Formula 4)}$$

Shell calcium (%), which was determined from a mineral solution through atomic absorption spectrometry, excreted calcium (%) at daytime (18 hours) and excreted calcium at nighttime (7 hours), which were determined based on the calcium intake and the calcium determined in the feces, and the economic efficiency index (EEI %).

To determine the economic viability of adding CF to the diet, the cost of the diet per kg of eggs produced (Y_i) was determined using the equation adapted from the methodology of Bellaver et al. (1985; Formula 5).

$$Y_i = \frac{P_i * Q_i}{E_i} \quad \text{(Formula 5)}$$

where Y_i is the diet cost per kg of eggs produced in the ith treatment (control, HN), P_i is the price per kg of the diet used in the ith treatment, Q_i is the quantity of diet consumed in the ith treatment, and E_i is the kg of eggs produced. The economic efficiency index (EEI) was determined using the following equation (Formula 6).

$$EEI = \left(\frac{LCe}{CTei}\right) \times 100 \quad \text{(Formula 6)}$$

where LCe is the lowest diet cost per kg of eggs produced with different treatments, and CTei is the cost of the ith treatment.

Statistical analyses

All statistical analyses were conducted using SAS version 9.4, 2020 (SAS Institute Inc, Cary, NC, USA). Before data analysis, the normality of the distribution and homogeneity of variance for the residuals were determined using PROC UNIVARIATE. The Shapiro–Wilk test was used to determine normality, whereas the Bartlett test was used to determine homogeneity. The data were analyzed using a completely randomized design with PROC GLM (model determined by likelihood ratio test). The hen was taken as the experimental unit. The model included diet as a fixed effect and hen as a random effect for statistical analysis. Correlations between feed intake and calcium consumption and the variables associated with productive performance and economic efficiency, egg quality, and calcium kinetics were analyzed using the Pearson correlation test (PROC CORR). Differences between means were determined using the least squares means method with p-value ≤ 0.05. The values were represented as least squares mean ± standard error of the mean.

RESULTS

Data analysis revealed a diet effect ($p < 0.001$), with the CF-fed hens exhibiting a significantly higher weight at the end of the experiment compared to the control group (Table 3). This increase in weight was attributed to a significant weight gain ($p < 0.05$), with CF-fed hens gaining an average of 49.7 g more than the control (Table 3). Egg production and mass were significantly higher in the CF-fed hens ($p < 0.05$; 88.6 %/hen/day and 60.1% g/hen/day, respectively) compared with the control (85.7 %/hen/day and 52.9 g/hen/day for egg production and mass, respectively). Higher egg production resulted in significantly lower ($p < 0.05$) FCI (1.8 kg/kg) and higher ($p < 0.05$) EEI (94.8%) in the CF-fed hens compared to the control group (2.1 kg/kg and 90.2% for FCI and EEI, respectively; Table 3).

Further diet analysis revealed that the CF-fed hens exhibited significantly higher egg weight ($p < 0.05$; 68.5 vs. 62.2 g), shell weight (5.8 vs. 5.1 g), and calcium levels in the shell (35.6 vs. 33.4%) than the hens in the control group (Table 4). Evaluation of the diets revealed that CF significantly increased the calcium consumption of hens ($p < 0.05$; 5.4 g/day) compared to other experimental diets (4.1 g/day; Table 5). Calcium levels in daytime excreta were significantly higher in the hens that did not consume CF ($p < 0.05$; 7.6 vs. 5.7%); however, no significant differences in the calcium levels in nocturnal excreta were observed between the groups ($p > 0.05$; Table 5).

The results also indicated that the feed and calcium intake were differently associated with variables affecting productive and economic efficiency, egg quality, and calcium kinetics. For the variables affecting productive and economic efficiency, feed intake was significantly associated with weight gain in both the control and the CF-fed hens ($r = 0.34$ and 0.52; $p < 0.05$; Figure 1a). Calcium intake was also significantly associated with egg production (egg mass) in both the control ($r = 0.64$; $p < 0.05$) and the CF-fed hens ($r = 0.53$; $p < 0.05$). Similarly, egg production (egg mass) was significantly associated ($p < 0.05$) with both FCI and EEI, showing correlations

greater than 0.80 in both groups. However, the correlation between egg mass and feed conversion ratio was negative in both groups (Figure 1a). Next, correlations between variables affecting egg quality and feed and calcium intake were analyzed (Figure 1b). Feed intake was significantly associated with egg weight and shell thickness in the control and the CF-fed hens ($r \geq 64$; $p < 0.05$; Figure 1b). Calcium intake was also significantly associated ($p < 0.05$) with the shell thickness in both the control and the CF-fed hens ($r = 0.59$ and 0.67, respectively). Additionally, egg production was significantly associated with egg weight ($r \geq 72$; $p < 0.05$) in both groups. In the control group, feed intake was not significantly associated ($p > 0.05$) with any variable determining calcium mobilization (Figure 1c). In the CF-fed hens, feed intake was associated with shell percentage ($r = 0.40$; $p < 0.05$) and shell calcium ($r = 0.37$; $p < 0.05$). The CF-fed hens exhibited stronger associations ($p < 0.05$) of calcium levels in the shell with calcium levels in the daytime and nighttime excreta than the hens that did not consume CF (Figure 1c).

Table 3. Effect of cactus flour intake on productive indicators in laying hens (mean ± SD)

Indicator	Control diet	Cactus flour diet	P - value
Initial body weight (kg)	1.817 ± 0.19	1.952 ± 0.21	0.1507
Final body weight (kg)	1.887 ± 0.18[a]	2.072 ± 0.16[b]	< 0.0001
Weight gain (g)	70.3 ± 3.28[a]	120.0 ± 4.11[b]	0.0104
Feed intake (g/hen/day)	112.9 ± 6.23	112.6 ± 5.33	0.8459
Production (%/hen/day)	85.7 ± 2.04[a]	88.6 ± 2.46[b]	0.0019
Egg mass (g/hen/day)	52.9 ± 6.29[a]	60.1 ± 5.09[b]	0.0123
Feed conversion rate (kg/kg)	2.1 ± 0.19[b]	1.8 ± 0.09[a]	0.0062
Economic efficiency index (%)	90.2 ± 1.76[a]	94.8 ± 2.17[b]	0.0018

[a-b]Means in the same row with superscripts differ significantly ($p < 0.05$).

Table 4. Effect of cactus flour intake on egg quality indicators in laying hens (mean ± SD)

Indicator	Dieta control	Cactus flour diet	P - value
Egg weight (g)	62.2 ± 6.2[a]	68.5 ± 4.09[b]	0.0493
Shell weight (g)	5.1 ± 0.70[a]	5.8 ± 0.31[b]	< 0.001
Longitudinal diameter (cm)	6.0 ± 0.14[a]	6.1 ± 0.17[b]	0.0580
Transverse diameter (cm)	4.3 ± 0.09	4.4 ± 0.15	0.3509
Shell thickness (mm)	0.27 ± 0.006[a]	0.31 ± 0.003[b]	0.0019
Shell percentage (%)	8.2 ± 0.83[a]	8.5 ± 0.40[b]	0.0431
Calcium in shell (%)	33.4 ± 2.52[a]	35.6 ± 1.44[b]	< 0.001

[a-b]Means in the same row with superscripts differ significantly ($p < 0.05$).

Table 5. Effect of cactus flour intake on indicators of calcium mobilization in laying hens (mean ± SD)

Indicator	Dieta control	Cactus flour diet	p-value
Calcium consumption (g/hen/day)	4.1 ± 0.21[a]	5.4 ± 0.55[b]	< 0.001
Calcium in diurnal excreta (%)	7.6 ± 1.17[a]	5.7 ± 0.70[b]	< 0.001
Calcium in nocturnal excreta (%)	10.0 ± 0.57	9.6 ± 0.76	0.2962

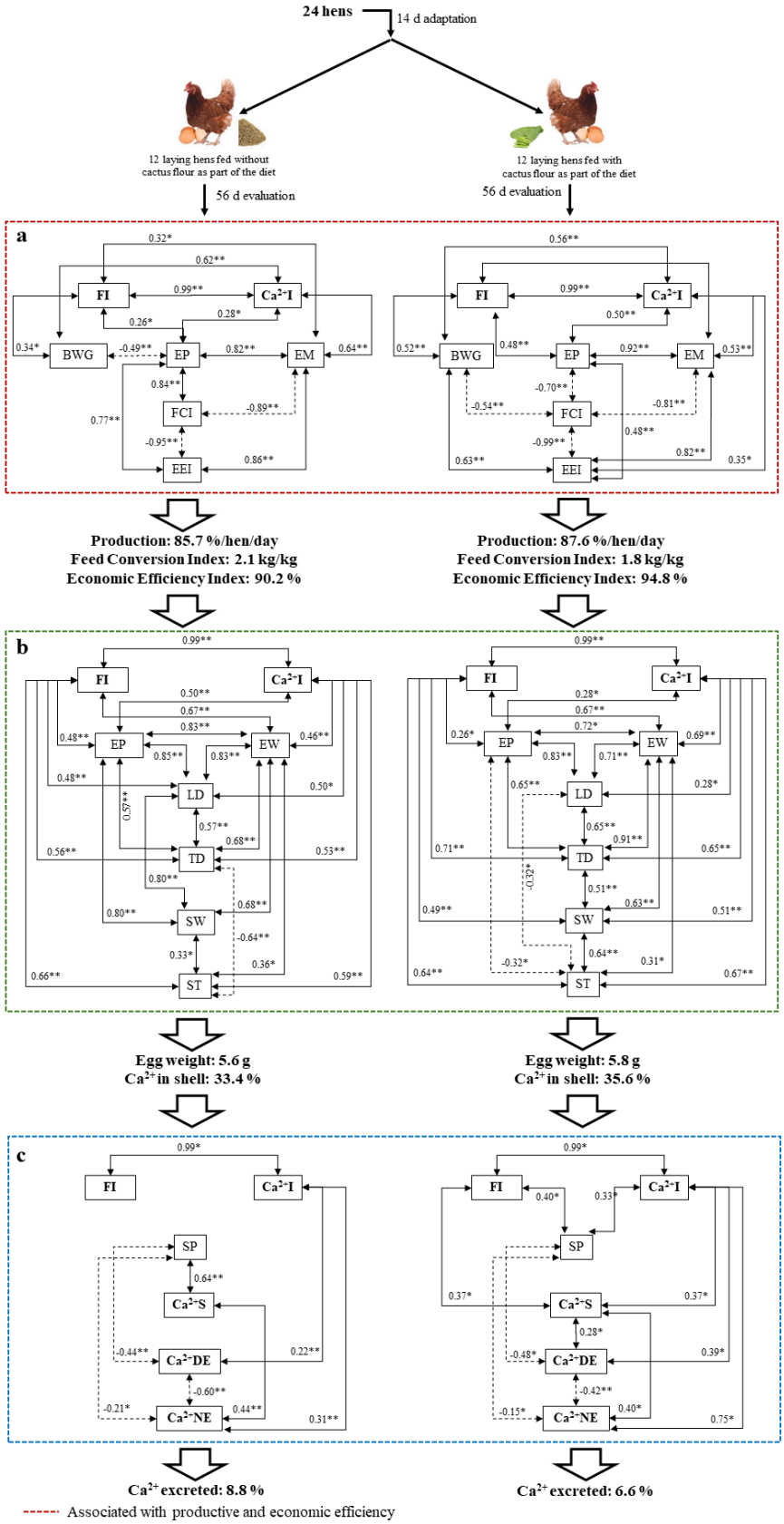

Figure 1. Schematic representation of the Pearson correlation coefficients for feed intake and calcium intake in laying hens fed with cactus flour with variables that affect productive and economic efficiency (**a**), egg quality (**b**), and calcium mobilization (**c**). FI: Feed intake, Ca^{2+}I: Calcium intake, BWB: Body weight balance, EP: Egg production, EM: Egg mass, FCI: Feed conversion index, EEI: Economic efficiency index, EW: Egg weight, LD: Longitudinal diameter of the egg, TD: Transverse diameter of the egg, SW: Shell weight, ST: Shell thickness, SP: Shell percentage, Ca^{2+}S: Calcium in shell, Ca^{2+}DE: Calcium in diurnal excretion, Ca^{2+}NE: Calcium in nocturnal excretion. *: Significant ($p < 0.05$); **: Highly significant ($p < 0.001$).

DISCUSSION

Calcium is a vital nutrient for laying hens, especially for bone formation and as an enzyme cofactor (Li et al., 2016). Calcium is essential for blood coagulation, eggshell formation, and muscle and nerve function (Li et al., 2017). However, the impact of dietary calcium levels on laying hen's productivity remains a topic of debate. Some studies suggested hens can produce adequate eggs and operate productively with as little as 3.2% calcium in their diet (Świątkiewicz et al., 2015). According to Cufadar et al. (2011), adding 3.0, 3.6, or 4.2% of calcium to the diet did not significantly affect egg production, egg weight, egg mass, or feed conversion index (FCI). Safaa et al. (2008) plotted the egg production curve and reported that hens require more than 3.5% calcium in their diet for optimal laying performance and that increasing the level of dietary calcium to 4.0% improved egg production, egg mass, and FCI.

Cactus flour (CF) is considered a good source of minerals, including high concentrations of calcium, potassium, magnesium, and phosphorus (Santana et al., 2021). Sousa et al. (2024) reported that the addition of 3.0, 6.0, and 9.0% CF to the diet did not affect the productive variables of laying hens. The authors attributed these findings to the presence of calcium oxalate, an anti-nutritional factor in CF that binds to calcium, making it unavailable, thus impacting the bioavailability of calcium for animal absorption (McConn and Nakata, 2004; Batista et al., 2009). Notably, older cactus plants tend to have lower concentrations of calcium oxalate (Rodríguez-Garcia et al., 2007). Sousa et al. (2024) observed no effect of CF intake on the productivity of laying hens when cladodes aged two years were used likely due to a decrease in phosphorus content as cladodes mature, which alters the calcium/phosphorus ratio and subsequently reduced the bioavailability of calcium (Rodríguez-García et al., 2007; Hernández-Urbiola et al., 2010). Furthermore, as the cladode ages, the levels of cellulose, hemicellulose, and lignin increase, compromising the digestibility of nutrients, as the gut does not synthesize the enzymes needed for their degradation (Castellano et al., 2021).

Regarding egg quality variables, such as shell thickness and percentage, and productive variables, many studies have reported contradictory results using the calcium levels outlined by NRC (1994). For instance, one study found that laying hens fed diets with high concentrations of calcium (4.4%) exhibited reduced shell thickness than the hens in the control group (3.7% calcium; Jiang et al., 2013). However, in another study, eggshell quality (shell weight and thickness) improved when the dietary calcium level was increased from 3.5 to 4.0% (Safaa et al., 2008). Studies using laying hens between 58 and 93 weeks of age have reported higher requirements of calcium for optimal eggshell quality than those outlined by the NRC (1994).

In the present study, enhanced eggshell quality in CF-fed hens was associated with the quantity and bioavailability of minerals (calcium, phosphorus, and magnesium) found in nopal, which aligns with previous reports (Hernández-Urbiola et al., 2010; Rojas-Molina et al., 2015; Quintero-García et al., 2020). The bioavailability of calcium was closely associated with the phosphorus content of the diet. Owing to the low availability of phosphorus and its antagonism with calcium, increasing the concentration of calcium without considering the phosphorus levels can adversely affect the egg quality and health in hens, increasing the risk of fractures (Sinclair-Black et al., 2023). The total calcium/phosphorus ratio in the diet should be 2:1 for all species; however, in laying hens, this ratio can be increased up to > 7:1. In terms of available phosphorus, this ratio (calcium/available phosphorus) can reach up to 10-12:1 (de Araújo et al., 2015). Here, the calcium/phosphorus ratio in the CF versus control diet was 11.7 vs. 9.0, thus leading to greater bioavailability of calcium in the CF-fed hens. Furthermore, the molar ratio of oxalate/calcium according to age was approximately 0.12, indicating that molar ratios of oxalate/calcium ≥ 1.0 indicated a lack of calcium availability (Bhandari and Kawabata, 2004; Rojas-Molina et al., 2015). Therefore, based on the oxalate/calcium ratio, the bioavailability of calcium in the CF-fed group was not compromised.

Increased bioavailability of calcium in the CF-containing diet possibly influenced bone matrix formation, thereby minimizing the use of endogenous calcium and enhancing the eggshell quality. Changes in eggshell quality showed correlations between calcium consumption and eggshell weight and thickness, with stronger correlations observed in the CF-fed hens than in the control. Similarly, higher intake and bioavailability of calcium in the CF diet resulted in higher calcium levels in the shell (higher weight, thickness, and calcium percentage in the shell) and lower calcium levels in the excreta compared to those in the control group (6.6 vs. 8.8%). These results showed that the CF-fed hens exhibited stronger correlations between calcium intake and calcium

levels in the shell and calcium levels in the daytime and nighttime excreta compared to controls.

CONCLUSION

The results indicated that the inclusion of cactus flour in the diet of laying hens enhanced productivity and eggshell quality. The best egg quality and feed conversion rate per kilogram of produced eggs were obtained at 1% CF, which also improved the economic efficiency index (EEI) per kilogram of produced eggs. Nevertheless, further research is needed to determine the optimal level of CF in the diets of laying hens.

DECLARATIONS

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Availability of data and materials

The data that support the findings of this study are available on request from the corresponding author.

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Authors' contributions

AJ and GO designed the experiment, EG and CV conducted the experiment, AJ, and GO completed data analysis; AJ supplied resources, AJ and GO wrote the original draft manuscript, AJ and EG corrected the manuscript, AJ and CV supervised the project. All authors have checked the collected, and analyzed data and agreed on the submission of this article.

Competing interests

The authors assert that they have no competing interests.

Ethical considerations

All authors have reviewed ethical concerns, including data fabrication, double publication and submission, redundancy, plagiarism, consent to publish, and misconduct before being published in this journal.

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Antibiotic Resistance Pattern of *Escherichia coli* Isolated from Layer Chicken in Bali-Indonesia

I Nengah Kerta Besung[1*], Putu Henrywaesa Sudipa[1], I Gusti Ketut Suarjana[1], and Ni Ketut Suwiti[2]

[1]*Department of Veterinary Medicine, Faculty of Veterinary Medicine, Udayana University, Denpasar 80234, Bali, Indonesia*
[2]*Department of Veterinary Basic Science, Faculty of Veterinary Medicine, Udayana University, Denpasar 80234, Bali, Indonesia*

*Corresponding author's E-mail: kerta_besung@unud.ac.id

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ABSTRACT

Antibiotics have been used as growth promoters in the poultry industry worldwide, which might lead to the emergence of anti-microbial resistant bacterial strains. Theoretically, older animals should have been exposed to antibiotics and anti-microbial resistant (AMR) strains for longer periods, which may result in the discovery of more resistant strains. The present study aimed to evaluate the antibiotic resistance of *Escherichia coli* isolated from fecal samples of layer chicken that showed signs of watery diarrhea. In the current study, 134 fecal samples were taken from the layer chicken farms in Penebel village, Tabanan District, Bali, Indonesia. The chickens were classified into three groups including Group 1 under 7 days of age, group 2 aged 7-30 days, and Group 3 chickens older than one month. The samples were cultured in Eosin Methylene Blue agar. The suspected colonies were stained, and subjected to biochemical tests. *Escherichia coli*-positive colonies were subjected to a bacteria sensitivity test using multiple antibiotic discs. The result demonstrated multi-drug resistance (MDR) of *Escherichia coli*, while the isolated *Escherichia coli* was resistant to the most common antibiotics in layer farms in the study area including kanamycin, enrofloxacin, sulfamethoxazole-trimethoprim, streptomycin, and enrofloxacin. In addition, the present study confirmed that although all sample groups were sensitive to bacitracin, oxytetracycline, and clindamycin, they were resistant to sulfamethoxazole-trimethoprim, kanamycin, and ampicillin.

Keywords: Antibiotic resistance, Bali, Colibacillosis, *Escherichia coli*, Layer chicken

INTRODUCTION

Implementing strict biosecurity measures is essential to protect the health of chickens and ensure the sustainability of poultry farming. The risk of disease can be reduced by keeping the coop clean, avoiding high chicken population density, and limiting the number of people entering the coop. These efforts will help maintain the health of chickens and support the productivity and profitability of poultry farms (Islam et al., 2023).

One species of bacteria that often causes economic loss and is resistant to antibiotics is *Escherichia coli*, the causal agent of Colibacillosis. This disease is a serious threat to the poultry industry worldwide (Watts and Wigley, 2024). The overall total loss in Indonesia reached more than 10% of total national poultry assets (Wibisono et al., 2018). This bacterium is also zoonotic (Babazadeh and Ranjbar, 2021; Hu et al., 2022).

Colibacillosis is caused by *Escherichia coli* bacteria, which are rod-shaped and Gram-negative (Giubelan et al., 2024). The prevalence of colibacillosis infection in broiler chicken was 5%, with a mortality rate of 1.25% and the case fatality rate was 33.3% (Santoso et al., 2020). Up to 40% of colibacillosis occurs under three weeks of age, while 60% of colibacillosis infections happen in older poultry (Santoso et al., 2020). Colibacillosis occurs more often in young chickens, which is caused by the Avian Pathogenic *Escherichia coli* (APEC) type. The APEC bacteria are dominated by three serotypes, which are O1, O2, and O3. Approximately 10-15% of all *Escherichia coli* found in the intestines of healthy chickens are classified as the APEC type. The APEC type causes

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various clinical manifestations, such as respiratory, systemic, and reproductive tract infections in layer and broiler chickens (Peighambari et al., 2000; Ribeiro et al., 2023).

Antibiotics have been used as growth promoters in the poultry industry worldwide, which might lead to the emergence of anti-microbial resistant (AMR) bacterial strains. Many cases have been reported where bacteria were initially sensitive to antibiotics but gradually became resistant. Previously, we found that *Escherichia coli* isolated from layer chicken was sensitive to kanamycin, while to oxytetracycline, ampicillin, streptomycin, and sulfamethoxazole was resistant, and to chloramphenicol was intermediate resistant (Besung et al., 2019). Another study found that *Escherichia coli* isolated from broiler chickens in Subang, West Java, was 90% resistant to at least three or more types of antibiotics (Niasono et al., 2019). The most common resistance was against tetracycline 97.3%, sulfamethoxazole 87.8%, trimethoprim 74.3%, ampicillin 68.9%, nalidixic Acid 64.8%, ciprofloxacin 45.9%, enrofloxacin 40.5%, gentamicin 28.4%, and chloramphenicol 10.8%. Almost the same incident also occurred in China (Wang et al., 2021), in Iran (Jahantigh et al., 2020), and in Central Nigeria (Akanbi et al., 2022).

With the increasing development of resistance in poultry, the bacteria sensitivity situation becomes unclear, and bacteria sensitivity testing is needed. This testing is one of the ways to determine the bacteria's resistance to antibiotics. Theoretically, the older animal should have been exposed to antibiotics and AMR strains for a longer period, so more resistant strains might be discovered. The objective of this study was to find out the pattern of the antibiotic-resistant *Escherichia coli* isolated from fecal samples of layer chicken showing clinical signs of watery diarrhea in Bali, Indonesia.

MATERIALS AND METHODS

Ethical approval

Ethical clearance for this study was approved by the Animal Ethics Committee of the Faculty of Veterinary Medicine Udayana University, Denpasar, Indonesia No. B/168/UN/14.2.9/PT.01.04/2023.

Sampling

All layer farms in Tabanan District Bali, Indonesia, were visited in August and September 2023. The swab samples were collected from the cloaca of chicken that had watery diarrhea using a bacterial Stuart transport medium (Oxoid). In one farm, three groups of chickens namely chickens under 7 days, 8-30 days, and over 30 days, if available, were observed separately. The clinical signs were observed and two cloacal swabs were collected from chickens in the same group. The total numbers of samples were 44, 41, and 49 for groups 1, 2, and 3, respectively. The number of samples was dependent on the availability of chicken in the respective group and the presence of suspect colibacillosis.

Bacteria isolation and identification

Bacteria in feces were cultured in Eosin Methylene Blue Agar (EMBA). A metallic green colony was classified using Gram staining and a series of biochemical tests, such as the Sulphide Indole Motility (SIM) test, Methyl Red (MR), Voges Proskauer (VP), and Simmons Citrate tests (SC) as previously described (Abu-Sini et al., 2023). The colonies were also tested in Triple Sugar Iron Agar (Koutsianos et al., 2020) Glucose, and Lactose Test.

Antibiotic sensitivity test

The colonies that were identified as *Escherichia coli* were tested by a sensitivity test using the agar diffusion method from Kirby-Bauer. Three colonies were taken from the EMBA media and grown in Mueller Hinton Broth (MHB) and incubated for 2 hours at 37°C. The culture turbidity should reach 0.5 MacFarland standard. The cultures were then spread in the Mueller Hinton Agar (MHA). Multiple antibiotic discs that contain bacitracin, oxytetracycline, clindamycin, sulfamethoxazole-trimethoprim, streptomycin, kanamycin, ampicillin, and enrofloxacin (Oxoid), which are commonly used for human and animal in Bali, were then attached to the MHA. The agar media was incubated for 18-24 hours, and the inhibitory diameter was measured and compared with the Clinical and Laboratory Standards Institute (CLSI) sensitivity test standard (Magiorakos et al., 2012; Weinstein and Lewis, 2020). The diameter of the zone was measured manually using a caliper.

Data analysis

The number of colonies that were resistant, intermediate, and sensitive against respective antibiotics were tabulated and analyzed descriptively. The comparison of the *Escherichia coli* inhibition zones against each antibiotic in layer chickens Group 1, Group 2, and Group 3 layers were analyzed using IBM SPSS Statistics software version 27, 2020. The level of significance measured was 5% ($p < 0.05$).

RESULTS AND DISCUSSION

An example of the result of the antibiotics sensitivity tests using the Kirby-Bauer agar diffusion method is presented in Figure 1. The size of the inhibition diameter is standardized to determine the sensitive, intermediate, and resistant categories (Magiorakos et al., 2012; Weinstein and Lewis, 2020). In the picture, an isolate from G-1 resistant to discs 1, 3, 5, and 7 (left); an isolate from G-2 to 1, 2, 3, 4, and 6 (center); as well as an isolate from G-3 to 1, 2, 3, 4, and 5 (right) are presented. The discs 1-8 contained antibiotics (1) bacitracin, (2) oxytetracycline, (3) clindamycin, (4) sulfamethoxazole-trimethoprim, (5) streptomycin, (6) kanamycin, (7) ampicillin, and (8) enrofloxacin. The size of the inhibition shown in Figure 1 indicates isolated *Escherichia coli* from the samples is resistant to ≥ 3 antibiotics from different classes and was referred to as multi-drug resistant (MDR).

Improper and excessive use of antibiotics can cause bacteria to become resistant. Resistant bacteria will survive and reproduce, while sensitive bacteria will die (Van Boeckel et al., 2015). Resistant bacteria often avoid detection or attack the immune system through mechanisms. Some bacteria can produce enzymes that destroy antibodies or change their surface structure to avoid recognition by immune cells (Thomson et al., 2021).

The average diameters of antibiotic inhibition zones to isolates of *Escherichia coli* originating from different chicken age groups are presented in Table 1. The two lowest inhibition zones (tend to be resistant) were demonstrated in bacitracin and clindamycin, while the highest (sensitive) was in sulfamethoxazole-trimethoprim. The proportion (%) of *Escherichia coli* isolates from three different groups of chicken showing sensitive (S), intermediate resistant (I), and resistant (R) to various antibiotics are presented in Table 2. The result showed variation in the three most antibiotics that isolate resistant against, which listed from the highest were in group 1 K (kanamycin), E (enrofloxacin) and ST (sulfamethoxazole-trimethoprim), in group 2 K (kanamycin), S (streptomycin), ST (sulfamethoxazole-trimethoprim), in group 3 were ST (sulfamethoxazole-trimethoprim), K (kanamycin), and E (enrofloxacin).

The prevalence of antibiotic resistance among *Escherichia coli* isolates from layer chickens of different age groups in the Tabanan District, Bali, Indonesia, is presented in Table 2. Notably, *Escherichia coli* isolates from chickens less than 7 days old exhibited resistance to sulfamethoxazole-trimethoprim, kanamycin, and enrofloxacin with resistance rates exceeding 10%. However, resistance to other antibiotics, such as oxytetracycline, bacitracin, and ampicillin, was below 10%. As the chickens aged, the resistance changed. *Escherichia coli* isolates from chickens aged 8-30 days and over 30 days showed increased resistance to streptomycin, kanamycin, and ampicillin, while resistance to bacitracin, clindamycin, sulfamethoxazole-trimethoprim, and enrofloxacin decreased. Notably, none of the *Escherichia coli* isolates from any aged group exhibited resistance to certain antibiotics. Changes in antibiotic resistance on this farm were related to the unwise use of antibiotics. The antibiotic selection was determined by their availability in the market. Antibiotics that are often given to laying hens as treatments or growth stimulants can lead to the development of resistance. Conversely, antibiotics that are never used may result in increased bacterial sensitivity (Mann et al., 2021).

The prevalence of antibiotic resistance in layer chickens within breeding facilities poses a significant challenge to the livestock industry. The indiscriminate use of antibiotics as a preventive measure, rather than solely for treatment, exacerbates the emergence of resistant bacterial strains (Rahman and Hollis, 2023). Previous studies indicated that *Escherichia coli* and *Salmonella* isolated from layer chickens frequently exhibit resistance to commonly used antibiotics, such as ampicillin, tetracycline, and sulfonamides (Zhao et al., 2021). Comprehensive surveys conducted in breeding centers reveal a substantial prevalence of resistance, with some studies reporting resistance rates exceeding 50% for specific antibiotics (Ahmed et al., 2024).

The comparative analysis presented in Table 3 demonstrates an absence of statistically significant differences ($p > 0.05$) between the inhibition zones of bacitracin and clindamycin antibiotics against *Escherichia coli* strains isolated from layer chicken farms, specifically from chickens aged 1-7 days, 8-30 days, and over 30 days. This observation can be attributed to the presence of a lipopolysaccharide layer in the outer membrane of *Escherichia coli*, which acts as a barrier, limiting the penetration of various antibiotics, including clindamycin and bacitracin. Clindamycin is typically effective against Gram-positive bacteria, while bacitracin works well on bacteria with a thick peptidoglycan cell wall. However, since *Escherichia coli* has a thin peptidoglycan layer protected by an outer membrane, these antibiotics are less effective (Puvaca and de Llanos Frutos, 2021). The inhibition zones for the antibiotics oxytetracycline and sulfamethoxazole-trimethoprim against *Escherichia coli* from farms with chickens older than 30 days were significantly lower compared to those with chickens aged 1-7 days and 8-30 days ($p < 0.05$).

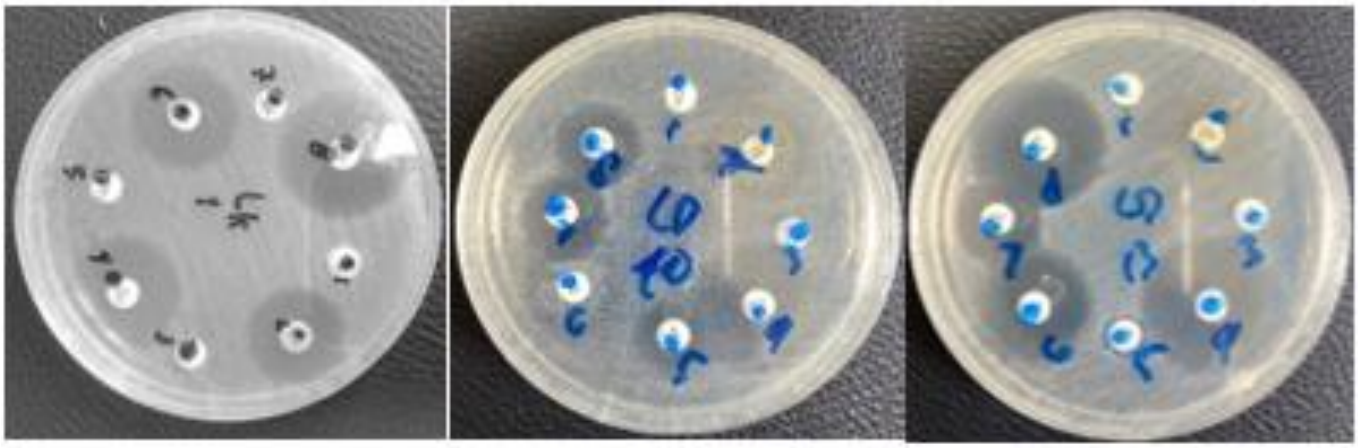

Figure 1. The antibiotics sensitivity tests using the Kirby-Bauer agar diffusion method. Left was a colony from a sample of chicken group 1, middle from group 2, and right from group 3, respectively. Handwritten numbers 1-8 were discs 1 to 8 that contained antibiotics bacitracin, oxytetracycline, clindamycin, sulfamethoxazole-trimethoprim, streptomycin, kanamycin, ampicillin, and enrofloxacin.

Table 1. Average diameters of antibiotic inhibition zone to isolates of *Escherichia coli* originated from different layer chickens in Tabanan District, Bali, Indonesia

	B	O	C	ST	S	K	A	E
Group 1	8.57 ± 6.11	17.07 ± 3.46	7.83 ± 2.91	27.36 ± 5.32	18.93 ± 4.76	19.12 ± 2.03	20.10 ± 3.49	23.40 ± 3.11
Group 2	7.13 ± 0.51	18.10 ± 4.02	7.48 ± 2.53	20.33 ± 6.59	21.28 ± 1.92	22.75 ± 2.38	23.05 ± 2.50	24.78 ± 4.07
Group 3	7.10 ± 0.47	15.44 ± 3.99	7.40 ± 2.32	26.04 ± 4.14	20.08 ± 2.27	22.10 ± 1.97	19.29 ± 3.58	22.04 ± 4.86
Average of respective antibiotics	7.60	16.87	7.57	24.58	20.10	21.32	20.81	23.41

B: Bacitracin, O: Oxytetracycline, C: Clindamycin, ST: Sulfamethoxazole-Trimethoprim, S: Streptomycin, K: Kanamycin, A: Ampicillin, E: Enrofloxacin.
Group 1: Layer chickens under 7 days old, Group 2: 8-30 days old, Group 3: Over 30 days old

Table 2. Proportion (%) of sensitive *Escherichia coli* isolated from layer chickens of Tabanan District, Bali, Indonesia to various antibiotics

Antibiotics	Proportion (%) of isolates that were sensitive (S), intermediate resistant (I) and resistant (R)								
	Group 1			Group 2			Group 3		
	S	I	R	S	I	R	S	I	R
Bacitracin	93.02	2.33	4.65	97.56	2.44	0.00	100	0.00	0.00
Oxytetracycline	90.70	9.30	0.00	95.12	4.88	0.00	100	0.00	0.00
Clindamycin	95.35	0.00	4.65	97.56	0.00	2.44	100	0.00	0.00
Sulfamethoxazole-Trimethoprim	69.77	0.00	30.23	70.73	0.00	29.27	14.29	0.00	85.71
Streptomycin	88.37	0.00	11.63	29.27	26.83	43.90	48.98	10.20	40.82
Kanamycin	34.88	2.33	62.79	14.63	0.00	85.37	18.37	0	81.63
Ampicillin	90.70	0.00	9.30	75.61	0.00	24.39	18.37	20.41	61.22
Enrofloxacin	46.51	16.28	37.21	85.37	0.00	14.63	12.24	30.61	57.15

S: Sensitive; I: Intermediate; R: Resistant to various antibiotics.

Table 3. The analysis of *Escherichia coli* sensitivity to various antibiotics based on the age of layer chickens in Tabanan District, Bali, Indonesia

Inhibition zone (mm) / Antibiotics	Group 1	Group 2	Group 3	Average	p-value
Bacitracin	8.50 ± 0.92[a]	7.12 ± 0.80[a]	7.10 ± 0.67[a]	7.57 ± 0.307	0.105
Oxytetracycline	18.12 ± 1.628[a]	17.00 ± 1.522[a]	15.45 ± 1.570[b]	16.78 ± 1.342	0.005
Clindamycin	7.80 ± 0.438[a]	7.46 ± 0.395[a]	7.39 ± 0.331[a]	7.54 ± 0.222	0.729
Sulfamethoxazole- Trimethoprim	27.14 ± 1.464[a]	25.92 ± 1.990[a]	20.34 ± 1.020[b]	24.61 ± 3.963	0.000
Streptomycin	18.86 ± 1.762[b]	20.06 ± 1.268[a]	21.22 ± 1.917[a]	20.02 ± 1.344	0.004
Kanamycin	19.14 ± 2.030[c]	22.61 ± 2.376[b]	22.96 ±1.972[a]	21.31 ± 2.605	0.000
Ampicillin	19.20 ± 1.576[b]	20.11 ± 1.486[b]	22.95 ± 1.500[a]	20.65 ± 1.595	0.000
Enrofloxacin	21.92 ± 0.864[b]	23.41 ± 1.113[a]	24.16 ± 1.067[a]	23.28 ± 1.243	0.006

[a, b, c] Means with different superscript letters in the column represent significant differences at $p < 0.05$. Group 1: Layer chickens under 7 days old, Group 2: 8-30 days old, Group 3: Over 30 days old.

Conversely, the inhibition zones for the antibiotics streptomycin, kanamycin, ampicillin, and enrofloxacin against *Escherichia coli* from chickens older than 30 days were significantly greater ($p < 0.05$) compared to those from chickens aged 1-7 days and 8-30 days. Accordingly, resistance to oxytetracycline and sulfamethoxazole-trimethoprim has increased, whereas resistance to streptomycin, kanamycin, ampicillin, and enrofloxacin has decreased. The changes in resistance on these farms are suspected to be due to the use of antibiotics in disease treatment or prevention.

Usage of antibiotics, whether to treat or prevent disease, can lead to increased antibiotic resistance. Over time, bacteria exposed to these antibiotics may develop mechanisms to survive their effects, reducing the antibiotics' effectiveness. This resistance can spread, posing significant challenges for managing infections and ensuring effective treatment in animal and human health contexts. The emergence of antibiotic resistance is a complex issue that requires a holistic approach, including the regulation of antibiotic use, improved management practices, and education for farmers. Integrated efforts are needed to mitigate the risks and impacts of antibiotic resistance in livestock farming (Ahmed et al., 2024).

Antimicrobial resistance (AMR) is a growing global health crisis that occurs when microorganisms evolve to resist the effects of medications used to treat infections (Tang et al., 2023). Antimicrobial resistance due to veterinary use, including in layer chicken, is a significant contributor to the global AMR crisis (Caneschi et al., 2023; Hedman et al., 2020). Poor biosecurity in layer chicken farms in Bali and uncontrolled antibiotic administration have led to the emergence of resistant bacteria. It is also believed that antibiotic resistance in these farms may have originated from surrounding farms.

The results of the present study showed that all three isolates were resistant to three or more antibiotics, which is referred to as MDR (Magiorakos et al., 2012). The MDR cases have been reported from various environments including chicken farms in Dakahlia and Sharkia Governorates, Egypt (Awad et al., 2023), in Malang Regency, Indonesia (Dameanti et al., 2022), and in Karatu, Northern Tanzania (Sonola et al., 2021). Furthermore, the current study revealed that resistance to certain antibiotics, including bacitracin and clindamycin, was observed across all age groups. Notably, resistance to bacitracin has been previously reported in *Escherichia coli* isolates obtained from chicken in Malang Regency, Indonesia (Dameanti et al., 2022), Farnham, Quebec, Canada (Thibodeau et al., 2008), and Zambia (Mudenda et al., 2023). Additionally, multidrug-resistant *Escherichia coli* O157 has been identified in poultry farms located in eastern Ethiopia (Shecho et al., 2017).

Antibiotic resistance in veterinary medicine significantly impacts animal health and public safety. Since antibiotics are commonly used to promote growth and prevent disease in livestock, their use can lead to the development of resistant bacteria in animals, which can then be transmitted to humans through the food chain. It results in serious risks, as resistant infections are more difficult to treat, leading to increased morbidity and healthcare costs (Van Boeckel et al., 2015). Additionally, the presence of antibiotic-resistant bacteria complicates treatment protocols and may result in higher treatment failure rates. There is a need for more responsible use of antibiotics, including enhanced monitoring and regulation. Addressing this issue is critical not only for maintaining animal welfare but also for protecting public health from the impacts of antibiotic resistance (Ajayi et al., 2024).

CONCLUSION

It can be concluded that the *Escherichia coli* isolated from layer chicken in the study area were sensitive to bacitracin, oxytetracycline, and clindamycin, but resistant to sulfamethoxazole-trimethoprim, streptomycin, kanamycin, and ampicillin. As the age of the chicken increases, the resistance to some antibiotics increases. Further research is needed to cover a wider study area. Biosecurity should be strictly implemented in farms that have a history of infection.

DECLARATIONS

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Authors' contributions

I Nengah Kerta Besung and Ni Ketut Suwiti designed the research, collected the samples, and provided the media for the research. Putu Henrywaesa Sudipa and I Gusti Ketut Suarjana conducted the research process in the laboratory. All authors reviewed the analyzed data and approved the final draft of the manuscript.

Competing interests

The authors have disclosed no conflicts of interest.

Ethical considerations

The authors confirm that all authors have reviewed and submitted the manuscript to this journal for the first time. Additionally, all authors checked the originality of data and sentences via plagiarism checkers.

Availability of data and materials

The original data presented in the study are included in the article. For inquiries, please contact the corresponding author.

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Effects of Fermented and Non-fermented Green Seaweed Supplementation on Performance, Caecal Bacterial Population, and Blood Constituents of Japanese Quails

Salma Hashem Abu Hafsa[1]*, Sawsan Abd-Ellatif[2], Mohamed Elsayed Eid Farag[3], Mervat, Ali Breakaa[3], Elsayed Shaaban Abdel Razik[1], and Ayman Abdelmohsen Hassan[3]

[1]*Livestock Research Department, Arid Lands Cultivation Research Institute, City of Scientific Research and Technological Applications, New Borg El-Arab, Alexandria 21934, Egypt*
[2]*Bioprocess Development Department, Genetic Engineering and Biotechnology Research Institute, City of Scientific Research and Technological Applications, New Borg EL-Arab, Alexandria 21934, Egypt*
[3]*Animal Production Research Institute, Agricultural Research Center, Ministry of Agriculture, Dokki, Giza 12619, Egypt*

*Corresponding author's E-mail: hashim_salma@yahoo.com

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ABSTRACT

Green Seaweeds are a valuable feedstock that can be utilized in poultry feed. Due to their recalcitrant polysaccharide structure, their use is still limited in poultry farming. This structure can be broken by a biotechnological approach such as solid-state fermentation (SSF) such as *Trichoderma reesei*, which simultaneously increases the nutritional value of the biomass. The current study aimed to investigate the effect of supplementation of fermented and non-fermented green seaweed (*Ulva lactuca*) on growth performance, nutrient digestibility, carcass characteristics, caecal microbiota, serum biochemistry, and antioxidant status in growing Japanese quails. Japanese quails (n = 375; one day old) were divided into five groups, with three replicates per group (25 quails in each replication). The quails were fed with five experimental diets, namely a control diet (basal diet without supplement), a basal diet supplemented with 1% and 2% green seaweed (GS) as well as 1% and 2% fermented green seaweed (FGS) for 42 days. The results showed that the groups fed FGS had a greater body weight gain and better feed conversion ratio than the other groups. The FGS groups showed the highest digestibility of crude protein and crude fiber, followed by the GS groups. FGS supplementation decreased abdominal fat percentage while increasing the bursa of Fabricius weight. The count of *Lactobacillus* was significantly increased in quails fed either GS or FGS, while *Clostridium perfringens* and *Escherichia coli* were decreased. The green seaweed-fed groups had significantly greater total protein, albumin, and globulin levels than the control group. Total lipids, triglycerides, cholesterol, HDL, and LDL were decreased in quails-fed diets containing 1% and 2% FGS. The quails in FGS diet groups had higher levels of total antioxidant capacity, catalase, and superoxide dismutase than the other groups, but lower levels of MDA. In conclusion, adding up to 2% fermented *Ulva lactuca* to the basal diet of Japanese quail promotes the growth and health of quails.

Keywords: Antioxidant status, Caecal microbiota, Fermentation, Green seaweed, Performance, Lipid profile, Japanese quail

INTRODUCTION

There is currently a great interest in using seaweed in poultry feed. Seaweed, as a rich source of bioactive components, can improve poultry health and performance while it increases the quality of poultry products (meat and eggs) when incorporated into feed (Holdt and Kraan 2011; Michalak and Mahrose, 2020). Seaweeds offer a lot of

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promise as mineral feed additives, especially Ca, Cu, Fe, I, Mg, Mn, P, Se, and Zn (Abu Hafsa et al., 2019; 2021). Seaweed-chelated micrometals are more readily available to animals (including poultry) than inorganic compounds (Evans and Critchley, 2014). Green seaweed species, such as *Ulva lactuca*, contain a variety of compounds that are not found in terrestrial plants, such as ulvan, alginate, and fucoidan, that have various biological functions, including the ability to modulate gut health due to their prebiotic, antimicrobial, antioxidant, anti-inflammatory, and immunomodulatory effects (Li et al., 2018; Cañedo-Castro et al., 2019). *Ulva* sp. may have higher levels of protein (15-20%), essential amino acids, fiber (290-670 g/kg), with both soluble and insoluble fibers (Øverland et al., 2018), polysaccharides, minerals, vitamins (A, B12, C, D, E, and K), lipids, polyunsaturated fatty acids including omega-3 fatty acid, pigments, and many antioxidant compounds, including polyphenols (Holdt and Kraan, 2011; Li et al., 2018; Øverland et al., 2018). El Basuini et al., (2023) reported that incorporating Arthrospira or Chlorella in the diet of rabbits improved performance, nutrient utilization, intestinal efficacy, and antioxidants. Fermentation of feed with microorganisms can result in functional feeds formulated to enhance gut microbiome, performance, and health, with the potential to improve feed quality for poultry and animals by increasing the availability of fiber and nutrients (Al-Harthi and El-Deek, 2012) and elongating the storage period (Choi et al., 2014; Dewi et al., 2019). Fermentation is an environmentally friendly safe technique that has various advantages, including reducing the risk of microbial contamination during storage and allowing obtaining a wide variety of bioactive compounds (Missotten et al., 2013; Xie et al., 2016), improving the anticoagulant, and anti-inflammatory properties of seaweed, as well as increasing the antioxidants and bioactive potential (Dordević et al., 2010). Thus, fermentation techniques, primarily those involving fungus, are increasingly being used to apply particular enzymes using materials such as substrates. According to a review of the literature, *Trichoderma reesei* (*T. reesie*) is one of the most well-known organisms capable of producing high amounts of enzymes, including cellulases, hemicellulases, and xylanases. Furthermore, beneficial microorganisms utilized in microbial fermentation can improve poultry performance by acting as probiotics. Thus, fermentation of seaweed by microorganisms can lead to synergistic effects (Lin et al., 2016). The findings obtained by Choi et al. (2014) suggested that feeding broilers 0.5% fermented seaweed improved body weight gain, feed conversion ratio (FCR), and immunological status, compared to the control group. It has been reported that fermented feeds improved animal performance and gut morphology (Chiang et al., 2010; Sun et al., 2013), enhance the immunity of animals (Gao et al., 2009; Sugiharto and Ranjitkar, 2018), and modulate the gut microbiota, partially by the selective inhibition of intestinal pathogens (Canibe and Jensen, 2012; Missotten et al., 2015), and improve nutrient digestibility and neutralization of anti-nutritional agents in the feed (Missotten et al., 2015; Wang et al., 2019). *Ulva* sp. has been intensively studied as a feed ingredient for broiler chickens. Abudabos et al. (2013) reported incorporating 3.0% *Ulva lactuca* into broiler diets improved breast muscle yield and dressing percentage, as well as lowered abdominal fat and mortality. These improvements were due to the availability of soluble fibers and sulfur-containing essential amino acids, such as methionine and cysteine. Cañedo-Castro et al. (2019) found that increasing the length of the intestinal villi led to an increase in the intestinal surface area as well as higher brush-border enzyme activity, resulting in a larger surface area for absorption and digestion capacity. In addition, *Ulva* treatments had significantly lower serum triglycerides and total cholesterol levels than the control (Abudabos et al., 2013; Cañedo-Castro et al., 2019). Obviously, whilst promising, further work in this field of application is required. Recently, feed additives pre-fermented with probiotic organisms have attracted attention for their ability to enhance growth performance and gut health in poultry production. The objective of this study was to investigate the effects of supplementation of fermented and non-fermented green seaweed byproducts on growth performance, nutrient digestibility, carcass traits, caecal bacterial population, and serum biochemistry and antioxidants status parameters in growing Japanese quail.

MATERIALS AND METHODS

Ethical approval

All experimental procedures of the study were performed according to the Animal Care and Use Committees and approved by the ethics committee of the City of Scientific Research and Technological Applications (Protocol No. 33-2B-0621), Alexandria, Egypt.

Collection and preparation of green seaweed (*Ulva lactuca*)

Green seaweed (*Ulva lactuca*) was handpicked from submerged rocks in Abu Qir Bay, on the Mediterranean

Sea coast of Alexandria, Egypt. The collected *Ulva lactuca* was washed and rinsed three times in fresh water to remove sand and debris before being sun-dried for three days. The dried samples were ground into powder and placed in airtight bags for further chemical analyses. The chemical analysis of *Ulva lactuca* powder as a feed additive is presented in Table 1.

Table 1. The chemical composition of non-fermented and fermented green seaweed

Items	Non-fermented green seaweed	Fermented green seaweed
Chemical analysis (Percentage on a dry matter basis)		
Organic matter	81.67	78.59
Crude protein	21.05	31.77
Crude fiber	9.88	7.69
Ether extract	3.18	2.87
Nitrogen free extract	47.56	36.6
Ash	18.33	21.41
Neutral detergent fiber	38.44	35.52
Acid detergent fiber	24.28	22.06
Acid detergent lignin	7.36	7.02
Hemicellulose	14.16	13.46
Cellulose	16.92	15.04
Neutral detergent fiber nitrogen	34.26	30.11
Non-fiber carbohydrate	23.18	13.84
Mineral composition (mg/kg)		
Sodium	193.8	211.7
Potassium	96.9	102.3
Calcium	72.4	78.6
Magnesium	200.1	210.5
Phosphorus	306.4	311.9
Iodine	188.9	174.6
Lead	0.052	0.047
Cadmium	0.029	0.021
Iron	2.06	2.11
Cobalt	0.10	0.04
Manganese	0.08	0.04
Selenium	1.11	0.99
Zinc	0.84	0.91

Fermentation of green seaweed with *Trichoderma reesei*

In this study, the fermentation process was a solid-state fermentation type (SSF). Fermentation of green seaweed (*Ulva lactuca*) was performed using *T. reesie* (ATCC 28217) for 96 h at 37°C and under aerobic conditions. The *T. reesie* was obtained from the Microbiology Research Center (MIRCEN), Faculty of Agriculture, Ain Shams University, Egypt. The total substrate consisted of *Ulva lactuca* seaweed powder, 4% molasses, 0.4% urea (46.5% N), 0.2% KH2PO4, 0.03 MgSo4 (7H2O), and water; the ratio of green seaweed powder to water was 1:2 from the total volume of the substrate. The fermentation endpoint was determined from total sugar, reduced sugar, and pH according to Abd Ellatif et al., (2019). After fermentation, green seaweed was dried in an oven and then ground into a powder in a blender. The chemical composition analysis was performed for FGS to compare GS byproducts before and after fermentation (Table 1). Fermentation conditions were handled according to the optimum growth conditions in Abd Ellatif et al (2019). The fermented green seaweed byproduct was stored at –20°C until supplementation.

Experimental design, diets, and laboratory analyses

A total of 375 unsexed one-day-old Japanese quail quails (average weight of 8.53 ± 0.34) were distributed randomly into five equal experimental treatments (75 quails each) and three replicates (25 quails each). Dietary treatments included the control (basal diet without any supplementation), while the second and the third treatments received the basal diet containing green seaweed (GS) at 1% and 2% of the diet respectively. The fourth and fifth treatment groups received the basal diet containing fermented green seaweed (FGS) at 1% and 2% of the diet respectively. All quails were reared in wire battery cages in a well-ventilated room and kept under the

same managerial, hygienic, and environmental conditions. In the first 4 days, the brooding temperature was 33°C. Then, it was lowered to 30°C until the end of the first week, and it was reduced to 28°C in the second week. From the third week, the ambient temperature was maintained at around 25°C and the relative humidity was between 60 and 70%, with 23 hours/day light throughout the experimental period. Feed and fresh water were available *ad libitum* throughout the entire 42-day experimental period. The experimental diets were formulated according to the NRC (1994) and presented in Table 2.

Table 2. Ingredients and chemical composition of the basal diet of growing Japapnese quail.

Ingredients (%)	Starter phase (0-3 weeks)	Finisher phase (4-6 weeks)
Yellow corn	54.1	60.4
Soybean meal (44% CP)	28.5	22.3
Protein concentrate*	10.00	9.1
Wheat bran	6.00	6.8
Vegetable oil	0.50	0.50
Dicalcium phosphate	0.20	0.20
Vitamin and mineral premix**	0.30	0.30
L-lysine	0.15	0.15
Dl- methionine	0.25	0.25
Total	100	100
Proximate chemical analysis (%)		
Crude protein	23.07	20.46
Crude fiber	3.42	3.71
Ether extract	4.73	4.94
Calculated nutritional values		
Metabolizable energy (MJ /kg)	14.712	14.80
Calcium (%)	0.83	0.79
Available phosphorus (%)	0.33	0.29

*Protein concentrate contained: 52% Crude protein, 2.03% Crude fiber, 6.17% Ether extract, ME 2080 (Kcal/Kg), 1.50 % Methionine, 2.00% Methionine and Cystine, 3.0% Lysine, 7.00% Calcium, 2.93% available Phosphorus, and 2.5% Nacl. **Each 3 kg of vitamin and mineral premix contains (per ton of feed), Vit. A 12000000 IU, Vit. D3 2000000 IU, Vit. E I0g, Vit. K3 1000 mg, Vit. B1 1000 mg, Vit. B2 5g, Vit. B6 1.5g, Vit. B12 10 mg, Pantothenic acid 10g, Niacin 30g, Folic acid 1g, Biotin 50 mg, Iron 30g, Manganese 60g, Choline chlorite 10g, Iodine 300 mg, Copper 4g, Zinc 50g, and Selenium 100 mg.

Growth performance

Individual quail weights were recorded weekly to determine the final body weight (FBW), and the feeder in each cage was recorded daily to determine daily feed intake (DFI). Body weight gain (BWG) is the final body weight (g) - initial body weight (g), and feed conversion ratio (g feed/g gain) was calculated. The mortality rate was recorded daily, and at the end of the experiment, the percentage was recorded for each group.

Nutrient digestibility

During the final week of the trial, 8 quails (4 males and 4 females) were chosen from each replicate/treatment and placed in individual battery cages for the digestibility study. Quails were allowed to acclimate for 2 days, then the feed intake was recorded and faeces samples were gathered daily from each cage before feeding in the morning for 5 consecutive days. All the fresh samples were stored at -20°C for further analysis. Before being pulverised for chemical analysis the feed and excreta samples were oven-dried for 48 h at 70°C.

Carcass characteristics and caecal microbes

At the end of the experimental period (at 42 days of age), 24 quails (12 males and 12 females) per treatment were randomly chosen and the quails were weighed and slaughtered using the Islamic Halal method after 12 hours of feed deprivation. Carcass traits were assessed after complete bleeding, and the weights of abdominal fat, gizzard, and bursa of Fabricius were weighed and calculated as a percentage of the live body weight. Dressing percentage was computed as carcass weight relative to slaughter weight. The lengths (cm) of the intestine and caecum were determined. To determine caecal bacterial counts, another three quails from each replicate/treatment were euthanized by cervical dislocation. Caeca were quickly removed and their contents were collected in sterile tubes. Tenfold dilutions of 1 g of each sample were serially prepared in phosphate buffer solution and poured directly onto Petri dishes containing culture media. *Lactobacillus sp*. and anaerobic bacteria were cultured in de Man–Rogosa–Sharpe (MRS) agar and incubated in anaerobic conditions for 48 hours at 37°C. Total aerobic and *Escherichia coli* bacteria were plated on MacConkey agar and incubated aerobically for 24 h at 37°C. *Clostridium perfringens* was plated on a Perfringens agar base (Oxoid) mixed with 400 mg of D-cycloserine/liter and incubated under anaerobic conditions for 48 h at 37°C according to (Abu Hafsa and Hassan, 2022). Bacterial colony-forming units (CFU) in the Petri

dishes were counted using a range of 30-300 cfu/g, depending on the growth characteristics of the bacterial species. The counts were expressed as log cfu/g.

Chemical analyses

Green seaweed, feed, feces, and meat samples were finely pulverized in a Cyclotec mill (Cyclotec 1093; Foss, Germany) and stored before chemical analysis. Moisture was determined in oven-dried samples at 70°C to constant weight. The content of CP (N 6.25) was determined according to Kjeldahl's method (Method No. 978.04, AOAC, 2005). The ether extract was determined according to the Soxhlet extract method using petroleum ether as an extracting agent (40-60°C, AOAC, 2005). Ash content was determined by incinerating the samples in a muffle furnace at 550°C. The contents of neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL) were determined using a Tecator Fibretic System, according to the method of Van Soest et al. (1991). The content of nitrogen free extract (NFE) was calculated as NFE (%) = 100 % – (EE perncetage + CP perncetage + Ash perncetage + CF perncetage). Cellulose was measured as Cellulose (%) = ADF percentage - ADL percentage and hemicellulose was calculated as hemicellulose (%) = NDF percentage - ADF percentage. Mineral elements such as calcium, cadmium, cobalt, iron, lead, magnesium, manganese, phosphorus, potassium, sodium, and zinc were analyzed after samples were dissolved in 1 $MHNO_3$ and H_2O_2 and microwaved. The elements' concentrations in dried seaweed samples were determined with an atomic absorption spectrophotometer (Unicam 919; Unicam Ltd., Cambridge, UK), whereas phosphorus was colorimetrically determined using molybdovanadate reagent. Nitschke and Stengel (2015) method was used to determine Iodine. Selenium was determined by inductively coupled plasma–mass spectrometry (ICP-MS, Lavu et al., 2013).

Blood biochemical parameters and antioxidant status

At the end of the experiment, 24 quails from each treatment were slaughtered by severing the jugular vein to collect blood samples into sterile tubes. Subsequently, blood samples were immediately centrifuged for 15 minutes at 2000 × g by a centrifuge (T32c; Janetzki, Wallhausen, Germany), and stored at -20°C for further biochemical analysis. Total protein, albumin, glucose, total lipids, triglyceride, total cholesterol, low-density lipoprotein (LDL), and high-density lipoprotein (HDL), commercial kits from the biodiagnostic company (Giza, Egypt) were determined calorimetrically using standard kits supplied by the biodiagnostic company (Cairo, Egypt), according to the manufacturer's instructions. The total globulin fraction is generally determined by subtracting the albumin from the total protein. Total antioxidant capacity (TAC), catalase, superoxide dismutase (SOD), and malondialdehyde (MDA) were determined using commercial kits from Biodiagnostic Company (Giza, Egypt) and a spectrophotometer (Shimadzu, Japan) according to the manufacturer's instructions.

Statistical analyses

Data were subjected to statistical analyses in a randomized complete block design using the general linear model procedures of SAS/STAT (Statistical Analysis System, version 9.3, SAS Institute Inc., Cary, NC, USA) (2011). The obtained data were tested by an analysis of variance with a one-way design to test the treatment at each sampling, according to the following formula:

$$Yij = \mu + Ti + Eij$$

Where Yij denotes the represented observation in the j^{th} non-fermented or fermented green seaweed, μ is the overall mean effect, Ti signifies the effect of the j^{th} non-fermented or fermented green seaweed, and Ɛij refers to the random error associated with the j^{th} birds assigned to the i^{th} non-fermented or fermented green seaweed treatment. The means were compared using Duncan's Multiple Range Test (Duncan, 1955). Significant differences among means were considered to be significant at the $p < 0.05$. All results are presented as the least squares mean.

RESULTS

Chemical composition of fermented and non-fermented green seaweed

The chemical and mineral compositions of non-fermented green seaweed (GS) and fermented green seaweed (FGS) are shown in Table 1. Contents of organic matter, crude fiber, ether extract, NFE, ADF, ADL, NDFn, NFC, cellulose, and hemicellulose were decreased after fermenting green seaweed with *T. reesie*. Fermenting green seaweed with *T. reesie* increased crude protein ash content. An appreciable increase in mineral content was observed in green seaweed when fermented with *T. reesie* compared to non-fermented seaweed.

Growth performance

Table 3 shows that quails fed the control diet and those provided a diet supplemented with GS and FGS had

significant differences ($p < 0.05$) in final body weight, body weight gain, and FCR. Final body weight, body weight gain, and FCR were highest ($p < 0.05$) in quails fed a diet supplemented with 1% and 2% FGS, followed by those fed a diet supplemented with 1% and 2% GS. However, there were no significant variations in DFI between the treatment groups ($p > 0.05$). The increase in the birds' general health is shown in the lower mortality rate of birds fed the diet with GS or FGS compared with the control diet.

Nutrient digestibility

The nutrient digestibility of the experimental diets provided to Japanese quail is shown in Table 4. In general, dry matter, organic matter, crude protein, and crude fiber digestibility values increased significantly ($p < 0.05$) with quail-fed diets supplemented with GS or FGS compared with the control group, except NFE, which did not differ significantly ($p > 0.05$). Quails fed 1% and 2% FGS had the best CP and CF digestibility, followed by GS-fed quails.

Carcass characteristics and caecal microbes

Adding green seaweed, either non-fermented or fermented, to the basal diet significantly increased carcass yield, intestinal and cecum lengths, and bursa of Fabricius weight ($p < 0.05$, Table 5). Quails fed the 1% and 2% FGS diet had the longest ($p < 0.05$) intestinal and cecum lengths and the highest ($p < 0.05$) weight of bursa of Fabricius, followed by those fed 1% and 2% GS. Quails fed the 1% and 2% FGS diet had the lowest ($p < 0.05$) abdominal fat percentage followed by those fed 1% and 2% GS compared to the control diet. The experimental treatments did not affect the weights of the liver and gizzard. Quails fed either GS or FGS had reduced meat moisture compared with the control group ($p < 0.05$, Table 5). Quails fed 1% and 2% FGS diets had the highest CP content but the lowest EE content, followed by those fed 1% and 2% GS. The meat ash content was higher in quails fed either GS or FGS than those in the control ($p < 0.05$).

In groups fed 1% or 2% of GS or FGS, total anaerobic bacteria counts increased, but total aerobic bacteria count decreased significantly ($p < 0.05$, Table 5). In quails fed with either 1% or 2% of GS or FGS, the *lactobacillus* count increased dramatically, whereas the *Escherichia coli* and *Clostridium perfringens* counts declined significantly ($p < 0.05$). Quails fed 1% and 2% FGS had the highest *Lactobacillus* count but the lowest *Escherichia coli* and *Clostridium perfringens* counts.

Blood biochemistry and antioxidant status

The effect of the experimental diets on serum biochemical parameters and antioxidant status is shown in (Table 6). Total protein, albumin, and globulin in the serum were significantly greater in the green seaweed treatment groups than in the control group ($p < 0.05$). However, quails fed either the GS or FGS diet had significantly lower AG ratio, total lipids, triglycerides, cholesterol, HDL-c, and LDL-c concentrations than those on the control diet ($p < 0.05$). Quails fed the 1% and 2% FGS diet exhibited the lowest reduction in total lipids, triglycerides, cholesterol, HDL-c, and LDL-c levels compared with the control group, while there were no variations in glucose levels between treatment groups. Quails fed 1% and 2% FGS diets had the highest ($p < 0.05$) levels of TAC, catalase, and SOD but the lowest level of MDA, followed by those fed 1% and 2% GS ($p < 0.05$, Table 6). However, no significant differences were observed between quails fed 2% GS and quails fed 1% GS, or between those fed 1% and 2% FGS ($p > 0.05$).

Table 3. Effects of dietary supplementation of fermented or non-fermented green seaweed on growth performance of Japanese quail

Parameters \ Treatments	Control	GS 1 %	GS 2 %	FGS 1 %	FGS 2 %	SD	P-value
Initial body weight (g)	8.52	8.44	8.61	8.58	8.49	0.34	0.873
Final body weight (g)	198.34^{c}	209.52^{b}	211.61^{b}	215.73^{a}	218.54^{a}	2.17	0.024
Body weight gain (g)	4.52^{c}	4.79^{b}	4.83^{b}	4.93^{a}	5.00^{a}	0.05	0.001
Daily feed intake (g)	14.45	14.33	14.36	14.44	14.39	0.42	0.788
Feed conversion ratio	3.20^{a}	2.99^{b}	2.97^{bc}	2.93^{c}	2.88^{c}	0.05	0.002
Mortality rate (%)	4.00^{a}	1.33^{b}	1.33^{b}	2.67^{b}	1.33^{b}	1.14	0.016

$^{a-c}$ Different superscript letters in the same row differed significantly at $p < 0.05$. GS: Green seaweed. FGS: Fermented green seaweed. SD: Standard deviation

Table 4. Effects of dietary supplementation of fermented or non-fermented green seaweed on the nutrient digestibility of Japanese quail

Parameters \ Treatments	Control	GS 1 %	GS 2 %	FGS 1 %	FGS 2 %	SD	P-value
Dry matter (%)	75.39 [b]	78.35 [a]	78.42 [a]	79.33 [a]	79.39[a]	1.45	0.029
Organic matter (%)	76.41 [b]	77.95 [a]	78.05 [a]	78.88 [a]	78.82 [a]	0.78	0.016
Crude protein (%)	73.26 [c]	74.53 [b]	74.66 [b]	75.84 [a]	75.97 [a]	0.36	0.001
Crude fiber (%)	45.18 [c]	49.07 [b]	50.16 [b]	52.58 [a]	52.85 [a]	1.12	0.011
Nitrogen free extract (%)	78.02[b]	90.29[a]	90.26[a]	90.83[a]	90.77[a]	2.68	0.721

[a-c] Different superscript letters in the same row differed significantly at $p < 0.05$. GS: Green seaweed. FGS: Fermented green seaweed, SD: Standard deviation

Table 5. Effects of dietary supplementation of non-fermented or fermented green seaweed on carcass traits and caecal bacterial population of Japanese quail

Parameters \ Treatments	Control	GS 1 %	GS 2 %	FGS 1 %	FGS 2 %	SD	*P*-value
Carcass yield (%)	69.27[b]	70.73[a]	70.66[a]	70.83[a]	70.88[a]	0.32	0.026
Liver weight (%)	2.21	2.15	2.09	2.13	2.06	0.18	0.726
Abdominal fat (%)	1.42[a]	1.26[b]	1.19[b]	1.12[c]	1.06[c]	0.05	0.031
Gizzard weight (%)	2.57	2.61	2.48	2.55	2.64	0.22	0.835
Intestinal length (cm)	57.34[c]	60.44[b]	60.16[b]	62.32[a]	63.06[a]	0.74	0.001
Cecum length (cm)	9.11[c]	9.86[b]	9.88[b]	10.21[a]	10.22[a]	0.11	0.006
Bursa of fabricius (%)	0.08 [c]	0.11 [b]	0.11 [b]	0.14 [a]	0.15 [a]	0.01	0.001
Chemical composition of meat (%)							
Moisture	72.39[a]	71.06[b]	70.97[b]	70.83[b]	70.99[b]	0.27	0.021
Crude protein	20.05[b]	20.88[a]	20.82[a]	20.94[a]	21.01[a]	0.22	0.033
Ether extract	4.29[a]	3.96[b]	3.84[b]	3.91[b]	3.81[b]	0.25	0.007
Ash	1.45	1.51	1.55	1.53	1.56	0.13	0.578
Caecal bacterial count (log cfu/g)							
Total anaerobic bacteria	5.87 [c]	6.43 [b]	6.59 [ab]	6.77 [a]	6.89 [a]	0.21	0.026
Total aerobic bacteria	5.73 [a]	5.24 [b]	5.04 [cb]	4.92 [c]	4.75 [c]	0.17	0.018
Lactobacillus	6.41 [c]	6.92 [b]	7.19 [b]	7.47 [a]	7.58 [a]	0.10	0.001
Escherichia coli	4.55 [a]	4.16 [b]	4.08 [b]	3.89 [c]	3.77 [c]	0.13	0.001
Clostridium perfringens	2.67 [a]	2.22 [b]	2.15 [b]	1.88 [c]	1.76 [c]	0.11	0.001

[a-c] Different superscript letters in the same row differed significantly at $p < 0.05$. GS: Green seaweed. FGS: Fermented green seaweed, SD: Standard deviation

Table 6. Effects of dietary supplementation of non-fermented or fermented green seaweed on serum biochemical parameters and antioxidant status of Japanese quail.

Parameters \ Treatments	Control	GS 1 %	GS 2 %	FGS 1 %	FGS 2 %	SD	P-value
Biochemical parameters							
Total protein (g/dl)	3.37 [b]	3.81 [a]	3.87 [a]	3.89 [a]	3.92[a]	0.06	0.017
Albumin (g/dl)	1.86 [b]	2.04 [a]	2.07 [a]	2.06 [a]	2.09 [a]	0.07	0.024
Globulin (g/dl)	1.51 [b]	1.77 [a]	1.80 [a]	1.89 [a]	1.84 [a]	0.09	0.031
A/G ratio	1.23 [a]	1.15 [b]	1.15 [b]	1.09 [b]	1.14 [b]	0.04	0.012
Glucose (mg/dl)	108.76	111.05	109.76	110.35	110.74	2.73	0.853
Total lipids (mg/dl)	389.37 [a]	278.46 [b]	289.04 [b]	244.61 [c]	248.36 [c]	24.93	0.027
Triglycerides (mg/dl)	96.83 [a]	89.66 [b]	88.04 [b]	68.85 [c]	66.77 [c]	2.06	0.007
Cholesterol (mg/dl)	143.29 [a]	129.73 [b]	121.76 [b]	99.97 [c]	94.06 [c]	12.04	0.004
HDL-c (mg/dl)	84.71 [a]	76.26 [b]	75.33 [b]	66.16 [c]	63.05 [c]	1.52	0.001
LDL-c (mg/dl)	39.21 [a]	35.54 [ab]	28.82 [b]	20.04 [c]	17.66 [c]	7.77	0.004
Antioxidant status							
TAC (U/L)	10.46 [c]	14.64 [b]	15.83 [ab]	16.44 [a]	17.89 [a]	1.78	0.002
Catalase (U/L)	104.53 [c]	140.86 [b]	145.06 [ab]	149.55 [a]	154.42 [a]	5.05	0.039
SOD (U/L)	28.58 [c]	36.26 [b]	38.27 [ab]	40.07 [a]	43.66 [a]	3.16	0.004
MDA (U/L)	5.73 [a]	4.02 [b]	3.94 [bc]	3.78 [c]	3.58 [c]	0.29	0.001

[a-c] Different superscript letters in the same row differed significantly at $p < 0.05$. GS: Green seaweed; FGS: Fermented green seaweed. HDL-c: High-density lipoprotein concentration, LDL-c: Low-density lipoprotein concentration, TAC: Total antioxidant capacity, SOD: Superoxide dismutase activity, MDA: Malondialdehyde

DISCUSSION

The results showed that the organic matter, crude fiber, NFE, NDF, ADF, ADL, cellulose, and hemicellulose contents of the FGS with *T. reesie* were lower than the GS. This result may be due to the fact that *T. reesie* consumes carbohydrates particularly soluble carbohydrates and crude fiber and its fraction as carbon sources to produce CO_2 and energy and then uses this energy to proliferate and convert nitrogen sources in the media to microbial protein. Moreover, crude protein and ash contents of FGS were higher than GS. The biological treatment increased crude protein from 21.05 to 31.77% for GS and FGS, respectively. The enhancement in the content of CP could be due to fungus growth (El-Ashry et al., 2002). El-Menniawy (2008) reported that treatment of sugar cane bagasse by *T. viride* resulted in a decrease in NFE. Abdel-Azim et al. (2011) found that treated rice straw and corn stalks with *T. viride* had higher CP, ash, and EE content than the untreated substrate. These results might be attributed to the breakdown of lignocellulose bonds where the cellulose can be hydrolyzed by fungi (El-Ashry et al., 2002; Bilal, 2008; Abdel-Azim et al., 2011). The result of the present study demonstrates the potential use of solid-state fermentation (SSF) to biovalorize the nutritional value of seaweeds and produce high-value carbohydrates (data not shown). Especially in the green seaweed *U. lactuca*, *T. reesei* successfully produced enzymes that modified the structure of the seaweed, resulting in increased protein. Therefore, SSF is an environmentally friendly, economical and sustainable biovalorization approach that may lead to nutritionally suitable seaweed for poultry feed.

In this study, green seaweed (*Ulva lactuca*) affected growth performance. The nutritive value of *Ulva lactuca*, which is an enriched natural source of various components including protein, fatty acids, polysaccharides, minerals, and nearly all essential vitamins, has favorable effects due to its palatability and high nutrient content, which can stimulate body metabolism and enhance digestion and nutrient absorption, improved the body weight gain of groups treated with GS or FGS compared with the control group. The considerable enhancement in growth performance found in quails given the green seaweed diet is thought to be due to an increase in necessary amino acids, particularly sulfur-containing amino acids (Burtin, 2003); green seaweed offers remarkable nutritional qualities and could, thus, serve as a feasible alternative source of nutrients for poultry (Al-Harthi and El-Deek, 2012). Furthermore, macroalgae such as green seaweed are an excellent source of iodine, and the results in Table 1 show that *Ulva lactuca* can accumulate large amounts of iodine, which is necessary for thyroid function and health (Burtin, 2003). *Ulva rigida* was utilized in broiler diets at 2%, 4%, and 6% as a prebiotic enriched with microelements cobalt, copper, chromium, manganese, and zinc to improve broilers growth performance (Cañedo-Castro et al., 2019). Erum et al. (2017) reported that adding 5%, 10%, or 15% *Sargassum muticum* as a feed supplement to broiler feeds improved final body weight, average daily gain, FI, and FCR. Because of their prebiotic properties, seaweed polysaccharides can help increase poultry performance and improve general gut health (Kulshreshtha et al., 2014). However, Abu Hafsa and Hassan (2022) reported that the *Sargassum siliquastrum*-supplemented diet did not influence final body weight, average body gain, or average feed intake, but it improved FCR and resulted in a lower mortality rate. Similarly, Bai et al. (2019) reported that supplementing broilers' diet with 1% *L. japonica* enhanced FCR compared with the control. The findings of this study are in agreement with those of previous studies on laying quails (Abu Hafsa et al., 2019) and laying hens (Rizk et al., 2017) and demonstrated that the supplementation of seaweed enhanced their productive performance. A study by Abu Hafsa et al. (2021) showed that 4% of seaweed supplements improved rabbit growth performance. Rabbit diets with Arthrospira at 500 mg/kg and Chlorella at 300 and 500 mg/kg had the highest feed conversion ratio and improved nutrient utilization, according to El Basuini et al. (2023). Sweeney et al. (2012) found that extracts of macroalgae can improve growth performance and health by altering the gut structure, increasing nutrient digestion and absorption, gut microbiota, and/or modulating immune function, thereby enhancing the gut barrier function. Polysaccharides found in macroalgae can act as prebiotics, promoting animal growth and overall health through positive effects on the digestive tract (Vidanarachchi et al., 2009).

In the present study, the digestibility of a diet containing green seaweed was improved using biological treatment, except for NFE. Biological treatments of seaweed and agro-industrial byproducts have been reported to improve palatability and digestibility in several species (Fayed et al., 2009; Okab et al., 2013; Choi et al., 2014). The enhancement upon pretreatment of green seaweed by-product could also be attributed to the production of enzymes by *T. reesie* during fermentation,

which plays a significant role in the efficacy of nutrient breakdown and digestion in the birds' digestive system (Singh et al., 2019). The significant differences between the control group and treatment groups on quail growth performance further confirm the efficacy of the pretreatment of green seaweed to improve the utility of the green seaweed. Abu Hafsa and Hassan (2022) reported that *Sargassum siliquastrum* supplementation improved the digestibility of nutrients in Japanese quails. El Basuini et al., (2023) reported that incorporating Arthrospira or Chlorella in the diet of rabbits improved performance and nutrient utilization.

In this study, a significant increase in carcass yield and a significant decrease in abdominal fat were associated with feeding *Ulva lactuca*. These results are in agreement with those of Abudabos et al. (2013) who found that birds given 3% *Ulva lactuca* had a higher dressing percentage but less abdominal fat than the control group. Wang et al. (2013) found that adding 2%, 3%, or 4% dry green algae enhanced the quality of the breast meat: the fat content was dramatically reduced, and the abdominal fat rate was reduced. Erum et al. (2017) found that supplementation of broiler diets with *Sargassum muticum* improved carcass traits and increased the dressing percentage; however, it decreased the fat content of the carcass. The presence of soluble fibre in *Ulva lactuca*, which was reported to have 21.3% of soluble fibre (Lahaye and Jegou, 1993); could also be responsible for the decreased abdominal fat percentage observed. This is supported by the present findings of serum total lipids and cholesterol analysis. The hypocholesterolemic impact is associated with various properties characteristic of soluble fibre such as fermentability, viscosity, and bile salt binding capacity (Davidson and McDonald, 1998). Another reason contributing to the low content of fat is that *Ulva lactuca* has a high composition of polyunsaturated fatty acids, especially in terms of omega 3 and 6 fatty acids (Wahbeh, 1997). The intestine and cecum lengths of the S. siliquastrum-fed quails were much longer than those of the control group (Abu Hafsa and Hassan, 2022). In general, seaweed has a good impact on meat composition, which is usually improved as a result of fat reduction. Broiler chickens fed a diet containing seaweed had higher protein content but a lower fat content than the control birds (Zahid et al., 2001). *Spirulina* supplementation may improve meat quality by enhancing the integrity of muscle fibers and, as a result, the ability of muscles to retain water (Dal Bosco et al., 2014).

Green seaweed (*Ulva lactuca*) supplementation increased *Lactobacillus* abundance while reducing *Escherichia coli* and *Clostridium perfringens* counts in the quail caecum. Seaweed polysaccharides could be used as prebiotic ingredients in animal health applications, promoting the growth and/or activity of beneficial gut microbiota like *Lactobacillus* sp., which, in turn, helps the host by reducing pathogen invasion and disease (O'Sullivan et al., 2010; Ford et al., 2020). *Lactobacillus* bacteria produce lactic and acetic acids, which decrease the gut pH and render it unsuitable for pathogen growth. *Lactobacillus* bacteria also boost immunity by upregulating the synthesis of intestinal mucins, which limit pathogen adherence to the intestinal epithelium and so prevent pathogen translocation (Gibson and Roberfroid (1995) and Dhama et al. (2008). The observed improvements in the microbial community could be due to a rise in *Lactobacillus* bacteria count, which leads to the establishment of resistance to *Escherichia coli* and *Clostridium perfringens* colonization through a competitive exclusion mechanism. Marine seaweeds contain a wide range of active components and proteins (Øverland et al., 2018); phlorotannins (Gupta and Abu-Ghannam, 2011); and pigments, such as carotenoids (O'Sullivan et al., 2011), which function as prebiotics to promote the growth of beneficial bacteria while preventing the growth of pathogenic bacteria, hence, improving overall health (Vidanarachchi et al., 2009).

Total protein intake has a significant impact on blood protein profile. As a result, the higher total serum protein level of quails fed GS or FGS in the diet could be an indication of high protein content. An alteration in normal systemic protein utilization is usually indicated by abnormal serum albumin. Furthermore, a low albumin/globulin ratio in GS or FGS groups showed higher disease resistance and immunological response in growing quails, implying that GS or FGS groups' immunity was improved over the control group. Rizk et al. (2017) found that adding dried green seaweed to the diet of Sinai hens (0.1 and 0.2 %) reduced total lipids, triglycerides, cholesterol, and LDL levels when compared to the control group. Al-Harthi and El-Deek (2012) stated that *Sargassum* utilized as a feed additive in different forms (sun-dried, autoclaved, or boiled) at a dose of 3% or 6% significantly lowered triglycerides, cholesterol, and HDL levels when compared with the control hens. El Basuini et al., (2023) found that arthrospira- or chlorella-containing algae groups had higher serum total protein levels and lower total cholesterol. Ulvan contains a significant amount of sulfate, which has the ability to deconstruct cholesterol and could be used as an antihyperlipidemic medication, contributing to the

reduction in cholesterol content (Qi and Sheng, 2015). Cañedo-Castro et al. (2019) suggested that dietary seaweed can be an alternative for improving intestinal integrity and lowering serum cholesterol concentrations.

A bird's antioxidant status is important for its resistance to infection, health maintenance, and productivity (Surai, 2002). Abdel-Daim et al. (2015) found that increases in TAC, catalase, and SOD and decreases in MDA, were indicators of decreased levels of lipid peroxidation. The most essential antioxidant enzyme, superoxide dismutase (SOD), is required for the elimination of superoxide in animals (Vijayavel et al., 2007). The significant rise in SOD values in *Ulva lactuca*-supplemented groups could imply a strong association between seaweed supplementation and better antioxidant capacity, as reported by Droge (2002). Seaweed is a promising source of bioactive peptides with numerous beneficial properties, including antioxidant potential (Chandini et al., 2008; Fan et al., 014). Li et al. (2018) reported that the concentrations of TAC and SOD were considerably greater in the groups supplemented with 0.5% to 1% ulvan than in the control group; nevertheless, MDA concentration was lower. Abu Hafsa and Hassan (2022) reported that quail treated with *Sargassum siliquastrum* had higher TAC and SOD than the control group. El Basuini et al., (2023) found that the groups fed an algal diet including either Arthrospira or Chlorella had the best GPx, whereas excellent SOD and CAT efficiency occurred at 500 mg/kg of Arthrospira and 300 and 500 mg/kg of Chlorella. Sulphfated polysaccharides, specific to marine algae, are structurally analogous to animal glycosaminoglycans, which explains their high reactivity and specific biological activities when administered to animals (Suarez, 2019). Sulfated polysaccharides have an excellent antioxidant effect in vitro, including the ability to scavenge radicals (Wang et al., 2016).

CONCLUSION

Supplementation of up to 2% fermented green seaweed to the basal diet of Japanese quail promotes the growth and health of quail. *T. reesei* successfully produced enzymes that modified the structure of the seaweed, resulting in increased protein, especially in green seaweed. This finding led to an increase in growth performance, the beneficial caecal bacterial population, immunity, and antioxidant status, while lowering triglycerides, cholesterol, and pathogenic bacteria and improving the intestinal health of treated birds. These findings suggested that SSF has a promising future in biovalorizing the nutritional value of green seaweed and producing high-value carbohydrates.

DECLARATIONS

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This study was conducted at the Noubaria experimental station, Animal Production Research Institute, and the Agricultural Research Centre. All experimental procedures of the study were performed according to the Animal Care and Use Committees and approved by the ethics committee of the City of Scientific Research and Technological Applications (Protocol No. 33-2B-0621), Alexandria, Egypt.

Authors' contributions

Abu Hafsa and Hassan created the idea and designed the study, collected data, wrote the paper, and performed the statistical analysis. Formal analysis and methodology carried on by Abu Hafsa, Abd-Ellatif, Abdel Razik, and Hassan. Abu Hafsa drafted the manuscript and approved the final manuscript. All authors checked and confirmed the final analysis data and the last revised manuscript before publication in the journal.

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Competing interests

The authors declared that they have no competing interests.

Availability of data and materials

The data presented in this study are available on request from the corresponding author.

Ethical considerations

Ethical issues (including plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/submission, and redundancy) have been checked by the authors before the submission. The final results of the statistical analysis have been also checked and confirmed by all authors.

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Epidemiological Investigation of *Eimeria* Species and Effectiveness of Togolese Medicinal Plants Used Against Chicken Coccidiosis

Ferdinand G. Tchodo[1,2]*, Hervé B. Dakpogan[2], Banfitebiyi Gambogou[3], Ombortime N'nanle[1], Benjamin Adjei-Mensah[1], Kokou Tona[1], and Batomayena Bakoma[4]

[1]*Regional Center of Excellence in Poultry Science, University of Lome, BSP 1515, Lome, Togo*
[2]*School of Animal Production System Management P.O.BOX 43 Ketou, National Agricultural University, 01 P.O.BOX 55 Porto-Novo, Benin*
[3]*Institut Togolais de Recherche Agronomique, BSP 1163, Lome, Togo*
[4]*Pharmaceutical Sciences Research Laboratory, University of Lome, BSP 1515, Lome, Togo*

*Corresponding author's E-mail: gregoiretchodo@gmail.com (F.G. Tchodo)

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ABSTRACT

Eimeria species cause coccidiosis, a poultry disease that occurs worldwide. Infection is linked to decreased feed efficiency and body weight increase. The present study aimed to assess the prevalence of coccidian species in Togolese poultry farms and evaluate the anticoccidial efficacy of three local medicinal plants. From July to September 2023, two hundred and ninety-five fecal samples were randomly collected using a cross-sectional observational study in the maritime region of Togo, specifically in Vo, Lacs, Zio, and Grand-Lomé districts. Data on risk factors were collected through an interview with the poultry farmers. All fecal samples collected were subjected to *Eimeria* oocyst counting using the standard McMaster technique. The anticoccidial activity of the extract of *Azadirachta indica* leaves *Carica papaya* seeds, and *Sarcocephalus latifolius* roots in a completely randomized design was evaluated on 23-day-old male Isa brown chicks infected with 30.10^4 oocysts. Body weight gain, feed efficiency, lesion score, proportion of bloody droppings, anticoccidial index, and excretion of coccidia oocysts were assessed. The results revealed an overall prevalence of 39.66% (117/295) for coccidiosis, with 75% of positive samples having fewer than 10,000 oocysts/g. The logistic regression test indicated that the interval between two anticoccidial prophylaxis applications, age, management, and breed were significant risk factors associated with coccidial infection, with young chicks (≤ 8 weeks) being 5.66 times more susceptible than those older ones (8 weeks) with 0.86 as an odd ratio. Six *Eimeria* species were identified, with *E. maxima* (54.17%), *E. brunetti* (33.33%), and *E. tenella* (25%) being the most common. The anticoccidial efficacy of *Azadirachta indica* leaves, *Carica papaya* seeds *Sarcocephalus latifolius* roots extract, and amprolium was demonstrated by a reduction in lesion scores, bloody diarrhea, and oocysts per gram in feces (OPG) as well as an improvement in body weight, feed conversion ratio, and production efficiency factor when compared to infected and untreated groups. The anticoccidial index was marked in the chickens treated with *Sarcocephalus latifolius* roots extract (170) and amprolium (176). The findings of this large-scale epidemiological study and anticoccidial efficacy tests revealed that these Togolese medicinal plants can be sustainable and cost-effective strategies for coccidiosis control.

Keywords: Anticoccidial drug sensibility, Coccidiosis, Prevalence, Risk factor, Togo

INTRODUCTION

Despite breakthroughs in preventative and control measures through chemotherapy, diet, management, and genetics, coccidiosis remains one of the most costly and pervasive diseases that impact poultry and other domestic species (Tanweer et al., 2014; Kadykalo et al., 2018). It is a gastrointestinal tract (GIT) disease caused by a microscopic protozoan parasite (coccidia) from the genus *Eimeria* in the phylum Apicomplexa (Gilbert et al., 2011).

Eimeria acervulina, *Eimeria brunetti*, *Eimeria maxima*, *Eimeria mitis*, *Eimeria necatrix*, *Eimeria praecox*, *Eimeria tenella*, *Eimeria lata*, *Eimeria nagambie*, *Eimeria zaria* have all been identified and classified as domestic species affecting chickens (Abbas et al., 2012; Blake et al., 2021). The oocyst, expelled with the feces sporulates two days later in the environment and remains as the infectious form of the parasite. This sporulation occurs under conditions of poor litter management (wet or water-soaked litter promotes sporulation), contaminated drinkers and feeders, poor ventilation, and high stocking density. Chicks become infected by the ingestion of sporulated oocysts in contaminated water and feed (McDougald, 2003). *Eimeria* invade the intestinal mucosa and damage the epithelium (anorexia, difficulty in digestion and nutrient absorption, dehydration, diarrhea, and blood loss at three to five days post-infection), which can lead to the leakage of serum into the gut and stimulation of mucus production, both of which can provide a rich source of nutrients for opportunistic bacteria (*C. perfringens*) proliferation (Moore, 2016). The proliferation of sporocysts in the epithelial cells of the intestine leads to anorexia, difficulty in digestion and nutrient absorption, dehydration, and blood loss at three to five days post-infection (Hauck et al., 2019). The macroscopic lesions in the digestive tract predispose poultry to many bacterial gastrointestinal diseases, such as *clostridiosis*, *salmonellosis*, and *colibacillosis* (Dakpogan et al., 2018). Immunosuppressive viral diseases, including infectious bursal disease, Marek's disease, and infectious viral anemia of chicks also exacerbate coccidiosis (Lanckriet et al., 2010). According to Blake et al. (2020), the yearly cost of controlling coccidiosis in hens in Brazil, Egypt, Guatemala, India, New Zealand, Nigeria, and the United States for anticoccidial medications (e.g., ionophores and synthetic chemicals) in feed or water is £10.36 billion (recalculating internationally). In West Africa and especially in Togo, the poultry industry has recently experienced growth in terms of meat and egg production according to the Food and Agriculture Organization of the United Nations (FAO, 2015). The estimated poultry population in Togo increased from 18 million in 2014 to more than 25 million in 2017 with the establishment of parent farms and/or day-old chick producers (DSID, 2018). This subsector has played a significant role in poverty alleviation and food security, particularly in rural regions, by providing direct or indirect employment to both male and female communities (Islam et al. 2012). However, poultry production systems are still confronting several infectious diseases, such as coccidiosis, which have a negative impact on their performance and create barriers to maximum production (Sharma et al., 2013; Zhang et al., 2013; Zhuang et al., 2014). Given the unfavorable clinical and economic impact of the disease, special attention should be paid to coccidiosis and regular epidemiological assessment of *Eimeria* species present in poultry farms should be conducted for control and prevention strategies other than expensive synthetic anticoccidials, which may leave residues in poultry-derived food that are harmful to the health of consumers. Researchers recently focused on therapeutic plants and their derivatives, such as papaya, garlic, neem and moringa. They obtain beneficial phytochemicals, substances against induced eimeriosis and innocuous consumers for chicken products (egg and meat) (Wunderlich et al. 2014; Bauri et al., 2015; Muthamilselvan et al. 2016; Thagfan et al. 2017; Dakpogan et al. 2019). Therefore, the purposed of this study was to offer an up-to-date status of coccidiosis in Togo by studying the occurrence of *Eimeria* infection in poultry farms and comparing the anticoccidial activity of *Azadirachta indica*, *Sarcocephalus latifolius*, and *Carica papaya* decoction with synthetic medications in broiler chickens challenged with *Eimeria tenella*.

MATERIALS AND METHODS

Ethical approval

The ethics and scientific committee of the Regional Center of Excellence in Poultry Sciences, University of Lome (CERSA/UL) and the Livestock Directorate (DE) of Togo's Ministry of Agriculture, Livestock, and Rural Development (MAEDR) with reference number 328/DE dated June 16, 2023, had approved the current study.

Study area

The study was carried out in the Maritime Region of Togo, specifically in Vo, Lacs, Zio, and Grand-Lome district. Geographically, Togo is a West African country located approximately between 6°N and 1°E at a minimum altitude of 0m from the Atlantic Ocean and a maximum altitude of 983m from Mount Pic d'Agou. This Southern region of Togo experiences annual temperature variations (from 23°C to 32°C) with an average rainfall of up to 1200 mm per year. The sub-equatorial or Guinean climate characterizes this region with two rainy seasons (March to July or monsoon, and September to October) and two dry seasons (November to February or Harmattan, and July to September). According to FAO (2011), Togo is characterized by a traditional and commercial poultry production system, with the majority (82%) of farms located in the Maritime region (Figure 1).

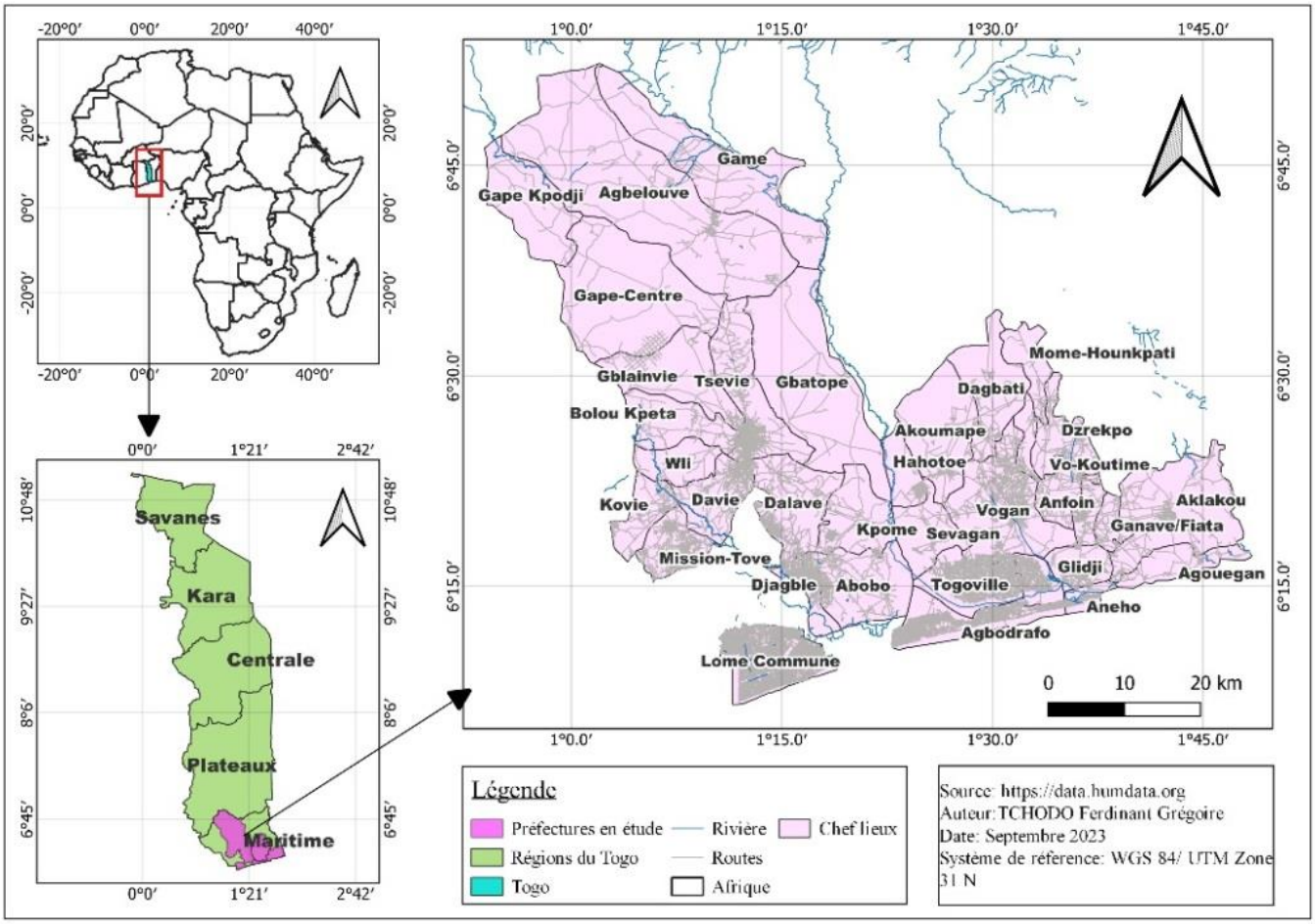


Figure 1. Map of the maritime region, Togo

Epidemiological study design

Sampling procedure

A cross-sectional observational study was conducted in poultry farms in the maritime region to assess the prevalence and intensity of coccidian infection and identify prevalent *Eimeria* oocyst species in the region. The optimal sample size of 42 poultry farms was determined by considering the theoretical coccidiosis prevalence (31%) in a litter-based layer-rearing system (Lunden et al., 2010). To achieve this, a sampling method combining stratified sampling with weighting and convenience sampling within each district of the region was employed.

Sample collection

Two-hundred ninety-five (295) samples of chicken feces were collected from randomly selected poultry farms in the Maritime Region of Togo between July and September 2023. All studied farms have no history of using the coccidiosis vaccination in their programs. The 295 fecal samples were collected from broiler farms, laying hen farms, local chicken farms, and broiler breeder farms. Additionally, the type of farms management consisted of cage (6) litter-based system (260) and free ranged (29). Each sample consisted of freshly egested manure collected from several chickens or a pooled fecal sample from different areas in the poultry house. A survey questionnaire was used to collect information regarding age, farming system, chicken type, environment, and other risk factor-related information through direct interviews/oral conversations. The questionnaire was in French, however, it was translated to farmers by a native speaker. The collected samples were put into screw-cap containers, kept on ice in a cooler and then sent to the Poultry Production Techniques Laboratory at CERSA, University of Lome in Togo for microscopic evaluation.

Parasitological examination

Three grams of each fecal sample were suspended in 42ml of NaCl-saturated water, homogenized, and filtered through a tea strainer to remove coarse elements. Using a

pasteur pipette, 0.15 ml of this mixture was placed in each chamber of the Mac Master slide. After a 5-minute rest, the eggs stuck under the upper glass were observed using a microscope (Olympus, Japan) at a magnification of x40. The number of oocysts was presented as the number of oocysts per gram of feces (OPG; Haug et al., 2008).

Morphology and morphometry oocysts identification

Flores et al. (2022) described the oocyst sporulation process, which was applied in this work. Positive fecal samples (117) were diluted five times with phosphate-buffered saline (PBS, pH 7.4), homogenized using a vortex mixer, and filtered through a mesh sieve. The filtrate was transferred to a polypropylene container and centrifuged for 10 minutes at 1,000 × g. The sediment containing the oocysts was re-suspended with PBS and washed twice using centrifugation (1,000 × g, 10 minutes). After washing, the two ml of sediment was re-suspended with two ml of potassium dichromate solution (2.5%) in small petri dishes. This was done to maintain adequate moisture while also killing other microbes in the samples that competed for oxygen and nutrients with the oocysts. To accomplish sporulation, the samples were incubated in an oven at 28 degrees celsius for 1-3 days with aeration. Microscopy at 100 × magnification was used to evaluate oocyst sporulation. Photographs were acquired for identification using an compound microscope (Olympus, Japan) equipped with an IX73 digital camera. The primary morphological traits were described, according to the key provided by McDougald (2003). Measurements of oocyst size were carried out using a calibrated ocular micrometer.

Anticoccidial activity of Togolese medicinal plants Herb extract and anticoccidial drug

Azadirachta indica leaves, *Carica papaya* seed in the flowering stage, and fresh root of *Sarcocephalus latifolius* were acquired at a local market (Gbossime, Lome in Togo). They were washed and then dried at room temperature (30 °C) for two hours. They were weighed after a period of partial drying. For each 100 g of plant, one liter of boiled water was utilized. Each plant powder was mixed with 100 °C tap water. After 30 minutes, the infusion was filtered, allowed to cool to room temperature (30°C), and given to the chicks. This procedure was performed every morning for the whole five-day therapeutic session (Dakpogan et al., 2019). The infected chicks received the infusion *ad libitum* for five days following infection, which corresponded to the period of oxidant assault generated by the coccidian parasite (Ogwiji et al., 2024), while the uninfected groups received water. The typical anticoccidial chemical was amprolium, which was administered at a dose of 0.6 g per liter of water.

Experimental design

A total of 125-day-old Isa-brown male chicks from a local hatchery were housed in a deep litter carpeted beginning enclosure, under 22 hours of lighting, and held at initially 35 °C with a decline up to 22 day-olds before being divided into the experimental groups. The chicks had unlimited access to feed (Table 1) and water. The main biosecurity precautions were vaccinations against newcastle disease, infectious bronchitis, and infectious bursal disease. The chicks were randomly assigned to five treatment groups. Each treatment group had 25 chicks, with 5 replicates per group and 5 chicks per replication. The experimental groups consisted of the group of chicks treated with *Azadirachta indica* infusion, the group of chicks treated with *Sarcocephalus latifolius* infusion, the group of chicks treated with *Carica papaya* infusion, the group of chicks treated with amprolium, and the group of untreated control chick*s'* roots extracts (infusion), amprolium, and untreated control chicks.

Table 1. Calculated composition of experimental feed during the starter (0-10 days of age) phase

Ingredient (%)	Starter (0-8 weeks)
White maize	57
Wheat bran	4
Roasted soybeans	12
Brewery by product	4
Oyster shell	1.5
Fish meal[1]	16
Broiler concentrate	5
Dl-methionine	0.1
L-lysine	0.2
Sodium chloride	0.2
Chemical nutritional characteristics	
Metabolizable energy (kcal/kg)	2920.99
Crude protein (%)	21.29
Crude fiber (%)	3.30
Lysine	1.21
Methionine ((%)	0.50
Methionine + cysteine (%)	0.76
Calcium	0.92
Total phosphorus	0.58

[1]A commercial fish meal with 60% crude protein made in Senegal and utilized by West African breeders.

Unsporulated *E. tenella* oocysts were obtained from the feces of naturally infected hens at 7 days postinfection

(DPI) in Togo's seaside region. According to El-Ashram and Suo's (2017) procedure, these unsporulated oocysts were first sporulated, purified, and kept in a 2.5% potassium dichromate solution at 25°C for 72 hours. The key to oocyst species identification established by McDougald (1998) was used by observing lesions and utilizing morphologic features and morphometry. The sporulated oocysts were kept at 4°C. Following parasite species confirmation, oocysts were collected, sporulated, and cleaned before the dose was adjusted to 3. 10^4 /mL/chick. All feces produced by each group of chicks in the 24 hours preceding the experimental infection were examined to guarantee the absence of any oocyst. At 23 days old, all chicks were orally gavaged with a one mL distilled water suspension containing 30,000 *E. tenella* sporulated oocysts adjusted using the standard method of McMaster.

Assessment of anticoccidial effectivity of Togolese medicinal plants

The efficacy of herbal extracts was evaluated using bloody diarrhea, survival rate, oocyst excretion, lesion score, body weight increase, and feed conversion ratio. Clinical symptoms and death were reported at each DPI. From the third to seventh day after inoculation, the proportion of blood in feces was assessed by counting bloody excreta twice a day. According to Abbas et al. (2010), the degree of bloody diarrhea was classified into one of four categories ranging from zero to three, based on the average of bloody excreta fragments rounded to the nearest integer. Briefly, the numbers 0, 1, 2, and 3 signified 0, 33, 33-66, and 66-99% of total feces, respectively. The survival rate was calculated by dividing the number of surviving chicks by the total number of chicks. Oocyst excretion was measured and counted between 6 and 14 days after inoculation using the approach outlined by Haug et al. (2008). Lan et al. (2016) cited the method for calculating oocyst value and decrease rate. The lesion scores were determined on the sixth day after infection (Johnson and Reid, 1970). Chick body weight and feed consumption were determined before the startlement of the experiment, as well as after the first and second weeks of infection. Lan et al. (2016) procedure was used to estimate the anticoccidial index (ACI) for each treatment using the formula shown below.

$$ACI = (\text{relative ratio of BWG} + \text{survival rate}) - (\text{lesion scores} + \text{oocyst value}) \quad \text{(Formula 1)}$$

Morisawa et al. (1977) method was followed to assess the anticoccidial index (ACI) values. An ACI > 180 indicated excellent anticoccidial impact, 160-180 indicated marked, 140-160 indicated moderate, 120-140 indicated mild, and < 120 indicated inactive.

Statistical analysis

The collected data were statistically processed using SPSS software version 26 (2018). Prevalence was calculated for all data by dividing the number of positive samples by the total number of samples examined, then multiplied by one hundred. The association between disease prevalence and hypothetical risk factors was evaluated using the chi-square test. Univariate logistic regression was used to calculate odds ratios of associated risk factors. The anticoccidial and performance indicators were expressed as mean ± SEM and a 1-way variance analysis was used to determine differences in those parameters between groups. Using the General Linear Model (GLM) procedure, the bloody diarrhea and lesion scores of each treatment were compared. The significant level was set at $p < 0.05$ using the Tukey test.

RESULTS

The overall prevalence of coccidiosis

The prevalence of chicken coccidiosis in poultry farms in the Maritime Region of Togo showed that the majority of the fecal samples examined were negative (60.34%). Only 39.66% of the 295 collected and examined fecal samples from the poultry farms were positive for chicken *Eimeria* oocysts during the period of July to September 2023.

Prevalence of chicken coccidiosis associated with risk factors

Table 2 shows a significant variable difference ($p < 0.05$) in the prevalence of coccidiosis among different age groups, management types, chicken types, and disease prevention frequency. The prevalence in the age group was (83.16%) in young chickens aged 3 to 8 weeks compared to adults (36.63%) aged over 8 weeks, indicating that young chicks were more susceptible than adults ($p = 0.0192$). No cases of coccidiosis were observed in cage-reared chicks. However, 34.27% of the samples from the litter-reared farms tested positive compared to 97.02% from the free-range farms ($p < 0.0001$). With regards to the breed type, the prevalence was 0 and 27.10% in parent stock and layers compared to broilers and local chickens, which had a prevalence of 57.69% and 93.56%, respectively ($p = 0.0002$). Farms that used drug to prevent the disease every two to three weeks had a lower prevalence (0% and 13.37%) compared to those that prevent the disease every four weeks or beyond (34.78% and 62.37%; $p = 0.0031$).

Table 2. Prevalence of coccidiosis associated with risk factors in maritime region of Togo

Risk factors	Category	No. Samples (%)	Positive (%)	Prevalence (%)	Chi-square ($\chi 2$)	P-value
Age	Adult	276 (93.48)	101	36.63	5.482	0.0192
	Young	19 (6.52)	16	83.16		
Type of management	Cage	6 (2.17)	0	0.00	20.8	< 0.0001
	Litter	260 (88.04)	89	34.27		
	Free range	29 (9.78)	28	97.02		
Type of breed	Lay hen	196 (66.30)	53	27.10	19.65	0.0002
	Broiler	64 (21.74)	37	57.69		
	Local chicken	29 (9.78)	27	93.56		
	Broiler breeder	6 (2.17)	0	0.00		
Frequency of disease prevention	2 weeks	35 (11.96)	0	0.00	13.87	0.0031
	3 weeks	45 (15.22)	6	13.37		
	4 weeks	83 (28.26)	29	34.78		
	Onset of disease	131 (44.57)	82	62.37		

The odds ratio associated with risk factor

Figure 2 illustrates the analysis of risk factors according to the epidemiological status of farms. Chickens aged less than eight weeks were 5.66 times more susceptible ($p = 0.019$) to the presence of *Eimeria* oocysts than those over eight weeks old (0.86). Broiler chickens that received classic anticoccidial preventive drugs with a frequency of ≤ 2 weeks (0.11) were less susceptible ($p = 0.003$) than those with a frequency of more than 2 weeks (1.48). Farmers who formulate their feed were 1.52 times more at risk of disease ($p = 0.040$) than those who used commercial feed (0.21). Depending on the rearing system, chickens raised in a traditional free-range system were 22 times more susceptible ($p < 0.0001$) than those raised in confinement (0.67).

Degree of infection according to the number of oocysts per gram of feces

The degree of *Eimeria* oocyst shedding is presented in Table 3. Out of the 295 samples examined, 117 were positive for *Eimeria* oocysts. Seventy-five percent (75%) of the samples had a low infection level (< 10,000 oocysts/g of feces) compared to 12.5% for moderate infection (10,000-15,000 oocysts/g of feces) and high (12.5%) infection (>15,000 oocysts/g of feces).

Infection level associated with risk factors

Figure 3 illustrates the status of oocyst shedding according to risk factors. The number of oocysts in the samples was < 500/g (29.17%), 500-999/g (8.833%), 1000-9999/g (37.50%), and > 10,000 (25%, Figure 3a). On the other hand, Figure 3b shows that irrespective of the management system (litter-based or free-range farming), only 25% of the samples had high infection (> 10,000 oocysts/g). In contrast, 26% of broilers raised on litter showed an infection > 10,000 compared to a load < 10,000 in broilers raised free-range (Figure 3c).

Distribution of chicken coccidiosis in the Maritime region of Togo

The distribution of the prevalence of chicken coccidiosis was geographically depicted on the map in Figure 4. The prevalence was 20% in the Zio district, 23.08% in the Lacs district, 20% in the Vo district, and 43.86% in the Grand-Lome district. The infection was low (<10,000 oocysts/g) in the farms of the Zio, Vo, and Lacs districts. However, it was moderate (10,000-15,000 oocysts/g) in the farms of the Grand-Lome district.

Presumptive *Eimeria* species identification

All the positive samples analyzed presented oocysts of *Eimeria* spp. In total six different species of *Eimeria* were identified. The most prevalent were the large oocyst with *E. maxima* (54.17%), the medium one with *E. brunetti* (33.33%), and *E. tenella* (25%), followed by *E. acervulina* (8.33%), *E. praecox* (8.33%), and the small oocyst with *E. mitis* (4.17%). Table 4 shows the morphometric features and linear regression of the *Eimeria* species. Except for *E. brunetti*, *E. mitis*, *E. acervuline*, and *E. praecox*, the mean length of all *Eimeria* species was greater than the mean width ($p < 0.05$). The species infection status is presented in Table 5. Species infection ranged from a single species to four different species per sample. The species *E. maxima* was the most prevalent in all farms at over 87%. The prevalence of infection with single species was 25% compared to 75% for mixed species infection (37.5%, 25%, and 12.5% respectively for double, triple, and quadruple infections).

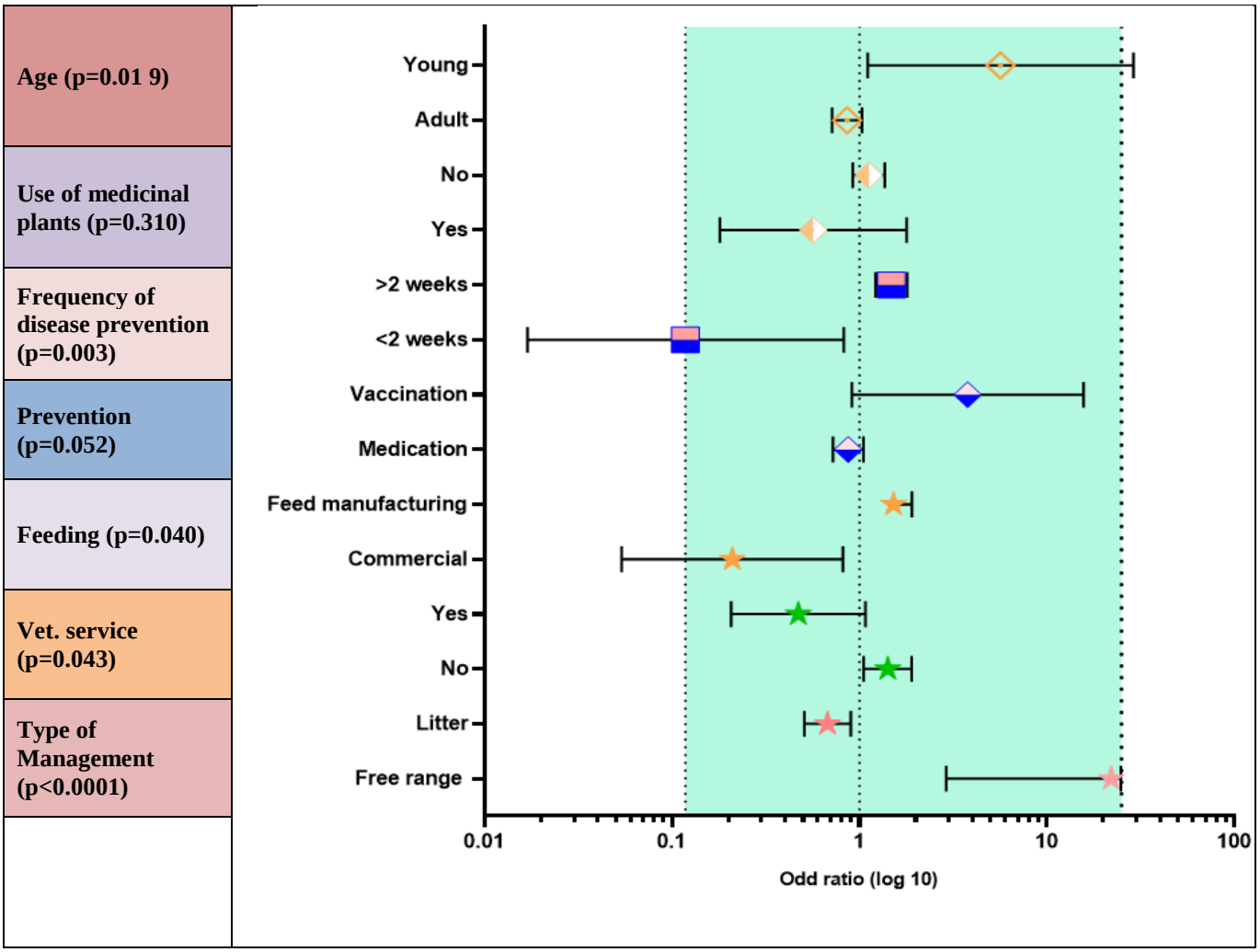
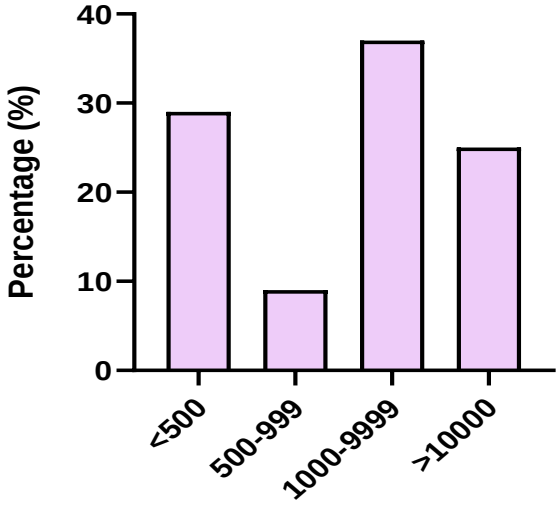


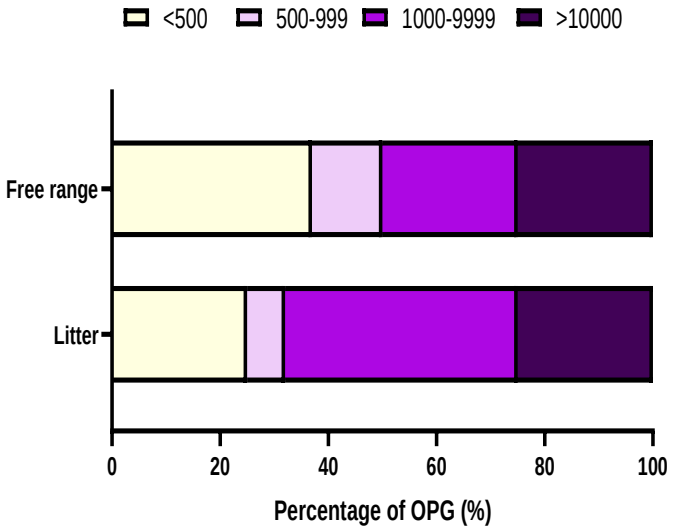
Figure 2. Graphical presentation of potential risk factors associated with coccidiosis in the Maritime region of Togo

Table 3. Level of infection based on the oocytes per gram count of coccidiosis in the Maritime region of Togo

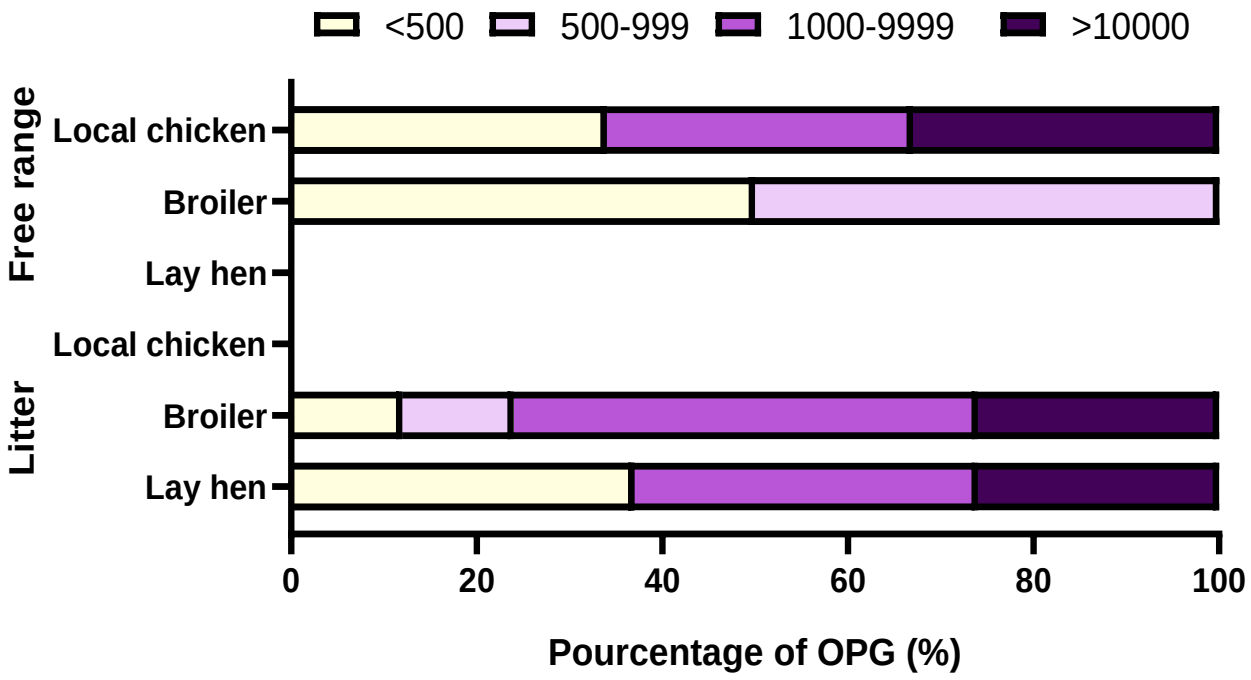
Oocyst count/g of feces	Degree of infection	No. positive samples	Percentage (%)
< 10 000 oocysts	Low	87	75
10 000-15 000 oocysts	Moderate	15	12.5
> 15 000 oocysts	High	15	12.5



a: Oocyst number/g of feaces



b: Level of infection based on management system



c: Level of infection based on management system and breed

Figure 3. Infection level associated with risk factors of coccidiosis in the Maritime region of Togo

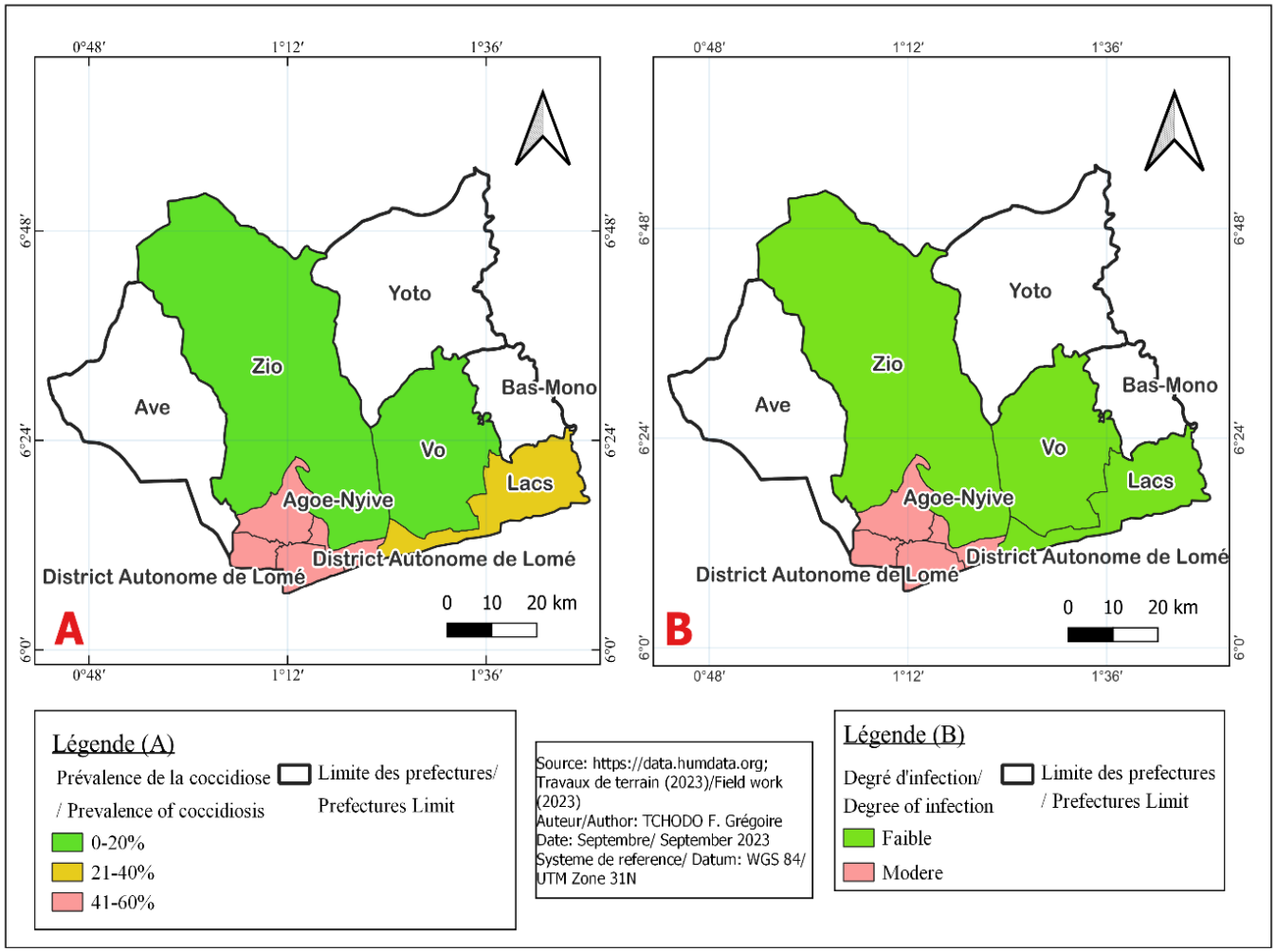


Figure 4. Prevalence of coccidiosis in the Maritime region of Togo. **A**: Prevalence by district; **B**: Degree of infection by study district

Table 4. Morphometry and linear regression of *Eimeria* spp. identified in the maritime of Togo

Species	%[a]	Shape	Oocysts		Linear regression [b]	
			Length (µm)	Width (µm)	R^2	Equation
E. maxima	54.17	Ovoid	31.6(27.0 - 41.1)	21.7(23.3 – 31. 6)	0.609	y = 8.143 + 0.904x
E. brunetti	33.33	Ovoid	25.7(24.0 – 29.9)	19.1(18.5 – 25.8)	0.144	y = 22.070 + 0.246x
E. mitis	4.17	Subspherical	14.5(6.8 – 9.3)	13.8(5.9 – 7.39)	0.205	y = 4.098 + 0.648x
E. praecox	8.33	Ovoid	21.4(20.7 – 22.3)	18.3(17.0 – 19. 5)	0.206	y = 15.542 + 0.322x
E. acervulina	8.33	Ovoid	19.1(18.1 – 19.8)	17.8(15.9 – 18.8)	0.550	y =11.776 + 0.414x
E. tenella	25.00	Ovoid	23.1(22.0 – 28.8)	20.9(18.8 – 24.7)	0.390	y =9.641 + 0.696x

[a] Percentage of oocysts. [b] Linear regression of the width and length of the oocysts

Table 5. Mixed infection of *Eimeria spp* in the Maritime region of Togo

Status of infection	Species	Prevalence (%)
Simple	*E. maxima*	31.25
	E. mitis	6.25
Double	*E. maxima, E. brunetti*	18.75
	E. tenella, E. brunetti	6.25
	E. tenella, E. maxima	31.25
Triple	*E. praecox, E. brunetti, E. tenella*	6.25
	E. brunetti, E. maxima, E. tenella	18.75
	E. acervulina, E. brunetti, E. maxima	6.25
	E. maxima, E. tenella, E. brunetti	6.25
Quadruple	*E. maxima, E. acervulina, E. tenella, E. praecox*	18.75
Total		100

Figure 5. Effect of medicinal plant used against coccidiosis on disease expression (clustering together, ruffled feathers, and bloody droppings), in the different groups of chickens in Togo

Behavior changes and bloody diarrhea

All infected chicks showed clinical signs of coccidiosis, such as clustering, ruffled feathers, and depression (Figure 5). However, no mortality was ever recorded during the study (Table 2). A complete absence of bloody droppings and a lower proportion of bloody dropping was observed in infected chicks treated with amprolium *Sarcocephalus latifolius* and *Azadirachta indica* extract respectively (Table 6).

Lesion scores

Figure 6 (A-D) shows the pathological lesion scores of the caeca of the experimental groups, which ranged from normal to severe (Fig6.A-D). Chicks treated with amprolium and infusion of Sarcocephalus latifolius root showed significant improvement ($p < 0.05$) in cecal morphology and a reduction in length (fig6.A-B). The ceca of the infected unmedicated group showed significant pathologic abnormalities (Figure 6, D), including ceca length shrinkage (atrophy), wall thickening, erosion, and blood clotting. The medicated *Azadirachta indica* group showed considerable improvement ($p < 0.05$) in cecal morphology and reduced length.

Comprehensive evaluation of anticoccidial efficacy

Figure 7 depicts oocysts shed in chicks' feces from 5 days post-infection to 7 days post-infection across all treatment groups. The number of fecal oocysts shed increased with the duration of the infection, with the fewest on day 5 and the most on day 7. Infected treated chicks had considerably decreased *Eimeria tenella* oocyst excretion ($p < 0.05$) compared to the infected untreated control chick group. The lowest oocyst excretions were recorded in the amprolium treated chick group (1.32×10^5)

followed by *Sarcocephalus latifolius* roots extract (1.49 x 10^5) oocysts shed per gram of feces.

Anticoccidial index

The infected unmedicated and infected and treated with *Azadirachta indica* demonstrated passive anticoccidial effects with the lowest Anticoccidial index value (ACI; Figure 8). Amprolium-treated and the *Sarcocephalus latifolius*-treated group demonstrated significant anticoccidial effects, with ACI indices of 176 and 170, respectively. The *Carica papaya* group's ACI index was 135, indicating a mild anticoccidial effect.

Performance

The average daily body weight gain of infected chicks treated with amprolium and medicinal plant extracts was significantly higher ($p < 0.05$) among all the groups (Table 7). The lowest body weight gains in the second week post-inoculation period were observed in infected untreated chicks' groups. The feed conversion ratio did not vary among the experimental groups in the first week. However, in the second week, the lowest feed conversion ratio ($p < 0.05$) was observed in *Sarcocephalus latifolius* treated chicks' group compared to other groups (Table 7).

Table 6. Effect of medicinal plant used against coccidiosis in bloody diarrhea, lesion score, and morbidity in the different groups of chickens in Togo

Groups	Bloody diarrhea	Lesion scores	Survivability (%)	Morbidity (%)
Control	$2.91^{a} \pm 1.97$	$4^{a} \pm 0$	100	100
Amprolium	$0.00^{b} \pm 0.00$	$0.60^{b} \pm 0.40$	100	100
Azadirachta indica	$1.81^{b} \pm 0.18$	$3^{a} \pm 0.34$	100	100
Carica papaya	$2.48^{a} \pm 1.78$	$2.4^{ab} \pm 0.7$	100	100
Sarcocephalus latifolius	$0.61^{b} \pm 0.16$	$1^{b} \pm 0.7$	100	100
P-value	$p < 0.0001$	$p < 0.0001$	-	-

[a, b] Means within columns with different superscript letters differ significantly ($p < 0.05$)

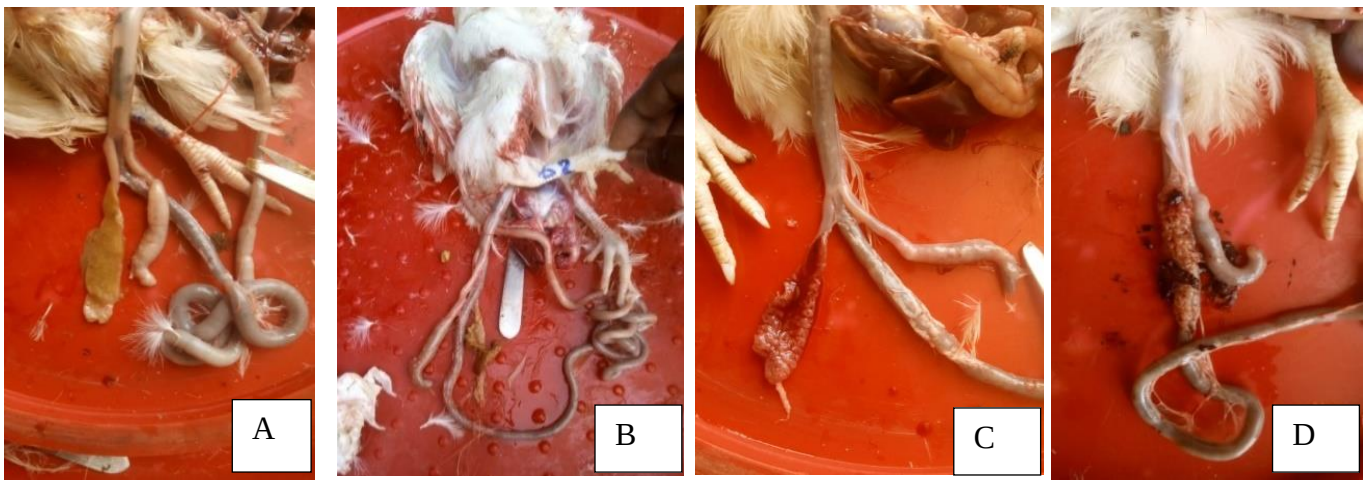


Figure 6. Effect of the medicinal plant used against coccidiosis on lesion scores of chicken's challenges with *Eimeria* tenella oocysts. Caeca was collected from each group on day 6 of the infection. **A, B, C,** and **D** mean scores of 0, 2, 3, and 4 of lesions found respectively.

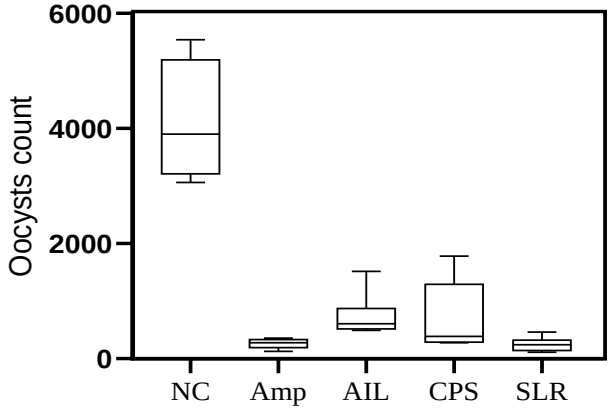


Figure 7. Effect of medicinal plant used against coccidiosis on oocysts shed per gram of feces from 5 days post inoculation (DPI) to 7 DPI. NC, infected unmedicated; Amp, Amprolium group; AIL, CPS, and SLR, infected and treated respectively with extract of *Azadirachta indica* leaves, *Carica papaya* seeds, and *Sarcocephalus latifolius* roots.

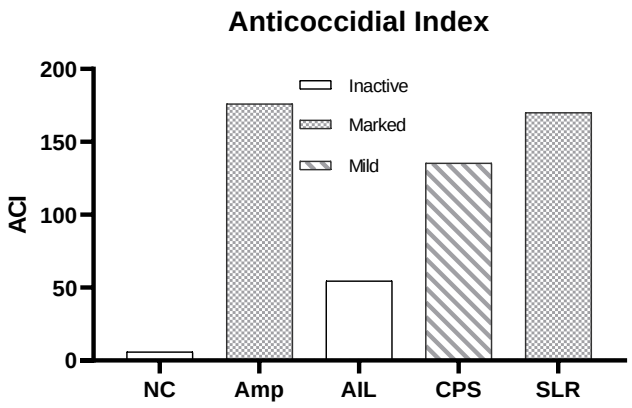


Figure 8. Effect of medicinal plant used against coccidiosis on the anticoccidial index (ACI). NC, infected unmedicated; Amp, Amprolium group; AIL, CPS, and SLR, infected and treated respectively with extract of *Azadirachta indica* leaves, *Carica papaya* seeds, and *Sarcocephalus latifolius* roots.

Table 7. Effect of medicinal plant used against coccidiosis in body weight gain and feed conversion ratio (M ± SE) of infected chicks in Togo

Groups	Body weight gain (g)		Feed conversion ratio	
	D 0 – D 6	D 6 – D 14	D 0 – D 6	D 6 – D 14
Control	$6.61^{a} \pm 0.31$	$7.24^{a} \pm 0.17$	$4.31^{a} \pm 0.09$	$4.09^{a} \pm 0.05$
Amprolium	$10.15^{bc} \pm 0.43$	$11.29^{b} \pm 0.40$	$3.42^{b} \pm 0.27$	$3.61^{ac} \pm 0.22$
Azadirachta indica	$9.55^{b} \pm 0.32$	$11.47^{b} \pm 0.37$	$3.23^{b} \pm 0.11$	$3.52^{bc} \pm 0.07$
Carica papaya	$10.04^{b} \pm 0.31$	$12.03^{bc} \pm 0.17$	$3.38^{b} \pm 0.16$	$3.74^{ac} \pm 0.23$
Sarcocephalus latifolius	$11.03^{c} \pm 0.34$	$12.44^{c} \pm 0.31$	$3.61^{b} \pm 0.23$	$3.46^{bc} \pm 0,14$
p-value	< 0.0001	< 0.0001	0.0042	< 0.0001

[a, b] Means within columns with different superscript letters differ significantly ($p < 0.05$).

DISCUSSION

Chicken coccidiosis is considered one of the most widespread intestinal diseases in poultry production systems, thus holding significant economic importance. Therefore, from July to September 2023, the overall prevalence of chicken coccidiosis in the Maritime Region poultry farm of Togo was estimated at 39.66%. This rate (36.6%) was comparable to that reported by Dakpogan and Salifou (2013) in litter-based, high stocking density layer rearing system in Benin. However, it was greater than the values reported by Adem and Ame (2023) in Ethiopia's Haramaya district (27.01%) and Abera et al. (2016) in poultry farms in Addis Ababa (27.6%). This prevalence was also lower than that seen in Nigeria (42.7%), Algeria (63.26%), and Turkey (54.3%) as reported by Adang and Isah (2016), Debbou-louknane et al. (2018), and Karaer et al. (2012) respectively. This variation in coccidia infection prevalence may be attributed to factors such as epidemiology of coccidia infection, knowledge of the disease, climatic conditions, and geographical environment. One observed practice during sample collection was the perpetual pouring of water onto the litter from drinkers or during water serving, which promotes *Eimeria* accumulation, sporulation, and subsequent infection. This fluctuation in prevalence may also be attributed to the development of immunity against coccidia due to the frequent use of anticoccidial drugs or other preventive measures (Haug et al., 2008). This could explain the low prevalence rate (4%) observed in farms

that frequently use anticoccidial drugs prophylactically at intervals less than three weeks compared to those with a frequency or interval of more than three weeks (34.33%). Logistic regression revealed that coccidia infection was significantly higher in chickens aged less than 8 weeks (66.67%) compared to chickens older than eight weeks (23.67%). Therefore, the risk of young chickens contracting the disease was 5.66 times greater than for adults. This could be explained by an immature immune system in young birds, making them more susceptible to infection even with less pathogenic *Eimeria* strains. The low rate observed in adult chicks reveals that as the chick's age increases, they become immune and resistant to infections. This was consistent with the findings of Adem and Ame (2023), who also found a high prevalence (31.8%) in young chicks (2-8 weeks) compared to adults (22.9%) over 8 weeks old. Similarly, Abera et al. (2016) and Adang and Isah (2016) have shown that coccidia infection was related to the age of chickens in poultry farms in Addis Ababa, Ethiopia, and in traditional chicken farming in the Gombe metropolis in Nigeria. However, these results were inconsistent with those obtained by Abadi et al. (2012), Dakpogan and Salifou (2013), and Bachaya et al. (2015), who reported higher prevalence in adults than in young chickens. In this study, no cases of coccidian infection were noted in samples from cage or battery farming systems. However, the prevalence of coccidiosis was higher in the traditional extensive free-range farming system (88.89%) than in the deep litter or semi-intensive confinement farming system (19.75%). The results revealed that the high prevalence of coccidiosis according to the farming system and poultry type was associated with broiler chickens (100%) and local chickens (85.71%) raised in the traditional extensive free-range farming system compared to those raised in confinement (44.44% for broilers and 0% for local chickens). This lower rate observed in confinement farming could be explained by the particular attention given to chickens in this system with the adoption of prophylactic programs, preventive measures, and adherence to hygiene rules that were lacking in the traditional extensive free-range system. The determination of oocyst per gram (OPG) using the McMaster technique allowed for the assessment of the current degree of coccidian infection in the field and the parasite's reproduction rate in the chicks' intestines. The OPG values in the collected samples varied considerably, with 75% of the samples showing a low OPG (< 10,000 oocysts per gram of feces), 12.5% moderate (10,000-14,999 oocysts/g of feces), and 12.5% high (> 15,000 oocysts/g of feces). This was in line with the results found by Adem and Ame (2023), who recorded over 96% of samples positive for coccidian infection with an OPG < 10,000/g of feces. This suggested a considerable production of oocysts in the field, but the use of anticoccidials and other preventive measures reduced the oocyst number. The morphological identification of prevalent *Eimeria* species in poultry farms in the Maritime Region of Togo revealed six different species with an average mixed infection of 2.125 *Eimeria* species; 75% of positive samples contained 2 to 4 *Eimeria* species in a fecal sample. This high prevalence of mixed-species infection was observed in other studies, indicating the widespread nature of this phenomenon. *E. maxima* (54.17%), *E. brunetti* (33.33%), and *E. tenella* (25%) were the most prevalent species, with *E. maxima* being the most widespread. This high prevalence of *E. maxima* infection can be attributed to its high potential to affect the poultry's small intestine compared to other *Eimeria* species. This was consistent with the results observed in South Korea, Southeast Nigeria, and Romania, respectively by Ola-Fadunsin (2017), and Györke et al. (2013). Furthermore, poultry researchers were interested in finding alternatives to synthetic anticoccidial medications (Dakpogan et al. 2018; Alhotan and Abudabos, 2019; Al-Quraishy et al., 2020; Qaid et al. 2021). The current study also investigated the efficacy of natural products versus the standard synthetic anticoccidial product on Isa-brown male chicks subjected to *Eimeria* oocyst challenge and raised on litter. Anticoccidial medications, such as amprolium 20%, were the most often used ionophores in Togolese poultry farms due to their action on the chicken *Eimeria* parasites. Bloody diarrhea, intestinal lesion scores, anticoccidial index, body weight gain, and oocyst shedding were all evaluated, as well as mortality and morbidity rates. Coccidiosis was well-known for producing severe illness in poultry farms, including bloody diarrhea, by destroying intestinal epithelial cells. The present study discovered typical clinical symptoms of poultry coccidiosis, such as clustering together, ruffled feathers, depression, and anorexia, which were in line with the findings of Qaid et al. (2021). Treatments were considered protective when chicks continue to gain weight and their lesion scores and bloody diarrhea score were zero or at a low number (Allen et al., 2004). Present studies revealed that infected treated chicks had lower lesion scores, a reduced proportion of bloody droppings and a lower excretion of *Eimeria tenella* oocysts. Amprolium and *Sarcocephalus latifolius* had no effect on sick chicks' bloody diarrhea, despite the knowledge that reduced bleeding can protect infected

chicks from secondary infections, inflammatory reactions, and hazardous chemical absorption (Havrlentová et al., 2011). In intensive farming, the spread of oocysts shed in feces poses a risk for coccidiosis (Wondimu et al., 2019). This study found that *Sarcocephalus latifolius* root decoction significantly reduced oocysts/g output compared to amprolium groups, suggesting that *Sarcocephalus latifolius* roots may play a significant role in controlling large-scale avian coccidiosis epidemics in poultry farms, as *Cinnamomum verum* suggested by Qaid et al. (2021). The findings were congruent with those of Dakpogan et al. (2019), who found that *Senna siamea* and *Khaya senegalensis* extract treatment against chicken coccidiosis reduced lesion scores, bloody diarrhea, and oocysts per gram of feces (OPG). Furthermore, Qasem et al. (2020) found that Rumex nervosus leaf extracts reduced the quantity of oocysts in a chick's challenge with *Eimeria* tenella. Li-Yun et al. (2021) discovered that continuous administration of various doses of natural garlic essential oil could significantly reduce clinical symptoms, cecal lesions, and oocyst count. Several studies have shown that the avian cecum was one of the most essential digestive organs, and when *Eimeria* kills the epithelial cells of the cecum, the chicken suffers from malabsorption, resulting in poor body weight gain (Blake and Tomley, 2014; Lan et al., 2016). Then, treatment with *Azadirachta indica* leaves, *Carica papaya* seeds, and *Sarcocephalus latifolius* root extract enhanced the sick chick's body weight increase and feed conversion ratio, which were comparable to the results produced by the traditional anticoccidial medicine. We also utilized a conventional approach to estimate total drug sensitivity (known as the ACI). The current study found that the ACI of *Sarcocephalus latifolius* roots and amprolium were 170.15 and 176.11, respectively, indicating a good anti-coccidiosis effect.

CONCLUSION

The study revealed a notable prevalence of coccidiosis (39.66%) with *E. maxima* being the most prevalent species in the Maritime region of Togo from July to September 2023. Factors, including age, management, breed, and disease prevention frequency were statistically identified as the main risk factors for coccidiosis. Young chickens (< 8 weeks) were found to be more susceptible to infection compared to adults (> 8 weeks) and mixed species infection with multiple *Eimeria* species was common. Furthermore, the results indicated that *Sarcocephalus latifolius* root extract may successfully alleviate clinical symptoms, cecal lesions, and oocyst discharge. At the same time, it was also required to establish adequate dosage and methods to formulate *Sarcocephalus latifolius* root into a new, beneficial, and harmless pharmacological agent for both illness prevention and therapy.

DECLARATIONS

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Authors' contributions

Tchodo Ferdinand Gregoire contributed to the experimental design, data collection, analysis, and manuscript writing. Hervé Brice Dakpogan, Banfitebiyi Gambogou, Ombortime N'nanle, and Benjamin Adjei-Mensah assisted in data collection and text revision. Tona Kokou and Batomayena Bakoma worked on the experiment's design and supervision, as well as manuscript revision. The final manuscript has been read and approved by all authors.

Competing interests

The authors declare no conflicts of interest.

Ethical considerations

The authors have ensured that the work meets the journal's ethical guidelines (including plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publishing and/or submission, and redundancy) for submission and publication.

Availability of data and materials

The necessary data will be provided by authors according to reasonable requests.

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Performance of Quail (*Coturnix Japonica*) Fed Diets with Fish Meal Substituted by Catfish Offal Flour *(Pangaius hypopthalmus)*

Edi Erwan[1]*, Rendi Pratama[2], Irdha Mirdhayati[1], Jordi Aditiya Prameswara[3], Ilyas Husti[4], and Mozhdeh Emadi[5]

[1]*Department of Animal Science, Faculty of Agriculture and Animal Science, State Islamic University of Sultan Syarif Kasim Riau (Universitas Islam Negeri Sultan Syarif Kasim Riau), Pekanbaru, Riau, Indonesia*
[2]*Alumni of Department of Animal Science, Faculty of Agriculture and Animal Science, State Islamic University of Sultan Syarif Kasim Riau (Universitas Islam Negeri Sultan Syarif Kasim Riau), Pekanbaru, Riau, Indonesia*
[3]*PT.CJ Feed and Care Indonesia, Serang Plant, Banten, Indonesia*
[4]*Graduate School of State Islamic University of Sultan Syarif Kasim Riau (Universitas Islam Negeri Sultan Syarif Kasim Riau), Pekanbaru, Riau, Indonesia*
[5]*Department of Animal Science, Faculty of Agriculture, Payam-e-Noor University, Tehran-Iran*

*Corresponding author's E-mail: erwan_edi@yahoo.com

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ABSTRACT

Catfish offal flour (COF; *Pangaius hypopthalmus*) has the potential to replace fish meal (FM) due to its high crude protein content. The present study aimed to investigate the effects of substituting FM with COF in basal diets on food intake (FI), body weight gain (BWG), and feed conversion ratio (FCR) in quails. A total of 100 male quails were randomly assigned to five treatment groups, each with four replicates. The treatment groups were fed with basal diet + 0% COF and 100% FM (T0), basal diet + 25% COF and 75% FM (T1), basal diet + 50% COF and 50% FM (T2), basal diet + 75% COF and 25% FM (T3), and basal diet + 100% COF and 0% FM (T4). Feed intake, BWG, and FCR were measured from 0 to 35 days of age. The findings indicated that substituting FM with COF up to 100% did not significantly affect FI, BWG, and FCR. It can be concluded that COF has the potential to replace FM in basal diets while maintaining performance in quails.

Keywords: Catfish offal flour, Body weight gain, Feed intake, Feed conversion ratio, Quail

INTRODUCTION

Quail farming is a popular practice in many communities and has long been a significant source of animal protein in Indonesia, particularly through the production of its meat and eggs. The population of quails in Indonesia increased from 13,932,649 to 14,819,755 in 2020 (Directorate General of Livestock and Animal Health, 2020). The management of quail maintenance is similar to that of other poultry, encompassing both breeding and feeding strategies. Among these practices, feed management is a crucial aspect of the maintenance of quails. Each developmental stage in quails requires feed with a specific protein content. During the starter period, the feed should contain a maximum of 24% crude protein (CP) and 2,800 Kcal/kg of metabolizable energy (ME). In the grower period, the feed should contain up to 20% of CP and 2,600 Kcal/kg of ME. Finally, for the layer period, the feed should contain a maximum of 22% CP and 2,700 Kcal/kg of ME (SNI 01-3907, 2006). Catfish offal is a protein source derived from fishery waste that can be utilized as animal feed. In 2018, the production of catfish in Riau, Indonesia was 36,554.82 tons (Directorate General of Livestock and Animal Health, 2020). Moreover, fish offal constitutes 10-15% (depending on the species) of the fish biomass (Bhaskar and Mahendrakar, 2008). Utilizable catfish offal includes intestines, swim bladders, liver, and gonads, which account for approximately 7.5% of the whole fish weight (Prabosasongko, 2003). According to Prabosasongko (2003), catfish offal silage contains 54.17% protein, 21.79% fat, 4.29% ash, 1.81% crude fiber, and 17.95% nitrogen-free extract. Previous studies have reported that catfish offal meal contains 53.38% CP, 18% crude fat, and 2.04% crude fiber, making it a potential substitute for fish meal in animal feed. Based on the aforementioned considerations, this study aimed to investigate the effects of utilizing catfish (*Pangasius*) offal waste as a substitute for fish meal in fulfilling protein requirements in livestock feed. In particular, the objective of this study was to examine the effects of substituting fish

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meals with catfish offal meals on the performance of quails (*Coturnix japonica*).

MATERIALS AND METHODS

Ethical approval

This investigation was performed under strict regulations by the recommendations in the Guide for the Care and Use of Animals, at the Faculty of Agriculture and Animal Science, State Islamic University of Sultan Syarif Kasim Riau, Pekanbaru, Indonesia.

Formulation of feed

The feed used in this study was custom-formulated, with its nutritional content tailored to meet the requirements of quails during the starter and growth phases, as specified by the NRC (1994). The nutritional composition of the feed ingredients and the formulation of the experimental diets are presented in Table 1.

Table 1. Composition and feed content of starter-phase of dietary with catfish offal flour in quail.

Treatment (%) / Feed Ingredients	T0	T1	T2	T3	T4
Yellow corn	26	26	26	26	27
Rine bran	49	49	49	49	48
Soybean Meal	13	13	13	13	13
Fish meal	10	7.5	5	2.5	0
COF	0	2.5	5	7.5	10
Top Mix	2	2	2	2	2
Total	100	100	100	100	100
Chemical analysis					
EM (Kcal/kg)	2892.47	2893.72	2894.97	2896.22	2899.5
CP (%)	19.94	19.95	19.97	19.99	19.97
CFr(%)	5.8	5.71	5.63	5.55	5.40
CF(%)	2.46	2.70	2.93	3.16	3.43
Calsium (%)	5.41	4.13	2.86	1.58	0.31
Phosphor (%)	0.62	0.55	0.48	0.41	0.35

COF: Catfish offal flour; CP: Crude Protein; CFr: Crude Fiber; CF: Crude Fat; Top Mix compound minerals and vitamins. Crude Fat; Top Mix compound minerals and vitamins. T0: Basal diet consisted of 100% FM + 0% COF, T1: Basal diet + 75% FM+25% COF, T2: Basal diet + 50% FM + 50% OF, T3: Basal diet + 25% FM + 75% COF, T4: Basal diet + 0% FM + 100% COF.

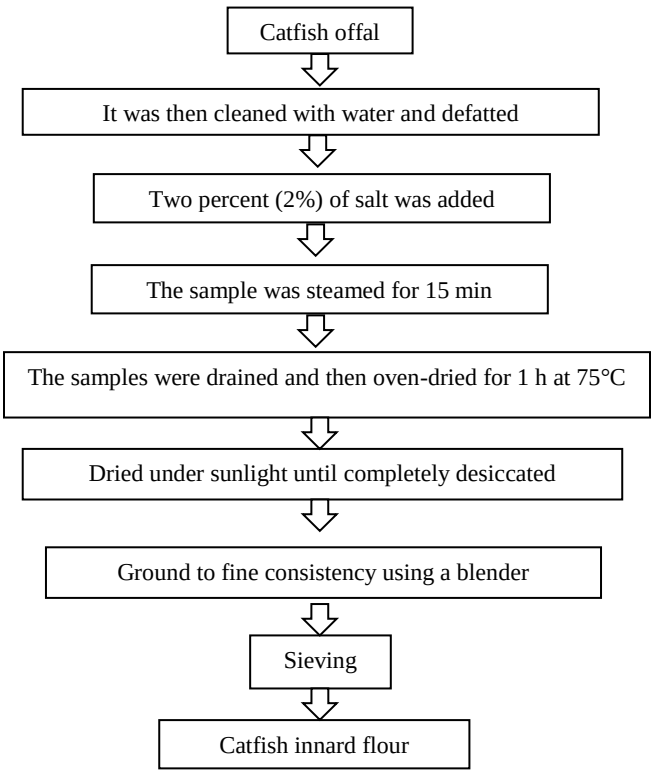

Figure 1. The production processing of Catfish innard flour

Research procedure

This study was conducted from July to August 2021 at the Livestock Production Technology Laboratory, Faculty of Agriculture and Animal Science, State Islamic University of Sultan Syarif Kasim Riau, and the Garuda Sakti Quail Farm, Jalan Sepakat, Gang Keluarga, Pekanbaru, Indonesia. The experiment involved 100 male Japanese quails, divided into five treatment groups with four replications each.

The quails were housed in cages maintained at a constant temperature of 30 ± 1^0C and provided with continuous lighting throughout the experiment. Treatments were administered from day-old quails (DOQ) until they reached 63 days of age. The treatments tested the use of catfish offal flour (COF) as a substitute for fish meal (FM) in the basal diet in quails (Figure 1). The treatment groups were as follows included T0 (basal diet with 100% FM + 0% COF; control), T1 (basal diet with 75% FM + 25% COF), T2 (basal diet with 50% FM + 50% COF), T3 (basal diet with 25% FM + 75% COF), and T4 (basal diet with 0% FM + 100% COF).

Measured parameters

Feed intake

Feed intake (FI) was determined as the amount of feed consumed by the quails, calculated by subtracting the amount of leftover feed from the total feed provided

(Maknun et al., 2015). The feed intake over the period from 0 to 35 days was determined by measuring the reduction in the amount of feed consumed from a pre-weighed feeder.

Body weight gain

The body weight gain (BWG) of the quails was calculated as the difference between their initial body weight and their final body weight during each weighing period.

Feed conversion ratio

The feed conversion ratio (FCR) was determined by dividing the total feed intake (FI) by BWG during each period, following the methods of Wen et al. (2018).

Data analysis

This study was designed as a completely randomized design (CRD). Data were analyzed using the StatView software package (Version 5, SAS Institute, Cary, USA, 1998). Analysis of variance (ANOVA) was performed, and the Tukey test was done as a post hoc test. Prior to the analysis, raw data were examined using Thompson's method at a significance level of $p < 0.05$ to identify outliers. Once the data were confirmed to meet the assumptions of ANOVA, statistical comparisons were conducted.

RESULTS AND DISCUSSION

Feed intake

The average feed intake (g/quail) of quails fed a ration containing COF as a replacement for fish meal in the basal diet over a 35-day experimental period was presented in Table 2. The results showed that substituting fish meal with COF at levels ranging from 25% to 100% did not have a significant effect ($p > 0.05$) on feed intake in treatments. The average feed intake across treatments ranged from 238.75 to 283.60 grams.

The non-significant effect of substituting commercial fish meal with COF was likely due to the relatively similar nutritional content, including crude protein, crude fiber, and metabolizable energy across all treatments, resulting in comparable feed intake levels among quails. Some factors may influence feed use including appetite, intestinal digestion, and energy metabolism (So et al., 2007; Byrne et al., 2015).

The non-significant difference in feed intake observed in this study was likely because the substitution of commercial fish meal with COF at levels from 25% to 100% in the diet met the nutritional requirements, particularly for protein. This finding was supported by Dauhi et al. (2021), who reported that substituting commercial fish meal with fish offal meal up to 12% in the diet resulted in no significant difference in feed intake due to the low protein content in Japanese quails.

Table 2. Average feed intake (g/quail) of quail from 0 to 35 days of age fed by substitution of fish meal with Catfish offal flour in basal diets

Treatment	Feed intake (g/quail/35 days)
T0	257.05 ± 54.76
T1	238.75 ± 77.46
T2	269.80 ± 22.48
T3	283.60 ± 11.98
P-value	0.79

T0: Basal diet consisted of 100% FM + 0% COF, T1: Basal diet + 75% FM+25% COF, T2: Basal diet + 50% FM + 50% OF, T3: Basal diet + 25% FM + 75% COF, T4: Basal diet + 0% FM + 100% COF.

Body weight gain

The average body weight gain (g/quail) of quails-fed diets containing COF as a substitute for fish meal in the basal diet over a 35-day study period was shown in Table 3. The results indicated that substituting fish meal with COF at levels ranging from 25% to 100% in quails aged from 0-35 days did not significantly affect ($p > 0.05$) body weight gain in treatments. Moreover, the results demonstrated that the body weight gain of quails was not affected by the substitution of commercial fish meal with COF in their diet. The average weight gain in this study ranged from 80.79 to 84.14 grams.

The lack of significant differences in body weight gain can be linked to feed intake, which remained unaffected by the substitution of commercial fish meal with COF. According to Richards and Proszkowiec-Weglarz (2007), increases in body weight in commercial chickens were often accompanied by unintended increases in feed intake. Hence feed intake is very important for increasing body weight gain and better feed efficiency (Wen, et al., 2018; Yan, et al., 2019). They also stated that body weight gain was primarily a result of metabolic accumulation, which was supported by the quantity of feed consumed by livestock and the optimization of feed utilization.

Table 3. Average Body weight gain (g/quail) of 35-day-old quails fed by substitution of fish meal with Catfish offal flour in basal diets

Treatment	Weight gain (g/quail/35 days)
T0	80.79 ± 2.14
T1	82.81 ± 1.80
T2	83.68 ± 0.93
T3	82.20 ± 1.75
T4	84.14 ± 2.87
P-value	0.32

T0: Basal diet consisted of 100% FM + 0% COF, T1: Basal diet + 75% FM+25% COF, T2: Basal diet + 50% FM + 50% OF, T3: Basal diet + 25% FM + 75% COF, T4: Basal diet + 0% FM + 100% COF.

Feed conversion ratio

The average feed conversion ratio (g/quail) of quails fed a diet containing COF as a substitute for fish meal in the basal diet over a 35-day study period was presented in Table 4. The results demonstrated that substituting fish meal with COF at levels ranging from 25% to 100% did not significantly affect ($p > 0.05$) the feed conversion ratio among treatments. The mean FCR ranged from 2.90 to 3.30.

Table 4. Average feed conversion ratio of 35-day-old quails fed by substitution of fish meal with Catfish offal flour in basal diets

Treatment	Feed Conversion ratio
T0	3.19 ± 0.72
T1	2.90 ± 0.97
T2	3.23 ± 0.28
T3	3.45 ± 0.11
T4	3.30 ± 0.54
P-value	0.85

T0: Basal diet consisted of 100% FM + 0% COF, T1: Basal diet + 75% FM+25% COF, T2: Basal diet + 50% FM + 50% OF, T3: Basal diet + 25% FM + 75% COF, T4: Basal diet + 0% FM + 100% COF.

The results of this study were lower than those reported by Dauhi et al. (2021), who observed no significant differences in FCR when adding up to 12% catfish offal meal to quail diets ($p > 0.05$). It was hypothesized that the non-significant difference in feed conversion ratio observed in this study may be attributed to the lack of significant effects on both feed intake and body weight gain, which also showed no significant differences.

This finding was consistent with that of Richards and Proszkowiec-Weglarz (2007) who reported that the higher BWG of broiler chickens was accompanied by improved feed utilization efficiency. Increased body size in broiler chickens has been accompanied by unintended increases in FI. Therefore, high FI was crucial for higher BWG and better FCR (Wen et al., 2018; Yan, et al., 2019). Mahmudah et al. (2015) emphasized this important point that the quality of the diet can change by the balance of dietary protein in quails.

CONCLUSION

Based on the findings of this study, it was concluded that substituting fish meal with COF up to 100% in the basal diet can effectively support the performance of Japanese quails. It was suggested that further research be conducted to explore the use of COF in diets for other poultry species.

DECLARATIONS

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Authors' contributions

Edi Erwan, Rendi Pratama, and Irdha Mirdhayati conducted the experiments, prepared and analyzed the data, and drafted the manuscript. Jordi Aditiya Prameswara, Mozhdeh Emadi, and Ilyas Husti reviewed and edited the manuscript. All authors have checked and approved the final version of the manuscript.

Competing interests

The authors declare no conflicts of interest.

Availability of data and materials

All the data and materials are available on request from the corresponding author.

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Ethical consideration

The authors affirm that all ethical issues have been addressed, including plagiarism, consent to publish, misconduct, double publication and/or submission, and redundancy.

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Assessing the Productivity of BLRI-Developed Native Ducks at the Community Level Compared to Indigenous Ducks in Conventional Farming Systems

Syidul Islam[1], Md. Ashraful Islam[1], Sharmin Sultana[1], Rezwanul Islam[2], Md. Habibur Rahman[3], and Razia Khatun[1*]

[1]*Farming System Research Division, Bangladesh Livestock Research Institute, Savar, Dhaka, Bangladesh*
[2]*Bangladesh Livestock Research Institute, Regional Station, Bhanga, Faridpur, Bangladesh*
[3]*Animal Health Research Division, Bangladesh Livestock Research Institute, Savar, Dhaka, Bangladesh*

*Corresponding author's E-mail: rkbaby96@yahoo.com

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ABSTRACT

Duck farming is a profitable business in low-lying areas of Bangladesh. The present study aimed to disseminate Bangladesh Livestock Research Institute (BLRI) developed native ducks BLRI-1(Rupali) and BLRI-2 (Nageswari) and validate their production ability compared to indigenous ducks under existing farming conditions in Bhanga upazila of Faridpur. An experiment was done at the community level where 45 farmers were selected based on their duck type. Data on the productive potentials of BLRI-developed native ducks were recorded and compared with the local germplasm of ducks. Among 45 duck-rearing farmers, with an average age of 38.58 years and farming experience of 12.38 years. Ducks were raised under scavenging conditions where 82.2% of farmers used separate duck houses and regular house cleaning was practiced by 68.89% of farmers. Ducks were consistently fed paddy, rice, and rice bran whereas 82.2% of farmers provided supplement feed with duckweed, and 15.6% supplied ready-made feed. The highest growth performance was observed for Rupali ducks growing to 1505.62 g by 24 weeks, compared to 1486.07 g for Nagesawri ducks. The highest egg production was 192.00 ± 5.70 eggs in Nageswari ducks followed by 181.33 ± 7.55 eggs for Rupali. Statistically significant differences were observed in adult male and female weights, eggs per clutch, and egg weight among the three breeds. Most of the farmer (84.4%) vaccinate their duck, against Duck Plague and Duck Cholera. The highest incidences of Duck Plague and Duck Cholera were observed in Native duck farms in comparison to BLRI-developed duck farms. Farmers obtained the highest Net income 8149.00 BDT (68.04 USD) and Benefit-Cost Ratio (BCR) of 1.60 in Rupali ducks compared to the Indigenous ducks at 1.30 whereas the overall BCR in duck rearing was 1.49. Major constraints regarding duck farming were disease outbreaks (73.3%) and high feed prices (64.4%). Thus, the study highlighted the significant variations in the performance and economic viability of ducks and emphasized farmers' training and breed-specific management strategies such as improved housing; feeding, and disease management practices to boost the profitability of duck farming.

Keywords: Benefit-cost ratio, BLRI duck, Disease outbreak, Growth performance, Native duck, Profitability

INTRODUCTION

Raising poultry plays a crucial role in livestock farming providing nutrition and a source of household earnings for small-scale, marginal, and landless poor farmers (Rahman et al., 2020). Many farmers who cannot afford to keep large animals such as cattle and goats can more easily raise poultry. Duck is one of the second largest poultry species that is raised in the southern and harbor regions of Bangladesh. The environment and climate of Bangladesh are favorable to duck rearing. Duck raising is popular worldwide, but more than 75% of the ducks were reared in Asia (Ahmed et al., 2021). The total duck population in Bangladesh was 68.261 million and is the second in number among poultry species in Bangladesh (DLS, 2024). The population of ducks is increasing in Bangladesh. According to the Food and Agricultural Organization (FAO), Bangladesh reared a lot of ducks and obtained positions 11th and 4th in duck meat and egg production among Asian countries (Pingle, 2011). Native duck germplasm has been reared in Bangladesh for both meat and egg purposes. Duck rearing in the traditional way has been practiced for different centuries in Asia (Parvez et al., 2020; Jalaludeen and Churchil, 2022).

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Ducks are reared in rice fields, canals, and rivers to access their feeds (Khatun et al., 2020). Duck plays a crucial role in income generation, nutrition supplement, and job opportunity creation for the people living in low-lying areas of Bangladesh. Ducks offer several benefits, including strong disease resistance, exceptional foraging skills in wetland environments, and natural flocking tendencies. Native duck germplasm has been reared in Bangladesh for both meat and egg purposes (Ahmed et al., 2021). To empower small farmers and landless laborer families through a holistic and self-sufficient strategy that not only improves income, employment, and nutrition but also promotes community development, gender equality, and environmental protection, all within the broader scope of rural development, where ducks serving as a key resource (Caron et al., 2009). Duck farming is an important part of sustainable livelihood development for poor rural communities and also an additional source of household income (Islam et al., 2023). The growth performance of ducks is higher than chickens (Das et al., 2020). Duck farming can play a significant role in increasing egg and meat production in Bangladesh. The backyard duck is a greater source of human resource development in low-lying areas of Bangladesh. However, duck farming in Bangladesh decreased due to several reasons such as shrinking water bodies, pollution of grazing fields, difficulty in obtaining inputs like ducks, feed, and medications, difficulties with marketing, and disease outbreaks (Sheheli et al., 2023). Furthermore, Bangladesh Livestock Research Institute (BLRI) initiated two native ducks BLRI-1 (Rupali) and BLRI-2 (Nageshwari) by phenotypic, productive, and reproductive characteristics improvement through selective breeding of high-yielding native germplasm (Khatun et al., 2020). These duck breeds have been improved to meet the demand for eggs and meat in Bangladesh. Several studies suggest that exotic ducks are not well-suited and not showed always better performance (Ali, 2020; Ali and Islam, 2021). However, BLRI-improved ducks are much suited to Bangladeshi climatic conditions. This developed duck had a higher growth rate and egg production in comparison to local duck breeds. Many studies have been done at one station to identify the productive, reproductive, and phenotypic characteristics of the BLRI-1 and BLRI-2 ducks under intensive farming conditions (Islam et al., 2014; Khatun et al., 2020). No studies were carried out at the field level to evaluate the growth, productivity, and reproductive performance along with the profitability of farmers in BLRI-developed duck rearing compared to native ducks. *This study aims to compare the productivity and adaptability of BLRI-developed native ducks (BLRI-1 and BLRI-2) with indigenous ducks in conventional farming systems.* This study was undertaken with the following objectives: a) To know the socioeconomic conditions of farmers along with duck farming and health management at the community level; b) To know the growth and productive performance of BLRI improved native ducks in comparison to local germplasm; c) To know the profitability and constraints of duck farming in the selected community.

MATERIAL AND METHODS

Study areas and duration

The study was conducted at BLRI Technology Village, Jandi; a low-lying area of Bhanga upazila under Faridpur district in Bangladesh from June 2022 to July 2024. Bhanga Upazila is located at 23.3971°N (latitude) and 90.0036°E (longitude) where the average annual rainfall is 2000-2500 mm the temperature ranges from 12 to 40^0C and the humidity ranges between 55-75%. This is a low-lying area of Bangladesh where BLRI established a technology at Jandi village to disseminate BLRI-developed technologies. Duck farming is very suitable in the selected area.

Experimental design

The experiment was performed by categorizing the duck into 3 distinct groups: Rupali, Nageswari, and Indigenous duck. Each group was formed with 15 duck farmers. A total of 10 Rupali and 10 Nageswari ducks were distributed to each farmer where each farmer got 8 female and 2 male ducks. In this study, the local native duck-rearing farmers were considered the control group farmers with at least 10 ducklings. All ducks were reared under the scavenging system, and the farmers provided two times a day minimal feed or no supplemental feed (Figure 1). The experimental design was utilized with a completely randomized design.

Data recording

Experimental data was recorded by regular observation and other data was collected through a pretested questionnaire. Data were recorded on socioeconomic conditions, duck rearing, feeding and management system, productive and reproductive performance, health and operational biosecurity, and constraints of farmers in duck rearing. Both experimental and descriptive data were collected to highlight the farming system and productivity of farms in low-lying areas of Bangladesh.

Figure 1. Scavenging of ducks in water bodies and farmers' feed supplementation, Bhanga, Bangladesh

Data analysis

Collected data were entered, sorted, compiled, tabulated, and organized into a Microsoft Excel sheet (MS Excel, 2021). Then data were statistically analyzed by Statistical Package for the Social Sciences (SPSS, Version-25). One-way ANOVA was done by the Duncan method to know the significance at the 5% level. All data was then tabulated using descriptive statistics such as frequency distribution, percentage, mean, and standard error value for further interpretation.

The net return was calculated by using the following formula:

Net return = GR-GC

Where GR is Gross return and GC is Gross cost.

GC = TFC+TVC

Where TFC is Total fixed cost and TVC is Total variable cost.

To calculate the benefit-cost ratio the formula was as follows:

$$\text{Benefit-Cost ratio} = \frac{\text{Gross return (GR)}}{\text{Gross cost (GC)}}$$

The benefit-cost ratio was a relative measure used to compare benefit per cost. It helped to analyze the financial efficiency of the farms. The multiple regression model was used to determine the effects of key variables in overall duck farming. The relationship between Y and X was established through regression analysis, where the variation in Y due to changes in X was estimated using a Linear Multiple Regression model, which is represented as follows:

$$Y = a + b1X1 + b2X2 + b3X3 + b4X4 + b5X5 + \ldots\ldots\ldots\ldots\ldots + b11X11 + e \ldots\ldots\ldots\ldots\ldots$$

Where, Y: Profit of Duck-rearing farmers (BDT/year), a: Constant b: Regression coefficient, X1: Hatched duckling value (BDT/year), X2: Bought duckling value, X3: Bought duck value, X4: Stock value of duck, X5: Feed value, X6: Treatment cost, X7: Labor cost, X8: Housing cost with 10% depreciation, X9: Miscellaneous Cost, X10: Age, X11: Family size, e: Error term. Along with different costs, age, and family size have a great impact on production and profitability in duck farming.

To simplify the estimation of the above equation, it is converted into a multiple linear form by applying the logarithm. The logarithmic version of the equation is as follows:

$$\text{Log } Y = \text{Log } a + b1 \log X1 + b2 \log X2 + \ldots b6 \log X6 + e$$

The multi-collinearity is an important component of multiple regression analysis. The multi-collinearity test examines the correlation among independent variables (X1, X2, X3, …, X11). Multi-collinearity is indicated when the correlation coefficients between these variables exceed certain thresholds (typically 0.85). If the correlation is less than or equal to 0.60, it suggests no significant multi-collinearity (Wantasen et al., 2024).

RESULTS

Socio-economic status of the farmer

In the present study, the average age of a farmer was 38.58 ± 1.72 years. The typical family size was 4.91 ± 0.21 members, and the average number of earning members per family was 1.31 ± 0.12. Additionally, the farmers had an average of 12.38 ± 1.44 years of farming experience (Table 1). The educational levels of farmers showed that 8.9% are illiterate, while 42.2% have completed primary education (Table 1). Additionally, 33.3% have an education level below the Secondary School Certificate (SSC), 13.3% have completed SSC, and only 2.2% have completed the Higher Secondary Certificate (HSC). Concerning occupation, it was revealed that all the duck-rearing farmers were housewives.

Housing and feeding management

The data on housing facilities for ducks in Table 2 reveals that 82.2% had separate duck houses. 97.8% of the farmers utilized wood for duck house construction purposes, while 2.2% used mud. Regarding floor type for duck housing, 95.6% of the farmers used wood, while 4.4% used mud indicating a strong preference for wooden flooring among the selected farmers, likely due to its practicality and durability in maintaining hygienic conditions for ducks. In the case of wooden floored houses, farmers used plastic bags as litter and could easily clean the floor. The wooden floor had less chance of damping and prevented the duck from contact with soil-borne disease organisms such as *Pasturella multocida* which causes duck cholera disease. In this study, 100% of farmers reared their ducks under scavenging. Farmers revealed the house cleaning practices that 55.56% used brooms for cleaning, while 33.33% used water and 11.11% used disinfectant. Poor cleaning practices increase the susceptibility to diseases. The study revealed that all farmers (100%) consistently fed their ducks with paddy, rice, and rice bran (Table 2). Additionally, 82.2% of the farmers supplemented the ducks' diet with duckweed, 53.3% included snails, and 15.6% provided ready-made feed which indicates that farmers did not provide balanced feed to their ducks and they take nutritional feed mainly from the environment during scavenging.

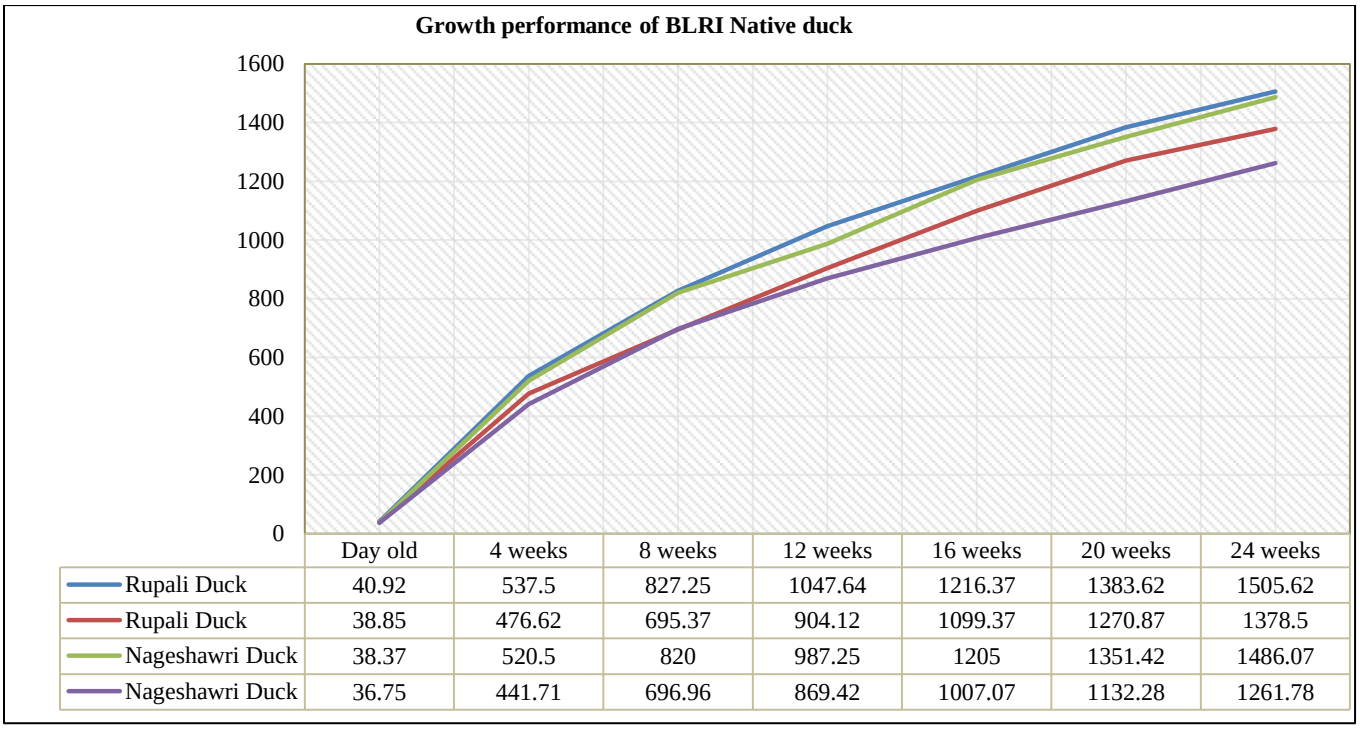
Table 1. Family status of duck-rearing farmer in the community of Bhanga, Bangladesh in 2024

Family Status of Farmers	Mean ± S. E. (n=45)	Parameter	Percent (n)
Age of farmer	38.58 ± 1.72	**Occupation:** Housewife	100.0 (45)
Family size	4.91 ± 0.21	**Training Facilities Received**	
Earning member	1.31 ± 0.12	DLS	4.4 (2)
Farming Experience	12.38 ± 1.44	BLRI	73.3 (33)
Education level	**Percent (n)**	**Total trained**	77.8 (35)
Illiterate	8.9 (4)	No training	22.2 (10)
Primary	42.2 (19)	**Purpose of duck earing**	
Below SSC	33.3 (15)	Extra Income	4.4 (2)
SSC	13.3 (6)	Family needs and extra income	95.6 (43)
HSC	2.2 (1)	Total	100.0 (45)
Total	100.0 (45)	-	-

S. E: Standard error, n: Number, SSC: Secondary school certificate, HSC: Higher secondary school certificate, DLS: Department of livestock services, BLRI: Bangladesh livestock research institute

Table 2. Housing facility and management system of duck in the community of Bhanga, Bangladesh during 2022-2024

Parameter	Percent (n)	Parameter	Percent (n)
Housing facility		**Floor-type**	
Separate duck house	82.2 (37)	Wood	95.6 (43)
Duck and chicken at the same house	17.8 (8)	Mud	4.4 (2)
System of rearing: Scavenging	100.0 (45)	**House cleaning practice**	88.89 (40)
Housing material		Cleaned by using a Broom	68.89 (31)
Mud and wood	2.2 (1)	Cleaned by Water	55.56 (25)
Wood and Tin	97.8 (44)	Cleaned by Disinfectant	33.33 (15)
Feed Ingredients			
Paddy	100 (45)	Duckweed	82.2 (37)
Rice	100 (45)	Snail	53.3 (24)
Rice bran	100 (45)	Ready feed	15.6 (7)



	Day old	4 weeks	8 weeks	12 weeks	16 weeks	20 weeks	24 weeks
Rupali Duck	40.92	537.5	827.25	1047.64	1216.37	1383.62	1505.62
Rupali Duck	38.85	476.62	695.37	904.12	1099.37	1270.87	1378.5
Nageshawri Duck	38.37	520.5	820	987.25	1205	1351.42	1486.07
Nageshawri Duck	36.75	441.71	696.96	869.42	1007.07	1132.28	1261.78

Figure 2. Growth performance of Rupali and Nageshwari duck at the community level of Bhanga, Bangladesh in 2022

The growth performance of BLRI improved native duck

The growth performance of BLRI-improved native ducks (Rupali and Nageshawri ducks) was documented across various stages of development in Figure 2. At day old, Rupali ducklings weighed 38.85 g and 40.92 g for females and males, respectively, while Nageshawri ducklings weighed 36.75 g and 38.37 g. By 24 weeks, Rupali male ducks reached an average weight of 1505.62 g, whereas Nageshawri male ducks weighed slightly less at 1486.07 g and then Rupali females were 1378.50 g and Nageshwari females were 1261.78 g, respectively. This data indicates that the Rupali duck had a higher growth rate than the Nageshwari duck.

Productive and reproductive performance of ducks at the community level

In the present study, it was examined adult female weights as 1.57 ± 0.06 kg for Rupali, 1.37 ± 0.06 kg for Nageswari, and 1.25 ± 0.11 kg for Indigenous Deshi ducks. Adult male weights averaged 1.78 ± 0.05 kg for Rupali, 1.56 ± 0.04 kg for Nageswari, and 1.45 ± 0.05 kg for Indigenous Deshi ducks (Table 3). Age at first laying was 210.00 ± 4.88, 205.33 ± 6.23, and 225.33 ± 5.70 days for Rupali, Nageswari, and Indigenous Deshi ducks respectively (Table 3, $p < 0.05$). Egg production per clutch was highest in Nageswari ducks with 36.73 ± 1.59 eggs, followed by Rupali with 34.33 ± 1.68 eggs and Indigenous Deshi with 26.67 ± 1.16 eggs. Overall egg production was highest in Nageswari ducks at 192.00 ± 5.70 eggs, followed closely by Rupali at 181.33 ± 7.55 eggs. Egg weight was significantly different among breeds, with Rupali eggs weighing 64.93 ± 0.67 g, Nageswari eggs 61.53 ± 0.58 g, and Indigenous Deshi eggs 62.06 ± 0.62 g ($p < 0.05$). Age at first laying and total egg production exhibited statistically significant differences ($p < 0.05$) where the adult boy weight of male and female ducks along with their egg production per clutches was significant at a 1% level ($p < 0.05$). These findings underscore the distinct performance characteristics observed among the different duck breeds. This variation was due to the improved variety of native ducks had higher performance than local germplasm.

Health, diseases, and biosecurity management of duck

The study documented the vaccination and deworming practices among duck farmers, revealing that 84.4% of farmers vaccinated their ducks overall, with 80.0% specifically vaccinating against Duck Plague and 40.0% against Duck Cholera. The majority of farmers (64.4%) administered vaccines every 6 months, while

20.0% opted for an annual schedule (Table 4). Additionally, 77.8% of duck farmers practiced deworming of ducks, primarily at 6-month intervals (71.1%). As all farmers didn't practice vaccination and deworming at regular intervals, disease outbreaks at a higher rate were observed during the study. Therefore, an awareness program needs to be applied to improve health management practices. Further study will be conducted to know the impact of vaccination and deworming on the health status of ducks.

Table 3. Productive and reproductive performance of different ducks at the community of Bhanga, Bangladesh in 2022-2024

Parameters	Rupali (Mean ± S.E.)	Nageswari (Mean ± S.E.)	Indigenous Deshi (Mean ± S.E.)	Overall (Mean ± S.E.)	Significant Level
Adult female weight	1.57 ± 0.06	1.37 ± 0.06	1.25 ± 0.11	1.39 ± 0.03	<0.001$^{(***)}$
Adult male weight	1.78 ± 0.05	1.56 ± 0.04	1.45 ± 0.05	1.59 ± 0.03	<0.001$^{(***)}$
Age at first laying	210.00 ± 4.88	205.33 ± 6.23	225.33 ± 5.70	213.55 ± 3.42	0.04$^{(**)}$
Weight at laying	1.64 ± 0.05	1.58 ± 0.08	1.64 ± 0.38	1.63 ± 0.13	0.98$^{(NS)}$
Egg per clutch	34.33 ± 1.68	36.73 ± 1.59	26.67 ± 1.16	32.58 ± 1.06	<0.001$^{(***)}$
Total egg production	181.33 ± 7.55	192.00 ± 5.70	168.00 ± 9.71	180.44 ± 4.66	0.11$^{(NS)}$
Egg weight	64.93 ± 0.67	61.53 ± 0.58	62.06 ± 0.62	62.84 ± 0.41	0.01$^{(**)}$

Different superscript letters in the same row differ significantly, $p < 0.05$; ***$p < 0.01$: Significant at 1% level; **$p < 0.05$: Significant at 5% level; NS > 0.05: Non-significant.; S.E: Standard error

Table 4. Vaccination and deworming practiced by community farmers of Bhanga, Bangladesh during 2022-2024

Parameter	Percent (n)	Parameter	Percent (n)
Vaccination practice	84.4 (38)	Deworming practice	77.8 (35)
Duck plague vaccination	80.0 (36)	**Deworming interval**	
Duck cholera vaccination	40.0 (18)	3 months	4.4 (2)
Vaccination interval		4 months	2.2 (1)
6 months	64.4 (29)	6 months	71.1 (32)
1year	20.0 (9)	-	-

Outbreak of diseases and biosecurity management

The experiment investigated disease outbreaks in duck farms shown in Table 5 revealing that the highest outbreak of duck plague (73.33%) and duck cholera (53.33%) was found for Native ducks while duck plague affected 46.66% and duck cholera affected 26.67% of Nageswari Duck farms. Moreover, 40.00% of duck plague and 33.33% of duck cholera outbreaks were observed in Rupali duck farms during the respective periods. In the study, farmers responded that the highest percentage of disease outbreaks predominantly occurred during winter (66.67%) in Nageswari Duck and were reduced in frequency during the following year, with outbreaks occurring in summer (13.33%) both in Nageswari and Native Duck. Additionally, the highest disease occurrence was about 60% or more in Nageshwari and local duck farms during the winter seasons whereas in Rupali duck farm 40% of disease outbreaks were observed during the summer month. The study examined biosecurity practices and management strategies for diseased and deceased ducks among farmers (Table 5). Results indicated that 97.8% of farmers reported that duck contact with wild birds was common due to rearing in a scavenging system. Moreover, the majority (91.1%) of farmer isolated and kept their diseased ducks in separate sheds. Regular cleaning of excrement was practiced by 57.8% of farmers. For deceased ducks, burial (53.3%) was the most common management method, followed by disposal in fields (26.7%), water (17.8%), and incineration (2.2%). Though the Department of Livestock Services was the main extension service worker to provide treatment facilities, the veterinarians from research teams provided treatment facilities to this selected community at a free cost to develop a livestock technology village. Their service rate was high in this community. That is why BLRI provides treatment support at 75.6%, with additional care provided

by quacks/ village doctors (33.3%), veterinary hospitals (4.4%), and farmers themselves (37.8%).

The benefit-cost ratio in duck farming

The study analyzed economic parameters across different duck breeds, revealing significant variations in financial metrics (Table 6). Rupali ducks demonstrated the highest net income of 8149.00 BDT (68.04 USD), with a Benefit-Cost Ratio (BCR) of 1.60, while Nageswari ducks showed a net income of 8048.47 BDT (67.20 USD) and a BCR of 1.59. Indigenous Deshi ducks exhibited a net income of 4303.67 BDT (35.93 USD) and a BCR of 1.30. The total average income of farmers from Rupali, Nageswari, and Indigenous Deshi ducks were 21721.33 BDT (181.37 USD), 21624.67 BDT (180.56 USD), and 18742.00 BDT (156.49 USD), respectively. The overall net income and BCR of the duck-rearing farmers were 6833.71 BDT (57.06 USD) and 1.49 in the chosen areas. These findings underscored the economic viability and profitability of duck farming, highlighting breed-specific differences in financial performance and efficiency.

Table 5. Diseases outbreak and biosecurity management by community duck farmers of Bhanga, Bangladesh in 2022-2024

Parameters (%)	**Rupali % (n)**	**Nageswari % (n)**	**Native duck % (n)**
Duck Plague	40.00 (6)	46.66 (7)	73.33 (11)
Duck Cholera	33.33 (5)	26.67 (4)	53.33 (8)
Season of disease outbreak			
Summer	40.00 (6)	20.00 (3)	26.67 (4)
Rainy	20.00 (3)	13.33 (2)	13.33 (2)
Winter	40.00 (6)	66.67 (10)	60.00 (9)
Parameters	**Percent (n)**	**Parameters**	**Percent (n)**
Overall Biosecurity management		**Diseased duck management**	
Contact with wild bird	97.8 (44)	Keep in the same shed	8.9 (4)
Cleaning of Excrement	57.8 (26)	Keep it in a separate shed	91.1 (41)
Death duck management		**Treatment facilities for duck**	
Throw in Field	26.7 (12)	By Veterinary Hospital	4.4 (2)
Buried	53.3 (24)	By Researcher of BLRI	75.6 (34)
Burnt	2.2 (1)	By Quack	33.3 (15)
Throw in Water	17.8 (8)	By Own self	37.8 (17)

Table 6. Benefit cost ratio in different types of duck farming in the community of Bhanga, Bangladesh during 2022-2024

Parameters	**Rupali**	**Nageswari**	**Indigenous Deshi**	**Total**
Hatched duckling value (BDT.)	753.33	826.67	913.33	831.11
Bought duckling value (BDT.)	580.00	503.33	673.33	585.56
Bought duck value (BDT.)	130.00	232.00	93.33	151.78
Stock duck value (BDT.)	1726.67	2253.33	3540.00	2506.67
Feed cost (BDT.)	4554.33	4608.87	3411.00	4191.40
Veterinary cost (BDT.)	860.00	483.33	473.33	605.56
Housing cost (10% depreciation) (BDT.)	318.00	280.67	275.33	291.33
Family labor cost (BDT.)	4346.67	4133.33	4780.00	4420.00
Other cost (BDT.)	303.33	254.67	278.67	278.89
Gross cost (BDT.)	13572.33	13576.20	14438.33	13862.29
Family needs duck value (BDT.)	2003.33	2123.33	2640.00	2255.56
Sold duck value (BDT.)	3753.33	4200.00	5373.33	4442.22
Stock duck value (BDT.)	4096.67	4533.33	4261.33	4297.11
Family needs egg value (BDT.)	7741.33	6550.67	4118.67	6136.89
Sold egg value (BDT.)	4126.67	4217.33	2348.67	3564.22
Gross income (BDT.)	21721.33	21624.67	18742.00	20696.00
Net Income (BDT.)	8149.00	8048.47	4303.67	6833.71
Benefit cost ratio (BCR)	1.60	1.59	1.30	1.49

Production function analysis (multiple regression test)

The results of the estimation of the model for multiple regression analysis on Duck rearing are shown in Table 7.

Interpretation of the estimated model

The analysis of the production function indicated that the values of hatched ducklings, feed costs, and labor expenses significantly affected the gross returns and profits from Duck production.

Value of hatched ducklings (X_1)

It was found that the regression coefficient for the value of hatched ducklings was estimated at 0.527 for Ducks, which is significant at the 1% probability level. This indicates a positive relationship between the value of hatched ducklings and gross returns (Table 7).

Feed value (X_5)

In the case of feed cost, the regression coefficient was 0.444 for the duck farmers which was significant at a 1% probability level. As a result, a positive relationship was found between feed value and gross returns.

Labor cost (X_7)

The estimated coefficient for labor costs was 0.262 for farmers raising ducks, and this result was significant at the 1% probability level.

Value of R^2

The R^2 value of 0.732 suggests that 73.2% of the total variation in gross returns among native duck-rearing farmers is accounted for by the variables included in the model which means that 26.8% of the variation remains unexplained, likely due to other factors that were not included in the model.

Value of adjusted R^2

The adjusted R^2 value of 0.643 is shown in Table 7 which indicates that 64.3% of the total variation in total income from native duck farming is explained by the variables in the model.

F-change

The F change of the model derived was 8.200. This value was significant at a 1% probability level implying that all the explanatory variables included in the model were important for explaining the variation in total return and profit for duck farming.

Multi-collinearity test of independent variables included in the regression analysis

The results presented in Table 8 revealed that all correlation coefficients were below 0.85, which represents that there is no multi-collinearity and no significant relationships among the independent variables.

Constraints of farmers in duck rearing

The study detected several constraints encountered by the farmers in duck rearing shown in Table 9. These included the outbreak of diseases was the highest ranking, affecting 73.3% of farmers, and high feed prices were the second-ranked, which were a concern for 64.4% of farmers. Other significant challenges included lack of training (28.9%), vaccine shortages (28.9%), and attacks by predatory animals (hawks, foxes, Mongoose, and other wild animals) 22.2%. Additionally, high duckling prices (17.8%), poor veterinary services (15.6%), and theft (11.1%) were reported as constraints. A smaller proportion of farmers cited lack of quality ducklings (13.3%), unavailability of ducklings (2.2%), and uncertainty in profitability (2.2%) as challenges in duck rearing.

Table 7. Profit-function analysis in duck farming through multiple regression in Bhanga, Bangladesh

Parameters	Regression coefficients	t-value	Significant level
(Constant)	1927.275	0.428	0.672
Hatched duckling value (X_1)	0.527	4.276	0.001***
Bought duckling value (X_2)	0.005	0.051	0.959
Bought duck value (X_3)	0.141	1.368	0.181
Stock value of duck (X_4)	0.010	0.099	0.922
Feed value (X_5)	0.444	4.560	0.001***
Treatment cost (X_6)	0.033	0.320	0.751
Labor cost (X_7)	0.262	2.488	0.018**
Housing cost with depreciation (X_8)	0.171	1.238	0.225
Other cost (X_9)	0.088	0.883	0.383
Age (X_{10})	-0.121	-1.071	0.292
Family size (X_{11})	-0.080	-0.763	0.451
R Square	0.732		
Adjusted R square	0.643		
F Change	8.200		0.001***

Different superscript letters in the same row differ significantly, $p < 0.05$; ***$p < 0.01$: Significant at 1% level; **$p < 0.05$: Significant at 5% level; NS > 0.05: Non- significant.

Table 8. Multi-collinearity analysis of independent variables included in the regression analysis

	X1 Log	X2 Log	X3 Log	X4 Log	X5 Log	X6 Log	X7 Log	X8 Log	X9 Log	X10 Log	X11 Log
X1 Log	0.00	0.03	0.59	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00
X2 Log	0.01	0.31	0.00	0.00	0.00	0.35	0.00	0.00	0.00	0.00	0.00
X3 Log	0.01	0.25	0.22	0.15	0.00	0.16	0.00	0.00	0.00	0.00	0.00
X4 Log	0.18	0.08	0.03	0.30	0.04	0.01	0.00	0.00	0.00	0.01	0.00
X5 Log	0.24	0.18	0.02	0.24	0.00	0.31	0.00	0.07	0.00	0.00	0.01
X6 Log	0.21	0.00	0.01	0.05	0.00	0.00	0.01	0.26	0.00	0.11	0.01
X7 Log	0.02	0.01	0.00	0.03	0.68	0.02	0.03	0.00	0.08	0.01	0.01
X8 Log	0.00	0.00	0.00	0.02	0.02	0.03	0.19	0.02	0.23	0.13	0.12
X9 Log	0.26	0.06	0.00	0.14	0.12	0.02	0.04	0.06	0.27	0.02	0.56
X10 Log	0.07	0.01	0.02	0.04	0.04	0.08	0.64	0.56	0.06	0.54	0.26
X11 Log	0.00	0.07	0.11	0.01	0.09	0.01	0.08	0.03	0.37	0.17	0.02

Table 9. Constraints of farmers in duck rearing at Bhanga, Bangladesh in 2022-2024

Parameters	Percent (n)	Ranking	Parameters	Percent (n)	Ranking
Outbreak of disease	73.3 (33)	I	Poor Veterinary Service	15.6 (7)	VI
High feed price	64.4 (29)	II	Lack of Quality Duckling	13.3 (6)	VII
Lack of training	28.9 (13)	III	Theft	11.1 (5)	VIII
Vaccine shortage	28.9 (13)	III	Profit not guaranteed	2.2 (1)	IX
Attacked by predatory animal	22.2 (10)	IV	Unavailability of Duckling	2.2 (1)	IX
High duckling price	17.8 (8)	V	-	-	-

DISCUSSION

The age of the farmers in the study area was similar to the farmer's mean age found by Jha et al. (2015) and Afrin et al. (2016). Jha et al. (2015) reported that 52% of farmers were young and the literacy rate was 48% that were closely similar to the present study. A literacy rate of 57% was reported by Parvez et al. (2020) which seemed to be lower but a higher value for family size was also recorded than the present study. Afrin et al. (2016) presented the average family size was 5.8 of duck rearing farmers along with a higher literacy rate of 94% in the Kishoreganj district which was close to the present study. Afrin et al. (2016) presented that the majority (73%) of the farmers had not received any training. However, a higher percentage of trained farmers was observed in the study area. The dissimilar results were due to farmers being trained by the research team before on BLRI native duck farming and management.

It was observed that most of the farmer used separate housing for their ducks which was made of tin and wood. The study of Rahman et al. (2009) mentioned that most of the duck farmers (93.5%) provide separate duck houses. They also narrate that the duck house was constructed with tin and wood was 65.5%. These data were strongly supported by the present study. A comparatively higher percentage of wood and tin-made duck is seen in the current study areas than in the findings of Jha et al. (2015), where they mentioned that 50% of houses were built with tin and wood. It was observed that duck houses made of tin and wood were durable and long-lasting with relatively low costs involved. On the contrary, Alam et al. (2014) reported that 55% of farmers housed their poultry (chicken and duck) in the same house in their living premises whereas 45% used shelters made of wood and tin or soil and tin or a combination of soil bamboo and wood. Rahima et al. (2023) expressed that most of the poultry houses were constructed from tin and bamboo (88.82%) which was strongly aligned with the current study. All farmers in this study reared their ducks in a scavenging system. The study of Jha et al. (2015) was slightly related to the present study where they stated during the rainy

season, ducks were raised only on natural feed resources under a scavenging management system. The current study was consistent with the outputs of Rahman et al. (2009), who stated that most of the farmers (67.5%) mainly utilized ponds as the scavenging place for ducks. A study carried out by Rahima et al. (2023) revealed that a maximum of 97.64% of farmers raised their poultry in semi-scavenging conditions under a backyard poultry production system. 88.89% farmer practiced their duck house cleaning. The findings of Rahman et al. (2009) were slightly lower than the present study where they mentioned that approximately 45% of duck-rearing farmers followed cleaning practices of their duck houses 2 to 3 times a month while only 10.50% performed daily cleaning practices. Jha et al. (2015) stated that a maximum of 35.50% of farmers regularly cleaned the duck house and a few portions (14%) of farmers never followed cleaning practices in their farms. Alam et al. (2014) also reported that 50-60% percent of farmers cleaned their poultry houses daily and 30% followed house cleaning once a week. 73.53% of farmers usually cleaned poultry houses reported by Rahima et al. (2023). Those studies were more or less related to the results of the current study. Rahman et al. (2009) indicated that 62% of farmers provide additional feed ingredients like rice polish, broken rice, and wheat bran which was closely supported by the present findings. Jha et al. (2015) reported 53.50% and Parvez et al. (2020) found 50% of farmers provided additional supplement feed to optimize the egg production of their ducks. The report of Rahima et al. (2023) mentioned that farmers supplied different additional feeds nearly supported the present study. The variations in output were observed due to the locations of the study, financial capability, knowledge of daily requirements and supply of supplemented feed were different in the present study. Therefore, farmers selected locally available feed ingredients and used them as supplemented feed for duck rearing in the studied area.

The body weight at eight weeks of age in different ducks at the community level was lower than the finding of Khatun et al. (2016), which presented higher live weights of Rupali, and Nageswari ducks because of providing a balanced supplementary diet. For Rupali and Nageswari ducks, the mean live weights at day old were nearly similar to the report of Morduzzaman et al. (2015) but higher at 4 weeks and 8 weeks of age, where the body weight gain was lower at 12 weeks of age. After 8 weeks of age farmer didn't provide supplementary feed to their duck and ducks take feed only from natural sources as a result poor growth was observed after that time. By the way, Rupali and Nageswari ducks had higher body weight gain in comparison to the study of Islam et al. (2012), who found poor growth rates of Indigenous Deshi ducks. In the study of Islam et al. (2014), it was stated that the mean body weights of BLRI-1 (Rupali) and BLRI-2 (Nageswari) ducks at day old 34.69 g, 34.54 g while 378.95 g and 359.22 g in 4th weeks of age that was lower than present study but slightly similar to the body weight at 8th weeks 846.71 g and 844.43 g whereas higher body weight at 12th weeks of 1399.91 g and 1313.05 g, respectively compared to the present findings. However, the higher adult body weight (1690 g) was indicated by Alam et al. (2014) concerning native ducks in the Mymensingh district. A comparatively lower average body weight of duck (1.22 ± 0.19 kg) than the current findings was observed by Rahima et al. (2023) in the Jhenidah district.

The study report is slightly different from the study report of Khatun et al. (2016), who reported first egg production age was 154, 147, and 161 days with the weight at first egg laying of 1437 g, 1455 g, and 1435 g for Rupali, Nageswari and Local ducks reared with supplementary feeding at farmer's level. The egg weights of the Rupali and Native ducks in the study of Khatun et al. (2016), were nearly similar but lower for the Nageswari duck in comparison to the current study. According to the study of Khatun et al. (2020), the average annual egg production of Rupali and Nageswari ducks was relatively higher than the present findings because they conducted their experiment under an intensive management system. They also stated the higher egg weight than the current outputs in Rupali and Nageswari duck. Moreover, higher egg weight was reported by Sharma et al. (2002) in Nageswari duck. According to Momu and Hossain (2022), Deshi black ducks got quick sexual maturity at a younger age compared to Deshi white ducks. Islam et al. (2014) reported a lower average age at sexual maturity for BLRI-1 ducks and BLRI-2 ducks than the present study. In the studies of Morduzzaman et al. (2015), the average egg production of a single Nageshwari duck was 140 to 160 per year which was lower than the present findings. Zaman et al. (2005) reported that Nageswari ducks had a relatively lower annual egg production compared to the present study findings. Alam et al. (2014) conducted a study with locally available ducks (Deshi, Khaki Campbell, and Jending) in the Mymensingh district where they found comparatively lower results than the current study in terms of the average age at sexual maturity, annual egg production and egg weight. Rahima et al. (2023) observed a lower average egg production and egg weight compared to the current study. The differences in

results may have arisen due to farmers rearing different ducks under scavenging conditions. Ducks consumed feed from natural sources and farmers were not to provide any definite supplementary feed to their ducks.

In the case of vaccination and deworming 84.4% and 77.8% of farmers practiced, however, all of them did not follow the vaccination schedule. A different result was observed in the study of Rahman et al. (2009) where they reported that most of the respondents (85.5%) did not provide vaccines to ducks. Approximately, 86% of farmers did not practice vaccination for their poultry (Chicken and Duck) owing to a lack of sufficient knowledge and facilities for vaccination reported by Alam et al. (2014). In addition, Jha et al. (2015) indicated that 65% of farmers were not aware of the importance of vaccination; they did not even vaccinate their ducks. Conversely, 30.50% of farmers did not regularly practice the scheduled vaccination and 14.50% followed the regular vaccination schedule whereas only 8.82% of farmers vaccinated their poultry under backyard poultry production stated by Rahima et al. (2023). These variations may have arisen due to the difference in location and year of the study conducted with duck-rearing farmers. Vaccination and deworming reduced the outbreak of disease but due to irregular vaccination and deworming practices, farmers faced some challenges of disease outbreak. So further research and extension work is necessary to know the impact of vaccination and deworming along with minimization of challenges regarding disease outbreaks.

Duck plague is the most prevalent disease reported by Khan et al. (2018) similar to current findings. The finding of seasonal outbreaks of disease was contrary to Khan et al. (2018), where authors found a higher incidence of diseases in the rainy season. The variation may occur due to different agroecological locations, climatic circumstances, animal-raising methods, housing systems, and also variations in sample numbers. Rahman et al. (2009), reported that duck plague and duck cholera outbreaks were the frequently observed diseases of ducks which supports the present study. However, Rahman et al. (2009) announced that the maximum outbreak of diseases was found at 34.18% in summer. 49% of farmers responded to duck cholera, 22% to duck plague, and 18% responded to no disease outbreak in their duck in the Mymensingh district (Alam et al., 2014). In addition, Jha et al. (2015) stated that the majority of the farmers (65%) had incomplete ideas about duck diseases. They also reported that the inadequate nutrient supply and poor management practices were the main reasons behind the elevated occurrence of diseases during summer followed by the other two seasons. A similar result was seen in the case of Rupali and Nageswari ducks where Rahima et al. (2023) reported that Duck plague (45.50%) and duck cholera (22.82%) were the more frequent diseases in ducks. The present study showed differences in results from the above findings because the study locations and sample size were different among those studies. Although, duck plague and duck cholera were causal diseases in different ducks but winter season was more susceptible to disease outbreaks for ducks due to drastically falling temperatures, cold weather and lack of sufficient nutrient consumption were challenging for ducklings and grower ducks to adapt to the ambient temperature during the winter season. This outbreak of several diseases around the year reduced the willingness of farmers to duck farming. That is why duck farming at the community level has declined.

In the case of biosecurity practices, contact with wild birds is the major source of duck plague (Henning et al., 2009; Elmberg et al., 2017). The dissimilar results in treatment were observed according to the findings of Rahman et al. (2009), where they obtained that only 7.25% of farmers isolated and medicated their diseased ducks. They also reported that 92.75% followed the traditional treatment method and only 7.25% practiced the modern mode of treatment. Furthermore, it was reported that only 9.75% of farmers followed the burring of dead ducks whereas 90.25% of farmers directly brought down dead ducks somewhere else which caused environmental hazards. The difference in results in the recent study indicated the gradual increase in of awareness the sick and dead duck management practices among farmers in the study area. It was a common practice that, very few farmers usually reported to the health center and utilized the treatment facilities until the situation became severe with a risen mortality rate (Debnath et al., 2020). These findings underscore the diverse approaches to biosecurity and disease management in duck farming communities.

In terms of net income, the present findings were consistent with the results of Khatun et al. (2016). Parvez et al. (2020) reported a lower net return of 6735 BDT (56.33 USD) from duck rearing with a BCR of 1.30. Comparatively higher net income and BCR were observed because of the variations in study location, year, and number of respondents considered for the current study than that of the above studies. In the present study, the overall BCR of duck-rearing farmers was lower than the outputs of Afrin et al. (2016), where they reported the BCR was 2.03.

The present study indicated several challenges encountered by the farmers in duck rearing. These findings were related to Alam et al. (2014), who reported that conventional rearing methods, feed scarcity, poor housing facilities, disease outbreaks, inadequate access to vaccines and medicine, and attacks of predatory animals indicated as the major constraint for backyard poultry (chicken and duck) farming in Mymensingh district. On the other hand, Rahman et al. (2009) indicated that almost 100% of duck owners stated that the outbreak of diseases and higher prices involved in getting quality feed (97%) emerged to be notable constraints for duck-rearing farmers which strongly supported the present study. They also reported that due to a lack of proper knowledge and training facilities, the majority of the farmers (95.7%) were not aware of taking special care of ducklings. However, a lower percentage (28.9%) of farmers reported that they faced challenges due to a lack of training in the present study. Additionally, they identified issues such as theft (37%), the attacks of predatory animals (23%), and major potential to harm the paddy fields (16%) as social problems. The most common constraints of disease outbreaks (54.12%) followed by a lack of adequate knowledge and predatory animal attack for poultry rearing in backyard systems reported by Rahima et al. (2023).

CONCLUSION

In the current study, ducks were raised under scavenging conditions with locally available feed supplements. However, 82.2% of farmers used separate duck houses whereas regular house cleaning was practiced by 68.89% of farmers was not satisfactory. In the case of growth rate, it was observed that Rupali male ducks weigh about 1505.6 g and females 1378.5 g compared to Nagesawri male ducks 1486.07 g and females 1262.8 g at 24 weeks. The highest egg production was 192 eggs in Nageswari ducks followed by 181 eggs in Rupali and the lowest 168 eggs in local indigenous ducks which indicates BLRI native ducks had higher productivity than local ducks. About 84.4% of farmers vaccinated their ducks against the Duck Plague and Duck Cholera due to the regular vaccination program continued by BLRI in this community. However, the biosecurity practice was not at an acceptable level. The BCR in duck farming was in Rupali 1.60 and Nageshwari 1.59 which was almost similar but lower in local ducks at 1.30 due to poor productivity. The overall BCR in duck farming was 1.49. This BCR will be improved if it is possible to mitigate the challenges mentioned by duck-rearing farmers like outbreaks of disease, high feed price, poor knowledge, and unavailability of improved duck/duckling variety. After all, duck farming is a profitable business in waterlogged low-lying areas of Bangladesh. The growth performance of the BLRI-1 native duck (Rupali) was better than BLRI-2 Native duck (Nageswari) and Indigenous duck breeds where the egg production of the Nageswari duck was better than Rupali and Indigenous duck. From this study, it can be concluded that the socioeconomic status of farmers along with housing, feeding, breeding, health, and biosecurity management is not satisfactory. Therefore, extension service along with technical intervention through the identification of research gaps is very necessary to mitigate the challenge in duck farming. Future research should be imposed on technology-based duck farming improvement in lowland areas of Bangladesh.

DECELERATIONS

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Competing interests

There is no competing of interest.

Availability of data and materials

The data are available upon request from the corresponding author.

Ethical considerations

For this article to be published with scientific research standards in the Journal of the World's Poultry Research, all authors have ruled and agreed on ethical issues, including fabrication of data, double publication and submission, redundancy, plagiarism, consent to publication, and misconduct.

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Authors' contributions

This study was carried out in collaboration among all authors. Syidul Islam and Md. Ashraful Islam

conceptualized and designed the study, did the experiment in the community, and wrote the protocol and manuscript. Syidul Islam and Sharmin Sultana wrote the methodology, completed the formal analysis, and wrote the manuscript. Rezwanul Islam helped in data collection. Md Habibur Rahman helped to write the original manuscript. Syidul Islam, Md Ashraful Islam, and Sharmin Sultana edited the manuscript for final submission. Razia Khatun provided guidelines for writing the manuscript and financial support for the manuscript. All Authors read and agreed to publish the last edition of the manuscript.

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Effects of Layer Breeder Age and Early Hypoxic Stimulation (ED 7-9) of the Chorioallantois Membrane on Eggshell Decalcification, Neovascularization of Heart Tissue, Mineralization and Morphometrics of Hatchlings

Richard Koblah Agbehadzi[1]*, Prince Sasu[1], Benjamin Adjei-Mensah[1,4], Nideou Dassidi[1], Yaah Aimee Emmanuelle Kouame[1], Achiamaa Asafu-adjaye Koranteng[1,2], Hezouwe Tchilabalo Meteyake[1], Jacob Alhassan Hamidu[1,3], and Kokou Tona[1]

[1]*Laboratory of Regional Center of Excellence for Poultry Science, University of Lomé, 01 BP 1515, Lomé, Togo*
[2]*CSIR-Animal Research Institute, P O Box AH 20, Achimota, Accra, Ghana*
[3]*Department of Animal Science, Faculty of Agriculture, Kwame Nkrumah University of Science and Technology, PMB, University Post Office, Kumasi, Ghana*
[4]*Department of Animal Science, University of Ghana, P.O. Box LG 25, Legon, Ghana*

*Corresponding author's E-mail: rk.agbehadzi@outlook.com

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ABSTRACT

Oxygen concentration (O_2) during incubation is crucial for embryo development, and hypoxic conditions can influence phenotypic plasticity in poultry. Although low O_2 (hypoxia) can be detrimental, it may also promote adaptive responses. Breeder age, a known genetic determinant of egg quality and embryonic development, is likely to interact with O_2 levels during incubation, however, this relationship remains understudied in layer breeders. This study examined how layer breeder age and reduced oxygen (O_2) levels during early embryonic development affect various factors, including eggshell decalcification (Dcal-SHL), chorioallantoic membrane (CAM) weight, heart tissue vascularization, egg weight loss (EWL), eggshell temperature (EST), and calcium (Ca) and phosphorus (P) content in bone and blood and tibia and femur morphometrics. A total of 900 eggs from 33 and 50-week-old ISA brown layer breeders were incubated in a 2x3 factorial design with O_2 levels of 15%, 17% (hypoxic), and 21% (control). Oxygen was reduced for 1hr/day from embryonic days (ED) 7-9 using air-N_2 flushing. Results showed increased CAM weight and heart tissue vascularization under hypoxia, especially in older breeders (50 weeks). Hypoxic conditions (15% and 17% O_2) reduced embryo weight loss and eggshell temperature compared to controls during the post-exposure phase (ED 15-18). There was an interaction between breeder age and O_2 levels on mineral absorption, with reduced oxygen leading to lower Ca and P absorption in bones, higher eggshell P retention, and decreased tibia morphometrics (weight, length, diameter, and seedor index) in hatchlings. Additionally, CAM weight correlated negatively with Dcal-SHL Ca at 15% O_2. The study concluded that reduced oxygen during early embryonic development increases CAM weight, heart neovascularization, and Ca mobilization from eggshell to blood. However, older flocks exhibited reduced Ca transfer to bones, likely due to homeostatic imbalance.

Keywords: Breeder age, Bone mineralization, Eggshell quality, Hypoxia, Oxygen level

INTRODUCTION

The poultry industry is a cornerstone for global food security, providing an essential source of animal protein through egg and meat production. As the demand for poultry products continues to rise, the industry must prioritize optimizing embryonic development and chick quality to sustain productivity and maintain the health of poultry flocks. A key aspect of this optimization involves understanding the factors that influence embryogenesis, particularly the genetic influence of breeder and hen age and the environmental conditions exposed to during incubation. Among these conditions, oxygen (O_2) levels in the incubator play a pivotal role in embryonic development and mineral absorption, which are critical for producing healthy chicks (Oviedo-Rondón et al., 2020; Tona et al., 2022; Yalçin and Oviedo-Rondón, 2023).

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Breeder age is a well-established genetic determinant of egg quality, embryonic development, and subsequent chick viability. As hens get older, they undergo significant physiological changes that affect their reproductive output, nutrient allocation, and eggshell quality (Park and Sohn, 2018). Typically, older hens produce larger eggs; however, these eggs may often have compromised eggshell integrity (Gu et al., 2021) and reduced calcium deposition (Al-Batshan et al., 1994). The decline in eggshell quality can adversely affect embryonic development, particularly bone strength in hatchlings (Kraus and Zita, 2019; Yenilmez and Atay, 2023; Varol Avcılar et al., 2024). During embryogenesis, changes in egg and eggshell traits like shell thickness and chemical properties need to be understood in order to make accurate assumptions about how embryos respond to different environmental conditions during incubation (Orłowski et al., 2019).

High mineral absorption from the eggshell during incubation is crucial for the developing embryo, as the eggshell is rich in calcium for bone mineralization. The genes responsible for mobilizing minerals from the egg during embryonic growth vary between the yolk sac and chorioallantoic membrane (CAM) in the embryo (Halgrain et al., 2021). Specialized genes in the chorioallantoic membrane facilitate the breakdown of the eggshell, allowing calcium to be absorbed into the bloodstream and transported to developing bones (Halgrain et al., 2022). Several other factors, including eggshell thickness, incubation conditions, and the embryo's metabolic activity, influence the efficiency of calcium absorption, directly impacting the embryo's skeletal development. Embryos with a greater ability to mobilize calcium from the eggshell would benefit from this pattern of embryonic calcium nutrition, which could enhance hatchling fitness by promoting growth (Stewart et al., 2019).

Oxygen concentration in the incubator during incubation is another critical environmental variable that directly influences embryonic development. Oxygen in the blood is essential for oxidative phosphorylation, the primary pathway for energy production in developing embryos (Almansa-Ordonez et al., 2020; May-Panloup et al., 2021; Deluao et al., 2022). Hypoxia, or low oxygen concentration in the blood can occur due to factors, such as high altitudes, inadequate ventilation, or suboptimal incubator settings. These conditions can significantly affect metabolic rates, thermoregulation, and overall growth. In response to hypoxia, embryos may exhibit adaptive changes in the chorioallantoic membrane, cardiovascular system, and mineral metabolism, which can alter bone formation and development (Druyan et al., 2012; Zhang et al., 2017; Haron et al., 2021). Hypoxic condition is also a vehicle for introducing phenotypic plasticity and adaptation in poultry, indicating that, although hypoxia may be detrimental, it has the potential to enhance embryo development (Hammarlund, 2020; Haron et al., 2021; Storz and Scott, 2021). Chan and Burggren (2005) reported that the timing and severity of hypoxia can significantly influence embryonic outcomes.

Despite the importance of oxygen during incubation, the interaction between breeder age and the level of oxygen concentration in the incubator remains poorly understood, especially concerning eggshell quality, mineral absorption, and bone morphometrics of hatchlings. Nangsuay et al. (2021) and several other researchers investigated the interaction between broiler breeder age and oxygen levels in the incubator, however, there is limited literature on the influence of oxygen levels during incubation on layer breeders and age effects. For example, older breeder hens tend to produce eggs with thinner shells, which may compromise calcium availability for embryonic bone development (Bain et al., 2011; Ahmed, 2016). The precise impact of varying oxygen levels on these processes is not fully elucidated, particularly concerning the role of the chorioallantoic membrane in facilitating gas exchange and mineral absorption under different oxygen tensions from the eggshell.

The incubation period of avian embryos is critical for their development, particularly during some specific windows when major physiological transformations occur. One such period is the early to mid-incubation phase, during which the chorioallantoic membrane develops and begins to play a major role in respiratory gas exchange and mineral absorption (Nowak-Sliwinska et al., 2014; Halgrain et al., 2022). The present study seeks to address these gaps by investigating the effects of layer breeder age and early reduced incubator oxygen (15% and 17% O_2) levels on chorioallantoic membrane development, eggshell decalcification, eggshell weight, embryo weight loss, eggshell temperature (EST), bone and blood Ca and P mineral absorptions, morphometrics of the chick's tibia and femur and neovascularization of the heart tissue. By integrating recent findings with correlative data, the present study aimed to provide a comprehensive understanding of the mechanisms underlying chorioallantoic membrane growth, eggshell decalcification, bone and blood mineralization, and skeletal development in chicks.

MATERIALS AND METHODS

Ethical approval

The current experimental protocols were approved in accordance with the guidelines of the Animal Ethics and Scientific Committee of the Regional Center of Excellence for Poultry Sciences at the University of Lomé (CERSA-UL), under approval number 008/2021/BC-BPA/FDS-UL.

Experimental site and facilities

The study was conducted at the Regional Center of Excellence for Poultry Sciences, University of Lomé (CERSA-UL). Facilities used included a hatchery and a laboratory unit. Field experimentation and laboratory analysis of samples was carried out from February to March 2024. The incubators were situated in an environment of latitude 6°1′95″N, longitude 1°2′53″E and an elevation of 26m above sea level.

Experimental design

A total of nine hundred (900) hatching eggs from ISA (Institut de Sélection Animale) Brown layer breeders aged 33 and 50 weeks with a respective average weight of 53.85 ± 2.40 g and 60.42 ± 2.02 g were incubated at three oxygen concentrations (O_2) levels that included 15%, 17% (experimental groups) and 21% (control group). From the total hatching eggs, 450 were allocated to each breeder age group. Each oxygen concentration level in a breeder age group had 150 hatching eggs which were subdivided into three replicates of 50 eggs on setting trays. On the embryonic day (ED) 7, 8, and 9, the 15% and 17% O_2 (experimental group) incubators were individually and sturdily flushed with an air-N_2 mixture (1hr/day) to maintain their respective O_2 levels. The oxygen concentration levels were continuously monitored with an O_2 gas detector (Model HFP-1201 BX, No. D6924, Xi'an Huafan Technology Co., Ltd., China) during flushing (Druyan et al., 2012; Zhang and Burggren, 2012). Following the 1-hour flushing on each day, experimental incubators were returned to normoxic incubation conditions as the controlled group.

Storage and incubation conditions

Hatching eggs from ISA Brown layer breeders were collected from a commercial farm in Lomé, Togo. The eggs were stored at 18°C and 75% relative humidity for 4 days, pre-warmed at 24°C for 6 hours, and weighed individually before being subjected to the 2 x 3 incubation design in three PAS REFORM SmartPro Combi incubators (PasReform, Zeddam, Netherlands) with a holding capacity of 600 eggs. The eggs were kept warm for 18 days at a temperature of 37.7°C and a relative humidity level of 56% while being rotated every hour at a 90° angle. On the 18th day, eggs containing live embryos were identified through candling, weighed and transferred to the hatcher in baskets for the three-day hatching period (until day 21 of incubation).

Pre-incubation egg quality measurement

Twenty-five (25) eggs were randomly selected from each breeder's age group (totaling 50) and examined for egg quality. Eggs selected for quality examination were excluded from the total experimental number.

Parameters determined

Egg quality measurement before setting

Prior to incubation, the characteristics of the eggs measured included egg weight (EW, g), egg length (L, mm), egg width (W, mm), eggshell weight (SW, g) and thickness (ST, mm), as well as yolk weight (YW, g). All weights were measured using an analytical scale balance, while L, W, and ST were measured using a digital Vernier caliper. Additional geometric qualities such relative eggshell weight (RSW, %), shape index (SI, %), geometric diameter (Dg, mm), egg volume (V, cm^3), elongation (Elong, mm), specific gravity (SSG, g/cm^3), eggshell surface area (SA, cm^2), eggshell sphericity (SP, %), eggshell volume (SV, cm^3), pore number (PN), eggshell density (SD, cm^2), eggshell weight/surface area (SW/SA) were estimated using the following formulas (Formula 1-11).

$RSW\ (\%) = [(SW)/(EW)] \times 100$ (Formula 1)

$SI\ (\%) = [(W/L)] \times 100$ - (Carter, 1968) (Formula 2)

$PN = 304W^{0.767}$ - (Rahn and Paganelli, 1989) (Formula 3)

$Dg = (L*W^2)^{1/3}$ - (Mohsenin, 2020) (Formula 4)

$EV\ (cm^3) = (0.6057 - 0.0018W) * LW^2$ - (Narushin, 2005) (Formula 5)

$SA\ (cm^2) = (3.155 - 0.0136L \pm 0.0115W) * LW$ - (Narushin, 2005) (Formula 6)

$SP\ (mm) = [(LW^2)^{1/3} / L] \times 100$ - (Severa et al., 2013; Kumbar et al., 2016) (Formula 7)

$SV = SV\ (cm^3) = ST \times S$ (Rahn and Paganelli, 1989) (Formula 8)

$SD\ (g/cm) = (SW / S \times ST)$ - (Rahn and Paganelli, 1989; Shafey, 2002) (Formula 9)

$Elong\ (mm) = L/W$ (Formula 10)

$SSG\ (g/cm^3) = (EW/V)$ - (Rahn and Paganelli, 1989) (Formula 11)

Chorioallantoic membrane weight

On embryonic day (ED) 11, post-exposure, six embryonated eggs were taken from each of the three replicates of each factorial group. The eggs were weighed, and the shells were broken in the air space region to remove the embryo. The chorioallantoic membrane (CAM) was weighed to estimate the relative CAM (rCAM) weight using the formula (Formula 12).

$rCAM\ weight\ (\%) = [(CAM\ weight)/(EW)] \times 100$ (Formula 12)

where; rCAM = relative chorioallantoic membrane weight, CAM = Chorioallantoic membrane, EW = egg weight

Egg weight loss

Egg weight loss (EWL) was estimated by weighing 30 viable eggs at embryonic day (ED) 0 ($EW_{ED\ 0}$) and reweighed daily from ED_6 to ED_{18}. Egg weight loss was calculated using the Formula 13.

EWL (%) = [(WL)/($EW_{ED\ 0}$)] × 100, where; WL = $EW_{ED\ 0} - EW_{ED\ 6-18}$ (Formula 13)

where; EWL = egg weight loss; WL = weight loss; EW = egg weight

Eggshell temperature

An infrared thermometer (XS-IFT002B, Ganzhou Xianshun Technology Co. Ltd, China) was used to measure eggshell temperatures (EST) for a total of 15 eggs per treatment from ED 11 to ED 18, following the procedure described by Olojede et al. (2016). During exposure to air-N_2 from ED 7-9, EST was measured before exposure, immediately after exposure, and two hours after exposure.

Blood sampling

At hatch (day 21), nine blood samples were collected from the hearts of nine alive chicks per group using a 27-G needle and 1 mL syringe into plain gel tubes. Samples were centrifuged with a BIOBASE Plus spectrophotometer for 15 minutes at 4,000 rpm and serum was transferred into Eppendorf tubes and stored at -21°C for calcium (Ca), phosphorus (P), and magnesium (Mg) testing.

Tibia and femur morphometric measurement

Twelve (12) chicks were cervically dislocated their tibia and femur. The chicks were killed before the tibia and femur were removed and air-dried for 72 hours. Weight, length, and diameter (width at the midpoint and endpoint) were determined with a sensitive scale (Ohaus STX8200 Scout, China) and a digital Vernier caliper respectively. The relative weight of the tibia or femur weight, seedor index (SI), and robusticity index (RI) were calculated using the specific formulas (Formula 14-16) by Riesenfeld (1972) and Evaris et al. (2021).

Relative tibia or femur weight (%) = [(tibia or femur weight)/(yolk free chick weight)] × 100 (Formula 14)

$SI = \frac{weight\ of\ bone\ (g))}{length\ bone\ (cm)}$, SI = seedor index (Formula 15)

$RI = \frac{length\ of\ bone\ (mm)}{\sqrt[3]{weight\ of\ bone\ (g)}}$, RI = robuscity index (Formula 16)

Determination of calcium and phosphorus in the eggshell, tibia femur bone, and blood of chicks

To determine the calcium (Ca) and phosphorus (P) content in the eggshells and bones of day-old chicks, six samples from each treatment group were cleaned with alcohol and benzene for 96 hours and dried in an oven (Memmert Universal Oven U, Germany) at 105°C until a constant weight was achieved. The specimens were burned to ashes at a temperature of 550°C for a period of 6 hours in a muffle furnace (Nabertherm GmbH, Bahnhofstr 20, 28865 Lilienthal/Bremen, Germany). The Ca content was determined by titration with $KMnO_4$ in a 0.02 N EDTA solution from a red to blue endpoint (Okalebo et al., 2002; Song et al., 2022). Calcium in samples was estimated by Formula 17.

Ca (mg) = Titer value of EDTA x 0.4008 (Formula 17)

$Ca\ (\%) = \frac{mg\ Ca}{\text{Sample wt x volume}}$ x 100

The phosphorus concentrations were measured on the Spectronic 20 spectrophotometer to give absorbance measurements at a wavelength of 420 nm. The observed absorbance was used to determine the P content from the standard curve (Okalebo et al., 2002). The percentage of P was calculated as Formula 18.

P content (g) in 100 g sample (P %) = $\frac{C\ x\ df\ x\ 100}{1\ 000\ 000} = \frac{C\ x\ 1000\ x\ 100}{1\ 000\ 000} = \frac{C}{10}$, (Formula 18)

where

C = concentration of P (µg/ml) as read from the standard curve; *df* = dilution factor, which is 100 *10 = 1000.

Magnesium (Mg), Ca, and P in the blood sample were determined using the enzyme-linked fluorescent assay (ELFA) method on VIDAS®. Glucose was determined at hatch using an ACCU-CHEK Active Glucometer.

Heart tissue sampling

After the period of hypoxic exposure, three heart tissues dissected from the embryos were individually fixed in 10% buffered formalin at ED 11. After undergoing alcohol (100, 96, 80, and 70%) and xylol treatments, a 5 µm thick portion was sliced from the paraffin-embedded blocks. The specimens were additionally treated with xylene to remove paraffin and then dyed with Hematoxylin and Eosin, following Al-Sabawy et al. (2021). A 100um microphotograph (100x magnification) image was taken under a light microscope (Thermo Fisher Scientific, Massachusetts, USA) and neo-vascularization in the tissue of the histopathological images was graded by a histopathologist using the scale of Okur et al. (2022). Four positives (++++) were very high (ectatic vessels with high congestion), three positives (+++) were high (vessels seat of moderate congestion) and two positives (++) were normal (normal tissues).

Statistical analysis

Egg weight and eggshell characteristics measured before incubation were analyzed using a one-way ANOVA with the general linear model (GLM).

Yijk = µ + Ai + eijk, (Formula 19)

where Yijk represented the measured variable, µ was the overall mean, Ai was the main effect of breeder age (33 or 50 weeks), and eijk was the random residual error.

Other data were analyzed using a completely randomized design with a 2 x 3 factorial arrangement and a two-way ANOVA according to the following formula:

Yijk = µ + Ai + Oj + AO_2ij + eijk, (Formula 20)

where Yijk was the measured variable, µ was the overall mean, Ai was the main effect of breeder age (33 or 50 weeks), O_2j was the effect of oxygen concentration levels (15%, 17%, or 21%), AO_2ij was the interaction

between breeder age and oxygen concentration, and eijk was the random residual error. All statistical analyses were performed using Minitab Statistical Software, version 21.2 (Minitab, LLC, NY, US, 2021). Data for egg weight loss was transformed using the square root of the arcsine before analysis. Correlation analysis was conducted between Ca and P content in decalcified eggshells, blood, bones and the morphometries of the tibia and femur. The Tukey post-hoc test was used to compare means, with significance set at $p < 0.05$. The results were presented as the mean ± the Standard Deviation (SD). GraphPad Prism, version 9.5.1 (2023) was used for charts and graphs.

RESULTS

Fresh egg weight and eggshell characteristics

Tables 1 and 2 present the results for egg weight, external geometry and eggshell Ca and P content from 33 and 50 weeks breeder flocks. In Table 1, eggs from 50 weeks flocks are significantly ($p < 0.001$) heavier by 6.57 grams than those from 33 weeks flocks. Egg length and width were significantly ($p = 0.01$, 0.008 respectively) greater in 50 weeks flocks compared to the 33 weeks breeder flocks. The calcium content was significantly higher ($p = 0.023$) in 33 weeks eggshells than in the 50 weeks eggshells. Table 2 shows that geometric diameter (Dg), egg volume (V), eggshell volume (SV), and pore number (PN) were significantly ($p < 0.001$) greater in 50 weeks flocks than in the 33 weeks breeder flocks. However, specific gravity (SSG) was significantly higher ($p < 0.001$) in 33 weeks than in the 50 weeks breeder flocks. However, phosphorus levels in the eggshell, relative eggshell weight, thickness, sphericity, shape index, eggshell density, and the ratio of eggshell weight to surface area were not significantly ($p > 0.05$) affected by the 33 and 50 weeks ages of breeder flocks used in the present study.

Chorioallantoic membrane and eggshell weight

The chorioallantoic membrane (CAM) and eggshell weights on embryonic day (ED) 11 following air-N_2 exposure are shown in Figure 1. Chorioallantoic membrane weight was significantly influenced ($p < 0.001$) by the interaction between breeder age and oxygen (O_2) levels in the incubator, with 50 weeks breeders having higher weights than the 33 weeks flocks. Chorioallantoic (CAM) weight was also significantly ($p < 0.001$) higher at 15% and 17% compared to 21% incubator oxygen level. No significant ($p > 0.05$) main effect of breeder age was observed on CAM weight. Figure 1b shows no interaction or main effects ($p > 0.05$) of breeder age or incubator oxygen level on eggshell weight on ED 11 post-exposure.

Egg weight loss

Figure 2 illustrates egg weight loss (EWL) during incubation. A significant interaction between breeder age and incubator oxygen levels was observed for EWL only at ED 10 ($p = 0.046$). However, breeder age consistently had a significant effect on EWL throughout the post-exposure incubation period up to ED 18 ($p < 0.001$), as 50 weeks breeders lost more weight compared to the 33 weeks breeder flocks. Egg weight loss under 15% O_2 level during early incubation was significantly different from the 17% and 21% (control group), specifically on ED 15, ED 16, ED 17, and ED 18, with p-values of 0.025, 0.002, 0.016, and < 0.001, respectively. Reduced oxygen levels (15% and 17%) led to less weight loss compared to the 21% O_2 (control group) across both breeder ages.

Eggshell temperature during and post-air-N_2 exposure period

Eggshell temperature (EST) measured before, immediately after and 2 hours after exposure during the oxygen reduction period is shown in Table 3. The interaction effect between breeder age and oxygen level on EST was not significant before exposure on embryonic days (ED) 7 to 9. However, a significant interaction was observed after exposure to ED 9 ($p = 0.011$). Eggshell temperature was higher at 21% oxygen compared to 15% and 17%, with 33 weeks breeders showing a more pronounced response on ED 8 ($p = 0.009$) and ED 9 ($p < 0.001$) compared to 50 weeks breeders. Two hours after exposure (2hrAE) on ED 9, EST remained significantly elevated at 21% ($p = 0.05$) compared to 15% but was not significantly different from the 17% incubator oxygen level. No main effect of breeder age was observed during the entire period and embryonic days of exposure. Figure 3 shows the post-exposure EST of incubated eggs from 33 and 50 weeks breeder flocks exposed to 15%, 17%, and 21% oxygen levels. No interaction effect ($p > 0.05$) between breeder age and oxygen levels was observed during the entire post-exposure incubation period until hatching. However, oxygen levels had a significantly decreased effect on ED 15 ($p = 0.010$), ED 16 ($p = 0.006$), ED 17 ($p < 0.001$), and ED 18 ($p = 0.005$) compared to 21% (control group). The main effect of breeder age was also noted, with 33 week breeder flocks recording higher EST at ED 17 ($p = 0.034$) and ED 18 ($p = 0.036$) compared to the 50 weeks breeder flocks. Calcium and phosphorus levels in decalcified eggshell, bone, and blood of chicks

Table 4 shows the calcium (Ca) and phosphorus (P) levels in the bone, and blood of chicks at hatch and decalcified eggshells from 33 and 50 weeks of breeder eggs. The current findings indicated that there was a significant ($p < 0.05$) interaction between breeder age and oxygen levels in an incubator on decalcified eggshells (Dcal-SHL) P, bone calcium (BNE-Ca), bone phosphorus (BNE-P), serum calcium (SER-Ca), serum phosphorus (SER-P), serum magnesium (SER-Mg) and plasma glucose (GLU). Breeder age had a significant effect ($p < 0.001$), as chicks hatched from 50 weeks breeder flocks had lower amounts of Ca in Dcal-SHL, lower BNE-P, higher SER-Ca and SER-P and lower GLU ($p = 0.003$) levels compared to 33 weeks flocks. Reduced oxygen

levels by 15 and 17% in the incubator resulted in a significantly ($p < 0.001$) lower BNE-Ca and P absorption and a slightly high but unclear significant trend for P retention in Dcal-SHL ($p = 0.03$) and SER-Mg ($p < 0.001$) compared to 21% incubator oxygen level.

Tibia and femur morphometrics

Tables 5 and 6 show the effect of breeder age and oxygen levels on the tibia and femur morphometrics of chicks at hatch. Table 5 indicates significant interaction effects ($p < 0.05$) of breeder age and oxygen levels on absolute and relative tibia weight, tibia length, diameter, robusticity, and seedor index. Embryos exposed to 15% and 17% oxygen levels in the incubator hatched into chicks which showed significantly ($p < 0.05$) reduced tibia absolute and relative weights, shorter lengths, diameters, and seedor index in comparison to embryos exposed to 21% oxygen levels. The main significant effect ($p = 0.015$) of breeder age was observed on only tibia robuscity as 33 weeks breeders were noted to be higher than 50 weeks breeders. All other tibia morphometrics were not affected by breeder age.

Table 6 shows a significant interaction effect ($p < 0.05$) of breeder age and oxygen levels on absolute and relative femur weight, femur robusticity, and seedor index. No main effect ($p > 0.05$) of oxygen levels (i.e. 15%, 17% and 21%) was observed on femur morphometry. Only absolute femur weight ($p = 0.025$) and the femur seedor index ($p = 0.039$) differed significantly by breeder age, with the 50-week breeder flocks recording heavier weight compared to the 33-week flock. Other femur morphometric parameters, including relative tibia weight, tibia length, diameter, and robusticity were not affected ($p > 0.05$) by the age of the layer breeders.

Correlation between calcium and phosphorus retained in decalcified eggshell, chorioallantoic membrane weight, calcium and phosphorus mobilized into the blood and bone, and tibia and femur morphometry under different oxygen concentration levels in the incubator

Tables 7 and 8 show correlations between calcium (Ca) and phosphorus (P) levels in decalcified eggshells, chick bone, serum (blood), CAM weights, and hatchling tibia and femur morphometrics under different oxygen levels, including 15% (below diagonal in Table 8), 17% (above diagonal in Table 8), and 21% (Table 7).

Under 21% oxygen level, a moderate positive correlation existed between decalcified eggshell calcium (Dcal SHL-Ca) and tibia robusticity (TB-rob; $r = 0.57$, $p = 0.05$), and between decalcified eggshell phosphorus (Dcal SHL-P) and tibia diameter TB-dm; $r = 0.63$, $p = 0.03$). Bone calcium (BNE-Ca) positively correlated with serum phosphorus (SER-P) and tibia seedor index (TB-SI; $0.58 \leq r \leq 0.62$, $p < 0.049$). Bone phosphorus (BNE-P) correlated positively with (TB-rob; $r = 0.58$, $p = 0.048$) and femur robuscity (FM-rob; $r = 0.72$, $p = 0.009$). Serum calcium (SER-Ca) was positively correlated with the femur seedor index (FM-SI; $r = 0.59$, $p = 0.042$). A negative correlation was observed between Dcal SHL-P and both SER-Ca and SER-P ($-0.75 \leq r \leq -0.58$, $p < 0.05$). Serum phosphorus showed a strong negative association with TB-rob and FM-rob ($-0.79 \leq r \leq -0.71$, $p \leq 0.02$), while it exhibited a positive correlation with relative tibia weight (rTB-wgt), TB-SI, and FM-SI ($0.62 \leq r \leq 0.79$, $p \leq 0.01$). There was a negative correlation between SER-Ca and FM-rob ($r = -0.69$, $p < 0.014$). Additionally, a moderately weak negative correlation was found between BNE-P and SER-P, TB-SI, and FM-SI ($-0.61 \leq r \leq -0.58$, $p < 0.05$). Chorioallantoic weight (CAM-wgt) was moderately positively correlated with Dcal SHL-P ($r = 0.70$, $p = 0.025$) and moderately negatively correlated with BNE-Ca ($r = -0.66$, $p = 0.036$).

At 15% O_2, strong positive correlations were observed between Dcal SHL-Ca and both BNE-Ca and BNE-P ($0.72 \leq r \leq 0.82$, $p < 0.01$). Decalcified eggshell phosphorus (Dcal SHL-P) had moderate to strong positive correlations with CAM-wgt and FM-SI ($0.64 \leq r \leq 0.89$, $p \leq 0.02$). In contrast, Dcal SHL-P had strong negative correlations with BNE-Ca and BNE-P ($-0.96 \leq r \leq -0.80$, $p < 0.01$), while BNE-P showed a moderately negative relationship with FM-SI ($-0.58 \leq r \leq 0.57$, $p \leq 0.05$). Chorioallantoic membrane weight (CAM-wgt) had a strong negative correlation with Dcal SHL-Ca and BNE-P ($-0.88 \leq r \leq 0.81$, $p \leq 0.005$), but a strong positive correlation with Dcal SHL-P ($r = 0.89$, $p = 0.001$).

At 17% oxygen level, a moderately strong negative correlation was observed between Dcal SHL-Ca and BNE-P ($r = -0.61$, $p = 0.04$). Decalcified eggshell phosphorus (Dcal SHL-P) showed very strong negative associations with BNE-P and SER-P ($-0.95 \leq r \leq -0.84$, $p \leq 0.001$). Bone phosphorus (BNE-P) had a moderate positive relationship with SER-Ca ($r = 0.63$, $p = 0.03$) and a very strong positive association with SER-P ($r = 0.93$, $p < 0.001$). Finally, CAM-wgt showed a moderate positive correlation with FM-SI ($r = 0.662$, $p = 0.037$) and a moderate negative correlation with FM-rob ($r = -0.62$, $p = 0.05$).

Heart tissue histology

According to Table 9 and Figure 4, the result is presented for the graded level of neo-vascularization in the heart tissue of an embryo at ED 11. The heart tissue from embryos incubated under hypoxic conditions of 15% and 17% oxygen levels for 50 weeks breeder flocks compared to the 33 weeks group and the control for 50 weeks were graded to be very high (ectatic vessels with high congestion, "++++") beyond normal tissue development. For the control (21%) and 17% O_2 level for the 33-week breeders, compared to the 50-week groups and 15% oxygen level for 33 weeks, vessel dilation was graded as high (vessels seat with moderate congestion, "+++").

Table 1. Fresh egg weight and shell qualities of 33 and 50 weeks ISA brown layer breeders into early hypoxic stimulation

Parameters	Egg weight (g)	Egg length (mm)	Egg width (mm)	Eggshell weight (%)	Shell thickness (mm)	Eggshell Ca (% DM)	Eggshell P (% DM)
33 weeks	53.85±2.40^{b}	36.96±1.41^{b}	27.36±0.98^{b}	10.14±0.80	0.37±0.05	31.32±2.81^{a}	0.39±0.04
50 weeks	60.42±2.02^{a}	40.32±0.84^{a}	28.85±0.52^{a}	9.82±0.52	0.40±0.05	29.93±3.22^{b}	0.41±0.03
P-value	< 0.001	0.001	0.008	0.418	0.314	0.023	0.232

^{a,b}: Values within the same column followed by different subscripts differ significantly ($p < 0.05$). All results are presented as mean ± SD, Ca: Calcium; P: Phosphorus

Table 2. Eggshell geometry of 33 and 50 weeks ISA brown layer breeders in early hypoxic stimulation

Parameters	Geometric diameter (mm)	Egg volume (cm^3)	Elongation (mm)	Specific gravity (g/cm^3)	Eggshell SA (cm^2)	Eggshell sphericity (%)	Eggshell volume (cm^3)	Pore number	Shape index (%)	Eggshell density (cm^2)	Eggshell wgt/SA
33 weeks	30.23±0.68^{b}	15.39±0.99^{b}	1.35±0.08	3.50±0.08^{a}	26.92±26.92^{b}	81.89±3.37	0.99±0.13^{b}	6465.90±220.90^{b}	74.14±4.57	5.59±0.55	2.00±0.02
50 weeks	32.25±0.54^{a}	18.59±0.91^{a}	1.40±0.03	3.25±0.05^{b}	30.48±0.92^{a}	80.00±1.00	1.21±0.16^{a}	7062.90±181.30^{a}	71.56±1.33	4.96±4.96	1.98±0.01
P-value	< 0.001	< 0.001	0.236	< 0.001	< 0.001	0.217	0.022	< 0.001	0.213	0.126	0.113

^{a,b}: Values within the same column followed by different subscripts differ significantly ($p < 0.05$); All results are presented as mean ± SD, wgt: Weight; SA: Surface area

Table 3. Eggshell temperature of 33 and 50 weeks ISA brown layer breeders taken before, immediately after, and two hours after exposure to early hypoxic stimulation (ED 7-9)

		Before Exposure			After Exposure			2 Hours After Exposure		
		ED-7	ED-8	ED-9	ED-7	ED-8	ED-9	ED-7	ED-8	ED-9
Breeder age (A)	33 weeks	36.81±0.10	36.94±0.05	36.96±0.05	36.75±0.13	36.75±0.17	36.80±0.18	36.79±0.15	36.87±0.25	36.95±0.06
	50 weeks	36.80±0.04	36.91±0.02	37.99±0.11	36.68±0.20	36.71±0.22	36.83±0.14	36.71±0.20	36.77±0.11	36.94±0.09
Oxygen level (O_2)	15%	36.78±0.04	36.91±0.03	36.98±0.09	36.64±0.14	36.63±0.24^{b}	36.75±0.13^{b}	36.75±0.15	36.77±0.31	36.89±0.03^{b}
	17%	36.80±0.08	36.93±0.02	36.96±0.11	36.70±0.22	36.66±0.07^{b}	36.71±0.10^{b}	36.69±0.25	36.77±0.12	36.96±0.08^{ab}
	21%	36.82±0.11	36.94±0.07	36.99±0.07	36.81±0.11	36.92±0.09^{a}	36.99±0.07^{a}	36.81±0.11	36.92±0.09	36.99±0.07^{a}
Interaction (A * O_2)	33 weeks * 15%	36.81±0.04	36.92±0.02	36.95±0.02	36.68±0.13	36.62±0.02	36.70±0.17^{ab}	36.77±0.23	36.79±0.48	36.90±0.02
	33 weeks * 17%	36.78±0.12	36.93±0.03	36.93±0.07	36.73±0.10	36.66±0.05	36.71±0.16^{ab}	36.78±0.07	36.84±0.04	36.97±0.08
	33 weeks * 21%	36.83±0.16	36.97±0.09	36.99±0.03	36.83±0.16	36.97±0.09	36.99±0.03^{a}	36.83±0.16	36.97±0.09	36.99±0.03
	50 weeks * 15%	36.76±0.03	36.91±0.04	37.00±0.13	36.59±0.16	36.63±0.38	36.80±0.05^{ab}	36.73±0.06	36.75±0.10	36.88±0.05
	50 weeks * 17%	36.83±0.03	36.92±0.02	36.99±0.14	36.67±0.32	36.65±0.10	36.69±0.01^{b}	36.60±0.36	36.71±0.14	36.96±0.10
	50 weeks * 21%	36.80±0.05	36.91±0.02	36.98±0.10	36.80±0.05	36.86±0.04	36.98±0.10^{ab}	36.80±0.05	36.71±0.04	36.98±0.10
P-value	A	0.786	0.154	0.383	0.430	0.704	0.766	0.337	0.321	0.795
	O_2	0.808	0.587	0.894	0.186	0.009	< 0.001	0.504	0.371	0.052
	A * O_2	0.887	0.455	0.926	0.583	0.104	0.011	0.741	0.710	0.375

^{ab}: Values within the same column followed by different subscript letters differ significantly ($p < 0.05$); All results are presented as mean ± SD; ED: Embryonic day

Table 4. Calcium and phosphorus levels in both tibia and femur bone, blood serum of chicks at hatch, and decalcified eggshell of 33 and 50 weeks ISA brown layer breeder eggs incubated in different incubator oxygen concentration levels (ED 7-9)

Parameters	Dcal SHL-Ca (% DM)	Dcal SHL-P (% DM)	Bone Ca (% DM)	Bone P (% DM)	Ser Ca (mmol/L)	Ser P (mmol/L)	Ser Mg (mmol/L)	GLU (mmol/L)
Breeder age (A)								
33 weeks	26.24±2.34^{a}	0.33±0.0314	10.73±0.39	8.70±1.44^{a}	11.68±0.10^{b}	7.20±0.67^{b}	3.74±0.04	197.53±12.64^{a}
50 weeks	22.01±3.12^{b}	0.33±0.0219	10.51±0.91	7.86±1.32^{b}	11.80±0.10^{a}	8.39±0.68^{a}	3.73±0.02	180.00±19.18^{b}
Oxygen level (O_2)								
15%	24.24±3.27	0.33±0.0293^{ab}	10.09±0.46^{b}	7.92±1.16^{b}	11.75±0.09	7.87±0.98	3.71±0.02^{b}	193.10±11.81
17%	22.55±3.18	0.35±0.0259^{a}	10.35±0.46^{b}	7.00±0.11^{c}	11.77±0.14	8.16±0.96	3.76±0.04^{a}	180.65±22.32
21%	25.59±3.48	0.31±0.0068^{b}	11.42±0.21^{a}	9.92±0.46^{a}	11.70±0.12	7.34±0.57	3.73±0.01^{b}	192.54±17.94
Interaction (A * O_2)								
33 weeks * 15%	26.75±0.32^{a}	0.30±0.0028^{e}	10.43±0.11^{b}	9.00±0.02^{c}	11.70±0.11^{ab}	7.38±1.02^{bc}	3.70± 0.01^{b}	203.58±3.32^{a}
33 weeks * 17%	24.68±0.16^{ab}	0.37±0.0023^{a}	10.50±0.01^{b}	6.90±0.01^{d}	11.70±0.09^{ab}	7.33±0.51^{bc}	3.79±0.04^{a}	196.25±19.75^{a}
33 weeks * 21%	27.29±3.72^{a}	0.31±0.0073^{cd}	11.24±0.12^{a}	10.21±0.52^{a}	11.64±0.12^{b}	6.88±0.24^{c}	3.73±0.01^{b}	192.75±8.31^{a}
50 weeks * 15%	21.73±2.89^{b}	0.36±0.0025^{b}	9.74±0.41^{c}	6.85±0.40^{d}	11.80±0.01^{ab}	8.37±0.70^{ab}	3.72±0.02^{b}	182.61±5.67^{ab}
50 weeks * 17%	20.43±3.37^{b}	0.32±0.0060^{c}	10.20±0.63^{bc}	7.10±0.03^{d}	11.84±0.15^{a}	8.98±0.36^{a}	3.74±0.01^{b}	165.06±11.04^{b}
50 weeks * 21%	23.88±2.42^{ab}	0.31±0.0052^{de}	11.60±0.09^{a}	9.64±0.01^{b}	11.75±0.10^{ab}	7.81±0.37^{bc}	3.73±0.01^{b}	192.33±25.30^{a}
P-value								
A	< 0.001	0.968	0.371	< 0.001	0.002	< 0.001	0.303	0.003
O_2	0.096	0.003	< 0.001	< 0.001	0.284	0.077	< 0.001	0.172
A * O_2	< 0.001	< 0.001	< 0.001	< 0.001	0.034	< 0.001	< 0.001	0.002

^{a,b,c}: Values within the same column followed by different subscript letters differ significantly ($p < 0.05$); All results are presented as mean ± SD; ED: Embryonic day; Dcal SHL-Ca: Decalcified eggshell calcium; Dcal SHL-P: Decalcified eggshell phosphorus; Ser Ca: Serum calcium; Ser P: Serum phosphorus, Ser Mg: Serum magnesium; GLU: Glucose.

Table 5. Effects of breeder age and incubator oxygen concentration levels on tibia morphometry of chicks from 33 and 50 weeks ISA brown layer breeders in early hypoxic stimulation (ED 7- 9)

Parameters	Absolute weight (g)	Relative weight (%)	Length (mm)	Diameter (mm)	Robusticity (mm/g)	Seedor index (g/mm)
Breeder age (A)						
33 weeks	0.05±0.0069	0.17±0.03	25.15±1.02	2.53±0.22	6.86±0.23^{a}	0.020±0.0023
50 weeks	0.05±0.0108	0.16±0.03	24.92±1.28	2.55±0.22	6.63±0.30^{b}	0.022±0.0037
Oxygen level (O_2)						
15%	0.05±0.0075^{b}	0.15±0.03^{b}	24.26±0.97^{b}	2.41±0.16^{b}	6.73±0.21	0.020±0.0025^{b}
17%	0.05±0.0069^{b}	0.17±0.03^{ab}	25.21±0.98^{ab}	2.49±0.17^{b}	6.85±0.28	0.020±0.0024^{ab}
21%	0.06±0.0100^{a}	0.19±0.02^{a}	25.64±1.10^{a}	2.71±0.19^{a}	6.66±0.34	0.023±0.0036^{a}
Interaction (A * O_2)						
33 weeks * 15%	0.05±0.0075^{b}	0.16±0.04^{ab}	24.63±0.83^{ab}	2.37±0.10^{b}	6.79±0.23^{ab}	0.020±0.0025^{b}
33 weeks * 17%	0.05±0.0078^{b}	0.18±0.03^{ab}	25.56±1.30^{ab}	2.51±0.15^{ab}	6.91±0.30^{a}	0.020±0.0026^{b}
33 weeks * 21%	0.05±0.0063^{b}	0.18±0.02^{ab}	25.27±0.80^{ab}	2.71±0.23^{a}	6.88±0.15^{ab}	0.020±0.0020^{b}
50 weeks * 15%	0.05±0.0082^{b}	0.14±0.02^{b}	23.89±1.02^{b}	2.44±0.20^{ab}	6.67±0.18^{ab}	0.019±0.0026^{b}
50 weeks * 17%	0.05±0.0065^{b}	0.15±0.02^{b}	24.86±0.35^{ab}	2.47±0.19^{ab}	6.80±0.27^{ab}	0.020±0.0025^{b}
50 weeks * 21%	0.07±0.0049^{a}	0.20±0.01^{a}	26.01±1.30^{a}	2.72±0.16^{a}	6.44±0.34^{b}	0.025±0.0023^{a}
P-value						
A	0.168	0.446	0.553	0.825	0.015	0.078
O_2	0.010	0.008	0.007	< 0.001	0.238	0.027
A * O_2	< 0.001	0.002	0.015	0.008	0.035	0.001

^{a,b}: Values within the same column followed by different subscript letters differ significantly ($p < 0.05$); All results are presented as mean ± SD; Rel: Relative; ED: Embryonic day

Table 6. Effects of breeder age and incubator oxygen concentration levels on femur morphometry of chicks from 33 and 50 weeks ISA brown layer breeders in early hypoxic stimulation (ED 7-9)

Parameters	Absolute Weight (g)	Relative Weight (%)	Length (mm)	Diameter (mm)	Robusticity (mm/g)	Seedor index (g/mm)
Breeder age (A)						
33 weeks	0.03±0.006^{b}	0.11±0.03	17.60±0.63	2.33±0.19	5.59±0.33	0.018±0.003^{b}
50 weeks	0.04±0.007^{a}	0.12±0.03	18.08±0.88	2.27±0.14	5.46±0.25	0.020±0.003^{a}
Oxygen level (O_2)						
15%	0.03±0.005	0.11±0.02	17.87±0.80	2.29±0.10	5.50±0.24	0.019±0.002
17%	0.04±0.007	0.12±0.03	17.70±0.67	2.28±0.22	5.47±0.30	0.020±0.004
21%	0.03±0.009	0.11±0.03	17.94±0.92	2.34±0.16	5.61±0.34	0.019±0.004
Interaction (A * O_2)						
33 weeks * 15%	0.03±0.004^{ab}	0.11±0.01^{b}	17.66±0.22	2.28±0.10	5.60±0.25^{b}	0.02±0.002^{ab}
33 weeks * 17%	0.04±0.008^{ab}	0.13±0.03^{a}	17.59±0.75	2.32±0.28	5.36±0.25^{c}	0.02±0.004^{ab}
33 weeks * 21%	0.03±0.004^{b}	0.10±0.01^{bc}	17.54±0.85	2.41±0.14	5.82±0.33^{a}	0.02±0.002^{b}
50 weeks * 15%	0.04±0.004^{ab}	0.12±0.02^{ab}	18.08±1.13	2.31±0.12	5.39±0.19^{c}	0.02±0.001^{ab}
50 weeks * 17%	0.03±0.008^{ab}	0.11±0.03^{b}	17.80±0.64	2.24±0.16	5.58±0.33^{b}	0.02±0.004^{ab}
50 weeks * 21%	0.04±0.009^{a}	0.12±0.03^{ab}	18.35±0.86	2.27±0.16	5.41±0.19^{bc}	0.02±0.004^{a}
P-value						
A	0.025	0.675	0.064	0.243	0.172	0.039
O_2	0.932	0.763	0.752	0.676	0.463	0.812
A * O_2	0.039	0.045	0.457	0.595	0.044	0.031

^{abc}: Values within the same column followed by different subscript letters differ significantly ($p < 0.05$). All results are presented as mean ± SD; ED: Embryonic day

Table 7. Correlation between calcium and phosphorus in decalcified eggshell after incubation, calcium, and phosphorus absorbed by chick's tibia and femur bone, blood serum, glucose and tibia and femur morphology of chicks hatched from 21% (control) oxygen concentration level of 33 and 50 weeks ISA brown layer breeders

Parameters	Dcal SHL-Ca	Dcal SHL-P	BNE-Ca	BNE-P	SER-Ca	SER-P	rTB-wgt	TB-leng	TB-dm	TB-rob	TB-SI	rFM-wgt	FM-leng	FM-dm	FM-rob	FM-SI
Dcal SHL-P	0.22															
BNE-Ca	-0.41	-0.51														
BNE-P	0.54	0.21	-0.51													
SER-Ca	-0.43	-0.75[b]	0.27	-0.30												
SER-P	-0.52	-0.58[a]	0.58[a]	-0.58[a]	0.82											
rTB-wgt	-0.48	0.06	0.46	-0.45	0.14	0.62[a]										
TB-leng	0.12	0.11	0.43	-0.10	-0.37	0.03	0.37									
TB-dm	-0.22	0.63[a]	-0.22	-0.51	-0.22	-0.02	0.22	-0.02								
TB-rob	0.57[a]	0.28	-0.41	0.58[a]	-0.53	-0.79[b]	-0.78	0.19	-0.16							
TB-SI	-0.49	-0.26	0.62[a]	-0.58[a]	0.36	0.79[b]	0.92	0.27	0.08	-0.89						
rFM-wgt	-0.25	-0.06	0.25	-0.44	0.50	0.57	0.17	0.00	0.46	-0.22	0.17					
FM-leng	0.11	0.22	0.18	-0.10	0.21	0.46	0.38	0.17	0.39	-0.27	0.31	0.79				
FM-dm	0.07	-0.07	-0.39	0.30	0.17	-0.23	-0.52	-0.90	-0.20	0.06	-0.46	-0.10	-0.19			
FM-rob	0.47	0.55	-0.47	0.72[b]	-0.69[b]	-0.71[b]	-0.14	-0.08	-0.23	0.33	-0.33	-0.71	-0.28	0.28		
FM-SI	-0.34	-0.29	0.47	-0.61[a]	0.59[a]	0.74[b]	0.29	0.16	0.35	-0.35	0.38	0.93	0.70	-0.29	-0.87	
CAM-wgt	-0.14	0.70[a]	-0.66[a]	0.32	-0.32	-0.44	-0.03	-0.22	0.44	0.17	-0.29	-0.16	-0.11	0.11	0.46	-0.40

[a]: $p \leq 0.05$; [b]: $p \leq 0.01$; [c]: $p \leq 0.001$; Dcal SHL-Ca: Retained eggshell calcium; Dcal SHL-P: Retained eggshell phosphorus; BNE-Ca: Absorbed bone calcium; BNE-P: Absorbed bone phosphorus; SER-Ca: Absorbed serum calcium; SER-P: Absorbed serum phosphorus; rTB-wgt: Relative tibia weight; TB-leng: Tibia length; TB-dm: Tibia dm; TB-rob: Tibia robusticity; TB-SI: Tibia seedor index; rFM-wgt: Relative femur weight; FM-rob: Femur robusticity; CAM-wgt: CAM weight. No correlation within minerals or tibia or femur morphometrics was considered.

Table 8. Correlation between calcium and phosphorus remains in decalcified eggshell after incubation, calcium and phosphorus absorbed by chick's tibia and femur bone and blood serum at hatch, glucose and tibia and femur morphology of chicks in early 15% (below diagonal) and 17% (above diagonal) hypoxic stimulation of 33 and 50 weeks ISA brown layer breeders

Parameters	Dcal SHL-Ca	Dcal SHL-P	BNE-Ca	BNE-P	SER-Ca	SER-P	rTB-wgt	TB-leng	TB-dm	TB-rob	TB-SI	rFM-wgt	FM-leng	FM-dm	FM-rob	FM-SI	CAM-wgt
Dcal SHL-Ca		0.75	0.37	-0.61[a]	-0.15	-0.52	0.41	0.29	0.06	0.05	0.08	0.12	-0.32	-0.09	0.01	-0.09	-0.07
Dcal SHL-P	-0.80		0.29	-0.95[c]	-0.45	-0.84[c]	0.56[a]	0.34	0.10	0.12	0.09	0.34	-0.25	0.15	-0.29	0.13	-0.23
BNE-Ca	0.72[b]	-0.80[b]		-0.41	-0.43	-0.42	-0.01	0.26	-0.24	0.47	-0.36	0.41	0.34	-0.05	-0.39	0.43	0.50
BNE-P	0.82[c]	-0.96[c]	0.84		0.63[a]	0.94[c]	-0.44	-0.38	-0.03	-0.33	0.09	-0.38	0.16	-0.21	0.37	-0.22	0.13
SER-Ca	-0.41	0.57	-0.42	-0.55		0.75	0.02	-0.42	0.15	-0.53	0.29	0.06	0.04	0.12	-0.11	0.12	0.34
SER-P	-0.47	0.49	-0.27	-0.55	0.84		-0.41	-0.54	-0.16	-0.42	0.10	-0.33	0.01	-0.10	0.28	-0.19	0.05
rTB-wgt	0.41	-0.43	0.45	0.36	-0.53	-0.25		0.51	0.14	-0.48	0.80	0.39	-0.06	-0.32	-0.35	0.25	-0.01
TB-leng	0.39	-0.43	0.52	0.38	-0.51	-0.11	0.80		0.32	0.33	0.26	-0.14	-0.15	-0.54	0.08	-0.19	0.07
TB-dm	-0.08	0.23	-0.36	-0.37	-0.08	0.20	0.17	0.41		0.03	0.16	-0.08	0.22	0.24	0.14	-0.12	0.24
TB-rob	0.02	-0.26	0.24	0.36	-0.06	-0.13	-0.51	-0.07	-0.25		-0.83	-0.14	-0.06	0.14	0.01	-0.10	0.18
TB-SI	0.24	-0.09	0.16	-0.02	-0.30	0.01	0.88	0.71	0.46	-0.75		0.05	-0.05	-0.43	0.02	-0.01	-0.16
rFM-wgt	-0.31	0.34	-0.29	-0.23	<0.001	-0.30	-0.13	-0.47	-0.60	-0.22	-0.17		0.64	0.31	-0.89	0.96	0.53
FM-leng	-0.49	0.31	-0.41	-0.25	0.28	-0.08	-0.65	-0.83	-0.57	0.29	-0.76	0.55		0.16	-0.43	0.71	0.61
FM-dm	-0.11	0.19	0.05	0.01	-0.19	-0.43	0.06	-0.10	-0.58	0.02	-0.08	0.83	0.31		-0.42	0.35	0.28
FM-rob	0.11	-0.44	0.24	0.42	<0.01	-0.07	-0.19	-0.17	-0.25	0.54	-0.49	-0.40	0.40	-0.38		-0.92	-0.62[a]
FM-SI	-0.41	0.64[c]	-0.51	-0.57[a]	0.17	-0.03	-0.27	-0.43	-0.18	-0.35	-0.04	0.82	0.30	0.63	-0.76		0.66[a]
CAM-wgt	-0.88[c]	0.89[c]	-0.52	-0.81[b]	0.48	0.51	-0.40	-0.31	-0.01	0.03	-0.22	0.29	0.38	0.26	-0.21	0.46	

[a]: $p \leq 0.05$; [b]: $p \leq 0.01$; [c]: $p \leq 0.001$; Dcal SHL-Ca: Retained eggshell calcium; Dcal SHL-P: Retained eggshell phosphorus; BNE-Ca: Absorbed bone calcium; BNE-P: Absorbed bone phosphorus; SER-Ca: Absorbed serum calcium; SER-P: Absorbed serum phosphorus; rTB-wgt: Relative tibia weight; TB-leng: Tibia length; TB-dm: Tibia dm; TB-rob: Tibia robusticity; TB-SI: Tibia seedor index; rFM-wgt: Relative femur weight; FM-rob: Femur robusticity; CAM-wgt: CAM weight No correlation within minerals or tibia or femur morphometrics was considered.

Table 9. Neo-vascularization in heart tissue of embryos from 33 and 50 weeks ISA layer breeder at ED 11 after early hypoxic stimulation

Groups		Heart tissue
Breeder age (A_b)	**Oxygen level (O_2)**	**Neo-vascularization score**
33 weeks	15%	++++
	17%	+++
	21%	+++
50 weeks	15%	++++
	17%	++++
	21%	+++

Breeder age and oxygen level (A * O_2): 33 weeks * 15% O_2 (33 weeks breeder eggs incubated in 15% O_2); 33 weeks * 17% O_2 (33 weeks breeder eggs incubated in 17% O_2), 33 weeks * 21% O_2 (33 weeks breeder eggs incubated in 21% O_2), 50 weeks * 15% O_2 (50 weeks breeder eggs incubated in 15% O_2), 50 weeks * 17% O_2 (50 weeks breeder eggs incubated in 17% O_2), 50 weeks * 21% O_2 (50 weeks breeder eggs incubated in 21% O_2). Neo-vascularization grading; ++++: Very high (ectatic vessels with high congestion); +++: high (vessels seat of moderate congestion); ++: Normal (normal tissues).

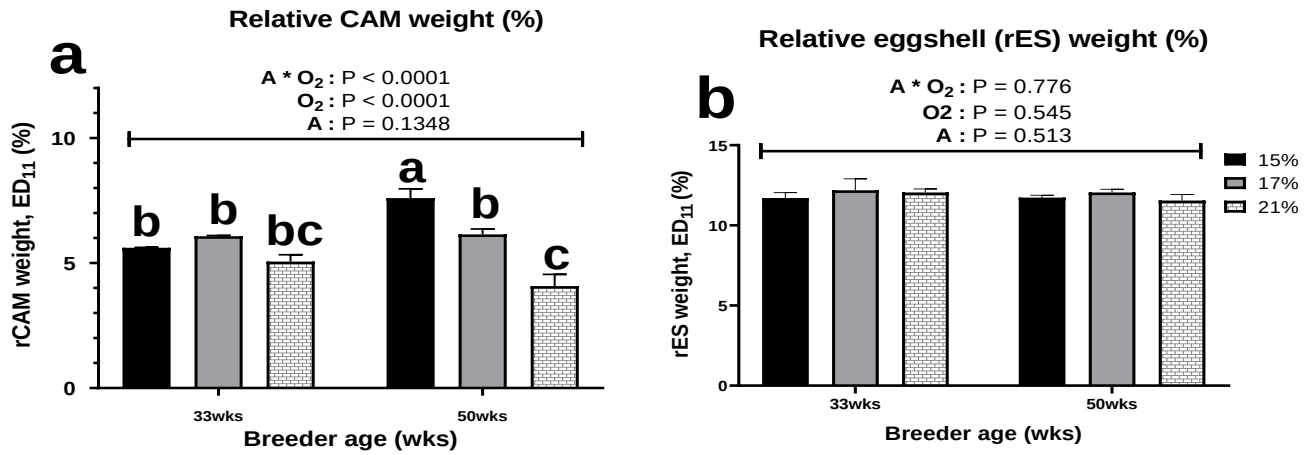

Figure 1. Chorioallantoic membrane (CAM) and eggshell (ES) weight at ED 11 of 33 and 50 weeks ISA brown layer breeder flocks exposed to early hypoxic stimulation (ED 7-9). [a,b,c]: Bar charts with different letters indicate significant differences ($p < 0.05$).

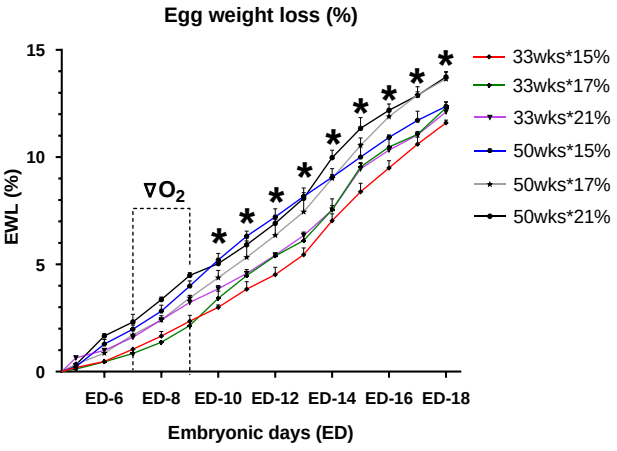

Figure 2. Egg weight loss of 33 and 50 weeks ISA brown layer breeder flocks exposed to early hypoxic stimulation (ED7-9). *: ED with asterisk symbol indicates significant differences ($p < 0.05$), vO_2: A period of hypoxic stimulation; ED: Embryonic day; EWL: Egg weight loss.

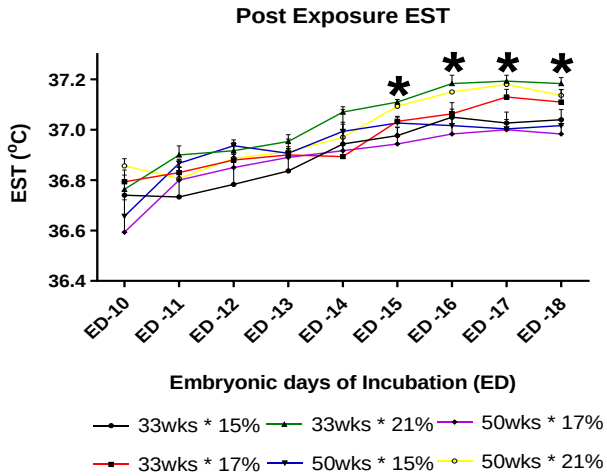

Figure 3. Post-exposure eggshell temperature (EST) of 33 and 50-week embryos exposed to early hypoxic stimulation (ED 7-9). *: An asterisk symbol indicates significant differences ($p < 0.05$); ED: Embryonic day.

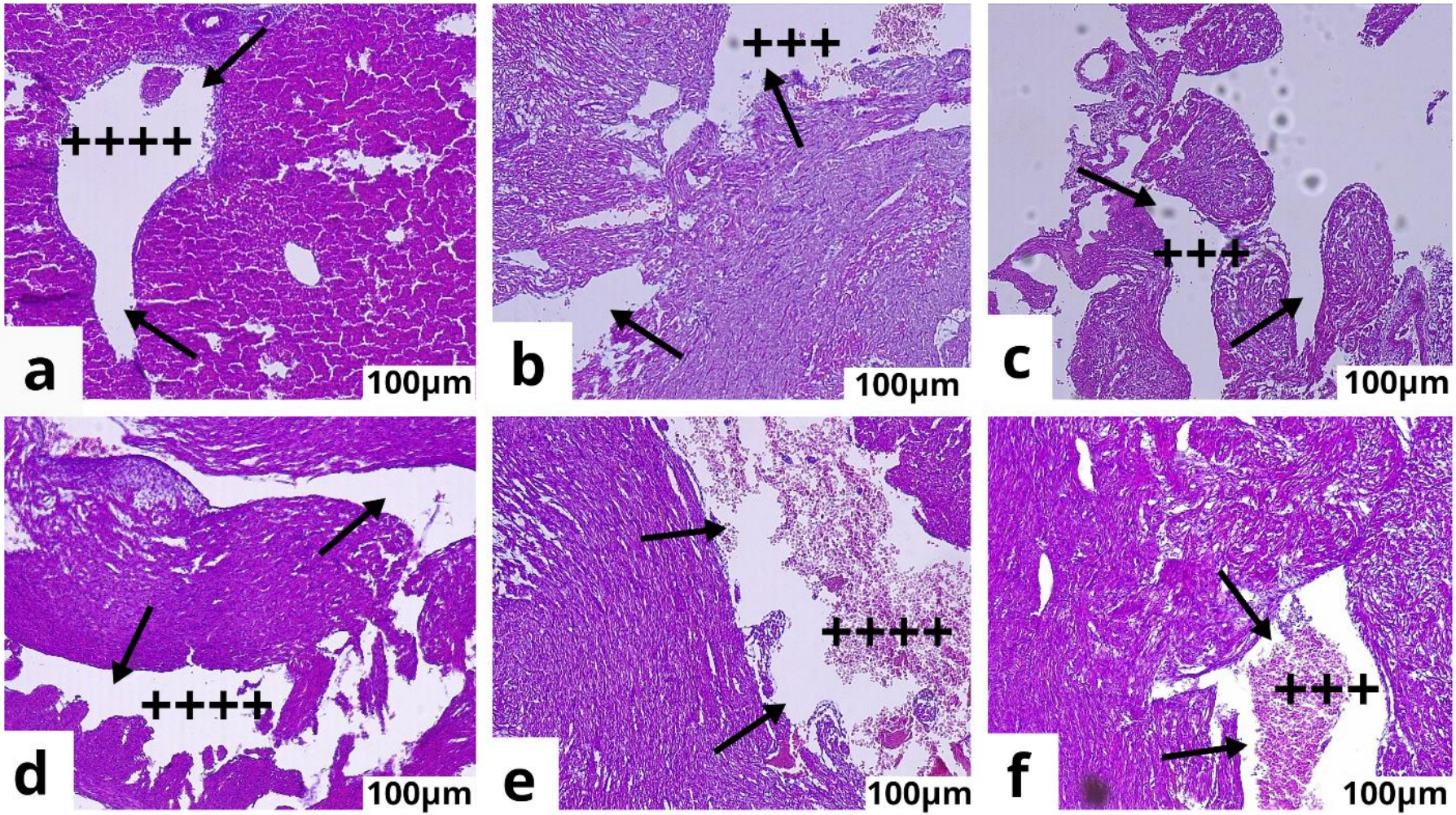

Figure 4. The heart tissue (100x magnification) showing neo-vascularization at ED11 of 33 and 50 weeks ISA breeder eggs in early hypoxic stimulation.
abcdef: Breeder age and Oxygen concentration (A * O_2); **a:** 33 weeks * 15% O_2 (33 weeks breeder eggs incubated in 5% O_2), **b:** 33 weeks * 17% O_2 (33 weeks breeder eggs incubated in 17% O_2), **c:** 33 weeks * 21% O_2 (33 weeks breeder eggs incubated in 21% O_2), **d:** 50 weeks * 15% O_2 (50 weeks breeder eggs incubated in 15% O_2), **e:** 50 weeks * 17% O_2 (50 weeks breeder eggs incubator 17% O_2), **f:** 50 weeks * 21% O_2 (50 weeks breeder eggs incubated in 21% O_2). Neo-vascularization grading: ++++: Very high (ectatic vessels with high congestion); +++: High (vessels seat of moderate congestion); ++: Normal (normal tissues).

DISCUSSION

Eggshell quality is pivotal in embryonic development, influencing factors, such as gas exchange, moisture loss, and hatching success. The current study highlighted those eggs from older breeders (50 weeks) tended to be heavier with larger dimensions and more pores compared to those from younger breeders (33 weeks). The observation corroborated existing findings that egg size generally increases with breeder age due to alterations in the reproductive system and nutrient allocation (Park and Sohn, 2018). Interestingly, eggshells from younger breeders were found to have higher calcium content, suggesting a greater allocation of calcium to counterbalance the smaller egg sizes (Yair and Uni 2011; Santos et al., 2021). The current finding of higher Ca in younger breeders compared to older breeders aligned with studies indicating that older hens often produce eggs with thinner shells due to reduced calcium absorption and metabolism (Ahmed, 2016; Bain et al., 2016; Ketta and Tůmová, 2016). On the contrary, Dolgorukova et al. (2022) reported that older hens tend to deposit more calcium for embryonic development. Torres (2013) also indicated that differences in dietary calcium intake or physiological conditions between breeder ages could influence the amount of Ca deposited in the eggshell.

The chorioallantoic membrane (CAM) plays a critical role in gas exchange and calcium absorption during embryonic development. The current study observed an increase in CAM weight under reduced oxygen levels (15% and 17%), suggesting a compensatory mechanism to enhance gas exchange in a hypoxic environment (Zamudio, 2003; Nowak-Sliwinska et al., 2014; Zhang et al., 2020). The increase in chorioallantoic weight under reduced oxygen level in the present study agrees with the results of Druyan et al. (2012) and Haron et al. (2017; 2021) who found these increases as an adaptive response to growth under hypoxic stress. The increased CAM weight in older flocks may also be attributed to the larger dimension and pore distribution around the older eggs allowing enough gaseous exchange. The absence of a significant effect of breeder age on eggshell weight underscored that oxygen concentration levels during incubation are more crucial for CAM development than breeder age, although eggshell weight generally decreases under normal incubation conditions (Halgrain et al., 2021; 2022)

Unlike Nangsuay et al. (2021) who found an interaction between breeder flock age and oxygen concentration at ED18 of incubation for yolk-free body mass, the present study reported an interaction between breeder age and oxygen level on egg weight loss immediately after exposure to ED 10. A notably lesser egg weight loss under hypoxic conditions (15% and 17% O_2) through to ED 18, suggesting suppressed metabolic activity. The reduction in egg weight loss supports the idea that increased carbon dioxide (CO_2) which was an alternative to lower oxygen levels reduces metabolic rate, thereby decreasing water loss and conserving energy under suboptimal conditions (Bilalissi et al., 2022). Heavier eggs from older breeders typically exhibited greater weight loss during incubation (Lourens et al., 2006; Ahmed, 2016). Additionally, eggs from chickens adapted to high altitudes showed lower water loss when incubated under hypoxic conditions (Zhang et al., 2008).

Concurrently with embryo weight loss in the present study, the eggshell temperature (EST) under hypoxic conditions, especially from ED 15 to ED 18 was lower compared to the control group (21% oxygen level). The lower EST obtained from reduced oxygen levels (15% and 17% O_2) confirmed a state of lower metabolic rates and thermoregulation in embryos. Haron et al. (2017) reported a decrease in EST after returning hypoxic-treated embryos to normal conditions. Younger breeder (33 weeks) eggs demonstrated higher ESTs at later developmental stages (ED 17 and ED 18) and glucose levels of chicks at hatch compared to older (55 weeks) breeders' flocks. The current finding of an increased glucose level of chicks of the 33 weeks breeders compared to the 50 weeks breeders was consistent with the studies of Güz et al. (2020) and Nasri et al. (2020) who also reported similar results, suggested that the younger flocks had stronger thermoregulatory response to hypoxic stress during the time of pipping compared to the older flocks. In broilers, Nangsuay et al. (2021), reported no interaction between breeder age and oxygen concentration on embryonic heat production. However, in layers, the interaction between breeder age and incubator oxygen concentration on embryonic heat production may differ due to varying embryonic development trajectories (Tona et al., 2001; Hamidu et al., 2011).

Bone development in chicks is heavily dependent on calcium and phosphorus from the eggshell during incubation. The present study found significant interactions between breeder age and incubator oxygen level on bone mineral contents, with younger breeders and 21% controlled levels showing a higher Ca absorption by the bone. Reduced absorption of bone calcium and phosphorus under hypoxic conditions aligns with previous findings that suggested hypoxia impaired calcium

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metabolism and deposition in developing bones, leading to weaker skeletal structures (Chen et al., 2022; Wawrzyniak and Balawender, 2022). Older breeders exhibited lower bone phosphorus but retained higher serum calcium and phosphorus in chicks compared to the younger flocks. Ahmed (2016) attributed higher plasma Ca and P levels to greater eggshell calcium content in chicks from younger breeders. Some genes also play a critical role in Ca and P absorption during incubation. These genes involved in the mobilization of egg minerals during embryonic development are different between yolksac and CAM extraembryonic structures (Halgrain et al., 2021; 2022; 2023).

Hypoxic conditions (15% and 17% oxygen level) were associated with lower absolute and relative weights of the tibia and femur, along with shorter tibial lengths, diameters and seedor index, indicating that reduced oxygen during the early chorioallantoic development during embryogenesis can impair bone development during embryogenesis, likely due to decreased oxidative phosphorylation and resulting energy deficits in developing tissues (Oviedo-Rondón et al., 2008). The impact of oxidative stress on endochondral ossification could influence the rate and extent of bone mineralization in growing long bones (Glimcher, 2006). The interaction between breeder age and oxygen concentration was particularly evident in tibia than in femur morphometries. In chicks, the quality of bone development is largely determined by the quality of tibia development. The tibia bone was frequently observed for negative changes and was therefore examined in chicks for their skeletal growth, mineralization, and strength (Aguado et al., 2015). As Almeida Paz and Bruno (2006) indicated seedor density determines bone density. The current study showed lower seedor index values observed under hypoxic conditions (15% and 17%) compared to the control (21%) oxygen level groups, indicating a less dense bone of chicks possibly due to the less calcium availability.

Limited knowledge existed about the correlative impact of trace mineral bioavailability in eggshells and yolks and its effect on chick skeletal growth. The current study showed that calcium and phosphorus levels in decalcified eggshells, chick bones, and blood serum correlated with the tibia and femur morphometrics, confirming a dynamic relationship between mineral metabolism and skeletal development. Positive correlations between eggshell calcium and tibia robusticity, and between serum phosphorus and tibia seedor index, highlighted the importance of mineral absorption in supporting bone strength and structure. Conversely, negative correlations between serum calcium and femur robusticity under hypoxic conditions suggested that an impaired metabolism of Ca and P from the blood to the bone was possible and could likely cause a weak bone structure. Insufficient calcium intake and vitamin D deficiency are positively correlated with osteoporosis prevalence (Voulgaridou et al., 2023).

It was fascinating to report from the present research that there was a strong positive correlation between decalcified eggshell calcium and bone Ca and P under 15% oxygen level. The positive correlation between the amount of calcium remaining in the eggshell and those absorbed by the chick's bone reflects the importance of calcium transfer from the eggshell to the embryo, even under suboptimal conditions. Calcium mobilization from the eggshell was positively correlated with the number of mammillary tips (Karlsson and Lilja, 2008). The decrease in Ca and P levels in the eggshell under 15% hypoxic condition was likely due to increased CAM weight and vascularization. The decrease in eggshell Ca and increased CAM weight and vascularization under the 15% hypoxic conditions seemed to be more evident in older breeder flocks compared to the younger breeder flocks (Agbehadzi et al., 2024). An increase in CAM weight strongly correlated with a decrease in Ca retained in decalcified eggshell but unfortunately, calcium was not ionized into the chick bone. Increasing CAM weight potentially increases vascularization which may have resulted in more Ca absorption from the eggshell into the blood during embryogenesis. Sys et al. (2013) found that Ca^{2+} was transported to the embryonic circulation by chorionic epithelial cells at 100 nanomoles per hour for one square centimeter of CAM surface. The inability of Ca to be ionized from the blood into the bone before hatch time confirms the report that many calcium signaling pathways may be altered by hypoxia, especially when it is chronic (Pearce, 2006; Quan et al., 2021). Besides, during embryo development, incubating eggs at 39°C also decreased the accessibility of blood-ionized Ca to bone mineralization (Sgavioli et al., 2016). The mechanism for this occurrence under different incubation conditions needs to be further investigated.

The high level of vessel dilation observed in the heart tissue of embryos incubated under hypoxic conditions suggests an adaptive response to increase O_2 supply to embryonic tissues (Hsia et al., 2013; Ramanlal and Gupta, 2023; Agbehadzi et al., 2024). Vascular dilation occurs due to the activation of adenosine monophosphate-activated protein kinase in vascular endothelial cells (Rodríguez et al., 2021). The direct role of increased

vasodilation on mineral transfer from the eggshell to bone tissue is unknown. However, the current study's findings assumed that the adaptive response causes an increased rate of Ca transport from the eggshell into the blood through the adaptive regulatory supply of oxygen.

CONCLUSION

Eggs from older breeders (50 weeks) were heavier, had larger external dimensions, and exhibited a more porous distribution compared to those from younger breeders (33 weeks). However, eggshells from younger breeders contained more calcium, indicating age-related differences in calcium allocation. The reduction in oxygen levels, particularly under hypoxic conditions (15% and 17%) oxygen level notably affected the quality of eggs from different breeder ages. This effect led to compensatory mechanisms of the embryo, such as increased chorioallantoic membrane (CAM) weight, increased neo-vascularization in heart tissue, reduced metabolic activity, lower egg weight loss, and altered eggshell temperature (EST) during late incubation. Early hypoxic stimulations resulted in lower absolute and relative weights of the tibia and femur, shorter tibial lengths, diameter, and seedor index, and impaired calcium and phosphorus absorption from the blood into the bone, especially in older breeders. The study also identified strong negative correlations between CAM weight and Ca in decalcified eggshells under 15% oxygen level, indicating that increased vascularization had the potential to increase Ca absorption from the eggshell into the blood. However minimal Ca was ionized from the blood into the bone of chicks, particularly for the 50-week breeders. Future studies could investigate the molecular mechanisms behind the role of oxygen-sensing pathways in regulating mineral absorption from the blood into the bone under different environmental conditions.

DECLARATIONS

Authors' contributions

Richard Koblah Agbehadzi designed, executed, analyzed data, validated results, drafted, and edited the manuscript. Prince Sasu, Benjamin Adjei-Mensah, Hezouwe Tchilabalo Meteyake, Nideou Dassidi, Yaah Aimee Emmanuelle Kouame, and Achiamaa Asafu-adjaye Koranteng supported in reviewing and editing the manuscript. Jacob Alhassan Hamidu and Kokou Tona conceptualized, affirmed experimental design, validated results, and edited the manuscript. All authors approved the final version before publication.

Ethical consideration

The authors used original analyzed data obtained from the present study to write the article and submitted only to this journal. The content of the article is checked for plagiarism before submission to the journal.

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Availability of data and materials

All the data generated on the field and analyzed during this study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare no competing interests.

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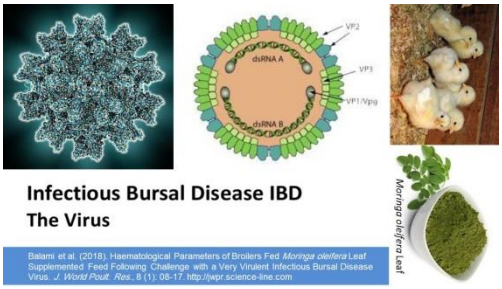

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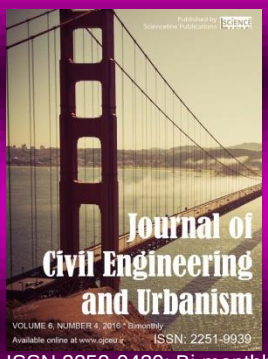

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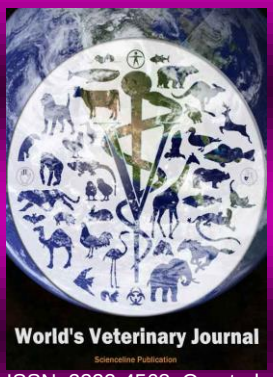

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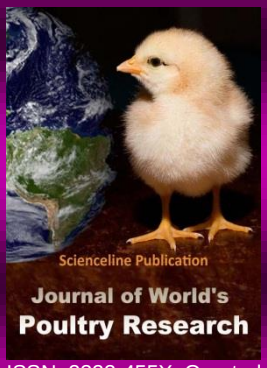

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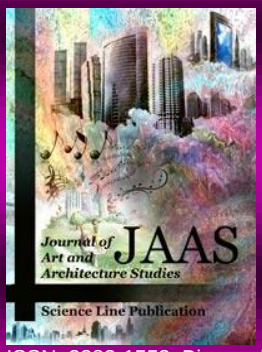

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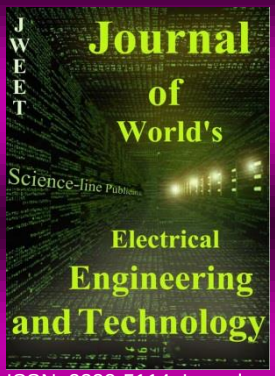

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