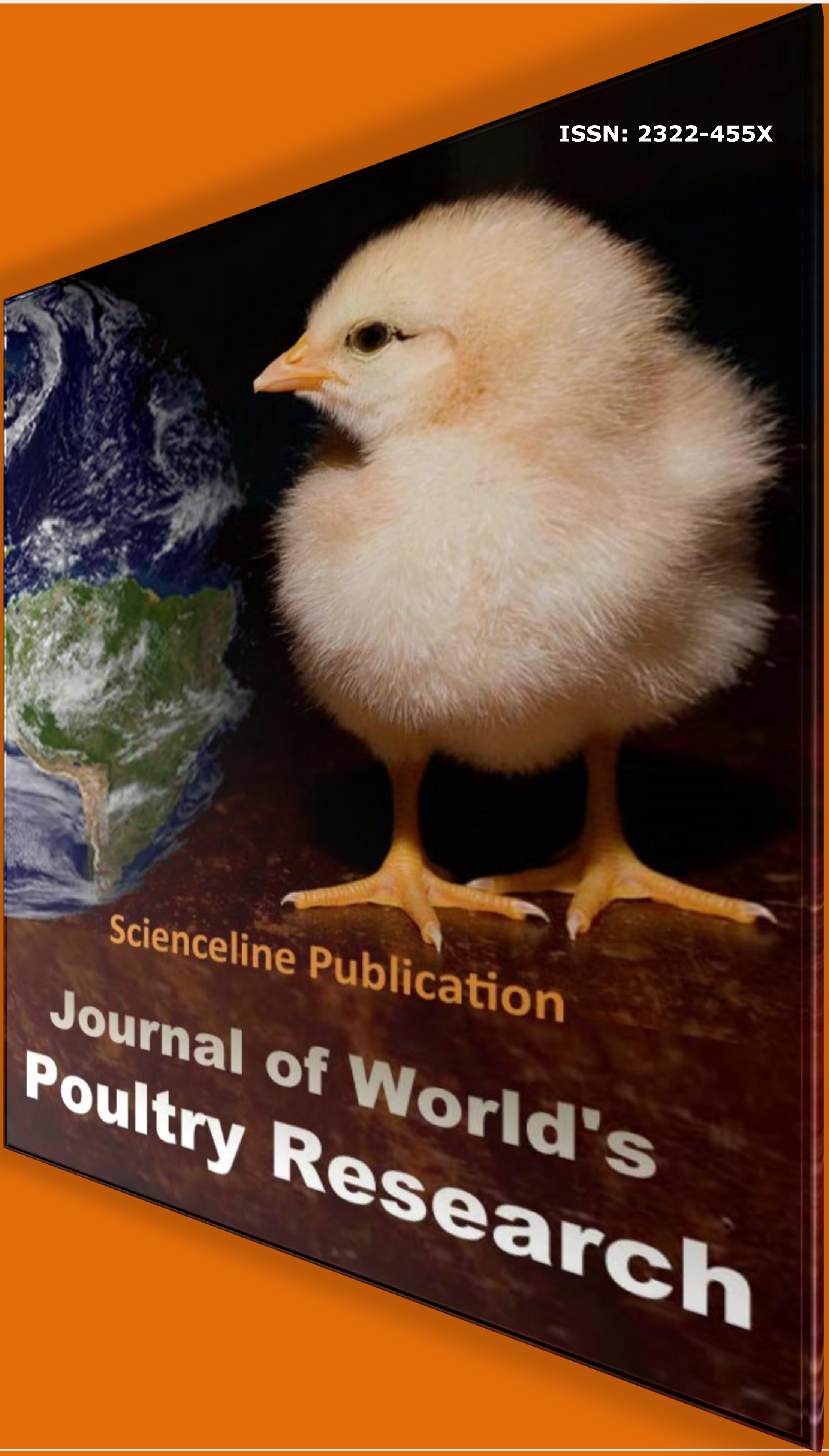
Journal of World's Poultry Research

BOOKLET



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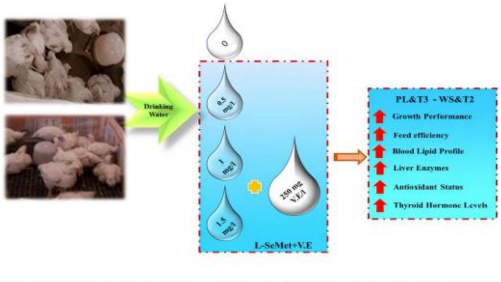
Volume 15 (2); June 2025

Research Paper

The Interplay of Litter Types, Blends of L-selenomethionine, and Vitamin E in Broiler Chicken's Performance and Health

Rady EM, EL-Gendi G, El-Garhy O, and Okasha H.

J. World Poult. Res. 15(2): 139-150, 2025; pii: S2322455X2500014-15
DOI: https://dx.doi.org/10.36380/jwpr.2025.14



Rady EM, EL-Gendi G, El-Garhy O, and Okasha H (2025). **The Interplay of Litter Types, Blends of L-selenomethionine, and Vitamin E in Broiler Chicken's Performance and Health.** *J. World Poult. Res.*, 15(2): 139-150. DOI: https://dx.doi.org/10.36380/jwpr.2025.14

ABSTRACT: The choice of litter type (LT) in broiler chicken production can significantly influence overall performance and health. Adding specific combinations of L-selenomethionine and Vitamin E (L-SeMet + V.E) to drinking water can impact broiler chickens' health. This study aimed to explore the effects of two LT types, wood shavings (WS) and perforated plastic litter (PL) as well as water supplementation with L-SeMet + V.E, on growth performance, blood parameters, antioxidant capacity, and thyroid hormone levels in broiler chickens. A total of 312 one-day-old male broiler chickens were randomly assigned to two different LT groups (WS and PL), with 156 chicks in each. Within each LT group, the chickens were further divided into four subgroups: a control group (T0) that received plain water, and three treatment groups that received drinking water supplements. Each subgroup consisted of three replicates, with 13 broiler chickens per replicate. The water treatments involved varying levels of supplementation with L-SeMet and vitamin E: T1 (0.5 mg/L + 250 mg/L), T2 (1 mg/L + 250 mg/L), and T3 (1.5 mg/L + 250 mg/L). Chickens were assessed for growth performance, feed efficiency, and blood biochemical parameters including lipids, liver enzymes, antioxidants, and thyroid hormones. Chickens raised on PL and receiving T3 exhibited significantly enhanced performance and feed efficiency, outperforming those raised on WS and given different supplementation levels. Significant enhancements were observed in the blood lipid profile and liver enzyme levels across the different LT groups, with the highest values recorded in the PL group. Additionally, broiler chicken in the T2 group, along with the WS × T2 and PL × T3 interactions, showed a notable improvement in blood biochemical parameters. Similarly, chickens raised on PL and given T3-supplemented water, along with the interactions WS × T2 and PL × T3, showed a significant improvement in antioxidant status and thyroid hormone levels in comparison to chickens raised on WS and the other treatment combinations. The use of PL and supplemenation with L-SeMet + V.E in drinking water were found to enhance growth performance, improve blood parameters, increase antioxidant capacity, and influence thyroid hormone levels in broiler chickens.

Keywords: Antioxidant, Broiler chicken, L-selenomethionine and Vitamin E, Productivity, Thyroid hormone

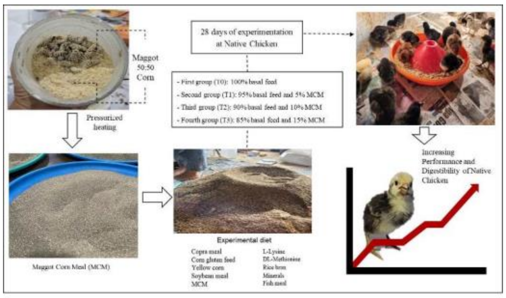
[Full text-PDF]

Research Paper

Effects of Dietary Inclusion of Maggot (*Hermetia illucens*) and Corn on Productivity of Starter-Stage Native Chickens

Panuntun S, Utama C, and Sulistiyanto B.

J. World Poult. Res. 15(2): 151-157, 2025; pii: S2322455X2500015-15
DOI: https://dx.doi.org/10.36380/jwpr.2025.15



Panuntun S, Utama C, and Sulistiyanto B (2025). **Effects of Dietary Inclusion of Maggot (*Hermetia illucens*) and Corn on Productivity of Starter-Stage Native Chickens.** *J. World Poult. Res.*, 15(2): 151-157. DOI: https://dx.doi.org/10.36380/jwpr.2025.15

ABSTRACT: Native chickens require innovative feeding strategies to enhance productivity. This study aimed to examine the dietary effects of using maggot and corn meal (MCM) on the performance and feed digestibility of native chickens. The study used a complete randomized design, with a total of 200 one-day-old native chicks randomly allocated into four groups, each with five replicates. The first group received 100% basal feed (T0). The second group received 95% basal feed and 5% MCM (T1), the third group received 90% basal feed and 10% MCM (T2), and the fourth group received 85% basal feed and 15% MCM (T3). Group T1 exhibited significantly higher live weight, daily body weight gain, crude protein digestibility, crude fiber digestibility, and crude fat digestibility compared to other treatments. The T3 group showed the highest feed intake, whereas the best feed conversion ratio (FCR) was observed in the T0 group. It is concluded that a 5% inclusion of MCM in the feed has beneficial effecta on growth performance and digestibility of native chickens.

Keywords: Black soldier fly, Combination feed, Corn, Digestibility, Native chicken, Performance

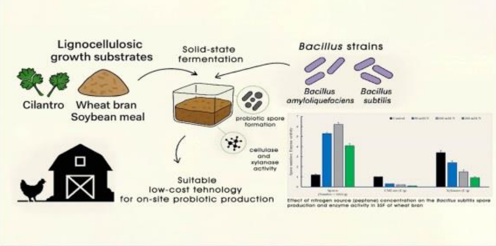
[Full text-PDF]

Research Paper

Obtaining High Yields of *Bacillus* species During Solid-State Fermentation of Plant Raw Materials for Use as a Feed Additive

Khardziani T, Metreveli E, and Elisashvili V.

J. World Poult. Res. 15(2): 158-165, 2025; pii: S2322455X2500016-15
DOI: https://dx.doi.org/10.36380/jwpr.2025.16



Khardziani T, Metreveli E, and Elisashvili V (2025). **Obtaining High Yields of *Bacillus* species During Solid-State Fermentation of Plant Raw Materials for Use as a Feed Additive**. *J. World Poult. Res.*, 15(2): 158-165. DOI: https://dx.doi.org/10.36380/jwpr.2025.16

ABSTRACT: *Bacillus* spp. are a natural alternative to antibiotic therapy and are most suitable for use as probiotic feed additives to improve the growth and productivity of farm animals. The present study aimed to establish culture conditions suitable for the maximal production of probiotic bacteria spores and enzymes of *Bacillus subtilis* IMB-73 and *Bacillus amyloliquefaciens* IMB-79 in solid-state fermentation (SSF) of wheat bran, soybean flour, and cilantro. The tested lignocellulosic materials ensured rapid and abundant growth of bacilli; however, wheat bran proved to be the most suitable growth substrate, yielding the highest spore counts of 5.1 × 10^{11}/g and 5.5 × 10^{11}/g in the cultivation of *B. amyloliquefaciens* IMB-79 and *B. subtilis* IMB-73, respectively. Supplementation of an additional nitrogen source to the wheat bran medium resulted in a significant increase in spore productivity. In particular, peptone at a concentration of 67 mg/g substrate provided the maximum spore yield in the fermented product. Both strains secreted high endoglucanase and xylanase activities in the SSF of wheat bran. Additional nitrogen sources slightly suppressed the secretion of enzymes during the SSF of wheat bran by *B. subtilis* IMB-73 but increased cellulase activity in cilantro SSF by approximately 2-5 times. The ease of production and the high spore yield achieved by scaling up production in polypropylene bags demonstrated the feasibility of using the developed technology for commercialization on local agricultural farms.

Keywords: *Bacillus*, Cellulase, Lignocellulose fermentation, Probiotic, Spore production

[Full text-PDF]

Research Paper

Biosecurity and Health Management Practices in Duck Farming in Coastal and Haor Regions of Bangladesh

Islam S, Islam MA, Sultana S, Sarker MSK, and Khatun R.

J. World Poult. Res. 15(2): 166-174, 2025; pii: S2322455X2500017-15
DOI: https://dx.doi.org/10.36380/jwpr.2025.17

- Poor biosecurity practices observed in both areas
- Health management practice is often reactive not preventive
- Disease outbreak, lack of vaccine, poor duckling, high feed price etc. are major challenges

- Feed price should be affordable
- Ensure quality duckling, availability of vaccine and extension of veterinary services
- Training program for development of skill and awareness build up

Islam S, Islam MA, Sultana S, Sarker MSK, and Khatun R (2025). **Biosecurity and Health Management Practices in Duck Farming in Coastal and Haor Regions of Bangladesh**. *J. World Poult. Res.*, 15(2): 166-174. DOI: https://dx.doi.org/10.36380/jwpr.2025.17

ABSTRACT: Duck farming has become a profitable venture in Bangladesh due to its economic potential and adaptability. This study aimed to assess the current state of native duck health, disease prevalence, and biosecurity management in selected regions of Bangladesh. Data were collected from 180 duck-rearing farmers from six upazilas of the Coastal and Haor regions using a standard questionnaire. The study investigated vaccination practices, disease prevalence, biosecurity management, and available treatment facilities. Approximately 60% of farmers in the Haor regions and 40 % in the Coastal regions reported vaccinating their ducks, while 56.67% in Haor and 34.44% in Coastal regions practiced deworming. Duck plague was the most prevalent disease in duck farms, with 83.33% and 90.00% prevalence rates in the Haor and Coastal areas, respectively. In the Haor region, 81.11% of farms reported disease incidence among growing ducks. Disease outbreaks (Duck plague and Cholera) were highest during the monsoon season at 77.78% of farms in Haor areas. In the Coastal region, 63.33% of farmers reported a higher duckling mortality rate. The frequent contact between ducks and wild birds was more common in Haor areas, as indicated by 32.22% of farmers. Disease outbreaks were identified as the major constraint to duck farming, and affordable feed price was the most pressing concern in both study areas. Despite these challenges, improvements in disease management, vaccination coverage, and biosecurity measures, alongside efforts to reduce feed costs, could significantly enhance the sector’s growth. It can be concluded that duck farming holds considerable potential and promising opportunities in the Haor and Coastal regions of Bangladesh.

Keywords: Biosecurity practice, Constraint, Deworming, Disease management, Vaccination

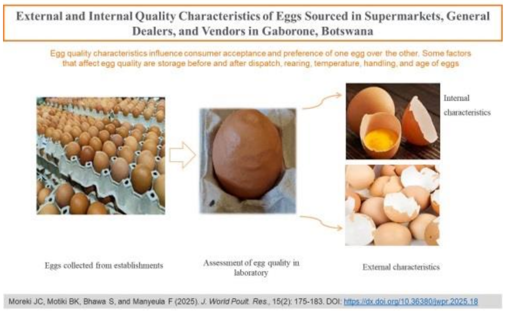
[Full text-PDF]

Research Paper

External and Internal Quality Characteristics of Eggs Sourced in Supermarkets, General Dealers, and Vendors in Gaborone, Botswana

Moreki JC, Motiki BK, Bhawa S, and Manyeula F.

J. World Poult. Res. 15(2): 175-183, 2025; pii: S2322455X2500018-15
DOI: https://dx.doi.org/10.36380/jwpr.2025.18



ABSTRACT: Egg quality characteristics influence consumer acceptance and preference of one egg over another. Several factors that impact egg quality include storage before and after dispatch, rearing conditions, temperature, handling, diseases, and the age of the eggs. The present study evaluated internal and external quality traits of eggs sourced from supermarkets, general dealers, and vendors in Gaborone, Botswana. One hundred and twenty eggs (24 eggs per location) were sourced from four supermarkets, seven general dealers, and 10 vendors in Gaborone. Parameters measured were egg weight (g), length (mm), width (mm), average shell thickness (mm), shell weight (g), surface area (cm^2), volume (cm^3), shape index, Haugh unit (HU), and shell weight per unit surface area (SWUSA, mg/cm^2) of the eggs. The current results indicated that egg weight and surface area were the highest for eggs sourced from supermarkets. Heavier eggs correlated with better HU scores, indicating richer and denser yolk, while surface area plays a role in moisture loss and potential shell strength. Eggs purchased from supermarkets and general dealers had noticeably greater egg weights, egg volumes, shell percentages, and SWUSA. Eggs from supermarkets had the greatest egg content weight, whereas those purchased from vendors had the lowest. The HU was highest for supermarket eggs compared to other egg sources. It was observed that eggs bought from supermarkets had superior internal and external quality traits compared to those from general dealers and vendors. It was concluded that eggs from vendors had lower quality due to inadequate storage and cooling facilities compared to supermarkets and general dealers.
Keywords: External quality, Haugh unit, Internal quality, Supermarket, Vendor

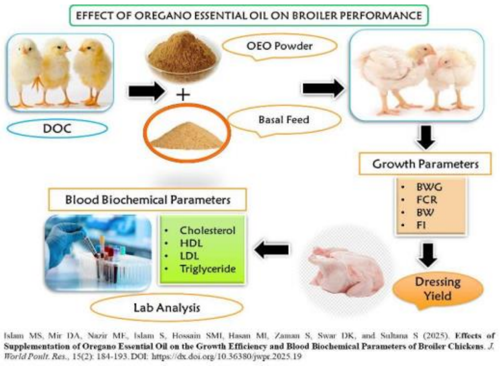
[Full text-PDF]

Research Paper

Effects of Supplementation of Oregano Essential Oil on the Growth Efficiency and Blood Biochemical Parameters of Broiler Chickens

Islam MS, Mir DA, Nazir ME, Islam S, Hossain SMI, Hasan MI, Zaman S, Swar DK, and Sultana S.

J. World Poult. Res. 15(2): 184-193, 2025; pii: S2322455X2500019-15
DOI: https://dx.doi.org/10.36380/jwpr.2025.19



ABSTRACT: Oregano essential oil (OEO) has emerged as a safe, effective, and bioactive additive, increasingly incorporated into feed formulations to enhance the growth performance and overall well-being of broiler chickens. This experiment aimed to investigate the consequences of the dietary supplementation of OEO regarding the growth efficiency, carcass characteristics, and blood biochemical profiles of broiler chickens. The 35-day feeding trial involved 200 unsexed Hubbard Classic broiler chickens with an average initial body weight of 42.79 g. The broiler chicks were randomly assigned to four experimental groups, each comprising five replicates of ten birds. The treatment groups were fed basal diets supplemented with 300 mg/kg (OEO$_{-1}$), 400 mg/kg (OEO$_{-2}$), and 500 mg/kg (OEO$_{-3}$) of OEO, while the untreated group (OEO$_{-0}$) was fed a basal diet without any additives. Weekly assessments of growth performance metrics were conducted for 5 weeks, and blood parameters were examined once on day 35. The results revealed that OEO$_{-3}$ treatment had a significant impact on the body weight, feed intake, and feed conversion ratio of broilers. A considerably increased dressing percentage was observed in the OEO$_{-3}$ group. The findings indicated that OEO-supplemented groups significantly influenced both high-density lipoprotein (HDL) and low-density lipoprotein (LDL) levels, especially OEO$_{-3}$, which showed higher HDL and lower LDL levels compared to other treatment and control groups. However, no significant effect was observed on total blood cholesterol and triglyceride concentrations in the experimental broilers. Incorporating OEO into the diet suggested that the higher doses (500 mg/kg) enhanced growth efficiency, increased HDL cholesterol, and decreased LDL and triglycerides in the blood of broiler chickens.
Keywords: Blood parameter, Broiler chicken, Feed conversion ratio, Growth efficiency, Oregano oil

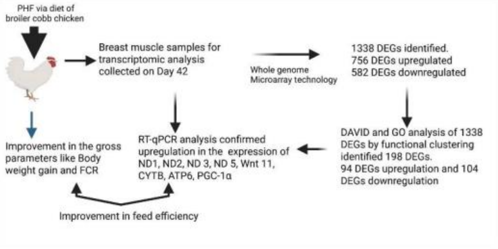
[Full text-PDF]

Research Paper

Impact of Polyherbal Formulation on Transcriptome Profiling of Chicken Breast Muscle: Elucidation of Molecular Mechanisms for the Enhanced Cellular Feed Efficiency in Broiler Chickens

Marimuthu S, Suresh S, and D'Souza P.

J. World Poult. Res. 15(2): 194-207, 2025; pii: S2322455X2500020-15
DOI: https://dx.doi.org/10.36380/jwpr.2025.20



Marimuthu S, Suresh S, and D'Souza P (2025). **Impact of Polyherbal Formulation on Transcriptome Profiling of Chicken Breast Muscle: Elucidation of Molecular Mechanisms for the Enhanced Cellular Feed Efficiency in Broiler Chickens.** *J. World Poult. Res.*, 15(2): 194-207. DOI: https://dx.doi.org/10.36380/jwpr.2025.20

ABSTRACT: To elucidate the cellular mechanisms underlying feed efficiency (FE) enhanced by polyherbal formulation (PHF), a combination of *Andrographis paniculata, Punica granatum,* and *Emblica officinalis*, the microarray technology was used to identify the genetic pathways related to feed utilization through genomical profiling of breast muscle in Cobb broiler chickens supplemented with or without PHF. 300 male day-old chicks were randomly distributed into two groups of 6 replicates with 25 each, including control (basal-diet) and PHF (Basal-diet+PHF-400g/ton), and their impact on performance parameters was assessed on day 42. Similarly, the global gene expression of breast muscles collected in each group was profiled using Agilent chicken whole genome microarray technology. Further, the Database for Annotation Visualization and Integrated Discovery (DAVID) Bioinformatics Resources and gene ontology (GO) analysis were used to identify the functional clustering of differentially expressed genes (DEGs) and gene network pathways associated with FE. RT-qPCR was subsequently conducted to cross-validate the expression of genes identified by DAVID. The PHF supplementation significantly improved the body weight gain (BWG) and feed conversion ratio in broilers compared to the control. Totally, 1338 DEGs (756 up-regulated and 582 down-regulated) were identified, of which 732 DEGs of PHF were significantly different from the control group. However, bioinformatics analysis revealed a significant modulation of 198 DEGs (94 up-regulated and 104 down-regulated) after hierarchical clustering, whose collective expression indicates significant enrichment of FE-related biological processes in the PHF-treated group. Further, a deeper understanding of the following DEGs (*ND1, ND2, ND3, ND4, ND4L, ND5, ND6 CYTB, COX1, COX2, COX3, ATP6, PGC1-a, PPAR, MEF2, PARK2, Wnt3A, Wnt11, Golgb1*, and *IGF1*) was established by mapping with Kyoto-Encylopedia-of-Genes-and-Genomes (KEGG) pathway in relation to mitochondria cellular respiration, mitochondrial biogenesis, mitophagy, energy metabolism and muscle growth in the breast muscle. Moreover, validation of microarray analysis of selected genes using RT-qPCR showed that the genes (*ND1, ND2, ND3, ND5, CYTB, ATP6, PGC1-a,* and *Wnt11)* were expressed in the same direction as that of GO analysis. In conclusion, supplementation of PHF resulted in transcriptional modulation in the mitochondrial functions, which was correlated to the improvements of corresponding phenotypic traits (FE and BWG) in broiler chickens.
Keywords: Differentially expressed gene, Feed efficiency, Genome microarray, Mitochondrial respiration

[Full text-PDF]

Research Paper

The Evaluation of MAPK/ERK Signaling Pathway in Chicken Necrotic Enteritis Based on Microbiomics and Metabolomics

Xie C, Sun L, Li Y, Chu Y, Liu X, Tong Q, and Gu C.

J. World Poult. Res. 15(2): 208-223, 2025; pii: S2322455X2500021-15
DOI: https://dx.doi.org/10.36380/jwpr.2025.21

ABSTRACT: Chicken necrotic enteritis is a prevalent intestinal disease caused by *Clostridium perfringens* (*C. perfringens*) in chickens. Previous research has confirmed the close relationship between the gut microbiota and its metabolites in connection with chicken necrotic enteritis. However, it remains unclear how the gut microbiota of the host influences host metabolism following the onset of necrotic enteritis (NE). The close relationship between gut microbiota and their metabolites in chicken necrotic enteritis (NE) has been established, yet the metabolic influence of microbiota post-NE onset remains unclear. In this study, 1-day-old White Leghorn chickens were divided into three groups (n=10/group), a negative control (CON) fed a basal diet, a fishmeal-supplemented group (F) receiving 50% fishmeal, and an NE group inoculated orally with *C. perfringens* alongside fishmeal supplementation. Growth performance, intestinal lesions, and morphological changes were recorded. Cecal contents were subjected to 16S rDNA sequencing for microbiota profiling, while serum metabolomics was analyzed via LC-MS.No noticeable damage was observed in the small intestines of the F group, whereas the NE group exhibited marked body weight reduction. Cell necrosis and jejunal mucosal shedding were identified, accompanied by ileal villi atrophy and significant reductions in tight junction proteins (Claudin-1 and ZO-1). Both F and NE groups showed decreased cecal abundances of *Lactobacillus* and *Blautia*, alongside increased *Clostridium* and *Escherichia coli*. Serum metabolomics revealed distinct glycerophospholipid and arginine-proline metabolism alterations in the F group versus CON. In contrast, NE-associated metabolic shifts were linked to pathways regulating cell proliferation, differentiation, and migration, particularly MAPK signaling. Downregulation of MAPK/ERK pathway genes was detected in the jejunal mucosa of infected chickens compared to CON and F groups. Concurrently, jejunal PCNA expression was quantified and found to be significantly reduced in the NE cohort relative to controls. Drawing upon the experimental results, it was concluded that necrotic enteritis in chickens was linked to a disruption in the intestinal epithelial barrier. Additionally, alterations in the gut microbiota hindered the activation of the MAPK/ERK signaling pathway, which in turn reduced the proliferation of intestinal epithelial cells and impaired the repair processes crucial for intestinal barrier restoration.

Keywords: Chicken, Necrotic enteritis, Microbiomics, Metabolomics, MAPK/ERK, Signaling pathway

[Full text-PDF]

Research Paper

Assessing the Efficiency of Different Coccidiostats against *Eimeria* spp. in Broiler Chickens Grown in the Floor Pen System

Rahman MA, Wasef A, Das NC, Islam MR, Islam MN, Mannan MA, Sarker NB, Aftabuzzaman M, and Mohanta UK.

J. World Poult. Res. 15(2): 224-232, 2025; pii: S2322455X2500022-15
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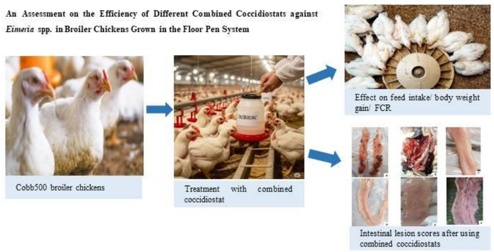


Rahman MA, Wasef A, Das NC, Islam MR, Islam MN, Mannan MA, Sarker NB, Aftabuzzaman M, and Mohanta UK (2025). **Assessing the Efficiency of Different Coccidiostats against *Eimeria* spp. in Broiler Chickens Grown in the Floor Pen System.** *J. World Poult. Res.*, 15(2): 224-232. DOI: https://dx.doi.org/10.36380/jwpr.2025.22

ABSTRACT: Coccidiosis is a critical parasitic disease that affects poultry through severe impairment in growth and yield. The present study aimed to evaluate the efficacy of combined coccidiostats in broiler chickens reared in floor pen systems. A total of 1200 Cobb500 broiler chickens were divided into five treatment groups, each group consisting of eight replications, which were based on different coccidiostats, including Treatment 1 (Control), Treatment 2 (Maduramicin-Nicarbazin), Treatment 3 (Narasin-Nicarbazin), Treatment 4 (Semduramicin-Nicarbazin), and Treatment 5 (Monensin-Nicarbazin). Performance parameters were recorded on days 7, 14, 21, 28, and 33. Treatment 3 exhibited the lowest feed consumption per chicken (2.797 kg), followed by Treatment 5 (2.825 kg) and Treatment 2 (2.835 kg). The control group consumed the highest amount of feed (2.880 kg), followed by Treatment 4 (2.888 kg). During week five, Treatment 5 demonstrated the highest body weight gain (1915 g), whereas Treatment 2 showed the lowest (1808 g). The lowest feed conversion ratio (FCR) was observed in Treatment 5 (1.500), followed by Treatment 3 (1.504), Treatment 4 (1.547), Treatment 2 (1.571), and the control group (1.576). However, the maximum intestinal lesion scores were noted in the control group, compared to the experimental groups observed on the culling day. Treatment 5 indicated the lowest lesion scores, followed by treatments 3, 4, and 2. Among the observed species, *Eimeria (E.) tenella* exhibited the highest lesion scores compared to E. maxima and E. acervulina in all the groups. Therefore, the effective use of these coccidiostats is crucial for preventing and managing coccidiosis outbreaks in poultry.

Keywords: Broiler chicken, Eimeria, Feed conversion ratio, Floor pen system, Monensin, Narasin

[Full text-PDF]

Research Paper

Molecular Characterization of Infectious Laryngotracheitis Virus Circulating in Egypt during 2023

Yehia N, Omar S, Hassan W, Mossad Z, Said D, and Abdelhalim A.

J. World Poult. Res. 15(2): 233-250, 2025; pii: S2322455X2500023-15
DOI: https://dx.doi.org/10.36380/jwpr.2025.23

Yehia N, Omar S, Hassan W, Mossad Z, Said D, and Abdelhalim A (2025). **Molecular Characterization of Infectious Laryngotracheitis Virus Circulating in Egypt during 2023.** *J. World Poult. Res.*, 15(2): 233-250. DOI: https://dx.doi.org/10.36380/jwpr.2025.23

ABSTRACT: Since its discovery in Egypt in 1983, the infectious laryngotracheitis virus (ILTV) has continued to spread, leading to substantial losses for poultry farms. This study aimed to identify and molecularly characterize the ILTV currently in circulation in Egypt in 2023. Fifteen pooled trachea and lung samples were collected from affected vaccinated and non-vaccinated layer farms (Shiver, Lohhman, Brown layer, and Aviagel) aged from 10-30 weeks old spread throughout eight governorates in Egypt. A real-time polymerase chain reaction (PCR) is used to detect ILTV. Ten positive samples representing various governorates were chosen for partial sequencing of the *ICP4* gene, and four positive samples were chosen for sequencing of the *TK, gD, and gG* genes. Using phylogenetic analysis, the *ICP4* clusters into chicken embryo origin vaccine and vaccine-like strains (CEO) and tissue culture origin vaccine and vaccine-like strains (TCO) and wild type. The GAHV-1-Egy-WO5-2023, GAHV-1-Egy-WO7-2023, and GAHV-1-Egy-WO8-2023 strains cluster in the CEO group with the CEO vaccine and vaccine-like strains with 98.9%–100% amino acid identity (A.A.); However, the remaining strains (GAHV-1-Egy-WO1-2023, GAHV-1-Egy-WO2-2023, GAHV-1-Egy-WO3-2023, GAHV-1-Egy-WO4-2023, GAHV-1-Egy-WO6-2023, GAHV-1-Egy-WO9-2023, and GAHV-1-Egy-WO10-2023) were clustered in the TCO group with TCO vaccine strains and vaccine-like strains with 100 % A.A. By analysis of *gD, gG, and Tk* genes, there is no difference between the TCO and CEO groups. Compared with several reference CEO and TCO vaccine strains and vaccine-like strains, some new specific mutations (Q161H and Q182H) were recorded in the

ICP4 in GAHV-1- Egy-WO8-2023. In addition, A34G, and P276L were recorded in the *gD* gene in the GAHV-1-Egy-WO1-2023 and GAHV-1-WO4-2023. Also, the R115I, G126A, and S163I were recorded in the *TK* gene in GAHV-1-Egy-WO8-2023 and A99E in GAHV-1-Egy-WO5-2023, which can affect the virulence and pathogenicity of the virus, which may be due to the reactivation of the vaccine strain by the bird-to-bird transmission or viral recombination. In conclusion, the ILTV outbreaks in poultry farms across several regions of Egypt during 2023 were induced by vaccine strains derived from TCO and CEO. Thus employing innovative vector vaccines and reassessing the current vaccination regimens is necessary.

Keywords: Infectious laryngotracheitis, Diagnosis, isolation, Genetic characterization

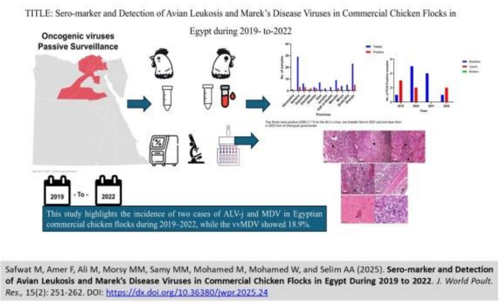
[Full text-PDF]

Research Paper

Sero-marker and Detection of Avian Leukosis and Marek’s Disease Viruses in Commercial Chicken Flocks in Egypt During 2019 to 2022

Safwat M, Amer F, Ali M, Morsy MM, Samy MM, Mohamed M, Mohamed W, and Selim AA.
J. World Poult. Res. 15(2): 251-262, 2025; pii: S2322455X2500024-15
DOI: https://dx.doi.org/10.36380/jwpr.2025.24

ABSTRACT: In the poultry industry, oncogenic disease viruses result in significant financial losses. This study aimed to update incidence data in Egyptian chicken flocks from 2019 to 2022. The prevalence of Marek’s disease virus (MDV) and Avian leukosis virus (ALV) was monitored during the passive surveillance program in commercial poultry flocks, including (43) breeders, (45) layers, and (7) broiler flocks during 2019-2022 in Egypt. This passive surveillance was adopted depending on molecular detection of MDV and ALV viruses using PCR tests and serological detection of ALV using ELISA test kits. The histological and post-mortem results for clinically diseased birds have been recorded in different organs, including the Liver, spleen, kidney, ovary, intestine, and brain. Ninety-five flocks were reported and tested using PCR for monitoring ALV viruses (subtypes A, B, C, D, and J) and vvMDV. Samples used for PCR were prepared from liver and spleen tissues (5 chickens/pool). The tested flocks were negative for ALV subtypes A, B, C, and D, while only two cases were positive for ALV-J (2/95, 2.1%), including one breeder flock in 2021 and one-layer flock in 2022, both from Sharqia governorate. Although the overall detection rate of vvMDV was 18.9% (18/95), breeder flocks showed the highest detection rate (25.6%), compared to layer flocks (15.6%). Furthermore, the vvMDV virus detection rate varied from one year to another, including 4/13 (30.8%), 7/43 (16.3%), 4/24 (16.7%), and 3/15 (20 %) in 2019, 2020, 2021, and 2022, respectively. The vvMDV-positive cases were reported in eight governorates, including Sharqia, Menofia, Daqahlia, Gharbia, Qualiobia, Al Beheira, Giza, and Damietta. Moreover, the study indicated that vvMDV was detected in chickens aged 5 to 61 weeks. The ELISA test was used to detect ALV antibodies serologically. The seroprevalence of ALV was 4.7% in the study area. In conclusion, among the tested samples, only two cases of ALV-J were reported in Egyptian commercial chicken flocks during 2019-2022. During the same period, vvMDV showed an 18.9% prevalence rate. Further studies are recommended to evaluate the MDV vaccination program, including vaccine quality and efficacy.



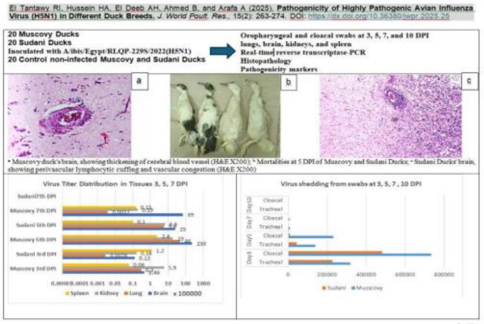
Keywords: Avian leukosis virus, Detection rate, Egypt, Marek’s disease virus, Prevalence
[Full text-PDF]

Research Paper

Pathogenicity of Highly Pathogenic Avian Influenza Virus (H5N1) in Different Duck Breeds

El Tantawy RI, Hussein HA, El Deeb AH, Ahmed B, and Arafa A.
J. World Poult. Res. 15(2): 263-274, 2025; pii: S2322455X2500025-15
DOI: https://dx.doi.org/10.36380/jwpr.2025.25

ABSTRACT: Avian influenza viruses (AIVs) pose a global threat, with wild waterfowl serving as key reservoirs for transmission to poultry. The present study investigated the pathogenicity, viral shedding patterns, tissue distribution, and pathological effects of a highly pathogenic avian influenza virus (HPAIV) in two duck breeds, including Muscovy and Sudani ducks. An Egyptian H5N1 strain (A/ibis/Egypt/RLQP-229S/2022), originally isolated from a wild ibis, was used. Forty ducks (20 Muscovy and 20 Sudani) were divided into infected and control groups (10 per group per breed). At four weeks of age (Average weight of 1.2 ± 0.1 kg),



each infected duck received a single intranasal dose of 10^6 EID_{50}. Cloacal and oropharyngeal swabs were collected at 3, 5, 7, and 10 days post-infection (DPI) to monitor viral shedding, while clinical signs were recorded daily. Mortality was higher in Muscovy ducks, which exhibited higher mortality (70%) than Sudani ducks (50%), with both breeds showing neurological signs and lethargy. Viral load analysis of cloacal swabs via RT-PCR (Targeting the AIV M gene), exceeded oropharyngeal shedding, peaking by five DPI and persisting longer in Muscovy ducks (Seven DPI compared to five DPI in Sudani ducks), suggesting that fecal-oral transmission is the primary route of spread and that viral replication is more active in the intestinal tract. Tissue distribution analysis revealed broader viral dissemination in Muscovy ducks, particularly in the brain, lung, kidney, and spleen. These findings demonstrated differential susceptibility between breeds, with Muscovy ducks posing a higher transmission risk due to prolonged viral shedding and tissue tropism. The virus used in the present study carried pathogenicity markers across several proteins, including hemagglutinin (HA), neuraminidase (NA), polymerase basic 1 (PB1), polymerase basic 2 (PB2), nucleoprotein (NP), non-structural protein 1 (NS1), and polymerase acidic (PA) protein. Overall, while both duck breeds are vulnerable to the circulating H5N1 HPAI strain, their susceptibility and clinical outcomes differ. These findings demonstrated that both Muscovy and Sudani ducks are susceptible to H5N1 HPAIV infection, Muscovy ducks showing higher mortality and more extensive viral shedding and histopathological alterations. However, both duck breeds are variable in their susceptibility to H5N1 infection.

Keywords: Avian influenza viruses, Duck breeds, H5N1, Histopathological changes, Pathogenicity, Virus shedding

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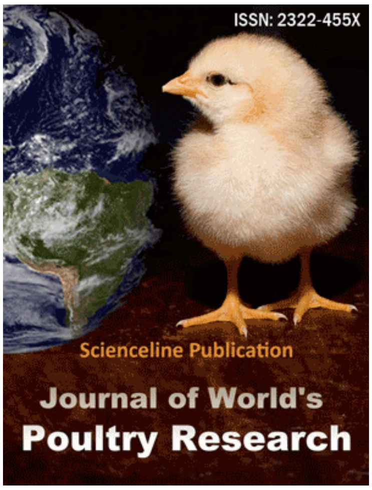

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The Interplay of Litter Types, Blends of L-selenomethionine, and Vitamin E in Broiler Chicken's Performance and Health

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ABSTRACT

The choice of litter type (LT) in broiler chicken production can significantly influence overall performance and health. Adding specific combinations of L-selenomethionine and Vitamin E (L-SeMet + V.E) to drinking water can impact broiler chickens' health. This study aimed to explore the effects of two LT types, wood shavings (WS) and perforated plastic litter (PL) as well as water supplementation with L-SeMet + V.E, on growth performance, blood parameters, antioxidant capacity, and thyroid hormone levels in broiler chickens. A total of 312 one-day-old male broiler chickens were randomly assigned to two different LT groups (WS and PL), with 156 chicks in each. Within each LT group, the chickens were further divided into four subgroups: a control group (T0) that received plain water, and three treatment groups that received drinking water supplements. Each subgroup consisted of three replicates, with 13 broiler chickens per replicate. The water treatments involved varying levels of supplementation with L-SeMet and vitamin E: T1 (0.5 mg/L + 250 mg/L), T2 (1 mg/L + 250 mg/L), and T3 (1.5 mg/L + 250 mg/L). Chickens were assessed for growth performance, feed efficiency, and blood biochemical parameters including lipids, liver enzymes, antioxidants, and thyroid hormones. Chickens raised on PL and receiving T3 exhibited significantly enhanced performance and feed efficiency, outperforming those raised on WS and given different supplementation levels. Significant enhancements were observed in the blood lipid profile and liver enzyme levels across the different LT groups, with the highest values recorded in the PL group. Additionally, broiler chicken in the T2 group, along with the WS × T2 and PL × T3 interactions, showed a notable improvement in blood biochemical parameters. Similarly, chickens raised on PL and given T3-supplemented water, along with the interactions WS × T2 and PL × T3, showed a significant improvement in antioxidant status and thyroid hormone levels in comparison to chickens raised on WS and the other treatment combinations. The use of PL and supplemenation with L-SeMet + V.E in drinking water were found to enhance growth performance, improve blood parameters, increase antioxidant capacity, and influence thyroid hormone levels in broiler chickens.

Keywords: Antioxidant, Broiler chicken, L-selenomethionine and Vitamin E, Productivity, Thyroid hormone

INTRODUCTION

To enhance poultry health, it is important to concentrate on both management environment and dietary approaches in order to achieve optimal results. The success of broiler chicken production is influenced by a range of environmental factors, one of the most crucial being the type and proper management of litter material (Pepper and Dunlop, 2021). The use of high-quality bedding materials in commercial poultry production has attracted considerable attention due to its impact on productive performance (Bilgili et al., 2009). The performance and well-being of broiler chickens is greatly influenced by the type of litter employed in their production (Okasha et al., 2021a; b). It is common knowledge that litter is utilized to

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reduce birds' exposure to excrement and to absorb excess moisture (Farghly et al., 2018). However, poor litter quality can lead to the development of pododermatitis in broiler chickens, resulting in discomfort and welfare concerns (Shepherd and Fairchild, 2010), as well as influencing immunological responses, performance, and vulnerability to diseases (De Jong et al., 2014; Wei et al., 2015).

Although litter is traditionally employed in poultry farming, particularly in broiler production, improvements in management practices can enhance both the environmental factors and the well-being of the poultry. One option is to utilize raised plastic flooring, commonly found in broiler chicken barns (Li et al., 2017). Previous study has indicated that broiler chicken productivity can be maintained by utilizing perforated plastic flooring (Almeida et al., 2017), while also improving broiler chicken health (Okasha et al., 2021b). Plastic litter offers durability and cost-effectiveness, as it does not degrade, require replacement, or involve high maintenance costs. It is easy to install and clean plastic litter types, with the potential to reduce occurrences of foot pad injuries (Farghly et al., 2018). Maintaining ideal litter conditions is essential for broiler chicken performance, as excessive moisture, ammonia levels, and the presence of pathogenic organisms can have negative consequences (De Toledo et al., 2020). These include footpad dermatitis, respiratory disorders, and reduced performance such as an unfavorable feed conversion ratio (FCR), decreased body weight (BW), and body weight gain (BWG) (Abougabal and Taboosha, 2023). Therefore, it is essential to meticulously choose and oversee the appropriate litter type in order to ensure optimal broiler chicken health, welfare, and overall performance.

Selenium (Se) is a vital element found in at least 25 selenoproteins that regulate various biological functions in the body (Wang et al., 2017). Selenium's main biological roles include antioxidant defense, adjustment of the immune system, and control of the body's inflammatory reaction (Rayman and Stranges, 2013; Selim et al., 2015). These roles may positively impact the efficiency and metabolic function of broiler chickens. Poultry feed supplements can include both types of selenium, specifically sodium selenate (Na_2SeO_4) and sodium selenite (Na_2SeO_3) (Wickramasuriya et al., 2023). A major advantage of supplying selenomethionine (SeMet) over utilizing inorganic selenium or alternative organic compounds is that SeMet is metabolized with methionine, creating a reserve of selenium in broiler chicken tissues (Surai et al., 2018). According to study of Gangadoo et al. (2020), organic selenium is absorbed and retained more effectively as compared to synthetic forms, with higher concentrations in the duodenum, ileum, and spleen, indicating increased uptake, while lower levels are found in the brain, liver, and breast tissues. To prevent Se deficiency and meet the poultry demands, dietary enrichment with natural selenium is widely practiced due to its superior bioavailability (Surai et al., 2018).

Supplementing broiler chicken diets with selenium has demonstrated favorable impacts on growth, feed markers, immunity, and decreased mortality (Habibian et al., 2016; Calik et al., 2022a). Organic selenium compounds such as hydroxyselenomethionine (OH-SeMet) and L-selenomethionine (L-SeMet) improve selenium reserves in birds, enhance antioxidant protection, and boost resilience to stress in breeder nutrition (Surai and Fisinin, 2014; Wang et al., 2021a; Babazadeh and Ahmadi Simab, 2022). The enhancement of antioxidant capacity has been found to be associated with the activation of selenium-dependent antioxidant enzymes (Liu et al., 2021). In addition, selenium plays a vital role as a component of glutathione peroxidases (Huang et al., 2019). These enzymes, together with vitamin E, contribute to the protection of cells from free radicals (Yoon et al., 2007). Vitamin E is the primary antioxidant that is soluble in lipids, and it holds significant importance in cell membranes (Packer et al., 2001; Gao et al., 2010; Traber and Stevens, 2011). It acts as a crucial lipid antioxidant by interrupting chain reactions and scavenging free radicals within cell membranes and subcellular structures (Young et al., 2003). The studies has shown that adding vitamin E to broiler chicken diets can provide notable benefits due to its potent antimicrobial properties and its capacity to bolster the immune functions (Dalia et al., 2018; Calik et al., 2022a). It can be hypothesized that providing preeminent levels of vitamin E as a supplement can offer benefits in preventing the negative impact of husbandry-related stressors such as transportation, handling, and vaccination, all leading to reduced broiler chicken performance (Perez-Carbajal et al., 2010; Calik et al., 2022b).

The study aimed to evaluate the effects of adding L-selenomethionine and vitamin E (L-SeMet + V.E) supplementation in drinking water on growth performance, feed efficiency, blood profiles, antioxidant enzymes, and T3 and T4 levels. It also explored the impact of this supplementation on broiler chickens raised on either wood shavings or perforated plastic litter, focusing on how it may improve performance and health and mitigate stress effects.

MATERIALS AND METHODS

Ethical approval

The animal care and experimental protocols were approved by the Research Ethics Committee at the Faculty of Agriculture, Moshtohor, Benha University (REC-FOABU/No.16000/3), were observed. The experimental broiler chickens were subjected to the utmost care, and all required actions were implemented to minimize discomfort.

Key features of the different litter types

The study utilized a washable perforated plastic floor (Al-Ekhlass for Plastics Industry and Trading, Qalyubia, Egypt), measuring 100 × 100 × 15 mm (length × width × height), and weighing 1.75 kg with a resistance of up to 200 kg/m2 per unit. The wood shavings (beech) used for the poultry litter were obtained from a nearby tree processing facility. These soft, dry, and non-toxic shavings were spread at a depth of 7 cm. The materials exhibited high absorbency and were resistant to clumping.

Animals and study design

All broiler chicks (Ross 308) were obtained from a commercial hatchery (Association of Al-Tanmia for Hatching and Poultry Production, Qalyubia, Egypt). The chicks were transported in a temperature-controlled vehicle for about one hour to the experimental farm located at the Poultry Research Unit, Faculty of Agriculture, Benha University, Egypt. Upon arrival, the chicks were housed in research enclosures within a controlled-geographical area, where stringent measures were implemented to maintain optimal experimental and hygienic conditions. All pens, each measuring 1 × 1 meter, were placed in the same location to ensure consistency. The stocking density in each pen was maintained at 13 chicks per square meter. Continuous electric lighting was provided for the first five days, after which the lighting schedule was adjusted to 20 hours of light and 4 hours of darkness for the following 30 days. Lighting intensity was applied in accordance with Directive 2007/43/EC in order to enhance the welfare of broiler chickens. Gas-powered space heaters were used in this experiment. The air temperature and relative humidity were kept at 33°C and 63%, respectively, for as long as three days before the chicks were placed in the pens. Throughout the initial week of the trial, the temperature was maintained at 33°C. Following this, the temperature was gradually reduced by 2-3°C per week until it reached the target range of 25°C by day 28. The broiler chickens were vaccinated according to a specified schedule: On day 8, they were administered the Newcastle Disease Virus (NDV) Clone 30 vaccine via eye drop, in addition to a bivalent NDV vaccine administered through injection under the neck membrane. The Gumboro intermediate vaccine was given at 12 days of age, followed by the NDV Clone 30 vaccine again at 21 days of age. The inactivated Avian Influenza (AI) H5N2 vaccine was administered at 10 days of age (Nobilis, Intervet, Boxmeer, The Netherlands). The immunization process followed established guidelines and protocols, and a licensed veterinarian supervised its implementation.

For a 35-day experiment, a total of 312 one-day-old male Ross 308 broiler chicks with an average initial body weight of 46.22 ± 0.1 g were used. The study employed a 2 × 4 factorial arrangement of treatments with three replicates, resulting in 24 experimental groups of chickens (n = 13 per group). Each group served as an experimental unit for the study. The chicks were randomly divided into two primary groups based on litter types: wood shavings and perforated plastic litter (n = 156 chicks per litter type). The chicks from each litter type were randomly allocated into a control group and three experimental groups (n = 39 chicks per group), which received different levels of L-SeMet + V.E into their drinking water. The experimental sets were labeled as follows: T1, (0.5 mg L-SeMet + 250 mg V.E); T2, (1 mg L-SeMet + 250 mg V.E); and T3, (1.5 mg L-SeMet + 250 mg V.E) mg/L, respectively. In this study, the supplementation level of vitamin E was increased in alignment with the guidelines provided by Aviagen (Aviagen, 2019). This finding aligns with earlier study, where an average supplementation of 200 mg/kg of vitamin E was shown to enhance oxidative status, improve performance parameters, and positively influence meat quality in broiler chickens (Mazur-Kuśnirek et al., 2019). In the current study, the organic selenium (Se) content in the diet was adjusted to the maximum permissible level, ranging from 1 to 3 mg/kg, consistent with the levels utilized in prior studies (Calik et al., 2022b; Kazem and Al-Khafaji, 2023). The vitamin E product added to the water was a misallied liquid solution that could disperse in water. It contained 250 mg of α-tocopherol per liter (L-selenomethionine® and vitamin E®, Alcon Biosciences Pvt. Ltd, Mumbai, India). Table 1 details the nutritional makeup of the experimental diets meticulously computed and analyzed, and then utilized in various stages of the experiment. The broiler chickens were fed a pelleted basal diet formulated to meet and slightly exceed the nutrient requirements specified by the National Research Council (NRC, 1994). Both feed and water were provided *ad libitum* for the entire duration of the study.

Table 1. The composition of ingredients and nutrients in the basal starter and grower diets in broiler chickens (Ross 308)

Ingredients (%)	**Starter diet (0-3 weeks)**	**Gower diet (3-5 weeks)**
Yellow Corn	60.56	65.4
Soybean meal (40%)	22	16.1
Corn gluten meal (60%)	12.6	13.2
Calcium hydrogen phosphate	1.5	1.7
Limestone	2.01	2.32
Salt (NaCl)	0.42	0.42
VIT and Min premix [1]	0.3	0.3
Choline chloride (50%)	0.2	0.2
L-lysine (78%)	0.24	0.24
Methionine	0.17	0.12
Total	100	100
Energy and nutrient composition		
ME (kcal/kg)	3000	3053
Crude protein (%)	22.29	20.77
EE (%)	2.80	3.00
Crude fiber,%	3.00	2.77
Ca (%)	1.21	1.19
T. phosphorus (%)	0.65	0.62
Av. phosphorus (%)	0.42	0.40
Lys (%)	1.11	0.95
Met (%)	0.50	0.40
Met + Cys (%)	0.95	0.80

[1] This feed formulation comprises a mineral and vitamin premix, providing the following concentrations (mg/3kg): Iron 80, Manganese 100, Copper 8, Zinc 75, Selenium 0.15, and Iodine 0.35. Additionally, it includes vitamin A (12,500 IU), vitamin D3 (2,500 IU), vitamin E (30 IU), vitamin K3 (2.65 mg), vitamin B1 (2 mg), vitamin B2 (6 mg), vitamin B3 (50 mg), vitamin (B512 mg), vitamin B7 (0.0325 mg), vitamin B9 (1.25 mg), and vitamin B12 (0.025 mg) per kilogram. These values were calculated based on feedstuff data as shown in the NRC (1994). Abbreviations: ME (kcal/kg): Metabolisable Energy (kcal/kg), EE (%): Ether Extract (%), T. phosphorus (%): Total phosphorus (%), Av. phosphorus (%): Available phosphorus (%), Met (%): Methionine, Met + Cys (%) Methionine and Cystine (%)

Taking samples and measurements

Growth performance and feed efficiency

Broiler chickens were weighed weekly to individually assess their growth performance, including body weight (BW) and body weight gain (BWG). Additionally, daily feed intake (FI) was recorded in replicates to calculate the ratio between (FI/g: BWG/g) expressed as feed conversion efficiency ratio (FCR). The European Production Efficiency Factor (EPEF) was calculated using the following equation: EPFE = (Livability[%] × BW [kg])/(FCR × Trial duration) × 100.

Blood samples

Twelve broiler chickens were chosen at random from each main group at the end of the 35-day trial (four per replicate). After a 12-hour period of fasting, 5 mL of blood was extracted from the wing vein. The samples were carefully placed into sterile tubes containing heparin and serum separator for subsequent analysis of thyroid hormones. The tubes were centrifuged for ten minutes at 4°C at 3500 × g to isolate the plasma. The plasma lipid profile, including total lipids, cholesterol (CHO), triglycerides (TRIG), low-density lipoproteins (LDL), and high-density lipoproteins (HDL), was analyzed using commercially available kits (Meikang Incorporated, Ningbo, China) following the methodology outlined by Gornall et al. (1949). Additionally, liver enzymes such as aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured using a colorimetric method as described by Sirois (2019). The enzymes were measured using kits procured from the Nanjing Jiancheng Institute of Bioengineering (Jiangsu, China).

Evaluating antioxidant capacity

To assess the concentration of total antioxidants (T-AOC) in plasma, a commercially available kit (Randox, UK) was utilized, which was based on the method developed by Miller et al. (1993). The activity of blood glutathione peroxidase (GPx, EC 1.11.1.9) was assessed using commercially available GPx kits from Randox (Crumlin, UK), adhering to the manufacturer's instructions. The method for GPx measurement was based on the procedure established by Paglia and Valentine (1967). Additionally, superoxide dismutase (SOD, EC 1.1.5.1) activity in erythrocyte lysates was measured using kits provided by Randox Laboratories Ltd. (Crumlin, UK), following the protocols outlined by Woolliams et al. (1983). Moreover, plasma malondialdehyde (MDA) levels were measured using a modified fluorometric technique as detailed by Jo and Ahn (1998).

Measuring thyroid hormone levels

Serum samples were used to assess the levels of thyroid hormones (T3 and T4) using radioimmunoassay (RIA) kits, following the manufacturer's instructions. The techniques outlined by Renden et al. (1994) were employed for this analysis.

Statistical analysis

The pen served as the experimental unit (n = 24), and group means were subjected to statistical analysis. The data underwent analysis utilizing the General Linear Models (GLM) procedure of two-way ANOVA in SPSS (IBM SPSS, Version 27.0. Armonk, NY). Tukey's test was utilized to differentiate between treatments and assess the differences. The linear model was specified as:

$$Yijk = \mu + LTi + Tj + (LTT)\ ij + eijk$$

where Yijk represents the kth observation, and μ stands for the overall mean. The term LTi refers to the effect of the ith litter type (wood shavings and plastic litter), whereas Tj represents the effect of the jth L-SeMet + V.E treatment level (0, 0.5, 1, 1.5 + 250 V.E per mg/L). Additionally, the term (LTT) ij represents the interaction effect between the ith litter type and the jth L-SeMet + V.E treatment. The term eijk refers to the experimental error, assuming a mean of zero ($\bar{X} = 0$) and a variance of σ^2e. Statistical significance was assessed using a P-value threshold of 0.001, unless otherwise specified.

RESULTS

Growth performance and feed efficiency

Table 2 highlights the effects of litter type and L-SeMet + V.E supplementation on broiler chickens' growth and feed efficiency up to 35 days. Significant improvements ($p < 0.001$) were observed in growth performance (BW and BWG) and feed efficiency (CFI, CFCR, and EPEF) due to both litter type and L-SeMet + V.E inclusion. While initial body weights showed no differences ($p = 0.648$), supplementation had a notable impact on growth traits during later periods. The addition of of 1.5 + 250 mg/l L-SeMet + V.E to the drinking water resulted in a significant increase in BW, BWG EPEF and decreased CFCR ($p < 0.001$). Interactions between WS with T2 and PL with T3 showed the most significant improvements in growth, feed efficiency, and European Production Efficiency Factor (EPEF) ($p < 0.001$).

Blood biochemical analysis

Table 3 summarizes the findings of the blood lipid profile and liver enzyme analysis. Statistically significant differences ($p < 0.001$) were detected in the plasma concentrations of CHO, TRIG, LDL, HDL, and AST among broiler chickens reared on WS and PL litters. The incorporation of 1 + 250 mg/l L-SeMet + V.E into the drinking water significantly altered all blood parameters ($p < 0.001$), with increased HDL levels and reduced lipid profiles and liver enzymes. Some interactions such as WS × T2 increased HDL, while others such as PL × T3 reduced CHO, TRIG, and LDL levels. Both PL × T3 and WS × T2 significantly decreased AST and ALT levels, respectively, as compared to other interactions ($p < 0.001$).

Antioxidants and thyroid hormones

Table 4 and Graph 1 show significant differences ($p < 0.001$) in plasma antioxidants (TAOC, GPX, MDA) and serum thyroid hormones (T3, T4) among broiler chickens raised on different litter types. Chickens on PL litter exhibited higher antioxidant levels and thyroid hormones with lower MDA, as compared to those on WS litter. Supplementation with L-SeMet + V.E at 1.5 + 250 mg/L significantly improved antioxidant status and thyroid hormone levels across all parameters ($p < 0.001$). Interaction analysis revealed that chickens raised on both litter types and given T2 or T3 had the highest antioxidant capacity (TAOC, GPX, SOD) and thyroid hormones (T3, T4), with the lowest MDA levels ($p < 0.001$).

Table 2. Effects of different litter types, L-Selenomethionine + Vitamin E, and their interactions on broiler chickens' growth and feed efficiency

Items		BW (g)		BWG (g)	CFI (g/bird)	CFCR	EPEF
		0	5 WKS	0-5 WKS	0-5 WKS	0-5 WKS	0-5 WKS
Litter types (LT)	WS	46.26	2175.47^{b}	$2129.0.5^{b}$	3094.50^{b}	1.46^{a}	425.72^{b}
	PL	46.49	2307.66^{a}	2261.48^{a}	3137.67^{a}	1.39^{b}	453.72^{a}
	SEM	0.342	3.930	16.604	6.057	0.014	6.593
L-SeMet + V.E (T) mg/l	T0	47.22	2218.66^{c}	2171.44^{b}	3132.79^{b}	1.44^{b}	428.44^{b}
	T1	46.34	2048.00^{d}	2001.65^{c}	3072.73^{c}	1.53^{a}	398.52^{c}
	T2	46.29	2282.52^{b}	2236.23^{b}	3100.05^{bc}	1.38^{bc}	464.30^{a}
	T3	45.66	2417.09^{a}	2371.74^{a}	3165.80^{a}	1.35^{c}	484.84^{a}
	SEM	0.484	5.558	23.481	9.464	0.20	8.847
LT × T	WS × T0	47.56	2149.33^{e}	2101.77^{cde}	3156.73^{b}	1.50^{ab}	408.86^{cd}
	WS × T1	45.86	2084.00^{f}	2038.14^{de}	3050.87^{d}	1.50^{ab}	406.77^{cd}
	WS × T2	46.52	2335.71^{b}	2289.17^{b}	3090.50^{cd}	1.35^{cd}	483.32^{b}
	WS × T3	46.00	2132.86^{e}	2087.14^{cde}	3104.93^{bcd}	1.49^{abc}	409.63^{cd}
	PL × T0	46.88	2288.00^{c}	2241.12^{bc}	3104.53^{bcd}	1.39^{bc}	451.28^{bc}
	PL × T1	46.83	2012.00^{g}	1965.17^{e}	3094.60^{cd}	1.57^{a}	390.27^{d}
	PL × T2	46.05	2229.33^{d}	2183.29^{bcd}	3109.60^{bcd}	1.42^{bc}	$445..23^{bc}$
	PL × T3	45.31	2701.33^{a}	2656.35^{a}	3226.67^{a}	1.21^{d}	560.05^{a}
	SEM	0.684	7.861	33.207	13.382	0.280	10.339
p-value	LT	0.648	<.001	<.001	<.001	0.007	<.001
	T	0.193	<.001	<.001	<.001	<.001	<.001
	LT × T	0.572	<.001	<.001	<.001	<.001	<.001

The data sets reflect the mean values obtained from three replications per treatment. Different letters denote significant differences ($p < 0.001$) among means within the same column for each factor and interactions. Abbreviations: BW: Body weight, BWG: Body weight gain, CFI: Cumulative feed intake, CFCR: Cumulative feed conversion ratio, EPEF: European production efficiency factor, L: Litter types, WS: Wood shavings, PL: Perforated plastic litter, T: Treatments, WKS: Weeks, L-SeMET: L-selenomethionine, V.E: Vitamin E, SEM: Standard erorr mean, T0: Control group (plain water), T1: 0.5 + 250 mg/l L-SeMET + V.E, T2: 1 + 250 mg/l L-SeMET + V.E, T3: 1.5 + 250 mg/l L-SeMet + V.E

Table 3. Effects of different litter types, L-Selenomethionine + Vitamin E, and their interactions on broiler chickens' lipid profile and liver enzymes

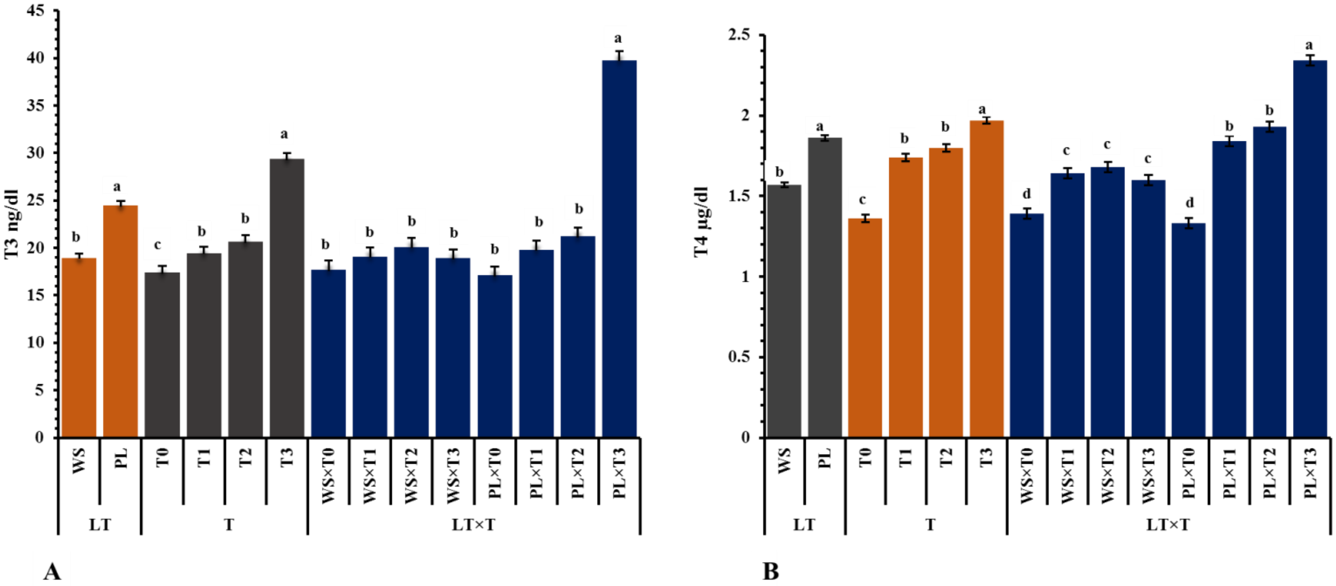
Items		**TL (mg/dl)**	**CHO (mg/dl)**	**TRIG (mg/dl)**	**LDL (mg/dl)**	**HDL (mg/dl)**	**AST (U/l)**	**ALT (U/l)**
Litter Types (LT)	WS	851.98	216.66^{a}	74.07^{a}	156.92^{a}	44.92	152.64^{a}	57.96
	PL	779.76	123.21^{b}	58.02^{b}	70.14^{b}	41.46	144.40^{b}	58.29
	SEM	7.208	2.699	2.430	2.083	0.768	1.067	1.578
L-SeMet + V.E (T) mg/l	T0	1130.95^{a}	198.81^{a}	101.23^{a}	140.56^{a}	35.06^{b}	1.66.79^{a}	72.42^{a}
	T1	821.42^{b}	173.81^{ab}	77.775^{b}	112.70^{b}	39.47^{b}	143.59^{b}	57.11^{b}
	T2	549.21^{c}	146.43^{c}	42.590^{c}	98.445^{d}	49.75^{a}	141.26^{b}	44.91^{c}
	T3	761.90^{b}	160.71^{bc}	42.595^{c}	102.44^{bc}	48.49^{a}	142.45^{b}	58.06^{b}
	SEM	10.312	3.862	3.436	2.981	1.084	1.526	2.231
LT × T	WS × T0	1071.43^{ab}	242.86^{a}	106.17^{a}	183.42^{a}	32.33^{d}	178.52^{a}	74.30^{a}
	WS × T1	857.14^{bcd}	228.57^{a}	87.65^{ab}	160.66^{ab}	38.21^{cd}	140.28^{bcd}	44.63^{b}
	WS × T2	550.81^{e}	176.19^{b}	49.38^{cd}	133.24^{b}	58.78^{a}	139.26^{cd}	42.56^{b}
	WS × T3	928.57^{abc}	219.05^{a}	53.09^{cd}	150.39^{b}	50.38^{ab}	152.52^{bc}	70.36^{a}
	PL × T0	1190.48^{a}	154.76^{bc}	96.30^{a}	97.71^{c}	37.79^{cd}	155.07^{b}	70.54^{a}
	PL × T1	785.71^{cde}	119.05^{cd}	67.90^{bc}	64.74^{d}	40.73^{bcd}	146.91^{bcd}	69.60^{a}
	PL × T2	547.62^{e}	116.67^{cd}	35.80^{d}	63.65^{d}	40.73^{bcd}	143.26^{bcd}	47.26^{b}
	PL × T3	595.24^{de}	102.38^{d}	32.10^{d}	54.94^{d}	46.60^{bc}	132.38^{d}	45.76^{b}
	SEM	14.583	5.461	4.860	4.215	1.533	2.16	3.156
Two-way ANOVA								
p-value	LT	0.086	<.001	<.001	<.001	0.054	0.001	0.885
	T	<.001	<.001	<.001	<.001	<.001	<.001	<.001
	LT × T	0.007	0.01	<.001	0.054	<.001	<.001	<.001

The data sets reflect the mean values obtained from three replications (4 chickens in each replicate) per treatment. Different letters denote significant differences ($p < 0.001$) among means within the same column for each factor and interactions. Abbreviations: L: Litter types, WS: Wood shavings, PL: Perforated plastic litter, T: Treatments, L-SeMET: L-selenomethionine, V.E: Vitamin E, T0: Control group (plain water), T1: 0.5 + 250 mg/l L-SeMET + V.E, T2: 1 + 250 mg/l L-SeMET + V.E, T3: 1.5 + 250 mg/l L-SeMET + V.E, SEM: Standard error mean, TL: Total lipids, CHO: Cholesterol, TRIG: Triglycerides, LDL: Low density lipoproteins, HDL: High density lipoproteins, AST: Aspartate aminotransferase, ALT: Alanine aminotransferase

Table 4. Effects of different litter types, L-Selenomethionine + Vitamin E, and their interactions on broiler chickens' antioxidant levels

Items		**TAOC (mM/L)**	**GPX (U/GT)**	**SOD (U/ml)**	**MDA (nmol/mL)**
Litter Types (LT)	WS	0.77^{b}	43.67^{b}	3.48	3.65^{a}
	PL	0.89^{a}	48.62^{a}	3.52	2.37^{b}
	SEM	0.011	0.452	0.112	0.052
L-SeMet + V.E (T) mg/l	T0	0.74^{b}	19.21^{d}	2.81^{b}	5.28^{a}
	T1	0.77^{b}	29.22^{c}	3.27^{b}	3.25^{b}
	T2	0.83^{b}	58.35^{b}	3.94^{a}	2.05^{c}
	T3	0.97^{a}	77.81^{a}	3.99^{a}	1.43^{c}
	SEM	0.016	0.414	0.158	0.074
LT × T	WS × T0	0.67^{c}	19.45^{e}	2.88^{c}	6.30^{a}
	WS × T1	0.77^{bc}	38.90^{d}	3.30^{bc}	1.96^{c}
	WS × T2	0.88^{b}	97.26^{a}	4.38^{ab}	1.15^{c}
	WS × T3	0.77^{bc}	38.90^{d}	3.38^{bc}	5.20^{ab}
	PL × T0	0.82^{bc}	18.98^{e}	2.75^{c}	4.26^{b}
	PL × T1	0.77^{bc}	19.55^{e}	3.25^{c}	2.21^{c}
	PL × T2	0.79^{bc}	58.36^{c}	3.50^{bc}	1.72^{c}
	PL × T3	1.18^{a}	77.80^{b}	4.60^{a}	1.31^{c}
	SEM	0.023	0.586	0.224	0.105
Two-way ANOVA					
p-value	LT	<.001	<.001	0.804	<.001
	T	<.001	<.001	<.001	<.001
	LT × T	<.001	<.001	0.002	<.001

The data sets reflect the mean values obtained from three replications (4 chickens in each replicate) per treatment. Different letters denote significant differences ($p < 0.001$) among means within the same column for each factor and interactions. Abbreviations**:** L: Litter types, WS: Wood shavings, PL: Perforated plastic litter, T: Treatments, L-SeMET: L-selenomethionine, V.E: Vitamin E, SEM: standard error mean, T0: Control group (plain water), T1: 0.5 + 250 mg/l L-SeMET + V.E, T2: 1 + 250 mg/l L-SeMET + V.E, T3: 1.5 + 250 mg/l L-SeMet + V.E, TAOC: Total antioxidants (mM/L), GPX: Glutathione peroxidase (U/GT), SOD: Superoxide dismutase (U/ml), MDA: Malondialdehyde (nmol/ml)



Graph 1. Effects of litter types, L-Selenomethionine + Vitamin E, and their interactions on serum levels of T3 (A) and T4 (B) in broiler chickens. The data represent mean values from three replicates (4 chickens in each replicate) per treatment. The use of distinct letters placed above the columns indicates significant variances, established by Tukey's test with a significance level of $p < 0.001$. Abbreviations: L: Litter types, WS: Wood shavings, PL: Perforated plastic litter, T: Treatments, L-SeMET: L-selenomethionine, V.E: Vitamin E, T0: Control group (plain water), T1: 0.5 + 250 mg/l L-SeMET + V.E, T2: 1 + 250 mg/l L-SeMET + V.E, T3: 1.5 + 250 mg/l L-SeMet + V.E, T3: Triiodothyronine hormone (ng/dl), T4: Thyroxin hormone (µg/dl)

DISCUSSION

Growth performance and feed efficiency

There is a notable emphasis on enhancing the productive performance of broiler chickens. Achieving better performance requires the implementation of efficient management practices, ensuring optimal housing conditions, and carefully formulating their diet (Bist et al., 2024). The findings of the current investigation unveiled that broiler chickens raised on PL litter type exhibited significantly superior growth performance compared to those housed on other WS litter systems. This was evident in parameters of growth performance (BW and BWG), and feed efficiency (CFI, FCR, and EPEF). These results are attributed to the fact that broiler chickens reared on perforated plastic litter types consumed a higher amount of feed. This is likely due to a reduced likelihood of pecking in the litter, which leads to increased feed intake. Consequently, this positively affects body weight (BW) and leads to an increase in body weight gain (BWG). Chickens raised on plastic flooring exhibited comparatively greater weight gains but had a lower FCR compared to chickens reared solely on wood shavings (Almeida et al., 2017; Çavuşoğlu et al., 2018). This improvement may be attributed to the fact that WS bedding absorbs broiler chicken feces, resulting in increased moisture and interaction with ammonia. Consequently, the surface temperature of the bedding is somewhat elevated, which affects the environmental comfort for the broiler chickens. This leads to a decrease in feed intake and adversely affects feed conversion efficiency. However, this is not the case with PL litter type, where broiler chickens kept in deep litter systems exhibited the lowest FI and BW (Abd El-Wahab et al., 2020). Broiler chickens raised on plastic litter exhibit a lower pecking at litter, which results in increased feed intake. The higher observed feed intake could account for the variations in growth performance and feed efficiency among different litter types. Reducing contact with fecal matter improves the overall health of broiler chickens, leading to enhanced growth performance (Wang et al., 2015). In the present study, broiler chickens reared on PL litter exhibited significantly higher values for body weight gain, feed intake, and feed conversion ratio compared to those raised on WS litter. Without a doubt, the factors encompassed within the European Production Efficiency Factor (EPEF), including bird livability percentage, body weight (kg), FCR, and days until slaughter, as discussed and examined earlier, demonstrate superior performance in broiler chickens housed in PL systems, resulting in a higher EPEF score.

The findings of this study show that supplementing broiler chickens' drinking water with organic L-SeMet combined with vitamin E is more effective in preserving growth performance and improving feed efficiency (Table 2). The higher supplementation rates (1 and 1.5 mg/L L-SeMet + 250 mg/L V.E) yielded more pronounced positive effects. This finding indicates that the combination of organic L-SeMet was effective in enhancing energy utilization of feed. The findings from this study align with the results of Calik et al. (2022b), suggesting that incorporating organic selenium + V.E into broiler chickens' diet at a level of 1:250 mg/kg can significantly enhance both BWG and FI. The observed enhancement in productive performance in this investigation can be attributed to antioxidant properties of organic L-SeMet and V.E, which help protect against potential stressors encountered during standard husbandry practices. Selenium plays crucial roles in the body, primarily as a key component of selenoproteins (Wang et al., 2021a). These selenoproteins participate in various functions, comprising the metabolism of thyroid hormones. Specifically, they facilitate the transformation of T4 into its active state T3 (Schomburg, 2012), which has a crucial function in controlling energy and protein assimilation in the body (Wickramasuriya et al., 2023). The current study suggests that increased thyroid hormone levels significantly enhance growth performance and feed efficiency in broiler chickens. However, limited published studies exists on the effects of L-SeMet + V.E supplementation via drinking water under standard husbandry practices, indicating a need for further investigation.

Blood biochemical analysis

The assessment of blood biochemical factors serves as a valuable tool for identifying metabolic and nutritional changes, which are crucial for assessing the overall health condition in poultry (Ghasemi et al., 2013). The study revealed significant effects of litter type, L-SeMet + V.E supplementation, and their interaction on blood biochemical factors. Broiler chickens raised on both litter types and given T2 or T3 showed increased HDL levels and reduced plasma lipids and liver enzymes. The findings of the current studyare in line with a former experiment conducted by Okasha et al. (2021b) on broiler chickens. Similarly, broiler chickens reared on wood shavings and sand showed significantly lower levels of CHO, TRIG, LDL compared to those reared on plastic litter. Additionally, higher levels of HDL were obverved in broiler chickens reared on WS litter compared to those raised on sand litter (Lasheen et al., 2023). The study found higher AST activity in broiler chickens raised on wood shavings compared to perforated plastic litter, though it did not impact broiler chickens' well-being. These results are consistent with previous findings, with AST activity highest in the avian heart, followed by the liver and skeletal muscle (Ognik and Krauze, 2016; Costa et al., 2021).

Incorporation of L-SeMet + V.E into drinking water led to decreased plasma lipid profile and liver enzymes, alongside increased plasma HDL levels in broiler chickens raised on both types of litter, as indicated in Table 3. These findings align with Kang et al. (2000) who proposed that selenium has a cholesterol-lowering effect, decreasing both cholesterol and triglycerides levels. Additionally, Amer et al. (2019) discovered that incorporating Se, irrespective of its origin, resulted in a reduction in TRIG levels in rabbits. Selenium plays a role in metabolism of lipids (Zhang et al., 2018). The study suggests that L-SeMet + V.E supplementation improves lipid metabolism by increasing HDL levels and reducing LDL levels, which is beneficial as lower LDL minimizes the risk of oxidase-low-density lipoproteins (ox-LDL) formation and cholesterol buildup in arterial walls (Toth, 2005). Therefore, selenium has a crucial function in safeguarding cells against oxidative stress by boosting the activity of glutathione peroxidase (Negis et al., 2006). Incorporation of L-SeMet + V.E had a significant impact on the serum ALT and AST levels in broiler chickens. This is reinforced by the discovery of Dalia et al. (2017)**,** who observed a notable reduction in the liver enzymes in chickens fed organic selenium. Moreover, Biswas et al. (2010) documented comparable results, noting a reduction in ALT and AST concentrations in broiler chickens that received a diet enriched with Se at a level of 0.5 and 1 mg/kg. A decrease in lipid profile and liver enzyme levels indicates improved protection against oxidative damage by enhancing the redox state. These findings are supported by the antioxidant status results obtained in this study.

Antioxidants and thyroid hormones

In avian physiology, monitoring oxidative status plays a crucial role in assessing health changes (Surai et al., 2019). Assessing antioxidant levels in broiler chickens typically involves analyzing parameters such as T-AOC and MDA in the bloodstream, as well as the activity of enzymatic scavengers SOD and GPx (Ghasemi et al., 2020). Broiler chickens raised on PL litter and supplemented with 1.5 mg/L of L-SeMet + V.E showed improved serum antioxidant status and thyroid hormones, highlighting its protective role against oxidative stress. In line with the present results, Ghanima et al. (2020) and El-Maaty et al. (2023) demonstrated that broiler chickens reared on perforated PL exhibited improved antioxidant status in contrast to those reared on floor litter or in batteries. According to Okasha et al. (2021b), substantial differences were noted in the plasma GPX and MDA levels of broiler chickens as a result of the litter type. Antioxidant and thyroid hormone levels in broiler chickens raised on wood shavings may be influenced by stress factors such as mold, iodine interference, and ammonia exposure (Şen et al., 2023). Further research is needed to fully understand the effects of different litter types on broiler chicken production under standard practices.

Chickens' antioxidant system is regulated by numerous crucial enzymes, comprising T-AOC, GPx, SOD, and MDA (Wang et al., 2021b). Antioxidant properties in selenium stem from its role in GSH-Px, where it forms part of the enzyme's active center, aiding in the removal of unstable molecules generated during metabolism (Huang et al., 2019; Deng et al., 2022). The findings suggest reduced lipid oxidation, likely due to the higher bioavailability of organic selenium combined with V.E., which enhances selenium retention in the body. Consequently, this increased retention contributes to elevated levels of GSH-Px (Zhang et al., 2014). Likewise, Dalia et al. (2017) found that bacterial organic selenium (BOSe) is a superior selenium source for broiler chickens, enhancing antioxidant levels and glutathione peroxidase gene activation more effectively than inorganic selenium. Thyroid hormones (T3 and T4) play a vital role in gluconeogenesis and glycogen synthesis in poultry, facilitating the transformation of non-carbohydrate substrates into glucose and promoting the production and storage of glycogen in the liver (Duntas and Brenta, 2018). The current study revealed that L-SeMet + V.E supplementation elevated T3 and T4 levels in broiler chickens, likely attributed to the functions of selenium in promoting the conversion of T4 to T3 and sustaining selenium proteins. Similarly, Lin et al. (2014) demonstrated that selenium deficiency hinders the conversion of T4 to T3 in chickens by decreasing the activity of critical metabolic enzymes (Iodothyronine Deiodinase Type-1, Iodothyronine Deiodinase Type-2, and Iodothyronine Deiodinase Type-3) and selenoproteins (Thioredoxin Reductase 2, Selenoprotein I, Selenoprotein U, Glutathione peroxidase 1, and Glutathione Peroxidases 2), thereby indirectly disrupting thyroid hormone metabolism. Enhancing antioxidant response and regulating T3 and T4 through nutrition is of growing interest. The study hypothesizes that supplementing L-SeMet and Vitamin E in drinking water may improve antioxidant and thyroid hormone levels, boosting broiler chickens’ performance and feed efficiency. Supplementing L-SeMet and Vitamin E in drinking water could lead to cost savings through improved feed efficiency, reduced health issues, and enhanced broiler chickens' welfare due to improved health and growth. These benefits make it a promising strategy for poultry farming.

CONCLUSION

Broiler chickens raised on perforated plastic litter and supplemented with L-SeMet + Vitamin E in drinking water showed improved growth, higher feed efficiency, enhanced antioxidant status, and elevated T3 and T4 levels. The supplementation also improved blood parameters, increasing HDL and reducing lipid profile and liver enzyme levels. This approach demonstrates significant benefits for broiler chicken productivity and health under standard husbandry practices. By enhancing strategies for organic supplementation, assessing their long-term impacts, and investigating the interactions between L-SeMet and other nutrients, broiler chicken farming practices could be significantly advanced. These initiatives would contribute to sustainable, efficient, and welfare-focused poultry production in the future.

DECLARATIONS

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Authors’ contributions

Hamada Okasha, Eman Rady, Osama El-Garhy, and Gaafar EL-Gendi authored the original text, contributed to the experimental design, carried out the experiments, and conducted the statistical analysis. Hamada Okasha and Eman Rady contributed to both the statistical analysis and the design of the experiments. Hamada Okasha, Eman Rady, Osama El-Garhy, and Gaafar EL-Gendi analyzed and reviewed the results and then composed the final essay. All authors have reviewed and approved the final version of the manuscript for publication.

Competing interests

The authors declare no conflicts of interest.

Ethical considerations

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

Availability of data and materials

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

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Effects of Dietary Inclusion of Maggot (*Hermetia illucens*) and Corn on Productivity of Starter-Stage Native Chickens

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ABSTRACT

Native chickens require innovative feeding strategies to enhance productivity. This study aimed to examine the dietary effects of using maggot and corn meal (MCM) on the performance and feed digestibility of native chickens. The study used a complete randomized design, with a total of 200 one-day-old native chicks randomly allocated into four groups, each with five replicates. The first group received 100% basal feed (T0). The second group received 95% basal feed and 5% MCM (T1), the third group received 90% basal feed and 10% MCM (T2), and the fourth group received 85% basal feed and 15% MCM (T3). Group T1 exhibited significantly higher live weight, daily body weight gain, crude protein digestibility, crude fiber digestibility, and crude fat digestibility compared to other treatments. The T3 group showed the highest feed intake, whereas the best feed conversion ratio (FCR) was observed in the T0 group. It is concluded that a 5% inclusion of MCM in the feed has beneficial effecta on growth performance and digestibility of native chickens.

Keywords: Black soldier fly, Combination feed, Corn, Digestibility, Native chicken, Performance

INTRODUCTION

According to the BPS-National Statistics (2023), the total population of native chickens in Indonesia has recently been increasing, serving as an important alternative protein source. The population of native chickens in Indonesia rose from 300.1 million in 2018 to 308.6 million in 2022 (BPS, 2023). Despite their significance, native chickens have exhibited relatively slow growth and high feed conversion values (Promket and Ruangwittayanusorn, 2021). Feed quality is the main factor that supports the growth of native chickens. Low feed quality prevents native chickens to grow and develop optimally (Jachimowicz et al., 2022). The high price of manufactured feed encourages farmers to seek affordable yet nutritionally adequate alternatives (Alshelmani et al., 2021). Developing innovative feeds using the advantages of locally available ingredients presents a viable solution. As an energy source, maize can be combined with protein sources to meet nutritional sufficiency (Barszcz et al., 2022). Black Soldier Fly (BSF) larvae (*Hermetia illucens*), also known as maggots, are a promising protein source, containing 44.26% protein content and equipped with various types of essential amino acids (Schiavone et al., 2019). Compared to soybean meal, maggot exhibits superior amino acid content, including histidine (2.91% vs. 1.23%), leucine (6.79% vs. 3.65%), lysine (8.25% vs. 2.88%), methionine (1.84% vs. 0.62%), threonine (3.86% vs. 1.84%), and valine (5.54% vs. 2.3%) (Surendra et al., 2020; Aguirre et al., 2024). However, the Black soldier fly maggot has a limiting factor in chitin content derived from the exoskeleton (Purkayastha and Sarkar, 2020). High-pressure heating is expected to loosen the bonds in chitin and bring out the resistant starch of maize. Chitin will then look like a surface with large pores and dense piles of microfibrils. After steaming, the surface of chitin becomes smooth without microfibrils, and the pores increase in depth and spread over the entire surface (Tan et al., 2015). Resistant starch can be utilized by intestinal microflora as a food substrate during the digestive process, so it has the potential to become a prebiotic (Qin et al., 2020).

The current study aimed to evaluate the dietary effects of BSF maggot and corn, processed by high-pressure heating, on the productivity of native chicks. This study utilizes maize as an energy source and BSF maggot as a protein source, combined via high-pressure heating.

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MATERIALS AND METHODS

Ethical approval

All procedures involving animals were approved by the Faculty of Animal and Agricultural Science, Diponegoro University, Semarang, Indonesia (Approval No. 61-04/A-08/KEP-FPP).

Study design

The study employed a complete randomized design with 200 one-day-old unsexed native chicks sourced from Balai Pembibitan Ternak Maron, Indonesia, having an initial body weight of 29.01 ± 2.16 g. They were randomly allocated into four groups with five replicates. The first group received 100% basal feed (T0). The second group received 95% basal feed and 5% MCM (T1), the third group received 90% basal feed and 10% MCM (T2), and the fourth group received 85% basal feed and 15% MCM (T3). The study was conducted for 28 days at the starter-stage native chicken (Nuningtyas et al., 2024). One-day-old chicks were divided into 20 pens (4 groups with 5 replications; 10 chickens/1 m^2). Rearing was conducted at the Faculty of Animal and Agricultural Science's teaching farm (Diponegoro University, Semarang, Indonesia). Postal cages were used by applying sanitation and health measures, with no vaccinations or antibiotics administered during the rearing period. The day-old-chicks (DOCs) were weighed on arrival to obtain the initial weight and then divided into pens according to the groups. They were given sugar water to restore body energy lost during transportation. Feed and water were provided *ad libitum* during the treatment. Residual feed was weighed every day, and the chicks were weighed every week. Lighting duration was set for 20 hours/day, using a 2700K bulb per pen. The experiment was carried out for 28 days before data collection. The variables measured in this study included the final weight, Daily Body Weight Gain (DBWG), Feed Intake (FI), Feed Conversion Ratio (FCR), crude protein digestibility, crude fiber digestibility, crude fat digestibility, and nitrogen retention.

Treatment feed preparation

The MCM feed was made using 18-21-day-old fresh maggot (prepupal stage) and ground corn in a 50:50 ratio. Fresh maggot was obtained from STIP Farming, Semarang, for IDR 4500/kg. Maggot and corn were blended using a blender. Then they were put into 0.8 thickness plastic bags and autoclaved based on the method suggested by Utama et al. (2017), i.e., placing the feed in the autoclave until the temperature reaches 121ºC under 1.5 atm pressure. After reaching this stage and waiting for 15 minutes, the autoclave was turned off. Upon cooling the autoclave, the feed was removed from the plastic bags and dried in an oven dryer at 50°C for ± 48 hours. After drying, the dried MCM was reground to pass through a 30-mesh sieve to ensure consistent particle size before dietary incorporation. Diet formulations adhered to the nutritional requirements for starter-phase native chickens (Indonesian National Standard SNI 7783.1:2013). Proximate analysis was conducted to verify nutritional content (Table 1).

Table 1. Feed composition and nutrient content of diet in starter-stage native chickens per treatment

Treatments **Feed**	**T0**	**T1**	**T2**	**T3**
		--------------%--------------		
Copra meal	10	10	10	9
Corn gluten feed	29	18	19	25
Yellow corn	18	21	15	8.5
Soybean meal	22	20	15	10
MCM	0	5	10	15
L-Lysine	0.25	0.25	0.25	0.25
DL-Methionine	0.25	0.25	0.25	0.25
Rice bran	17	19	21	21
Minerals	1.5	1.5	1.5	1.5
Fish meal	2	5	8	9.5
Nutrient content				
Crude protein* (%)	21.17	21.04	21.28	21.18
ME* (kkal/kg)	2924	2914	2990	3107
Crude fiber* (%)	6.87	6.75	6.6	6.74
Crude fat* (%)	3.49	3.94	3.93	3.42

The composition was formulated according to the ration needs of starter-stage native chickens: SNI 7783.1:2013. *The results of the Proximate Analysis of Biology Laboratory, Atma Jaya University Yogyakarta (2024). The first group received 100% basal feed (T0). The second group received 95% basal feed and 5% MCM (T1), the third group received 90% basal feed and 10% MCM (T2), and the fourth group received 85% basal feed and 15% MCM (T3).

Final weight

The final body weight measurement was carried out by weighing via a digital scale at the end of the rearing period (on the 28th day).

Daily body weight gain

Body weight was measured using digital scales. Measurement of Daily Body Weight Gain (DBWG) in g/chicken/day was calculated following Formula 1 (Utama et al., 2020):

$$\text{DBWG} = \frac{\text{Final weight} - \text{initial weight}}{28\ (\text{days of observation})} \qquad \text{(Formula 1)}$$

Feed intake

Feed intake data were collected daily by calculating the difference between the given feed and the residual feed. Weighing of residual feed was done before feeding in the morning.

Feed conversion ratio

Feed Conversion Ratio (FCR) was calculated based on the ratio between the amount of ration consumed and the measured weight gain.

$$\text{FCR} = \frac{\text{Amount of feed consumed (g)}}{\text{Body weight gain (g)}} \qquad \text{(Formula 2)}$$

Digestibility values

Measurement of digestibility values was performed on 28-day-old chicks by sampling 2 chickens per replicate. Total collection methods were conducted according to Utama et al. (2020). Each replicate group of chickens was kept in a battery cage (2 chickens/repetition/cage), and 6 chickens were placed in each cage to get endogenous excreta. Chickens were moved to the battery cage and allowed to adjust to the conditions for 12 hours by providing feed and water. The transfer of chickens to battery cages was done at night and under low temperatures to minimize stress on the chickens. Afterwards, all treated and endogenous chickens were fasted for 24 hours to eliminate feed residues in the digestive tract. Chickens were fed 100 g of treatment feed, and the excreta were collected for 48 hours. Chickens for endogenous excreta collection were kept fasted and given water *ad libitum*. Endogenous chicken excreta were collected for 44 hours. Excreta were sprayed once every hour with 1N HCl to capture and reduce nitrogen evaporation. The collected excreta were cleaned of contaminants, then dried by natural drying for 2 days at 28-34°C. During the drying process, the excreta were covered using an insect net to prevent flies and other contaminants from entering, and were still routinely sprayed with HCl. The samples were then ground and put into plastic clips according to the replication to be tested for crude protein, crude fiber, crude fat, and nitrogen content.

Crude Protein Digestibility (CPD), Crude Fiber Digestibility (CFD), and Crude Fat Digestibility/Ether Extract Digestibility (EED) were calculated following Formulas 3-5, respectively (Moningkey et al., 2019). The nitrogen retention (NR) was calculated using Formula 6 (Sibbald, 1980):

(Formula 3)

$$\text{CPD (\%)} = \frac{(\text{CP consumption} - \text{CP excreta})}{\text{CP consumption}} \times 100\%$$

(Formula 4)

$$\text{CFD (\%)} = \frac{(\text{CF consumption} - \text{CF excreta})}{\text{CF consumption}} \times 100\%$$

(Formula 5)

$$\text{EED (\%)} = \frac{(\text{EE consumption} - \text{EE excreta})}{\text{EE consumption}} \times 100\%$$

(Formula 6)

$$\text{NR (\%)} = \frac{(\text{Fd} \times \text{Nf}) - ([\text{E} \times \text{Ne}] - [\text{En} \times \text{Nen}])}{(\text{Fd} \times \text{Nf})} \times 100\%$$

where Fd is the feed consumed (g), Nf is the nitrogen feed (%), Ne is the nitrogen excreta (%), E is the total excreta (g), En is the total excreta endogenous (g), and Nen is the nitrogen endogenous (%).

Data analysis

Data were analyzed using SPSS, version 23.0. The data were tested for normality and homogeneity and were analyzed using analysis of variance (ANOVA). Significant differences ($p < 0.05$) identified via one-way ANOVA were further analyzed using Duncan’s Multiple Range Test (DMRT).

RESULT AND DISCUSSION

Growth performance

The dietary inclusion of maggot-corn meal (MCM) significantly influenced ($p < 0.05$) the final weight, daily body weight gain (DBWG), feed intake (FI), and feed conversion ratio (FCR) of starter-stage native chickens (Table 2).

Table 1. Effects of different dietary MCM levels on final weight, daily body weight gain, feed intake, and feed conversion in the starter-stage of native chickens

Treatment Parameter	T0	T1	T2	T3
Final weight (g/chicken) *	374.7 ± 26.14^{ab}	384.6 ± 4.32^{a}	367.6 ± 7.72^{ab}	359.3 ± 18.6^{b}
Daily body weight gain (g/chicken/day) *	12.71 ± 0.94^{ab}	12.34 ± 0.16^{a}	12.06 ± 0.28^{ab}	11.8 ± 0.65^{b}
Feed intake (g/chicken/day) *	21.21 ± 0.32^{d}	24.38 ± 0.17^{c}	25.51 ± 0.18^{b}	26.1 ± 0.12^{a}
Feed conversion*	1.59 ± 0.01^{a}	1.78 ± 0.03^{b}	1.95 ± 0.04^{c}	2.04 ± 0.1^{c}

Source: Primary research data (2024); *Different superscript letters in each row show significant differences ($p < 0.05$). The first group received 100% basal feed (T0). The second group received 95% basal feed and 5% MCM (T1), the third group received 90% basal feed and 10% MCM (T2), and the fourth group received 85% basal feed and 15% MCM (T3).

Final weight and daily body weight gain

The various levels of MCM in the diets had a significant effect ($p < 0.05$) on the final weight and daily body weight gains of starter-stage native chicks. The highest final weight and DBWG values were found in the second group (T1) (384.6 ± 4.32 g/chicken and 12.34 ± 0.16 g/chicken/day) whereas the lowest final weight and DBWG indexes were found in the fourth treatment group (T3) (359.3 ± 18.6 g/chicken and 11.8 ± 0.65 g/chicken/day). This suggests that MCM is effective as an alternative feed only up to a 5% inclusion level. Factors that can affect final weight are body weight gain and feed conversion. Utama et al. (2024) noted that the final weight is related to both feed conversion and body weight gain, as the more feed nutrients are converted into tissues that form organs, which can increase body weight gain, so that the final weight increases. Maggot and corn meal levels of 10 and 15% in the diet decreased the final weight. This can be attributed to the chitin content in MCM. Kastalani et al. (2021) stated that chitin can inhibit protein digestion because it can form complex bonds with proteins and bind nitrogen in amino acids. According to Ramdani et al. (2018), chickens need protein for metabolism in the meat formation process. Warisman et al. (2024) also stated that the 5.4% of chitin in steamed maggot decreased the digestibility value and nutritional parameters of the chicken ration. A decreased level of digestibility will affect the feed conversion.

Feed intake

The effect of different levels of MCM on the diet had a significant effect ($p < 0.05$) on the feed intake of starter-stage native chicken. The addition of MCM to the diet tends to increase the ration consumption of native chickens. Feed intake increased with MCM inclusion, peaking in T3 (26.1 g/head/day) and then T2 (25.51 g/head/day) and T1 (24.38 g/head/day) compared to the control (21.21 g/head/day). It is assumed that the chitin content is increased by increasing the content of MCM in the diet. According to Daniar and Herdyastuti (2019), chitin is grouped among the animal fibers whose structure is similar to cellulose, with a crude fiber content of 72.52%. An increase in crude fiber will trigger the rate of digesta in the intestine so that feed quickly leaves the digestive tract, the livestock feed hungry, and continue to consume rations (Jha and Misshra, 2021). Chitin will increase the digesta rate, so that the chickens will quickly feel hungry and continue to consume feed.

Feed conversion ratio (FCR)

The feed conversion ratio of starter-stage native chickens was also influenced by various MCM levels. The control group (T0) achieved the best FCR (1.59 ± 0.01), while T3 had the poorest (2.04 ± 0.1). The usage of MCM in the ration tends to increase FCR. This can be attributed to the values of nitrogen retention. Abun et al. (2023) stated that higher nitrogen retention results in more efficient feed conversion. Utama et al. (2024) added that high nitrogen retention values indicate high quality feed protein containing balanced essential and non-essential amino acids, which are easily absorbed and converted into body weight. This is relevant to the nitrogen retention value in Table 3, showing that higher nitrogen retention values lead to higher FCR values.

Table 3. Effect of different dietary MCM levels on crude protein, crude fiber, crude fat digestibility, and nitrogen retention in the starter-stage of native chickens

Treatment Parameter	T0	T1	T2	T3
Crude protein digestibility*	80.73 ± 0.69^{b}	83.35 ± 0.9^{a}	81.75 ± 0.8^{b}	78.27 ± 1.11^{c}
Crude fiber digestibility*	45.72 ± 1.82^{b}	53.01 ± 1.49^{a}	47.6 ± 0.85^{b}	39.64 ± 1.55^{c}
Crude fat digestibility*	81.78 ± 0.95^{c}	92.33 ± 1.35^{a}	91.5 ± 0.69^{a}	85.48 ± 1.39^{b}
Nitrogen Retention*	88.10 ± 1.38^{a}	88.75 ± 0.4^{a}	83.26 ± 0.68^{b}	80.71 ± 2.75^{b}

Source: Primary research data (2024); *Different superscript letters in each row show significant differences ($p < 0.05$). The first group received 100% basal feed (T0). The second group received 95% basal feed and 5% MCM (T1), the third group received 90% basal feed and 10% MCM (T2), and the fourth group received 85% basal feed and 15% MCM (T3).

Feed digestibility

The findings revealed that there were significant effects ($p < 0.05$) arising from the type of treatment on crude protein digestibility, crude fiber digestibility, crude fat digestibility, and retention of nitrogen, as presented in Table 3.

Crude protein digestibility

Dietary MCM inclusion significantly affected ($p < 0.05$) Crude Protein Digestibility (CPD) in starter-stage native chicks. with the highest CPD observed in T1 (83.35 ± 0.9%) and the lowest in T3 (78.27 ± 1.11%). This shows that MCM is effective up to 5%, and the higher levels will reduce protein digestibility. In comparison, increasing the composition of MCM and reducing the composition of soybean meal in the diet also affected protein digestibility. This is thought to be due to the chitin content in MCM. Chitin can form complex bonds with proteins and bind amino acid nitrogen (Kastalani et al., 2021). A study by Warisman et al. (2024) showed that the steamed maggot decreased the digestibility values and the nutritional parameters of the chicken ration due to the chitin content. Sánchez-Muros et al. (2014) stated that poultry lack chitinase enzymes, limiting their ability to absorb chitin.

Crude fiber digestibility

Different MCM levels in the diet had a significant effect ($p < 0.05$) on Crude Fiber Digestibility (CFD) among starter-stage native chicks. As presented in Table 3, the highest CFD was observed in the second group (T1) at 53.01%t, with the third group (T2) at 47.6%, the first group (T0) at 45.72%, and finally reaching at 39.64% in the fourth group (T3) as the lowest CFD. This finding shows that the addition of MCM at 10% and 15% levels tends to reduce the level of crude fiber digestibility in native chickens. It seems the reason is the fact that the chitin content at 10% and 15% MCM level causes an increase in digesta rate, so that the process of digesting crude fiber by intestinal microbes only lasts for a short time. Chitin in feed will accelerate the rate of digesta; the faster the digesta rate is. the shorter the digestion process will be (Has et al., 2013; Nirwana et al., 2021). Morgan et al. (2022) noted that an extremely short digestion rate leads to a short time available for digestive enzymes to degrade nutrients, which can reduce feed digestibility.

Crude fat digestibility

Varying MCM levels in diets significantly affected ($p < 0.05$) Crude Fat Digestibility (CFD) in starter-stage native chicks. The highest CFD was obtained in the second group (T1) at 92.33%, followed by the third group (T2) at 91.5%, and then the fourth group (T3) at 85.48%, finally achieving 81.78% as the lowest in the first group (T0). Using MCM in the ration effectively increased crude fiber digestibility up to 10%, but it decreased CDF at a 15% level. The MCM feeding significantly increased crude fat digestibility compared to the control treatment ($p < 0.05$). This is probably because maggot has a type of fat that is easily digested. Black soldier fly larvae contain 37.34% monounsaturated fatty acids and 20.45% polyunsaturated fatty acids and are dominated by medium-chain fatty acids (C8-C12) (Kim and Rhee, 2016; Alifian et al., 2019). Unsaturated fatty acids can also affect the performance of lipase enzymes to increase fat absorption (Adhami et al., 2021).

Nitrogen retention

Nitrogen retention (NR) was highest in T1 (88.75%) and lowest in T3 (80.71%). According to Sibbald and Wolynetz (1985), a factor that helps increase nitrogen retention value is the production of quality feed, which is easily converted into weight. This indicates that the use of MCM at the 5% level can improve feed quality, seen from the nitrogen retention value perspective. The decrease in nitrogen retention value in T2 and T3 is thought to be due to the chitin content in the MCM. The addition of MCM results in an increase in the chitin content in the feed. Kastalani et al. (2021) stated that chitin can bind nitrogen from amino acids, reducing the digestibility value of protein. Belluco et al. (2013) and Lee et al. (2022) argue that chitin causes a decrease in the digestibility of feed ingredients and can bind the nutrients needed.

CONCLUSION

It is suggested that maggot and corn meal be replaced with soybean meal up to 5% to increase live weight, daily weight gain, and digestibility values of crude protein, crude fiber, and crude fat of native chickens. Further studies on changes in the micronutrient structure of MJS are needed to determine its potential as a functional feed ingredient.

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Author's contributions

Sabrang Panuntun conducted the research, collected and analyzed the data, and drafted the manuscript. Cahya Utama and Bambang Sulistiyanto reviewed and edited the manuscript. All authors have read and approved the final version of the manuscript.

Competing interests

The authors have declared that no competing interests exist.

Ethical considerations

Plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy have been checked by all the authors.

Availability of data and materials

All data generated during the study are included in this article. Any additional information is available upon reasonable request.

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Obtaining High Yields of *Bacillus* species During Solid-State Fermentation of Plant Raw Materials for Use as a Feed Additive

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ABSTRACT

Bacillus spp. are a natural alternative to antibiotic therapy and are most suitable for use as probiotic feed additives to improve the growth and productivity of farm animals. The present study aimed to establish culture conditions suitable for the maximal production of probiotic bacteria spores and enzymes of *Bacillus subtilis* IMB-73 and *Bacillus amyloliquefaciens* IMB-79 in solid-state fermentation (SSF) of wheat bran, soybean flour, and cilantro. The tested lignocellulosic materials ensured rapid and abundant growth of bacilli; however, wheat bran proved to be the most suitable growth substrate, yielding the highest spore counts of 5.1×10^{11}/g and 5.5×10^{11}/g in the cultivation of *B. amyloliquefaciens* IMB-79 and *B. subtilis* IMB-73, respectively. Supplementation of an additional nitrogen source to the wheat bran medium resulted in a significant increase in spore productivity. In particular, peptone at a concentration of 67 mg/g substrate provided the maximum spore yield in the fermented product. Both strains secreted high endoglucanase and xylanase activities in the SSF of wheat bran. Additional nitrogen sources slightly suppressed the secretion of enzymes during the SSF of wheat bran by *B. subtilis* IMB-73 but increased cellulase activity in cilantro SSF by approximately 2-5 times. The ease of production and the high spore yield achieved by scaling up production in polypropylene bags demonstrated the feasibility of using the developed technology for commercialization on local agricultural farms.

Keywords: *Bacillus*, Cellulase, Lignocellulose fermentation, Probiotic, Spore production

INTRODUCTION

One of the primary challenges in agriculture in Georgia is the use of antibiotics to prevent diseases in farm animals and poultry. Other challenges facing the economy and agriculture include the lack of local production of probiotics and dependence of the agri-food industry on imports, and the generation of large quantities of by-products and waste by the food industry, which are mostly dumped in open areas, leading to environmental problems.

Research in recent years has shown that *Bacillus* spp. bacteria can prevent gastrointestinal infections and are a natural alternative to antibiotic therapy for use in animal and poultry farms to enhance their growth, productivity, and all performance characteristics (Popov et al., 2021; Herrmann et al., 2023; Liao et al., 2023). These spore-forming bacteria are the most suitable for use as probiotic feed additives. In their spore form, *Bacillus* spp. can withstand harsh physical and chemical environmental conditions during industrial processes, storage, and application. In their vegetative form, the bacteria manifest immunomodulatory properties and the ability to synthesize a wide range of biologically active compounds that provide high efficacy against pathogens.

Given these circumstances, there is a need to develop a simple, low-cost, and highly effective technology for obtaining probiotics using the biosynthetic potential of individual bacilli and their ability to utilize lignocellulose (Mahariawan et al., 2021; Herrmann et al., 2023). The

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nutrient medium should generally support rapid growth and high cell density and ensure their subsequent sporulation. For example, under submerged fermentation conditions, mandarin peel at a concentration of 40 g/L was found to be the best substrate among ten lignocellulosic materials for maximum production of *Bacillus subtilis* KATMIRA 1933 spores (Khardziani et al., 2017). Mahariawan et al. (2021) proved that wheat flour is a promising growth substrate for submerged fermentation to produce spores of *Bacillus megaterium*. Grigs et al. (2023) developed a low-cost medium using locally available broad bean flour and molasses and achieved high spore yields of *Bacillus subtilis* MSCL 897 with a sporulation efficiency of 80-90%.

Compared with submerged fermentation, solid-state fermentation (SSF) of plant materials is known to have several advantages, such as relatively high volumetric productivity and product concentration, simpler downstream processing, less wastewater generation, and the need for simple fermentation equipment (Rodríguez-Couto, 2019). Besides, the SSF is the most appropriate for organizing on-site tailor-made probiotics production, when the whole fermented products are directly used as feed additives (Siqueira et al., 2020). Of course, to achieve the maximum yield of spores, it is necessary to optimize the nutrient medium and cultivation conditions to ensure the maximum formation of bacterial biomass and spores.

Recent studies have shown that *B. subtilis* IMB-73 and *B. amyloliquefaciens* IMB-79 spores stimulate broiler growth and improve feed conversion (Chistyakov et al., 2015; Chkuaseli et al., 2021). Moreover, toxicogenic and mutagenic tests, as well as the ability to coaggregate with *Escherichia coli* and *Pseudomonas aeruginosa*, tolerance to incubation in 0.3% bile salts and pH 2.0-3.0, showed that these strains are promising for food and medical (Algburi et al., 2016).

This study aimed to elucidate the cultivation conditions suitable for the growth and probiotic spore production by *B. subtilis* IMB-73 and *B. amyloliquefaciens* IMB-79 during SSF of abundantly available plant materials to develop a simple technology that could be used and commercialized in local agricultural farms.

MATERIALS AND METHODS

Strains and inoculum preparation

Bacillus subtilis IMB-73 (former strain KATMIRA, 1933) and *B. amyloliquefaciens* IMB-79 (former strain B-1895) obtained from the Russian National Collection of Industrial Microorganisms (Moscow, Russia) were used for the probiotic bacteria production. For inoculum preparation, the bacteria were grown for 24 hours on a rotary shaker at 160 rpm, 37°C in the nutrient medium of the following composition (g/L): Glucose (2.0), yeast extract (2.0), peptone (2.0), KH_2PO_4 (1.0), $MgSO_4$ (0.5). The cells were then harvested, resuspended in sterile physiological saline, and served as the inoculum for SSF.

Lignocellulosic materials

Wheat bran and soybean meal were obtained from the feed company Nutrimax (Georgia), which is interested in enriching these materials with probiotics. Coriander residue was obtained from the essential oil company AromaAmbra (Georgia), which is interested in valorizing this by-product. This material was tested for the first time as a substrate for bacterial growth. These plant residues were oven-dried at 50°C, ground, and sieved through standard-mesh sieves to obtain particles ranging from 0.5 mm to 3 mm.

Cultivation conditions

The SSF of selected plant materials was carried out in 100 mL flasks containing 4 g of substrates moistened with 13 mL of the medium (g/L): KH_2PO_4 (1.0), $MgSO_4 \cdot 7H_2O$ (0.5), peptone (10.0), NaCl (2.0). Three inorganic (physiologically base salt KNO_3, physiologically acid salt $(NH_4)_2SO_4$, and NH_4NO_3) and three organic (peptone, yeast extract, and casein hydrolysate) nitrogen sources were tested for the optimum spore production. Control lacking an additional nitrogen source was run in parallel. The initial pH of all media for cultivating bacteria was adjusted to 7.0 before sterilization at 121^oC for 30 minutes. One mL of properly diluted bacterial suspension (to have in inoculated medium $1\text{-}2 \times 10^6$ colony-forming units [CFU]/g substrate) was used to inoculate each flask. The contents of the flasks were well mixed and incubated at 37^oC for 4 days.

After 3 and 4 days of SSF, the total fermented biomass was weighed and divided into three parts. One part was used for spore count; for this purpose, 1 g of the biomass was placed into sterile 100 mL flasks, mixed with 10 mL of sterile physiological saline supplemented with 0.1% Tween 80, and homogenized by vigorous vortexing for 1-2 minutes. The second part was utilized for the measurement of the enzymatic activity; specifically, 2 g of the biomasses were extracted twice with 15 mL of distilled water (total volume 30 mL), the solids were separated by centrifugation at 10,000 g for 5 minutes at 4^oC, and the supernatants were analyzed for pH, reducing sugars (RS) and cellulase activity. The remaining biomass was weighed and dried at 105^{o}C to determine the biomass's dry weight.

To scale up the probiotic production, 0.5 kg of milled wheat bran or cilantro was soaked in the optimized nutrient medium to 75% saturation and placed in polypropylene gas-permeable bags, Microsac PPB75/BEU6/X33-57 (SACO2, EKE, Belgium) for sterilization by autoclaving at 121°C for 1 hour. After cooling, the bags were inoculated with bacterial suspension to receive 1×10^{6} CFU/g substrate and well mixed to distribute the inoculum evenly. Inoculated bags were incubated for 4 days in the dark in a climate camera at 32°C.

Spore count

The flasks with suspended biomass were heated at 80°C for 10 minutes to kill the remaining vegetative cells. Then 100 µL samples were taken from the 10^{7}-10^{9} diluted suspensions and spread plated onto the sterile agar medium of the following composition (g/L): glucose (2.0), KH_2PO_4 (1.0), $MgSO_4$ (0.5), peptone (2.0), yeast extract (2.0), agar (17.0), pH (7.0). The plated samples were incubated at 37^{o}C for 24 hours and 48 hours, and the grown colonies were counted. The average values from 3 independent plates were used in all experiments to calculate the number of spores per g of dry fermented biomass.

Cellulase and xylanase activity assay

The supernatants obtained after biomass separation were analyzed for carboxymethyl cellulase (CMCase, EC 3.2.1.4) activity according to the IUPAC recommendations, with 1% of low viscosity carboxymethyl cellulose in 50 mM citrate buffer (pH 5.0) at 50^{o}C for 10 minutes (Ghose, 1987). Xylanase (EC 3.2.1.8) activity was determined in the same conditions using birchwood xylan (Roth 7500, 1% w/v) as an enzyme substrate (Bailey et al., 1992). Glucose and xylose standard curves were used to calculate cellulase and xylanase activities. The release of reducing sugars was measured using the dinitrosalicylic acid reagent method. One unit of CMCase or xylanase activity was defined as the amount of enzyme required to release 1 µmol of glucose or xylose, respectively, per minute. Activities were referred to per biomass dry weight (U/g).

Statistical analysis

All experiments were performed twice in three replicates. The results obtained are expressed as the mean ± standard deviation. The mean values and standard deviations were calculated using Excel. Student's t-test was used to perform a statistical analysis of the differences among the experimental groups, and p-values < 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Effect of lignocellulosic growth substrate

At the initial stage of the study, *B. subtilis* IMB-73 and *B. amyloliquefaciens* IMB-79 spore yield and enzyme activity in the fermented products were assessed during the SSF of selected materials. Unlike wheat bran and soybean meal, which are common components of culture media for microorganism cultivation and animal feed formulations, cilantro was used as a growth substrate for spore-forming bacteria cultivation for the first time.

The results depicted in Table 1 allow for drawing several important conclusions. Firstly, the tested lignocellulosic materials are suitable growth substrates, ensuring rapid and abundant visual growth of bacilli and the formation of many spores. Secondly, these findings are consistent with previous studies indicating that the spore yield significantly depends on the growth substrate available in the nutrient medium (Ren et al., 2018; Elisashvili et al., 2019). In particular, the wheat bran was the most appropriate growth substrate, providing the highest yield of spores, 5.1×10^{11}/g and 5.5×10^{11}/g in the cultivation of *B. amyloliquefaciens* IMB-79 and *B. subtilis* IMB-73, respectively. Conversely, cilantro and especially soybean meal appeared to be poorer growth substrates for spore formation by both bacterial species. It is likely that, compared to wheat bran, both substrates are richer in nutrients that remain available at the end of bacterial cultivation; therefore, the transition of vegetative cells to sporulation is delayed. Thirdly, all fermented products contain low concentrations of reducing sugars after four days of SSF lignocellulose. Low sugar concentrations are a prerequisite for the initiation of abundant sporulation by most *Bacillus* species studied, including *B. amyloliquefaciens* IMB-79 and *B. subtilis* IMB-73 (Posada-Uribe et al., 2015; Elisashvili et al., 2019). It should be noted that regardless of the bacterial species and the type of growth substrate, the maximum spore yield was observed after 4 days of SSF. This is probably explained by the fact that during the cultivation of bacteria, the availability of nutrients in the medium decreased, and only on the fourth day did the vegetative cells go into starvation and sporulation.

It is known that several *Bacillus* species, among them *B. amyloliquefaciens* and *B. subtilis*, are capable of producing a high amount of industrially important enzymes (Yadav et al., 2020; My et al., 2022; Mushtaq et al., 2024). The results obtained show that both bacteria produce cellulase and xylanase during the SSF process. This ability of the bacilli allows them to hydrolyze lignocellulosic polysaccharides into metabolizable sugars and use them as carbon sources. Interestingly, during fermentation, the sugar content remains low enough to prevent catabolite repression of cellulase and xylanase synthesis by the bacteria. The results depicted in Table 1 indicated that compared to *B. amyloliquefaciens* IMB-79, the enzymatic activity of *B. subtilis* IMB-73 was less dependent on the lignocellulosic material in the medium, but in both bacterial cultures, xylanase activity was 4-12 times higher than endoglucanase activity. Interestingly, Mazanko et al. (2018) showed that feeding soy products fermented with *B. subtilis* KATMIRA1933 and *B. amyloliquefaciens* B-1895 to laying hens and roosters resulted in increased sperm production, egg production, quality, and hatchability. It is suggested that the hydrolases produced by the bacilli contributed to increased nutritional value and better feed digestibility.

Table 1. *Bacillus subtilis* IMB-73 and *Bacillus amyloliquefaciens* IMB-79 spore production and enzyme activity in solid-state fermentation of lignocellulosic growth substrates

Growth substrate	**Reducing sugars (mg/g)**	**Spores (Number × 10^{11}/g)**	**CMCase (U/g)**	**Xylanase (U/g)**
B. amyloliquefaciens IMB-79				
Cilantro	0.5 ± 0.2^{4}	$0.3 \pm 0^{4*}$	0.2 ± 0^{3}	2.2 ± 0.3^{4}
Wheat bran	1.7 ± 0.2^{4}	5.1 ± 0.2^{4}	0.5 ± 0.1^{4}	6.1 ± 0.5^{4}
Soybean meal	1.8 ± 0.2^{4}	0.2 ± 0.1^{4}	0.5 ± 0.1^{3}	3.7 ± 0.4^{4}
B. subtilis IMB-73				
Cilantro	0.4 ± 0.1^{4}	0.6 ± 0.1^{4}	0.6 ± 0.1^{3}	2.7 ± 0.3^{4}
Wheat bran	1.8 ± 0.2^{4}	5.6 ± 0.2^{4}	0.6 ± 0.1^{4}	2.8 ± 0.2^{4}
Soybean meal	1.3 ± 0.1^{4}	0.3 ± 0.1^{4}	0.4 ± 0^{3}	2.0 ± 0.2^{4}

* The numbers indicate the days of the maximum values. CMCase: Carboxymethyl cellulase

Effect of nitrogen source

The nature and concentration of nitrogen sources are important nutritional factors influencing both *Bacillus* growth and spore formation (Elisashvili et al., 2019). For example, a significant increase in spore productivity was observed during submerged fermentation of broad bean flour and molasses with *B. subtilis* MSCL 897 and the enrichment of the medium with yeast or corn extract (Grigs et al., 2023). It was indicated that all organic nitrogen sources favored spore production in the submerged fermentation of mandarin peels by *B. subtilis* KATMIRA1933 (now IMB-73), and peptone ensured almost a three-fold increase in the spore count as compared with the control medium (Khardziani et al., 2017). Therefore, several inorganic salts and organic compounds in the concentration of 40 mM as nitrogen were tested as nitrogen sources in addition to the nitrogen present in cilantro and wheat bran to determine their effect on spore production by *B. subtilis* IMB-73. The data revealed several general features (Tables 2 and 3). Firstly, adding organic nitrogen sources to the control medium resulted in an increase in the medium pH at the end of fermentation. Secondly, compared to cilantro, wheat bran without an additional nitrogen source is an excellent growth substrate, accumulating up to 1 × 10^{11} spores/g of fermented product. It is possible that processing cilantro seeds for essential oil extraction results in a reduction in the nutritional value of the residue or the formation of bacterial growth inhibitors. Thirdly, although spore yields varied significantly when bacilli were grown in the presence of nitrogen sources, all supplements were favorable for sporulation. Among them, inorganic salts increased the spore yield by 2.4-3 times, whereas organic nitrogen sources ensured at least a 5-fold increase in the spore count compared with the control medium. Fourthly, the effect of an additional nitrogen source on cellulase and xylanase production significantly depended on the lignocellulosic growth substrate in the nutrient medium. Adding any nitrogen source to the control medium containing wheat bran suppressed the secretion of enzymes, especially CMCase, by *B. subtilis* IMB-73. On the contrary, in the SSF of cilantro, the cellulase productivity of the same bacterial strain increased 2-5 times with supplementation of the control medium with additional nitrogen sources. It should be noted that although the xylanase activity of *B. subtilis* IMB-73 varied depending on the chemical nature of the nitrogenous compound, compared to endoglucanase activity, its activity was less dependent on the presence of an additional nitrogen source in the nutrient medium. The data obtained showed that compared to the medium based on soybean meal and cilantro, *B. subtilis* IMB-73 performs better in the wheat bran-containing medium in the presence of peptone. Therefore, it was important to establish the concentration of this nitrogen source that is optimal for the maximum formation of probiotic spores. The results showed that adding peptone to the wheat bran medium at any concentration used can significantly increase ($p \leq 0.05$) spore productivity (Figure 1). The maximum spore yield was observed when the concentration of peptone in the nutrient medium was 100 mM nitrogen (specifically, 67 mg peptone/g substrate). A further twofold increase in the peptone content in the medium reduced the concentration of spores in the fermented product. It is known that sporulation generally occurs in unfavorable environments, such as nutrient starvation (Monteiro et al., 2014; Elisashvili et al., 2019). It is possible that increasing the peptone concentration beyond a certain point (100 mM) prevents nitrogen depletion and thus hinders sporulation, reducing the productivity of the culture. Undoubtedly, depletion of nutrients in the medium is necessary for effective sporulation (Monteiro et al., 2014).

A different response of the bacterial culture was revealed when measuring the enzymatic activity of *B. subtilis* IMB-73 depending on the nitrogen concentration. In this case, the maximum activity of endoglucanase and xylanase was detected in the control medium (Figure 1). The activity of both enzymes in the fermented product decreased as the concentration of the additional nitrogen source in the nutrient medium increased. Notably, the concentration of reducing sugars in the fermented products decreased from 5.8 in the control to 1.4-1.6 mg/g at a concentration of 100-200 mM nitrogen. Therefore, catabolite repression by sugars cannot be the reason for the decrease in cellulase and xylanase secretion during bacterial growth in the presence of high nitrogen concentrations. It is possible that the presence of excess peptone as a nitrogen source caused metabolic stress in vegetative cells and suppressed their biosynthetic activity (Mahariawan et al., 2021). This phenomenon requires further research.

Table 2. Effect of nitrogen sources on the *Bacillus subtilis* IMB-73 spore production and enzyme activity in solid-state fermentation of cilantro

Nitrogen source	**Final pH**	**Reducing sugars (mg/g)**	**Spores (Number × 10^{11}/g)**	**CMCase (U/g)**	**Xylanase (U/g)**
Control	7.3 ± 0.1	0.5 ± 0.1^{3}	0.1 ± 0.03^{4}	0.3 ± 0^{3}	2.4 ± 0.6^{3}
KNO_3	7.5 ± 0.1	0.2 ± 0^{3}	0.3 ± 0.05^{4}	0.7 ± 0.4^{4}	2.3 ± 0.3^{3}
NH_4NO_3	7.3 ± 0.1	0.2 ± 0.1^{4}	0.3 ± 0.02^{4}	1.6 ± 0.2^{3}	3.2 ± 0.5^{3}
$(NH_4)_2SO_4$	7.1 ± 0.1	0.2 ± 0.1^{4}	0.3 ± 0.03^{4}	0.7 ± 0.4^{4}	2.7 ± 0.4^{4}
Peptone	7.9 ± 0.1	0.4 ± 0.1^{3}	0.6 ± 0.07^{4}	0.7 ± 0.2^{4}	2.6 ± 0.3^{3}
Casein hydrolysate	7.7 ± 0.1	0.4 ± 0.1^{3}	0.5 ± 0.07^{4}	0.5 ± 0.2^{4}	2.5 ± 0.2^{4}
Yeast extract	7.8 ± 0.1	0.3 ± 0.1^{3}	0.6 ± 0.09^{4}	1.3 ± 0.4^{3}	2.9 ± 0.2^{3}

$^{3-4}$ The numbers indicate the days of the maximum values. CMCase: Carboxymethyl cellulase

Table 3. Effect of nitrogen sources on the *Bacillus subtilis* IMB-73 spore production and enzyme activity in solid-state fermentation of wheat bran

Nitrogen source	**Final pH**	**Reducing sugars (mg/g)**	**Spores (Number × 10^{11}/g)**	**CMCase (U/g)**	**Xylanase (U/g)**
Control	7.8 ± 0.1	6.1 ± 0.4^{4}	1.0 ± 0.2^{4}	1.1 ± 0.2^{3}	3.7 ± 0.3^{3}
KNO_3	7.7 ± 0.1	2.0 ± 0.2^{3}	2.4 ± 0.2^{4}	0.8 ± 0.1^{3}	4.3 ± 0.2^{4}
NH_4NO_3	7.8 ± 0.1	3.2 ± 0.3^{4}	2.9 ± 0.3^{4}	0.7 ± 0.1^{4}	2.8 ± 0.2^{4}
$(NH_4)_2SO_4$	7.6 ± 0.1	1.7 ± 0.2^{4}	2.6 ± 0.2^{4}	0.9 ± 0.2^{3}	2.9 ± 0.2^{4}
Peptone	8.2 ± 0.1	1.6 ± 0.2^{4}	5.4 ± 0.2^{4}	0.7 ± 0.1^{3}	2.5 ± 0.2^{3}
Casein hydrolysate	8.4 ± 0.1	2.9 ± 0.3^{4}	4.7 ± 0.3^{4}	0.8 ± 0.1^{3}	4.7 ± 0.4^{4}
Yeast extract	8.3 ± 0.1	1.7 ± 0.3^{3}	5.3 ± 0.2^{4}	1.0 ± 0.1^{3}	2.9 ± 0.1^{4}

$^{3-4}$ The numbers indicate the days of the maximum values. CMCase: Carboxymethyl cellulase

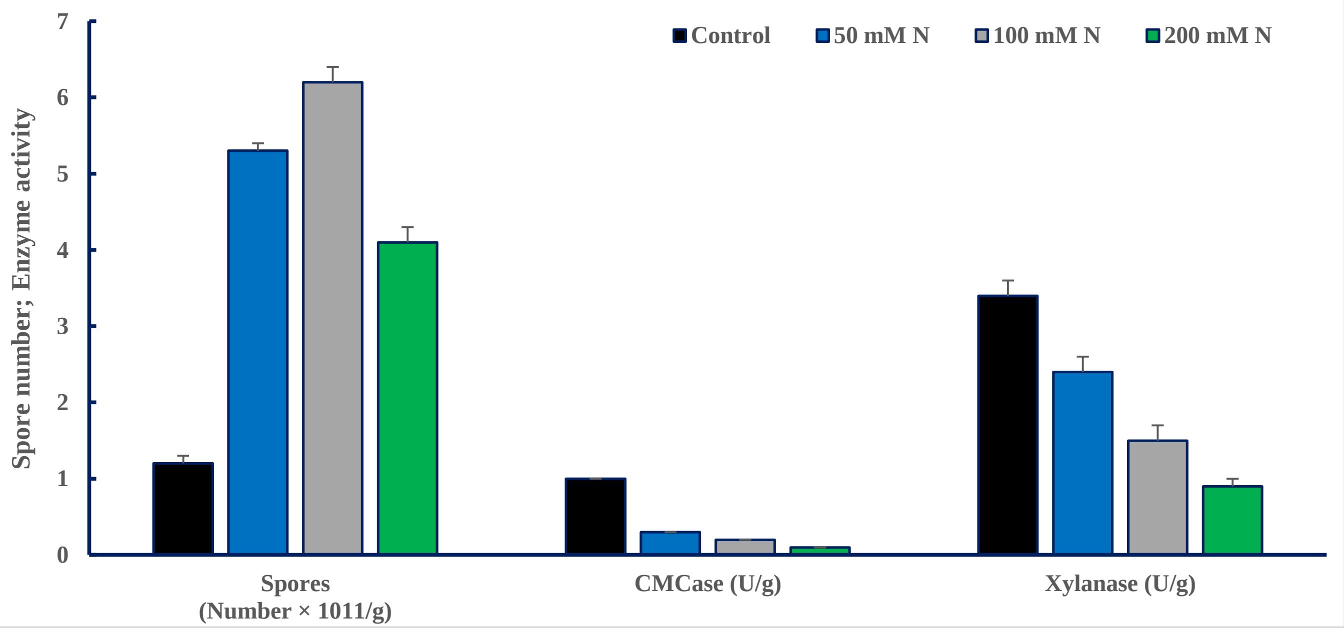

Figure 1. Effect of the peptone concentration on the *Bacillus subtilis* IMB-73 spore production and enzyme activity in solid-state fermentation of wheat bran

At the final stage, to test the feasibility of the conducted in-flask SSF process for pilot scale, cultivation conditions optimized for maximum probiotic spore production by *B. subtilis* IMB-73 were applied to both wheat bran and cilantro SSF in polypropylene bags. Analyses of the spore count after 4 days of culturing the bacteria revealed that the spore yield in the fermented product reached 5.5 × 10^{11}/g and 0.6 × 10^{11}/g of dry biomass, respectively, during fermentation of bran and cilantro. Thus, the mass production experiment confirmed the results obtained when culturing bacteria in flasks. These promising results indicate that SSF of locally available renewable feedstock supplemented with appropriate carbon and nitrogen sources provides high yields of *B. subtilis* IMB-73 spores and is the most suitable and low-cost method for establishing on-site probiotic production without requiring significant capital investment. If produced on site, poultry and livestock farms can directly use the entire fermented product as a feed additive.

CONCLUSION

The findings presented in this study provide new knowledge on the physiology of *B. amyloliquefaciens* IMB-79 and *B. subtilis* IMB-73 in the SSF of plant raw materials. Specifically, various lignocellulosic materials may be successfully exploited as growth substrates for cultivating spore-forming bacteria. Nevertheless, a strain-specific growth substrate should be revealed to ensure the highest spore productivity. During the fermentation of lignocellulose, *B. amyloliquefaciens* IMB-79 and *B. subtilis* IMB-73 secrete cellulase and xylanase activity to ensure hydrolysis of polysaccharides into metabolizable sugars to provide the bacterial cultures with the carbon and energy sources. Supplementing the nutrient medium with lignocellulosic growth substrate with an additional nitrogen source at an optimal concentration favors spore production with high yield. Further studies are needed to evaluate the effects of these probiotic bacteria on egg production parameters of laying hens and on growth, productivity, and feed conversion efficiency of broiler chickens.

DECLARATIONS

Authors' contributions

Tamar Khardziani and Eka Metreveli conducted the experiments and formalized and analyzed the data. Vladimir Elisashvili supervised the study and prepared the manuscript. All authors read and approved the last edition of the manuscript.

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Competing interests

The authors declare that there is no conflict of interest.

Availability of data and materials

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

Ethical considerations

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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Biosecurity and Health Management Practices in Duck Farming in Coastal and Haor Regions of Bangladesh

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ABSTRACT

Duck farming has become a profitable venture in Bangladesh due to its economic potential and adaptability. This study aimed to assess the current state of native duck health, disease prevalence, and biosecurity management in selected regions of Bangladesh. Data were collected from 180 duck-rearing farmers from six upazilas of the Coastal and Haor regions using a standard questionnaire. The study investigated vaccination practices, disease prevalence, biosecurity management, and available treatment facilities. Approximately 60% of farmers in the Haor regions and 40 % in the Coastal regions reported vaccinating their ducks, while 56.67% in Haor and 34.44% in Coastal regions practiced deworming. Duck plague was the most prevalent disease in duck farms, with 83.33% and 90.00% prevalence rates in the Haor and Coastal areas, respectively. In the Haor region, 81.11% of farms reported disease incidence among growing ducks. Disease outbreaks (Duck plague and Cholera) were highest during the monsoon season at 77.78% of farms in Haor areas. In the Coastal region, 63.33% of farmers reported a higher duckling mortality rate. The frequent contact between ducks and wild birds was more common in Haor areas, as indicated by 32.22% of farmers. Disease outbreaks were identified as the major constraint to duck farming, and affordable feed price was the most pressing concern in both study areas. Despite these challenges, improvements in disease management, vaccination coverage, and biosecurity measures, alongside efforts to reduce feed costs, could significantly enhance the sector's growth. It can be concluded that duck farming holds considerable potential and promising opportunities in the Haor and Coastal regions of Bangladesh.

Keywords: Biosecurity practice, Constraint, Deworming, Disease management, Vaccination

INTRODUCTION

Bangladesh is often described as a waterlogged country due to its geography. One-third of the country is covered with wetlands. The southern boundary of Bangladesh borders the Bay of Bengal and is connected with numerous rivers, canals, haors, and beels (lake like wetlands with stagnant water). The Haor Basin is an internationally important wetland ecosystem. Bangladesh is a riverine country where 16,488 km² of haors, canals, ponds, and low-lying water reservoirs can be efficiently utilized for duck production (Parvez et al., 2020). Duck farming is particularly profitable in the southern Coastal belt and Haor-based ecosystems (Pervin et al., 2013; Parvez et al., 2020). In these areas, many farmers are engaged in family and commercial duck rearing. Duck production plays a vital role in supporting rural farmers by providing employment and income sources for Asian communities (Adzitey and Adzitey, 2011). It is considered an important avenue for the marginalized and rural communities in the Haor areas of Bangladesh (Khan et al., 2018).

Although duck farming is a lucrative business, it faces several obstacles, most notably the frequent outbreaks of infectious diseases (Khan et al., 2018). Duck plague is a major infectious disease that can affect all ages and is associated with high morbidity and mortality rates ranging from 5% to 100% (Chakraborty et al., 2024). According to Islam et al. (2016), 52% of duck farms in Mymensingh were infected with duck cholera. Duck plague and duck cholera are more prevalent in areas with poor biosecurity practices, particularly in the Coastal regions (Rahman et al., 2009). These diseases result in high mortality, reduced growth rates, and decreased egg production in flocks, which negatively impact the economic livelihood of

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farmers, particularly that of small-scale farmers in rural areas. Biosecurity involves a set of preventive measures to control the outbreak and spread of infectious diseases and is a crucial component of disease management (Adel et al., 2023). However, small-scale poultry farms often lack sufficient biosecurity measures (East, 2007), and their practices are often inadequate (Yitbarek et al., 2016). Many duck farmers report poor biosecurity practices, limited vaccination, and insufficient access to treatment facilities (Agbolosu and Aawona 2021). In the Sylhet areas of Bangladesh, the major challenges include improper house cleaning, irregular vaccination, inappropriate disposal of dead ducks, and poor veterinary facilities (Jha et al., 2015). The mortality rate of ducks due to diseases was 29.71% in Hatia, where 21.1% died due to duck plague, and 32.1% due to a bacterial disease called duck cholera (Hoque et al., 2011). Moreover, the production cost, ranging from 72% to 87%, is influenced by the seasonal availability of natural feed resources (Uddin et al., 2020).

Despite the fact that several studies have examined duck-rearing methods, feeding strategies, disease outbreaks, and health management practices in Bangladesh, there remains a lack of region-specific research on biosecurity and health management practices in duck farming. Therefore, the present study was carried out to assess the current state of duck health and biosecurity management, to identify the major challenges faced by duck farmers in the Haor and Coastal areas, and to explore farmers' expectations for addressing and mitigating these challenges.

MATERIALS AND METHODS

Study area

The study was conducted in six districts of Bangladesh: Patuakhali, Pirojpur, Gopalgonj, Kishoregonj, Netrokona, and Sunamgonj. These districts were selected from six divisions, with three representing the Haor areas and three representing the Coastal areas. Within each district, at least one upazila (sub-district) was chosen for data collection. These areas were selected based on the high density of small-scale duck farms. Prior to data collection, consent was obtained from the farmers, and coordination was established with local leaders and Upazila Livestock Officers.

Methodology

A baseline survey was carried out in the selected six districts. Primary data were collected from duck farmers through face-to-face interviews, key informant interviews (KIIs) with Livestock Officers, and Focus group discussions. Secondary data were obtained from the Department of Livestock Services, relevant journals, reports, and official publications, from July 2023 to June 2024. In each district, a total of 30 duck-raising farmers were interviewed using a structured questionnaire. Additionally, direct observations were made at farmers' households to know the current state of health, biosecurity practices, disease outbreaks, operational challenges, and the needs of duck-rearing farmers. In total, data from 180 farmers were collected in the selected districts. Simple and clear questions were used to ensure easy understanding by the farmers and maximize the accuracy of the obtained data.

Statistical analysis

Microsoft Excel sheets were used to enter, sort, assemble, tabulate, and arrange the collected data for statistical analysis. Descriptive statistical analyses, including frequency distribution and percentages, were conducted using the Statistical Package for the Social Sciences (SPSS), Version 25.

RESULTS

Health and disease management of ducks

Table 1 summarizes the vaccination and deworming practices of duck-raising farmers in the Haor and Coastal regions. The majority of farmers in the Haor area used both duck plague and duck cholera vaccines (31.11%), whereas most farmers in the Coastal areas used only the duck plague vaccine (25.56%) to protect their birds. Deworming of ducks was carried out using levamisole or piperazine to protect the ducks against nematodes, particularly infections from roundworms. It was also observed that 41.11% of the farmers in the Haor region and 12.22% of the farmers in the Coastal region vaccinated their ducks every six months. Similarly, 40.00% of Haor farmers and 7.78% of Coastal farmers dewormed their ducks at three-month intervals. In addition, 18.89% of Haor and 20% of Coastal farmers reported vaccination at six-month intervals, with 16.67% and 21.11%, respectively, deworming their ducks over the same period. Annual vaccination and deworming practices were followed by only 7.78% and 5.56% of farmers in the Coastal region, respectively. Regarding overall practices, 60.00% of farmers in Haor and 40.00% in Coastal areas practiced vaccination. Deworming was reported by 56.67% of Haor farmers and 34.44% of those in the Coastal regions.

Table 1. Vaccination and deworming of ducks from July 2023 to June 2024 in selected Haor and Coastal regions

Parameters	Percentage (number)	
	Haor	Coastal
Type of vaccine		
Duck plague	28.89 (26)	25.56 (23)
Both duck plague and cholera	31.11 (28)	14.44 (13)
Vaccination interval		
6 Months	41.11 (37)	12.22 (11)
9 Months	18.89 (17)	20 (18)
12 Months	-	7.78(7)
Overall vaccination	60.00 (54)	40.00 (36)
Deworming of ducks		
3 Months	40.00(36)	7.78 (7)
6 Months	16.67 (15)	21.11 (19)
12 Months	-	5.56 (5)
Overall deworming	56.67 (51)	34.44 (31)

Table 2 presents the farmers' responses regarding disease prevalence and mortality rates in ducks, revealing notable regional differences between the Haor and Coastal areas. The most prevalent disease was Duck Plague, impacting 83.33% of flocks in the Haor regions and 90.00% in the Coastal regions. Duck Cholera exhibited a higher prevalence, affecting 30.00% of duck flocks in the Haor and 64.44% in the Coastal areas. Avian Influenza was less frequently reported, affecting 1.11% of flocks in the Haor and 3.33% in the Coastal regions. Other diseases were also reported, including Brooder Pneumonia and poisoning. Brooder Pneumonia affects 12.22% of duck farms in the Haor and 21.11% in the Coastal regions. Poisoning was observed only in the Coastal areas, affecting 2.22% of the flocks. The present finding indicates a need for targeted disease management strategies to address the specific challenges faced in the studied regions. The age of disease outbreak was a significant concern, with ducklings in the Coastal areas experiencing higher outbreaks reported by 71.11% of farmers compared to the Haor farmers at 33.33%. About 81.11% of the farmers in the Hoar areas reported higher outbreaks observed in growing ducks (8 to 16 weeks of age) as compared to 32.22% of the farmers in the Coastal regions. Adult ducks were less frequently affected in both regions, with 3.33% of farmers reporting outbreaks in the Haor and 18.89% in the Coastal areas. Seasonally, the majority of outbreaks occurred during the monsoon season (June to October) in the Haor regions mentioned by 77.78% of farmers, while more incidents were reported by farmers in the summer (46.66%) and winter (56.67%) in the Coastal areas.

Mortality rates also varied across regions and age groups. Among ducklings, the Coastal regions had a higher mortality rate as reported by 63.33% of farmers, compared to the Haor areas with 37.78% of farmers reporting so. In contrast, growers had a higher mortality rate reported by 50.00% of farmers in the Haor regions, compared to 28.89% of farmers in the Coastal areas. The feedback on adult mortality rates by farmers was relatively low, with 12.22% in the Haor and 7.78% in the Coastal regions. Regular excrement cleaning was more commonly practiced by 74.44 % of farmers in the Coastal region than in the Haor practiced only by 64.44% of farmers. Contact with wild birds was also more frequently reported by farmers in the Haor regions (32.22%) than those in the Coastal areas (6.67%).

Table 2. Farmers' responses on disease prevalence and mortality rates from July 2023 to June 2024 in Haor and Coastal regions, Bangladesh

Disease prevalence	Percentage (n)		Age wise disease prevalence	Farmers' response % (n)	
	Haor	Coastal		Haor	Coastal
Avian influenza	1.11 (1)	3.33 (3)	Duckling (below 8 weeks)	33.33 (30)	71.11 (64)
Duck cholera	30.00 (50)	64.44 (58)	Growing (8 to 16 weeks)	81.11 (73)	32.22 (29)
Duck plague	83.33 (75)	90.00 (81)	Adult (above 24 weeks)	3.33 (3)	18.89 (17)
Brooder pneumonia	12.22 (11)	21.11 (19)	**Season of incidence**		
Poisoning	-	2.22 (2)	Summer (March-June)	24.44 (22)	46.66 (42)
Others	8.89 (8)	2.22 (2)	Monsoon (June-October)	77.78 (70)	22.22 (20)
Mortality rate			Winter (November - February)	52.22 (47)	56.67 (51)
Ducklings	37.78 (34)	63.33 (57)	**Other parameters**		
Growers	50.00 (45)	28.89 (26)	Contact with wild bird	32.22 (29)	6.67 (6)
Adults	12.22 (11)	7.78(7)	Regular excrement cleaning	64.44 (58)	74.44 (67)

Biosecurity and duck farm management

The use of disinfectants during cleaning was notably low in both areas, with only 3.33% of Haor farmers and 1.11% of Coastal farmers practicing this method (Table 3). In the Haor region, 91.11% of farmers disposed of duck excreta by dumping, compared to 76.67% in the Coastal area. Using excreta as fertilizer was more common in the Coastal areas, practiced by 23.33% of the farmers, whereas only 8.89% of the farmers in the Haor regions adopted this method.

The findings revealed notable differences in sick birds and mortality management practices between the Haor and Coastal areas (Table 4). For sick bird management, a majority of farmers in both regions isolated sick ducks in separate sheds, with 56.67% in the Haor and 35.56% in the Coastal regions. However, 6.67% of Haor farmers and 2.22% of Coastal farmers kept sick birds in the same shed. Interestingly, 18.89% of Coastal farmers sold sick birds, while only 2.22% of Haor farmers did so. The slaughtering of sick birds by farmers was more common in the Coastal region (38.89%) than in the Haor region (34.44%). To manage dead ducks, 44.44% of Haor farmers and 43.33% of Coastal farmers buried dead ducks. Meanwhile, 36.67% of Coastal farmers disposed of dead ducks by throwing them into open fields, compared to 16.67% in the Haor regions. Additionally, 10.00% of Haor farmers burned the carcasses, while this practice was absent in the coastal regions.

Treatment facilities and vaccine sources

Farmers in both Haor and Coastal regions utilized a range of sources for duck health treatments, with veterinary hospitals being the primary facility, followed by pharmacies, quack practitioners, and self-treatment methods. Overall, 70% of farmers in Haor and 87.78% of farmers in the Coastal areas obtained treatment facilities for their ducks from the aforementioned sources (Table 5). The main source of vaccine was veterinary hospitals (44.44% in Haor and 47.78% in Coastal areas) as there was only limited availability of duck vaccines in the local market (with 5.56% both in the Haor and the Coastal areas).

Operational constraints regarding duck farming

Duck-rearing farmers faced different challenges in duck rearing, as outlined in Table 6. The most critical issue across both regions was the outbreak of diseases, cited by 66.67% of farmers in Haor and 87.78% in Coastal areas.

Reported diseases included duck plague, duck cholera, avian influenza, and brooder pneumonia. In the Haor areas, the second most frequently reported constraint was the high price of feed (63.33%), followed by lack of vaccines (50.00%), unavailability of ducklings (48.89%), lack of treatment facilities (47.78%), theft of ducks (32.22%), high mortality of ducklings/ducks (31.11%), lack of quality ducklings (30.00%), predator attacks (25.56%), high cost of ducklings (22.22%), and the risk of failing to obtain the desired profit (3.33%).

In contrast, farmers in the Coastal areas identified the lack of quality ducklings as the second major constraint (66.67%), as there were not enough hatcheries for incubation, hatching, and brooding. This was less of an issue in the Haor areas, where some hatcheries are locally available.

Other major constraints for Coastal farmers included the high mortality rate of ducklings/ducks (63.33%), high feed price (62.22%), lack of vaccine (43.33%), predatory animal attacks and profit uncertainty (30.00%), lack of treatment facilities (21.11%), high price of ducklings (17.78%), unavailability of ducklings year-round (12.22%), and theft (8.89%).

Farmers' demands and opinions regarding challenges in duck farming

To mitigate the mentioned operational challenges faced by the farmers, some opinions and demands obtained from policymakers are presented in Table 7. The reduction of feed prices and making it affordable for farmers were demanded by the highest number of farmers in both the Haor and the Coastal areas at 70.00% and 66.67%, respectively. As feed cost is the major cost in duck farming, it is urgently needed to make feed prices more affordable.

In the Haor regions, the second most common demands were ensuring the availability of quality ducklings and improving access to treatment facilities, both cited by 52.22% of respondents. These were closely followed by the adequate supply of vaccines (50.00%), training opportunities to improve farming skills (44.44%), and access to low-interest loans to support commercial duck farming initiatives (25.56%). In contrast, in the Coastal areas, the second most cited demand was for training and capacity-building programs, mentioned by 53.33% of farmers. The least prioritized issue in this region was the availability of low-interest loans, requested by only 5.56% of farmers (n = 5).

Table 3. Duck farm cleaning system in Haor and Coastal regions, Bangladesh from July 2023 to June 2024

Parameter	**Percentage (n)**	
	Haor	**Coastal**
Cleaning materials used		
Broom	81.11 (73)	80.00 (72)
Water and broom	15.56 (14)	18.89 (17)
Water, disinfectant, and broom	3.33 (3)	1.11 (1)
Excreta management method		
Dumping	91.11 (82)	76.67 (69)
Used as fertilizer	8.89 (8)	23.33 (21)

Table 4. Biosecurity and farm management from July 2023 to June 2024 in Haor and Coastal regions, Bangladesh

Parameters	**Percentage (n)**	
Sick duck management	**Haor**	**Coastal**
Kept in the same shed	6.67 (6)	2.22 (2)
Kept in separate sheds	56.67 (51)	35.56 (32)
Sold	2.22 (2)	18.89 (17)
Slaughtered	34.44 (31)	38.89 (39)
Death duck management		
Thrown into open fields	16.67 (15)	36.67 (33)
Buried	44.44 (40)	43.33 (39)
Burnt	10.00 (9)	-
Left in water	28.89 (26)	24.44 (18)

Table 5. Treatment facilities, vaccine source, and women's contribution from July 2023 to June 2024 in Haor and Coastal regions, Bangladesh

Parameters	**Percentage (n)**	
Source of treatment	**Haor**	**Coastal**
Veterinary hospital	44.44 (40)	47.78 (43)
Quack	4.44 (4)	21.11 (19)
Own	2.22 (2)	1.11 (1)
Veterinary pharmacy	18.89 (17)	17.79 (16)
Total treatment facility obtained	70.00 (63)	87.78 (79)
Source of vaccine		
Veterinary hospital	50.00 (45)	42.22 (38)
Market/company	5.56 (5)	5.56 (5)

Table 6. Operational constraints regarding duck farming in Haor and Coastal regions, Bangladesh

Farmers' constraints in duck rearing	**Haor percentage (n)**	**Ranking**	**Coastal percentage (n)**	**Ranking**
Disease outbreak	66.67 (60)	I	87.78 (79)	I
Higher feed price	63.33 (57)	II	62.22 (56)	IV
Lack of quality duckling	30.00 (27)	VIII	66.67 (60)	II
High mortality rate of ducklings/ducks	31.11 (28)	VII	63.33 (57)	III
Lack of vaccines	50.00 (45)	III	43.33 (39)	V
Lack of treatment facilities	47.78 (43)	V	21.11 (19)	VII
Unavailability of ducklings year-round	48.89 (44)	IV	12.22 (11)	IX
Attacks by predatory animals	25.56 (23)	IX	30.00 (27)	VI
Theft	32.22 (29)	VI	8.89 (8)	X
High prices of ducklings	22.22 (20)	X	17.78 (16)	VIII
high risk of obtaining the desired profit	3.33 (3)	XI	30.00 (27)	VI

Table 7. Farmers' demands and opinions regarding challenges in duck farming in the Haor and Coastal regions, Bangladesh

Farmers' opinions/demands	Haor percentage (n)	Coastal percentage (n)
Affordable feed prices	70.00 (63)	66.67 (60)
Sufficient training opportunities	44.44 (40)	53.33 (48)
Ensuring the availability of quality ducklings	52.22 (47)	44.44 (40)
Vaccine availability	50.00 (45)	43.33 (39)
Proper treatment facilities	52.22 (47)	36.67 (33)
Low-interest loan facilities for duck farming	25.56 (23)	5.56 (5)

DISCUSSION

The findings of the present study provide valuable insights into the health and disease management, biosecurity practices, operational challenges associated with duck farming, and the demands of farmers for duck farming in the Haor and Coastal areas of Bangladesh. These findings are consistent with earlier studies, while also offering updated and region-specific data. Previous research by Jha et al. (2015) revealed that approximately 30.50% of farmers in the Sylhet district did not follow a regular vaccination schedule. Interestingly, only 14.50% of farmers consistently adhered to a regular vaccination schedule. The vaccination profile was observed in 35% of cases, while vaccines were predominantly used only in commercial farms. According to Ahmed et al. (2021), 85% of small-scale farmers did not vaccinate their ducks, although around 50% were vaccinated against Duck Plague and 40% against Duck Cholera.

In the Coastal regions, Rahman et al. (2009) also noted low vaccination rates (14.5%), and Islam et al. (2023) observed similar practices in India, where a lack of vaccine availability and farmer training contributed to the absence of vaccination. The findings of the current study are closely similar to the reported data, as most of the farmers in the Haor and Coastal areas had poor knowledge of vaccination and deworming practices. Duck plague occurred in 8% of the farms, and other duck diseases were reported in 3.11% of the farms, as recorded by Khan et al. (2018) in the Haor region, which was lower than the current findings. Islam et al. (2016) observed that Duck Cholera affected 52% of flocks, Duck Plague 26%, limber neck poisoning 12%, and Avian Influenza 2%, which aligns with the present findings, although the farmers' perceptions of duck plague disease were much higher. Seasonality appears to influence disease outbreaks; Khan et al. (2018) noted a higher prevalence of duck plague during the summer, with 12.9% at the farm level and 5.82% at the flock level in the Haor areas. During the rainy season, 10.1% prevalence was observed at the farm level and 4.96% at the flock level, as compared to other seasons. Rahman et al. (2009) reported a maximum prevalence of 100% for duck cholera and duck plague in their study, with disease outbreaks most frequent in the summer (34.18%), followed by the rainy season (2.25%). Gosh et al. (2012) observed seasonal variations in the prevalence of duck plague and duck cholera in the southern coastal areas of Bangladesh: 13.3% in summer, 23.3% during the monsoon, and 63.3% in winter. Similarly, Debnath et al. (2020) identified duck plague and duck cholera as the most prevalent diseases in duck farms in India. These variations in prevalence rates can be attributed to differences in geographic location, duration of the studies, and data collection methods, including reliance on farmers' feedback from diverse socio-economic conditions**.** In the present study, young adult ducks in the Haor areas were more frequently affected by diseases, whereas ducklings were more susceptible in the coastal regions. Khan et al. (2018) similarly found higher disease outbreaks among young adult ducks in the Haor areas. In these regions, most farmers purchase ducklings from hatcheries or commercial farms and initially follow recommended guidelines. However, after a few weeks, they release the growing ducks into natural water bodies with minimal feed supplementation. Other causes include overcrowding, poor ventilation, temperature fluctuations, improper disposal of dead ducks into water bodies, contact with migratory birds, rising water levels during the rainy season, poor nutrition supplies for young ducks after proper management of ducklings, and the introduction of unvaccinated new flocks.

In the Coastal areas, poor brooding systems, inadequate vaccination practices, and management-related problems are key causes of disease outbreaks and high duckling mortality. However, ducks that survive to adulthood tend to adapt to harsh environmental conditions. Environmental challenges such as floods and low winter temperatures also impact duck health and agricultural productivity in the Haor regions (Ferdushi et al., 2019). Flash floods damage farms and pollute water sources, endangering duck health and safety. Sudden temperature changes, especially during cold winters, may also affect duck productivity and overall well-being. Furthermore, overcrowding due to high stocking densities can hinder growth, elevate stress levels, and increase the risk of disease among ducks. According to Delaporte and Maurel (2016), two-thirds of Bangladesh lies below 5 meters above mean sea level, making it highly vulnerable to riverine and rainwater flooding, and in coastal areas, to tidal flooding during storms. Nearly one-third of the country is susceptible to tidal inundation, and nearly 70% of the country is flooded during heavy monsoons (Delaporte and Maurel, 2016). Increased salinity in Coastal water bodies makes water unsuitable for ducks, reducing their productivity. Environmental changes also elevate the risk of disease outbreaks and duck mortality due to natural disasters such as floods and storm surges. Jha et al. (2015) found in the Haor area that 50.50% of farmers did not clean their farmhouses regularly, 35.50% maintained regular cleaning practices, and 14% never cleaned them at all. These findings align with the current study. In the context of biosecurity, regular cleaning is widely practiced; however, the use of disinfectants remains limited. Khan et al. (2018) found that only 3.73% of farmers regularly vaccinated their ducks, no farms used disinfectants, and only 0.3% practiced any form of biosecurity in the Haor areas. In the case of sick bird management, Agbolosu and Aawona (2021) reported that 24.1% of farmers slaughtered their sick ducks, 33% treated them, and 12.1% kept them in the flock till death. Practices such as isolation or proper burial were not followed, which was similarly noted by Khan et al. (2018) in the Haor areas. Jha et al. (2015) found that 18% of farmers in the Haor areas disposed of dead ducks outside, while Rahman et al. (2009) found that only 9.75% of farmers buried dead ducks; the remaining 90.25% disposed of them in open fields or water bodies such as ponds. These improper disposal methods pose environmental risks to humans, livestock, and poultry species. The biosecurity and disposal practices observed in the present study are consistent with these prior findings. Alam et al. (2012) also reported that an average of 71% of farmers relied on village doctors for veterinary services, while only 18% had the facility to consult directly with the nearest veterinary doctors. Moreover, 11% of farmers never sought veterinary advice for the treatment and medication of their diseased ducks.

In the present study, the challenges faced by the farmers varied across different geographical locations and specific parameters, though some key constraints were consistent in both regions. These findings align with Sheheli et al. (2023), who identified disease outbreaks as the primary constraint in duck farming. Currently, outbreaks of different duck diseases, such as duck plague, duck cholera, and avian influenza, have been reported to cause significant economic losses in duck production (Ajieh and Oyibojoba, 2018; Churchil and Jalaludeen, 2022; Sankaralingam and Mahanta, 2022). Such outbreaks lead to high mortality rates among ducks and ducklings, impair production performance, and ultimately reduce farm profitability in both study areas. Operational challenges, such as high feed prices, emerged as the second most significant constraint, consistent with the second study of Sheheli et al. (2023). Farmers in both areas complained about the escalating prices of feed, a concern also highlighted by Begum et al. (2020) and Zannat et al. (2018) in studies conducted in the North-Eastern Region and the Haor areas of Netrokona district in Bangladesh. Additionally, the scarcity of ducklings was identified as a critical issue, negatively affecting production and leading to economic losses. Inadequate veterinary service further exacerbated challenges, becoming particularly critical during disease outbreaks with high mortality rates. Other constraints, ranked in order of severity, included a lack of training and difficulties in protecting ducks from theft. It is important to note that the last problem ranked by the farmers was the risk of obtaining profit in the Haor areas, whereas theft was a more pressing concern in the Coastal areas. Roughly similar operational challenges have been documented in other duck farming regions, especially in the Haor areas (Kishoregonj district, Jaintiapur Upazila, Sylhet district), and southern Coastal areas of Bangladesh (Afrin et al., 2016; Ahmed et al., 2021; Sheheli et al., 2023). Vignesh et al. (2018) also reported comparable issues in Tamil Nadu, India, citing the unavailability of quality ducklings, non-availability of feed at affordable prices, diseases, high mortality, predatory animal attacks, theft, lack of veterinary services, and vaccination. Similarly, Wongtangtintharn et al. (2025) identified disease outbreaks and feed costs as key challenges in Thailand’s poultry industry. Ahmed et al. (2021) suggested several strategies to diminish the challenges faced by the farmers in duck farming, including farmer training programs, government investment in hatcheries, and monitoring for quality duckling production, adequate production and proper distribution of vaccines to ensure year-round availability and financial support such as the provision of low-interest loans. These recommendations align with the findings of the current study. Alam et al. (2012) conducted a study on the socio-economic profile of duck farmers and duck management practices in the Rajshahi region of Bangladesh and reported similar farmer demands and opinions, further supporting the present research.

CONCLUSION

The findings of the present study highlight several critical issues affecting duck farming in the Haor and Coastal areas of Bangladesh. Chief among them are poor vaccination and deworming practices, a high prevalence of Duck Plague and Duck Cholera, and deficiencies in biosecurity, treatment facilities, and modern farming knowledge. Due to these factors and challenges, farmers demanded several steps to improve overall management and duck farming practices. Considering these factors, it is essential to provide hands-on training on vaccination practices, waste management, disease control, biosecurity measures, disposal methods of dead ducks, and enhancement of treatment facilities in duck-rearing areas.

DECLARATIONS

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Author’s contributions

This study was carried out in collaboration among all authors. Syidul Islam conceptualized and designed the study and wrote the protocol and manuscript. Syidul Islam and Md. Ashraful Islam wrote the Methodology, completed the formal analysis, and wrote the manuscript. Md. Ashraful Islam and Sharmin Sultana helped in data collection. Md Sazedul Karim Sarker 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 the last version of the manuscript.

Availability of data and materials

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

Ethical considerations

All authors affirm compliance with the ethical standards for scientific research and publication as required by the Journal of the World's Poultry Research, including fabrication of data, double publication and submission, redundancy, plagiarism, consent to publication, and misconduct.

Competing interests

There is no conflict of interest regarding this article.

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External and Internal Quality Characteristics of Eggs Sourced in Supermarkets, General Dealers, and Vendors in Gaborone, Botswana

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ABSTRACT

Egg quality characteristics influence consumer acceptance and preference of one egg over another. Several factors that impact egg quality include storage before and after dispatch, rearing conditions, temperature, handling, diseases, and the age of the eggs. The present study evaluated internal and external quality traits of eggs sourced from supermarkets, general dealers, and vendors in Gaborone, Botswana. One hundred and twenty eggs (24 eggs per location) were sourced from four supermarkets, seven general dealers, and 10 vendors in Gaborone. Parameters measured were egg weight (g), length (mm), width (mm), average shell thickness (mm), shell weight (g), surface area (cm^2), volume (cm^3), shape index, Haugh unit (HU), and shell weight per unit surface area (SWUSA, mg/cm^2) of the eggs. The current results indicated that egg weight and surface area were the highest for eggs sourced from supermarkets. Heavier eggs correlated with better HU scores, indicating richer and denser yolk, while surface area plays a role in moisture loss and potential shell strength. Eggs purchased from supermarkets and general dealers had noticeably greater egg weights, egg volumes, shell percentages, and SWUSA. Eggs from supermarkets had the greatest egg content weight, whereas those purchased from vendors had the lowest. The HU was highest for supermarket eggs compared to other egg sources. It was observed that eggs bought from supermarkets had superior internal and external quality traits compared to those from general dealers and vendors. It was concluded that eggs from vendors had lower quality due to inadequate storage and cooling facilities compared to supermarkets and general dealers.

Keywords: External quality, Haugh unit, Internal quality, Supermarket, Vendor

INTRODUCTION

The functional properties of eggs are intriguing, and nutritionally fortified eggs, also referred to as nutrition-enriched or functional eggs, are one of the products that have experienced phenomenal growth worldwide in recent years (Mesías et al., 2011). Functional foods increase the quality of the human diet, lower the risk of developing certain chronic illnesses, and effectively and affordably promote public health, all of which support current health efforts (Tian et al., 2022). The demand for functional foods has increased over the years due to their ability to reduce the risk of certain diseases and address socio-demographic factors, such as the rise in life expectancy. The rising demand for functional foods among health-conscious consumers has motivated an innovation in the production of omega-3 eggs (Miranda et al., 2015).

When presented with new products that lack sufficient evidence to support their purchasing decisions, consumers typically adopt a conservative, risk-averse stance, although they are sensitive to new information regarding such products (McFadden and Huffman, 2017). Information is crucial in influencing how consumers perceive innovative products, as it raises awareness, disseminates knowledge, and shapes or alters a person's pre-existing attitude and ways of thinking (Rondoni et al., 2020).

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Over the past few decades, the egg industry has faced several challenges that have impacted the financial viability of eggs. Increased animal breeding and husbandry practices are raising societal concerns, as they are perceived to compromise animal well-being (Malone and Lusk, 2016; Montossi et al., 2018). For instance, most eggs produced globally come from cage-based systems, which present significant animal welfare concerns as hens are confined to facilities with little room to move around (Buller and Roe, 2014). Consumer preferences and real egg consumption are impacted by the growing number of human health conditions linked to the nutritional components of eggs, such as allergies (Loh and Tang, 2018) and excessive cholesterol (Zhu et al., 2018).

Consequently, the egg industry has responded to the critical issues and challenges, as well as the complex and growing consumer demand for sustainable and healthy food products (Grunert et al., 2014). These responses include among others bringing a wide range of new eggs to the market that differ in intrinsic and extrinsic attributes, such as organic, free-range, enriched eggs (Barnkob et al., 2020), and to create new ways to improve animal welfare criterion in egg farms, such as eliminating the practice of beak trimming (Hester and Shea-Moore, 2005) or avoiding male culling by employing the novel dual-purpose poultry system (Krautwald-Junghanns et al., 2018). Heng et al. (2013) reported that consumer polls indicated that environmental concerns are less important than animal welfare issues. Some studies have suggested a desire for more natural and animal-friendly egg production methods (Texeira et al., 2018).

Egg external and internal quality characteristics influence consumer behavior, such as the acceptance and preference for one egg over another (Venkatesh et al., 2019). Egg quality characteristics are crucial for the egg industry, as they influence grading, price, hatchability, chick weight, and consumer preferences, all of which are affected by the configuration of eggs (Kumar et al., 2022). Indicators of the exterior quality of eggs include egg size, shell color, breaking strength, shell deformation, shell weight, shell percentage, shell thickness, and ultrastructure (Roberts, 2004). Recently, Silva Neto et al. (2024) reported that the most used conventional parameters to evaluate egg quality include the Haugh unit (HU), the yolk color index, yolk and albumen ratios, and shell thickness and resistance. Several factors influence egg quality, including storage before and after dispatch, rearing conditions, temperature, handling, diseases, and egg age. Additionally, egg quality may be influenced by factors such as the hen's age, breed, induced molting, production system, nutritional status, and stressors, including heat stress. Other factors that influence egg quality are genetics, lighting, medications, diseases, and management practices (Ahmadi and Rahimi, 2011).

Storage conditions and ambient temperature can affect egg quality. Tabidi (2011) recommended that eggs can be preserved by refrigeration for a maximum of 30 days or storage at room temperature for no more than 15 days. According to the FAO (2003), the ideal temperature for storing eggs in the tropics is 13°C or lower, typically between 10°C and 13°C. Information about egg quality from different market segments in Botswana has not been documented. Egg quality challenges are common in summer due to elevated temperatures, which cause egg spoilage. Therefore, the present study evaluated the internal and external quality characteristics of eggs sourced from supermarkets, general dealers, and vendors in Gaborone, the capital city of Botswana, to ascertain if there was variation in egg quality from these egg segments.

MATERIALS AND METHODS

Ethical approval

No ethical approval was required as no animals were used in the experiment.

Study area

The study was conducted at the Meat Science Laboratory of the Department of Animal Science, Botswana University of Agriculture and Natural Resources (BUAN), Gaborone, Botswana. A total of 120 eggs (24 eggs per location) were purchased from vendors, general dealers, and supermarkets at five sites in Gaborone from January to February 2024 (two months). After purchase, eggs were stored at room temperature overnight, and measurements were performed the following day. Thereafter, eggs were individually evaluated using non-destructive and destructive methods.

Sample preparation

A total of 120 eggs were purchased from supermarkets, general dealers, and vendors in Gaborone (Table 1) and assessed for egg quality traits in the Meat Science Laboratory at BUAN. After purchase, eggs were stored at room temperature overnight until measurements including egg weight (g), shell thickness (mm), egg width (mm), egg length, egg contents weight (g), shell percentage and egg surface area (cm^2), egg volume (cm^3), egg shape index (%), shell weight per unit surface area (SWUSA, mg/cm²), and HU were performed the following day.

Table 1. The number of supermarkets, retailers, and vendors in Gaborone, Botswana, from which eggs were purchased

Category	Number
Supermarket	4
General Dealers	7
Vendors	10
Total	21

Data collection

External egg quality traits

Eggs were individually weighed using Adam's electronic scale sensitive to 0.01 g (Adam scale Pty Ltd, Gaborone, Botswana), and their weights were recorded. Thereafter, individual egg weights were combined, and the means computed. Egg length (mm) and width (mm) were measured using an electronic digital Vernier Caliper (Ingco, South Africa), sensitive to 0.01 mm. These measurements were used to calculate the egg shape index (ESI) and the egg volume (cm³). The ESI was estimated using the following equation.

ESI = egg width/egg length × 100 (Gwaza and Elkanah, 2017; Alkan, 2023).

Egg volume (EV) was calculated using Formula 1.

$EV = 0.51 \times L \times B^2$. (Formula 1)

L is the egg length, and b is the breadth (width) of the egg (Hoyt, 1979).

Internal egg quality traits

After measuring the external characteristics of each egg, the eggs were carefully broken individually using a scalpel to allow the passage of the albumen and the yolk without mixing their contents. Thereafter, the Vernier callipers and an electronic scale sensitive to 0.01 mm were used to determine shell thickness (mm) and weight (g) with intact membranes (Monira et al., 2003). The egg yolk and albumen were carefully separated and placed in separate Petri dishes and then individually weighed. After weighing each parameter, the Petri dishes were washed with clean water and wiped dry with a paper towel before the subsequent weighings.

The yolk diameter and height, albumen height and albumen diameter were measured using electronic callipers sensitive to 0.001 mm (Reddy et al., 1979). The yolk ratio, albumen ratio, and eggshell ratio were expressed as yolk weight/egg weight × 100, albumen weight/egg weight × 100, and eggshell weight /egg weight × 100, respectively (Yang and Luu, 2009; Alkan, 2023). Haugh unit was calculated using Formula 2.

HU = 100 log (H + 7.57-1.7W0.37) (Formula 2)

where H means albumen height (mm) while W means egg weight in grams (Altan et al., 1998).

Eggshells were washed under gentle running water to remove adhering albumen (Kul and Seker, 2004) and wiped with a paper towel to remove excessive moisture. Thereafter, shell thickness was measured using Vernier callipers sensitive to 0.01 mm (Carter, 1975). Two measurements from each of three regions (i.e., sharp end, equator, and broad/blunt end) were averaged to give three eggshell thickness values (Ehtesham and Chowdhury, 2002). The shell weight (mm) with intact membranes was carefully obtained and weighed using an electronic scale. The egg content weight (ECW) was obtained by subtracting the eggshell weight from the egg weight (Moreki, 2005; Phitsane, 2006). The egg surface area (cm^2) of each egg was calculated using the formula $3.9782W^{0.7056}$, where W is the egg weight in grams (Carter, 1975).

Statistical analysis

Data collected for the egg quality traits were subjected to analysis of variance (ANOVA) using the general linear model (GLM) procedures of SAS (version 9.2) (SAS, 2008). Duncan‘s multiple range test was used to test significant differences among the means.

RESULTS AND DISCUSSION

Data on egg quality characteristics (egg weight, shell percentage, shell thickness, ECW, egg volume, HU, albumen ratio, yolk ratio, ESI, ESA, and SWUSA) from supermarkets, general dealers, and vendors are presented in Table 2. The egg quality traits of supermarkets, general dealers, and vendors differed significantly ($p < 0.05$). Egg weight significantly differed ($p < 0.05$) among egg sources or marketing outlets (Table 2). Eggs from supermarkets were heavier ($p < 0.05$) than those from other sources, with vendors' eggs being lighter. The weight of eggs from supermarkets, general dealers, and vendors was 61.58 ± 0.70 g, 59.51 ± 0.54 g, and 56.97 ± 0.44 g, respectively.

Table 2. A comparison of least squares means and standard errors of egg quality traits from three retail levels in Gaborone, Botswana

Egg quality trait	**Supermarket**	**General dealer**	**Vendors**	**P-value**
Egg weight (g)	61.58 ± 0.70^{a}	59.51 ± 0.54^{b}	56.97 ± 0.44^{c}	< 0.05
Egg shape index (%)	74.00 ± 1.18^{b}	76.30 ± 0.90^{a}	73.55 ± 0.75^{b}	< 0.05
Shell weight (g)	8.33 ± 0.35^{a}	8.00 ± 0.27^{a}	5.96 ± 0.22^{b}	< 0.05
AST (mm)	1.68 ± 0.59^{b}	1.79 ± 0.45^{a}	1.22 ± 0.37^{c}	< 0.05
SHPCT (%)	13.51 ± 0.68^{a}	13.48 ± 0.52^{a}	10.64 ± 0.43^{b}	< 0.05
Egg content weight (g)	53.25 ± 0.87^{a}	51.51 ± 0.67^{ab}	51.00 ± 0.55^{b}	< 0.05
Egg volume (cm^3)	1060.05 ± 18.95^{a}	1090.25 ± 14.50^{a}	969.48 ± 11.99^{b}	< 0.05
ESA (cm^2)	72.83 ± 0.60^{a}	71.08 ± 0.46^{b}	68.89 ± 0.38^{c}	< 0.05
SWUSA (mg/cm^2)	114.30 ± 5.4^{a}	112.75 ± 4.19^{a}	87.50 ± 3.46^{b}	< 0.05
Haugh unit	85.90 ± 1.37^{a}	77.94 ± 1.16^{b}	74.52 ± 0.96^{b}	< 0.05
Albumen ratio (%)	56.10 ± 1.05^{b}	$53.60- \pm 0.06^{a}$	51.79 ± 1.86^{ab}	< 0.05
Yolk ratio (%)	32.33 ± 1.42^{b}	34.20 ± 1.09^{b}	41.02 ± 0.90^{a}	< 0.05

^{abc}Means that within a row that do not share common superscript letters differ significantly ($p < 0.05$). The ESA: Egg surface area, SHPCT: Shell percentage, SWUSA: Shell weight per unit surface area, AST: Average shell thickness.

Table 2 demonstrated that vendors had lower ($p < 0.05$) egg weights, probably due to moisture loss during storage and transportation to the market, resulting from a lack of a cold chain. The current results were not in line with those of Brito et al. (2020), who reported that open street market eggs had a higher egg weight ($p < 0.05$, 60.48 g) and the lowest shell percentage (9.23%) compared to supermarket eggs. The differences in egg weights might be related to the genetic make-up of the hens, age, management practices, and poor storage conditions (Vlčková et al., 2019). The current findings did not align with those of Tebesi et al. (2012), who observed an average egg weight of 42.03 g due to prolonged storage time. Previous studies by Brake et al. (1997) and Jones and Musgrove (2005) reported that prolonged storage of eggs led to decreases in egg weight. Tůmová et al. (2016) evaluated the interactions in performance and eggshell quality of Lohmann (LSL) and a traditional breed (the Czech hen), housed in conventional cages and reared on litter, and fed two levels of dietary calcium (3.5% *vs.* 3.0%). The authors found that an increase in dietary Ca resulted in an increase in egg weight in Czech hens housed in cages and LSL hens housed on the floor. Increased calcium intake leads to an increase in egg weight, shell thickness, and ESA. On the contrary, Roland and Bruant (1994) and An et al. (2016) found that dietary calcium had no significant effect on egg weight.

The ESI for the general dealers statistically differed ($p < 0.05$) from that of supermarkets and vendors. However, ESI for supermarkets and vendors was similar. Contrary to the present results, Venkatesh et al. (2019) indicated no significant ($p > 0.05$) differences in the ESI of eggs from wholesale, retailers, and interior vendors. Jayasena et al. (2012) assessed egg quality traits from the wholesale market in Sri Lanka and obtained an average ESI of 75.03, indicating that eggs had a normal shape. According to Duman et al. (2016), eggs with ESIs of <72, 72-76, and >76 are sharp, normal, and round, respectively. For Alkan (2023), the ESI of a standard egg ranges between 72 and 76, with an average of 74. The present results indicated that the eggs from wholesale, retailers, and interior vendors had similar normal shapes (ESI = 72-76).

The shell weight of eggs from supermarkets and general dealers was heavier ($p < 0.05$) than that of vendors. However, the shell weight for eggs sourced from supermarkets and general dealers was not significantly different ($p > 0.05$) from one another. The lower shell weight observed in vendors' eggs correlated with the smaller eggs sold in this market segment. The current result disagreed with Hussain et al. (2013), who indicated that the shell weight of indigenous chicken eggs was five to six grams. Farhad and Fariba (2011) observed that eggshell quality decreases as the hen ages, due to increased egg weight without a corresponding increase in calcium carbonate deposition on the shell. Shell thickness was statistically affected ($p < 0.05$) by egg sources. The shell thickness values for eggs from supermarkets, general dealers, and vendors were 1.68 ± 0.59 mm, 1.78 ± 0.45 mm, and 1.22 ± 0.37 mm, respectively. The present finding on shell thickness disagreed with Castilla et al. (2009), who reported eggshell thickness of 0.25 to 0.338 mm in the red-legged partridge. The difference could be ascribed to the species difference. In agreement with the current results, Venkatesh et al. (2019) observed that the shell thickness values of chicken eggs obtained from interior vendors were significantly lower ($p < 0.05$) than those from grocery shops.

In this study, shell percentage for eggs sourced from supermarkets and general dealers was higher ($p < 0.05$) than that of vendors (Table 2). However, the shell percentage for supermarkets and general dealers' eggs was not statistically significant ($p > 0.05$). The shell percentage for vendors, supermarkets, and general dealers' eggs was 10.64 ± 0.43%, 13.51 ± 0.68%, and 13.48 ± 0.51%, respectively. The lower shell percentage for eggs from vendors suggests that eggs sold in this market segment originated from older hens or hens fed diets deficient in certain nutrients, such as calcium and phosphorus. Peebles and Brake (1987) and Manyeula et al. (2021) stated that shell percentage decreases with the increasing age of the hen. Furthermore, as hens age, the quality of their shells declines, becoming thinner, thus impacting the eggs' ability to withstand breaking. Bovera et al. (2014) posited that this decrease in shell quality happens due to an increase in egg weight with the hen's age, and the shell weight failing to keep up with this growth. Several factors contribute to thinner shells, including high temperatures, age, poor nutrition, high water salinity, and diseases. The present results indicated that eggs sourced from vendors had poor shell quality ($p < 0.05$).

Sources of eggs significantly affected ECW ($p < 0.05$). The ECW for the supermarkets was significantly higher ($p < 0.05$) than that of vendors. However, the ECW for supermarkets and general dealers was similar. The ECW for eggs obtained from the supermarket, general dealers, and vendors was 53.25 ± 0.8 g, 51.51 ± 0.67 g, and 51.00 ± 0.55 g, respectively. The present findings disagreed with the study of Hussain et al. (2013), who reported an average ECW of 47.9 g. The egg volume values for the supermarket, general dealer, and vendor eggs were 1060.05 ± 18.95 cm^3, 1090.25 ± 14.50 cm^3, and 969.48 ± 11.99 cm^3, respectively. A significantly lower ($p < 0.05$) egg volume was observed from the vendors, whereas the highest was observed from supermarkets and general dealer sources. However, the egg volume for supermarkets and general dealers was similar. These results suggest that supermarkets and general dealers sold larger eggs, while vendors sold smaller eggs, due to their limited financial resources. Sedghia and Ghaderi (2023) reported that egg volume is a more reliable predictor of egg size.

Egg surface area differed significantly ($p < 0.05$) between egg sources. The ESA mean values for supermarkets, general dealers, and vendors' eggs were 72.83 ± 0.60 cm^2, 71.08 ± 0.46 cm^2, and 68.89 ± 0.38 cm^2, respectively. The supermarket eggs had a higher ESA ($p < 0.05$) than those from general dealers and vendors. Eggs from vendors had lower ESA. The ESA values in this study were similar to those reported by Rodríguez et al. (2016), who found ESA values ranging from 64.23 cm^2 to 71.71 cm^2. The SWUSA differed significantly ($p < 0.05$) among egg sources. The SWUSA values for supermarkets, general dealers, and vendors were 114.30 ± 5.48 mg/cm^2, 112.75 ± 4.19 mg/cm^2, and 87.50 ± 3.4 mg/cm^2, respectively. However, the highest ($p < 0.05$) SWUSA was observed in eggs sourced from vendors, while the lowest was observed in eggs from supermarkets and general dealers. An increase in egg weight could have contributed to a decrease in SWUSA. According to Alsobayel and Albadry (2011), the storage period causes significant increases ($p < 0.05$) in SWUSA.

In the present study, HU significantly ($p < 0.05$) differed among marketing outlets. The HU values for eggs from supermarkets, general dealers, and vendors were 85.90, 77.94, and 74.52, respectively. The higher HU value (85.90) indicated that supermarket eggs had superior quality to general dealer and vendor eggs, while the lowest quality was observed in the eggs sold by the vendors. Leandro et al. (2005) also indicated a lower HU value (44.91) for eggs sold in open street markets. USDA (2020) stated that an HU of 72 or higher indicates superior egg quality, suggesting that the eggs in the three market outlets in this study had good quality. The lower HU value observed in the eggs from general dealers and the vendors could be due to eggs being exposed to long storage and unfavorable storage conditions, or a lack of a cold chain. Cağlayan et al. (2009) reported that HU declines with lengthened storage time. Similarly, Moreki et al. (2023) observed that the HU for ostrich eggs decreased with the prolonged storage time. The current result disagreed with Bell et al. (2001), who indicated that the albumen quality of the brown shell eggs ranged from 62.8 to 71.5 HU. The findings of the present study demonstrated that an increase in egg weight results in an increase in HU.

Albumen ratio differed significantly ($p < 0.05$) between egg sources. The albumen ratio for eggs from supermarkets, general dealers, and vendors was 56.10 ± 1.05%, 53.60 ± 0.86%, and 51.79 ± 1.37%, respectively. The supermarkets' eggs had significantly higher ($p < 0.05$) albumen ratios than the general dealers' eggs. However, eggs sourced from vendors did not differ significantly ($p > 0.05$) from those sourced from supermarkets and general dealers in terms of albumen ratio. These results suggested that eggs sourced from supermarkets had better quality.

The present results were consistent with those of Brito et al. (2020), who observed that supermarket-sold eggs had higher albumen percentage levels than those sold in grocery stores. Conversely, Leandro et al. (2005) found no noteworthy variations in the albumen percentage among any facilities they assessed. Higher albumen values for supermarket eggs in the present study might be due to improved storage conditions.

The yolk ratio differed significantly ($p < 0.05$) between egg sources. The yolk ratios for supermarkets, general dealers, and vendors were 32.33 ± 1.42, 34.20 ± 1.09, and 41.02 ± 0.90, respectively. Eggs sourced from vendors had the highest yolk ratio ($p < 0.05$) compared to those from general dealers and supermarkets. The yolk ratio of chicken eggs in the present study was slightly lower than that reported by Cağlayan et al. (2009) for partridge eggs, which ranged from 34.01 to 36.82. However, the current results were inconsistent with Kgwatalala et al. (2013), who reported an average yolk ratio of 44.94 in helmeted Guinea fowl. The difference in yolk ratio values could be attributed to the fact that guinea fowl eggs were used in the study by Kgwatalala et al. (2013), whereas the present study used chicken eggs. The present study had higher yolk and albumen ratios, indicating the freshness of eggs. A lower yolk ratio might be due to high ambient temperatures and egg shaking during transportation from the farm to the market, thus causing deterioration of yolk quality. King'ori (2012) stated that egg quality parameters significantly decrease in the summer due to exposure of eggs to high temperatures.

Factors such as storage and transport affect egg quality (FAO, 2003; Tan et al., 2023). In the present study, eggs from vendors were not refrigerated, whereas eggs from general dealers might have been refrigerated. Supermarket eggs were stored under refrigeration. Eggs might have been transported to supermarkets and general dealers using refrigerated trucks, whereas vendors might have been supplied by small-scale farmers who lack access to a cold chain. However, the large-scale poultry farmers might have supplied eggs to vendors.

CONCLUSION

Based on the Haugh unit values, the quality of eggs from supermarkets was higher than that from general dealers and vendors. Vendors had the lowest egg quality compared to other market segments. It is concluded that eggs from vendors had lower quality due to a lack of a cold chain. As the sample size in the present study was small, a more extensive study with a larger sample size of eggs, alongside additional factors such as specific gravity, albumen viscosity, yolk color, vitelline membrane strength, elasticity, and egg solids, is necessary to explore egg quality in these crucial market segment for furthur studies.

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Authors' contributions

John Cassius Moreki conceived the idea and wrote the manuscript. Boineelo Katie Motiki collected and analysed data and also wrote the manuscript. Shame Bhawa and Freddy Manyeula helped with data interpretation and edited the manuscript. All authors checked and approved the last edition of the submitted article.

Competing interests

The authors declared that there are no competing interests.

Ethical considerations

All authors have examined ethical issues, including plagiarism, consent to publish, errors, data fabrication and/or deception, duplicate publication and/or submission, and redundancy.

Availability of data and materials

Data will be made available to the corresponding author upon request.

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Effects of Supplementation of Oregano Essential Oil on the Growth Efficiency and Blood Biochemical Parameters of Broiler Chickens

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ABSTRACT

Oregano essential oil (OEO) has emerged as a safe, effective, and bioactive additive, increasingly incorporated into feed formulations to enhance the growth performance and overall well-being of broiler chickens. This experiment aimed to investigate the consequences of the dietary supplementation of OEO regarding the growth efficiency, carcass characteristics, and blood biochemical profiles of broiler chickens. The 35-day feeding trial involved 200 unsexed Hubbard Classic broiler chickens with an average initial body weight of 42.79 g. The broiler chicks were randomly assigned to four experimental groups, each comprising five replicates of ten birds. The treatment groups were fed basal diets supplemented with 300 mg/kg (OEO_{-1}), 400 mg/kg (OEO_{-2}), and 500 mg/kg (OEO_{-3}) of OEO, while the untreated group (OEO_{-0}) was fed a basal diet without any additives. Weekly assessments of growth performance metrics were conducted for 5 weeks, and blood parameters were examined once on day 35. The results revealed that OEO_{-3} treatment had a significant impact on the body weight, feed intake, and feed conversion ratio of broilers. A considerably increased dressing percentage was observed in the OEO_{-3} group. The findings indicated that OEO-supplemented groups significantly influenced both high-density lipoprotein (HDL) and low-density lipoprotein (LDL) levels, especially OEO_{-3}, which showed higher HDL and lower LDL levels compared to other treatment and control groups. However, no significant effect was observed on total blood cholesterol and triglyceride concentrations in the experimental broilers. Incorporating OEO into the diet suggested that the higher doses (500 mg/kg) enhanced growth efficiency, increased HDL cholesterol, and decreased LDL and triglycerides in the blood of broiler chickens.

Keywords: Blood parameter, Broiler chicken, Feed conversion ratio, Growth efficiency, Oregano oil

INTRODUCTION

Poultry production is a key component of animal husbandry, which is facing new challenges and requires innovative strategies to maintain sustainability (El-Hack et al., 2022). Regardless of age or religion, chicken meat is a widely accessible and affordable animal protein, favored across all social classes. Meat and eggs from poultry are essential components of the human diet (Regar et al., 2019). To increase productivity and prevent disease, the majority of Bangladesh's rural chicken producers regularly use antibiotics on everything from day-old chicks to market-ready chickens (Haque et al., 2020). Nonetheless, the extensive application of antibiotic growth promoters (AGP) contributes to antibiotic resistance as well as the residues of antibiotics in animal-derived products. These antibiotic residues pollute the natural environment through excrement and feces, which pose a hazard to community health security (Suresh et al., 2018; Chen et al., 2022). Due to health concerns, the application of antibiotics to promote growth in the animal breeding sector has already been outlawed, and in many countries, using antibiotics as a growth promoter is forbidden.

Drug-resistant bacteria may proliferate as a result of the protracted and unchecked abuse of antibiotics in chicken farms (Simitzis, 2017; Egbule, 2022). But

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customers now demand healthier alternatives to traditionally raised broiler meat, which has driven a global transition towards antibiotic-free broiler meat production (Haque et al., 2020). Since the widespread acceptance of broiler meat as a healthier substitute, which contains lower fat and higher protein than other meats, as well as its economic value, it has high demand among consumers. In addition to advancements in genetics and management, numerous feed additives have been used to increase productivity and other qualities to meet the demands of the broiler sector (Hussein et al., 2020). The use of essential oils has been demonstrated to be an efficient and promising alternative to antibiotics for both pre- and post-harvest antibacterial methods (Micciche et al., 2019). According to the study by Nehme et al. (2021), plant-based essential oils are beneficial for the physical state of pigs (Chen et al., 2024), chickens (Gopi et al., 2014), with large/small ruminants, such as cattle and goats (Wells, 2023). It is known that the primary ingredients of Oregano Essential Oil (OEO), extracted from origanum plants, are thymol and carvacrol (Oniga et al., 2018). When given to broiler chickens, oregano oil has been demonstrated to possess antimicrobial, antioxidant, antiviral, anti-parasitic, and immune-modulatory properties (Alagawany et al., 2018). Moreover, oregano may enhance productivity (Ding et al., 2020) and stimulate the immune system (Rashidian et al., 2021). The aromatic plant oregano (*Origanum spp.)* has been used in poultry feed in place of pharmaceutical antibiotics due to its abundance of active ingredients such as carvacrol, thymol, rosmarinic acid, flavonoids, terpenes, and caffeic acid, among others (Oniga et al., 2018). Several studies demonstrated that supplementing oregano essential oils to chicken diets increases body weight and feed conversion ratio, enhances digestion, reduces disease incidence, boosts productive performance, and diminishes economic loss (Alagawany et al., 2018). Recently, many efforts have been devoted to studying the cholesterol-decreasing function of essential oil or plant-based extracts (Cross et al., 2003) along with their immune stimulatory effects (El-Faham et al., 2015). The present study lies in its investigation of OEO as a potential growth promoter in the dietary supplementation for poultry, especially in the production of broiler chicken. Therefore, this study was designed to evaluate the effects of OEO on the growth performance and blood biochemical indicators of broiler chickens.

MATERIALS AND METHODS

Ethical approval

All the chicks used in this experiment were treated and managed under the rules outlined by the Bangladesh Veterinary Council Act 2019, Government of the People's Republic of Bangladesh. Broiler care instructions and use regulations established by the institutions and countries have been followed strictly. All precautionary measures were taken into consideration to reduce pain and distress during the experimental period.

Experimental birds' selection

In the present study, a total of 200-day-old, unsexed broiler chicks (Hubbard Classic, with a mean initial body weight of 42.79 g) were utilized, which were purchased from a commercial hatchery in the Gazipur district of Bangladesh. Strict selection criteria were implemented prior to purchase to ensure consistent body size and the absence of visible deformities. These criteria included evaluation of physical characteristics, such as feather condition, leg structure, and general alertness of chickens.

Study location and experimental framework

The experimental poultry house of the Department of Poultry Science, Khulna Agricultural University, Khulna, Bangladesh, was utilized to conduct the feeding trial. Firstly, all the chicks were weighed, and then they were randomly allocated to four feeding treatments, each with five replicates, each containing 10 broiler chicks, using a completely randomized design (CRD). Figure 1 shows how the experiment is designed. The following four treatment groups were included in the experimental design. In the first group, chickens were fed a basal diet without any supplement (OEO_{-0}). In the second group, chickens were fed a basal diet supplemented with 300 mg/kg of oregano essential oil (OEO_{-1}). While in the third group, chickens were fed a basal diet supplemented with 400 mg/kg of oregano essential oil (OEO_{-2}). Finally, in the fourth group, chickens were fed a basal diet supplemented with 500 mg/kg of oregano essential oil (OEO_{-3}).

Housing and management of chicks

The experimental chickens were housed in an open-sided broiler rearing facility with rice husks used as bedding on a deep litter floor system. Following the manufacturer's guidelines, a phenyl solution was used to clean and disinfect housing areas, feeders, drinkers, heaters, and other relevant equipment. During the first week, the brooding temperature was maintained at 33°C, after which it was reduced by 2°C per week until it reached 24°C at the end of the trial. A relative humidity of 65% was regulated throughout the farm. Continuous lighting was provided for 24 hours a day during the entire experimental period. As a preventive measure, all chickens were administered the recommended vaccinations against Newcastle Disease (ND) on days 5 and 22, while chickens received the routine vaccination against Infectious Bursal Disease (IBD) on days 10 and 17, and these vaccines are produced in Bangladesh. Throughout the investigation, the chickens were managed according to standard husbandry practices following the breeder's instructions.

Experimental diets

The birds were fed with a mix bought from the nearby market, primarily consisting of corn and soybean meal, along with other ingredients. A starter diet, containing 22.08% crude protein and 3003.82 kcal ME/kg, was given during the first 14 days of the trial, and a ration for growing chicks was provided from day 15 to day 35, containing 21.05% crude protein and 3102.32 kcal ME/kg. The experimental diets were free from growth promoters, antibiotics, or anti-coccidial drugs. Throughout the study period, chickens had ad libitum access to fresh and clean water. The ingredients, compositions, and nutrient profiles of basal diets were formulated to meet the nutritional requirements at the growing stages and are presented in Table 1. The proximate composition of the basal diet was formulated according to AOAC (1990). All chickens were allowed unrestricted access to feed and water during the trial.

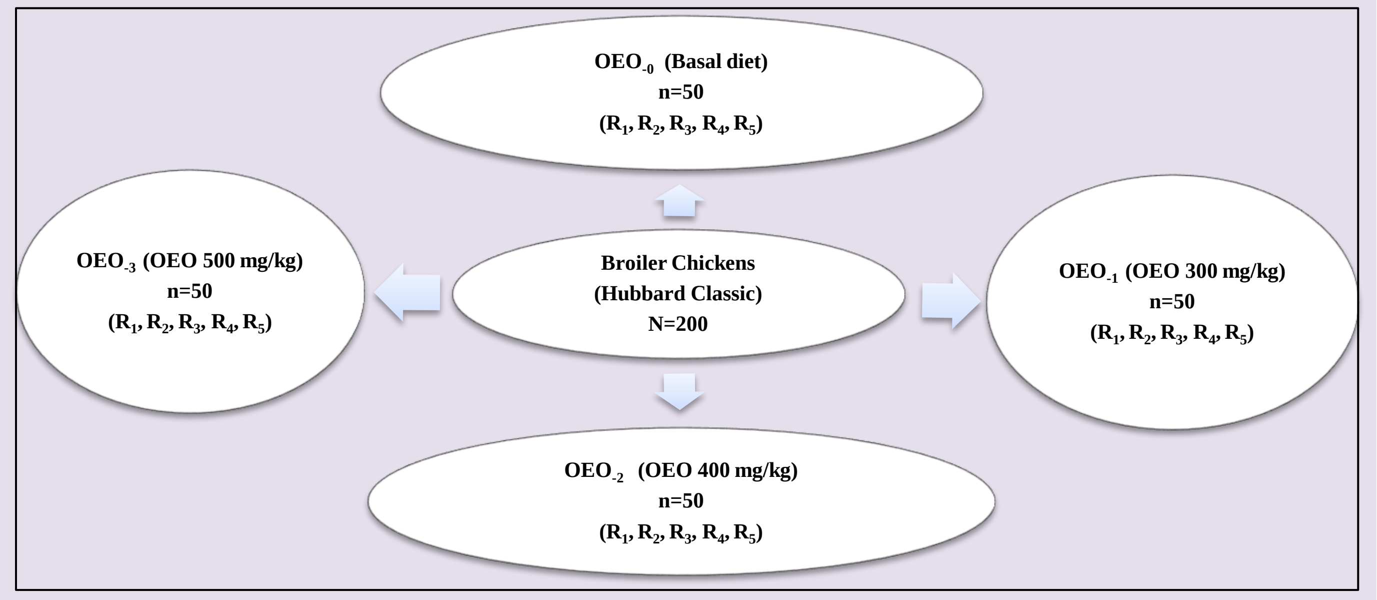

Figure 1. An overview of the experimental design with the Hubbard Classic breed of broiler chicken from September 2022 to June 2023.
***** OEO$_{-0}$: Basal diet; OEO$_{-1}$: Oregano essential oil (300 mg/kg); OEO$_{-2}$: Oregano essential oil (400 mg/kg); OEO$_{-3}$: Oregano essential oil (500 mg/kg); mg: Unit at milligram; kg: Kilogram**;** R: Replicates; n: Number of experimental chickens; N: Total number of experimental samples

Table 1. Feed ingredients and compositions of nutrients supplied to broiler chickens

Ingredients	**Starter (0-14 days) (%)**	**Grower (15-35 days) (%)**	**Ingredients**	**Starter (0-14 days) (%)**	**Grower (15-35 days) (%)**
Maize	57.80	58.00	Meat and bone meal	3.00	2.40
Wheat bran	0.00	2.26	Fish meal	0.60	1.00
Rice polish	1.80	2.00	Limestone	0.80	0.90
Full-Fat Soybean	2.00	2.00	Di-calcium phosphate	0.49	0.10
Vegetable oil	2.00	3.00	Common salt	0.25	0.20
Molasses	1.00	0.70	Vitamin mineral premix	0.20	0.01
Soybean meal	28.00	26.20	L-Lysine	0.02	0.01
Protein concentrate	2.00	1.20	L-Methionine	0.03	0.01
Antioxidant	0.01	0.01	Total metabolic energy (kcal/kg)	3003.82	3102.32
Calculated composition (%)					
CP (Crude Protein)	22.08	21.05	Ash	4.28	4.14
CF (Crude Fat)	3.54	3.72	Lysine	1.33	1.24
EE (Ether Extract)	5.39	6.36	Methionine	0.35	0.33
Available phosphorus	0.78	0.69	Calcium	1.08	0.92

*CP: Crude protein; CF: Crude fiber; EE: Ether extract

Experimental essential oil

The oregano essential oil (*Origanum vulgare)* utilized in this study was sold as Orego-Stim$^{®}$ powder. It is made up of more than thirty chemicals in a complex and natural matrix. It was purchased from the local market. Carvacrol (82%) and thymol (2.4%) are the two phenolic chemicals that account for over 85% of the makeup (Calislar et al., 2009).

Growth performance parameters

As soon as the day-old broiler chickens arrived at the experimental farm, each bird was weighed separately, and their weight was recorded. Thereafter, until the completion of the experiment, weekly records of body weight (BW), body weight gain (BWG), and feed intake (FI) in grams (g) were made in order to determine the feed conversion ratio (FCR) for each replicate within each treatment.

Body weight

To evaluate the effects of diet on body weight and growth patterns, the broilers were weighed at the start of the experiment and then weekly, in the morning before being given access to feed or water.

Feed intake

The daily feed consumption per chick was calculated by subtracting the weight of the leftover feed after 24 hours from the amount of initially provided feed, then dividing the difference by the total number of chickens in each group. The supplied feed weight was taken in the morning, and the leftovers were measured at night.

$$\text{Feed Intake (FI)} = \frac{\text{Weight of feed supplied (g) - Weight of leftover feed (g)}}{\text{Number of chickens in each group}}$$

Feed conversion ratio

The feed conversion ratio (FCR) was calculated by dividing the chickens' total weekly feed consumption (g) by their corresponding body weight gain (g). Improved efficiency is indicated by a decreased FCR, which means that birds need less feed to achieve one unit of body weight. The following formula by Dissanayake and David (2017) was used for calculating the FCR in Broiler chickens:

$$\text{FCR} = \frac{\text{Feed intake (g) / Chicken/week}}{\text{Body weight (g) / Chicken/week}}$$

Body weight gain

The body weight gain of the broiler was calculated by finding the difference between the final and initial body weight at a specific period of time.

Carcass characteristics

Five chickens per treatment were randomly selected at the end of the trial and fasted for 12 hours before slaughter. Studies have indicated that a fasting period of 8 to 12 hours is ideal for reducing the risk of contamination while maintaining carcass yield (Schneider and Gewehr, 2023). Slaughter was performed using the Halal method, following local religious practices. The carcass dressing percentage was then calculated using the following formulas provided by Wu et al. (2020).

$$\text{Dressing percentage} = \frac{\text{Carcass weight (g) / bird}}{\text{Live body weight (g) / bird}} \text{ x } 100$$

Analysis of blood biochemical parameters

By the end of the 35-day experiment, a sterile plastic syringe (5 ml) was used to aseptically collect 3 ml of blood from the wing veins of five randomly selected broilers in each group. To extract the serum, the blood samples were centrifuged immediately for 20 minutes at a velocity of 3000 rpm. Following that, the separated serum samples were transferred to 1.5 ml cryovial tubes (red top) and stored at -20 °C until they were analyzed. Total cholesterol, HDL, LDL, and triglyceride concentrations in the serum were assessed using commercially available standard kits (BioMereux, France) and an automatic analyzer (Humalyzer 300, Merck$^{®}$, Germany), following the guidelines provided by the manufacturer.

Statistical analysis

All the performance data were entered into a spreadsheet program of Microsoft Office Excel 2010. Using the Statistical Package for the Social Sciences (SPSS) Version 26, the data management and analysis were performed using one-way analysis of variance (ANOVA). To ascertain whether the treatment variations were significant, Duncan's Multiple Range Test was employed. The Means±Standard Error of the Means (M±SEM) were used to express the results. If the probability Value was less than 0.05 ($p < 0.05$), it was considered statistically significant.

RESULTS

The impact of adding OEO to the diet on the growth efficiency measures of broiler chickens is displayed in Table 2. The results of this investigation demonstrated that oregano essential oil (OEO) decreased the feed conversion ratio (FCR) of broiler chicks and improved growth efficiency compared to the control group ($p < 0.05$).

Throughout the experiment, broiler chickens fed varying amounts of dietary OEO exhibited a significant increase in feed intake compared to the control group on days 14, 21, 28, and 35 ($p < 0.05$). Notably, chickens supplemented with 500 mg/kg of OEO consumed significantly more feed than birds in all other dietary treatments, including the control group.

Table 2. Effects of dietary supplementation of oregano essential oil on the growth performance parameters of broiler chickens

Age (weeks)	Dietary treatments (Mean ± Standard Error of the Mean)				P-Value
	OEO$_{-0}$	**OEO$_{-1}$**	**OEO$_{-2}$**	**OEO$_{-3}$**	
Feed intake (g)					
1	184.51^{a}±4.82	196.52^{a}±4.20	233.54^{b}±6.18	219.86^{b}±5.16	<0.01
2	546.32^{a}±8.29	578.72^{a}±10.20	613.15^{b}±14.03	629.76^{b}±9.95	<0.01
3	1132.31±18.26	1180.75±21.50	1228.07±31.87	1237.91±36.70	0.062
4	2048.79^{a}±19.77	2114.35^{ab}±42.61	2145.66^{b}±27.14	2195.16^{b}±22.93	0.020
5	2590.33^{a}±16.72	2679.84^{b}±28.23	2723.22^{b}±36.12	2753.17^{b}±23.68	0.004
Body weight (g)					
1	197.31^{a}±2.61	238.30^{bc}±3.96	234.93^{b}±3.66	247.56^{c}±4.85	<0.01
2	494.84^{a}±6.98	541.35^{b}±9.06	576.13^{c}±8.08	592.56^{c}±7.98	<0.01
3	930.21^{a}±18.54	1038.67^{b}±20.83	1051.88^{b}±28.95	1085.57^{b}±14.40	<0.01
4	1387.81^{a}±14.00	1473.28^{b}±21.31	1527.18^{b}±31.70	1548.13^{b}±26.93	<0.01
5	1558.61^{a}±21.53	1677.60^{b}±14.01	1734.79^{bc}±15.28	1775.83^{c}±27.43	<0.01
Dressing yield (%)	69.76^{a}±0.73	71.36^{a}±0.80	71.87^{a}±0.72	74.77^{b}±1.35	0.013
Body weight gain (g)					
1	154.52^{a}±2.61	195.51^{bc}±3.96	192.14^{b}±3.66	204.77^{c}±4.85	<0.01
2	297.53^{a}±5.48	303.05^{a}±6.49	341.20^{b}±5.97	344.99^{b}±7.43	<0.01
3	435.36±24.94	497.32±22.90	475.74±34.04	493.01±20.59	0.352
4	457.59±14.84	434.61±11.90	475.30±14.74	462.55±38.17	0.645
5	170.80±23.15	204.31±17.14	207.61±37.95	227.70±48.84	0.702
Feed conversion ratio					
1	0.94^{bc}±0.036	0.82^{a}±0.025	0.99^{c}±0.030	0.89^{ab}±0.032	0.011
2	1.10±0.012	1.07±0.030	1.06±0.032	1.06±0.012	0.654
3	1.21±0.033	1.13±0.019	1.17±0.053	1.14±0.029	0.389
4	1.47±0.024	1.43±0.042	1.40±0.020	1.42±0.026	0.423
5	1.66^{b}±0.027	1.59^{ab}±0.018	1.56^{a}±0.021	1.55^{a}±0.021	0.014

* OEO$_{-0}$: Basal diet; OEO$_{-1}$: Oregano essential oil (300 mg/kg); OEO$_{-2}$: Oregano essential oil (400 mg/kg); OEO$_{-3}$: Oregano essential oil (500 mg/kg); g: Unit at gram; ^{a,b,c} Means within the same row with different superscript letters are significantly different ($p < 0.05$).

The results of this investigation demonstrated that the OEO decreased the FCR of broiler chicks and improved the growth efficiency compared to the control group ($p < 0.05$). Throughout the experiment, broiler chickens fed varying amounts of dietary OEO exhibited a significant increase ($p < 0.05$) in feed intake in comparison to the control group on days 14, 21, 28, and 35. Notably, chickens supplemented with 500 mg/kg of OEO consumed significantly more feed than birds in all other dietary treatments, including the control group. The body weight of the experimental birds is shown in Table 2. At the end of 35-day experiment, the group OEO$_{-3}$ receiving 500 mg/kg of oregano essential oil in their diet exhibited a considerably higher final body weight compared to the control group, followed by the groups OEO$_{-2}$ and OEO$_{-1}$ that received 400 mg/kg and 300 mg/kg of OEO in their diets, respectively. On the other hand, the treatment with OEO$_{-3}$ showed the highest weekly body weight gain of 493.01±20.59 g during 3rd week of age in broiler chicken, followed by OEO$_{-2}$ (475.74±34.04 g) and OEO$_{-1}$ (497.32±22.90 g). However, in the control group (OEO$_{-0}$), the highest average body weight gain of 457.59±14.84 g was found at the 4th week of age. An increasing trend in body weight gain at 1st, 2nd, and 3rd weeks was observed in all treatments (OEO$_{-3}$, OEO$_{-2}$, and OEO$_{-1}$) and also in the control group (OEO$_{-0}$). Additionally, the significant differences ($p < 0.01$) were observed only in terms of weekly average body weight gain (g) at the 1st and 2nd weeks of age in broiler chickens. At the 3rd, 4th, and 5th weeks of age, no significant differences were observed between treatment and control groups, indicating body weight gains across all dietary treatments by the later weeks (3rd to 5th) neither increased nor decreased remarkably. Furthermore, Table 2 demonstrates the impact of adding OEO to the diet and the FCR of broiler chickens. The findings of the current study indicated that the feed conversion ratio (FCR) of broiler chicken from day 1 to 35 was significantly reduced ($p < 0.05$) in the oregano essential oil (OEO) treated groups compared to the control group. However, chickens in the treatment OEO_{-3} group (500 mg/kg) exhibited the lowest FCR at day 35 compared to both the control (OEO_{-0}) and other OEO supplemented groups. Additionally, the data presented in the table showed that average feed intake was higher in the OEO supplemented chickens compared to the control group. In the control group, the feed intake was 184.51 g in the first week, which was increased to 219.86 g in the OEO_{-3} treatment group. In the last week, the average feed intake was 2590.33 g in the control group, followed by 2679.84 g in OEO_{-1}, 2723.22 g in OEO_{-2}, and 2753.17 g in the OEO_{-3} group. The chicken's body weight was 197 g at the first week and 1558.61 g at the fifth week of age in the control group of chickens, whereas in the treatment group OEO_{-1}, it was about 238.30 g and 1677.60 g in the first and fifth week, respectively. In addition to this, in the OEO_{-2} group, the growing weight of the broiler was 247.56 g and 1775.83 g recorded during the first and fifth week of age. Additionally, the OEO_{-3} treatment group showed the best growth performance in comparison to the control, OEO_{-1}, and OEO_{-2} groups. Regarding feed conversion ratio (FCR), the lowest value was observed in the OEO_{-3} group (1.55), and the highest FCR (1.66) was found in the control group. The highest dressing percentage was found in the OEO_{-3} group (74.77%), followed by OEO_{-2} (71.87%), OEO_{-1} (71.36%), and the control group (69.76%). Some of the blood biochemical parameters are displayed in Table 3. Based on the data from this investigation, OEO was not shown to have any influence on total cholesterol ($p > 0.05$). In contrast with the control group, the total serum cholesterol concentration was up in the OEO_{-2} and OEO_{-3} treatment, whereas it was decreased in the OEO_{-1} group. The total HDL cholesterol level was lowest in the control group (63.74 mg/dl) and highest in the OEO_{-3} group (77.52 mg/dl). LDL levels decreased from 74.85 mg/dl in the control group to 59.19 mg/dl in the OEO_{-3} group. In addition, the triglyceride level also declined in the treatment group at 41.69 mg/dl in OEO_{-1}, 38.27 mg/dl in OEO_{-2}, and 37.18 mg/dl in OEO_{-3}, whereas in the control group, it was about 44.25 mg/dl. In the present study, the overall cholesterol level was 131.86 mg/dl in the control group and 133.28 mg /dl in the treatment group OEO_{-3}. Furthermore, broilers supplemented with oregano essential oil (OEO) showed a significant increase in HDL levels compared to the control group at 35 days of age ($p < 0.05$). On the other hand, supplementing the diet with OEO caused a substantial reduction in LDL in comparison to control chickens ($p < 0.05$). However, the inclusion of different doses of OEO to the regular diet decreased the serum triglyceride levels, indicating the increased triglycerides (mg/dl) levels at OEO_{-1} (300 mg/kg) and gradually decreased in OEO_{-2} (400 mg/kg) and OEO_{-3} (500 mg/kg) groups. Although these decreasing levels were not statistically significant compared to the control group ($p > 0.05$).

Table 3. Effects of oregano essential oil supplementation in the diet of broiler chickens on the blood biochemical parameters at 35 days of age

Blood parameters	**Dietary treatments (Mean ± Standard Error of the Mean (SEM))**				**P-value**
	OEO_{-0}	**OEO_{-1}**	**OEO_{-2}**	**OEO_{-3}**	
Total cholesterol (mg/dl)	131.86±5.19	126.89±4.85	137.06±8.67	133.28±7.61	0.765
HDL (mg/dl)	63.74^{a}±3.39	68.65^{a}±2.10	71.37^{ab}±2.38	77.52^{b}±2.94	0.020
LDL (mg/dl)	74.85^{b}±2.88	67.45^{ab}±2.60	65.54^{ab}±4.03	59.19^{a}±1.96	0.015
Triglycerides (mg/dl)	44.25±1.50	41.69±2.49	38.27±1.86	37.18±3.36	0.188

OEO_{-0}: Basal diet; OEO_{-1}: Oregano essential oil (300 mg/kg); OEO_{-2}: Oregano essential oil (400 mg/kg); OEO_{-3}: Oregano essential oil (500 mg/kg); HDL: High density lipoprotein; LDL: Low density lipoprotein; mg/dl: Milligrams per deciliter $*^{a,b}$ indicates within the same row with different superscript letters are significantly different ($p < 0.05$).

DISCUSSION

In the current study, broiler performance was demonstrated by a considerable gain in BW and a decline in FCR following dietary OEO supplementation. Numerous studies have reported that oregano extract improves the growth efficiency of broiler chickens, consistent with the outputs of the present investigation (Amer et al., 2021; Irawan et al., 2021; Zhang et al., 2021). When compared to control chickens, Roofchaee et al. (2011) reported that broiler diets supplemented with 600 mg/kg of oregano during the grower stage considerably boosted ($p < 0.05$) body weight gain. In the opinion of Zaazaa et al. (2022) and Zhang et al. (2023), the broilers treated with 350 mg/kg and 700 mg/kg of oregano oil had the lowest FCR and considerably higher BW than the control group. When broiler chickens were treated with a mixture of oregano and thyme essential oil at 100+100 mg/kg, 200+200 mg/kg, and 300+300 mg/kg of diets, they showed increased body weight and reduced Feed Conversion Ratio (FCR) (Razaq et al., 2023). At the seventh week of life, broilers supplemented with 6% oregano had the heaviest BW, while broilers without the supplementation had the lowest BW (Marcos et al., 2020). Similarly, broilers that were given a supplement of EO at a dosage of 300-600 mg/kg of feed demonstrated a significant improvement in FCR, daily body weight gain, and average daily feed consumption (Peng et al., 2016). These findings support the results of the present study. Jamroz et al. (2005) demonstrated that supplementing the diet with OEO greatly enhanced the digestive system's chymotrypsin activity and improved crude protein digestibility. This, in turn, stimulates the digestive system to produce more digestive enzymes (Ciftci et al., 2005). Zhang et al. (2021) state that oregano essential oils act as an antioxidant, enhance intestinal health through improving gut micro flora and boosting the immune status, which are the major factors for improving the growth performance of broilers. Nevertheless, a few contradictory studies revealed that the incorporation of OEO as a dietary supplement had no appreciable impact on the broiler chicks' growth performance (Avila-Ramos et al., 2012; Kirkpinar et al., 2014; Hernandez-Coronado et al., 2019).

In the current investigation, the supplementation of OEO in the broiler feed did not have an impact on blood cholesterol concentrations. However, the inclusion of various levels of oregano essential oils in the diet decreased the serum triglyceride levels at 35 days of age. The study on poultry has yielded comparable findings, indicating that serum triglyceride concentrations decrease when oregano oil concentration is increased to 200 mg/kg (Mendoza-Ordonez et al., 2020). According to Kolodziej-Skalska et al. (2022), serum triglyceride and cholesterol concentrations decreased when they were supplemented with different levels of oregano oil. The primary constituent of oregano oil is carvacrol, which lowers plasma triglycerides (Lee et al., 2003). One probable explanation is that the functional chemicals in oregano essential oil influence lipid metabolism systems, as Abo Ghanima et al. (2020) ascribe one of the bioactive constituents in oregano essential oil, thymol, to reduce the synthesis of cholesterol. According to Moghrovyan et al. (2019), the plasma HDL concentrations of birds fed OEO were considerably higher ($p < 0.05$) compared to the control chickens. Flavonoids are additional chemical components found in OEO, just like essential oils. In comparison with results from the current study, Mendez-Zamora et al. (2017) observed that 400 mg of Mexican oregano oil/kg of feed raised the HDL and LDL levels in broiler blood. Haryanto et al. (2016), who fed broilers banana peel meal as a supplement, reported that the flavonoids derived from OEO increased the apolipoprotein A_1 production, which could be one reason for an increase in HDL. However, according to Kirkpinar et al. (2011), oregano oil in the diet did not affect the blood triglycerides or cholesterol of broiler chickens. Different inclusion rates, feeding schedules, or essential oil manufacturing techniques could be the cause of variations in cholesterol levels between the present study and earlier studies (Lim et al., 2006).

CONCLUSION

In conclusion, the findings from the current study suggest that OEO enhances the growth efficiency in broiler chicken. Specifically, adding 500 mg/kg of OEO to the basal diet showed a tendency to improve growth performance parameters and promote the carcass traits of broiler chickens. To completely investigate the mechanism of action of OEO on the growth performance of broiler chickens and to increase the efficiency of its application in broiler feed, more research on different supplementation amounts of OEO in different ingredients of diets is required.

DECLARATION

Ethical considerations

The authors have examined ethical issues, including plagiarism, permissions to publish, misconduct, and duplicate publishing, for publishing this scientific research in the Journal of the World's Poultry Research.

Author's contribution

Md. Sahidul Islam contributed to the conceptualization, formal analysis, investigation, methodology, software development, supervision, validation, visualization, and the writing of the original draft, as well as the review and editing process. Dilruba Akter Mir was involved in the investigation, supervision, validation, and visualization. Md. Emran Nazir focused on validation and visualization. Syidul Islam participated in the conceptualization, methodology, validation, visualization, and review and editing of the writing. S. M. Iqbal Hossain contributed to the methodology, supervision, and validation. Sharmin Zaman and Dabobrata Kumar Swar were responsible for visualizing the original draft and reviewing and editing the writing. Sharmin Sultana engaged in writing the original draft and the review and editing process. Md. Iftakharul Hasan worked on conceptualization, methodology, validation, visualization, and the review and editing of the writing. All authors have reviewed and approved the final version of the study.

Availability of data and materials

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

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Competing of interests

The authors declare no conflict of interest.

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Impact of Polyherbal Formulation on Transcriptome Profiling of Chicken Breast Muscle: Elucidation of Molecular Mechanisms for the Enhanced Cellular Feed Efficiency in Broiler Chickens

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ABSTRACT

To elucidate the cellular mechanisms underlying feed efficiency (FE) enhanced by polyherbal formulation (PHF), a combination of *Andrographis paniculata, Punica granatum*, and *Emblica officinalis*, the microarray technology was used to identify the genetic pathways related to feed utilization through genomical profiling of breast muscle in Cobb broiler chickens supplemented with or without PHF. 300 male day-old chicks were randomly distributed into two groups of 6 replicates with 25 each, including control (basal-diet) and PHF (Basal-diet+PHF-400g/ton), and their impact on performance parameters was assessed on day 42. Similarly, the global gene expression of breast muscles collected in each group was profiled using Agilent chicken whole genome microarray technology. Further, the Database for Annotation Visualization and Integrated Discovery (DAVID) Bioinformatics Resources and gene ontology (GO) analysis were used to identify the functional clustering of differentially expressed genes (DEGs) and gene network pathways associated with FE. RT-qPCR was subsequently conducted to cross-validate the expression of genes identified by DAVID. The PHF supplementation significantly improved the body weight gain (BWG) and feed conversion ratio in broilers compared to the control. Totally, 1338 DEGs (756 up-regulated and 582 down-regulated) were identified, of which 732 DEGs of PHF were significantly different from the control group. However, bioinformatics analysis revealed a significant modulation of 198 DEGs (94 up-regulated and 104 down-regulated) after hierarchical clustering, whose collective expression indicates significant enrichment of FE-related biological processes in the PHF-treated group. Further, a deeper understanding of the following DEGs (*ND1, ND2, ND3, ND4, ND4L, ND5, ND6 CYTB, COX1, COX2, COX3, ATP6, PGC1-α, PPAR, MEF2, PARK2, Wnt3A, Wnt11, Golgb1*, and *IGF1*) was established by mapping with Kyoto-Encylopedia-of-Genes-and-Genomes (KEGG) pathway in relation to mitochondria cellular respiration, mitochondrial biogenesis, mitophagy, energy metabolism and muscle growth in the breast muscle. Moreover, validation of microarray analysis of selected genes using RT-qPCR showed that the genes (*ND1, ND2, ND3, ND5, CYTB, ATP6, PGC1-α,* and *Wnt11)* were expressed in the same direction as that of GO analysis. In conclusion, supplementation of PHF resulted in transcriptional modulation in the mitochondrial functions, which was correlated to the improvements of corresponding phenotypic traits (FE and BWG) in broiler chickens.

Keywords: Differentially expressed gene, Feed efficiency, Genome microarray, Mitochondrial respiration

INTRODUCTION

Since the global population keeps increasing from 700 crores in 2011 to 800 crores in 2030 and 900 crores in 2050, the requirement for high-quality animal protein on the earth will be doubled by 2050 (Tian et al., 2016). Thus, improving the efficiency and sustainability of animal production systems depends heavily on features like feed efficiency (FE). Conventionally, the inclusion of antibiotics at subtherapeutic levels in a nutritionally balanced poultry diet maximizes the genetic potential for growth and FE in broilers (Basit et al., 2020). However, the feed grade antibiotic growth promoters were banned by the European Union due to the risk of possible multiple drug-resistance of human pathogenic bacteria and the issue

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of drug residues in meat products. In addition, the cost factor, possibility of the evolution of antibiotic-resistant microbes and condemnation by consumer associations as well as by scientists over antibiotic growth promoters enforced the poultry researchers and feed industries to find an alternative to antibiotics, which resulted in the evolution of probiotics, prebiotics, and organic acids supplements (Rahman et al., 2022). The feed manufacturers have recently adopted a new type of natural feed additives that are the result of contemporary science. These additives include botanicals, such as suitable blends of herbs or plant extracts, to maintain the health and good production of livestock that feed humanity (İpçak and Alçiçek, 2018; Opoola et al., 2019). Despite marked improvements in growth and FE using these feed additives (Havenstein et al., 2003), there remains a significant within and between-strain variation of these traits in broilers (Connor, 2015). This variation in relation to performance could be enlightened by recent findings of several authors (Ojano-Dirain et al., 2004), who proved that mitochondrial function and biochemistry were entangled with FE in broilers. Mitochondria, a well-defined cytoplasmic organelle of the cell, generate adenosine triphosphate (ATP) from the diet through a process called oxidative phosphorylation using oxygen and simple sugars (Picard et al., 2018). Being a powerhouse of the cell, mitochondria produce 90% of the energy utilized by the cell; an association of mitochondrial dysfunction with oxidative phosphorylation was presumed to have a substantial effect on the phenotypic expression of FE in animals. This was supported by Bottje et al. (2006), who demonstrated that the breast muscle of high FE birds showed higher activities of mitochondrial complexes and the respiratory control ratio when compared to low FE birds. However, there has been very scant information about specific genes or gene networks, as well as a very limited study of global gene expression profiling that underlies the interaction of mitochondrial function and FE, modulated by natural feed additives (Kong et al., 2011).

Polyherbal formulation (PHF), presumed to enhance cellular feed efficiency, is a combination of *Andrographis paniculata*, *Punica granatum*, and *Emblica officinalis* belonging to the families of Acanthaceae, Lythraceae, and Phyllanthaceae, respectively, in poultry (Mathivanan et al., 2008; Patel et al., 2016; Gosai et al., 2023; Jahja et al., 2023). These plants are well-known for betterment of feed conversion ratio (FCR), augmentation of body weight, and control of mortality rate in chickens (Tipakorn, 2002; Danet et al., 2013; Patel et al., 2016; Saleh et al., 2017). However, to date, there is no scientific data available on the effect of PHF on global gene expression that demonstrates the enhancing effect of FE in broiler chickens.

Nutrigenomics, a novel research field, is used to elucidate the cellular basis of the interaction between nutrients and other dietary bioactives using the genetic material (Ismail and Ergören, 2023). It is widely known that any change in nutrition impacts the patterns of genomic profiling (Lee et al., 2015; Resnyk et al., 2017; Martínez-Martínez et al., 2020), hence, these modified genomes can be targeted and identified to understand the impact of nutritional variations on the whole genome. Microarray is one of the most recent advances in technology in analysing the entire transcriptome that can be used to uncover the pathways and networks underlying feed efficiency. Therefore, the present study sought to elucidate the molecular basis for PHF effectiveness (growth and FE in broiler chickens) with the help of microarray technologies and two bioinformatics tools with Database for Annotation, Visualization and Integrated Discovery (DAVID) and gene ontology (GO), followed by further validation of target genes using RT-qPCR assay.

MATERIALS AND METHODS

Ethical approval

Institutional ethical committee approval (No. AHS/PR/03/2016) was obtained before the conduct of study and it was conducted by authorised, qualified and trained veterinarians, scientists and technicians in compliance with the guidelines laid down by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India.

Polyherbal formulation

The PHF is crude powder, indexed as Feed-X, which contains *Andrographis paniculata*, *Punica granatum*, and *Emblica officinalis*, and is manufactured by Natural Remedies Pvt., Ltd., Bengaluru, India. The PHF was supplemented with the diet at a dose of 400g/ton of feed.

Experimental design and inclusion level

Three-hundred-day-old Cobb-430 chicks, with an average weight of 45g, were randomly distributed into two groups of 150 each, including control (basal-diet [BD]) and PHF (BD+PHF-400g/ton), with six replicates of twenty-five birds each. Both the groups were raised on a basal diet (ME – 3100 kcal/kg and CP – 22, 20 and 18% in starter, grower and finisher phases, NRC, 1994) and the management practices were carried out as described by Selvam et al. (2017), except for the floor space. Birds were provided 0.027m^2 from day 1-21 and 0.055m^2 per bird from day 22-41. The performance parameters, including body weight gain (g), FCR (Marimuthu and D'Souza, 2019), and mortality (%), were assessed on day 39.

Sample collection

Following weighing on day 41, six chickens were chosen at random from each group, sacrificed by jugular vein exsanguination, and their breast muscles were sampled. After removing about 0.5 g of breast muscle, it was rinsed with prechilled phosphate-buffered saline (PBS), chopped into 5 x 5 mm pieces, and immediately stored in RNAlater® solution (Sigma-Aldrich, Canada) for 12 hours. It was then kept at -80°C until the RNA was extracted for transcriptome analysis. In addition, a piece of breast muscle (n = 10/group) was rapidly frozen using liquid nitrogen after prechilled PBS washing, and then stored at -80°C until avian uncoupling protein (avUCP) and protein carbonyl analysis were performed.

Preparation of tissue homogenate

A weighed quantity of breast muscle was added to four parts of PBS and homogenized using a tissue T18 digital ultra-turrax® homogenizer (IKA, Staufen, Germany). Then, it was centrifuged at 13800 RPM for one minute at 4℃, followed by sonication for 30 seconds. An aliquot was collected and stored at -20℃ for further analysis.

Chicken uncoupling protein assy

An uncoupling protein (UCP) level was measured using Nori® chicken avUCP ELISA kit (Genorise Scientific, Inc., USA). Briefly, breast muscle homogenate (1:4 dilution) was allowed to bind with immobilized chicken-specific (UCP2) antibody at room temperature for 2 hours. Then the samples were incubated with the detection antibody (specific for chicken UCP2) and detection reagent solution, followed by the addition of substrate solution for colour development, and its intensity was measured at 540 nm using Versamax Tunable Microplate Reader (BN02314 Model) purchased from Molecular Devices, LLC, USA.

Protein carbonyl assay

The protein carbonyl level was assessed by the protein carbonyl colorimetric assay kit (Cayman Chemical USA). Briefly, the breast muscle homogenate (1:5 dilution) was derivatized with carbonyl residues of dinitrophenylhydrazine, precipitated the derivatized protein using trichloroacetic acid, and washed with a 1:1 ethanol: Ethyl acetate mixture. Then it was resuspended in guanidine hydrochloride and measured spectrophotometrically at 370 nm using a Versamax Tunable Microplate Reader (BN02314 Model) obtained from Molecular Devices, LLC, US.

RNA isolation

Breast muscle samples were homogenized in QIAzol® Lysis Reagent Reagent, (Qiagen, USA) using a Smasher™ homogenizer (BioMerieux, USA), and isolated the total RNA with the help of RNeasy® Lipid Tissue Kit (Qiagen, Valencia, CA) method, and estimated the total RNA concentration of all 12 samples at 260/280 nm ratio using ND-2000 spectrophotometer (Nano Drop Technologies Inc., Wilmington, Delaware). Then it was subjected to RNA integrity assessment using Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA, USA), and subsequently used for microarray analysis.

cRNA generation, probe-labelling, and microarray-hybridization

The methodology used to synthesize cRNA, label it, and array hybridize it was followed as described by D'Souza et al. (2019).

Assemblage of microarray data and scrutiny

The schematic representation of the microarray scrutiny workflow is shown in Figure 1, and carried out as described in our previous work reported by D'Souza et al. (2019). GSE114769 could be used for the accession of microarray data from respective web sources.

Identification and hierarchical clustering

The data was normalized using logarithmic transformation, and the differentially expressed genes (DEGs) were selected using ± 0.6 as the cut-off criterion. Then the DEGs with similar expression profiles were clustered, which permits the user to form a group with the same profiles (Marimuthu et al. 2022).

Bioinformatics

The DAVID 6.7 database was used to identify the gene ontology (GO) terms related to the location and function of the significant DEGs, and the association of GO terms with other pathways using Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathways was also studied.

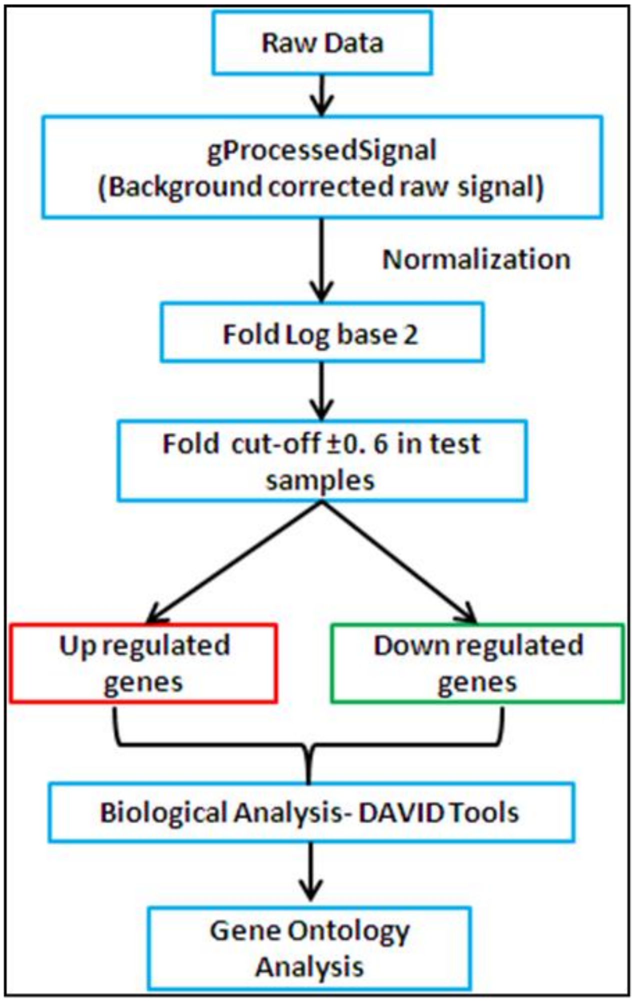

Figure 1. Schematic representation of the microarray analysis of breast muscle workflow

Quantitative Real-time PCR

The expression of selected genes involved in FE was reconfirmed using RT-qPCR. Three micrograms of total RNA from each sample of individual RNA from the control and test groups were used. The primers (Wang et al., 2019) were designed for selected DEGs using Primer 3 Plus online primer design software considering the exon and coding region of the transcripts. The total RNA was converted into cDNA using Affinity Script qPCR cDNA synthesis kit (Agilent Technologies, USA) as per the manufacturer`s protocol. In brief, approximately 1000 ng of RNA from each sample was taken for cDNA synthesis, and the first-strand cDNA was synthesized using universal oligo dT primers. Then, the expression levels of selected DEGs were analyzed using SYBR Green chemistry (Brilliant II SYBR Green qPCR master mix, Agilent Technologies, USA) in a Stratagene MX3005P instrument (Agilent Technologies, USA).

Statistical analysis

The significant DEGs with a false discovery rate (Benjamini–Hochberg test) of $p < 0.05$ were identified using unpaired t-test of GeneSpring version 7.2 (Agilent Technologies) software and the significance of growth performance was tested using unpaired t-test procedure of SPSS (version 9.3, SAS Institute Inc., Cary, NC, USA). The data were expressed as mean ± Standard Deviation (SD), and statistical significance was declared at $p < 0.05$.

RESULTS

Phenotypic traits

Following supplementation of PHF, there was a significant augmentation in body weight and improvement in FCR observed on day 42 when compared with the control group ($p < 0.05$, Table 1).

Assessment of avUCP and protein carbonyl levels

The results indicated that PHF inclusion showed a significant ($p < 0.05$) improvement in avUCP and protein carbonyl levels as compared to the control group (Figures 2 and 3).

Identification of differentially expressed genes

The genome-wide expression profiling of each probe was assigned to compare the PHF and control groups. The raw data files were intra-array normalized and then validated using GenespringGX software. A total of 756 genes were overexpressed and 582 genes were down-regulated (Table 2). Among them, 732 genes were modulated significantly at a threshold of $p < 0.05$, wherein 500 and 232 genes were expressed progressively higher and lower, respectively, when compared to the control group. Those identified DEGs differentiated the 2 experimental groups when represented on a heat map scale, in a hierarchical clustering analysis (Figure 4).

Bioinformatics

The PHF supplementation significantly modulated certain genes of breast muscle, and further, the biological complexity of gene expression was delineated using the DAVID 6.7 online tool, which is a GO term enrichment analysis software that highlights the most relevant GO terms associated with a given gene list ($p < 0.05$). There are three components to a GO annotation including cellular, biological process, and molecular function. In the entire data set, some of the mitochondrial DEGs were identified whose collective expression indicates a significant enrichment in mitochondria biological processes which includes system development, electron transport chain (ETC), multicellular organismal development, anatomical structure development, cellular developmental process, response to estrogen stimulus, cell differentiation, and nucleoside binding response in PHF treated group. The associated expressed and repressed genes with significant GO terms were tabulated in Tables 3 and 4, and were also represented as a heat-map in Figures 5 and 6. In addition, a deeper insight was made with respect to selective up-regulated DEGs (*ND1*, *ND2*, *ND3*, *ND4*, *ND4L*, *ND5*, *ND6 CYTB*, *COX1*, *COX2*, *COX3*, *ATP6*, *PGC1-α, PPAR, MEF2, PARK2, Wnt3A, Wnt11*, *Golgb1 and IGF1*) involved in mitochondrial respiratory chain, mitochondrial biogenesis, mitophagy, energy metabolism and muscle growth using KEGG database (Table 5).

Examination of specific differentially expressed genes involved in the electron transport chain with RT-qPCR

The profile of specific DEGs involved in mitochondrial biogenesis and ETC signalling pathway in the mitochondria of breast muscle, as well as in structural development, as demonstrated by DAVID, was further reconfirmed using qPCR assays. The qPCR values of *ND1*, *ND2*, *ND3*, *ND5*, *CYTB*, *ATP6*, *WNT11*, and *golgB1* genes in the tissues were almost similar to those of the microarray values (Table 6). Thus, the qPCR data basically substantiates the results of the GO analysis of the selected genes.

Table 1. Outcome of polyherbal formulation on performance parameters in broiler chickens aged 42 days

Parameters	Day	Control	PHF (400 g/ton)
BWG (g) (n=148-150)	42	1845.47 ± 20.73	***1968.87 ± 48.35
FCR (n=6)	42	1.722 ± 0.01	***1.649 ± 0.024
Mortality (%)		1.33	0.00
Breast muscle (g)		441.50 ± 13.91	**513.33 ± 34.24

Values are represented as mean ± SD, **p < 0.01 and *** p < 0.001 indicate the significant difference between the two groups; BWG: Body weight gain; FCR: Feed conversion ratio; PHF: Polyherbal formulation.

Table 2. Differentially expressed breast muscle genes of broiler chickens

Group	Up	Down
Control (breast muscle)	-	-
PHF (breast muscle)	756	582

PHF: Polyherbal formulation

Table 3. Up-regulated genes and associated significant gene ontology (GO) terms

GO Term	Count	P-Value	Genes
GO:0048731~system development	28	0.0005	*DRD1, PTGS2, MYBPC3, UCHL1, CLU, XRCC6, NINJ2, DAB1, MEIS2, GBX2, PRL, SPP1, TCF7, SMAD9, GSC, MYO6, IKZF1, LDB1, TBX5, MET, IGF1, EPHA2, ANXA2, SLIT3, HOPX, COL1A2, SEMA4D, BMP5*
GO:0022900~electron transport chain	6	0.0009	*ND1, ND5, ND2, ND3, CYTB, PPARGC1A*
GO:0007275~ multicellular organismal development	32	0.0015	*DRD1, PTGS2, UCHL1, CLU, XRCC6, MYBPC3, NINJ2, MEIS2, DAB1, GBX2, PRL, SPP1, DVL3, TCF7, MYO6, SMAD9, GSC, IKZF1, LDB1, TBX5, MET, IGF1, EPHA2, ANXA2, SLIT3, DKK3, ISL2, WNT9B, HOPX, COL1A2, SEMA4D, BMP5*
GO:0048856~ anatomical structure development	28	0.0016	*DRD1, PTGS2, MYBPC3, UCHL1, CLU, XRCC6, NINJ2, DAB1, MEIS2, GBX2, PRL, SPP1, TCF7, SMAD9, GSC, MYO6, IKZF1, LDB1, TBX5, MET, IGF1, EPHA2, ANXA2, SLIT3, HOPX, COL1A2, SEMA4D, BMP5*

Table 4. Down-regulated genes and associated significant gene ontology (GO) terms

GO Term	Count	P Value	Genes
GO:0048869~ cellular developmental process	27	0.0009	*RTN4, CDX2, FOXA2, ERBB4, MITF, NEO1, IL15, PIWIL1, POU2F1, AGRN, FGF1, EGR1, KIF3A, CREB1, SLIT1, STAT3, SLIT2, LAMA1, KRT19, LHFPL5, RGS2, KRT14, MYH11, GHRL, ADAM17, PBX3, DBN1*
GO:0043627~ response to estrogen stimulus	4	0.0011	*KRT19, SOCS2, GHRL, STAT3*
GO:0030154~cell differentiation	26	0.0013	*RTN4, CDX2, ERBB4, FOXA2, MITF, IL15, NEO1, PIWIL1, POU2F1, AGRN, FGF1, EGR1, CREB1, SLIT1, SLIT2, STAT3, LAMA1, KRT19, LHFPL5, RGS2, KRT14, MYH11, ADAM17, GHRL, PBX3, DBN1*
GO:0001883~ purine nucleoside binding	37	0.0054	*IFIH1, ERBB4, ASS1, TBK1, STK17B, BRSK2, MKNK1, ASNS, ITM2B, CAMKK2, RRAGC, GSS, GSR, NOS2, KIF3A, MAK, SWAP70, MAP2K3, MSH4, ACACA, RPS6KC1, MAPK11, ACLY, MCM3, SRPK1, MCM5, GART, GRK6, MYH11, ABCC3, ABCC4, MAPK8, ACAD11, AACS, KALRN, ATAD2B, DDX51*
GO:0001882~ nucleoside binding	37	0.0060	*IFIH1, ERBB4, ASS1, TBK1, STK17B, BRSK2, MKNK1, ASNS, ITM2B, CAMKK2, RRAGC, GSS, GSR, NOS2, KIF3A, MAK, SWAP70, MAP2K3, MSH4, ACACA, RPS6KC1, MAPK11, ACLY, MCM3, SRPK1, MCM5, GART, GRK6, MYH11, ABCC3, ABCC4, MAPK8, ACAD11, AACS, KALRN, ATAD2B, DDX51*

Table 5. Results of microarray for up-regulated genes in response to polyherbal formulation treatment in broiler chickens on day 41

Genes	Microarray fold change	p value	Gene Ensemble ID
ND1	1.7	0.0004	ENSGALG00000042750
ND2	1.6	0.0014	ENSGALG00000043768
ND3	1.7	0.0010	ENSGALG00000030436
ND4	1.5	0.0007	
ND4L	1.5	0.0066	
ND5	1.5	0.0049	ENSGALG00000029500
ND6	1.4	0.0058	
CYTB	1.6	0.0027	ENSGALG00000032079
COX1	1.4	0.1191	
COX2	1.3	0.0329	
COX3	1.1	0.0509	ENSGALG00000015591
ATP6	1.53	0.0104	ENSGALG00000015618
PGC1-α	1.9	0.0056	
PARK2	1.25	0.0380	
PPAR	1.3	0.028	
Wnt3A	1.5	0.033	ENSGALG00000042657
Wnt11	1.59	0.011	
GOLGB1	2.94	0.048	ENSGALG00000009783
IGF1	1.77	0.094	ENSGALG00000012755
WFIKKN2	1.32	0.5808	
MSTN	1.24	0.1252	
IGF1R	0.92	0.7742	

PHF: Polyherbal formulation

Table 6. Comparison of the results of microarray and qPCR analyses of selected genes in response to polyherbal formulation treatment in broiler chickens on day 41

Genes	qPCR log fold change (Log2)	Microarray log fold change (Log2)
ATP6	0.58	0.77
WNT11	0.44	0.84
Cyt-b	0.73	0.87
NAD1	0.45	0.79
NAD2	0.59	0.73
NAD3	0.81	0.87

PHF: Polyherbal formulation

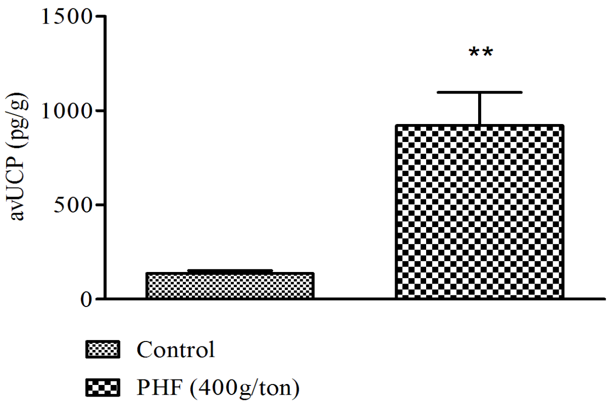

Figure 2. Effect of polyherbal formulation on avian uncoupling protein (avUCP). **p < 0.01 indicates the significant difference between the two groups; PHF: Polyherbal formulation.

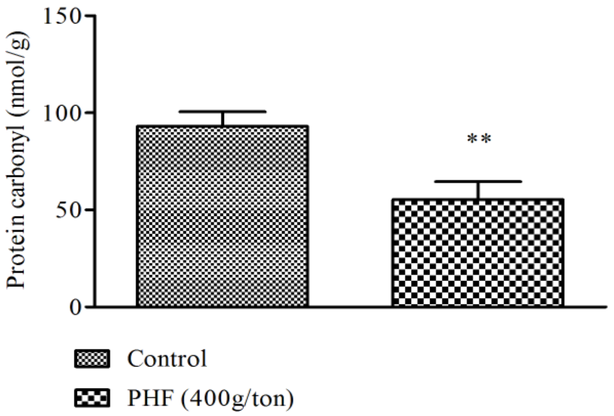

Figure 3. Effect of polyherbal formulation on protein carbonyl. ** indicates significant difference between the two groups (p < 0.01); PHF: Polyherbal formulation.

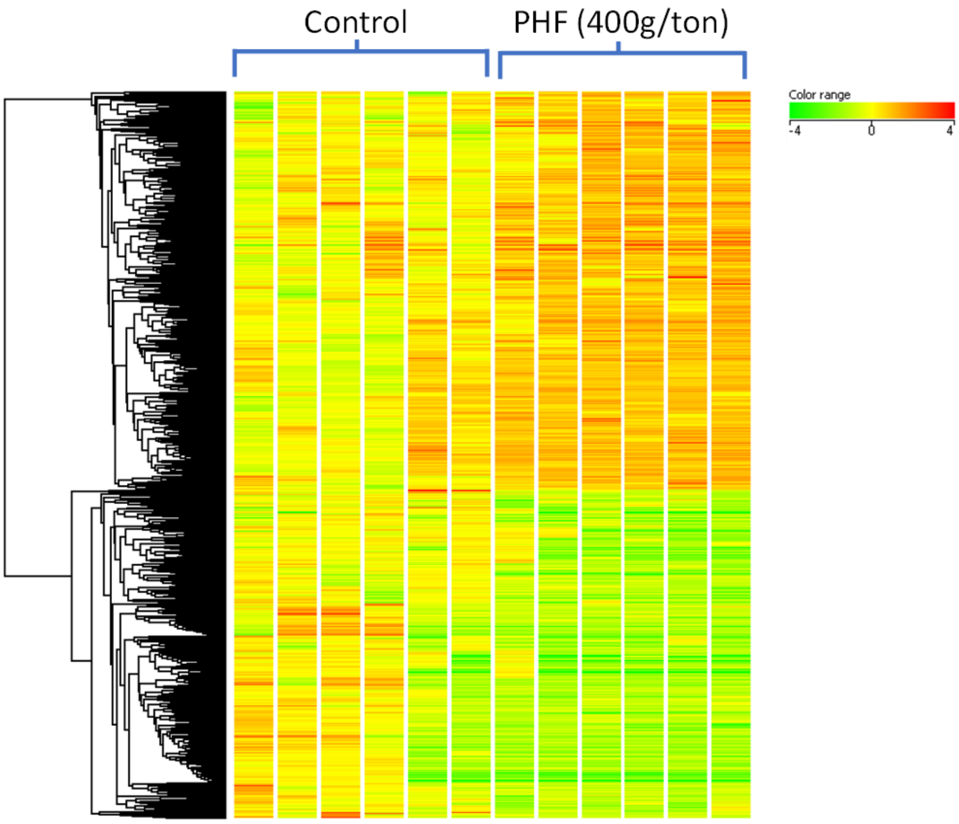

Figure 4. Cluster of differentially regulated genes. Up and down-regulated genes are represented by red and green colors, respectively, while yellow represents no change in fold expression values; PHF: Polyherbal formulation.

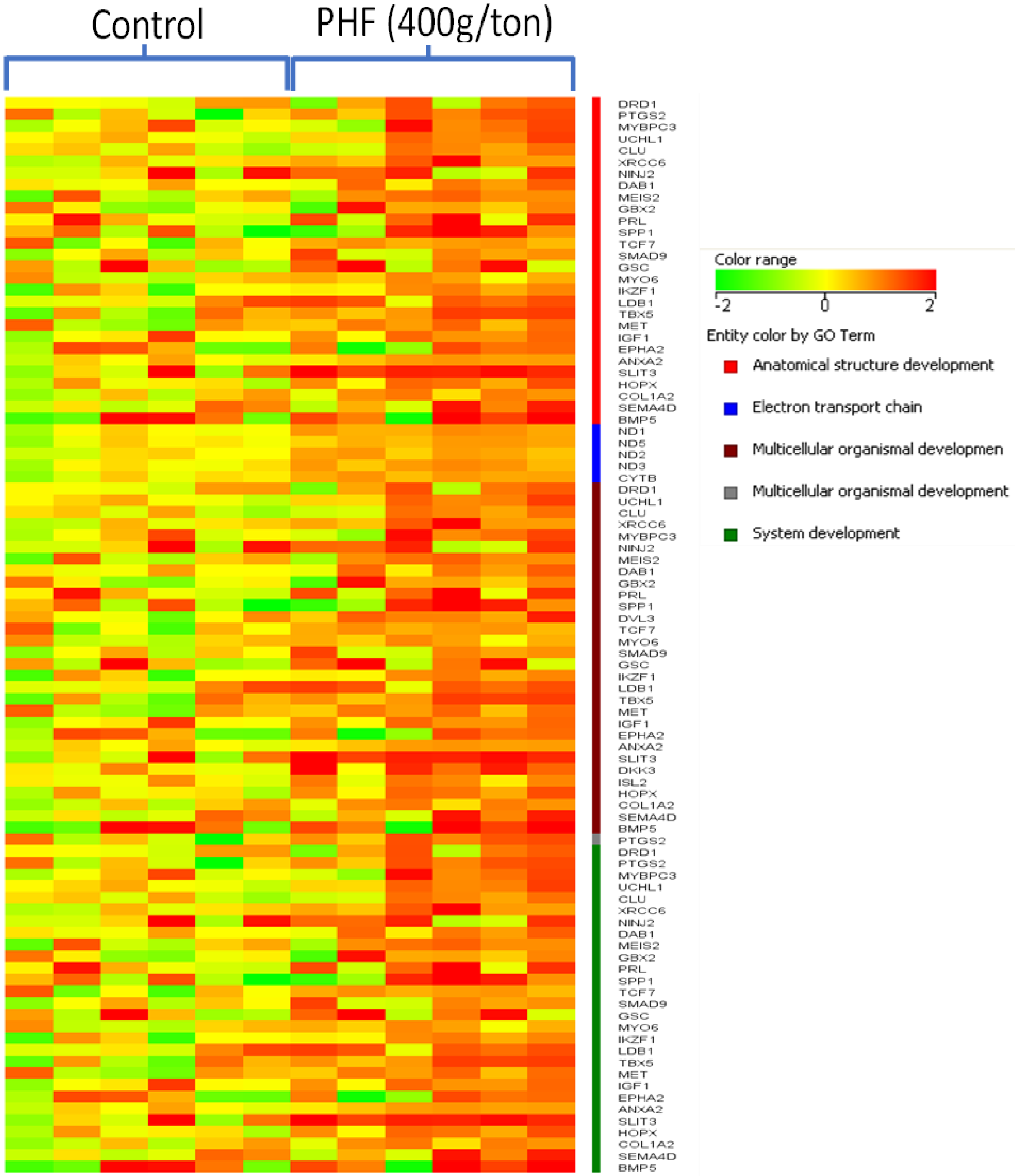

Figure 5. Heatmap showing expressed genes associated with the significant gene ontology terms. Up and down-regulated genes are represented by red and green colors, respectively, while yellow represents no change in fold expression values; PHF: Polyherbal formulation.

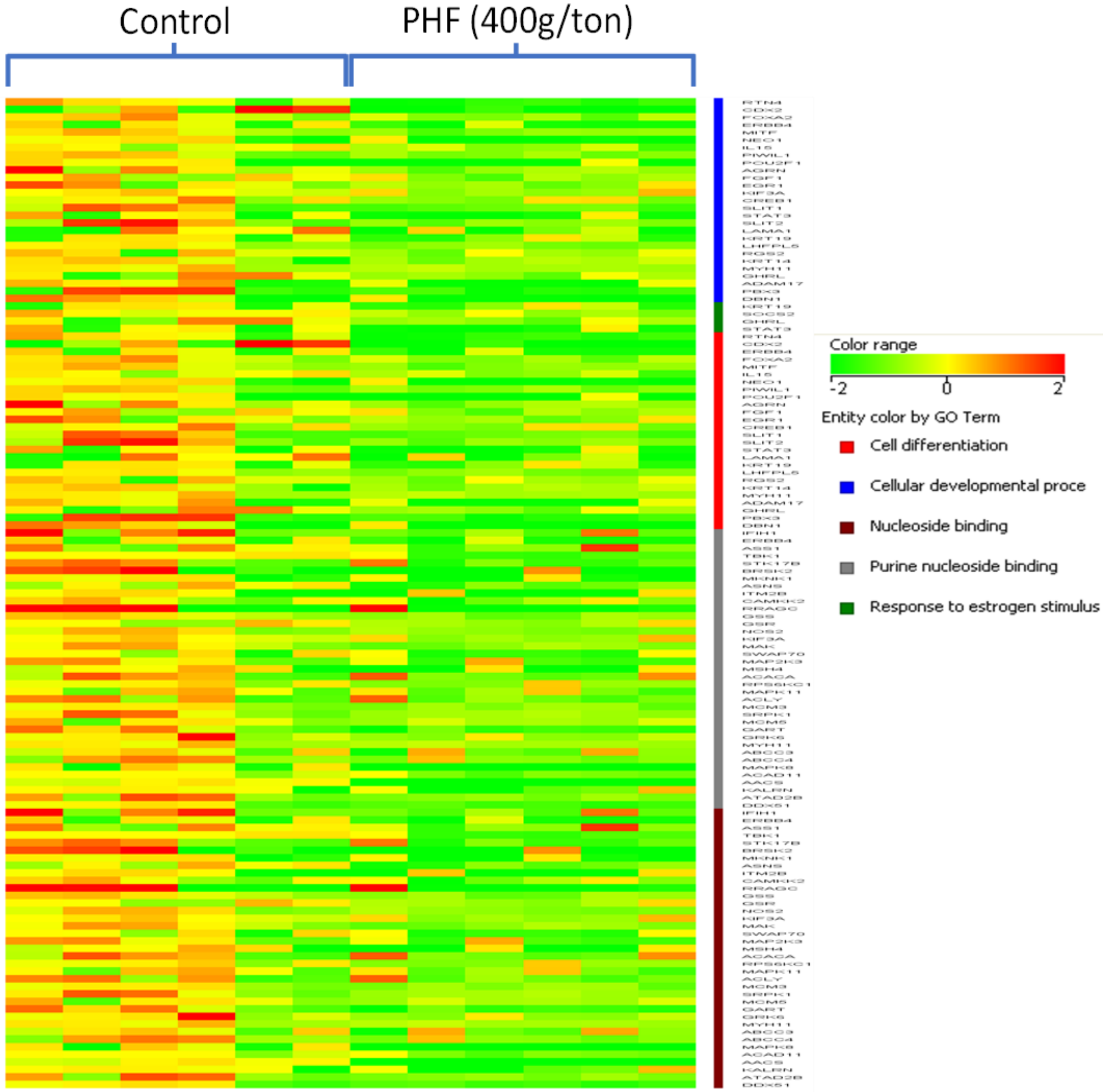

Figure 6. Heatmap showing repressed genes associated with the significant GO terms. Up and down-regulated genes are represented by red and green colors, respectively, while yellow represents no change in fold expression values; PHF: Polyherbal formulation

DISCUSSION

This study demonstrated that dietary inclusion of PHF enhanced the performance traits of broiler chickens. Microarray analysis indicated that PHF-mediated transcriptional changes associated with ETC, mitochondrial biogenesis, mitophagy, and structural development were reflected by enhanced FE in chickens. FE is a compound trait affected by two complex traits, such as feed intake and weight gain, which means the genomic profile of FE phenotypes may be different among the populations. Recent studies have proved that high feed efficiency (FE) broilers moderate their nutrient utilization and body energy expenditure based on the amount of gene products responsible for the function of the inner mitochondrial membrane protein. Correspondingly, the negative performance of low FE broilers was the outcome of disturbances in the electrons or protons gradient, leading to mitochondrial dysfunction, through the reduction of mitochondrial ATP synthesis and generation of higher reactive oxygen species (Iqbal et al., 2005; Krueger et al., 2008; Bottje and Carstens, 2009).

In addition, low FE may be related not only to the expression of genes that encode proteins of ETCs, but also to the lowered activity of the respiratory-chain complexes (Bottje and Carstens, 2009). Furthermore, Ojano-Dirain et al. (2007) discovered variations in the expression of mRNA encoding for mitochondrial transcription factors, proteins, and duodenal tissue between low and high FE broilers by analyzing the expression patterns of the genes involved in mitochondrial energy metabolism (Ojano-Dirain et al., 2007). The above observation demonstrated that FE of birds largely depends on mitochondrial function; however, generating a comprehensive picture of gene expression patterns involved in FE using a gene-by-gene approach would be difficult and time-consuming. Hence, the present study provides, apparently for the first time, a comprehensive analysis of the chicken breast muscle, supplemented with PHF, gene expression profile using Agilent microarray. This microarray technology was used to understand the mitochondrial physiology and phenotypic expression of FE in broilers supplemented with PHF by comparing thousands of mRNAs from a given tissue, and simultaneously provides a wide-ranging assessment of expression levels (Kong et al., 2011; Bottje et al., 2012; D'Souza et al., 2019).

There are four multi-protein complexes (I to IV) and adenosine triphosphate (ATP) synthase (Complex V) in the respiratory chain/oxidative phosphorylation system of the inner mitochondrial membrane. The complex I consists of *ND1, ND2, ND3, ND4, ND4L, ND5, and ND6* parts, which are a large enzyme (NADH dehydrogenase) complex and are responsible for the first step in the electron transport process. The *CYTB* gene encodes for a protein called cytochrome b, which is one of 11 components of a group of proteins called complex III. *COX1*, *COX2*, and *COX3* are the subunits and functional core of the enzyme Cytochrome c oxidase (complex IV) that catalyzes the reduction of oxygen to water (Braun, 2020). *ATP6* gene encodes for subunit A of the F0 functional domain that forms one part (subunit) of a large enzyme called ATP synthase (complex V), which is responsible for the last step of oxidative phosphorylation (Jonckheere et al., 2012). In the mitochondrial respiratory chain, the movement of electrons starts from the complexes I and II through the NADH- or FADH-linked substrates such as glutamate and succinate, respectively, and then travels down the respiratory chain from ubiquinone to the terminal electron acceptor, oxygen (O_2). During this electron transport, a proton (H_2) is also pumped from the matrix to the intermembrane space, which generates the proton-motive force. The resulting force gives the energy for ATP synthesis [from adenosine diphosphate (ADP) and Pi by the F0F1 complex] as protons move back through ATP synthase (Schormann et al., 2019). These explanations demonstrate the relationship of ETC with mitochondrial efficiency in ATP production, which is associated with converting nutrients into body components in birds supplemented with PHF. The current results of microarray analysis revealed that the expression levels of some important genes, including *ND1*, *ND2*, *ND3*, *ND4*, *ND4L*, *ND5*, *ND6*, *CYTB*, *COX1*, *COX2*, *COX3*, and *ATP6*, implicated in mitochondrial respiratory chain/oxidative phosphorylation, were significantly affected by PHF supplementation. It was corroborated by other studies, which showed that modulation of genes involved in ETC leads to alterations in the utilization of nutrients (Iqbal et al., 2005; Ojano-Dirain et al., 2007; Brito et al., 2016). Nevertheless, any leakage of electrons due to genetic alterations in the mitochondrial DNA causes partial reduction of oxygen to reactive oxygen species (ROS) such as superoxide (O^{2-}) and H_2O_2 (Nolfi-Donegan et al., 2020; Sies et al., 2022). This ROS creates oxidative stress, if not metabolized by antioxidants, and oxidizes the critical biomolecules (e.g., lipids, proteins, and DNA) in the cell, which leads to further mitochondrial DNA modifications and inefficiencies that accentuate additional ROS production. In these conditions, uncoupling protein (UCP) mediated proton conductance gets activated as a negative feedback loop to reduce the ROS generation in the respiratory chain complexes, which results in mild uncoupling (Brand et al., 2004). Interestingly, high avUCP and low protein carbonyl levels were observed in the current study, further signifying the better efficiency of mitochondria in the breast muscle of PHF supplemented birds. This was supported by Raimbault et al. (2001) and Bottje et al. (2006), who observed avUCP gene expression of high FE broilers was higher (Raimbault et al., 2001; Bottje et al., 2006). Similarly, low FE broilers had high protein carbonyl levels, which indicates protein oxidation due to greater oxidative stress in low FE mitochondria (Stadtman and Levine, 2000). Additionally, upregulation of the *Park2* gene in breast muscle substantiates the better mitochondrial function in the PHF treated group. This gene encodes one protein required for the PINK1/Parkin pathway of mitophagy (macroautophagy or bulk autophagy) that eliminates the damaged or superfluous mitochondria and preserves the population of healthy mitochondria. Greene et al. (2003) and Park et al. (2006) reported that loss of either mitochondrial PINK1/Parkin protein in Drosophila leads to mitochondrial respiratory chain dysfunction, which results in deterioration of flight muscles and dopaminergic neurons (Greene et al., 2003; Park et al., 2006). Surprisingly, the peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC1-α) gene, peroxisome proliferator-activated receptor (PPAR), and myocyte enhancer factor (MEF2) were also found to be up-regulated in the current study, indicating the enhancement of mitochondrial biogenesis and mitochondrial energy metabolism by PHF supplementation (Dorji et al., 2021). A key mechanism of cell adaptation and repair, mitochondrial biogenesis depends on transcriptional regulation controlled by multiple nuclear-encoded genes (PGC1-α) (Scarpulla, 2008; Piantadosi and Suliman, 2012). This was supported by Kong et al. (2011) and Bottje et al. (2012), who found that mitochondrial biogenesis was enhanced in birds with higher FE. PGC1-α also exerts strong effects on mitochondrial energy metabolism by coactivating both PPAR (nuclear receptor target) and MEF2 genes, which in turn regulate the mitochondrial fatty acid oxidation pathway (Vega et al., 2000) and glucose transport outside the mitochondria, respectively (Michael et al., 2001).

Furthermore, up-regulation of Golgin B1 (GOLGB1), WntA, Wnt11, and Insulin-like Growth Factors (IGF1) genes signifies that the protein accretion and muscle growth were improved in the breast muscle of PHF treated broilers. GOLGB1 is the Golgi integral membrane protein (gigantin), essential for the organization and retention of proteins in the Golgi apparatus (Linstedt and Hauri, 1993). This was supported by the results of Kong et al. (2011), who demonstrated that the up-regulation of GOLGB1 facilitates optimal packaging and transport of proteins to specific cell sites in high FE birds (Kong et al., 2011). Wnt signaling pathway is the positive regulator of myogenesis and is crucial for muscle, adipocytes, and bone development (Duprez, 2002; Yavropoulou and Yovos, 2007; Christodoulides et al., 2009). This was evidenced from the reports of Yue et al. (2011) who suggested that 7 genes (RHOA, CHP, Wnt3A, RAC1, Wnt11, Wnt9A, and MAPK9) on Wnt pathway were significantly associated with broiler weight gain and carcass paramaeters mainly by influencing muscle development rather than preadipocytes differentiation (Yue et al., 2011). IGF1 exerts a general effect on overall weight gain (Yoshida and Delafontaine, 2020), and overexpression in the muscle tissue leads to enhanced muscle growth in chicken (Mitchell et al., 2002). It was supported by Scanes et al. (1989), who stated that IGF-1 levels were significantly reduced in the low growth group in comparison to the high growth group at seven weeks of age.

The above discussions specify that PHF supplementation significantly improved the mitochondrial function in breast muscle as evidenced by the upregulation of molecular-function genes responsible for ETC, mitochondrial biogenesis, and mitochondrial energy metabolism. The PHF not only upregulates the genes at the nuclear level, but also influences the genes at the biological function level, including anatomical structure development (GO:0048856), multicellular organismal development (GO:0007275), and system development (GO:0048731). Interestingly, alteration in the expression of certain specific genes was cross-validated using RT-qPCR and substantiated by corresponding phenotypic traits such as feed efficiency and body weight gain improvement in broilers supplemented with PHF.

PHF also downregulates the genes involved in the apoptosis biological process that includes nucleoside binding (GO:0001882), purine nucleoside binding (GO:0001883), response to estrogen stimulus (GO:0043627), cell differentiation (GO:0030154), and cellular developmental process (GO:0048869).

CONCLUSION

In summary, whole genomic profiling of breast muscle demonstrated a significant modulation of genes involved in enhancing the mitochondrial cellular respiration for ATP generation, mitochondrial biogenesis, mitophagy, and energy metabolism in broiler chickens supplemented with PHF (400g/ton). This was supported by corresponding phenotypic traits such as FE and body weight gain improvement in chickens. These research findings confirm that PHF could be used as a natural cellular feed efficiency enhancer in poultry diets.

DECLARATIONS

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Authors` contributions

Saravanakumar Marimuthu, Subramaniyam Suresh, and Prashanth D'Souza participated in sample collection. Subramaniyam Suresh drafted the manuscript. Saravanakumar Marimuthu revised the manuscript. Prashanth D'Souza designed the study and analyzed the data. All authors read and approved the final manuscript.

Availability of data and materials

The published research paper included all the data collected and analyzed during the conduct of the study.

Ethical considerations

This manuscript has not been published, accepted for publication, or is undergoing editorial review elsewhere. It also does not contain any sentences that have been plagiarized.

Conflict of interests

All authors declare that there is no conflict of interest.

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The Evaluation of MAPK/ERK Signaling Pathway in Chicken Necrotic Enteritis Based on Microbiomics and Metabolomics

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ABSTRACT

Chicken necrotic enteritis is a prevalent intestinal disease caused by *Clostridium perfringens* (*C. perfringens*) in chickens. Previous research has confirmed the close relationship between the gut microbiota and its metabolites in connection with chicken necrotic enteritis. However, it remains unclear how the gut microbiota of the host influences host metabolism following the onset of necrotic enteritis (NE). The close relationship between gut microbiota and their metabolites in chicken necrotic enteritis (NE) has been established, yet the metabolic influence of microbiota post-NE onset remains unclear. In this study, 1-day-old White Leghorn chickens were divided into three groups (n=10/group), a negative control (CON) fed a basal diet, a fishmeal-supplemented group (F) receiving 50% fishmeal, and an NE group inoculated orally with *C. perfringens* alongside fishmeal supplementation. Growth performance, intestinal lesions, and morphological changes were recorded. Cecal contents were subjected to 16S rDNA sequencing for microbiota profiling, while serum metabolomics was analyzed via LC-MS.No noticeable damage was observed in the small intestines of the F group, whereas the NE group exhibited marked body weight reduction. Cell necrosis and jejunal mucosal shedding were identified, accompanied by ileal villi atrophy and significant reductions in tight junction proteins (Claudin-1 and ZO-1). Both F and NE groups showed decreased cecal abundances of *Lactobacillus* and *Blautia*, alongside increased *Clostridium* and *Escherichia coli*. Serum metabolomics revealed distinct glycerophospholipid and arginine-proline metabolism alterations in the F group versus CON. In contrast, NE-associated metabolic shifts were linked to pathways regulating cell proliferation, differentiation, and migration, particularly MAPK signaling. Downregulation of MAPK/ERK pathway genes was detected in the jejunal mucosa of infected chickens compared to CON and F groups. Concurrently, jejunal PCNA expression was quantified and found to be significantly reduced in the NE cohort relative to controls. Drawing upon the experimental results, it was concluded that necrotic enteritis in chickens was linked to a disruption in the intestinal epithelial barrier. Additionally, alterations in the gut microbiota hindered the activation of the MAPK/ERK signaling pathway, which in turn reduced the proliferation of intestinal epithelial cells and impaired the repair processes crucial for intestinal barrier restoration.

Keywords: Chicken, Necrotic enteritis, Microbiomics, Metabolomics, MAPK/ERK, Signaling pathway

INTRODUCTION

Necrotic enteritis (NE) in chickens, caused by *Clostridium perfringens* (*C. perfringens*), is a common disease in the poultry industry, often leading to significant economic losses (Skinner et al., 2010; Timbermont et al., 2010). NE has both clinical and subclinical forms. The clinical variant is characterized by sudden onset and high mortality rates, sometimes reaching 50% (Lee and Lillehoj, 2022; Timbermont et al., 2011), while the subclinical form primarily results in chronic intestinal mucosal damage in chickens, leading to nutrient malabsorption, lower growth rates, and inefficient feed consumption (Caly et al., 2015).

The development of NE requires predisposing factors, as infection with *C. perfringens* alone is often insufficient to induce the disease. The addition of high concentrations of fish meal in the feed is a major factor in the development of NE in chickens, as it supplies the nutrients required for the proliferation of CP (Drew et al., 2004a; Wu et al., 2010). It has also been shown that a diet exclusively consisting of fishmeal, when combined with a *C. perfringens* infection, leads to substantial alterations in

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the intestinal microbiota of chickens (Stanley et al., 2014). The intestinal flora actively participates in the regulation of numerous metabolic processes, including the metabolism of fatty acids, carbohydrates, and amino acids, and it closely interacts with the host's nutritional metabolism (Eckel, 2021). Metabolomic studies on hens with necrotic enteritis have primarily focused on the metabolites of gut microbiota, highlighting alterations in compounds such as bile acids, amino acids, and short-chain fatty acids (Bansal et al., 2020; Kidd et al., 2021; Wang et al., 2021a; Zaytsoff et al., 2022). Additionally, research using integrated metabolomics and transcriptomics analysis has revealed a strong correlation between intestinal damage in hens suffering from necrotic enteritis and the c-Jun N-terminal kinase (JNK) signaling pathway (Xiao, 2022). However, very few studies have delved into changes in serum metabolism and how it relates to gut flora in NE-affected chickens. Investigating the relationship between serum metabolites and gut microbiota can elucidate the systemic metabolic effects of NE, offering a more comprehensive understanding of its pathology and providing a clearer insight into the underlying disease mechanisms. This study aims to identify the potential mechanisms of NE in chickens, providing insights for its treatment. A NE model was established to analyze changes in the intestinal mechanical and chemical barriers, microbiota, and serum metabolomics following NE. Differential metabolites were identified, and altered metabolic pathways were constructed to elucidate the mechanisms of NE, offering valuable references for its treatment.

MATERIALS AND METHODS

Ethical approval

The animal use protocol in this study was approved by the Huazhong Agricultural University Animal Protection and Use Committee, located in Wuhan, China (Permit number: HZAUCH-2023-0009).

Bacterial strains

The *C. perfringens* type G strain was isolated from the liver of chickens naturally infected with NE and preserved by the laboratory of the Veterinary Pathology, Huazhong Agricultural University, Wuhan, China.

Animal experiment design and basal diet

Thirty one-day-old white Leghorn chickens with an average weight of 35.1 ±1.57 g was procured from Beijing Boehringer Ingelheim Vital Biotechnology Co., Ltd., situated in Beijing, China. Chickens are 15 males and 15 females. These one-day-old chickens were divided into three groups at random, each with ten chickens including the negative control group (CON), chickens were given basic feed; fish meal group (F), chickens were given high fish meal feed; infected group (NE), chickens were given high fish meal feed and gavaged with *C. perfringens*. From day 1 to day 14, all groups were fed a basic diet (Figure 1). From day 15 to day 23, groups F and NE were fed a high fishmeal diet. From day 19 to day 23, chickens in group NE were given $5\times10^{\wedge 8}$ to $5\times10^{\wedge 9}$ CFU of *C. perfringens* orally, while chickens in groups CON and F were given comparable amounts of sterile Fluid Thioglycollate (FTG) medium orally. The trial lasted for 24 days. Throughout the trial, the chickens were kept in wire cages with a 12-hour light cycle and unlimited access to food and water. The composition of the basal diet is shown in Table 1. And the vaccination and prevention protocols was shown in Table 2.

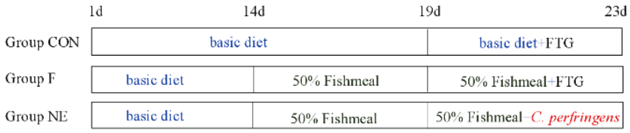

Figure 1. The experimental design of the present study. Group CON (n = 10): *C. perfringens* -uninfected chickens with basic diet; Group F (n = 10): *C. perfringens* uninfected chickens with high fishmeal diet; Group NE (n = 10): *C. perfringens* -infected chickens with high fishmeal diet.

Table 1. Guaranteed analytical value of product

Components:	**Content (%)**
Moisture: ≤	14.0
Crude protein: ≥	20.00
Calcium:	0.60-1.20
Total phosphorus: ≥	0.50
Sodium chloride:	0.20-0.80
Crude fiber: ≤	6.00
Crude ash: ≤	8.00
Methionine + cystine: ≥	0.74

Table 2. Vaccination and prevention protocols

Age (day)	**Disease(s)**	**Route**
1	IBD and MD	
5	Coccidiosis	Water
7	ND	Spray
7	IB	Spray
7	IC	Spray
12	Coccidiosis	Water
14	ND	Spray
14	IB	Spray
14	IC	Spray

IBD: Infectious bursal disease, MD: Marek disease, IC: Infectious Coryza

Small intestinal lesion score

Lesions were assessed on a scale ranging from 0 to 4, as follows: 0 = absence of observable lesions, 0.5 = pronounced congestion of serosa and mesentery engorged with blood,1 = intestines characterized by thin walls and friability with small red petechiae, 2 = localized necrotic lesions, 3 = areas presenting patches of necrosis, and 4 = widespread necrosis.

Sample collection and treatment

On the morning of day 24, every chicken's unique body weight (measured in grams) was recorded. And 2 mL blood samples were collected from the jugular or wing vein. The serum was separated from the clotted whole blood by centrifugation at 3000 g for 10 minutes within 2 hours of collection and stored at -80°C until further analysis. For histological examination, 2.0 cm segments from the duodenum, jejunum, and ileum were fixed in a 4% paraformaldehyde solution for 24 hours. Standard procedures were then followed for paraffin embedding, sectioning to approximately 4 μm, and staining using hematoxylin and eosin. Samples of the duodenal, jejunal, and ileal mucosa, and intestinal contents from the cecum, were collected and promptly frozen in liquid nitrogen at -196°C.

Morphological observation of the small intestine

Imaging of small intestinal tissue sections was performed utilizing a Nikon 80i light microscope (Nikon Corporation, Tokyo, Japan). For each experimental group, small intestinal tissue sections from five chickens were selected. The height of the villi (VL) and the depth of the crypts (CD) were assessed by measuring five villi in each section. Following these measurements, the ratio of villus height to crypt depth (VL/CD) was determined.

Analysis of cecal flora

The sample from the cecal contents was subjected to 16S rRNA sequencing at Majorbio Bio-Pharm Technology Co. Ltd. in Shanghai, China. Bioinformatics analysis was conducted utilizing the Majorbio Cloud Platform (He et al., 2021). Alpha diversity analysis was carried out using Mothur software (version 1.30.2). Beta diversity was assessed through Bray-Curtis principal coordinates analysis (PCoA), and the significance of bacterial segregation among groups was determined by ANOSIM analysis. Linear Discriminant Analysis (LDA) effect size (LEfSe) was used to examine differences in the gut microbiota composition between different groups (Zhao et al., 2024). PICRUSt analysis was executed to anticipate the potential functions of the microbiota (Langille et al., 2013).

Analysis of serum metabolomics

The serum samples were analyzed using LC-MS/MS at Majorbio Bio-Pharm Technology Co. Ltd. in Shanghai, China. Multivariate statistical analysis was conducted using the ropls (Version 1.6.2) R package from Bioconductor on the Majorbio Cloud Platform. Principle component analysis (PCA) was conducted using an unsupervised approach to provide an overview of the metabolic data, and visualize general clustering, trends, or outliers (Ringnér, 2008). Orthogonal partial least squares discriminant analysis (OPLS-DA) was employed for statistical analysis to discern global metabolic alterations among comparable groups (Gennebäck et al., 2013). Model validity was assessed based on model parameters R2 and Q2, which offer insights into the interpretability and predictability of the model, thereby reducing the possibility of overfitting. Variable importance in the projection (VIP) was calculated in the OPLS-DA model. p-values were estimated using a paired Student’s t-test in single-dimensional statistical analysis. VIP values larger than 1 and p-values less than 0.05 were used to identify statistically significant differences between the groups. By employing metabolic enrichment and database search for pathway analysis, the different metabolites between the two groups were compiled and mapped to their corresponding biochemical pathways.

Quantitative Real-Time PCR

Total RNA was extracted from the jejunal mucosa with the Trizol reagent provided by Nanjing Vazyme Biotech Co., Ltd., China. The amount and purity of RNA were assessed by measuring absorbance at 260 nm and 280 nm. For reverse transcription, 1 μg of the extracted RNA was processed using the HiScript II RT SuperMix for qPCR, which comes with a gDNA wiper from Nanjing Vazyme Biotech Co., Ltd., China.Quantitative Real - time PCR was performed following the manufacturer's guidelines, using the 2×SYBR Green PCR Mix from Aidlab Biotechnologies Co., Ltd., China. Each sample was measured in triplicate.The relative quantification of gene expressions was calculated using the 2 - △△Ct method, with 18S rRNA serving as the internal reference gene (Burge et al., 2012). The primer sequences were synthesized by Sangon Biotech Co., Ltd, located in Shanghai, China. The specific information about these primers is presented in Table 3.

Western blot

Jejunal mucosa protein was extracted from the protein lysate, and its concentration was measured using the bicinchoninic acid (BCA) protein assay kit (Beyotime, Shanghai, China). The Western blotting procedure followed the methodology described in previous studies (Lirong et al., 2019). Briefly, proteins were separated by SDS-PAGE and transferred onto a polyvinylidene difluoride (PVDF) membrane. The membrane was then blocked with 5% skimmed milk for 2 hours before being incubated overnight at 4°C with primary antibodies. Secondary antibodies conjugated with horseradish peroxidase were applied at room temperature for 1 hour (AS014, ABclonal Technology, Wuhan, China). Protein detection was performed using an ECL substrate, and the bands were visualized through electrochemical luminescence (ECL). The primary antibodies used included rabbit anti-Raf1 (A19638), rabbit anti-ERK1/2 (A16686), rabbit anti-p-ERK1/2 (AP0485), rabbit anti-MEK1/2 (A4868), rabbit anti-p-MEK1/2 (AP0209), and β-actin (AC026), all purchased from ABclonal Technology, Wuhan, China.

Immunohistochemistry

The distribution and expression of proteins in the jejunum were observed following the immunohistochemical staining procedures outlined in previous studies (Zhang et al., 2022a). *PCNA* (Proliferating Cell Nuclear Antigen) was procured from Gene Tech (Shanghai) Company Limited (GM087902, Gene Tech, Shanghai).

Statistical analysis

The data were analyzed using one-way ANOVA and Dunnett's multiple comparison test employing GraphPad Prism 7.0 software. Correlation analysis was performed using SPSS Statistics 27 (SPSS, Inc., Chicago, USA). The data are presented as means ± SD. A significance level of $p < 0.05$ indicates a significant difference.

Table 3. Primers used for quantitative real-time PCR

Gene	Primer sequence (5'-3')	Product size/bp	Accession Number	Reference
18S	F:CTCTTTCTCGATTCCGTGGGT R:CATGCCAGAGTCTCGTTCGT	96	XM_417846.8	(Simon et al., 2018)
Occludin	F: GCAGATGTCCAGCGGTTACT R: GCAGAGCAGGATGACGATGA	160	NM_205128.1	
Claudin-1	F: GTGTTCAGAGGCATCAGGTATC R: GTCAGGTCAAACAGAGGTACAA	107	NM_001013611.2	
Claudin-2	F: CTTTGCTTCATCCCACTGGT R: TCAAATTTGGTGCTGTCAGG	86	NM_001277622.1	(Xie, 2019)
ZO-1	F: GGAGTACGAGCAGTCAACATAC R: GAGGCGCACGATCTTCATAA	101	XM_413773	(Emami et al., 2019)
ZO-2	F: GCGTCCCATCCTGAGAAATAC R: CTTGTTCACTCCCTTCCTCTTC	89	NM_204918	(Emami et al., 2019)
MUC2	F: ACCAAGCAGAAAAGCTGGAA R: AAATGGGCCCTCTGAGTTTT	101	NM_001318434.1	(Xie, 2019)
Raf1	F: GCGAAATGGGATGACCTT R: TGTGTAGTGAGCGGAACG	201	XM_040646131.2	(Zhang et al., 2022b)
ERK	F: ACCTCAGCAACGACCACATT R: GAGCCAGTCCGAAGTCACAA	156	NM_204150.2	
MEK	F: GCAGGGCACCCATTACTC R: AGGTCGGCTGTCCATTCC	226	NM_205388.2	
PCNA	F: GCAGATGTTCCTCTCGTTGTGGAG R: GAGCCTTCCTGCTGGTCTTCAATC	95	NM_204170.2	(Xu et al., 2020)

PCNA: Proliferating Cell Nuclear Antigen is a nuclear protein that plays a crucial role in DNA replication, repair, and cell cycle regulation

RESULTS

Body weight and small intestinal lesion score

The body weight of chickens in the NE group was significantly reduced when compared to the CON and F groups (Figure 2A, $p < 0.05$). No significant difference in small intestinal lesion scores was observed between the F and CON groups (Figure 2B, $p > 0.05$), but the lesion score in the NE group was considerably higher than those in the CON and F groups (Figure 2B, $p < 0.05$).

Intestinal histopathology

In both the CON and F groups, the duodenum, jejunum, and ileum had maintained their structural integrity; no obvious pathological changes had been observed (Figure 3A-F). The villi structure of the duodenal intestine in the NE group exhibited clarity, and no evident histopathological alterations were discerned (Figure 3G). The jejunal intestinal epithelial cells displayed pronounced necrosis and shedding (Figure 3H, arrow), while the ileal villi exhibited atrophy, appearing shortened and thickened (Figure 3I, arrow).

Intestinal villus length and crypt depth

In the duodenum, compared with the CON group, the length of intestinal villi (VL) in both the F and NE groups had exhibited no statistically significant changes ($p > 0.05$), albeit with a marginal decrease in crypt depth (CD) and a slight increase in VL/CD (Figure 4). Moving to the jejunum, compared to the CON group, the variations in VL, CD, and VL/CD within the F group had been no significant difference ($p > 0.05$); whereas in comparison to the CON group, both VL and VL/CD in the NE group had demonstrated a reduction, albeit without a significant statistical significance ($p > 0.05$). Lastly, examining the ileum, in comparison to the CON group, both VL and VL/CD had exhibited a significant increase in the F group ($p < 0.05$); whereas VL/CD in the NE group, while lower than that of the CON group, had been markedly inferior to that of the F group ($p < 0.05$).

Relative mRNA expression of tight-junction protein and mucin in the jejunum of chickens

Compared to the CON group, the F group showed a significant upregulation of *OCLN* and *CLDN1* expression (Figure 5, $p < 0.05$), while *CLDN2* levels were significantly reduced ($p < 0.05$). Although *ZO1*, *ZO2*, and *MUC2* expression levels increased, the changes were not statistically significant ($p > 0.05$). In contrast, the NE group exhibited a non-significant decline in *OCLN*, *ZO2*, and *MUC2* expression ($p > 0.05$), whereas *CLDN1*, *CLDN2*, and *ZO1* levels were significantly lower than in the CON group ($p < 0.05$). Additionally, *OCLN*, *CLDN1*, *ZO1*, and *ZO2* expression in the NE group was markedly reduced compared to the F group ($p < 0.05$), while *MUC2* levels declined without statistical significance. These findings clearly suggest that the intestinal epithelial barrier integrity was compromised in the NE group.

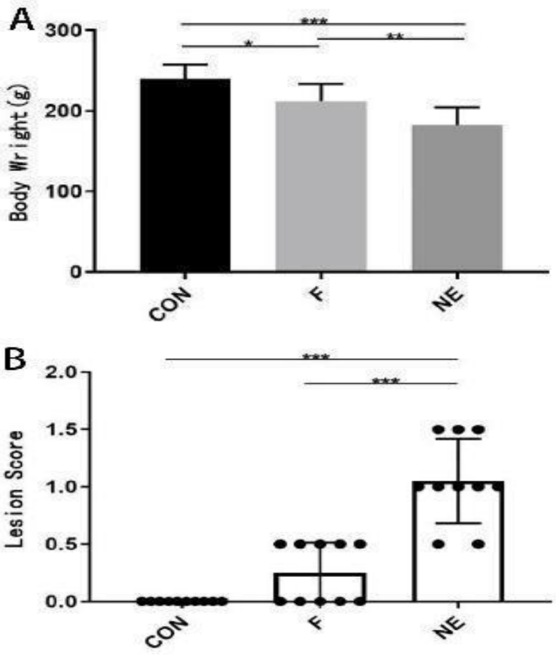

Figure 2. Body weight and small intestinal lesion score of 24-day-old chickens in each group. **A**: Body Weight; **B**: Lesion Score. CON: Negative control group; F: Fishmeal-supplemented diet group; NE: Necrotic Enteritis-induced group (*C. perfringens* challenge). Data are presented as means ± SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

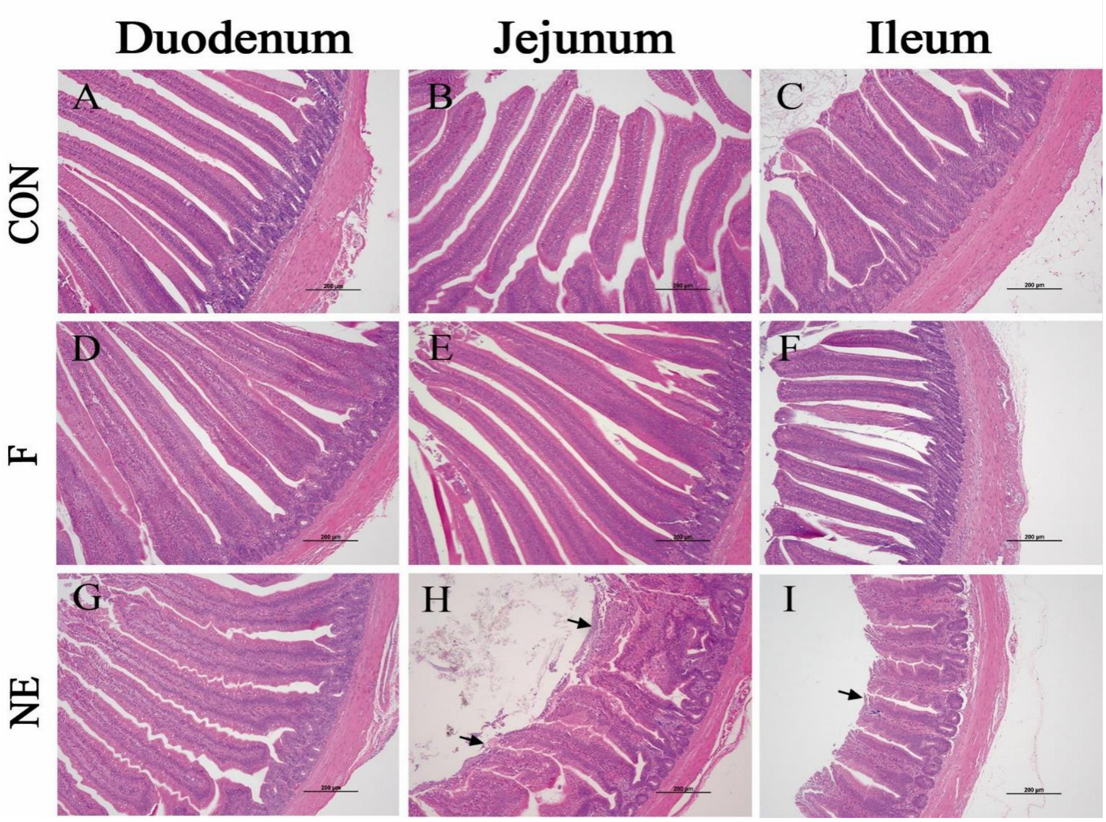

Figure 3. Histopathological observation of small intestine in 24-day-old chickens. **CON**: Negative control group; **F**: Fishmeal-supplemented diet group; NE: Necrotic Enteritis-induced group (*C. perfringens* challenge). Scale bars = 200 μm. **A, D, G**: Duodenum; **B, E, H**: Jejunum; **C, F, I**: Ileum.

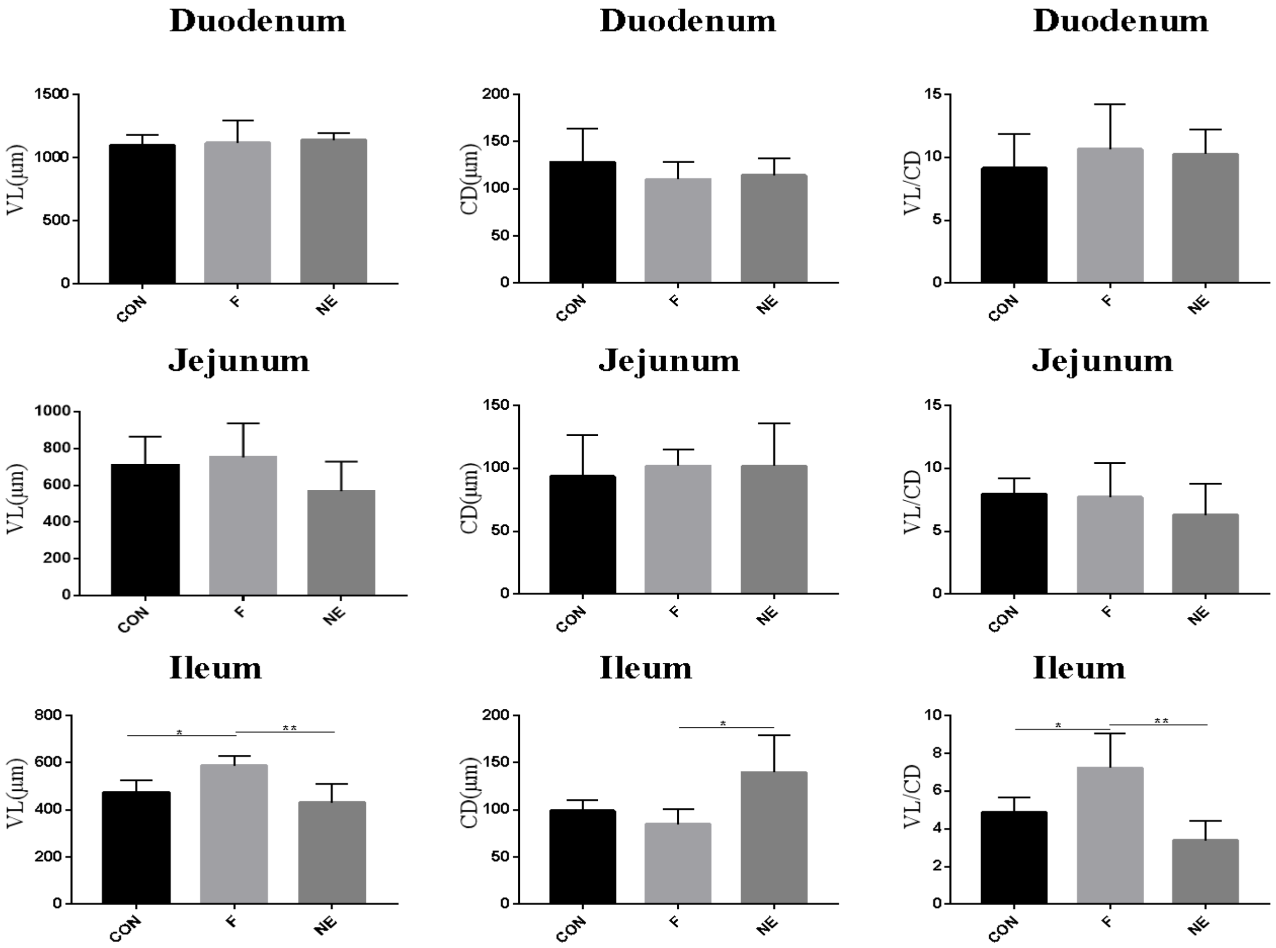

Figure 4. Length of intestinal villus and crypt depth of chickens in each group. **CON**: Negative control group; **F**: Fishmeal-supplemented diet group; **NE**: Necrotic Enteritis-induced group (*C. perfringens* challenge). Data are presented as means ± SD. * $P < 0.05$， ** $P < 0.01$.

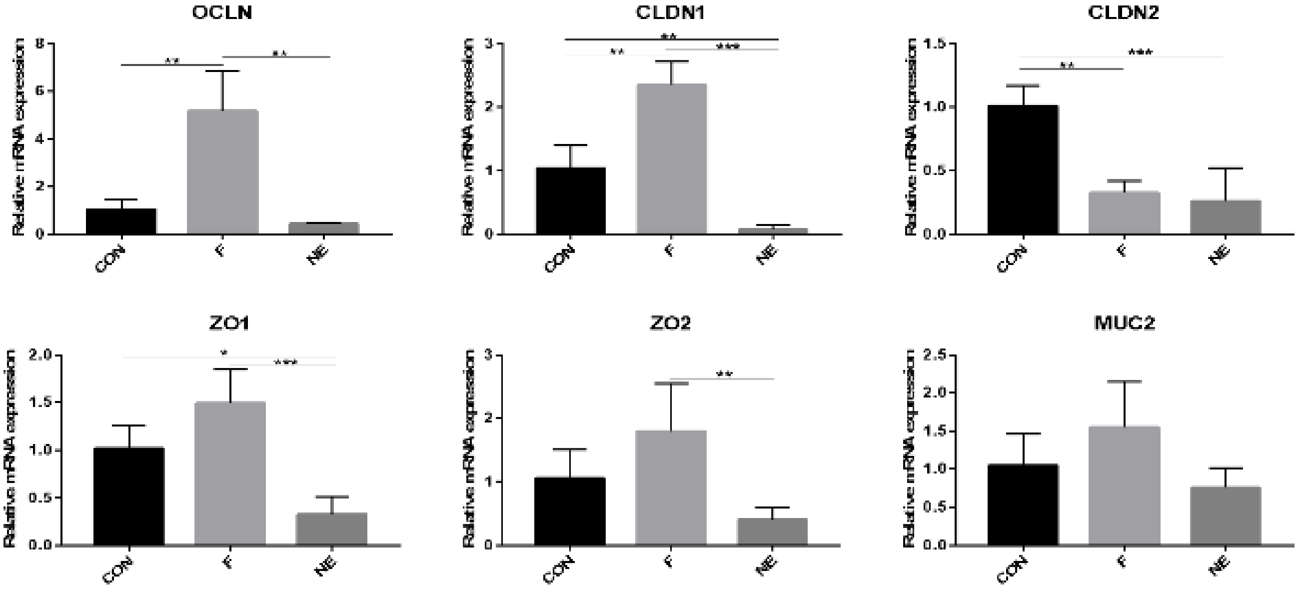

Figure 5. Expression of intestinal tight junction proteins and mucin genes in chickens of all groups. **OCLN**: Occludin; CLDN1: Claudin-1; **CLDN2**: Claudin-2; ZO1: Zonula occludins -1; **ZO2**: Zonula occludins-2; **MUC2**: Mucin2. **CON**: Negative control group; **F**: Fishmeal-supplemented diet group; **NE**: Necrotic Enteritis-induced group (*C. perfringens* challenge). Data are presented as means ± SD (n=3). * $P < 0.05$， ** $P < 0.01$， *** $P < 0.001$.

Intestinal flora diversity

The Chao and Shannon indices served as metrics for assessing α diversity, including the richness and diversity of the gut microbiota (Haegeman et al., 2013). The analysis indicated that microbial diversity in the NE group was significantly lower than in both the CON and F groups (Figures 6A, B). To assess β diversity, which reflects variations in gut bacterial composition, Principal Coordinate Analysis (PCoA) was conducted. The microbial community structure in the F group exhibited significant differences compared to the CON and NE groups ($p < 0.05$). However, the NE group's microbiota composition closely resembled that of the CON group (Figure 6C).

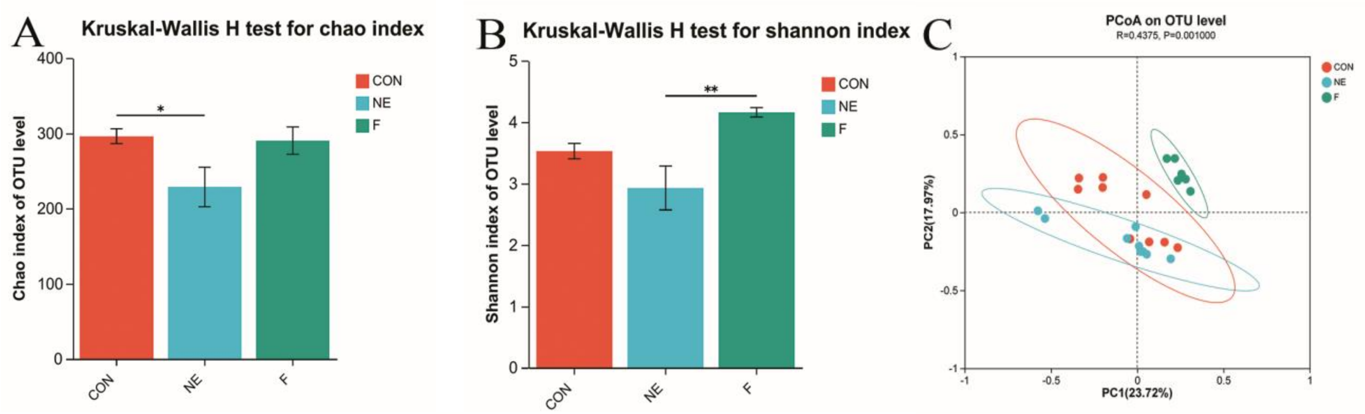

Figure 6. Diversity and structure of intestinal flora. **A**: Chao index; **B**: Shannon index; **C**: PCoA analysis of intestinal flora based on Bray Curtis distance. CON: Negative control group; **F**: Fishmeal-supplemented diet group; NE: Necrotic Enteritis-induced group (*C. perfringens* challenge)

Linear discriminant analysis effect size (LEfSe) analysis of intestinal flora

Linear discriminant analysis (LDA) effect size (LEfSe) analysis was used to detect significant differences in intestinal flora among the three groups at both the family and genus levels. The results revealed distinct taxa enrichment across the three groups. In group CON, taxa such as *Lactobacillus*, *norank_f__Ruminococcaceae*, *Blautia*, and *Ruminococcus gauvreauii group* were significantly enriched. In contrast, group NE exhibited a marked increase in *f__Clostridiaceae*, *Subdoligranulum*, *Erysipelatoclostridium*, *Anaerofilum*, and *Candidatus Arthromitus*. Meanwhile, group F showed notable enrichment in *norank_f__norank_o__Clostridia UCG-014*, *Negativibacillus*, *norank_f__norank_o__Clostridia vadinBB60 group*, *Colidextribacter*, *norank_f__Clostridium methylpentosum group*, and *Escherichia-Shigella* (Figure 7).

Analysis of PICRUSt

PICRUSt was employed to predict the gene functions of the cecal microbiota of chicks in the CON, NE, and F groups. The results showed a strong correlation between the intestinal microbiota and microbial metabolism in diverse environments (Figure 8).

Serum metabolomics

Serum metabolites were detected using the LC-MS/MS method. PCA analysis revealed significant differences ($p < 0.05$) in serum metabolites among all groups (Figure 9A, B). Furthermore, OPLS-DA analysis revealed significant differences ($p < 0.05$) in serum metabolites among all groups (Figure 9C-F).

Perform KEGG enrichment analysis and screen the differential metabolic pathways between group F and group CON, as well as between group NE and group CON. The outcomes demonstrated 14 distinct metabolic pathways between group F and group CON (Figure 10A), mainly centered on Arginine biosynthesis, Purine metabolism, and Glycerophospholipid metabolism. Fourteen distinct metabolic pathways were identified between the NE group and the CON group (Figure 10B), primarily focused on signal transduction pathways such as MAPK, VEGF, and ErbB signaling pathways. The findings indicated that differential metabolic pathways in group F primarily centered around metabolic pathways associated with amino acid, nucleotide, and lipid metabolism. In contrast, differential metabolic pathways in group NE were primarily focused on metabolic pathways related to cell proliferation, migration, and differentiation, such as the MAPK signaling pathway.

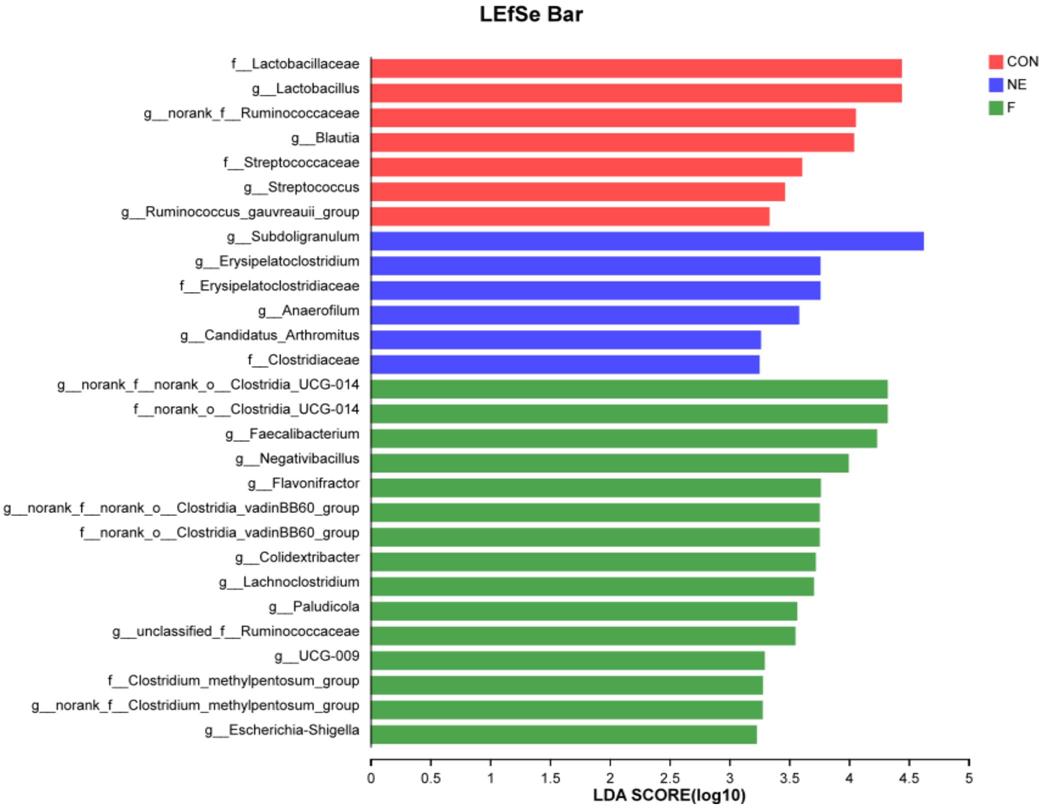

Figure 7. LefSe analysis of intestinal flora. LDA score > 3.2 was considered the screening condition to obtain differential intestinal flora.

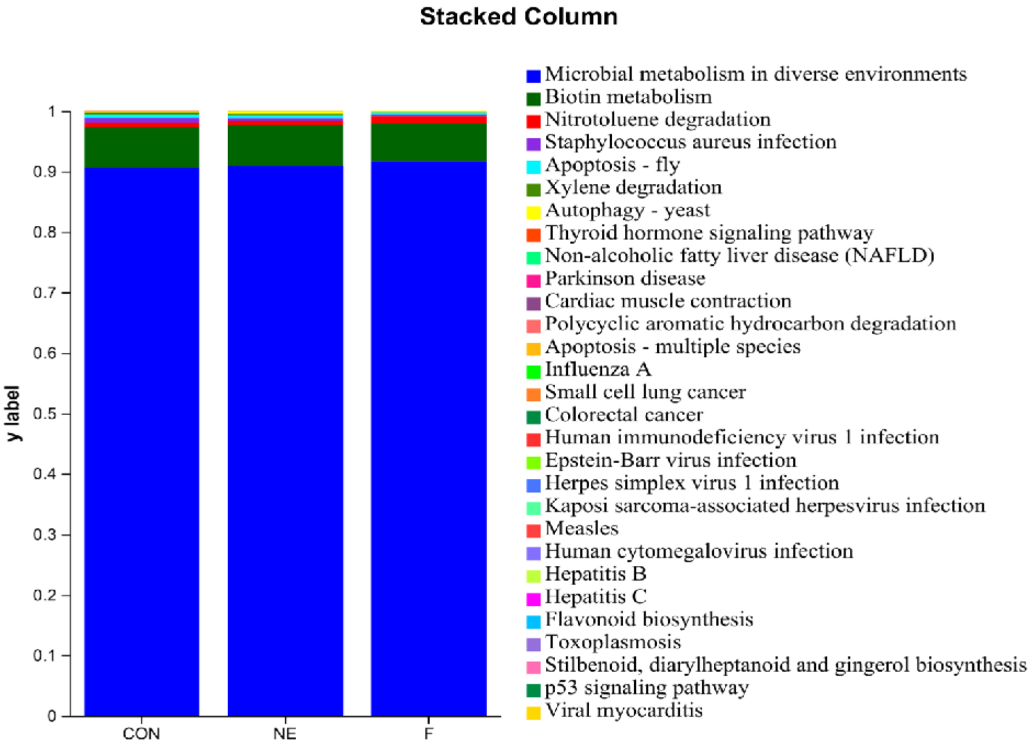

Figure 8. Functional gene prediction of gut microbiota in necrotic enteritis. Histogram Analysis of Metabolic Pathways by Strain (Abscissa: Experimental Groups, Ordinate: Pathway Abundance%) in White Lohmann Chickens.

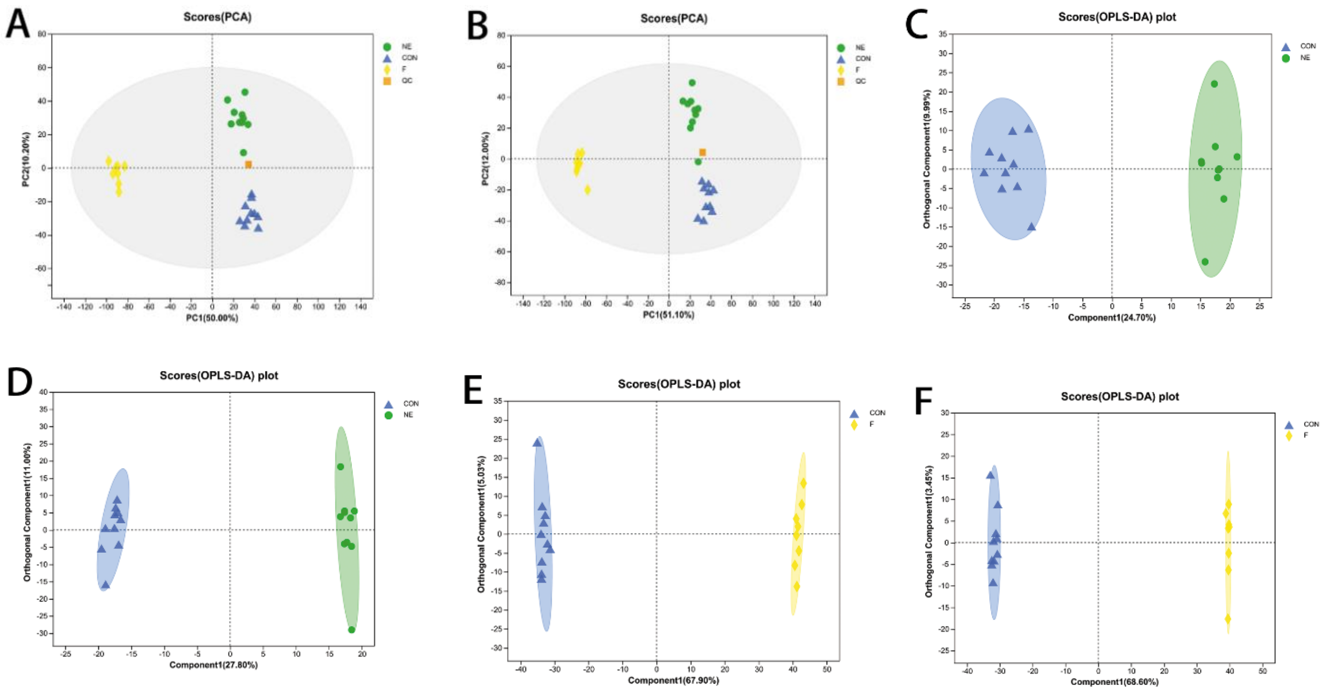

Figure 9. Metabolic profiling in chicken necrotic enteritis: PCA and OPLS-DA Scatter Plots. **A, B**: Principal component analysis (PCA) of chickens. **C-F**: Orthogonal partial least squares discrimination analysis (OPLS-DA) scatter plots of chickens.

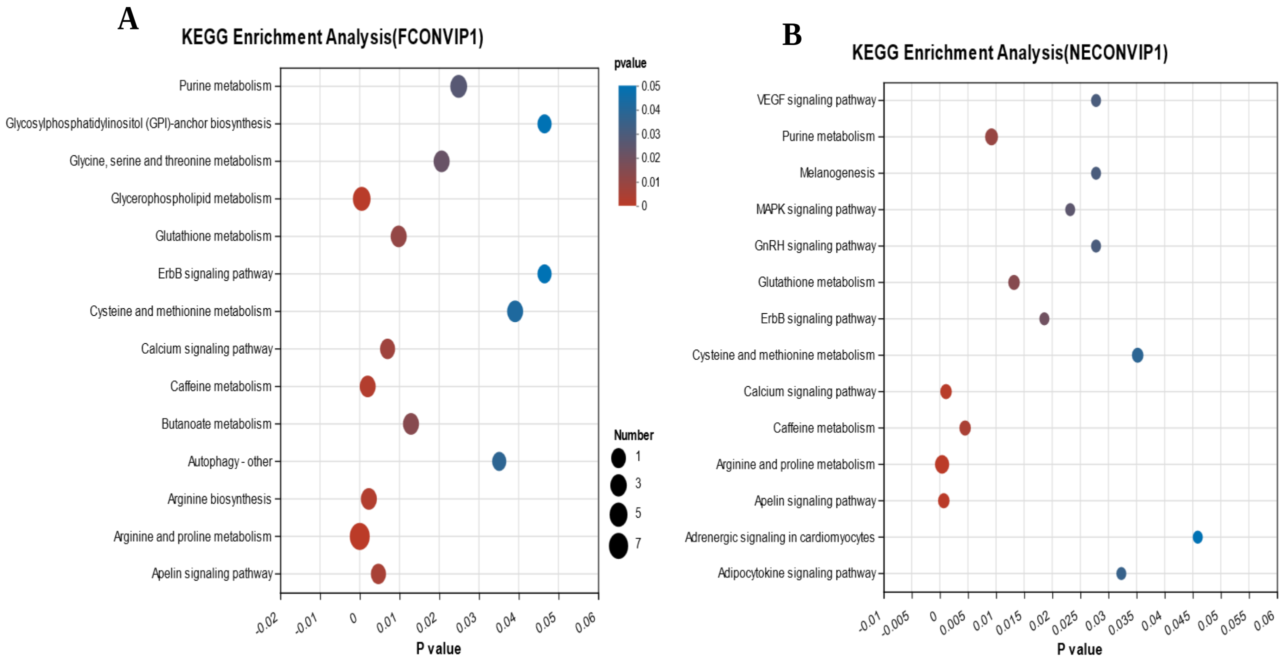

Figure 10. KEGG enrichment analysis reveals functional divergence in metabolic pathways. Amino Acid/Nucleotide Metabolism in Group F versus Cell Proliferation Signaling in Group NE (F/CON: n = 14 pathways; NE/CON: n = 14 pathways), **A**: Differential metabolic pathways between group F and group CON, **B**: Differential metabolic pathways between group NE and group CON. A p-value less than 0.05 was considered the screening condition to obtain differential metabolic pathways.

Intestinal flora and serum metabonomics

To elucidate the potential functional interplay between intestinal microbiota and serum metabolomics. An association analysis was conducted on the top 20 microbiota and metabolites exhibiting intergroup differences. In the F and CON groups, 10 metabolites were identified that demonstrated significant positive correlations with *g__Lactobacillus* and substantial negative correlations with *g__Negativibacillus*. These metabolites include diacylglycerols such as DG (18:0/18:2(9Z,12Z)/0:0), DG (18:2(9Z,12Z)/18:1(9Z)/0:0), DG (20:1(11Z)/18:4(6Z,9Z,12Z,13Z)/0:0) (Figure 11A). In both the NE and CON groups, four metabolites were observed to show negative correlations with *g__Anaerotruncus*, namely Malic acid, D-(+)-Malic acid, Xanthine, and Xanthosine. Moreover, Malic acid and D-(+)-Malic acid displayed significant positive associations with *g__Erysipelatoclostridium*, *g__Lachnospiraceae_NK4A136*_group, and *g__Subdoligranulum*, while showing marked negative correlations with *g__Fournierella* (Figure 11B).

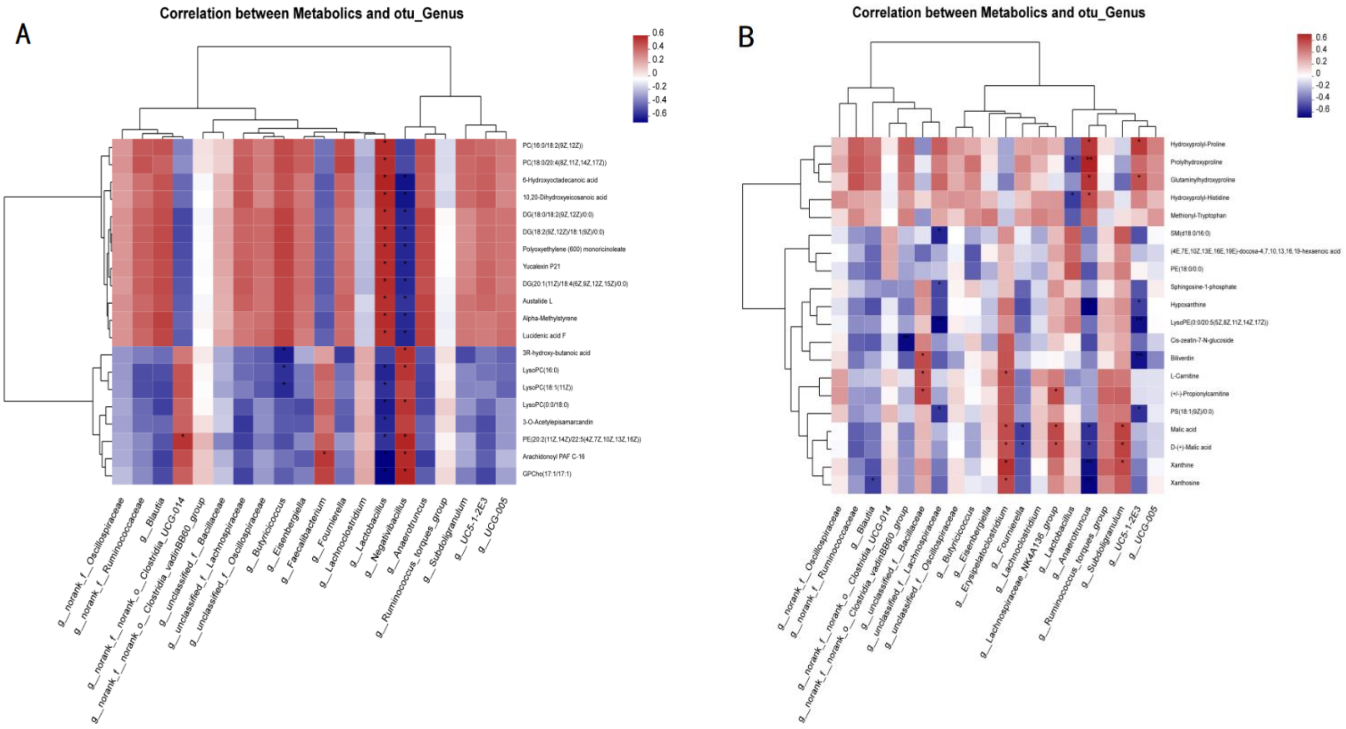

Figure 11. Integrated correlation analysis of gut microbiota and serum metabolites in 24-Day-Old white leghorn chickens with necrotic enteritis. **A**: Correlation analysis of intestinal flora and serum metabolites between group F and group CON; **B**: Correlation analysis of intestinal flora and serum metabolites between group NE and group CON. Red means positive correlation, and blue means negative correlation. Data are presented as means ± SD. * $P < 0.05$，** $P < 0.01$.

Expression of MAPK/ERK signaling pathway related genes in jejunum

The relative mRNA expression levels of *Raf1*, *ERK*, and *MEK* in the *MAPK/ERK* signaling pathway were measured using real-time fluorescence quantitative PCR. As shown in Figure 12, compared to the CON group, the mRNA expression levels of *Raf1*, *ERK*, and *MEK* were significantly increased in the F group ($p < 0.05$). In contrast, the NE group exhibited a decrease in Raf1 mRNA expression ($P > 0.05$) and a significant reduction in *ERK* and *MEK* mRNA levels ($p < 0.05$) compared to the CON group. Furthermore, when compared to the F group, the mRNA expression levels of *Raf1*, *ERK*, and *MEK* in the NE group were significantly lower ($p < 0.05$). These results indicate that the *MAPK/ERK* pathway is suppressed in the NE group.

Expression of proliferating cell nuclear antigen PCNA in the jejunum

The expression of the PCNA gene in the jejunum was assessed using qPCR and immunohistochemistry (IHC). As illustrated in Figure 13, compared to the CON group, chickens in the F group exhibited an elevated PCNA expression level in the jejunal mucosa, although this difference lacked statistical significance ($p > 0.05$). In contrast, the NE group demonstrated a marked reduction in PCNA immunostaining intensity compared to both the F and CON groups, with this decrease reaching statistical significance ($p < 0.05$).

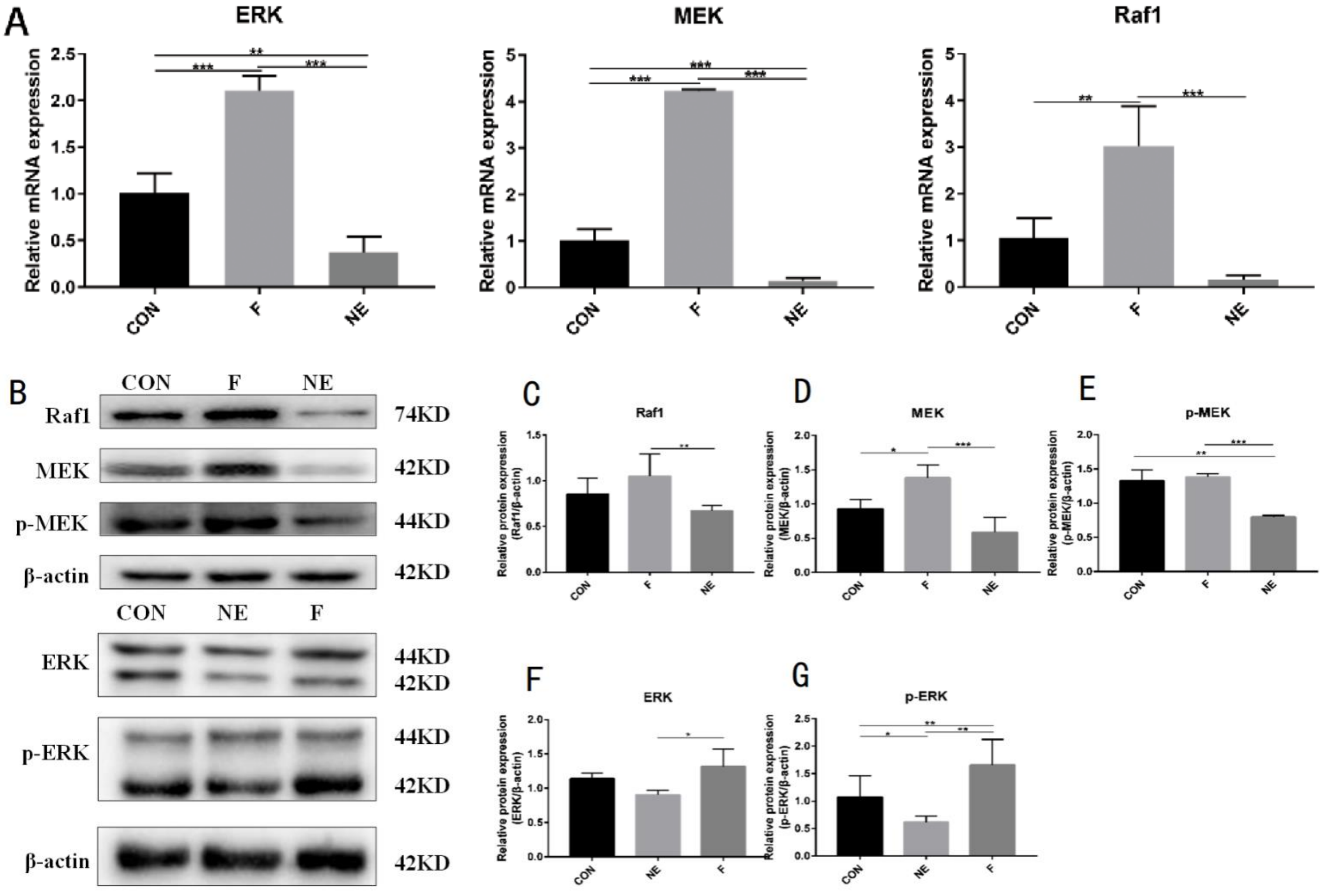

Figure 12. Expression of genes related to the MAPK/ERK signaling pathway. **A**: The relative mRNA expression of MAPK/ERK signaling pathway-related genes. **B**: Protein levels of MAPK/ERK signaling pathway-related genes. **C-G**: Results of the gray-scale analysis of the corresponding protein bands. β-actin served as the loading control. CON: Negative control group; F: Fishmeal-supplemented diet group; NE: Necrotic Enteritis-induced group (*C. perfringens* challenge). Data are presented as means ± SD. * P < 0.05，** P < 0.01，*** P < 0.001.

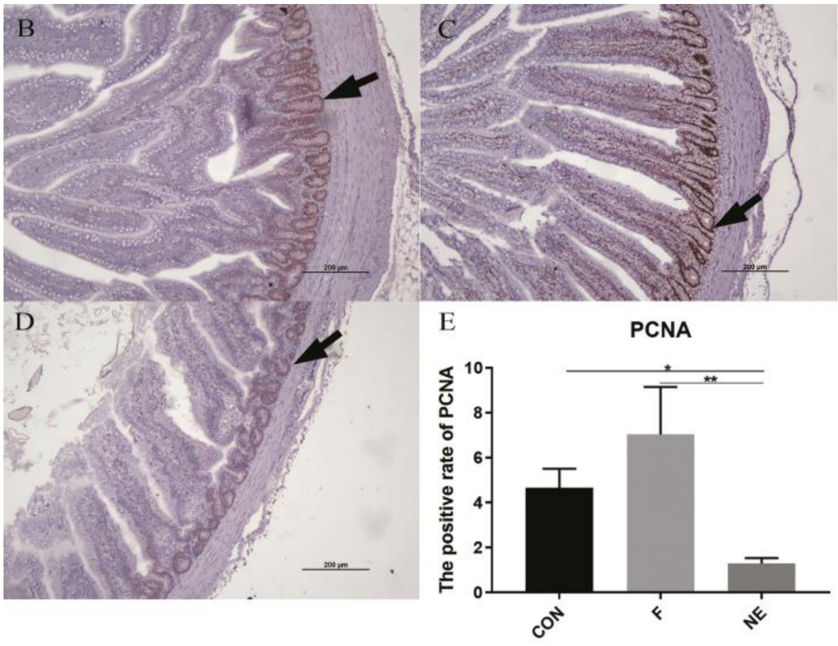

Figure 13. Expression of proliferating cell nuclear antigen (PCNA) gene in jejunum tissue of chickens. **A**: Relative mRNA expression of the PCNA gene. **B-C**: The protein distribution and expression levels of PCNA in the jejunum (IHC, 100×), PCNA positive staining shows a brown signal. **E:** Statistical analysis of PCNA positive rate. CON: Negative control group; **F**: Fishmeal-supplemented diet group; NE: Necrotic Enteritis-induced group (*C. perfringens* challenge). Scale bars = 200 μm. Data are presented as means ± SD. * P < 0.05,** P < 0.01.

DISCUSSION

Chicken necrotic enteritis stands as a recurrent enteric malady within the poultry sector, imposing an annual financial burden of approximately 600 million on the global poultry industry (Wade et al., 2015). The mere presence of *C. perfringens* is insufficient to instigate necrotic enteritis; prevalent modeling approaches encompass co-induction scenarios involving coccidia/*C. perfringens* or fishmeal/*C. perfringens* (Huang et al., 2018; Keerqin et al., 2021). Coccidiosis has the potential to inflict harm upon the intestinal mucosa, prompting the host to generate copious amounts of mucus, thereby fostering a conducive environment for the rapid colonization and multiplication of bacteria (Collier et al., 2008). Supplementation of the diet with high animal protein content(fishmeal) will escalate the reproductive rate of *C. perfringens* within the intestine, consequently leading to damage to the intestinal epithelium by the bacteria and their toxins(NetB toxin, alpha toxin and beta toxin) (Keerqin et al., 2021). Given that *coccidia* infection can induce substantial intestinal damage in chickens (Adhikari et al., 2020), making it indistinguishable from necrotic enteritis, the current study opted for high animal protein fish meal as the inducing agent (Drew et al., 2004b; Williams, 2005; Kaldhusdal et al., 2016).

Necrotic enteritis in chickens predominantly targets the small intestine, with the jejunal and ileal regions exhibiting the highest susceptibility to pathological damage (Goossens et al., 2020). The disease typically presents in a subclinical manner, characterized by minimal chicken mortality, yet causing intestinal damage resulting in compromised nutrient absorption and subsequent weight loss (Timbermont et al., 2011; Caly et al., 2015; Lee and Lillehoj, 2022). Throughout this study, there were no instances of mortality among the chickens in any experimental group. Relative to the other two groups, the body weight of chickens in the infected group exhibited a notable decrease, concomitant with significant small intestinal lesions. Within the infection group, the jejunum displayed shedding and necrosis of intestinal epithelial cells, while the ileum exhibited shortened villi. The integrity of the intestinal epithelial barrier is influenced by various factors, with intestinal tight junction proteins and mucus playing pivotal roles in preventing pathogen infiltration through the epithelial barrier. During the onset of chicken necrotic enteritis, *C. perfringens* generates a toxin that interferes with the tight junctions among intestinal epithelial cells, resulting in decreased expression of tight junction proteins such as Occludin, Claudin-1, and ZO-1, as well as mucin MUC2 in the chicken intestine (Gharib-Naseri et al., 2020; Park et al., 2022). Following necrotic enteritis progression, the infected group showed decreased expression of intestinal barrier-associated proteins, specifically Claudin-1, Occludin, ZO-1, ZO-2, and the mucin MUC2.

It has been reported that the inclusion of fish meal or a combination of fish meal and *C. perfringens* in the diet can lead to substantial alterations in the intestinal flora of chickens, thereby indirectly providing insights into the health status of poultry by examining the intestinal flora composition (Stanley et al., 2014). In the current study, high-throughput sequencing technology was used to analyze the cecal microbiota of chickens. The results showed a decrease in species diversity in the ceca of chickens fed a high fishmeal diet, with a notable reduction in beneficial bacterial genera such as *Faecalibacterium* and *Oscillospira*, and an increase in potentially harmful bacterial groups such as *Escherichia* and *Enterobacteriaceae*. These findings are consistent with previous studies on necrotic enteritis in chickens, indicating a disruption in the balance of the gut microbiota that may negatively impact intestinal health. *Lactobacillus*, a crucial probiotic residing in the intestines of humans and animals, is vital for enhancing the immune function of the intestinal mucosa, inhibiting intestinal inflammation, and curbing the growth of both intestinal pathogens and foodborne pathogens (Xu et al., 2018). *C. perfringens* infection results in a decreased relative abundance of *Lactobacillus*, *Blautia*, and *Ruminococcus*. Notably, *Rumenococcaceae* and *Blautia* are producers of short-chain fatty acids, crucial for providing energy to the intestinal epithelium and preserving epithelial integrity (Wang et al., 2021b; Koh et al., 2016; Bortoluzzi et al., 2019). In chickens suffering from necrotic enteritis, the relative abundance of Erysipelothrixaceae and Clostridium significantly increased. Notably, the abundance of Erysipelothrixaceae exhibited a negative correlation with body fat weight, colonic butyrate concentration, and overall intestinal health (Pham et al., 2020). In the present study, the abundance of *g__Lactobacillus*, *g__Blautia*, and *g__Ruminococcus_gauvreuii_group* decreased in both the F and NE groups, while the abundance of *g__norank_f__norank_o__Clostridia_UCG-014*, *g__norank_f__norank_o__Clostridia_vadinBB60_group*, *g__Colidextribacter*, and *g__Escherichia-Shigella* increased in the F group, and the abundance of *f__Clostridiaceae* and *g__Erysipelatoclostridium* increased in the NE group. These findings indicated that fish meal has the potential to alter the intestinal environment and disrupt the balance of intestinal flora, thereby promoting the growth and colonization of intestinal pathogens. Moreover, when fish meal is combined with *C. perfringens*, it leads to a superimposition of the intestinal flora composition in chickens with necrotic enteritis, causing a marked reduction in beneficial intestinal flora and a notable increase in harmful flora (Wu et al., 2014).

By conducting an enrichment analysis of differential metabolic pathways associated with distinct metabolites, it was discovered that the fishmeal group predominantly engaged in amino acid metabolism, including arginine and proline metabolism, nucleotide metabolisms like purine metabolism, lipid metabolism such as glycerophospholipid metabolism, and other various metabolic pathways. Furthermore, the differential metabolic pathway in the infection group primarily centered on the MAPK signaling pathway, which is closely associated with cell proliferation, differentiation, and migration. Analysis of gut microbiota gene function predictions revealed that the majority of microbiota alterations were linked to metabolism, suggesting the existence of substantial differences in metabolic function across treatments. An examination of the correlation between serum differential metabolites and intestinal flora among the groups revealed significant associations between *g__Lactobacillus* and *g__Negativibacillus* with the metabolite diglyceride (DG). DG, in turn, is implicated in various signaling pathways such as the MAPK, VEGF, and ErbB pathways, all of which play critical roles in regulating cell proliferation, differentiation, and migration (Din et al., 2025). Additionally, metabolites such as malic acid, D-Malic acid, and xanthine exhibited significant correlations with *g__Anaerotruncus*, *g__Erysipelatoclostridium*, *g__Lachnospiraceae_NK4A136_group*, *g__Subdoligranulum*, and *g__Fournierella*. Malic acid is intricately associated with the tricarboxylic acid cycle (TCA cycle), a pivotal component of the body's energy metabolism (MacCannell and Roberts, 2022). D-malic acid is intricately linked to the metabolism of butyrate. The energy sustenance of intestinal epithelial cells predominantly stems from butyrate, a factor pivotal for preserving the integrity of the intestinal barrier (Wang et al., 2021c). Xanthines actively participate in purine metabolism. The intestinal mucosa necessitates heightened concentrations of nucleotides to fuel processes such as energy production, cellular proliferation, and innate immunity. This demand for nucleotide substrates escalates markedly in response to events like tissue injury, infection, and wound healing, as these processes require extensive cell proliferation (necessitating DNA synthesis), heightened ATP production for energy-intensive repair mechanisms, immune cell activation and expansion to combat pathogens, and synthesis of structural proteins such as mucins to restore epithelial barrier integrity (Lee et al., 2020). The aforementioned observations illuminate that these microbial communities may be intricately engaged, either directly or indirectly, in the biosynthesis of these metabolites. Furthermore, these metabolites bear relevance to essential cellular processes encompassing survival, proliferation, and differentiation.

ERK (Extracellular Signal-Regulated Kinase), situated within the cytoplasm, belongs to the MAPK family. Following activation, it translocate to the nucleus, orchestrating the modulation of various transcription factors through phosphorylation. Moreover, it instigates gene expression in response to extracellular stimuli, playing a pivotal role in cellular processes such as proliferation, migration, differentiation, and apoptosis (Guo et al., 2020). Investigations revealed the inhibition of the ERK signaling pathway following intestinal injury. Some studies have also suggested that the ERK signaling pathway is involved in the proliferation and differentiation of epithelial cells following intestinal mucosal injury, and inhibition of this pathway exacerbates intestinal damage and apoptosis (ZHAN Yuanquan 2022). In the present study, suppression of ERK signaling pathway-related gene expression was observed in infected chickens, indicating impaired proliferation of intestinal epithelial cells in chickens with necrotic enteritis, which hindered post-injury intestinal repair.Notably, the MAPK/ERK signaling pathway intricately influences the expression of tight junction proteins; its inhibition correlates with a diminished expression of the tight junction protein Claudin-1. In both *in vivo* and *in vitro* experiments, it has been demonstrated that the activation of the ERK signaling pathway has the capability to enhance the expression of intestinal tight junction proteins (Dai et al., 2012). In the present study, it was observed that the expression of tight junction proteins in the intestinal tract of chickens in the infection group exhibited a decline, a phenomenon that could potentially be attributed to the mediation of the ERK signaling pathway. Proliferating cell nuclear antigen (PCNA) is a protein crucial to the cell cycle, predominantly localized in the base and middle regions of intestinal crypts (Y et al., 2016), where it actively engages in essential DNA metabolism processes, including replication and repair. The synthesis and expression of PCNA are intricately linked to the process of cell proliferation (Morris and Mathews, 1989; Cardano et al., 2020; Wang et al., 2022). The current investigation revealed a significant decrease in PCNA expression among chickens in the infection group in comparison to both the negative control group and the fish meal group, suggesting a hindrance in the proliferation of intestinal epithelial cells in chickens afflicted with necrotic enteritis, consequently leading to impaired renewal and repair mechanisms within the intestinal epithelium.

CONCLUSION

Feeding fish meal alone is insufficient to directly induce necrotic enteritis; however, it exerts a significant impact on the intestinal microbiota composition, leading to a reduction in beneficial flora such as *Lactobacillus* and *Blautia*. The change in the makeup of the intestinal flora encourages the growth of pathogenic *C. perfringens*, which in turn makes it more likely for subclinical necrotic enteritis to develop. Following the onset of necrotic enteritis, the mucosal layers of the jejunum and ileum in afflicted chickens sustain damage, consequently leading to impaired nutrient absorption capabilities. At the same time, an imbalance in the intestinal microbiota, known as dysbiosis, interferes with the MAPK/ERK metabolic pathway. As a result, this hinders the multiplication of intestinal epithelial cells and impairs the restoration of the intestinal epithelial barrier.Further research could focus on exploring potential interventions that can reverse the dysbiosis-induced disruption of the MAPK/ERK metabolic pathway. For example, studying the effects of specific probiotics or prebiotics on modulating the gut microbiota composition and its subsequent impact on the recovery of the intestinal epithelial barrier function through the regulation of the MAPK/ERK pathway. This may lead to the development of novel therapeutic strategies for intestinal diseases related to microbiota imbalance and epithelial barrier dysfunction.

DECLARATION

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Authors' contributions

Yunting CHU implemented the research and contributed to data collection, database creation, and preparation of the manuscript. Liyue SUN and Yanyi LI were also involved in preparing the manuscript and data analysis. Changqin GU guided the research and manuscript preparation. All authors checked and approved the final version of the manuscript for publishing in the present journal.

Ethical considerations

All authors have checked statistical analysis as well as the ethical issues, including plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy.

Availability of data and materials

The data of the article will be provided by the corresponding author according to reasonable requests.

Competing interests

There is no competing interest in this research.

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Assessing the Efficiency of Different Coccidiostats against *Eimeria* spp. in Broiler Chickens Grown in the Floor Pen System

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ABSTRACT

Coccidiosis is a critical parasitic disease that affects poultry through severe impairment in growth and yield. The present study aimed to evaluate the efficacy of combined coccidiostats in broiler chickens reared in floor pen systems. A total of 1200 Cobb500 broiler chickens were divided into five treatment groups, each group consisting of eight replications, which were based on different coccidiostats, including Treatment 1 (Control), Treatment 2 (Maduramicin-Nicarbazin), Treatment 3 (Narasin-Nicarbazin), Treatment 4 (Semduramicin-Nicarbazin), and Treatment 5 (Monensin-Nicarbazin). Performance parameters were recorded on days 7, 14, 21, 28, and 33. Treatment 3 exhibited the lowest feed consumption per chicken (2.797 kg), followed by Treatment 5 (2.825 kg) and Treatment 2 (2.835 kg). The control group consumed the highest amount of feed (2.880 kg), followed by Treatment 4 (2.888 kg). During week five, Treatment 5 demonstrated the highest body weight gain (1915 g), whereas Treatment 2 showed the lowest (1808 g). The lowest feed conversion ratio (FCR) was observed in Treatment 5 (1.500), followed by Treatment 3 (1.504), Treatment 4 (1.547), Treatment 2 (1.571), and the control group (1.576). However, the maximum intestinal lesion scores were noted in the control group, compared to the experimental groups observed on the culling day. Treatment 5 indicated the lowest lesion scores, followed by treatments 3, 4, and 2. Among the observed species, *Eimeria (E.) tenella* exhibited the highest lesion scores compared to *E. maxima* and *E. acervulina* in all the groups. Therefore, the effective use of these coccidiostats is crucial for preventing and managing coccidiosis outbreaks in poultry.

Keywords: Broiler chicken, *Eimeria*, Feed conversion ratio, Floor pen system, Monensin, Narasin

INTRODUCTION

Poultry meat holds significant global importance as a major source of nutrition, with an annual production of approximately 120 billion tons, predicted to double by 2050 (Alexandratos and Bruinsma, 2012). Poultry meat accounts for 78% of the total meat demand in Bangladesh and is highly regarded for its nutritional benefits (Rony et al., 2021). Despite the expansion of commercial poultry farming in Bangladesh, the poultry industry faces significant challenges, notably from diseases such as coccidiosis and Newcastle disease (ND), which severely impact productivity (Datta et al., 2025). Moreover, ND remains endemic in the country and further hampers production (Hossain et al., 2023). However, coccidiosis remains a critical parasitic disease affecting poultry worldwide. Globally, coccidiosis leads to an estimated annual economic loss of three billion dollars (Dalloul and Lillehoj, 2006; Noack et al., 2019), primarily affecting weight gain and feed efficiency of chickens, which collectively account for approximately 70% of production costs in poultry farming. Coccidiosis is a major threat to commercial broiler farms in Bangladesh, causing considerable financial losses due to mortality, retarded growth, and the prevention and treatment costs (Quiroz-Castañeda, 2018).

Protozoan parasites of the genus *Eimeria (E.)* are responsible for the disease, which affects the intestinal tract and is commonly found across different poultry

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species, particularly in chickens (Aarthi et al., 2010). Seven *Eimeria* spp., identified as *E. tenella*, *E. acervulina*, *E. maxima*, *E. necatrix*, *E. brunetti*, *E. mitis*, and *E. praecox*, infect poultry (Shirley et al., 1986). Among the *Eimeria* spp., *E. tenella*, *E. acervulina*, and *E. necatrix* are particularly problematic in Bangladesh (Iqbal et al., 2017; Alam et al., 2020). Avian coccidiosis is a highly infectious disease that can spread easily through insects, litter, apparatus, contaminated feed, and water. The disease is characterized by bloody diarrhea, enteritis, drooping wings, emaciation, and retarded growth (Abebe and Gugsa, 2018). Coccidiosis exhibited an average occurrence of 22.8% in the poultry industry, resulting in substantial economic losses in commercial poultry industries in Bangladesh (Rahman et al., 2019). The inadequate biosecurity and management practices have led to a very high prevalence of coccidiosis in many farms in Bangladesh (Datta et al., 2025). Being a tropical country, Bangladesh experiences a significant seasonal fluctuation in humidity and temperature, which accelerates the sporulation of *Eimeria* spp. Proper therapeutic measures are essential in controlling the disease as well as maintaining sustainable poultry production in the country. Biosecurity, sanitation protocols, and proper drug usage are crucial for sustainable poultry production in tropical environments (Chapman, 2018). The use of different coccidiostats, either separately or in combination, proves to be a successful substitute in the battle against coccidiosis (Quiroz-Castañeda and Dantán-González, 2015). Coccidiostats are divided into two main categories: chemical compounds and ionophores. Ionophores are commonly used as coccidiostats in poultry feed because they lead to slower development of resistance and can help enhance the host's immunity (Noack et al., 2019). However, prolonged use of coccidiostats triggers the emergence of drug-resistant strains, underscoring the need for effective control strategies (Hayajneh et al., 2024).

Gazipur is indeed a significant hub for poultry production in Bangladesh, especially for broiler chickens (Kamruzzaman et al., 2000), which not only supports local livelihoods but also meets the growing demand for poultry meat in urban areas. This industry contributes to the national economy and plays a vital role in food security for the country. Gazipur exhibits a wide tropical humid climate, seasonal variations in rainfall, high temperature, and high humidity, which are favorable for the survival and development of *Eimeria* spp. (Rony et al., 2021). As a result, thousands of birds die from severe coccidiosis every year, largely due to the indiscriminate use of coccidiostats by farmers (Martins et al., 2022). This indiscriminate use of coccidiostats contributes to the growing issue of drug resistance, making it immensely difficult to control outbreaks of coccidiosis. Therefore, the present study aimed to investigate the efficacy of four different combined coccidiostats against *Eimeria* spp. in broiler chickens.

MATERIALS AND METHODS

Ethical approval

All animal studies followed the ethical standards set by the Faculty of Animal Science and Veterinary Medicine, Sher-e-Bangla Agricultural University (SAU), Dhaka-1207, Bangladesh.

Study area

The current study was conducted at the Nourish Poultry and Hatchery Limited trial farm situated in Shreepur Upazila of Gazipur District, Bangladesh (24° 11' 60.00" N and 90° 28' 0.12" E) from April 2020 to May 2020.

Experimental design

A total of 1,200 day-old Cobb500 broiler chickens, being of mixed sex and having an average body weight of 38 g, were sourced from Nourish Hatchery, Bangladesh. The broiler chickens were reared in a floor pen system and maintained under standard nutrition and management practices of Cobb500 management guidelines until they reached 33 days of age (Hassan et al., 2016). The feeding program included pre-starter feed for the first 10 days, followed by starter feed from days 11 to 21, and concluded with grower rations (Toledo et al., 2011). The water and feed were supplied *ad libitum*. The feed ingredients included corn, soybean meal, rice bran, soybean oil, mono calcium phosphate (MCP), limestone powder, sodium bicarbonate, choline chloride, salt, vitamin and trace mineral premix, amino acids, and various feed additives (Table 1). The room temperature varied from 30°C to 34°C during the first week, gradually decreasing by the fourteenth day (Akter et al., 2023). Continuous lighting for twenty-four hours was provided for the entire period of the experiment. Vaccination was performed for ND (Nobilis® ND Clone 30, Germany) on days 5 and 25 through the intraocular route, and for Infectious bursal disease (IBD; Nobilis® Gumboro 228E, USA) on days 11 and 18 of age via drinking water. Since ND is endemic in Bangladesh (Hossain et al., 2023), and hatchery vaccination is not practiced, live ND vaccination is commonly used as a preventive measure on day five. Chlorinated water was provided, but no medications such as supplements or antibiotics were administered during the rearing period. No chickens were intentionally challenged with *Eimeria* spp., and no preventive measures were taken for coccidiosis, except for using coccidiostats in feed.

Table 1. Feed ingredients used in the pre-starter, starter, and grower rations for broiler chickens from day 1 to 33

Feed ingredients	**Pre-starter (kg)**	**Starter (kg)**	**Grower (kg)**
Maize	510	523	524.5
Soyabean meal 46%	350	318	296
Full Fat soya 34%	55	65	75
Rice polish	30	40	50
Soyabean oil	12	18	22
Limestone powder 37%	13	10	10
Salt	1.8	2.1	2.2
Sodium bi carbonate	3.5	3.2	3.3
MCP	10	8	6
DL Methionine	3.7	3.2	2.5
L Lysine	2.8	1.8	1.3
L Threonine	1.4	1	0.6
Vitamin premix	1	1	1
Mineral premix	0.5	0.5	0.5
Choline chloride 60%	0.8	0.7	0.6
Toxin binder	1	1	1
Coccidiostat	0.5	0.5	0.5
Probiotic	0.4	0.4	0.4
Prebiotic	0.5	0.5	0.5
Xylanase enzyme	0.1	0.1	0.1
Protease enzyme	0.2	0.2	0.2
Phytase enzyme	0.1	0.1	0.1
Emulsifier	0.5	0.5	0.5
Antioxidant	0.2	0.2	0.2
Betaine anhydrous	1	1	1
Total	1000	1000	1000
ME (Kcal/kg)	2952.60	3029.67	3075.25

MCP: Mono calcium phosphate, DL methionine: DL-2-amino-4-(methylthio) butanoic acid, ME: Metabolized energy

Coccidiostats

Since Nicarbazin was used in combination with other coccidiostats, four commercially available combined coccidiostats were used, including the Maduramicin-treated group (Treatment 2, Maduramicin 0.75 ppm + Nicarbazin 8 ppm; Nimax®, Huvepharma, Bulgaria), the Narasin-treated group (Treatment 3, Narasin 8 ppm + Nicarbazin 8 ppm; Maxiban®, Elancho, USA), the Semduramicin-treated group (Treatment 4, Semduramicin 3 ppm + Nicarbazin 8 ppm; Aviax® Plus, Phibrio, Brazil), the Monensin-treated group (Treatment 5, Monensin 8 ppm + Nicarbazin 8 ppm; Monimax®, Huvepharma, Bulgaria), and the Control group (Treatment 1, no coccidiostat was used). These coccidiostats were provided to the broiler chickens from day 1 to day 33 through the feed.

Trial design

The entire shed was divided into 40 pens, each fenced by iron netting. Thirty chickens were placed in each pen (36 sq ft). The five treatments were randomly assigned to eight pens throughout the shed. Chicken groups were assigned by treatment doses (Table 2).

Data collection

Data regarding mortality rates (%), body weight (g), feed consumption (kg), feed conversion ratio (FCR), and intestinal lesion scores were collected at different intervals on days 7, 14, 21, and 28 from all five groups. To assess body weight gain, eight chickens were measured weekly using a standard weight scale. Ten percent of chickens (Three) from each pen were weighed weekly, with all chickens being weighed on day 33.

Moreover, weekly FCR per pen was calculated and averaged for each group. The FCR was calculated by dividing the total feed intake by the live weight of the chickens (Mwale et. al., 2008). Ten chickens per group were sacrificed randomly for post-mortem examination on day 28. Then, the intestinal lesion scores were evaluated using the methods provided by Johnson and Reid (1970) and Kang et al. (2019).

Table 2. Experimental group of broiler chickens with treatment dosage for coccidiosis control

Groups	**Coccidiostats**	**Doses**	**Duration**
Treatment 1	Control/Without coccidiostat		1-33 days
Treatment 2	Maduramicin 0.75 ppm + Nicarbazin 8 ppm	500 g/MT	1-33 days
Treatment 3	Narasin 8 ppm + Nicarbazin 8 ppm	500 g/MT	1-33 days
Treatment 4	Semduramicin 3 ppm + Nicarbazin 8 ppm	500 g/MT	1-33 days
Treatment 5	Monensin 8 ppm + Nicarbazin 8 ppm	500 g/MT	1-33 days

Statistical analysis

One-way ANOVA was used to statistically analyze the data using the statistical program for social sciences (SPSS) version 16. With a significance level of $p < 0.05$, Duncan's multiple comparison test was employed to assess differences in means.

RESULTS

Weekly feed intake

During week 5 (Day 28 to Day 33), the average weekly feed intake per chicken was 2.880 kg for the control group, 2.835 kg for Treatment 2, 2.797 kg for Treatment 3, 2.888 kg for Treatment 4, and 2.825 kg for Treatment 5. Treatment 3 recorded the lowest feed consumption (2.797 kg) per chicken, followed by Treatment 5, Treatment 2, the Control group, and Treatment 4, respectively (Table 3), all of which were found statistically significant ($p < 0.05$).

Body weight gain

At week 5 (Day 28 to 33), the average body weight of broiler chickens treated with Treatment 5 documented the highest body weight gain (1915 g), followed by Treatment 3 (1883 g), Treatment 4 (1832 g), and Treatment 2 (1808 g) (Table 4). The differences in body weight gain across the treatment groups were statistically significant ($p < 0.05$).

Food conversion ratio

Treatment 5 demonstrated the lowest FCR (1.500), followed by Treatment 3 (1.504), Treatment 4 (1.547), Treatment 2 (1.571), and the control group (1.576) at the culling week for broiler chickens, which were statistically significant ($p < 0.05$). In this study, flocks raised on feed without coccidiostats showed a greater FCR compared to those raised on feed containing anticoccidials (Table 5).

Lesion score

On day 28, ten chickens from each dietary group were subjected to post-mortem examination to assess lesion scores (Figure 1; Johnson and Reid, 1970; Kang et al., 2019). In Treatment 5, *E. acervulina*, *E. tenella*, and *E. maxima* had lesion scores of 1, 3, and 4, respectively. For *E. acervulina*, *E. tenella*, and *E. maxima*, Treatment 3 had lesion scores of 1, 7, and 5, respectively. For the same species, Treatment 4 demonstrated lesion scores of 2, 6, and 9, whereas Treatment 2 had lesion scores of 2, 11, and 13, respectively. For these species, non-treated broiler chickens exhibited lesion scores of 4, 15, and 13, respectively. However, the highest lesion scores (4, 15, and 13) were recorded in the non-treated group, while the lowest scores (1, 3, and 4) were seen in broilers subjected to Treatment 5, followed by Treatments 2, 3, and 4. The lesion scores of *E. tenella* were higher than those of *E. maxima* and *E. acervulina* (Table 6).

Table 3. Feeding performance according to weekly feed intake per broiler chicken from day 1 to 33

Feed	Total flock	Week 1 (Kg)	Week 2 (Kg)	Week 3 (Kg)	Week 4 (Kg)	Week 5 (Kg)	p-value
Treatment 1 (Control group)	Cobb500 (30 × 8)	0.149	0.565	1.193	2.110	2.880*	0.05
Treatment 2 (Md + Nb)	Cobb500 (30 × 8)	0.154	0.574	1.156	2.075	2.835*	
Treatment 3 (Nr + Nb)	Cobb500 (30 × 8)	0.151	0.562	1.132	2.079	2.797*	
Treatment 4 (Sd + Nb)	Cobb500 (30 × 8)	0.150	0.572	1.220	2.131	2.888*	
Treatment 5 (Mn + Nb)	Cobb500 (30 × 8)	0.149	0.572	1.144	2.066	2.825*	

Md+Nb: Maduramicin + Nicarbazin, Nr+Nb: Narasin + Nicarbazin, Sd+Nb: Semduramicin + Nicarbazin, Mn+Nb: Monensin + Nicarbazin. *The values were significantly different at $p < 0.05$

Table 4. Weekly body weight gains in broiler chickens from day 1 to 33

Feed	Total flock	Week 1 (g)	Week 2 (g)	Week 3 (g)	Week 4 (g)	Week 5 (g)	p-value
Treatment 1 (Control group)	Cobb500 (30 × 8)	195	525	823	1412	1805*	0.05
Treatment 2 (Md + Nb)	Cobb500 (30 × 8)	197	534	850	1435	1808*	
Treatment 3 (Nr + Nb)	Cobb500 (30 × 8)	201	540	864	1468	1883*	
Treatment 4 (Sd + Nb)	Cobb500 (30 × 8)	199	535	862	1467	1832*	
Treatment 5 (Mn + Nb)	Cobb500 (30 × 8)	201	541	913	1517	1915*	

Md+Nb: Maduramicin + Nicarbazin, Nr+Nb: Narasin + Nicarbazin, Sd+Nb: Semduramicin + Nicarbazin, Mn+Nb: Monensin + Nicarbazin. *The values were significantly different at $p < 0.05$

Table 5. Weekly feed performance according to the feed conversion ratio in broiler chickens from day 1 to 33

Feed	Total flock	Week 1	Week 2	Week 3	Week 4	Week 5	p-value
Treatment 1 (Control group)	Cobb500 (30 × 8)	0.789	1.095	1.436	1.485	1.576*	0.05
Treatment 2 (Md + Nb)	Cobb500 (30 × 8)	0.757	1.070	1.405	1.470	1.671*	
Treatment 3 (Nr + Nb)	Cobb500 (30 × 8)	0.751	1.052	1.311	1.408	1.504*	
Treatment 4 (Sd + Nb)	Cobb500 (30 × 8)	0.756	1.060	1.327	1.417	1.547*	
Treatment 5 (Mn + Nb)	Cobb500 (30 × 8)	0.740	1.045	1.306	1.391	1.500*	

Md+Nb: Maduramicin + Nicarbazin, Nr+Nb: Narasin + Nicarbazin, Sd+Nb: Semduramicin + Nicarbazin, Mn+Nb: Monensin + Nicarbazin. *The values were significantly different at $p < 0.05$

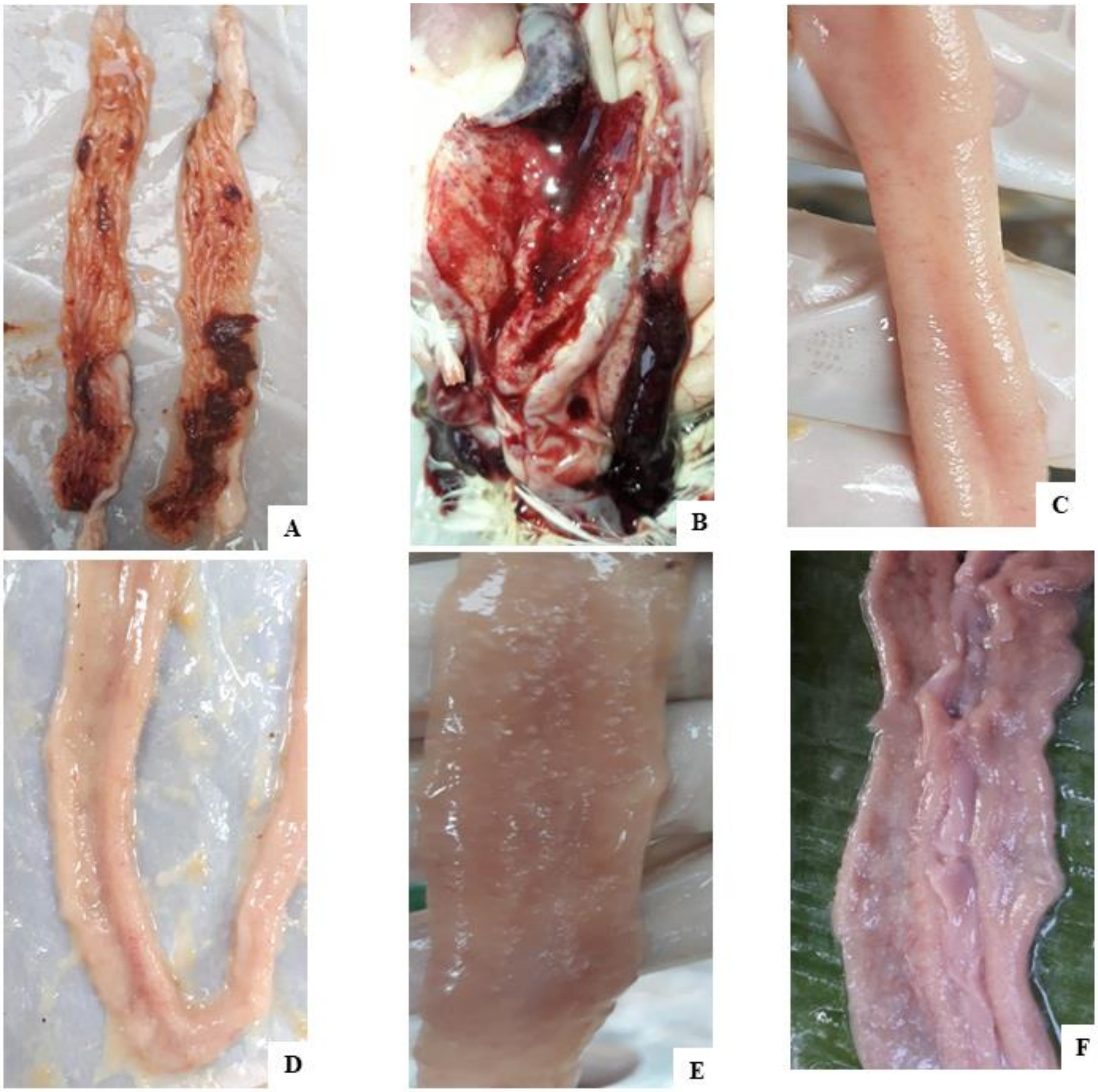

Figure 1. Post-mortem lesion score of *Eimeria* species in broiler chickens on day 28. **A** and **B**: Lesion score of *Eimeria tenella*, **C** and **D**: Lesion score of *Eimeria maxima*, **E** and **F**: Lesion score of *Eimeria acervulina*.

Table 6. Lesion score of different *Eimeria* species treated with different coccidiostats in broiler chickens on day 28

Treatment ***Eimeria* spp.**	**Lesion score of treatment 1 (Control group)**	**Lesion score of Treatment 2 (Md + Nb)**	**Lesion score of Treatment 3 (Nr + Nb)**	**Lesion score of Treatment 4 (Sd + Nb)**	**Lesion score of Treatment 5 (Mn + Nb)**
E. acervuline	4	2	1	2	1
E. maxima	13	13	5	9	4
E. tenella	15	11	7	6	3

Source of lesion score: Johnson and Reid (1970) and Kang et al. (2019). Md+Nb: Maduramicin + Nicarbazin, Nr+Nb: Narasin + Nicarbazin, Sd+Nb: Semduramicin + Nicarbazin, Mn+Nb: Monensin + Nicarbazin.

DISCUSSION

Eimeria species are responsible for causing substantial economic losses in the commercial poultry industry in Bangladesh. The reduced efficacy of anticoccidials has adverse effects on body weight gain and FCR in chickens (Abdelhady et al., 2021). The present study indicated the presence of variation in FCR, feed intake, lesion score, and body weight gain due to the use of coccidiostats in feed. Coccidiosis outbreaks can be avoided by using a proper combination of anticoccidial medicines in feed (Kabell et al., 2006). Ionophores are the most recommended treatment for coccidiosis due to their slower resistance development, enabling the host to gain immunity following initial exposure (Chapman et al., 2010). Although many coccidiostats have shown to develop resistance in the field, a field trial was conducted to test four specific combinations in broiler chicken feed: Monensin plus Nicarbazin, Narasin plus Nicarbazin, Maduramicin plus Nicarbazin, and Semduramicin plus Nicarbazin.

In the present study, the chickens treated with Narasin showed the lowest feed consumption per chicken, whereas the highest body weight gain (1915 g) was documented in the Monensin-treated group. These findings align with the observations of MacPherson (1978) and Vereecken et al. (2020), who recorded that Monensin contributes to enhancing body weight gain in broiler chickens. On the other hand, Jeffers et al. (1988) found that chickens treated with Monensin had lower body weight gain than those treated with Narasin. The difference in body weight gain might be due to the flock size of chickens, the composition of coccidiostats in feeds, geographical locations, and the feeding methods. The lowest FCR (1.500) was observed in the Monensin-treated group during the culling week, which was also supported by Vereecken et al. (2020) and MacPherson (1978), who recorded a reduced FCR in a floor pen system when Monensin was used in feed. The present study demonstrated that supplementing broiler feed with coccidiostats improved weight gain and reduced FCR compared to feed without them. The present findings aligned with those reported by Stallbaumer and Daisy (1988) in Europe (England, Netherlands, Spain, Germany, Italy) and El-Morsy et al. (2016) in Egypt.

Post-mortem examination findings in this study were based on naked-eye observations of the intestines. Chickens without coccidiostat treatment demonstrated the highest lesion scores, while the Monensin-treated group had the lowest lesion scores. The number of lesion scores in the control group was followed by the Maduramicin-treated group, the Semduramicin-treated group, and the Narasin-treated group. However, Ruff et al. (1980) found that Narasin was more effective than Monensin in reducing intestinal lesion scores in chickens. In this study, *E. tenella* showed higher lesion scores compared to *E. maxima* and *E. acervulina*, which was consistent with the findings of Amer et al. (2010). Alam et al. (2020) indicated that broiler chickens in Bangladesh commonly suffer from mixed infections of *E. tenella* and *E. acervulina*, reflecting the higher prevalence of coccidiosis due to inadequate farm management practices. The majority of anticoccidials in Bangladesh were found to be less effective in broiler production because of their overuse, abuse, or misuse, and occasionally, they are used as feed supplements for an extended period. Chickens are more susceptible to developing resistance to coccidiostats and medicines than other farm animals (Einstein et al., 1994). The combination of Monensin and Nicarbazin was used in the present study due to its slow resistance development against *Eimeria* spp., a fact supported by Vereecken et al. (2020).

In the present study, chickens were raised with different coccidiostats throughout the rearing period. However, some broiler chickens showed a lesion score of *Eimeria* spp., which was similar to that of Nematollahi et al. (2009). These lesion scores could result from resistance development or the abuse of coccidiostats (Hadipour et al., 2011). Subclinical coccidiosis, which is responsible for poor economic output, FCR, and body weight gain, is caused by drug resistance (Györke et al., 2013). Chemical coccidiostats exhibited a quick rise in resistance, whereas the opposite was true for ionophores (Noack et al., 2019).

The sole use of Nicarbazin exhibits delayed resistance development. Products containing both chemicals, coccidiostats and ionophores, develop resistance more slowly than those having merely one of the coccidiostats or ionophores due to their distinct mode of action (Chapman and Rathinam, 2022). Furthermore, Monensin and Nicarbazin have synergistic effects, and their cumulative strength is higher than the sum of the potencies of the individual components (Callender and Jeffers, 1980). Therefore, it is recommended to go through a rotational program to stop drug resistance, which is consistent with the findings of Györke et al. (2013).

CONCLUSION

Coccidiosis remains a major constraint for profitable broiler farming in Bangladesh. The widespread and prolonged use of coccidiostats has led to a global development of resistance. This is the first investigation on these coccidiostats in broilers in Bangladesh. In the present study, the Monensin-treated group (Treatment 5) recorded the highest body weight gain (1915 g), the lowest FCR (1.500), and the smallest lesion scores (1, 3, 4), whereas the Narasin-treated group (Treatment 3) documented the lowest feed intake. To minimize the incidence of resistance, the rotational approach of the coccidiostat in individual/shuttle programs could unlock a window for broiler farmers to control coccidiosis and promote sustainable poultry production. Further studies should explore the long-term impacts of rotational and integrated coccidiostat strategies, including natural alternatives, to enhance efficacy and minimize resistance development in broiler chickens.

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Author's contributions

Mohammad Arifur Rahman and Al Wasef collected the data and samples, designed the methodology, wrote the manuscript, and edited the manuscript. Nitol Chandra Das, Md. Rubel Islam, Md. Nooralam Islam, Nurul Basar Sarker, Muhammad Abdul Mannan, and Md. Aftabuzzaman contributed to the methodology and data collection. Uday Kumar Mohanta conceptualized, supervised, and reviewed the manuscript. All authors checked and confirmed the last edition of the submitted article.

Competing interests

The authors state that they have no conflicts of interest for publication.

Availability of data and materials

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All authors confirm that they have followed ethical guidelines, including those related to plagiarism, consent for publication, research misconduct, data falsification, duplicate submission, and redundancy.

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Molecular Characterization of Infectious Laryngotracheitis Virus Circulating in Egypt during 2023

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ABSTRACT

Since its discovery in Egypt in 1983, the infectious laryngotracheitis virus (ILTV) has continued to spread, leading to substantial losses for poultry farms. This study aimed to identify and molecularly characterize the ILTV currently in circulation in Egypt in 2023. Fifteen pooled trachea and lung samples were collected from affected vaccinated and non-vaccinated layer farms (Shiver, Lohhman, Brown layer, and Aviagel) aged from 10-30 weeks old spread throughout eight governorates in Egypt. A real-time polymerase chain reaction (PCR) is used to detect ILTV. Ten positive samples representing various governorates were chosen for partial sequencing of the *ICP4* gene, and four positive samples were chosen for sequencing of the *TK, gD, and gG* genes. Using phylogenetic analysis, the *ICP4* clusters into chicken embryo origin vaccine and vaccine-like strains (CEO) and tissue culture origin vaccine and vaccine-like strains (TCO) and wild type. The GAHV-1-Egy-WO5-2023, GAHV-1-Egy-WO7-2023, and GAHV-1-Egy-WO8-2023 strains cluster in the CEO group with the CEO vaccine and vaccine-like strains with 98.9%–100% amino acid identity (A.A.); However, the remaining strains (GAHV-1-Egy-WO1-2023, GAHV-1-Egy-WO2-2023, GAHV-1-Egy-WO3-2023, GAHV-1-Egy-WO4-2023, GAHV-1-Egy-WO6-2023, GAHV-1-Egy-WO9-2023, and GAHV-1-Egy-WO10-2023) were clustered in the TCO group with TCO vaccine strains and vaccine-like strains with 100 % A.A. By analysis of *gD, gG, and Tk* genes, there is no difference between the TCO and CEO groups. Compared with several reference CEO and TCO vaccine strains and vaccine-like strains, some new specific mutations (Q161H and Q182H) were recorded in the *ICP4* in GAHV-1- Egy-WO8-2023. In addition, A34G, and P276L were recorded in the *gD* gene in the GAHV-1-Egy-WO1-2023 and GAHV-1-WO4-2023. Also, the R115I, G126A, and S163I were recorded in the *TK* gene in GAHV-1-Egy-WO8-2023 and A99E in GAHV-1-Egy-WO5-2023, which can affect the virulence and pathogenicity of the virus, which may be due to the reactivation of the vaccine strain by the bird-to-bird transmission or viral recombination. In conclusion, the ILTV outbreaks in poultry farms across several regions of Egypt during 2023 were induced by vaccine strains derived from TCO and CEO. Thus employing innovative vector vaccines and reassessing the current vaccination regimens is necessary.

Keywords: Infectious laryngotracheitis, Diagnosis, isolation, Genetic characterization

INTRODUCTION

Infectious laryngotracheitis virus (ILTV) is an acute respiratory disease affecting chickens all over the world and leading to significant economic losses (Bagust, 2003; Hong et al., 2024; Abebe et al., 2024; Shao et al., 2025). The ILTV is identified as Gallid herpesvirus 1 (GaHV-1) and belongs to the Herpesviridae family and subfamily of Alphaherpesvirinae (Elshafiee et al., 2022). The ILTV causes varying rates of morbidity and mortality of about 90% and 70%, respectively, as previously described by Bagust et al. (2000) and Coppo et al. (2013) based on the circulating strains' pathogenicity and whether they are co-infected with other respiratory pathogens (Menendez et al., 2014). The ILTV genome is 150-155 kilobases of linear double-stranded DNA that codes for inverted internal (IR)

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and terminal (TR) repeats, unique long (UL), and unique short (US) repeats. UL and US sections encode 80 open reading frames (ORFs), whereas inverted repeats only contain three genes (*ICP4*, *US10*, and *sORF4/3*), as described by Lee et al. (2011) and Wu et al. (2022). The *ICP4* gene plays a crucial role in distinguishing distinct ILT strains (Creelan et al., 2006, Wang et al., 2021, Ponnusamy et al., 2022).

According to Lee (2011), ILTV encodes thymidine Kinase and 11 glycoproteins (*gL*, *gM*, *gH*, *gB*, *gC*, *gK*, *gG*, *gJ*, *gD*, *gI*, and *gE*) that are encoded by highly conserved ORFs. They have an essential role in the virulence and reproduction of viruses. The Glycoprotein G (*gG*) and the *TK* gene have been linked to ILTV pathogenicity (Han and Kim, 2001), and the *gD* protein functions as a virus-binding receptor to vulnerable cells (Di Giovine et al., 2011). Furthermore, the production of neutralizing antibodies is attributed to the glycoprotein B (*gB)* and glycoprotein D (*gD*) proteins (Rouse and Kaistha, 2006; Devlin et al., 2006; Lazear et al., 2012).

To prevent and control ILTV outbreaks, vaccines with modified live viruses are globally approved and utilized (Groves et al., 2019). Vaccine strains are propagated using either chicken embryos (CEO; chicken embryo origin) or cell culture (TCO; tissue culture origin). It has been seen that an attenuated virus vaccine can revert to virulence during bird-to-bird transmission (Groves et al., 2019; Kardoğan, Sarıçam, 2024). Numerous nations across the world, including the USA (Oldoni and García, 2007), Australia (Blacker et al., 2011), and Canada (Ojkic et al., 2006), have reported that modified live ILTV vaccinations, in particular CEO vaccines, are linked to illness outbreaks (Yi et al., 2024).

The most accurate and popular method for detecting ILTV infection is real-time PCR, which has good sensitivity, specificity, and speed (Davidson et al., 2015; Santander Parra et al., 2018; Asif et al., 2022). Viral isolation and serological assays can also identify an ILT virus infection. However, they are not very sensitive (Creelan et al., 2006; Zhao et al., 2013). Certain gene fragment sequencing appears helpful for defining field isolates and distinguishing vaccine strains from field isolates (Chacón and Ferreira, 2009; Shehata et al., 2013).

ILTV was first detected in Egypt in 1983 (Tantawy et al., 1983). Later, from 2011 to 2021, many field outbreaks were identified in various Egyptian governorates, leading to mortality rates and egg production declines of 20% to 35%, respectively. There was a noticeable sequence similarity between the detected viral strains and the vaccination strains utilized in Egypt. Some field isolates were confirmed to be CEO-like viruses (Shehata et al., 2013), and some detected both TCO-like and CEO-like viruses (Bayoumi et al., 2020; Mossad et al., 2022), and they can result in the creation of natural recombinants with each other (Lee et al., 2012). In field outbreaks, field and ILTV vaccination strains are hard to differentiate (Lee et al., 2012). Therefore, molecularly defining the circulating ILTV strains in affected poultry farms is essential.

Several molecular characterization methods are employed to differentiate the strains causing outbreaks. PCR followed by restriction fragment length polymorphism (PCR-RFLP) was used to effectively distinguish field and vaccine strains (Chang et al., 1997; Kirkpatrick et al., 2006; Hermann et al., 2024). However, this technique's applicability is limited by the substantial amount of DNA required (Chacón and Ferreira, 2009). Specific gene fragment sequencing appears to be useful for differentiating vaccine strains from field isolates and characterizing field isolates.

The present study aimed to detect, identify, and characterize four antigenic and functionally relevant genes (*ICP4*, *gD*, *gG*, and *TK*) of the ILT virus strains currently circulating in Egypt in 2023.

MATERIAL AND METHODS

Ethical approval

The study was conducted according to the Animal Health Research Institute guidelines of Benha Provincial Laboratory, Animal Health Research Institute, Agriculture Research Canter, Benha, Egypt**.**

Sample collection

During 2023, the tracheal, larynx, and lung field samples were collected from 50 freshly dead affected layer farms (Shiver, Lohhman, Brown layer, and Aviagel) aged from 10-30 weeks with average sizes (10000-15000) birds. The samples included 37 unvaccinated layer chicken farms and 13 vaccinated layer chicken farms. They were vaccinated with TCO (LT-IVAX, USA) and CEO (Nobilis® ILT, Netherlands), administered at 35 and 95 days of age, respectively. The samples were collected from eight Governorates in Egypt, including eight from El Sharkia, ten from Qalubia, fifteen from Dakahlia, seven from Monofia, three from Gharbia, two from Behira, two from Damietta, and three from Alexandria.

The affected chickens experienced severe respiratory symptoms, such as coughing, sneezing, gasping, expectoration of red exudates from respiratory orifices, difficulty in inspiration, infra-orbital sinus enlargement, and significant mortality (5-15% mortality). Upon post-mortem inspection, the field instances had congested and hemorrhagic tracheitis with blood clots and caseated material, and in a few cases, this led to a blocked laryngeal lumen. The 10 trachea, larynx, and lungs from each farm were homogenized in sterile phosphate buffer saline PBS and then centrifuged at 3000 rpm at 4ºC for 30 minutes to clarify them.

Screening and detection of infectious Laryngotracheitis virus by real-time PCR

DNA was extracted from the supernatant fluid using the QIAamp DNA Mini kit (QIAGEN, Hilden, Germany) in accordance with the manufacturer's instructions. In conclusion, 10 µl of proteinase K, 200 µl of lysis buffer, and 200 µl of sample solution were incubated at 56 °C for 10 minutes, then 200 µl of 100% ethanol was added. 50 µl of elution buffer was used to elute DNA from the lysate. Real-time PCR used specific primers and probes targeting the *gB* gene (Table 1). Using the Takara kit (Kusatsu, Japan) according to the manufacturer's instructions through the Stratagene Mx3005p Real-Time PCR System.

Virus isolation

Three specific pathogen-free (SPF) chicken eggs, ranging in age from ten to twelve days, and 0.2 ml of positive samples were inoculated in the chorioallantoic membrane (CAM). The inoculated eggs were incubated at 37ºC for a maximum of seven days with daily monitoring and investigation of the presence of distinctive pock lesions on the CAMs of dead embryos or even survival eggs in 7 days (Magouz et al., 2018; Wolfrum et al., 2020).

Genetic characterization of *ICP4, gD, gG, and TK* genes

Conventional PCR amplification

Using gene-specific primers, the *ICP4, gD, gG,* and *TK* genes of the isolated ILTV were partially amplified by PCR (Table 2). The PCR was carried out in an ABI thermocycler (ProFlexTM PCR System) using the EmeraldAmp GT PCR MasterMix from Takara, Kusatsu, Japan. Certain PCR products were electrophoresed on a 1.5% agarose gel, stained with ethidium bromide, and examined under an ultraviolet lamp. Their purification procedure used the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany).

Table 1. Real-time primers and probes used for the detection of infectious laryngotracheitis virus

Primers and probe	Sequence	Reference
gB-S	5' CAGTATCTGGCATCGCCTCAT '3	(Zhao et al., 2013)
gB-A	5' CCTGGGAACAGAACCTGAACT '3	
Probe	5' FAM-CTAACCCGTTCGCCGCACTCG-BHQ-1 '3	

Table 2. Primers used for amplification of *ICP4*, *TK*, *gD*, *gG* genes

Primer	Sequence	Weight (BP)	Reference
ICP4-1F	5'ACTGATAGCTTTTCGTACCAGCACG'3	688	(Chacón and Ferreira, 2009)
ICP4-1R	5'CATCGGGACATTCTCCAGGTAGCA'3		
TK1b-F	5'CTTAGCGGAACCTATGCAAG3'	781	The present study
TK-R	5'GAG GCC ATG TGC TGG TAA GT'3		
gD-F	5'ATG CAC CGT CCT CATC'3	1300	(Craig et al., 2017)
gD-R	5'TTA GCT ACG CGC GCAT'3		
gG-F	5'CCT TCT CGT GCC GAT TCA ATATG'3	1480	(Craig et al., 2017)
gG-R	5'AAC CAC ACC TGA TGC TTT TGTAC'3		

Gene sequencing and phylogenetic analysis

Using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, California, USA) and ABI 3500XL Genetic Analyzer (Life Technologies, California, USA) with gene-specific primers, amplicons of ten positive samples representing different governorates and vaccinated and unvaccinated flocks (Table 3) were partially sequenced for *ICP4.* Four of the ten isolates were chosen to be partially sequenced for the *gD, Gg, and TK* genes. The sequence was posted to the GenBank (NCBI) website using the accession number (Table 4). Nucleotide and amino acid sequences of sequenced samples against further strains of ILTV from different groups and vaccine strains obtained from the National Center for Biotechnology Information (NCBI) (Table 5) were aligned using DNASTAR software's MegAlign module and CLUSTAL-W tool (Lasergene version 7.2; DNASTAR, Madison, WI, USA, Burland, 2000). MEGA version 6 generated the phylogenetic tree using 1000 bootstrap repeats and a moderate-strength maximum likelihood approach (Tamura et al., 2013; Kumar et al., 2016). The pairwise amino acid identity percent was calculated using the DNAstar software (DNAStar, Madison, WI, Burland, 2000).

Table 3. The epidemiological data of sequenced isolates of the Infectious Laryngotracheitis virus

Name	**Governorates**	**Breed**	**Age (day)**	**Mortality percent (%)**	**Vaccination**
ILT-Egy-WO1-2023	El Sharkia	Shiver	70	5%	Non vaccinated
ILT-Egy-WO2-2023	Dakahlia	Lohhman	100	10%	Non vaccinated
ILT-Egy-WO3-2023	Behira	Brown layer	120	7%	Vaccinated
ILT-Egy-WO4-2023	Monofia	Aviagel	150	8%	Vaccinated
ILT-Egy-WO5-2023	Dakahlia	Lohhman	160	9%	Vaccinated
ILT-Egy-WO6-2023	Qalubia	Shiver	80	12%	Non vaccinated
ILT-Egy-WO7-2023	Gharbia	Lohhman	100	6%	Vaccinated
ILT-Egy-WO8-2023	Monofia	Aviagel	90	11%	Non vaccinated
ILT-Egy-WO9-2023	Domiatt	Lohamn	110	13%	Non vaccinated
ILT-Egy-WO10-2023	Qalubia	Aviagel	180	14%	Non vaccinated

TCO (LT-IVAX, USA) and CEO (Nobilis® ILT, Netherlands) vaccines were administered to the chickens at 35 and 95 days of age in vaccinated flocks.

Table 4. Accession number of sequenced strains of Infectious Laryngotracheitis virus

Accession number / **Name**	***ICP4***	***gG***	***gD***	***TK***
ILT-Egy-WO1-2023	OR900664	OR900656	OR900649	OR900660
ILT-Egy-WO2-2023	OR900665	-	-	-
ILT-Egy-WO3-2023	OR900666	-	-	-
ILT-Egy-WO4-2023	OR900667	OR900657	OR900650	OR900662
ILT-Egy-WO5-2023	OR900668	OR900658	OR900651	OR900661
ILT-Egy-WO6-2023	OR900669	-	-	-
ILT-Egy-WO7-2023	OR900670	-	-	-
ILT-Egy-WO8-2023	OR900671	OR900659	OR900652	OR900663
ILT-Egy-WO9-2023	OR900672	-	-	-
ILT-Egy-WO10-2023	OR900673	-	-	-

Table 5. Reference strains provided by the National Center for Biotechnology Information (NCBI)

Name	**Country**	**Virulent/ Vaccine**	**Accession Number**
GAHV1-SA2	Australia	Vaccine	JN596962
GAHV1-TCO-IVAX	USA	Vaccine	JN580312
GAHV1-CEO-TRVAX	USA	Vaccine	JN580313
GAHV1-81658	USA	Virulent	JN542535
GAHV1-USDA-REF	USA	Virulent	JN542534
GAHV1-LTI-IVAX	USA	Vaccine	FJ444832.1
GAHV1-TCO-low passage	USA	Vaccine	JN580315
GAHV1-TCO-high passage	USA	Vaccine	JN580314
GAHV1-TCO vaccine	USA	Vaccine	EU104908.1
GAHV1-40798	Korea	Virulent	MH937566.1
GAHV1-ACC78	Australia	Virulent	JN804826
GAHV1-CEO-high passage	USA	Vaccine	JN580316
GAHV1-V1-99	Australia	Virulent	JX646898
GAHV1-K317	China	Virulent	JX458824
GAHV1-Live attenuated serva (Nobilis)	Australia	Vaccine	HQ630064
GAHV1-CEO-low passage	USA	Vaccine	JN580317
GAHV1-Intervet	Italy	Vaccine	HM230782.1
GAHV1-LT-BLEN	USA	Vaccine	JQ083493
GAHV1-CL9	Australia	Virulent	JN804827
GAHV1-63140	USA	Virulent	JN542536
GAHV1-CEO Vaccine	USA	Vaccine	EU104900.1
GAHV1-Laryngo Vac	USA	Vaccine	JQ083494.2
GAHV1-Poulvac	Italy	Vaccine	KP677882.1

RESULTS

Clinical signs and post-mortem examination

The chicken had red exudates from respiratory orifices, sneezing, coughing, gasping, nasal and ocular discharges, difficulty inhaling, and swelling of the infra-orbital sinus, with a mortality rate of 5-15% (Figure 1).

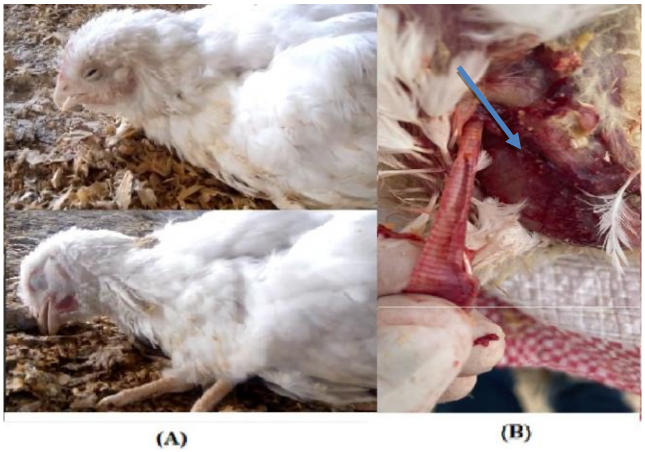


Figure 1. Clinical signs and post-mortem lesions were caused by the infectious laryngitis virus in affected chickens. **A:** Gasping and respiratory signs in affected chickens. **B:** The trachea had Fibrinohaemorrhagic exudate linked to hemorrhage and congestion in the lumen.

Post-mortem examination of the trachea and larynx indicated hemorrhage and congestion of the mucosa, along with a cast of fibrin-hemorrhagic blood obstructing the lumens. Caseous material was also discovered in the trachea and larynx. The lungs showed areas of congestion and consolidation (Figure 1).

Infectious Laryngitis virus screening by real-time PCR and viral isolation

Real-time PCR for 40 of the 50 samples was positive (5/8 El Sharkia, 8/10 Qalubia, 14/15 Dakahlia, 6/7 Monofia, 2/3 Gharbia, 2/2 Behira, 2/2 Domiatt, and 1/3 Alexandria) (Figure 2). The isolated positive samples on SPF-ECE indicated opaque, raised border pockets-like lesions distinguished by a grey core zone of necrosis and hemorrhagic chorioallantoic membrane. After inoculation, most embryos die 2-7 days later, and they are accompanied by slow development.

Gene amplification by conventional PCR

Ten isolates out of forty positive samples were selected according to different governorates to be amplified by conventional PCR for further sequencing of *ICP4* at 688 bp (Figure 3). Four isolates were partially amplified for *gD*, *gG*, and *TK genes*, gene sequencing at the expected weights 1300, 1480and 781 bp, respectively.

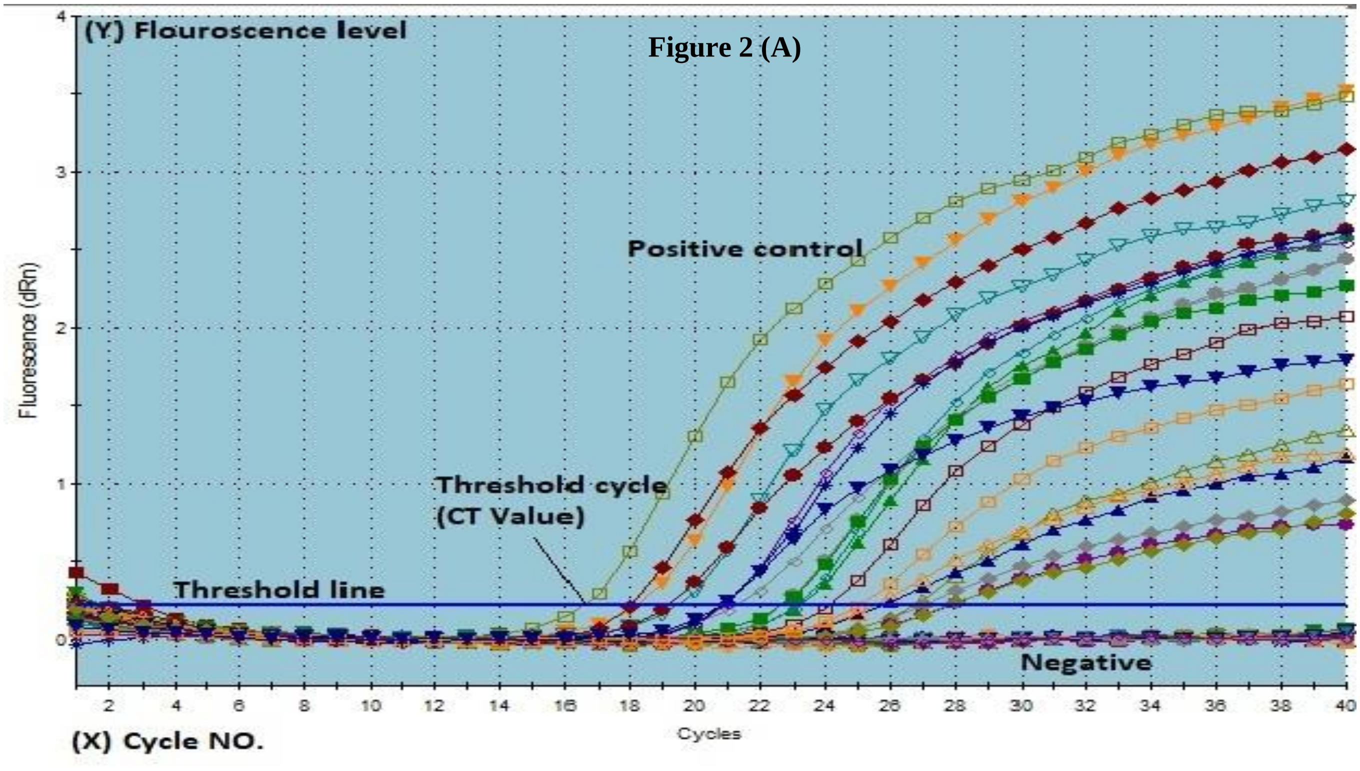


Figure 2 (A)

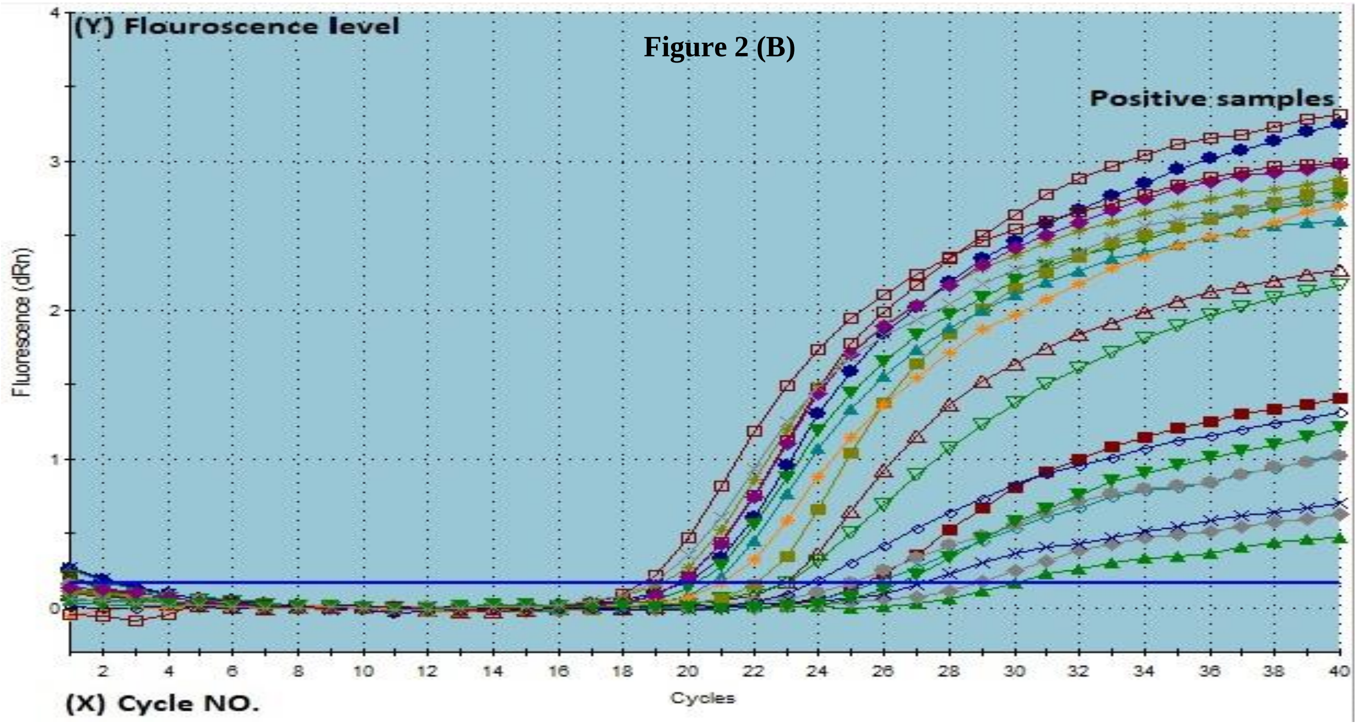


Figure 2. The colored amplification curves of the positive and negative controls, and 40 positive samples, in addition to the curve of 10 negative samples of the infectious laryngitis virus in affected chickens in Egypt (**A** and **B**)

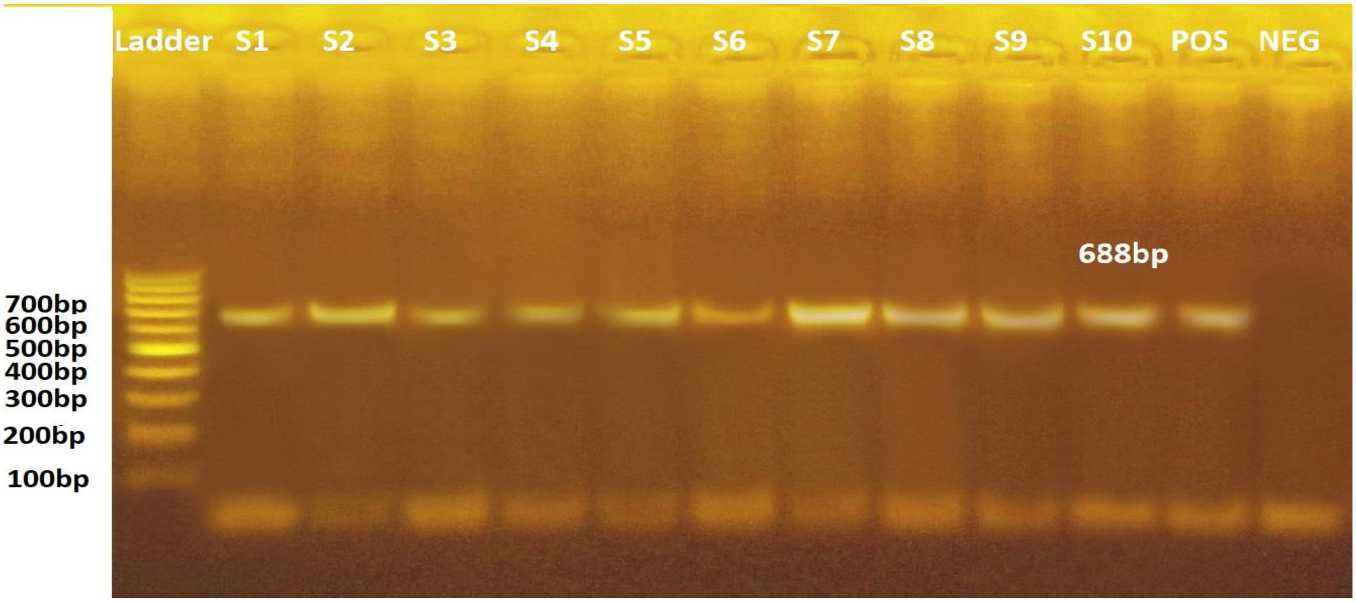


Figure 3. *ICP4* Genes amplification 10 positive samples by conventional PCR of the infectious laryngitis virus in affected chickens in Egypt.

Genetic characterization and phylogenetic analysis

Ten isolates were partially sequenced for the *ICP4* gene, while 4 isolates were selected for *gD, gG, and TK* partial sequencing. To detect similarity levels and analyze gene mutations, a multiple sequence alignment was created using sequenced strains in this study, other strains circulating in Egypt, strains from other countries, chicken embryo vaccine, and tissue culture origin vaccine (Table 5). The ILTV strains were clustered into two categories based on *ICP4* partial sequence-based phylogenetic analyses, including tissue culture origin (TCO) vaccines and vaccine-like strains, and chicken embryo origin (CEO) vaccines and vaccine-like strains. The third group (Wild type), including field isolates from Brazil and the United States, was identified (Figure 4).

The phylogenetic tree of *ICP4* gene (Figure 4) revealed that 7 of Egyptian strains under study (GAHV-1-Egy-WO1-2023, GAHV-1-Egy-WO2-2023, GAHV-1-Egy-WO3-2023, GAHV-1-Egy-WO4-2023, GAHV-1-Egy-WO6-2023, GAHV-1-Egy-WO9-2023 and GAHV-1-Egy-WO10-2023) belonged to the TCO group together with TCO vaccine strains (TCO low passage, TCO high passage, IVAX that used in Egypt) and virulent vaccine like strains from USA (USDA, 81658) and Egypian strains from 2015-2021 (Giza-2, Kaliobia-5, CLEVB-222, CLEVB-221). The remaining three strains in this study (GAHV-1-Egy-WO5-2023, GAHV-1-Egy-WO7-2023, and GAHV-1-Egy-WO8-2023) belong to the CEO group, along with virulent vaccine like strains from Australia (ACC78) and CEO vaccine strains (CEO low passage, CEO high passage, LT Blen, TRVX, and live attenuated serva vaccine that is used in Egypt).

The phylogenetic analysis of the *gD and gG* genes (Figures 5, 6) revealed no difference between the TCO and CEO strains. All strains in this study cluster with the TCO vaccine strains (IVAX), CEO vaccine strains (live attenuated serva, LT Blen, TRVX), and USDA, 81658, ACC78 virulent vaccine-like strains from the USA, Australia, as well as several Egyptian strains (Mansura-2019, Sharkia-2018). However, in the phylogenetic tree of the *gD* gene, the GAHV-1-Egy-WO1-2023 and GAHV-1-Egy-WO4-2023 were clustered on a separate branch with Fayoum_2019 and Qalubia_2018.

The thymidine kinase phylogenetic study shows no differences between TCO and CEO strains. All strains in this study were clustered with the TCO vaccine strain (IVAX), CEO vaccine strains (LT Blen, TRVX, live attenuated serva), various Egyptian strains (Sharkia11, Egypt-2015), and virulent vaccine like strains from the USA (USDA, 81658,) and Australia (ACC78) (Figure 7).

The *ICP4* gene of seven strains (GAHV-1-Egy-WO1, GAHV-1-Egy-WO2, GAHV-1-Egy-WO3, GAHV-1-Egy-WO4, GAHV-1-Egy-WO6, ILT-Egy-WO9 and ILT-Egy-WO10) had 100% amino acid identity percent with TCO vaccine strains (IVAX that used in Egypt), virulent vaccine like strains from USA (USDA, 81658) and Egyptian strains (Giza-2, Kaliobia-5, CLEVB-222, CLEVB-221). The other three isolates (GAHV-1-Egy-WO5, GAHV-1-Egy-WO7, and GAHV-1-Egy-WO8) had 98.9%-100% with CEO vaccine strains (live attenuated serva, that were used in Egypt, LT Blen, TRVX) and virulent vaccine like strains from Australia (ACC78) and 96.7%-97.8% with Egyptian strains (Monofia-1-2021 and Kaiobia-3-2021; Figure 8).

The *gD* gene of four strains(GAHV-1-Egy-WO1-2023, GAHV-1-Egy-WO4-2023, GAHV-1-Egy-WO5-2023 and GAHV-1-Egy-WO8-2023) had 99.8%-100% A.A. identity percent with -TCO vaccine (IVAX that used in Egypt) and CEO vaccine strains (LT Blen, TRVX, live attenuated serva that used in Egypt) and other virulent vaccine like strains from Australia (USDA, 81658, ACC78). While GAHV-1-Egy-WO5-2023 and GAHV-1-Egy-WO8-2023 had 99.8%-100% relation to many Egyptian strains (Alexandria-2018, Sharkia-2018, Mansura 2019, and Fayum-2019), GAHV-1-Egy-WO1-2023 and GAHV-1-Egy-WO4-2023 had 99.7-99.8% relation (Figure 9).

The *gG* gene of all sequenced strains had 100% A.A. identity percent with TCO vaccine strains (USDA, IVAX used in Egypt), CEO vaccine strains (LT Blen, TRVX, live attenuated serva used in Egypt), virulent vaccine like strains from the USA (81658) and Australia (ACC78) and several Egyptian isolates (Sharkia-2018; Mansura- 2019; Fayum-2019; Figure 10).

The *Tk* gene of GAHV-1-Egy-WO1-2023 and GAHV-1-Egy-WO4-2023 had 100% A.A. identity percent with TCO vaccine strain (IVAX that used in Egypt), CEO vaccine strains (LT Blen, TRVX, live attenuated serva), virulent vaccine like strains from USA (USDA,18685) and Australia (ACC78) and other Egyptian strains (Sharkia-11, Egypt-2015) but GAHV-1-Egy-WO5-2023 and GAHV-1-Egy-WO8-2023 had 97.2%-99.1% A.A. identity with TCO vaccine strain (IVAX that used in Egypt), CEO vaccine strains (LT Blen, TRVX, live attenuated seva), virulent vaccine like strains from USA (USDA,18685) and Australia (ACC78) and other Egyptian strains (Sharkia-11, Egypt-2015; Figure 11).

Mutation analysis

Comparing several reference CEO and TCO vaccine strains, and virulent vaccine-like strains from the USA, Australia, China, and Egypt between 2015 and 2021, listed in GenBank (Table 5). No mutations were detected in the *ICP4* in the seven strains related to TCO in this study (GAHV-1-Egy-WO1-2023, GAHV-1-Egy-WO2-2023, GAHV-1-Egy-WO3-2023, GAHV-1-Egy-WO4-2023, GAHV-1-Egy-WO6-2023, GAHV-1-Egy-WO9-2023, and GAHV-1-Egy-WO10-2023). All strain-related to CEO related strains in these studies (GAHV-1-Egy-WO5-2023, GAHV-1-Egy-WO7-2023, and GAHV-1-Egy-WO8-2023 had a deletion at 272 to 283 pb and V200M that is characteristic of the CEO vaccine and CEO vaccine-like strain. Also, the GAHV-1-Egy-WO8-2023 had Q161H and Q182H.

The *Gd* genes of GAHV-1-Egy-WO5-2023 and GAHV-1-Egy-WO8-2023 did not have any mutations, and GAHV-1-Egy-WO1-2023 and GAHV-1-Egy-WO4-2023 had A34G and P276L. No mutations were detected in the *Gg* gene in all sequenced strains in this study, and it had threonine at positions 67 and 103 was unique to the vaccination strain. The *TK* genes of GAHV-1-Egy-WO1-2023 and GAHV-1-Egy-WO4-2023 had no mutations; however, GAHV-1-Egy-WO5-2023 had A99E, and GAHV-1-Egy-WO8-2023 had R115I, G126A, and S163I.

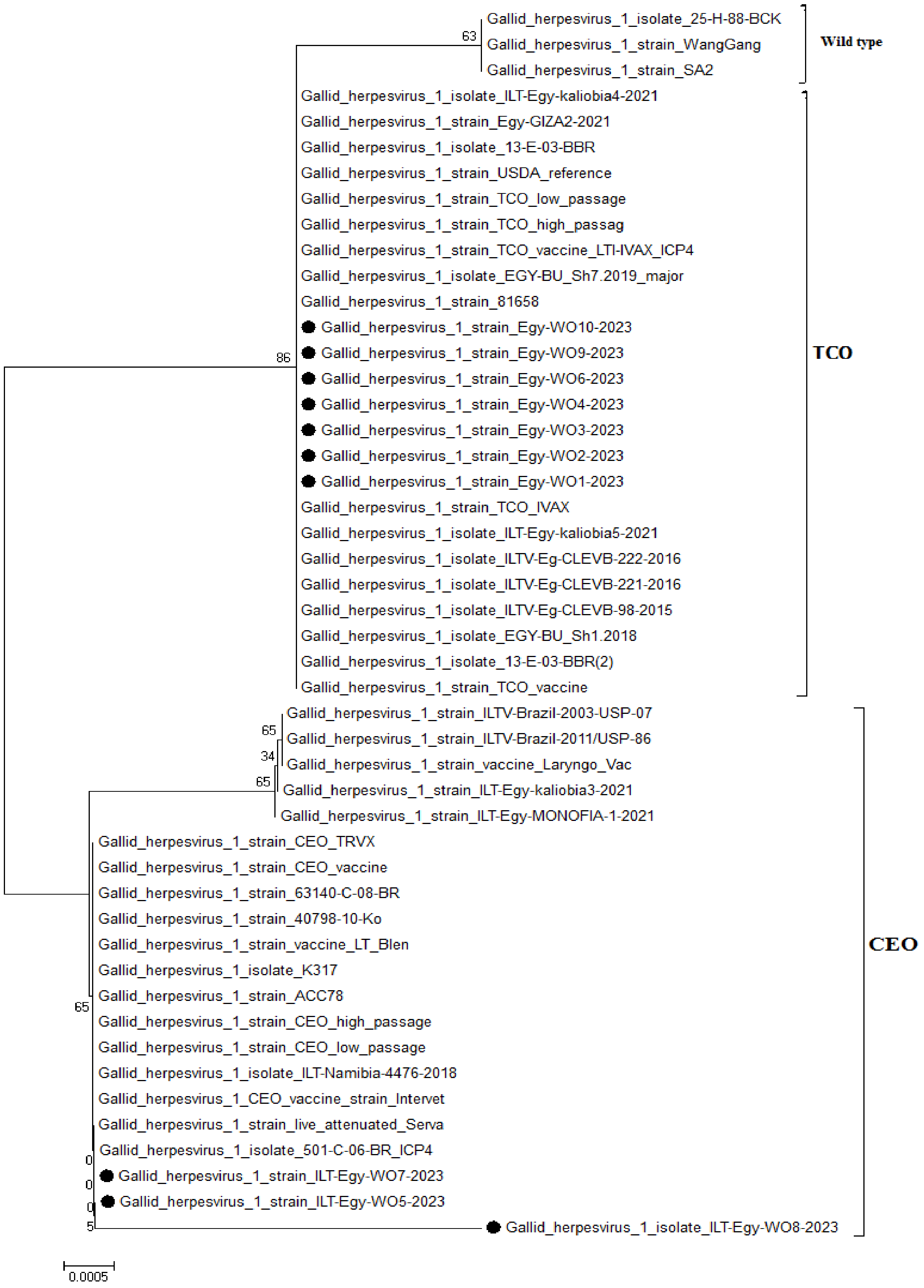


Figure 4. The phylogenetic analysis of the *ICP4* gene of the study strain in comparison to several field and vaccination strains of infectious laryngitis virus (ILTV) using the MEGA 6.0 program's maximum likelihood technique. The strains in this study are indicated by black dots.

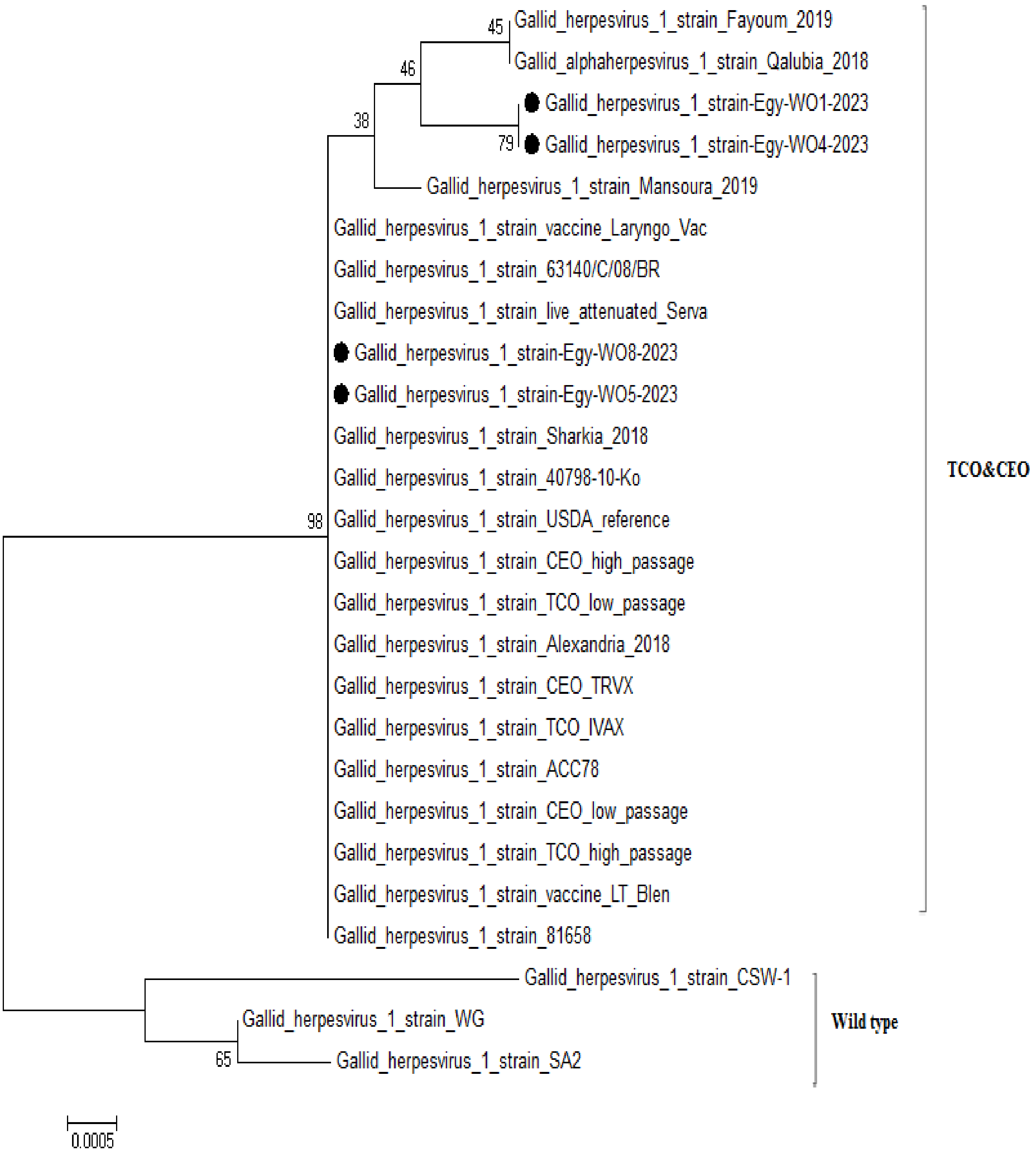


Figure 5. The phylogenetic analysis of the *gD* gene of the study strain in comparison to several field and vaccination strains of infectious laryngitis virus (ILTV) using the MEGA 6.0 program's maximum likelihood technique. The strains in this study are indicated by black dots.

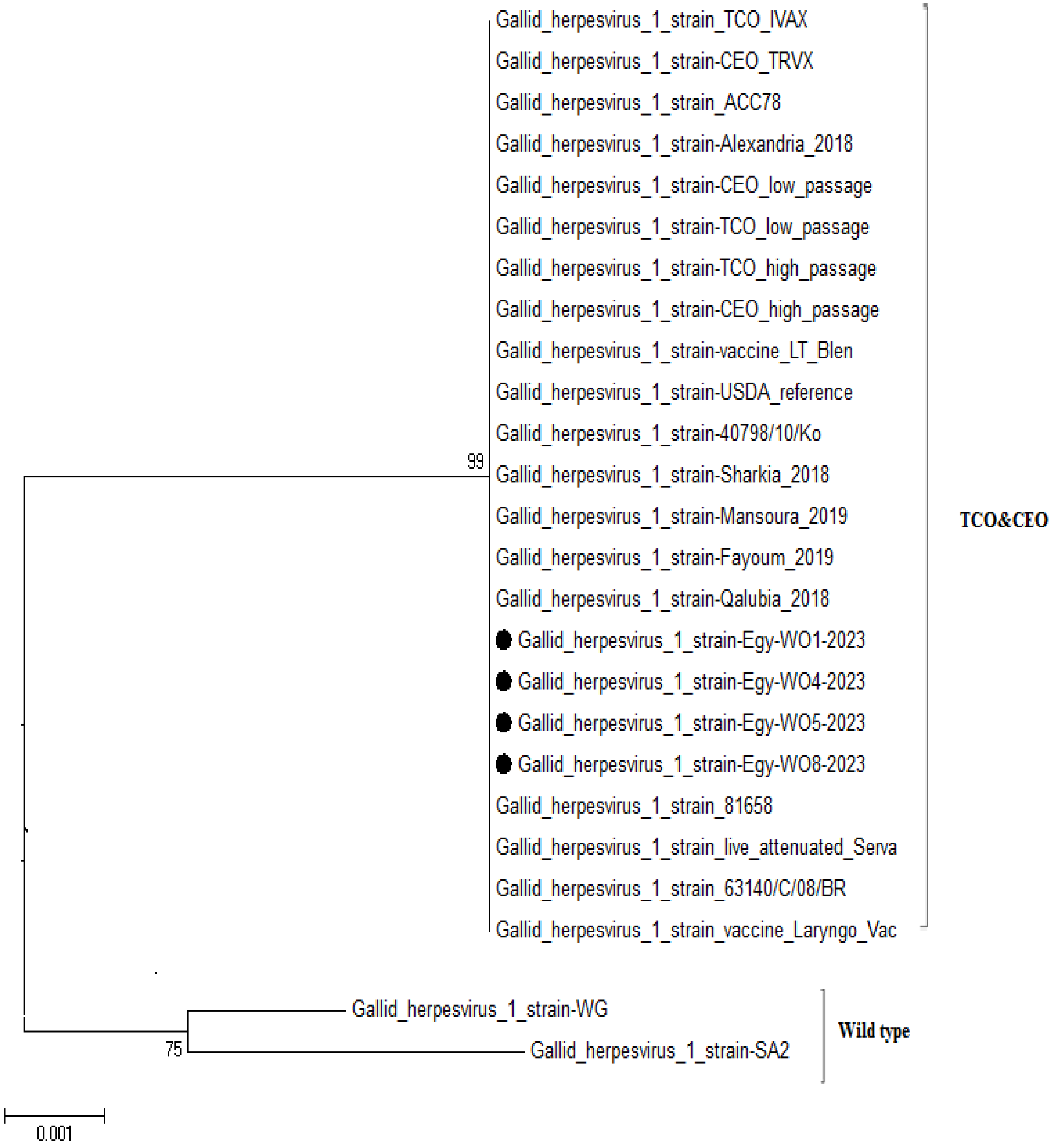


Figure 6. The phylogenetic analysis of the *gG* gene of the study strain in comparison to several field and vaccination strains of infectious laryngitis virus (ILTV) using the MEGA 6.0 program's maximum likelihood technique. The strains in the current study are indicated by black dots.

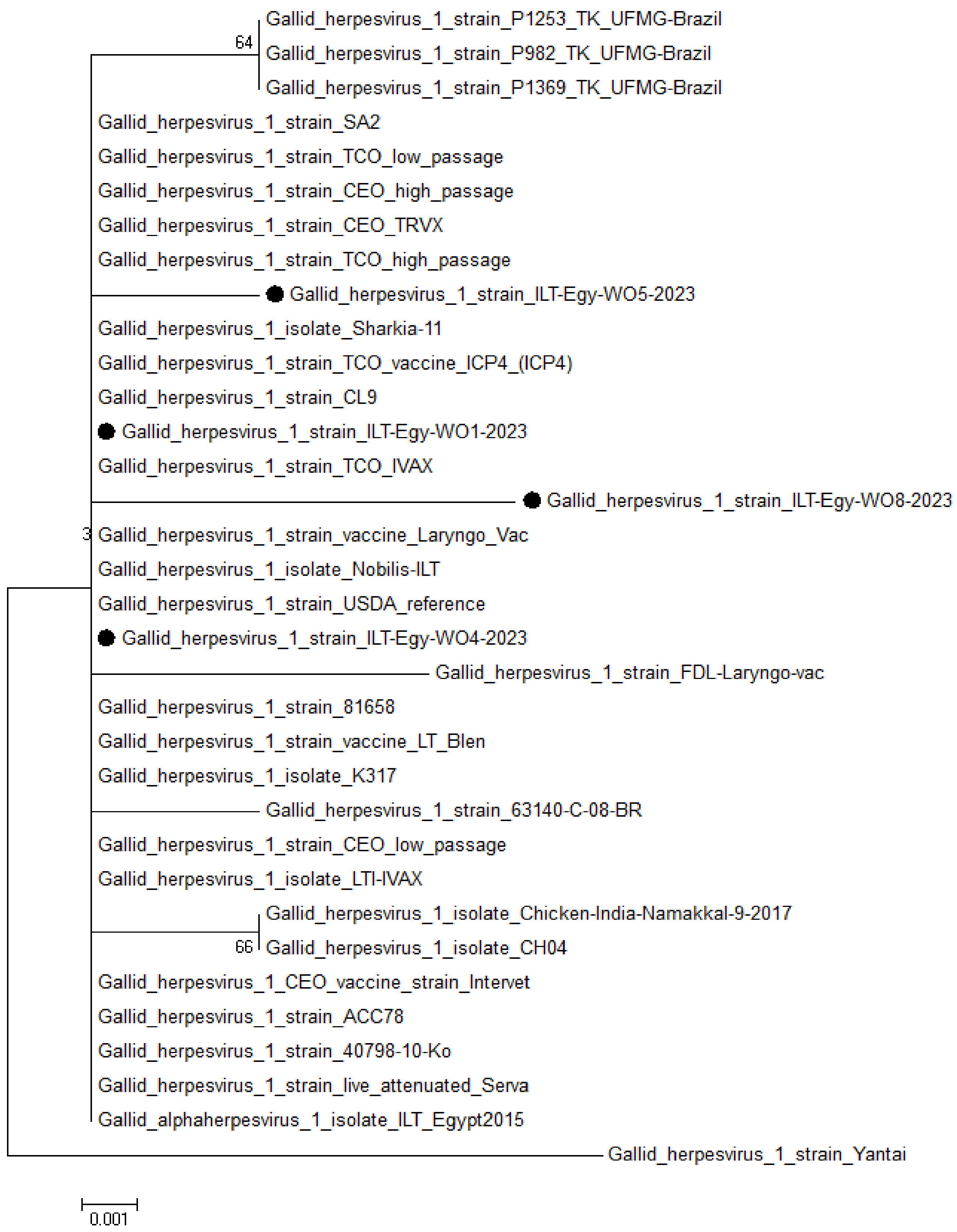
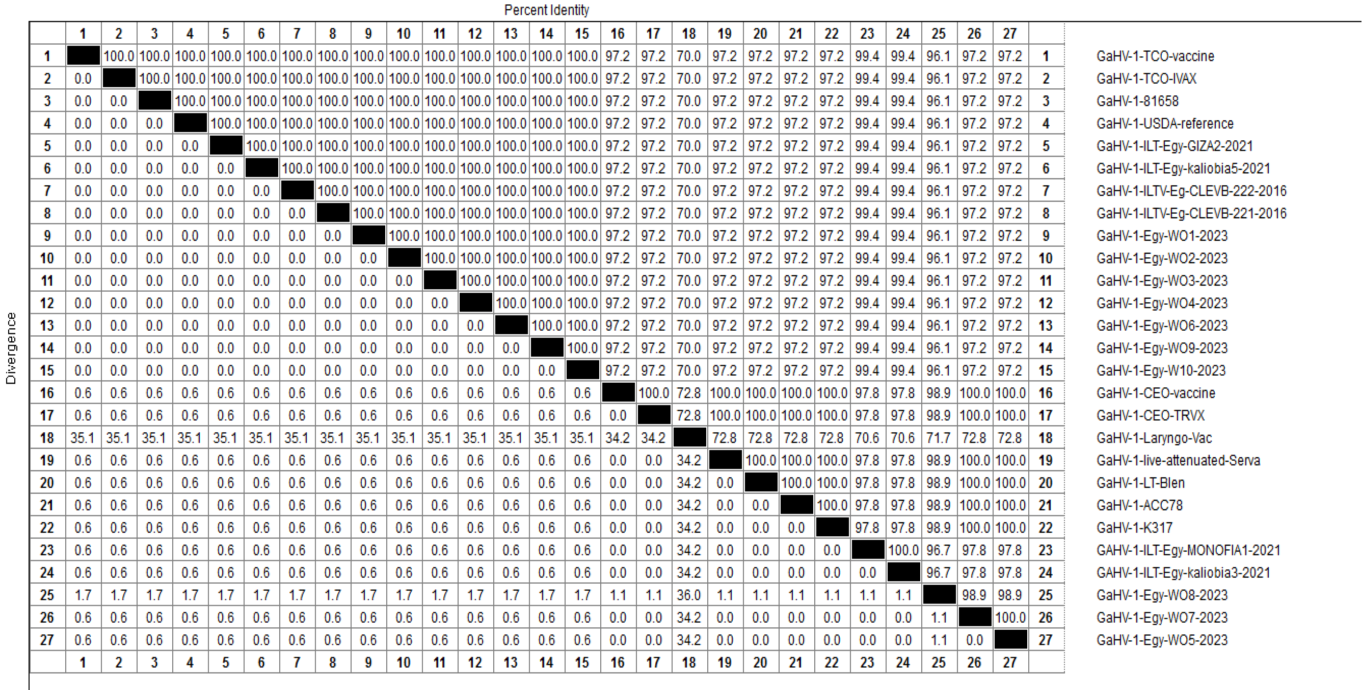


Figure 7. The phylogenetic tree of the *TK* gene of the study strain compared to several field and vaccination strains of infectious laryngitis virus (ILTV) using the MEGA 6.0 program's maximum likelihood technique. The strains in this study are indicated by black dots.

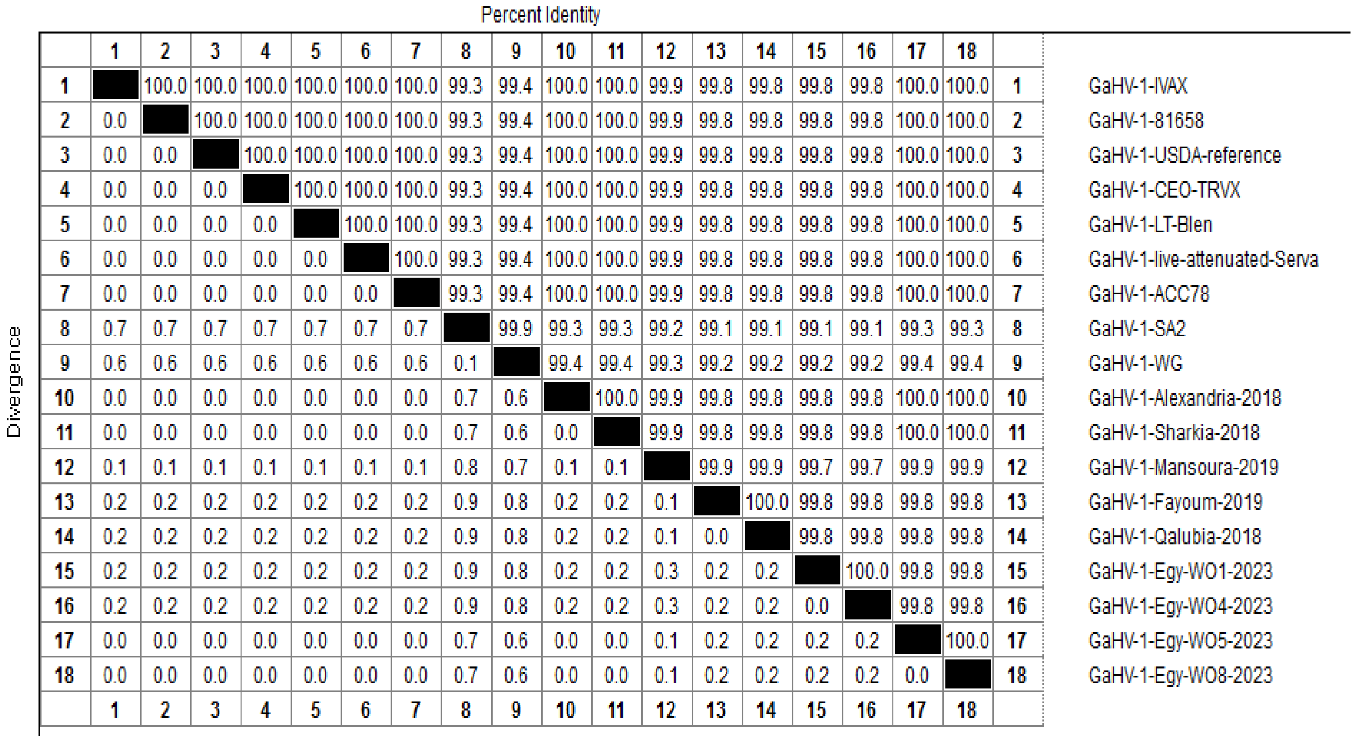


Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27		
1	■	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	97.2	97.2	70.0	97.2	97.2	97.2	97.2	99.4	99.4	96.1	97.2	97.2	1	GaHV-1-TCO-vaccine
2	0.0	■	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	97.2	97.2	70.0	97.2	97.2	97.2	97.2	99.4	99.4	96.1	97.2	97.2	2	GaHV-1-TCO-IVAX
3	0.0	0.0	■	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	97.2	97.2	70.0	97.2	97.2	97.2	97.2	99.4	99.4	96.1	97.2	97.2	3	GaHV-1-81658
4	0.0	0.0	0.0	■	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	97.2	97.2	70.0	97.2	97.2	97.2	97.2	99.4	99.4	96.1	97.2	97.2	4	GaHV-1-USDA-reference
5	0.0	0.0	0.0	0.0	■	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	97.2	97.2	70.0	97.2	97.2	97.2	97.2	99.4	99.4	96.1	97.2	97.2	5	GaHV-1-ILT-Egy-GIZA2-2021
6	0.0	0.0	0.0	0.0	0.0	■	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	97.2	97.2	70.0	97.2	97.2	97.2	97.2	99.4	99.4	96.1	97.2	97.2	6	GaHV-1-ILT-Egy-kaliobia5-2021
7	0.0	0.0	0.0	0.0	0.0	0.0	■	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	97.2	97.2	70.0	97.2	97.2	97.2	97.2	99.4	99.4	96.1	97.2	97.2	7	GaHV-1-ILTV-Eg-CLEVB-222-2016
8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	■	100.0	100.0	100.0	100.0	100.0	100.0	100.0	97.2	97.2	70.0	97.2	97.2	97.2	97.2	99.4	99.4	96.1	97.2	97.2	8	GaHV-1-ILTV-Eg-CLEVB-221-2016
9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	■	100.0	100.0	100.0	100.0	100.0	100.0	97.2	97.2	70.0	97.2	97.2	97.2	97.2	99.4	99.4	96.1	97.2	97.2	9	GaHV-1-Egy-WO1-2023
10	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	■	100.0	100.0	100.0	100.0	100.0	97.2	97.2	70.0	97.2	97.2	97.2	97.2	99.4	99.4	96.1	97.2	97.2	10	GaHV-1-Egy-WO2-2023
11	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	■	100.0	100.0	100.0	100.0	97.2	97.2	70.0	97.2	97.2	97.2	97.2	99.4	99.4	96.1	97.2	97.2	11	GaHV-1-Egy-WO3-2023
12	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	■	100.0	100.0	100.0	97.2	97.2	70.0	97.2	97.2	97.2	97.2	99.4	99.4	96.1	97.2	97.2	12	GaHV-1-Egy-WO4-2023
13	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	■	100.0	100.0	97.2	97.2	70.0	97.2	97.2	97.2	97.2	99.4	99.4	96.1	97.2	97.2	13	GaHV-1-Egy-WO6-2023
14	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	■	100.0	97.2	97.2	70.0	97.2	97.2	97.2	97.2	99.4	99.4	96.1	97.2	97.2	14	GaHV-1-Egy-WO9-2023
15	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	■	97.2	97.2	70.0	97.2	97.2	97.2	97.2	99.4	99.4	96.1	97.2	97.2	15	GaHV-1-Egy-W10-2023
16	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	■	100.0	72.8	100.0	100.0	100.0	100.0	97.8	97.8	98.9	100.0	100.0	16	GaHV-1-CEO-vaccine
17	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.0	■	72.8	100.0	100.0	100.0	100.0	97.8	97.8	98.9	100.0	100.0	17	GaHV-1-CEO-TRVX
18	35.1	35.1	35.1	35.1	35.1	35.1	35.1	35.1	35.1	35.1	35.1	35.1	35.1	35.1	35.1	34.2	34.2	■	72.8	72.8	72.8	72.8	70.6	70.6	71.7	72.8	72.8	18	GaHV-1-Laryngo-Vac
19	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.0	0.0	34.2	■	100.0	100.0	100.0	97.8	97.8	98.9	100.0	100.0	19	GaHV-1-live-attenuated-Serva
20	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.0	0.0	34.2	0.0	■	100.0	100.0	97.8	97.8	98.9	100.0	100.0	20	GaHV-1-LT-Blen
21	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.0	0.0	34.2	0.0	0.0	■	100.0	97.8	97.8	98.9	100.0	100.0	21	GaHV-1-ACC78
22	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.0	0.0	34.2	0.0	0.0	0.0	■	97.8	97.8	98.9	100.0	100.0	22	GaHV-1-K317
23	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.0	0.0	34.2	0.0	0.0	0.0	0.0	■	100.0	96.7	97.8	97.8	23	GAHV-1-ILT-Egy-MONOFIA1-2021
24	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.0	0.0	34.2	0.0	0.0	0.0	0.0	0.0	■	96.7	97.8	97.8	24	GAHV-1-ILT-Egy-kaliobia3-2021
25	1.7	1.7	1.7	1.7	1.7	1.7	1.7	1.7	1.7	1.7	1.7	1.7	1.7	1.7	1.7	1.1	1.1	36.0	1.1	1.1	1.1	1.1	1.1	1.1	■	98.9	98.9	25	GaHV-1-Egy-WO8-2023
26	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.0	0.0	34.2	0.0	0.0	0.0	0.0	0.0	0.0	1.1	■	100.0	26	GaHV-1-Egy-WO7-2023
27	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.0	0.0	34.2	0.0	0.0	0.0	0.0	0.0	0.0	1.1	0.0	■	27	GaHV-1-Egy-WO5-2023
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27		

Figure 8. Amino acid identity of the sequenced *ICP4* gene compared to vaccination strains and vaccine-like strains of both CEO and TCO, as well as other Egyptian strains.

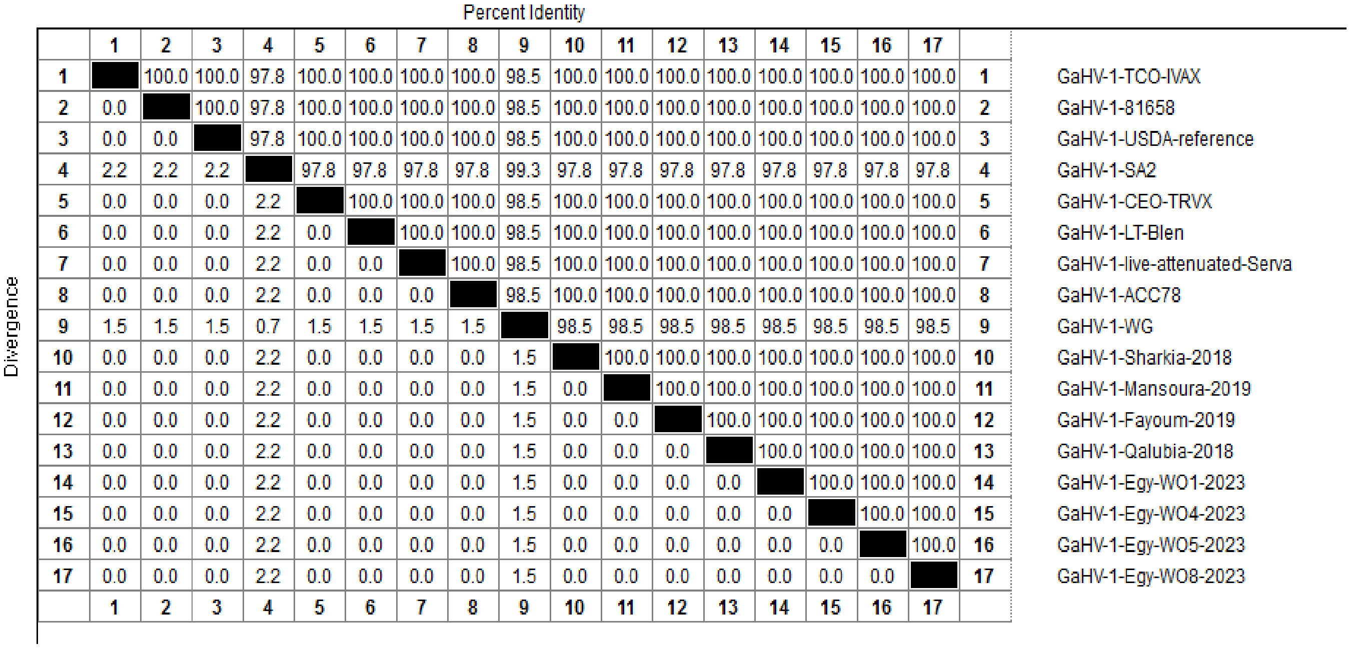


Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18		
1	■	100.0	100.0	100.0	100.0	100.0	100.0	99.3	99.4	100.0	100.0	99.9	99.8	99.8	99.8	99.8	100.0	100.0	1	GaHV-1-IVAX
2	0.0	■	100.0	100.0	100.0	100.0	100.0	99.3	99.4	100.0	100.0	99.9	99.8	99.8	99.8	99.8	100.0	100.0	2	GaHV-1-81658
3	0.0	0.0	■	100.0	100.0	100.0	100.0	99.3	99.4	100.0	100.0	99.9	99.8	99.8	99.8	99.8	100.0	100.0	3	GaHV-1-USDA-reference
4	0.0	0.0	0.0	■	100.0	100.0	100.0	99.3	99.4	100.0	100.0	99.9	99.8	99.8	99.8	99.8	100.0	100.0	4	GaHV-1-CEO-TRVX
5	0.0	0.0	0.0	0.0	■	100.0	100.0	99.3	99.4	100.0	100.0	99.9	99.8	99.8	99.8	99.8	100.0	100.0	5	GaHV-1-LT-Blen
6	0.0	0.0	0.0	0.0	0.0	■	100.0	99.3	99.4	100.0	100.0	99.9	99.8	99.8	99.8	99.8	100.0	100.0	6	GaHV-1-live-attenuated-Serva
7	0.0	0.0	0.0	0.0	0.0	0.0	■	99.3	99.4	100.0	100.0	99.9	99.8	99.8	99.8	99.8	100.0	100.0	7	GaHV-1-ACC78
8	0.7	0.7	0.7	0.7	0.7	0.7	0.7	■	99.9	99.3	99.3	99.2	99.1	99.1	99.1	99.1	99.3	99.3	8	GaHV-1-SA2
9	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.1	■	99.4	99.4	99.3	99.2	99.2	99.2	99.2	99.4	99.4	9	GaHV-1-WG
10	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.7	0.6	■	100.0	99.9	99.8	99.8	99.8	99.8	100.0	100.0	10	GaHV-1-Alexandria-2018
11	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.7	0.6	0.0	■	99.9	99.8	99.8	99.8	99.8	100.0	100.0	11	GaHV-1-Sharkia-2018
12	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.8	0.7	0.1	0.1	■	99.9	99.9	99.7	99.7	99.9	99.9	12	GaHV-1-Mansoura-2019
13	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.9	0.8	0.2	0.2	0.1	■	100.0	99.8	99.8	99.8	99.8	13	GaHV-1-Fayoum-2019
14	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.9	0.8	0.2	0.2	0.1	0.0	■	99.8	99.8	99.8	99.8	14	GaHV-1-Qalubia-2018
15	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.9	0.8	0.2	0.2	0.3	0.2	0.2	■	100.0	99.8	99.8	15	GaHV-1-Egy-WO1-2023
16	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.9	0.8	0.2	0.2	0.3	0.2	0.2	0.0	■	99.8	99.8	16	GaHV-1-Egy-WO4-2023
17	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.7	0.6	0.0	0.0	0.1	0.2	0.2	0.2	0.2	■	100.0	17	GaHV-1-Egy-WO5-2023
18	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.7	0.6	0.0	0.0	0.1	0.2	0.2	0.2	0.2	0.0	■	18	GaHV-1-Egy-WO8-2023
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18		

Figure 9. Amino acid identity of the sequenced *gD* gene compared to vaccination strains and vaccine-like strains of both CEO and TCO, as well as other Egyptian strains.

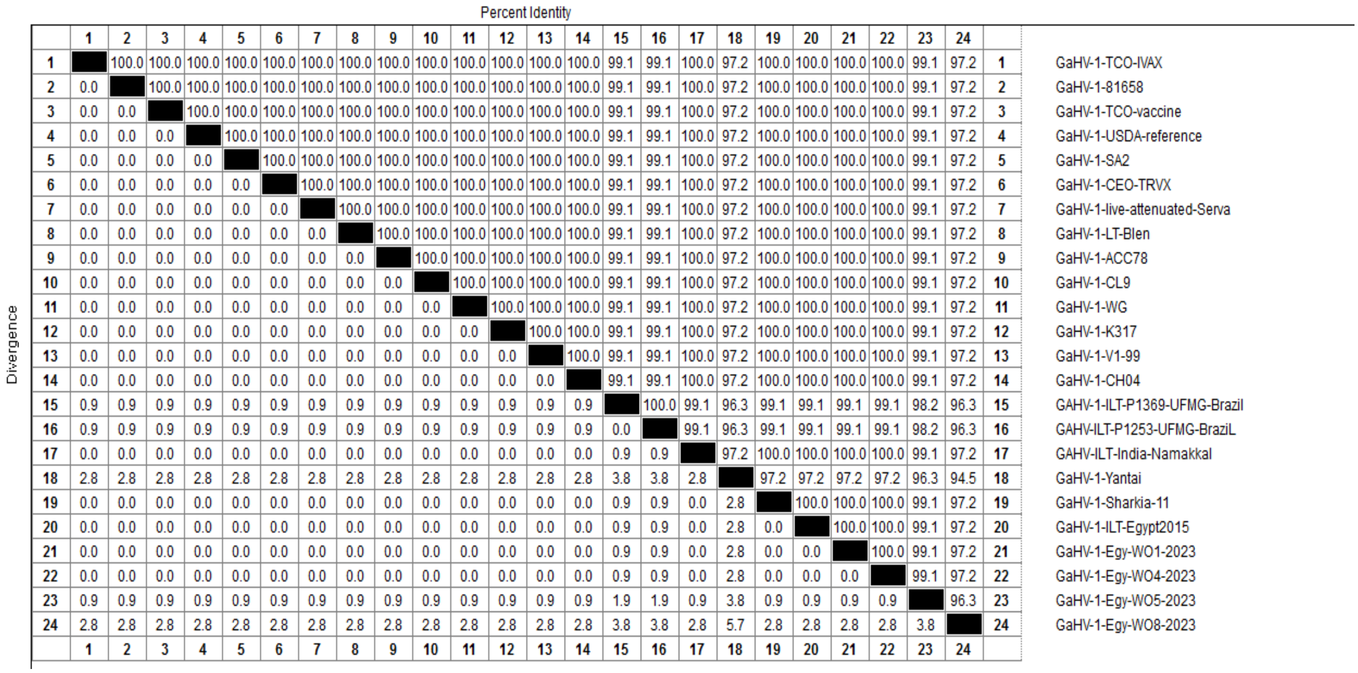


Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17		
1	■	100.0	100.0	97.8	100.0	100.0	100.0	100.0	98.5	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	1	GaHV-1-TCO-IVAX
2	0.0	■	100.0	97.8	100.0	100.0	100.0	100.0	98.5	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	2	GaHV-1-81658
3	0.0	0.0	■	97.8	100.0	100.0	100.0	100.0	98.5	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	3	GaHV-1-USDA-reference
4	2.2	2.2	2.2	■	97.8	97.8	97.8	97.8	99.3	97.8	97.8	97.8	97.8	97.8	97.8	97.8	97.8	4	GaHV-1-SA2
5	0.0	0.0	0.0	2.2	■	100.0	100.0	100.0	98.5	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	5	GaHV-1-CEO-TRVX
6	0.0	0.0	0.0	2.2	0.0	■	100.0	100.0	98.5	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	6	GaHV-1-LT-Blen
7	0.0	0.0	0.0	2.2	0.0	0.0	■	100.0	98.5	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	7	GaHV-1-live-attenuated-Serva
8	0.0	0.0	0.0	2.2	0.0	0.0	0.0	■	98.5	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	8	GaHV-1-ACC78
9	1.5	1.5	1.5	0.7	1.5	1.5	1.5	1.5	■	98.5	98.5	98.5	98.5	98.5	98.5	98.5	98.5	9	GaHV-1-WG
10	0.0	0.0	0.0	2.2	0.0	0.0	0.0	0.0	1.5	■	100.0	100.0	100.0	100.0	100.0	100.0	100.0	10	GaHV-1-Sharkia-2018
11	0.0	0.0	0.0	2.2	0.0	0.0	0.0	0.0	1.5	0.0	■	100.0	100.0	100.0	100.0	100.0	100.0	11	GaHV-1-Mansoura-2019
12	0.0	0.0	0.0	2.2	0.0	0.0	0.0	0.0	1.5	0.0	0.0	■	100.0	100.0	100.0	100.0	100.0	12	GaHV-1-Fayoum-2019
13	0.0	0.0	0.0	2.2	0.0	0.0	0.0	0.0	1.5	0.0	0.0	0.0	■	100.0	100.0	100.0	100.0	13	GaHV-1-Qalubia-2018
14	0.0	0.0	0.0	2.2	0.0	0.0	0.0	0.0	1.5	0.0	0.0	0.0	0.0	■	100.0	100.0	100.0	14	GaHV-1-Egy-WO1-2023
15	0.0	0.0	0.0	2.2	0.0	0.0	0.0	0.0	1.5	0.0	0.0	0.0	0.0	0.0	■	100.0	100.0	15	GaHV-1-Egy-WO4-2023
16	0.0	0.0	0.0	2.2	0.0	0.0	0.0	0.0	1.5	0.0	0.0	0.0	0.0	0.0	0.0	■	100.0	16	GaHV-1-Egy-WO5-2023
17	0.0	0.0	0.0	2.2	0.0	0.0	0.0	0.0	1.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	■	17	GaHV-1-Egy-WO8-2023
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17		

Figure 10. Amino acid identity of the sequenced *gG* gene compared to vaccination strains and vaccine-like strain of both CEO and TCO, as well as other Egyptian strains.



Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24		
1	■	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	99.1	99.1	100.0	97.2	100.0	100.0	100.0	100.0	99.1	97.2	1	GaHV-1-TCO-IVAX
2	0.0	■	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	99.1	99.1	100.0	97.2	100.0	100.0	100.0	100.0	99.1	97.2	2	GaHV-1-81658
3	0.0	0.0	■	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	99.1	99.1	100.0	97.2	100.0	100.0	100.0	100.0	99.1	97.2	3	GaHV-1-TCO-vaccine
4	0.0	0.0	0.0	■	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	99.1	99.1	100.0	97.2	100.0	100.0	100.0	100.0	99.1	97.2	4	GaHV-1-USDA-reference
5	0.0	0.0	0.0	0.0	■	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	99.1	99.1	100.0	97.2	100.0	100.0	100.0	100.0	99.1	97.2	5	GaHV-1-SA2
6	0.0	0.0	0.0	0.0	0.0	■	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	99.1	99.1	100.0	97.2	100.0	100.0	100.0	100.0	99.1	97.2	6	GaHV-1-CEO-TRVX
7	0.0	0.0	0.0	0.0	0.0	0.0	■	100.0	100.0	100.0	100.0	100.0	100.0	100.0	99.1	99.1	100.0	97.2	100.0	100.0	100.0	100.0	99.1	97.2	7	GaHV-1-live-attenuated-Serva
8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	■	100.0	100.0	100.0	100.0	100.0	100.0	99.1	99.1	100.0	97.2	100.0	100.0	100.0	100.0	99.1	97.2	8	GaHV-1-LT-Blen
9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	■	100.0	100.0	100.0	100.0	100.0	99.1	99.1	100.0	97.2	100.0	100.0	100.0	100.0	99.1	97.2	9	GaHV-1-ACC78
10	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	■	100.0	100.0	100.0	100.0	99.1	99.1	100.0	97.2	100.0	100.0	100.0	100.0	99.1	97.2	10	GaHV-1-CL9
11	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	■	100.0	100.0	100.0	99.1	99.1	100.0	97.2	100.0	100.0	100.0	100.0	99.1	97.2	11	GaHV-1-WG
12	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	■	100.0	100.0	99.1	99.1	100.0	97.2	100.0	100.0	100.0	100.0	99.1	97.2	12	GaHV-1-K317
13	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	■	100.0	99.1	99.1	100.0	97.2	100.0	100.0	100.0	100.0	99.1	97.2	13	GaHV-1-V1-99
14	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	■	99.1	99.1	100.0	97.2	100.0	100.0	100.0	100.0	99.1	97.2	14	GaHV-1-CH04
15	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	■	100.0	99.1	96.3	99.1	99.1	99.1	99.1	98.2	96.3	15	GAHV-1-ILT-P1369-UFMG-Brazil
16	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.0	■	99.1	96.3	99.1	99.1	99.1	99.1	98.2	96.3	16	GAHV-ILT-P1253-UFMG-BraziL
17	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.9	0.9	■	97.2	100.0	100.0	100.0	100.0	99.1	97.2	17	GAHV-ILT-India-Namakkal
18	2.8	2.8	2.8	2.8	2.8	2.8	2.8	2.8	2.8	2.8	2.8	2.8	2.8	2.8	3.8	3.8	2.8	■	97.2	97.2	97.2	97.2	96.3	94.5	18	GaHV-1-Yantai
19	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.9	0.9	0.0	2.8	■	100.0	100.0	100.0	99.1	97.2	19	GaHV-1-Sharkia-11
20	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.9	0.9	0.0	2.8	0.0	■	100.0	100.0	99.1	97.2	20	GaHV-1-ILT-Egypt2015
21	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.9	0.9	0.0	2.8	0.0	0.0	■	100.0	99.1	97.2	21	GaHV-1-Egy-WO1-2023
22	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.9	0.9	0.0	2.8	0.0	0.0	0.0	■	99.1	97.2	22	GaHV-1-Egy-WO4-2023
23	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	1.9	1.9	0.9	3.8	0.9	0.9	0.9	0.9	■	96.3	23	GaHV-1-Egy-WO5-2023
24	2.8	2.8	2.8	2.8	2.8	2.8	2.8	2.8	2.8	2.8	2.8	2.8	2.8	2.8	3.8	3.8	2.8	5.7	2.8	2.8	2.8	2.8	3.8	■	24	GaHV-1-Egy-WO8-2023
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24		

Figure 11. Amino acid identity of the sequenced *TK* gene compared to vaccination strains and vaccine-like strains of both CEO and TCO, as well as other Egyptian strains.

DISCUSSION

The ILTV has been identified in Egypt for the first time in 1983 (Tantawi et al., 1983), and outbreaks have continued until the most recent outbreaks in layer and broiler farms in 2021 (Ali et al., 2014; Bayoumi et al., 2020; Ibrahim et al., 2021; Mossad et al., 2022). ILTV still spreads throughout Egypt, causing outbreaks and significant losses in chicken farms (Bayoumi et al., 2020; Ibrahim et al., 2021; Mossad et al., 2022).

This study attempted to determine the detection and identification of the infectious laryngotrachitis virus that was an outbreak in Egypt during 2023 and the genetic characterization of four antigenic and functional genes (*ICP4, gD, gG, and TK*).

In this study, rapid detection of ILTV-suspected layer farms from 8 governorates suffered from respiratory signs such as Dysnea, gasping, coughing, red exudates from respiratory orifices, nasal discharge, and swelling of the infraorbital sinus and moderate mortalities (5-15%) as previously detected (Hughes et al., 1991; Ali et al., 2014; Bayoumi et al., 2020). Upon post-mortem examination, the suspected chicken had hemorrhagic tracheitis with caseated material and blood clots that may have led to a blocked laryngeal lumen, as previously recorded (Preis et al., 2013; Bayoumi et al., 2020).

The symptoms and post-mortem are not sufficient to diagnose ILTV, and the diagnosis was confirmed by a rapid molecular technique (real-time-PCR) (Pang et al., 2002) due to its rapid sensitivity and reproducibility (Mackay et al., 2002). In the current study, 40 out of 50 samples (80%) were positive for ILTV by real-time PCR in eight governorates (5/8 El Sharkia, 8/10 Qalubia, 14/15 Dakahlia, 6/7 Monofia, 2/3 Gharbia, 2/2 Behira, 2/2 Domiatt, and 1/3 Alexandria), in vaccinated and unvaccinated layer farms in Egypt during 2023.

To describe the genetic development of the ILT virus strains that are presently circulating in Egypt, partial sequencing and phylogenetic analysis of four genes (*ICP4, gD, gG, and TK)* were used. to determine whether an outbreak in Egyptian fields was caused by a wild field strain or a circulating vaccine strain. This was in line with earlier research that showed these genes were commonly used to differentiate between field and vaccine strains and identify the strains' virulence (Shehata et al., 2013; Craig et al., 2017; Bayoumi et al., 2020).

As *ICP4* is a critical viral protein that is typically employed in epidemiologic research to classify circulating virus strains and contribute to the regulation of gene expression of the virus (Shehata et al., 2013) additionally, the *ICP4* gene's nucleotide sequence was able to differentiate between vaccine strains and field isolates (Chacón and Ferreira, 2009). The Phylogenetic tree of the *ICP4* gene was split into two categories: TCO and CEO vaccine and vaccine-like strains, as previously reported (Oldoni and García, 2007; Couto et al., 2015; Bayoumi et al., 2020).

According to sequence and phylogenetic analysis of *ICP4* gene of the strains under study, 7 out of 10 Egyptian strains were clustered in TCO group with TCO vaccine strain (IVAX used in Egypt, TCO low passage, TCO high passage) and virulent vaccine like strains from USA (USDA, 81658) as well as Egyptian strains during 2015-2021 with 100% A.A. identity as previously described (Nagy et al., 2020; Ibrahim et al., 2021; Mossad et al, 2022). The remaining three strains in this study were grouped with the CEO vaccine strains (TRVX, live attenuated serva used in Egypt, CEO low passage, CEO high passage) and virulent vaccine like strains from Australia (ACC78), which had 98.9%-100% amino acid identity as recorded by Shehata et al. (2013), Bayoumi et al. (2020), and Mossad et al. (2022). However, this strain differed from the Egyptian strain that was recently circulated in Egypt in 2021, which had a 96.7%-97.8% A.A. identity. As a result of sequenced strains in the present study, the CEO and TCO-like virus strains were found and circulated in Egypt during 2023, causing severe outbreaks, most of which were TCO-like virus strains (70%). However, compared to TCO reverting, it has been demonstrated that chicken embryo origin reverting causes more serious respiratory illnesses and increased mortality (García, 2017).

According to earlier research by Chacón and Ferreira (2009), the CEO vaccine-like strains are identified by deletions in the 272–283 bp and V200M in the *ICP4* gene. These deletions were recorded in GAHV-1-Eg-WO5, WO7, and WO8 that cluster with CEO vaccine and vaccine like strains. Furthermore, the findings were consistent with earlier research conducted in Egypt by Nagy et al. (2020) and Mossad et al. (2022). Additionally, two novel A.A. mutations (Q161H and Q182H) unique to GAHV-1-Eg-WO8-2023 were discovered in this work. These mutations may impair the protein's structure and configuration, impacting its biological function (Sotomayor-Vivas et al., 2022). We need further research to study the impact of these mutations on the virulence of the virus.

The envelope Glycoproteins genes (G), such as *gG*, *gJ*, and *gD*, have been shown in previous studies to be crucial for the pathogenicity and antigenicity of ILTV in chickens. Additionally, field and vaccine strains can be differentiated from one another by using specific nucleotide mutations in the gG gene, such as 316, which serve as markers (Helferich et al., 2007; Craig et al., 2017). Additionally, Han and Kim (2001) found that the threonine at positions 67 and 103 was unique to vaccination strains. Previously, vaccination strains were distinguished from some Argentinean isolates using the nucleotide mutation at 163 in the gD gene (Craig et al., 2017).

According to *Gd*, *Gg* phylogenetic analysis, there is no difference in phylogenetic analysis between the CEO group and TCO group as previously recorded (Ali et al., 2014; Bayoumi et al., 2020). The strains in this study showed 99.7%, 100%, and 100% amino acid sequence identity with the TCO vaccine strain, IVAX, and CEO vaccine strains TRVX, live attenuated serva, virulent vaccine like strains from the USA and Australis (USDA, 81658, ACC78), and Egyptian strains in 2018-2019 respectively. Additionally, there was no variation between the Egyptian and vaccine strains in the nucleotide locations at position 163 of the gD gene and 316 of the Gg gene. These findings are in line with Ali et al. (2014) and Bayoumi et al. (2020), and they had threonine in the gG gene at 67 and 103 as vaccine and low virulent strain as previously detected (Ali et al., 2014). The GAHV-1-Eg-WO1 and GAHV-1-Eg-WO4 had new mutations at A34G and P276L in the gD gene that clustered them in the new branch that may affect the viral virulence (Huang et al., 1997; Bayoumi et al., 2020; Wu et al., 2022). Further studies need to determine their effectiveness on the virulence of the virus.

According to Han and Kim (2001), the *TK* gene is crucial for distinguishing between field isolates and ILTV vaccine strains and has been linked to ILTV pathogenicity and virulence. Different levels of pathogenicity have been associated with single-nucleotide polymorphisms in the TK gene. Furthermore, several Tk gene mutations were obtained to demonstrate viral attenuation for vaccine production. According to the current study's phylogenetically examined and sequenced TK gene from four isolates, there were no variations between the CEO and TCO groups. the strains showed 97.2% to 100% with CEO vaccine strain (LT Blen, TRVX, live attenuated seva, used in Egypt) and the TCO vaccine strain (IVAX, used in Egypt) along with virulent vaccine like strains from the USA, and Australia (USDA, 81658, ACC78) and Egyptian strains. According to the mutational investigation of the *Tk* gene, the GAHV-1-Eg-WO5 had A99E and GAHV-1-Eg-WO8 had R115I, G126A, and S163I that may have an impact on the virus pathogenicity (Han and Kim 2001; Santander-Parra et al., 2022). More research will be required to ascertain the efficacy of the pathogenicity of the virus.

The molecular characterization and phylogenetic findings of four genes (*ICP4, gD, gG, and Tk*) in strains isolated during 2023 indicated that outbreaks in Egyptian commercial broiler chicken flocks were related to ILTV vaccine strains. These findings align with other research on ILTV in Egyptian layer farms (Shehata et al., 2013; Ali et al., 2014; Bayoumi et al., 2020), which postulated that the CEO and TCO vaccination viruses would become more virulent following bird-to-bird transmissions, resulting in devastating epidemics among Egypt's susceptible chickens. Alternatively, the attenuated vaccine might have reactivated and caused outbreaks in susceptible chickens as a result of chicken-to-chicken transmission or the reactivation of latent infection brought on by stress, poor hygiene, and the spread of other pathogens. These results align with those obtained by Oldoni and García (2007), who demonstrated that the vaccination strains were closely linked to most commercial chicken ILTV isolates. According to Chang et al. (1997), outbreaks of ILTV occur when ILT vaccine viruses take the place of naturally occurring viruses circulating in the field. Additionally, it may be recombination that occurred between several attenuated vaccination strains, resulting in virulent recombinant viruses, which became the dominant strains in commercial poultry flocks as recorded by Lee et al. (2012). We recommend performing a full genome sequence of ILTV to detect any recombination occurrence.

CONCLUSION

According to molecular characterization of *ICP4, gG, Gd,* and *TK* genes, the ILTV outbreaks in poultry farms across several regions of Egypt during 2023 might be induced by vaccine strains derived from TCO and CEO, with some acquired mutations that may affect the virulence of the virus. It could be caused by reactivation of a viral vaccine strain from chicken to chicken transmission, or revival of a latent infection, or viral recombination of modified attenuated vaccines. Therefore, to stop ILT outbreaks in the future, there is a need to reevaluate current vaccination programs and use novel vector vaccines.

DECLARATIONS

Authors' contributions

Sabry Omar contributed to collecting samples. Ahmed Abdelhalim and Zienab Mossad performed PCR for TK, gG, gD, and ICP4. Nahed Yehia and Wessam Hassan made the sequence and phylogenetic analyses. Nahed Yehia and Wessam Hassan wrote the manuscript draft and revised it before submission. All authors checked and confirmed all data and the last draft of the manuscript before submission to the journal.

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Ethical considerations

The final version of this study has been reviewed by all authors and submitted for the first time to this publication.

Availability of data and materials

The study's original contributions are contained in the article and supplemental materials. Data is available upon reasonable request from the corresponding author.

Competing interests

The authors declare no conflict of interest.

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Sero-marker and Detection of Avian Leukosis and Marek's Disease Viruses in Commercial Chicken Flocks in Egypt During 2019 to 2022

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ABSTRACT

In the poultry industry, oncogenic disease viruses result in significant financial losses. This study aimed to update incidence data in Egyptian chicken flocks from 2019 to 2022. The prevalence of Marek's disease virus (MDV) and Avian leukosis virus (ALV) was monitored during the passive surveillance program in commercial poultry flocks, including (43) breeders, (45) layers, and (7) broiler flocks during 2019-2022 in Egypt. This passive surveillance was adopted depending on molecular detection of MDV and ALV viruses using PCR tests and serological detection of ALV using ELISA test kits. The histological and post-mortem results for clinically diseased birds have been recorded in different organs, including the Liver, spleen, kidney, ovary, intestine, and brain. Ninety-five flocks were reported and tested using PCR for monitoring ALV viruses (subtypes A, B, C, D, and J) and vvMDV. Samples used for PCR were prepared from liver and spleen tissues (5 chickens/pool). The tested flocks were negative for ALV subtypes A, B, C, and D, while only two cases were positive for ALV-J (2/95, 2.1%), including one breeder flock in 2021 and one-layer flock in 2022, both from Sharqia governorate. Although the overall detection rate of vvMDV was 18.9% (18/95), breeder flocks showed the highest detection rate (25.6%), compared to layer flocks (15.6%). Furthermore, the vvMDV virus detection rate varied from one year to another, including 4/13 (30.8%), 7/43 (16.3%), 4/24 (16.7%), and 3/15 (20 %) in 2019, 2020, 2021, and 2022, respectively. The vvMDV-positive cases were reported in eight governorates, including Sharqia, Menofia, Daqahlia, Gharbia, Qualiobia, Al Beheira, Giza, and Damietta. Moreover, the study indicated that vvMDV was detected in chickens aged 5 to 61 weeks. The ELISA test was used to detect ALV antibodies serologically. The seroprevalence of ALV was 4.7% in the study area. In conclusion, among the tested samples, only two cases of ALV-J were reported in Egyptian commercial chicken flocks during 2019-2022. During the same period, vvMDV showed an 18.9% prevalence rate. Further studies are recommended to evaluate the MDV vaccination program, including vaccine quality and efficacy.

Keywords: Avian leukosis virus, Detection rate, Egypt, Marek's disease virus, Prevalence

INTRODUCTION

Oncogenic disease viruses cause serious economic losses due to increased mortalities, carcass condemnation, decreased growth rates, reduced egg production, and changes in egg size and quality (Payne and Fadly, 1997). Avian leukosis viruses (ALVs) are one of the alpha retroviruses from the family Retroviridae (Zhang et al., 2020). According to the envelope gene, they are divided into six subgroups (A to E and J). All ALV subtypes are exogenous viruses that can induce B-cell malformation, leading to lymphoma, especially in susceptible chicken species, except for subgroup E, which consists of non-oncogenic endogenous viruses (Hollmann et al., 2021). The ALVs are transmitted through multiple routes, such as vertically (via eggs) and horizontally (direct or indirect contact between birds) (Tan et al., 2024). Infected chickens may exhibit a wide range of clinical signs, including general emaciation and paleness in the comb and wattle, ruffled feathers, recumbency, and decline in egg production in layer flocks (Eid et al., 2019). Since no effective vaccines or treatments exist to control leukosis, it

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is essential to identify and remove infected eggs and diseased chickens from breeding populations. In Egypt, ALV-J was confirmed in broiler breeder flocks (Arafa et al., 2007) and later spread rapidly (Kilany et al., 2015). The diagnosis of ALV infection is based on gross pathological lesions, serological, and molecular detection in infected chickens (Abdel Gayed et al., 2017).

Marek's disease (MD) is an infectious viral disease of chickens caused by the cell-associated Marek's disease virus (MDV), which belongs to the genus *Mardivirus*, subfamily *Alphaherpesvirinae*. It is also known as Gallid herpesvirus 2 (GaHV-2). There are three serotypes of MDV: MDV-1, which is pathogenic in chickens. MDV-2 and MDV-3 (herpesvirus of turkey-HVT), which are non-pathogenic and used in vaccine production (Witter and Schat, 2003).

MDV-1 strains are further classified into four pathotypes based on virulence: very virulent plus (vv+MDV), very virulent (vvMDV), virulent (vMDV), and mild (mMDV) (Witter et al., 2005).

The MDV-1 serotype is characterized by the presence of an oncogene and other unique genes, such as vIL8, PP38, and vTR, located in repeat regions, like TRL (Lee et al., 2000). Chickens are most susceptible at 4 weeks of age or older. The disease manifests in several forms in chickens, including lymphomatosis (in different organs like skin, eyes, and visceral organs), and neural form, which can cause temporary paralysis due to affection of the central nervous system (Nair, 2013).

Vertical transmission is rare, but horizontal transmission via airborne spread is well documented (Payne and Venugopal, 2000).

The first reported case of classical Marek's disease (MD) in Egypt was documented in 1953 (Soliman et al., 1954). Subsequent pathological, virological, and Sero-epidemiological studies confirmed that in Egypt. The MDV causes chicken disease complications, even in vaccinated flocks (Amin et al., 2001). Virulent MDV strains have been consistently detected in Egypt (Abdallah et al., 2018), and multiple studies by Hassanin et al. (2013) and Lebdah et al. (2017) have investigated the circulation of MDV strains in poultry flocks in Egypt. Through sequencing of the Meq protein, Abdallah et al. (2018) and Yehia et al. (2021) identified several amino acid mutations associated with MDV virulence. Several vaccines have been developed for MD control, including the Bivalent type (HVT and the serotype two strain SB-1), and the herpes virus of turkey (HVT) vaccine (Witter and Lee, 1984), or the "Rispens" vaccine (attenuated serotype one strain CVI988) (Rispens et al., 1972).

In Egypt, a bivalent vaccination strategy is recommended for broiler breeder flocks to enhance protection against virulent MDV strains (Eid et al., 2019). Polymerase chain reaction (PCR) is a rapid and reliable diagnostic tool to differentiate between vaccinated flocks and field strains of MDV serotype 1 (Handberg et al., 2001). The present study investigated the molecular prevalence of MDV and ALV using PCR and the seroprevalence of ALV (subtypes A and B) antibodies through ELISA testing. The study aimed to update incidence data in Egyptian chicken flocks from 2019 to 2022, with particular emphasis on geographical distribution patterns.

MATERIAL AND METHODS

Ethical approval

This study was conducted according to the guidelines of the Animal Health Research Institute (AHRI), the Agriculture Research Center (ARC), and the Ministry of Agriculture and Land Reclamation (MOALR) in Egypt.

Flocks' history

The Reference Laboratory of Veterinary Control of Poultry Production (RLQP), Animal Health Research Institute (AHRI), Giza, Egypt, received clinically diseased and /or healthy chickens with a history of variable mortalities, a decrease in growth rate and drop in egg production, and birds showing sings of emaciation, depression, ruffled feathers associated with nervous signs like paralysis and appearance of scattered visceral tumors for disease diagnosis. All chickens, ranging in age from 1 day to 604 days, had received commercial MD vaccines upon hatching.

Between 2019 and 2022, the authors received 1,594 different samples (638 dead chickens, 478 live chickens, and 478 blood samples) representing 95 flocks (15-20 bird samples/flock) from 12 different provinces in Egypt, to investigate the two major oncogenic viruses, MDV and ALV (subtypes A, B, C, D, and J).

The flocks included 45 breeders, 43 layers, and seven broilers. The provinces were Alexandria, Ismailia, Sharqiah, Menofia, Dakahilia, Gharbia, Qualiobia, Beheira, Giza, Damietta, Kafr El Sheikh, and Minya. Only 43 out of 95 flocks tested for ALV (subtypes A and B) by ELISA test from 9 different provinces of Egypt (Sharqia, Menofia, Dakahilia, Gharbia, Qualiobia, Beheira, Giza, Kafr El Sheikh, and Minya) are shown in Table 1.

Table 1. Geographical distribution of tested farms during 2019-to-2022 Passive Surveillance activities in Egypt using PCR and/or ELISA test.

[1]GOVERNORATE	YEAR				TOTAL NO. OF TESTED		
					FARMS	USING	
	2019	2020	2021	2022		[2]PCR	[3]ELISA
Beheira	5	7	8	9	29	29	18
Sharqiah	4	12	4	3	23	23	10
Menofia	0	8	1	0	9	9	3
Giza	0	3	4	0	7	7	3
Dakahilia	0	3	0	3	6	6	1
Qualiobia	1	1	3	0	5	5	4
Minya	0	2	2	0	4	4	2
Gharbia	1	2	0	0	3	3	1
Damietta	1	2	0	0	3	3	0
Kafr El Sheikh	1	2	0	0	3	3	1
Ismailia	0	0	2	0	2	2	0
Alexandria	0	1	0	0	1	1	0
Total	13	43	24	15	95	95	43

[1] The study covered 12 governorates from Egypt during the period 2019 to 2022. [2] PCR tests: Polymerase Chain Reaction test. [3] ELISA test: Commercially available Enzyme-linked immunosorbent Assay obtained from IDEXX.

Post-mortem examination and sample collection

Fifteen to twenty chickens from each flock were received at RLQP-AHRI for testing. The post-mortem examination for the received chickens was adapted according to RLQP standard operating procedures and protocols. Tissue specimens were directly collected from diseased organs, including the liver and spleen. They were collected and preserved frozen at -20°C until used for DNA extraction and PCR detection of the oncogenic viruses.

Ten to twenty blood samples from the wing veins of the available live birds admitted to the laboratory were taken using sterile 3 ml syringes and serum separation (centrifuged at 1000-2000 rpm). The serum was stored at -20°C in 2 ml collection tubes for ELISA serological testing.

Histopathological findings

For histopathological studies, tissue samples were directly collected from diseased organs, including the liver, spleen, lung, kidney, duodenum, intestine, proventriculus, brain, and ovary, and were preserved using 10% neutral-buffered formalin for an average of 3-4 days at room temperature.

Serological detection using the ELISA test

A total of 478 Sera samples (10-20 samples per flock) were collected from 43 chicken flocks (19 breeders and 24 layers) and tested for antibody detection against ALV (subtype A and B) using commercial antibody ELISA (IDEXX Laboratories, Inc., Maine, USA).

Molecular detection by PCR

The tissue samples from 95 chicken flocks were collected, prepared, and examined using PCR tests for different oncogenic viruses (MDV and ALV subtypes A, B, C, D, and J). Organ samples showing gross pathological lesions were selected from birds that had been previously examined. Only five chicken samples from each farm were pooled and treated as a single case sample. Briefly, the tissue was homogenized after being suspended in sterile phosphate-buffered saline (PBS) and centrifuged at 3000 rpm at 4°C for 15 minutes to obtain the supernatants. The specimens were stored at -20 °C until use, and DNA extraction was performed according to (Murray and Thompson, 1980).

Extraction of viral Nucleic acid

Tissue homogenate samples were subjected to whole nucleic acid extraction using the QIAamp MiniElute Virus Spin Kit (Qiagen, GmbH, Germany). Briefly, 200 µL of the sample suspension was treated with 25 µL of Qiagen protease and 200 µL of AL buffer for 15 min at 56°C. After incubation, 250 µL of absolute ethanol was added to the lysate. The sample was then purified by centrifugation following the manufacturer's instructions. DNA was eluted using 100 µL of elution buffer and stored at -20°C for further analysis

Amplification of viral nucleic acid using conventional PCR

PCR was performed using specific primers supplied by Metabion (Germany). The nucleotide sequences are listed in Table 2.

PCR amplification

A 25 µL total reaction mixture contained 12.5 µL of EmeraldAmp Max PCR Master Mix (Takara, Japan), 1 µL of each primer (20 pmol concentration), 5.5 µL of nuclease-free water, and 5 µL of DNA template. The process was performed using a Thermo Cycler 2720 (Applied Biosystems). About ALV A, B, C, and D, initial denaturation was performed at 94°C for 4 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s, with a final elongation at 72°C for 10 min (Fenton et al., 2005; Silva et al., 2007). For ALV J, the initial denaturation was conducted at 95°C for 5 min, followed Fby 35 cycles of 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s, with a final extension at 72°C for 10 min (Smith et al., 1979). The PCR conditions for MDV included one cycle of initial denaturation at 95°C for 15 min, 40 cycles of denaturation at 95°C for 45 s, annealing at 50°C for 45 s, extension at 72°C for 1 min, and a final extension at 72°C for 10 min (Handberg et al., 2001).

After amplification, the PCR products were separated by electrophoresis on a 1.5% agarose gel (AppliChem, Germany, GmbH) at 5 V/cm. For gel analysis, 15 µL of the PCR products were loaded into each well. Fragment sizes were determined using a 100 bp DNA ladder (Fermentas, Germany). The gel was imaged using a gel documentation system (Alpha Innotech, Biometra), and the data were analyzed using computer software.

Table 2. Primer nucleotide sequences are used for avian leukosis viruses (ALV) and Marek's disease virus in Egypt

Agent	Primer sequence (5'-3')	Amplified Product (bp)	Reference
ALV- A	H5-F GGATGAGGTGACTAAGAAAG EnvA-RAGAGAAAGAGGGGYGTCTAAGGAGA	694	Fenton et al. (2005)
ALV-B and D	BD-F CGAGAGTGGCTCGCGAGATGG BD-R AGCCGGACTATCGTATGGGGTAA	1100	Silva et al. (2007)
ALV-C	C-F CGAGAGTGGCTCGCGAGATGG C-R CCCATATACCTCCTTTTCCTCTG	1400	Silva et al. (2007)
ALV-J	H5-F GGATGAGGTGACTAAGAAAG H7-R CGAACCAAAGGTAACACACG	545	Smith et al. (1979)
MDV	ICP4 F GGATCGCCCACCACGATTACTACC ICP4 RACTGCC TCACACAACCTCATC TCC	434	Handberg et al. (2001)

RESULTS

Clinical and gross findings

Diseased chickens showed general signs of depression, stunted growth, prominent sternums, and mortality, reduced egg production in layers and breeders, and ruffled feathers in some birds; some affected flocks had neural lesions, such as paralysis of the legs, wings, and neck. In the case of ALV, the affected birds showed visceral tumors, diffuse or white nodules in the heart, proventriculus, liver, spleen, bursa of Fabricius, gonads, and kidney, and some birds had marked hepatosplenomegaly. For MD, the gross lesion was distinguished by congestion, hemorrhages, and splenomegaly with a nodular liver lesion and a sprain of the limb nerve.

Histopathological findings

Histological sections were prepared from different organs (liver, spleen, ovary, kidneys, intestines, and brain) from suspected cases and were submitted for histopathological examination. Most examined cases showed variable pathological alterations, ranging from mild inflammatory features to obvious neoplastic changes in confirmed cases.

In the case of ALV-J infection, examined organs (liver, spleen, kidney, ovary, and intestine) showed severe characteristic uniform lymphocytic and myelocytic cell infiltration. The liver revealed severe congestion of hepatic sinusoids and severe destruction of hepatocytes, which were replaced by diffuse lymphocytic and myelocytic cell infiltration, though one case showed only moderate hepatocellular necrosis with extensive lymphocytic cell infiltration (Figure 1.1). The spleen showed lymphocytic cell depletion and massive tumor lymphocytic cell infiltration associated with marked fibrous tissue proliferation (Figure 1.2). Kidneys revealed massive hemorrhages and intertubular lymphocytic cells infiltration with diffuse tubular degeneration and necrosis (Figure 1.3). The ovary was characterized by severe adenocarcinoma with abundant eosinophilic cells and tumor lymphocytic cell infiltration (Figure 1.4). The intestine revealed marked lymphocytic cell infiltration within the lamina propria with marked destruction of intestinal villi as well as a damaged muscular layer, which was replaced by massive lymphocytic cells (Figure 1.5).

The histopathological lesions of organs (brain and liver) suffering from MDV infection showed diffuse pleomorphic lymphocytic cell infiltration. The brain showed obvious perivascular cuffing, which was mild (Figure 1.6). The liver showed hepatocyte congestion, degeneration, infiltration of mononuclear cells, vasogenic edema, cell swelling, hyaline casts in renal tubules, and lymphoproliferative foci-diffuse necrosis, which infiltrated with pleomorphic lymphocytic cells mixed with fine fibrous tissue (Figure 1.7).

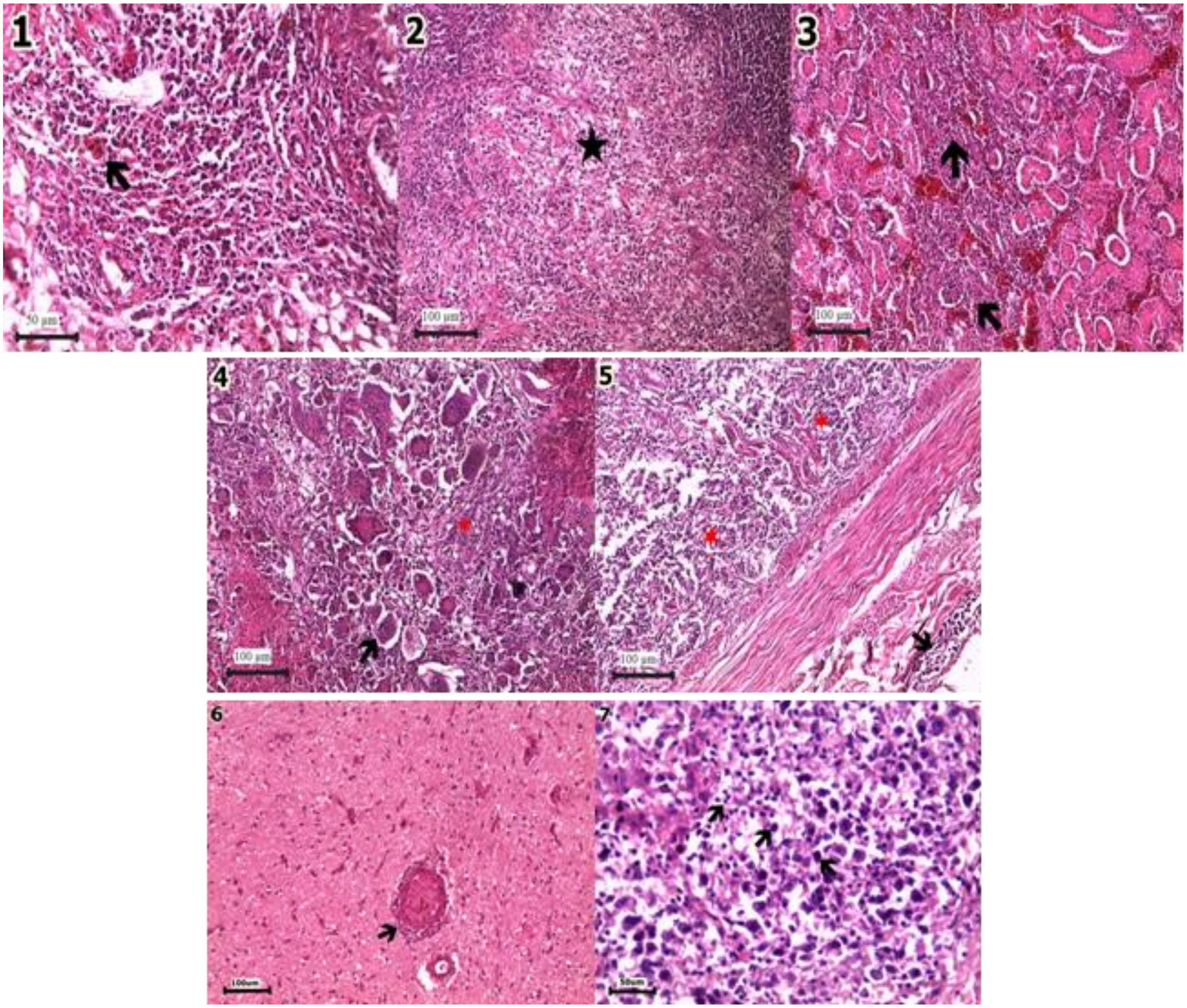


Figure 1. The histopathological analysis of infected chickens with ALV-J. **1:** Liver tissue showed diffuse infiltration of lymphocytic cells and marked myelocytic cells (arrow), **2:** Spleen with marked lymphocytic depletion and tumor cells infiltration mixed with fine fibrous tissue (star), **3:** Kidney revealed marked nephrosis with intertubular lymphocytic cells infiltration, **4:** Ovary showed disuse destruction and necrosis of ovarian parenchyma with marked adenocarcinoma and infiltration of lymphocytic cells and giant cells, **5:** Intestine showed severe enteritis with massive submucosal infiltration of lymphocytic cells (arrow) and within necrotic villi (star). The histopathological analysis of an infected chicken with MDV; **6:** Brain tissue showed marked perivascular cuffing (arrow) and gliosis; **7:** Hepatic tissue infiltrated with diffuse massive pleomorphic tumor cells (arrow). Hematoxylin and eosin stain (H&E).

Molecular detection using conventional PCR

A PCR assay was used to test 95 flocks of chickens from various production types (43 breeders, 45 layers, and seven broilers) for the molecular detection of MDV and ALV subtypes A, B, C, D, and J. The samples were taken from 2019 to 2022 (Table 3); only two flocks were positive (2/95), 2.1 % for the ALV-J virus, one breeder farm in 2021, and one-layer farm in 2022 from Sharqia governorate (Table 4).

The overall incidence of MDV during the study period was 18/95 (18.9%). The highest prevalence rate recorded among different years of the study was 30.8% in 2019, followed by 20% in 2022, 16.7% in 2021, and 16.3% in 2020, as shown in Table 3. The MDV prevalence rate in breeder flocks was 25.6%, while it was 15.6% in layer flocks; all broiler flocks were negative for MDV by PCR (Table 3). The MDV geographic prevalence was 66.7% (8/12) (Sharqia, Menofia, Dakahilia, Gharbia, Qualiobia, Beheira, Giza, Damietta) during the study period (2019 to 2022) (Table 4 and Figure 2). The age of MDV positivity in breeder flocks ranged from 35- 427 days (5-61 weeks), while for the case of the layer, flocks ranged from 60- 302 days (8.5-43 weeks) (Tables 5 and 6).

Serological investigation

Only 43 flocks (19 breeders and 24 layers) with a total of 478 sera samples were examined for antibodies against avian leukosis virus subgroups A and B (ALV) using a commercial ELISA test; samples were collected between 2019 and 2022 from 9 provinces. From 43 tested flocks, antibodies were detected in only two breeder flocks at age 61 weeks from Beheira governorate, with seroprevalence rates of 4.7% (2/43). The two flocks showed a 100% positivity rate, with a geometric mean (GMT) of 2848 and 1892, and the coefficient variation (CV) was 18 and 24, respectively.

Table 3. The positive flocks of Marek's disease virus during the 2019 -2022 passive surveillance program using PCR tests among different production sectors

Year	Number of Positive Farms/ Production Sectors (Pos/Total Tested)			*Yearly positivity rate
	Breeder	Layer	Broiler	
2019	1/1	3/12	0	4/13 (30.8%)
2020	5/20	2/18	0/5	7/43 (16.3%)
2021	4/14	0/9	0/1	4/24 (16.7%)
2022	1/8	2/6	0/1	3/15 (20 %)
**Sector Incidence	11/43 (25.6%)	7/45 (15.6%)	0/7	18/95 (18.9%)

*Yearly positivity rate: Shows the result of examined and positive flocks of MDV distribution from 2019 -2022 by PCR test during the passive surveillance program for oncogenic viruses in Egypt. **Sector Incidence: This shows the detection and distribution rate of MDV-positive flocks in relation to the production sectors.

Table 4. Geographical distribution of examined and positive flocks of Mark's Disease Virus and Avian Leukosis Virus-j by PCR during 2019-2022 in Egypt

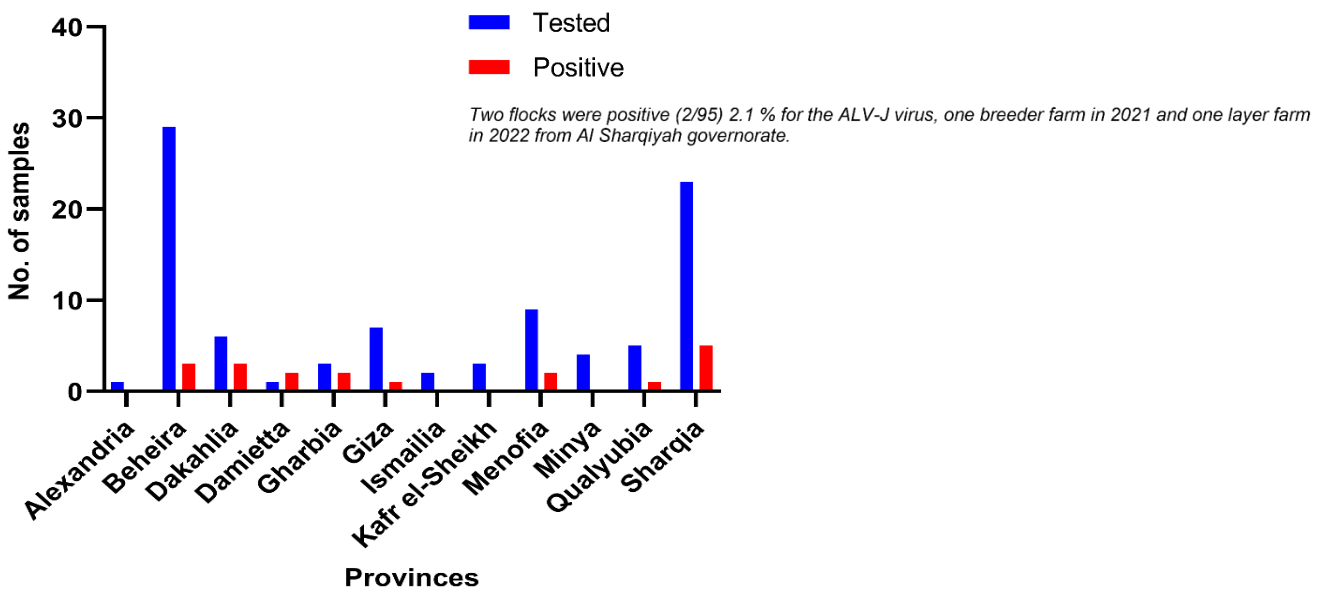
Governorate	Positive/Total Examined Farms	Positivity (%)
Alexandria	0/1	0
Ismailia	0/2	0
Sharqiah	*5/23	21.7%
Menofia	2/9	22.2%
Dakahilia	3/6	50%
Gharbia	2/3	66.7%
Qualiobia	1/5	20%
Beheira	3/29	10.3%
Giza	1/7	14.3%
Damietta	3/3	100%
Kafr El Sheikh	0/3	0
Minya	0/4	0
Total	20/95	21.1%

*Only two flocks were detected as positive (2/95) (2.1 %) for the ALV-J virus, one breeder farm in 2021, and one-layer farm in 2022, from Sharqiah governorate.

Table 5. Age distribution of positive Mark's Disease Virus flocks during 2019-2022 passive surveillance in Egypt

Age / Day **Type of production**	***≥ 100***	***100-200***	***200-300***	***≤ 300***	***Total***
Breeder	2	-	5	4	11
Layer	1	3	2	1	7
Total	3	3	7	5	18

All tested broiler cases were negative for MDV during the study time.



Two flocks were positive (2/95) 2.1 % for the ALV-J virus, one breeder farm in 2021 and one layer farm in 2022 from Al Sharqiyah governorate.

Figure 2. Geographical distribution of examined and positive flocks of Mark's Disease Virus (MDV) and Avian Leukosis Virus -J (ALV-J) by PCR during 2019-2022 in Egypt

Table 6. Descriptive data for 18 farms positive for Marek's Disease Virus and two farms positive for Avian Leukosis Virus-j by PCR test in Egypt during 2019-to-2022, passive surveillance

Flock ID	**Year**	**Governorate**	**Age (Day)**	**Type of production**	**ELISA**
1	2019	Sharqia	60	Layer	NEG
2			198	Layer	NEG
3		Gharbia	216	Layer	*ND
4		Damietta	218	breeder	ND
5	2020	Damietta	378	breeder	ND
6			399	breeder	ND
7		Menofia	210	Breeder	ND
8			35	breeder	ND
9		Sharqia	302	Layer	ND
10		Dakahilia	301	breeder	ND
11		Gharbia	216	Layer	NEG
12	2021	Beheira	427	breeder	POS
13			215	breeder	NEG
14		Qualyubia	273	breeder	NEG
15		Giza	35	breeder	NEG
16	2022	Dakahilia	120	Layer	ND
17			154	Layer	NEG
18		Beheira	280	breeder	NEG
19**	2021	Sharqia	80	breeder	ND
20**	2022		201	Layer	NEG

*Nd: Not done means blood samples were not collected. Pos: Positive – Neg: Negative. ** Farm positive for ALV-J by PCR test.

DISCUSSION

Oncogenic viruses, Marek's disease virus (MDV) and avian leukosis viruses (ALVs), are important neoplastic diseases that cause significant economic losses in the poultry industry due to immunosuppression, including chicken condemnation (Witter and Schat, 2003).

The current study offers updates on the molecular prevalence of MD and ALV subtypes A, B, C, D, and J, and antibody detection against ALV subtypes A and B in commercial chicken flocks during 2019-2022. Clinical signs in most studied cases included loss of body weight and thin abdomens with protruding sternums. The recorded gross lesions consisted of diffusion of focal tumors in different visceral organs, which appeared as white nodules in the spleen, liver, kidney, pancreas, lung, heart, proventriculus, and ovary with marked hepatosplenomegaly. The same clinical signs and gross lesions were recorded (Liu et al., 2019; Eid et al., 2019). Histopathological findings of ALV-J and MDV infection in different organs were previously recorded by Liu et al. (2019), Fotouh et al. (2020), and Soliman et al. (2023).

PCR is a suitable technique to determine the serotype specificity of ALV and to differentiate between vaccinal and field strains of MDV serotype-1 (Handberg et al., 2001; Silva et al., 2007). From 2019 to 2022, eighteen MDV cases and two ALV-J cases were detected by PCR in 95 Egyptian chicken farms, revealing the circulation of both viruses among commercial poultry flocks during the study period.

ALVs are serious oncogenic viruses that induce severe economic losses in the poultry industry in Egypt (Mousa and Abdel-Wahab, 2009; Yehia et al., 2021). Two flocks were positive (2/95), 2.1% for the ALV-J virus, one breeder farm in 2021, and one layer farm in 2022, from Sharqia governorate.

These results are consistent with Soliman et al. (2023), who reported that 1 of 6 breeder flocks (16.6%) in Sharqia governorate tested positive for ALV-J. However, Fotouh et al. (2024) detected 43 out of 57 positive samples (75.4%) from broiler flocks in Sharqia, Dakahilia, and Qalyubia Egyptian governorates from 2021 to 2023. ALVs are transmitted through multiple routes, including vertical transmission (hen to offspring) and horizontal transmission via direct or indirect contact between infected and healthy birds (Titan et al., 2024). Additionally, contamination of commercial Marek's disease vaccines with avian leukosis viruses has been documented as an important source of infection in vaccinated chickens (Silva et al., 2007; Mohamed et al., 2010).

MDV was detected in 18 out of 95 samples from 8 different provinces of Egypt from 2019 to 2022; 11/43 (25.6%) were breeders, and 7/45 (15.6%) were layers. All broiler flocks were negative for MDV by PCR test. The results agree with Ewies et al. (2020), who detected MDV in 5 out of 10 samples from 6- to 8-month-old vaccinated layer chicken farms from 6 governorates of Egypt from January 2019 to November 2020 and agree with Yehia et al. (2021), who detected MDV in 28 out of 40 samples from vaccinated layer and breeder chicken farms from 8 governorates of Egypt in 2020. In the current study, the detection of MDV field strains was differentiated from MDV-vaccinated strains by a PCR test. The MDV-positive flocks were detected in 8 different governorates of Egypt: Sharqia, Menofia, Dakahilia, Gharbia, Qualiobia, Beheira, Giza, and Damietta, showing a high geographical prevalence rate of 66.7% (8/12). Moreover, the incidence rates of MDV vary from one governorate to another and range from 100% in Damietta to a minimum of 10.3% in Beheira governorate. MDV was previously detected in the same governorates (El-Kenawy and El-Tholoth, 2019; Ewies et al., 2020; Yehia et al., 2021).

The apparent age of positive Marek's disease virus cases among breeder chicken flocks ranged from 35 to 427 days (5 to 61 weeks), and the most recorded positive flocks were at age 30-61 weeks. This complies with Zhuang et al. (2015), who confirmed an outbreak of Marek's disease in a vaccinated breeder flock during its highest egg-production period (24th and 30th weeks) in China. The age of Marek's disease virus-positive cases among layer flocks ranged from 60 to 302 days (8.5 to 43 weeks). Additionally, the most recorded positive flocks were from 14 to 43 weeks. This corresponds with Ewies et al. (2020), who identified MDV in vaccinated layer chickens aged between 24 and 32 weeks. Furthermore, the disease was demonstrated in chickens aged 3-4 weeks or older and is usually recorded between 12 and 30 weeks of age (OIE, 2010).

Marek's disease virus is one of the most transmissible diseases of fast lymphoproliferative changes in affected chickens. Although the Egyptian hatcheries follow an intensive MDV vaccination policy on the first day of age, the flocks still suffer from many cases of MDV infection, shedding, and subclinical virus circulation, which continues in farms. The vaccine breaks may occur due to the expanded virulence of MDV strains, which have developed the capability to vanquish immune responses convinced by vaccine application (Witter, 1997; Hassanin et al., 2013), the presence of immunosuppressive agents (Haridy et al., 2009; López-Osorio et al., 2017; Umar et al., 2017) or the challenges associated with the vaccine handling due to its cell-associated form, the attenuation procedure, and the incorrect dose and route of administration (Jarosinski et al., 2006; Geerligs et al., 2008; Abdul-Careem et al., 2014).

In recent years, Egypt has reported numerous cases of isolation of virulent and highly virulent plus MDV strains from immunized chickens. Many studies have documented the isolation of a very virulent strain of MDV type 1 from many poultry flocks that have been MDV-vaccinated (El-kenawy et al., 2019; Ewies et al., 2020; Yehia et al., 2021). The widespread use of vaccines may contribute to increased virulence in field strains. While vaccines protect chickens from clinical disease, they allow the virus to replicate and spread (Haq et al., 2013; Padhi and Parcells, 2016; Mescolini et al., 2020).

While sequencing the Meq proteins, Abdallah et al. (2018) and Yehia et al. (2021) from Egypt identified several mutations in amino acids associated with MDV virulence. Moreover, the direct proliferation that occurred in lymphocytes caused tumors because of Meq gene changes, which appear to be linked to increased virulence (Lupiani et al., 2004; Shamblin et al., 2004). The virulence of MDV has increased in recent years, and some recently isolated (vv and vv+) strains have been reported as more virulent for chickens than the previously isolated strains.

The severity of the disease varies, attributable to the viral genome mutation, which can generate novel pathotypes capable of evading vaccine-induced immunity. Yehia et al. (2021) found a low amino acid identity (as low as 82.5%) between field strains and vaccine strains (CVI988 and 3004), suggesting that current vaccines may have reduced efficacy in Egypt and highlighting the need for further research.

The enzyme-linked immunosorbent assay (ELISA) is a valuable tool for surveillance, enabling the detection of exogenous ALV infections in flocks. It can also identify subgroup-specific ALVs for eradication programs, allowing the removal of infected or carrier chickens. ELISA is sensitive, safe, rapid, and suitable for large-scale testing (Abdel Gayed et al., 2017). None of the 43 farms tested in this study had detectable antibodies against ALV (subtypes A and B), except for two breeder farms in Al Beheira governorate (61-week-old birds). Abdel Gayed et al. (2017) conducted serological studies on avian leukosis virus in broiler chickens in Egypt.

Avian leukosis virus subgroups A and B can persist in egg and meat-type chickens, often without causing significant economic losses. However, co-circulation of ALV and MDV may exacerbate disease severity compared to single infections (Wang et al., 2020).

CONCLUSION

The results demonstrate the sporadic incidence of only two cases of ALV-J, alongside a high prevalence of vvMDV (18.9%) in Egyptian commercial chicken flocks during 2019-2022. These findings underscore the urgency for further research to examine the genetic correlation of the circulating vvMDV strains and the currently used MDV vaccines. Furthermore, the authors recommend reviewing the current MDV vaccination program, including vaccine quality and efficacy, as well as maintaining the passive reporting program for oncogenic viruses for continuous monitoring.

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Authors’ contributions

Marwa Safwat contributed to the manuscript idea, serological testing, data analysis, and manuscript writing. Fatma Amer conducted the PCR and wrote the manuscript. Marwa Ali was responsible for epidemiological data collection, sorting, analysis, and histopathology. Mai M. Morsy conducted serology testing. Mohamed M. Samy conducted post-mortem, sample collection, sorting, preparation, and storage of field samples. Motaz Mohamed was responsible for field visits and sample collection. Both Wafaa Mohamed and Abdullah A. Selim designed the study, wrote the manuscript, and revised it. All authors read and approved the final version of the manuscript.

Availability of data and materials

The original contributions presented in the study are included in the article and will be available upon reasonable request from the corresponding author.

Competing interests

The authors declare no competing interests.

Ethical considerations

The authors confirm that this manuscript represents original study results that have not been previously published. All authors have reviewed and approved the manuscript before submission.

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Pathogenicity of Highly Pathogenic Avian Influenza Virus (H5N1) in Different Duck Breeds

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ABSTRACT

Avian influenza viruses (AIVs) pose a global threat, with wild waterfowl serving as key reservoirs for transmission to poultry. The present study investigated the pathogenicity, viral shedding patterns, tissue distribution, and pathological effects of a highly pathogenic avian influenza virus (HPAIV) in two duck breeds, including Muscovy and Sudani ducks. An Egyptian H5N1 strain (A/ibis/Egypt/RLQP-229S/2022), originally isolated from a wild ibis, was used. Forty ducks (20 Muscovy and 20 Sudani) were divided into infected and control groups (10 per group per breed). At four weeks of age (Average weight of 1.2 ± 0.1 kg), each infected duck received a single intranasal dose of 10^6 EID_{50}. Cloacal and oropharyngeal swabs were collected at 3, 5, 7, and 10 days post-infection (DPI) to monitor viral shedding, while clinical signs were recorded daily. Mortality was higher in Muscovy ducks, which exhibited higher mortality (70%) than Sudani ducks (50%), with both breeds showing neurological signs and lethargy. Viral load analysis of cloacal swabs via RT-PCR (Targeting the AIV *M* gene), exceeded oropharyngeal shedding, peaking by five DPI and persisting longer in Muscovy ducks (Seven DPI compared to five DPI in Sudani ducks), suggesting that fecal-oral transmission is the primary route of spread and that viral replication is more active in the intestinal tract. Tissue distribution analysis revealed broader viral dissemination in Muscovy ducks, particularly in the brain, lung, kidney, and spleen. These findings demonstrated differential susceptibility between breeds, with Muscovy ducks posing a higher transmission risk due to prolonged viral shedding and tissue tropism. The virus used in the present study carried pathogenicity markers across several proteins, including hemagglutinin (HA), neuraminidase (NA), polymerase basic 1 (PB1), polymerase basic 2 (PB2), nucleoprotein (NP), non-structural protein 1 (NS1), and polymerase acidic (PA) protein. Overall, while both duck breeds are vulnerable to the circulating H5N1 HPAI strain, their susceptibility and clinical outcomes differ. These findings demonstrated that both Muscovy and Sudani ducks are susceptible to H5N1 HPAIV infection, Muscovy ducks showing higher mortality and more extensive viral shedding and histopathological alterations. However, both duck breeds are variable in their susceptibility to H5N1 infection.

Keywords: Avian influenza viruses, Duck breeds, H5N1, Histopathological changes, Pathogenicity, Virus shedding

INTRODUCTION

The emergence of pandemic influenza outbreaks is frequently associated with the ability of influenza A viruses (IAV) to overcome species barriers and establish infection in novel host populations through substantial antigenic evolution (Horimoto and Kawaoka, 2001). Waterfowl serve as a natural reservoir for different avian influenza viruses (AIV) subtypes, playing a crucial role in maintaining viral diversity and facilitating transmission to domestic poultry through asymptomatic viral shedding (Blagodatski et al., 2021).

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The highly pathogenic avian influenza virus (HPAIV), first detected in poultry in Guangdong, China in 1996, has since evolved and spread globally, affecting domestic birds, wild avian species, and even humans. This lineage, known as Goose/Guangdong/1/96 (GS/GD), was able to cross species barriers and disseminate across Europe, Asia, Africa, and North America, primarily via migratory birds (Cui et al., 2022; Engelsma et al., 2022; Sagong et al., 2022).

Egypt reported its first H5N1 outbreak in 2005. Over the following years, multiple clades emerged and spread extensively in domestic poultry, with significant economic and public health impacts. The H5N1 strains isolated in Egypt have been classified into clades, such as 2.2, 2.2.1, 2.2.1.1, 2.2.1.2, and 2.3.4.4b (Arafa et al., 2015; El-Shesheny et al., 2021; Mosaad et al., 2023). The ongoing detection of these variants highlights the virus's persistence and adaptive capacity in the region.

Several HPAI H5N1 genotypes within clade 2.3.4.4b appeared in wild birds during late 2020 and were found in several African, Asian, European, and North American nations (Engelsma et al., 2022; Sagong et al., 2022). The AIV H5N1 causes significant morbidity and mortality in poultry and has been reported to cause human infection (Horimoto and Kawaoka 2001). Because domestic ducks can have close contact with wild birds and land poultry simultaneously, they represent a significant source of AIV transmission from wild waterfowl to terrestrial poultry (Kwon et al., 2019). The AIV is categorized into two groups based on pathogenicity in chickens, including HPAIVs, which cause high mortality (Up to 100%) and severe systemic disease; and low pathogenic avian influenza viruses (LPAIVs), which usually result in mild respiratory or enteric symptoms, with significantly lower mortality (Shriner and Root, 2020). Both forms circulate among domestic and wild birds.

Influenza A viruses, which belong to the Orthomyxoviridae family, are further classified into 18 hemagglutinin (H1–H18) and 11 neuraminidase (N1–N11) subtypes based on their surface glycoproteins. The HA glycoprotein represents the primary antigenic determinant of influenza viruses. While antigenic shift in HA can precipitate pandemic emergence through major antigenic changes, antigenic drift enables circulating strains to evade population immunity through gradual accumulation of mutations, as most HA-directed antibodies demonstrate strain-specific neutralization (Wu and Wilson, 2020). These viruses are susceptible to reassortment, particularly in waterfowl, which contributes to the emergence of novel strains with pandemic potential (Taylor et al., 2023). Previously, the H5N1 HPAI viruses of the Asian lineage did not cause significant harm or death in ducks (Perkins and Swayne, 2002). The ability of domestic ducks to harbor H5N1 HPAI viruses raises public health concerns, emphasizing the need to limit their further spread and circulation (Kim et al., 2009).

Ducks are a significant source of influenza viruses that can spread to humans and other birds and mammals. Ducks are a natural reservoir of AIV and can act as a reassortment host (Hassan et al., 2020). Although wild ducks can carry AIV without showing clinical disease (Abtin et al., 2022), newer H5N1 variants have caused more severe disease in domestic duck breeds. The virus can replicate systemically, leading to widespread tissue damage, organ-specific variation in virus titers, and increased mortality (Hulse-Post et al., 2005; Samir et al., 2019). Different viruses from the H5 subtype of clade 2.3.4.4b generated systematic infection and demonstrated efficient direct transmission in ducks (Sun et al., 2016).

The H5N1 virus (Clade 2.2.1.2) experimentally infected Sudani ducks (*Cairina moschata*), resulting in severe lung tissue damage and robust viral replication, but only slight alterations in brain histology and reduced viral replication (Samir et al., 2019). The present study aimed to investigate the pathogenicity, viral shedding, and histopathological effects of Egyptian HPAI H5N1 virus (A/ibis/Egypt/RLQP-229S/2022) on Muscovy and Sudani ducks to evaluate the breed-specific susceptibility and the potential risk for virus transmission.

MATERIALS AND METHODS

Ethical approval

The study protocol received approval from the Ethics Committee of the Animal Health Research Institute in Egypt (Approval No. AHRI-EG-2022-042). The experiment took place at the Experimental Animal House Facility, Animal Health Research Institute, Giza, Egypt, in accordance with institutional animal care guidelines.

Virus

The HPAI H5N1 isolate used in the present study, A/ibis/Egypt/RLQP-229S/2022, was originally isolated from a wild Ibis bird (*Threskiornis aethiopicus*) during active surveillance in 2022. It was confirmed as an HPAIV by sequencing the HA cleavage site (Mosaad et al, 2023). The virus was cultivated in the allantoic cavities of 9–11-day-old specific pathogen-free (SPF) embryonated chicken eggs. The harvested allantoic fluid was then clarified by centrifugation, filtered, and titrated to determine the median egg infectious dose (10^6 EID_{50}/mL). A 1:10 dilution was used for infection. The virus stock was tested by (Real time- polymerase chain reaction) RT-PCR and confirmed to be free of contaminants, including Newcastle disease virus, infectious bronchitis virus, and other AIV subtypes.

Study design

Housing

A total of 40 four-week-old ducks (20 Muscovy and 20 Native Sudani) were obtained from licensed commercial duck farms in Giza, Egypt. The ducks were divided into three groups, including 10 infected Muscovy ducks (Group 1), 10 infected Sudani ducks (Group 2), and a negative control of 20 non-infective ducks, 10 from each breed in 2 separate cages (Group 3). Duck serum samples were tested before the start of the study using Hemagglutination inhibition to confirm that all ducks were free from antibodies against avian influenza (AI).

All ducks were housed in animal Biosafety Level 3 (BSL-3) facilities at the Reference Laboratory for Veterinary Quality Control on Poultry Production (RLQP). Each group was housed in high-efficiency particulate air (HEPA-filtered) negative-pressure isolation units, with 0.5 m^2 space per duck, a 16:8 hours light-dark cycle, an ambient temperature of 25 ± 2°C, with relative humidity of 65%, *ad libitum* access to drinking water, and commercial pellet feed (Formulated for ducks, 22% protein) provided twice daily.

Inoculation and sampling

Ducks in infected groups received 0.1 mL intranasal inoculation of 10^6 EID_{50} of virus diluted 1:10. Oropharyngeal and cloacal swabs were collected on 3, 5, 7, and 10 days post-infection (DPI). Euthanasia was conducted using carbon dioxide inhalation as per ethical protocols. Tissue samples from lungs, brain, kidneys, and spleen were collected at each time point from 2 ducks per group. Ducks found dead or euthanized were immediately necropsied.

Internal organ viral shedding and replication

Tracheal and cloacal swabs were collected at 3, 5, 7, and 10 DPI from the inoculated and control groups with 10^6 EID_{50} virus to evaluate viral shedding from live ducks. To investigate viral replication in tissues, two ducks from each group were euthanized at 3, 5, 7, and 10 DPI. Lung, brain, spleen, and kidney were collected and processed for viral analysis.

Real-time reverse transcriptase-PCR

Swabs and tissue samples after grinding were processed for RNA extraction using Qiagen Viral RNA Mini Kit (Qiagen, Germany). For inactivation, 100 µL of each sample was mixed with 300 µL lysis buffer (Qiagen, Germany) supplemented with β-mercaptoethanol (100:1 v/v) and 3 mg RNA carrier (Qiagen, Germany). The mixture was incubated for 15 minutes at 22-25°C.

Quantitative RT-PCR targeting the conserved region of the *M* gene was carried out using the Qiagen OneStep RT-PCR kit (Qiagen, Germany) as described by Spackman et al. (2002). The amplification was carried out using the 7500 RT-PCR system (Applied Biosystems, USA) under the following cycling parameters. Reverse transcription (RT) at 50°C for 30 minutes, initial denaturation at 95°C for 15 minutes, followed by 40 PCR cycles consisting of denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 1 minute. Each RNA sample was tested in duplicate, and the assay was considered valid only if the cycle threshold (Ct) variations between replicates were less than 1, with a standard slope ranging from -3.2 to -3.7. The Ct values were then converted into equivalent 50% egg infectious dose per milliliter ($eqEID_{50}$/mL) using RNA standards derived from titrated virus.

Histopathology

Approximately 1 cm^3 samples of lung, brain, spleen, and kidney were fixed in 10% neutral buffered formalin. Tissues were dehydrated, embedded in paraffin, sectioned at 5 µm thickness, and stained with hematoxylin and eosin (H&E; Bancroft and Gamble, 2008). Slides were evaluated under a light microscope (Olympus BX50, Japan). Lesions were scored semi-quantitatively based on severity of inflammation, necrosis, and hemorrhage using a 0–3 scale in which 0 means no lesion, 1 means mild lesions, 2 means moderate lesions, and 3 means Severe.

Genetic markers for pathogenicity

The H5N1 virus (A/ibis/Egypt/RLQP-229S/2022) amino acid sequences accession numbers were retrieved from the Genbank database for the *HA* gene OP491851, the *NA* gene OP491854, the *PB2* gene OP491860, the *PB1* gene OP491859, the *NS1* gene OP491857, the *PA* gene OP491856, and the *NP* gene OP491855.For comparative analysis, closely related viral sequences were retrieved from the GenBank database. Multiple sequence alignments and pairwise comparisons were generated for each gene segment using the Clustal-V algorithm in *Mega 5* to identify genetic markers and determine their positions within the encoded proteins..

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics v20. A two-tailed Student's t-test was used to compare viral shedding data. Results were considered significant at $p \leq 0.05$.

RESULTS

Clinical signs and lesions

The control groups of Muscovy and Sudani ducks remained healthy throughout the current study, exhibiting no clinical signs or mortality. However, ducks experimentally infected with H5N1 (A/ibis/Egypt/RLQP-229S/2022) displayed neurological signs. Mortality rates differed significantly between species, with Muscovy ducks experiencing higher mortality (70%) compared to Native Sudani ducks (50%). The mean death time (MDT) was shorter in Sudani ducks (4 days) than in Muscovy ducks (5.2 days; Table 1). Postmortem findings revealed no pathological lesions in the control groups. In contrast, infected Muscovy ducks exhibited severe multisystemic involvement, including pericarditis, nephrosis, splenomegaly, pancreatitis, and petechial hemorrhages in the heart and spleen, along with brain congestion. Infected Native Sudani ducks displayed less extensive lesions, primarily pericarditis and nephrosis. Notably, Muscovy ducks showed more pronounced pathological changes compared to Sudani ducks, suggesting higher disease severity in this species (Table 1).

Virus shedding

Statistical analysis revealed no significant difference ($p > 0.05$) in viral shedding levels between Sudani and Muscovy ducks based on swab samples. However, significant temporal variations ($p \leq 0.05$) were observed in viral shedding across different days post-infection. The RT-PCR detection showed prolonged viral shedding in Muscovy ducks, which persisted until 10 DPI, while Sudani ducks cleared the virus earlier, with shedding detectable only until 7 DPI (Figure 1While both breeds showed early infection (3 DPI), only Sudani ducks excreted the virus through the oropharynx and cloaca on the 3, 5, and 7 DPI (Figure 1).

Table 1. Clinical signs and mortality of Muscovy and Sudani ducks at the end of the study

Breed	Mortality	MDT	Clinical signs	Post mortem
Muscovy	7/10 (70%)	5.2	Nervous manifestation	Pericarditis, petechial hemorrhage on the heart, brain hemolysis, pancreatitis, splenomegaly
Sudani	5/10 (50%)	4	Nervous manifestation	Pericarditis, nephrosis

MDT: Mean death time (days)

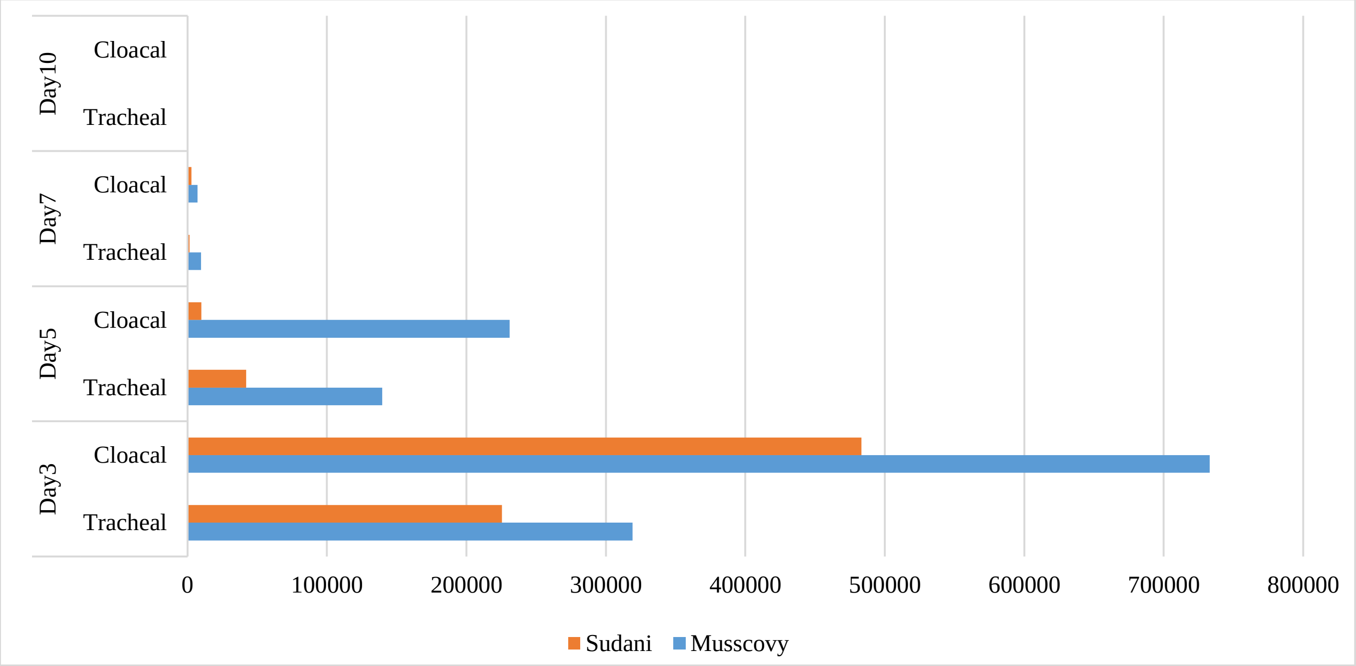


Figure 1. Virus shedding from swabs of both duck breeds at 3-, 5-, 7-, and 10-days post-infection. There was no significant difference between Sudani and Muscovy breeds in shedding results of H5 from tracheal swabs, $p > 0.05$, but there was a significant difference between both breeds in cloacal swabs, $p < 0.05$. Higher significant differences exist in shedding results on different days post-infection at $p \leq 0.05$. There was a gradual decrease in shedding from the 3rd to the 10th DPI.

Virus distribution in tissues

Widespread systemic infection was observed in Muscovy ducks, with viral detection in all examined tissues (Figure 2), and there was no statistically significant variation in viral distribution across different organs ($p > 0.05$); Both breeds demonstrated a highly significant difference in their shedding from tissues ($p \leq 0.05$). Additionally, the virus load was much higher in the brains and lungs than in other organs in both breeds (Table 2).

Histopathology

The histopathological changes in Muscovy ducks infected with H5N1 were observed in the brain tissue, featuring thickening of cerebral blood vessels, neuronal degeneration, and gliosis. In contrast, Sudani ducks exhibited perivascular lymphocytic cuffing in the brain along with gliosis, neuronal degeneration, and neuronophagia. The lungs of Muscovy ducks infected with H5N1 illustrated interstitial edema and infiltration of mononuclear inflammatory cells, accompanied by severe congestion of interstitial blood vessels. Interstitial capillary congestion was present in Muscovy ducks, and the bronchioles displayed hyperplasia of their lining epithelium. Comparatively, the lungs of Sudani ducks infected with H5N1 revealed diffuse interstitial edema with infiltration of mononuclear inflammatory cells, as well as hyperplasia of goblet cells lining the bronchial wall, along with bronchial and bronchiolar hyperplasia. The kidneys of Muscovy ducks infected with H5N1 exhibited vacuolar degeneration of the lining epithelium in some renal tubules, while others showed renal tubular necrosis. Interstitial capillary congestion and severe interstitial hemorrhage were noted in Muscovy ducks. Meanwhile, the kidneys of Sudani ducks infected with H5N1 demonstrated severe vacuolar degeneration and necrosis of the lining epithelium of renal tubules, alongside severe interstitial vascular congestion and interstitial hemorrhage. The spleen of Muscovy ducks infected with H5N1 indicated severe lymphoid depletion in the white pulp with splenic hemorrhage, whereas Sudani ducks infected with H5N1 presented with lymphoid depletion and diffuse hemorrhage in the spleen.

Molecular markers of pathogenicity

The pathogenicity markers of the challenge H5N1 virus used in the present study were compared to other related viruses from ducks in the same country but with different years (2012, 2016 and 2021) and different subtypes within the same or different clades (H5N1 and H5N8 of clade 2.3.4.4b and 2.2.1.2) as shown in Table 3.

The HA protein of the virus used in the present study has a cleavage site, *EKRRKR*, that is common in the same clade, indicating a highly pathogenic nature and its ability to replicate in different tissues in birds. The NA protein is expressed in different lengths among viruses. The shorter length protein is generally found in domestic ducks due to an amino acid deletion in the region from 49-68, which is found in the parent virus A/bar-headed goose/Qinghai/3/2005(H5N1). However, other H5N8 viruses had full-length NA.

The pathogenicity markers in the *PB1* protein are *V3* and *G622*; the pathogenicity of the used virus, A/ibis/Egypt/RLQP-229S/2022 (H5N1), is assumed to be varied compared to other H5N1 or H5N8 viruses found in ducks, as it contains *R3* and *A622*. At the same time, it shares with other viruses the same pathogenicity marker *E627* of the *PB2* protein. *PA* and *NP* proteins indicated the presence of pathogenicity markers *D383*, *V105*, and *K184*, respectively, while *NS1* has *S42* and *A149* markers.

Table 2. Virus distribution in tissues of Muscovy and Sudani ducks at 3-, 5-, and 7-days post-infection

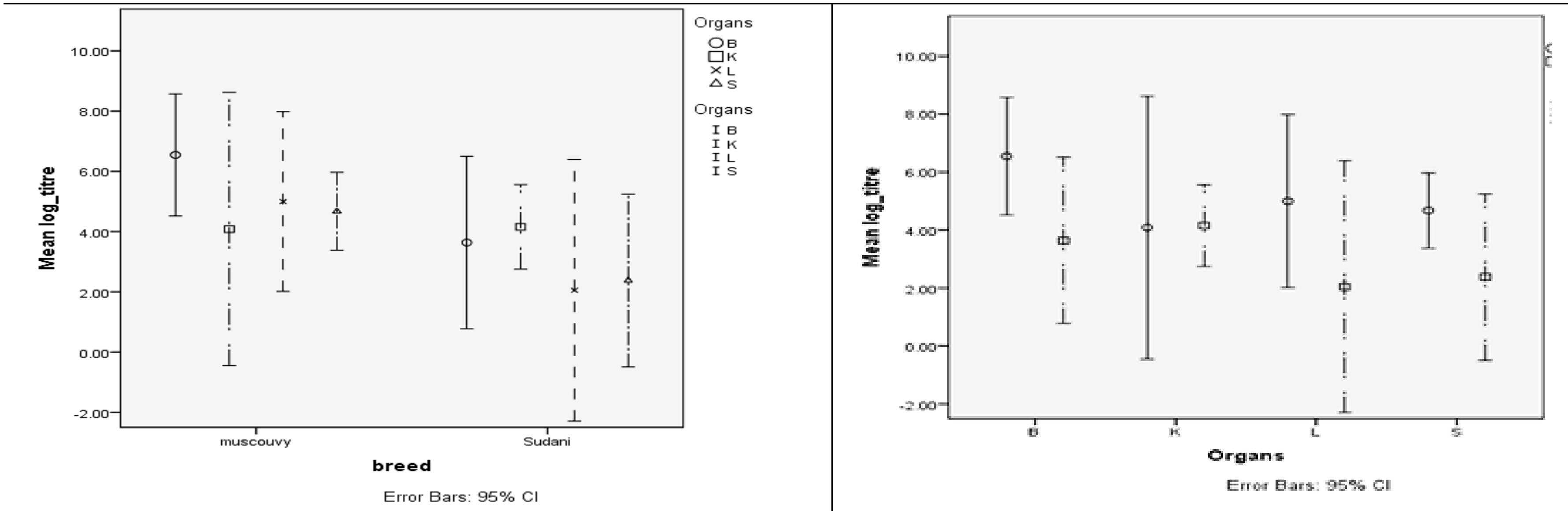
Organs	3rd DPI		5th DPI		7th DPI	
	Muscovy	Sudani	Muscovy	Sudani	Muscovy	Sudani
Brain	4.6×10^4	1.3×10^4	2.3×10^7	2.5×10^6	6.9×10^6	0
Lung	3×10^4	2.8×10^2	4.6×10^6	5.8×10^5	3.7×10^2	0
Kidney	5.9×10^5	1.6×10^4	1.9×10^6	6.6×10^5	1.9×10^4	0
Spleen	6×10^3	1.2×10^5	2.4×10^5	1×10^4	1.5×10^4	0

DPI: Days post-infection; The titers were higher in the brain than in other organs of Muscovy ducks at the 5th and 7th DPI. In Sudani ducks, the titer was higher in the brain than in other organs at the 5th DPI, with no shedding observed at the 7th DPI. The virus distribution in the organs of Muscovy ducks was greater at the 5th DPI than in Sudani ducks, while the virus persisted in the tissues of Muscovy ducks until the 7th DPI, but ceased in Sudani ducks as they recovered from clinical symptoms.

Table 3. Molecular markers of host adaptation and pathogenicity

Virus protein	**HA Cleavage site**	**NA Length**	**PB1**		**PB2**		**PA**	**NP**		**NS1**	
			G 622	**V 3**	**K 627**	**D 701**	**D 383**	**V 105**	**K 184**	**S 42**	**A 149**
A/ibis/Egypt/RLQP-229S/2022 (H5N1)	*EKRRKR*	460 (Δ49-68)	A	R	E	D	D	V	K	S	A
A/bar-headed goose/Qinghai/3/2005(H5N1)	*ERRRKKR*	460 (Δ49-68)	G	V	K	D	D	V	K	D	G
A/duck/Egypt/Q4596B/2012(H5N1)	*EKRRKKR*	449 (Δ49-68)	E	V	K	D	D	V	K	D	G
A/green-winged teal/Egypt/877/2016(H5N8)	*EKRRKR*	470	G	V	E	D	D	M	K	D	A
A/northern shoveler/Egypt/MB-D-817OP/2016(H5N8)	*EKRRKR*	567	G	V	E	D	D	V	K	D	A
A/duck/Egypt/SJCEIRR-BA19903OP/2021(H5N8)	*EKRRKR*	470	G	V	E	D	D	V	K	S	A
A/pintail/Egypt/RA19853OP/2021(H5N1)	*EKRRKR*	469	G	V	E	D	D	M	K	S	A

HA: Hemagglutinin, NA: Neuraminidase, PB1: Polymerase basic 1, PB2: Polymerase basic 2, NP: Nucleoprotein, NS1: Non-structural protein 1, and PA: Polymerase acidic protein. Δ: The complete length of the A/Goose/Guangdong/1/1996 genomic segments is used to number the deletions in NA.



○Muscovy, □Sudani
B: Brain, K: Kidney, L: Lung, S: Spleen

○ Muscovy, □Sudani
B: Brain, K: Kidney, L: Lung, S: Spleen

Figure 2. Virus distribution in organs of two duck breeds at day 5 post-infection. There was a highly significant difference between Sudani and Muscovy breeds in shedding the H5N1 virus from tissues ($p \leq 0.05$). There was no significant difference between different organs in the shedding results from the tissues ($p > 0.05$).

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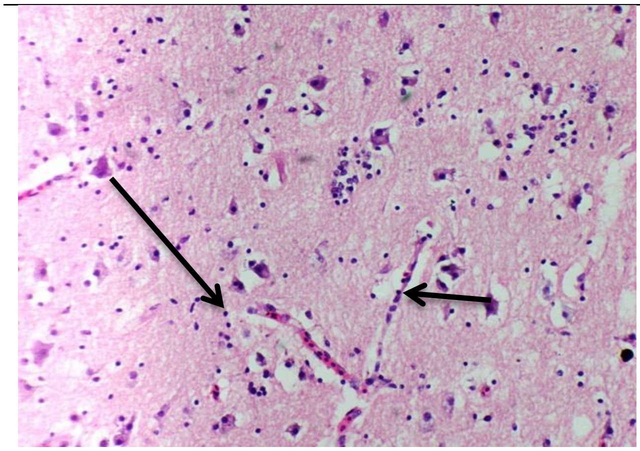
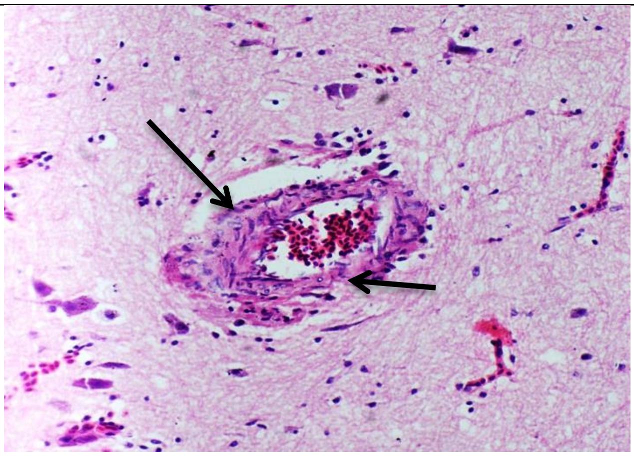
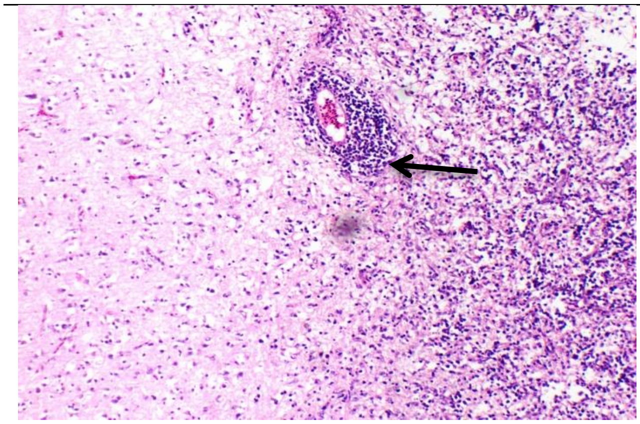
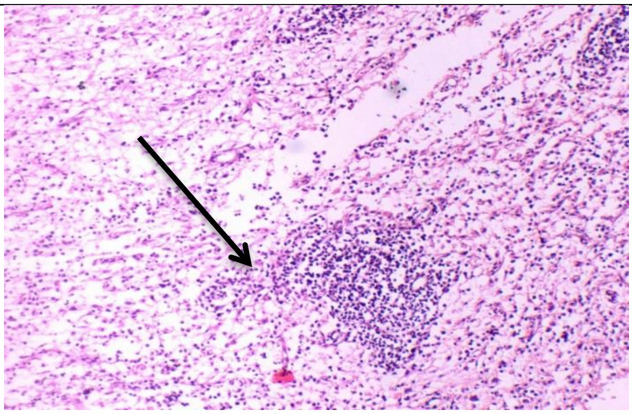
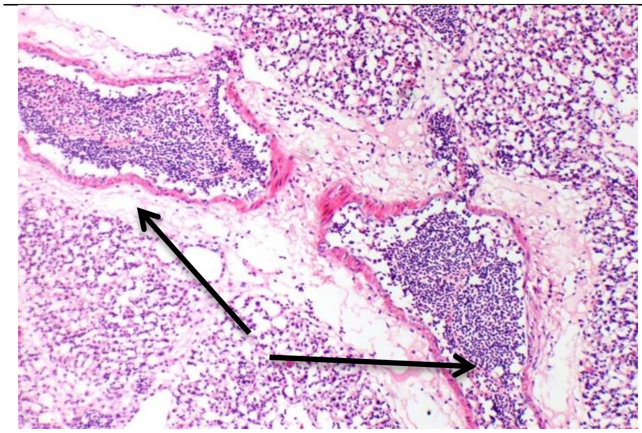
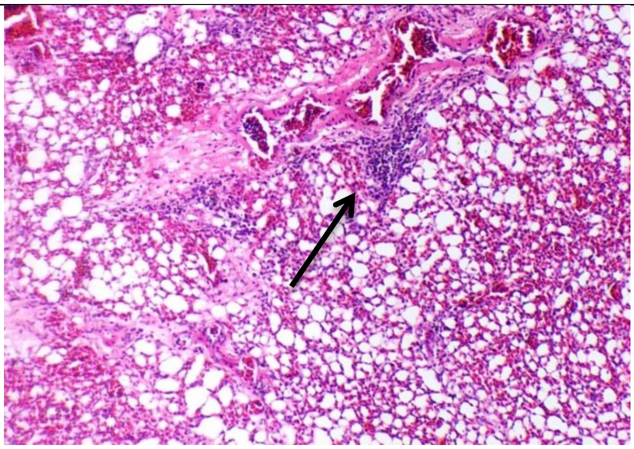
	
The Muscovy duck’s brain, showing neuronal degeneration (long arrow) and gliosis (short arrow, H&E ×200)	The Muscovy duck’s brain, showing thickening of cerebral blood vessel (black arrow, H&E ×200)
	
The Sudani duck’s brain, showing perivascular lymphocytic cuffing and vascular congestion (black arrow, H&E ×200)	The Sudani duck’s brain, showing focal gliosis (black arrow, H&E ×200)
	
The Muscovy duck’s lung, showing severe congestion of interstitial blood vessels (black arrows, H&E ×100)	The Sudani duck’s lung, showing vascular congestion with peri-vascular mononuclear infiltration (black arrow, H&E ×100)

Figure 3. Histopathological view of Sudani and Muscovy ducks infected with A/H5N1 from different organs at day 5 post-infection.

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DISCUSSION

The adaptability of H5N1 viruses to wild ducks plays a critical role in their widespread dissemination across continents. Asymptomatic shedding by wild waterfowl facilitates the reassortment and transmission of these viruses to domestic poultry, creating opportunities for the emergence of new and potentially more dangerous strains. Given the rapid evolutionary capacity of H5N1, implementing robust surveillance programs in poultry populations is critical for early detection and outbreak mitigation. Wild waterfowl, especially ducks, act as key natural reservoirs for AIV, frequently harboring and transmitting these pathogens without showing clinical symptoms (Blagodatski et al., 2021).

The present findings confirmed that both Muscovy and Native Sudani ducks are highly susceptible to infection with HPAI H5N1, as evidenced by virus shedding and histopathological changes. Muscovy and Native Sudani ducks showed marked viral replication and systemic dissemination. The current findings revealed a significant difference in the response of Muscovy and Native Sudani ducks to infection with a recent H5N1 AIV isolate. Muscovy ducks exhibited higher mortality rates and more severe clinical signs compared to their Sudani counterparts, suggesting a greater susceptibility to the H5N1 strain.

Duck species and breeds exhibit varying levels of susceptibility to H5N1 infection. While the immunological profiles of waterfowl prior to the emergence of highly pathogenic H5N1 strains remain poorly characterized, ducks are known to experience seasonal infections with LPAIV. These endemic LPAIV infections typically peak during autumn migration periods (Diskin et al., 2020; Kent et al., 2022). It is observed that the current H5N1 HPAIV has spread across continents largely due to its high adaptability to wild ducks, including migratory species (PAHO, 2023). In previous studies, H5N1 viruses found in wild waterfowl in Hong Kong induced severe neurological signs and resulted in mortality of ducks in both natural and controlled environments (Sturm-Ramirez et al., 2004; Beerens et al., 2021).

These differences in susceptibility could be attributed to several factors. Genetic variations between the two breeds may influence their immune response to the virus. It is possible that differences in behavior or physiology contribute to varying levels of viral replication and spread. For instance, Muscovy ducks might have behavioral patterns that lead to increased viral exposure or more efficient transmission. Specifically, the present study observed that Muscovy ducks shed more virus through their cloaca, suggesting a greater role for the fecal-oral route in transmission within this breed. In both breeds, the highest viral loads were found in the brain and lungs, suggesting a tendency for the virus to target these tissues (Szeredi et al., 2010). Interestingly, the H5N1 virus was detected in all tested organs of Muscovy ducks, with significantly higher viral loads compared to Sudani ducks.

The observed variations in pathogenicity in the present study aligned with previous studies, highlighting the diverse responses of different duck species to AIV. Some studies have shown that Muscovy ducks tend to experience more severe disease compared to other breeds, such as Pekin ducks (Pantin-Jackwood et al., 2013). In addition, Muscovy ducks were relatively shown to be susceptible to infection with H9N2 AIVs (Wang et al., 2019). This highlighted the importance of considering breed-specific susceptibilities in surveillance and control strategies.

In the present study, the MDT was four days in Sudani ducks, shorter than in Muscovy ducks (5.2 days). The H5N1 isolates exhibited variability in the MDT, ranging from 3.3 to 8.7 following intranasal injection, while other more recent isolates demonstrated an MDT between 1.7 and 4 after the same method of inoculation (Chen et al., 2004).

The mean cloacal titers were greater than the oropharyngeal titers in both breeds. These findings suggest that the virus primarily spreads via the fecal-oral route, with preferential replication occurring in the digestive tract. These current observations indicate that Muscovy and Native Sudani ducks faced a significant challenge in controlling HPAIV transmission, as they appear asymptomatic while shedding the virus for 7 days after infection. There was no significant difference between the Sudani and Muscovy breeds in the shedding results of H5 from swabs. More significant differences were observed in shedding results on different challenge days. Shedding gradually decreased from 3 to 10 DPI.

Comparison with earlier studies in domestic ducks supported the present findings. The duration and intensity of viral shedding through both cloacal and oropharyngeal routes aligned with reports on mule ducks or quails infected with HPAI H5N1 strains (Filaire et al., 2024; Bertran et al., 2013). However, the predominance of cloacal shedding in the current study underscored the fecal-oral route as a critical transmission pathway, especially in settings with a high risk of water contamination. The current observed shedding patterns aligned with known variations in H5N1 virulence across duck species, including mallards, Pekin ducks, and Muscovy ducks (Zhao et al., 2013; Uchida et al., 2019). These differences underscore the critical role of gastrointestinal replication in viral persistence within waterfowl populations and environmental transmission dynamics. While many H5N1 strains demonstrate high virulence in chickens attributed to their polybasic HA cleavage sites, mallards typically exhibit milder infections, reflecting significant host-specific differences in disease severity (Tang et al., 2009; Sun et al., 2016). Systemic viral spread to different organs reflected the pathophysiology and significant mortalities in both duck breeds. As previously demonstrated, the H5N1 clade 2.3.4.4b/2021 virus is highly infectious and transmissible in anseriformes but relatively poorly adapted to Galliformes (James et al., 2023).

The virulence of H5N1 in ducks is modulated by molecular features such as the HA cleavage site and polymerase complex proteins (Sonnberg et al., 2013). The presence of multiple basic amino acids at the HA cleavage site enhances host protease recognition, enabling systemic spread of the virus (Suguitan et al., 2012). The observed differences in tissue tropism and disease severity among duck breeds may be attributed to genetic variations in the HA cleavage sites of circulating H5N1 strains. The current isolate contained a polybasic motif consistent with the Gs/Gd/1/96 lineage, known for high virulence in poultry (Sonnberg et al., 2013).

The neuraminidase (NA) stalk length, another genetic factor, plays a role in host adaptation. Shortened NA stalks are often linked to adaptation from wild birds to domestic poultry, potentially enhancing viral fitness in terrestrial hosts (Li et al., 2011). The isolate used in the present study exhibited a shortened stalk, which may partially explain its efficient replication in both duck breeds.

In addition, several internal gene mutations have been implicated in host adaptation and pathogenicity. Viral virulence in poultry can be influenced by several polymerase protein alterations (Tada et al., 2011). One well-known mutation in the polymerase complex proteins is the substitution of lysine for glutamate at position 627 of the *PB2* protein (E627K), which is associated with a higher level of viral pathogenicity (Nilsson et al., 2017). The *E627K* mutation in the *PB2* protein enhances replication efficiency in mammalian cells by increasing polymerase activity at lower temperatures (Bogs et al., 2011). This mutation *E627K* raises viral polymerase activity for several AIV subtypes in mammalian cell lines and boosts the pathogenicity of H5N1 viruses (Bogs et al., 2011; Suttie et al., 2019). Combining this mutation with *D701N* of the *PB2* can increase viral polymerase activity, replication, and virulence (Tada et al., 2011; Taft et al, 2015).

The *NP* proteins indicated the presence of pathogenicity markers *V105* and *K184* (Tada et al., 2011). In previous studies, the *K184* mutation enhances viral replication and increases AIV virulence in chickens as well as improves viral replication in mammalian cells by enhancing the interaction between NP and importin-α isoforms (Wasilenko et al, 2009). The *V105* mutation appears to facilitate cross-species transmission from ducks to chickens by selectively enhancing viral replication efficiency in chicken embryonic fibroblasts, while maintaining unchanged replication capacity in duck cells (Tada et al., 2011). The *PA-N383D* mutation, found in some HPAI H5N1 isolates, has been associated with increased polymerase activity and enhanced replication in both mammalian and avian cell lines (Song et al., 2011; Lee et al., 2017). Similarly, *NS1-S42* and *A149* substitutions may suppress host interferon responses, facilitating immune evasion and higher virulence (Li et al., 2006; Hale et al., 2008).

These findings reveal the complex relationship between pathogen genetics and host biology in determining clinical outcomes. A deeper understanding of these molecular interactions enables better forecasting of viral spread and more precise intervention approaches in avian populations.

CONCLUSION

The present study confirmed that both Muscovy and Native Sudani ducks are highly susceptible to infection with HPAI H5N1, with significant viral shedding occurring via the cloacal route, pointing to a gastrointestinal tropism and fecal-oral transmission. The increased mortality observed in Muscovy ducks, despite comparable viral shedding levels, indicated the need for deeper investigation into breed-specific immune responses and neurotropism of H5N1 viruses. The present study underscored the complexity of H5N1 avian influenza and the importance of understanding breed-specific differences in susceptibility. Characterization of these differential susceptibilities enables optimized surveillance strategies and targeted control measures, enhancing protection for both poultry populations and public health.

Future studies should focus on comparative immunopathology among duck breeds, particularly evaluating cytokine responses and tissue-specific viral replication. Additionally, molecular surveillance of circulating HPAI strains in different avian hosts will be crucial for identifying key mutations associated with host adaptation and virulence. This information is critical for updating biosecurity, vaccination strategies, and outbreak response plans in both commercial and backyard poultry sectors.

DECLARATIONS

Authors' contribution

Hussein Ali Hussein designed the study. Riham I. El Tantawy collected the data. Riham I. El Tantawy and Abdelsatar Arafa conducted the data analysis and wrote the first draft of the manuscript, with Hussein Ali Hussein, Ayman El Deeb, and Basem Ahmed providing co-supervision. All authors confirmed the final edition of the study before submitting to the journal.

Availability of data and materials

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

Competing interests

The authors disclose no conflicts of interest.

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Ethical considerations

All authors acknowledge their adherence to ethical standards, including plagiarism, consent for publishing, research misconduct, data fabrication, duplicate publication, and redundancy.

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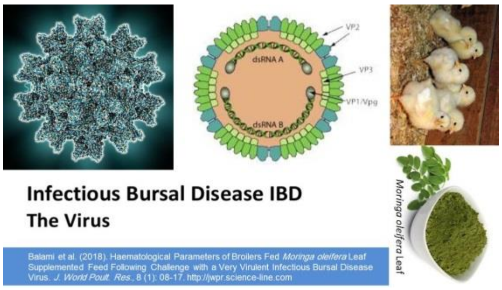

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4. Names of authors and title of journals, published in non-latin alphabets should be transliterated in English.
5. A sample of standard reference is "1th Author surname A, 2th Author surname B and 3th Author surname C (2013). Article title should be regular and 9 pt. Journal of World`s Poultry Research, Volume No. (Issue No.): 00-00." DOI:XXX."
6. Journal titles should be full in references. The titles should not be italic.
7. References with more than 10 authors should list the first 10 authors followed by 'et al.'
8. The color of references in the text of article is blue. Example: (Preziosi et al., 2002; Mills et al., 2015).
9. At least 35% of the references of any submitted manuscript (for all types of article) should include scientific results published in the last five years.

-Examples (at the text- blue highlighted)

Abayomi (2000), Agindotan et al. (2003), Vahdatpour and Babazadeh (2016), (Kelebeni, 1983), (Usman and Smith, 1992), (Chege, 1998; Chukwura, 1987a,b; Tijani, 1993, 1995), (Kumasi et al., 2001).

--Examples (at References section)

a) For journal:

Lucy MC (2000). Regulation of ovarian follicular growth by somatotropin and insulin- like growth factors in cattle. Journal of Dairy Science, 83: 1635-1647.

Kareem SK (2001). Response of albino rats to dietary level of mango cake. Journal of Agricultural Research and Development. pp 31-38. DOI:XXX.

Chikere CB, Omoni VT and Chikere BO (2008). Distribution of potential nosocomial pathogens in a hospital environment. African Journal of Biotechnology, 7: 3535-3539. DOI:XXX.

Tahir Khan M, Bhutto ZA, Abbas Raza SH, Saeed M, Arain MA, Arif M, Fazlani SA, Ishfaq M, Siyal FA, Jalili M et al. (2016). Supplementation of different level of deep stacked broiler litter as a source of total mixed ration on digestibility in sheep and their effects on growth performance. Journal of World`s Poultry Research, 6(2): 73-83. DOI: XXX

b) For symposia reports and abstracts:

Cruz EM, Almatar S, Aludul EK and Al-Yaqout A (2000). Preliminary Studies on the Performance and Feeding Behaviour of Silver Pomfret (Pampus argentens euphrasen) Fingerlings fed with Commercial Feed and Reared in Fibreglass Tanks. Asian Fisheries Society Manila, Philippine 13: 191-199.

c) For edited symposia, special issues, etc., published in a journal:

Korevaar H (1992). The nitrogen balance on intensive Dutch dairy farms: a review. In: A. A. Jongebreur et al. (Editors), Effects of Cattle and Pig Production Systems on the Environment: Livestock Production Science, 31: 17-27.

d) For books:

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

e) Books, containing sections written by different authors:

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

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

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Abbreviations of units should conform with those shown below:

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Percent	%		

Other abbreviations and symbols should follow the recommendations on units, symbols and abbreviations: in "A guide for Biological and Medical Editors and Authors (the Royal Society of Medicine London 1977).

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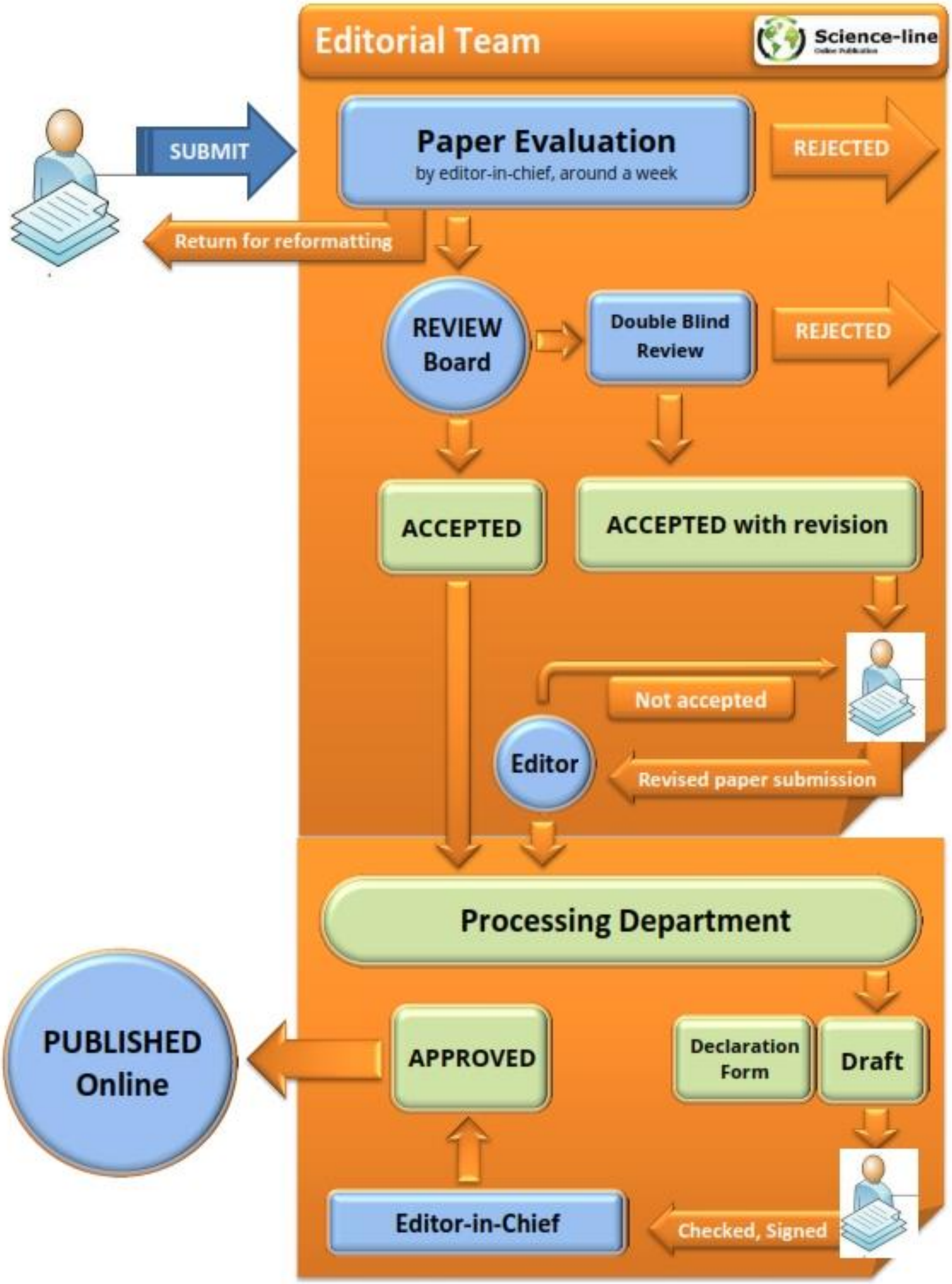

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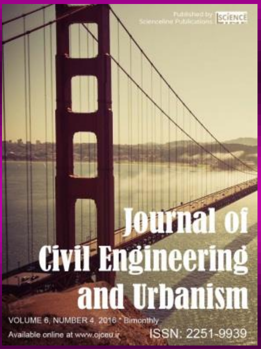

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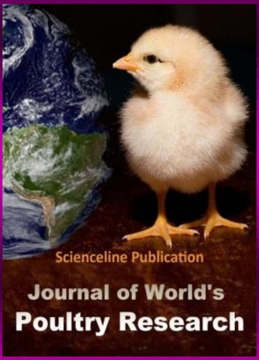

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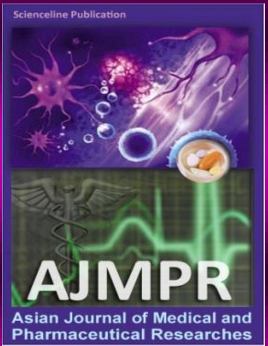

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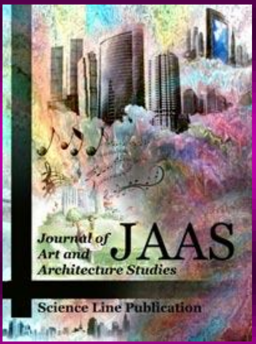

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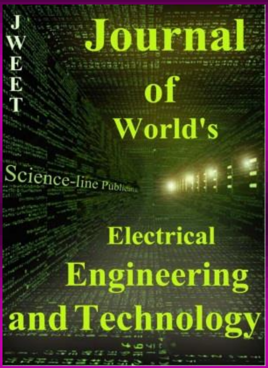

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