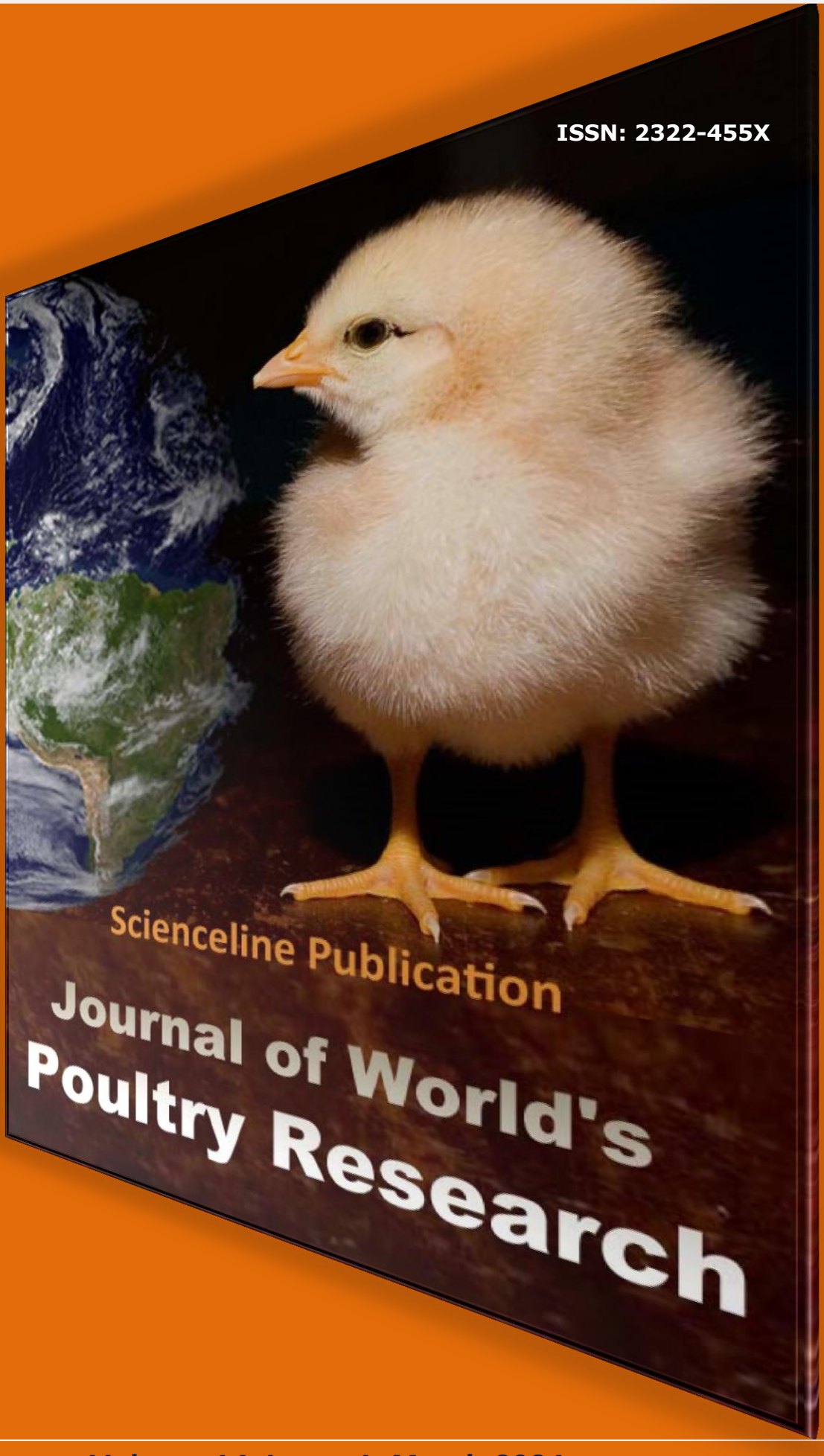
Journal of World's Poultry Research

BOOKLET



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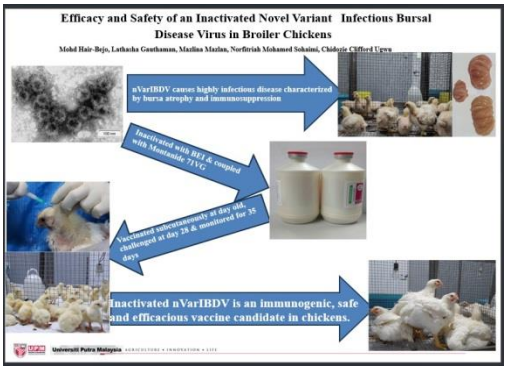
Volume 14 (1); March 25, 2024

Research Paper

Efficacy and Safety of an Inactivated Novel Variant Infectious Bursal Disease Virus in Broiler Chickens

Hair-Bejo M, Gauthaman L, Mazlan M, Sohaimi NM, and Ugwu CC.
J. World Poult. Res. 14(1): 1-11, 2024; pii: S2322455X2400001-14
DOI: https://dx.doi.org/10.36380/jwpr.2024.1



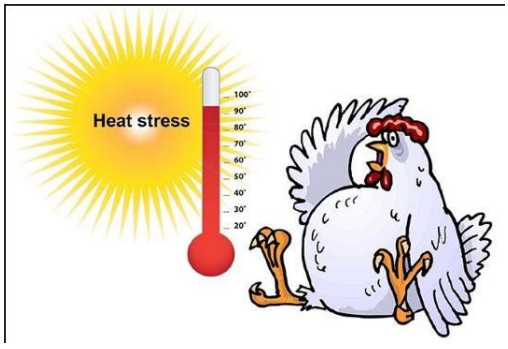
ABSTRACT: The infectious bursal disease virus (IBDV) is severe and highly contagious, causing high mortality and immunosuppression in chickens worldwide. A new novel variant, IBDV (nVarIBDV), has recently emerged in Asian countries, including Malaysia, highlighting the need to develop a new vaccine against this strain due to the inadequacy of existing commercial vaccines in protecting chickens from nVarIBDV infection. Therefore, the current study aimed to evaluate the efficacy and safety of inactivated nVarIBDV as a potential vaccine candidate in broiler chickens. A total of 65 one-day-old Arbo Acres broiler chickens were randomly divided into three groups (five animals in each group with four replications) before the challenge, namely A, B, and C. Groups A and B were immunized subcutaneously at day old with inactivated nVarIBDV (107 EID50/0.2 ml), and Group B was boosted at day 14. Group C was an unimmunized control. The experimental animals were divided into three subgroups and were challenged with pathogenic nVarIBDV (105 EID50/1.0ml) on day 28 post-inoculation through ocular and oral routes. The challenge sub-groups were named ACH, BCH, and CCH, respectively. The live body weight, bursa weight, and blood samples of the chickens were recorded. Gross lesions were examined, and samples of the bursa of Fabricius were collected from all the groups for histological evaluation. All the chickens appeared healthy and normal throughout the trial. Body weight increased in all groups without significant differences. The bursa weight and the bursa-to-body weight ratio of the booster group (Group B) were significantly higher than the non-booster and control groups. Gross lesions were not observed in the investigated groups. The challenged control group had higher bursa lesion scoring than the vaccinated groups. The IBDV antibody titer of challenged chickens in ACH, BCH, and CCH groups was higher than those of unchallenged groups A, B, and C at 35 days post-inoculation. The IBDV antibody titer of challenged chickens in group B was higher than challenged chickens in groups A and C (ACH and CCH). In conclusion, the inactivated nVarIBDV demonstrated safety and efficacy, with the booster Group (B) showing elevated humoral immune responses compared to the non-booster group.
Keywords: Antibody, Chicken, Efficacy, Inactivated vaccine, Novel variant infectious bursal disease virus

[Full text-PDF] [Crossref Metadata]

Research Paper

Effects of Palm (*Elaeis Guineensis*) Oil on Performance, Thermotolerance, and Welfare of Broiler Chickens in Heat Stress Condition

Yarkoa T, Songuine T, Kpomassé CC, Parobali T, Karou DS, and PItala W
J. World Poult. Res. 14(1): 12-22, 2024; pii: S2322455X2400002-14
DOI: https://dx.doi.org/10.36380/jwpr.2024.2



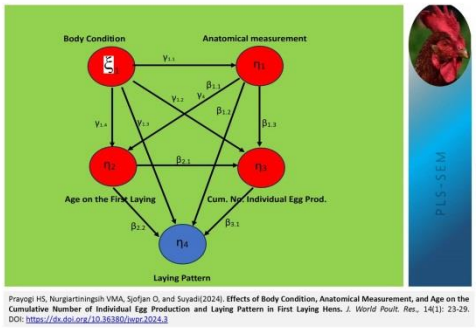
ABSTRACT: Heat stress negatively affects the broiler chickens' productivity and well-being. This study was carried out to assess the effect of dietary palm oil inclusion on the growth performance, thermotolerance, biochemical parameters, and welfare of broiler chickens raised in tropical climates. A total of 500 broiler chickens aged 15 days were divided into four treatments, each consisting of five replicates with 25 chickens per replicate in a randomized design. The control group was fed a standard diet without palm oil (T), and the remaining diets contained palm oil at the inclusion levels of 1% (P1), 2% (P2), and 3% (P3). During the 4 weeks of experimentation, daily temperature and relative humidity in the poultry house were measured by thermo-hygrometers, and growth performance was weekly recorded. At 45 days old, six broiler chickens were slaughtered with measurements taken for carcass compositions and intestinal length. At 42 days of age, blood samples were collected for the

Triiodothyronine (T3) and Thyroxine (T4) hormones, biochemical profiles, and Heterophil: lymphocyte (H/L) ratio assessment at the Regional Center of Excellence on Avian Sciences. Gait abnormality and litter quality were assessed at 38 days of age. The results indicated that the incorporation of 1% palm oil improved the growth performance of chickens compared to other groups. Similarly, the concentrations of T4 and T3 were higher in the 1% palm oil group. Triglycerides and total protein concentrations were higher in the broiler chickens of the control group, compared to other treatment groups. The dropping weight and gait score decreased with the increasing rate of palm oil. The results suggest that palm oil can be a beneficial dietary supplement for broiler chickens, particularly under heat-stress conditions. The incorporation of 1% palm oil contributes to the improvement of growth performance and the well-being of broiler chickens in tropical climates. However, it is crucial to consider the appropriate level of palm oil inclusion, as higher levels may have adverse effects, such as increased mortality.
Keywords: Energy, Feeding strategies, Heat stress, Palm oil, Welfare

[Full text-PDF] [Crossref Metadata]

Effects of Body Condition, Anatomical Measurement, and Age on the Cumulative Number of Individual Egg Production and Laying Pattern in First Laying Hens

Prayogi HS, Nurgiartiningsih VMA, Sjofjan O, and Suyadi.
J. World Poult. Res. 14(1): 23-29, 2024; pii: S2322455X2400003-14
DOI: https://dx.doi.org/10.36380/jwpr.2024.3



Prayogi HS, Nurgiartiningsih VMA, Sjofjan O, and Suyadi(2024). **Effects of Body Condition, Anatomical Measurement, and Age on the Cumulative Number of Individual Egg Production and Laying Pattern in First Laying Hens.** *J. World Poult. Res.*, 14(1): 23-29. DOI: https://dx.doi.org/10.36380/jwpr.2024.3

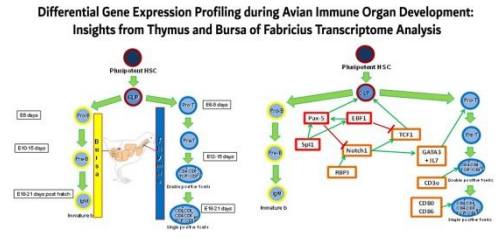
ABSTRACT: The individual egg production capacity of laying hens plays an outstanding role in achieving total production within a flock, which is affected by several internal and external factors. This study aimed to evaluate the effect of body condition, anatomical measurement, and age at the first laying (AFL) on the cumulative number of individual egg production (CNIEP) and laying pattern. Therefore, 172 Isa Brown laying chickens were investigated. Data on body condition and anatomical measurement were collected at the age of 16 weeks, while data on production was collected during 21 weeks of production (18-39 weeks). The obtained data were analyzed by Partial Least Square- Structural Equation Modeling (PLS-SEM) using smartPLS. The findings revealed that CNIEP could be predicted by body condition, anatomical measurement, and AFL. Compared to body condition and anatomical measurements, AFL was the most prominent factor in CNIEP. Body condition, anatomical measurement, AFL, and CNIEP had weak effects on the Isa Brown laying chickens' patterns.
Keywords: Anatomical measurement, Individual egg production, Laying pattern

[Full text-PDF] [Crossref Metadata]

Research Paper

Differential Gene Expression Profiling during Avian Immune Organ Development: Insights from Thymus and Bursa of Fabricius Transcriptome Analysis

El Helaly Goher MN, Moghaieb RE, El-Menawey MA-R, and Ramzy Stino FK.
J. World Poult. Res. 14(1): 30-40, 2024; pii: S2322455X2400004-14
DOI: https://dx.doi.org/10.36380/jwpr.2024.4



El Helaly Goher MN, Moghaieb RE, El-Menawey MA-R, and Ramzy Stino FK (2024). **Differential Gene Expression Profiling during Avian Immune Organ Development: Insights from Thymus and Bursa of Fabricius Transcriptome Analysis.** *J. World Poult. Res.*, 14(1): 30-40. DOI: https://dx.doi.org/10.36380/jwpr.2024.4

ABSTRACT: Pluripotent hematopoietic stem cells undergo maturation to regenerate blood cells. T-cell and B-cell maturation from common lymphoid progenitors (CLPs) is regulated by distinct transcription factors and signaling pathways. *Notch* signaling, *GATA3*, and *TCF1* drive T-cell fate, while *EBF1, PU.1, E2A, Pax5, TCF1*, and *Foxo1* regulate B-cell differentiation. Alterations in these factors can cause lineage deviations. Gene expression profiling of the chick thymus and bursa reveals dynamic expression patterns of transcription factors, cytokines, and signal molecules. *Notch1* and *Dll1* expression that increase during later stages indicate the ongoing role of *Notch* signaling in T-cell lineage maintenance. The current study aimed to identify differentially expressed genes during the development of the avian immune organs, focusing on the thymus and bursa using 24 Ross 308 avian breed. The mRNA libraries from these organs were analyzed using quantitative Real-time PCR analysis at six time points spanning the embryonic ages (days 15 and 18) and post-hatch age (days 3, 7, 14, and 28). The data for the gene expression indicated significant variations across different stages of immune organ development. Differential gene expression was observed between sorted T and B-cells, with *GATA3, CD3e, CD4*, and *Ptprc* showing higher expression in the T-cell population, and *Pax5* and *CD81* exhibiting higher expression in the B-cell population. Notably, *ENO1* and *IRF4* showed higher expression in T-cells at E15 and B-cells at E18. The study highlights the importance of regulatory factors and genes in maintaining cellular identity, furthers the understanding of avian immunology, and has the potential for improving poultry health and studying immune-related diseases in humans. These findings pave the way for further research on the role of biochemical components under important disease conditions in avian immunology and their potential applications for poultry health.

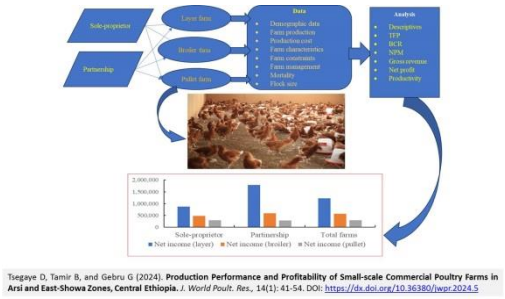
Keywords: Avian lymphocyte, B-cell maturation, Gene expression, Regulatory elements, T-cell

[Full text-PDF] [Crossref Metadata]

Research Paper

Production Performance and Profitability of Small-scale Commercial Poultry Farms in Arsi and East-Showa Zones, Central Ethiopia

Tsegaye D, Tamir B, and Gebru G.
J. World Poult. Res. 14(1): 41-54, 2024; pii: S2322455X2400005-14
DOI: https://dx.doi.org/10.36380/jwpr.2024.5



Tsegaye D, Tamir B, and Gebru G (2024). **Production Performance and Profitability of Small-scale Commercial Poultry Farms in Arsi and East-Showa Zones, Central Ethiopia.** *J. World Poult. Res.*, 14(1): 41-54. DOI: https://dx.doi.org/10.36380/jwpr.2024.5

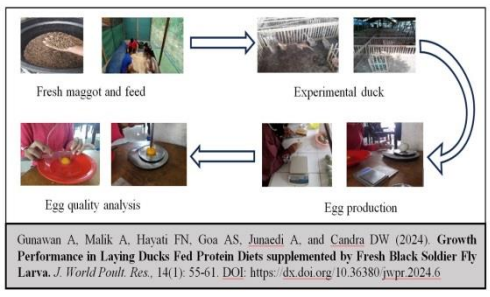
ABSTRACT: Poultry farming and the demand for chicken meat and eggs are growing due to their quality protein, essential nutrients, affordable price, and low investment cost. The current study was designed to investigate the productivity and profitability of small-scale commercial poultry enterprises. Data were collected from 221 poultry farms using a semi-structured questionnaire. The results were presented using descriptive statistics. Farm performances were measured by total-factor-productivity (TFP), benefit-cost-ratio (BCR), and net-profit-margin (NPM). The majority of the poultry farm owners were male (69.68%) and married (77.4%). Around 55.2% of poultry farmers aged 31-45 years. Group-owned farms had 4.59 ± 1.77 individual members. On average, a batch of layer chickens was kept for 17.0 ± 3.87 and 18.12 ± 4.25 months on individual and group-owned farms, respectively. Moreover, the mean selling ages of pullet and broiler chickens were 2.55 ± 0.71 and 2.18 ± 0.51 months, respectively. The flock size of layer chickens in sole-proprietor farms (562 ± 724) was significantly lower than in partnership farms (1165 ± 877). The average numbers of produced pullets by sole-proprietorship and partnership farms were 3177 ± 2360 and 3137 ± 1826, while the mean numbers of broilers produced were 2257 ± 1875 and 3269 ± 1669, respectively. The average egg and broiler weights in sole-proprietorship farms were significantly higher compared to group-owned farms. Annual egg production rates in individual and group-owned farms were 76.5% and 70.4%, respectively. The cost of feed, chicken, housing, labor, and medication were the top five production expenditures, while feed cost shares 60.4%. Egg producers had NPM, BCR, and TFP indices of 38.99%, 1.03, and 2.03, respectively, with notable differences by ownership types. The NPM, BCR, and TFP indices for broiler and pullet farms, respectively, were 42.78%, 0.93, and 1.93 for broilers and 35.21%, 0.92, and 1.67 for pullets. The results indicated that poultry firms performed optimally, regardless of ownership type. To further improve the efficiency and profitability of poultry enterprises, farmers need technical, finance, and management skills and input supply chains.
Keywords: Broiler, Layer, Poultry, Production, Profitability, Pullet

[Full text-PDF] [Crossref Metadata]

Research Paper

Growth Performance in Laying Ducks Fed Protein Diets supplemented by Fresh Black Soldier Fly Larva

Gunawan A, Malik A, Hayati FN, Goa AS, Junaedi A, and Candra DW.
J. World Poult. Res. 14(1): 55-61, 2024; pii: S2322455X2400006-14
DOI: https://dx.doi.org/10.36380/jwpr.2024.6



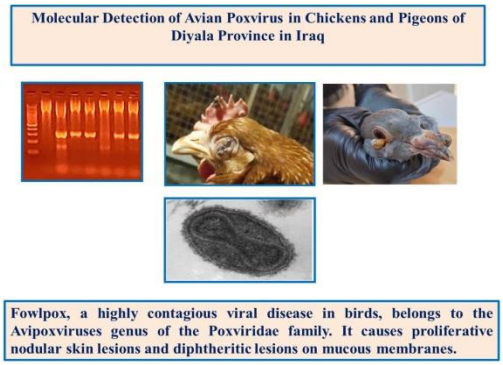
Gunawan A, Malik A, Hayati FN, Goa AS, Junaedi A, and Candra DW (2024). **Growth Performance in Laying Ducks Fed Protein Diets supplemented by Fresh Black Soldier Fly Larva.** *J. World Poult. Res.*, 14(1): 55-61. DOI: https://dx.doi.org/10.36380/jwpr.2024.6

ABSTRACT: Live black soldier fly (BSF) maggots serve as an alternative feed for ducks and other poultry, boasting a protein composition similar to fish meal. The current study aimed to evaluate the effect of live BSF maggot supplementation as a protein source on increasing duck daily production, feed conversion ratio, and egg quality. A total of 120 female Alabio ducks aged 7 months were randomly divided into 24 cages with four treatments and six replications. Each cage consisted of five ducks. Performance parameters such as daily egg production, feed conversion ratio, and egg mass production were evaluated, along with the quality of duck eggs, including egg weight, yolk weight, yolk crude protein, and albumen crude protein. The results revealed that protein levels did not significantly affect feed consumption during the laying phase for ducks. However, the comparison of duck day production between treatment groups indicated that ducks receiving lower protein levels (13.43%) exhibited lower production, compared to those with higher protein levels (18.29%). Furthermore, feed protein content had a notable impact on egg weight, yolk weight, and albumen crude protein. The study demonstrated a significant increase in egg yolk weight, while the percentages of egg yolk weight and eggshell weight showed no significant differences. In conclusion, this research suggests that supplementing duck diets with live BSF maggots can enhance egg quality and performance parameters.
Keywords: Black soldier fly, Duck, Egg quality, Maggot, Performance, Source of protein

[Full text-PDF] [Crossref Metadata]

Molecular Detection of Avian Poxvirus in Chickens and Pigeons of Diyala Province in Iraq

Faisal A, and Al-Azzawi A.
J. World Poult. Res. 14(1): 62-74, 2024; pii: S2322455X2400007-14
DOI: https://dx.doi.org/10.36380/jwpr.2024.7

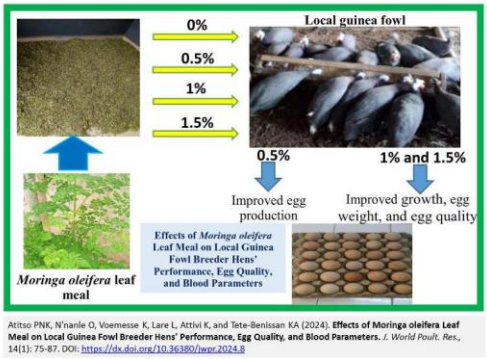


ABSTRACT: Fowlpox is an infectious viral disease affecting domestic and wild birds. The current study was conducted using PCR-based molecular detection to determine the avian pox virus (APV) and its genetic sequence. A total of 40 pigeons and 40 chickens suspected of APV were obtained from different bird markets. Following euthanasia, tissue samples were taken from the vents, eyes, wings, and beaks for molecular analysis. DNA extraction was focused on the core protein gene region (pb4). PCR results confirmed the presence of APV in all samples, generating specific cDNA bands of 329 bp. Four GenBank accession codes (OR670580, OR670581, OR670582, and OR670583) represented pigeon pox samples, whereas two GenBank accession numbers (OR619724 and OR619725) represented samples of fowlpox. A significant similarity of 99% was found by genetic analysis between the reference target sequences and the sequenced samples. In viral samples of fowlpox and avipoxvirus, nucleic acid variants (205 C > G and 204 T > C) were found with missense and silent effects on particular proteins (p.101Leu>Val and p.108Pro). Phylogenetic analysis organized the samples into clades representing fowlpox and pigeon pox viruses, showing close relationships with strains from different geographical regions. This study unequivocally demonstrates the susceptibility of both domestic and wild birds to avian pox, highlighting the pivotal role of phylogenetic analysis and molecular detection in elucidating novel perspectives on the genetic landscape of these viruses.
Keywords: Fowl pox, Gene, Pigeon pox, Polymerase Chain Reaction

[Full text-PDF] [Crossref Metadata]

Effects of Moringa oleifera Leaf Meal on Local Guinea Fowl Breeder Hens' Performance, Egg Quality, and Blood Parameters

Atitso PNK, N'nanle O, Voemesse K, Lare L, Attivi K, and Tete-Benissan KA.
J. World Poult. Res. 14(1): 75-87, 2024; pii: S2322455X2400008-14
DOI: https://dx.doi.org/10.36380/jwpr.2024.8

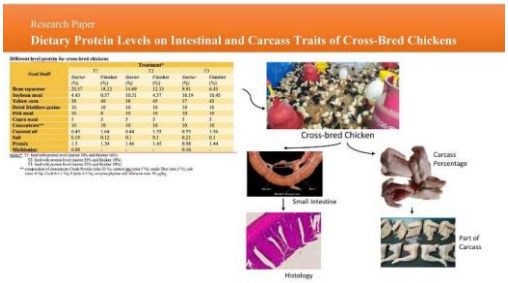


ABSTRACT: Breeding local guinea fowl has the potential to address protein malnutrition and alleviate poverty in West African countries. The current study aimed to examine the effects of incorporating *Moringa oleifera* leaf meal into the diet on hematology and biochemical parameters as well as the productive performance of local guinea fowl breeders in Togo. Thus, 512 local guinea fowls (22 weeks of age with an average weight of 1176.7 ± 2.9 g), comprising 384 females and 128 males, were examined for 31 weeks. The fowls were randomly assigned to four dietary groups, namely M0, M1, M2, and M3 containing 0%, 0.5%, 1%, and 1.5% of *Moringa oleifera* leaf meal in diets, respectively. Each group had 4 replicates of 32 fowls. During the study, feed intake, body weight, egg-laying rate, and feed conversion ratio were weekly recorded. Blood samples for hematology and biochemical analysis were taken from 12 females (3/replicate) at 34 and 50 weeks of age. Eggs were collected for the quality evaluation. Results showed that feed intake was comparable across all groups during the rearing period (23 to 33 weeks of age). However, it significantly decreased in fowls of the M2 and M3 during the laying period (34 to 50 weeks of age). The live weight of fowls in M3 was significantly higher than other groups before the laying period. The egg production, yolk ratio, and shell ratio of the birds in the M1 were like that of M0 and higher than that of M2 and M3. The feed conversion ratio was not significantly different between groups during the laying period. However, the albumen ratio and haugh unit were improved by feeding *Moringa* leaves especially at level 1.5%. The level of white blood cells and lymphocytes decreased by feeding the *Moringa* leaves at 1%. Neutrophils and platelet levels were comparable across groups. Total proteins, albumin, and transaminases increased in *Moringa* groups (M1, M2, M3), especially in M3. It was concluded that the use of *Moringa oleifera* leaves at 0.5% improved egg-laying performance in local guinea fowl. Moreover, 1% and 1.5% of incorporation improved the quality of eggs.
Keywords: Blood parameter, Breeder, Guinea fowl, Egg quality, *Moringa oleifera*

[Full text-PDF] [Crossref Metadata]

Dietary Protein Levels in the Small Intestine and Carcass Traits of Cross-Breed Chickens

Nuningtyas YF, Aulanni'am A, Sjofjan O, Nurgiartiningsih VMA, Furqon A, Lestari SP, and Natsir MH.
J. World Poult. Res. 14(1): 88-97, 2024; pii: S2322455X2400009-14
DOI: https://dx.doi.org/10.36380/jwpr.2024.9

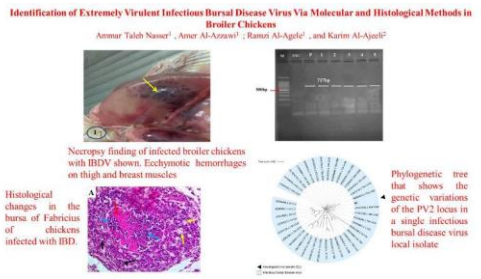


ABSTRACT: Protein is a source of nutrients that plays a significant role in biological processes. The current study aimed to evaluate the effects of feed with different protein levels on the pH and viscosity of the small intestine, ileum characteristics, and carcass traits of cross-breed chickens. A total of 160 cross-breed unsexed chicks aged 2 days were divided into three treatments (T1-T3), each consisting of 6 replications with 9 chicks per replication, consisting of a diet with protein level (T1; starter 18% and finisher 16%;), a diet with protein level (T2; starter 20% and finisher 18%), and a diet with protein level (T3; starter 22% and finisher 20%). Cross-breed chickens were crossed between Bangkok males and Lohmann laying hens. The variables were analyzed, including pH and viscosity of digesta, ileum characteristics consisting of total villous, height of villous, and depth of crypt. The carcass percentages consisting of the carcass, breast, thicks, wings, and back were measured. The research was analyzed using a completely randomized design. The results indicated that different protein levels in treatments were significantly different in total villous, height of villous, and depth of crypt but did not indicate a significant difference in pH and viscosity. Furthermore, the treatments have a significant difference in the carcass percentage and thick percentage but did not significantly affect the breast, wings, and back. It can be concluded that 22% crude protein in the starter and 20% crude protein in the finisher produced the greatest results in the intestinal characteristics and also in the carcass percentage of cross-breed chickens.
Keywords: Carcass, Cross-breed chicken, Feed, Intestinal characteristic, Protein

[Full text-PDF] [Crossref Metadata]

Identification of Extremely Virulent Infectious Bursal Disease Virus Via Molecular and Histological Methods in Broiler Chickens

Nasser AT, Al-Azzawi A, Al-Agele R, and Al-Ajeeli K.
J. World Poult. Res. 14(1): 98-112, 2024; pii: S2322455X2400010-14
DOI: https://dx.doi.org/10.36380/jwpr.2024.10



ABSTRACT: Infectious bursal disease (IBD) is caused by an RNA virus belonging to the Avibirnavirus genus within the Birnaviridae family. The global prevalence of infectious bursal disease virus (IBDV) is a significant concern, affecting birds of all ages. Birds infected with IBDV exhibit symptoms, such as depression, bleeding in the thighs and pectoral muscles, and enlargement of the bursa. This study aimed to identify predominant IBDV serotypes using molecular methods and to gain insights into the resulting pathological conditions in infected chickens. Additionally, the study investigated the viral sequence and the relationship between a local Diyala isolate and reference strains from the Genebank. In the current study, the IBDV was isolated from broiler chickens aged 2-3 weeks from 15 farms in the Diyala Governorate of Iraq. A total of 15 samples, each from a different farm, were collected. Necropsy samples were obtained from various organs of broiler chickens, including the bursa of Fabricius, lungs, liver, and kidneys. Specific primers targeting the VP2 gene were used for reverse transcription-polymerase chain reaction (RT-PCR) analysis. The RT-PCR analysis yielded a 727 bp fragment, confirming the presence of IBDV in 10 out of the 15 samples. One strain was assigned the accession number LC498531 in the NCBI database. Phylogenetic analysis using the neighbor-joining tree program revealed three distinct groups. All examined regional samples (S1) were situated within the constructed tree. Five samples formed a specific group, indicating a close relationship. Histological examination of the tissues showed visible alterations such as degeneration, necrosis, and infiltration of inflammatory cells, particularly heterophils, providing clear evidence of the disease. In conclusion, this study confirmed the presence of IBDV in broiler chickens from multiple farms in Iraq's Diyala Governorate, highlighting distinct clustering patterns in viral sequences. Moreover, the study confirmed the virus's presence using conventional RT-PCR, with histological examination supporting the findings.
Keywords: Broiler chicken, Bursal enlargement, Infectious bursal disease, RT-PCR

[Full text-PDF] [Crossref Metadata]

Effects of Natural Guard Liquid (an Essential Oil-Based Product) on Growth Performance, Hematological Profile, and Antibody Response to Newcastle Disease Virus in Broiler Chickens

Merdana IM, Maharanthi KN, Sudimartini LM, Sumadi IK, Babikian YHS, Babikian HY, Laiman H, Haliman RW, Yen TI, Kristina, Efendy HY, Setiasih NLE, and Ardana IBK.

J. World Poult. Res. 14(1): 113-123, 2024; pii: S2322455X2400011-14
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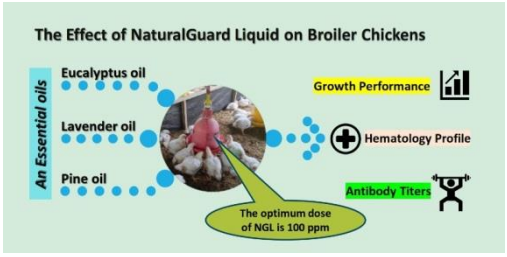

ABSTRACT: Natural guard liquid (NGL) is an immunomodulator consisting of an essential oil blend (lavender oil, eucalyptus oil, and pine oil) that can improve the immunity (IgG) of animals. The objective of this research was to assess the effectiveness of a mixture of NGL comprising of essential oil (eucalyptus, lavender, and pine oil) on growth performance, hematological profile, and antibody titer response to the Newcastle disease virus (NDV) in Lohman strain broiler chickens. A total of 400-day-old unsexed broiler chickens with an average weight of 42.48 ± 2.08 g were randomly distributed into four groups, each comprising two replications with 50 chicks. The control group, T0, received no essential oil, while other treatment groups, T1, T2, and T3, were administered NGL at 80, 100, and 200 ppm, respectively. The mixture was administered in drinking water for 30 days. The animals had received vaccinations at the Hatchery, including active NDV, inactive NDV, and Gumboro. Regular recordings were made for feed consumption, water intake, mortality, and body weight. Blood samples for routine hematological examination (hemoglobin, erythrocytes, packed cell volume, erythrocyte index, leucocytes, and differential leucocytes) and immune parameter (NDV antibody titer) assessment were collected at the onset as well as after 15 and 30 days of treatment. The hemoglobin levels, erythrocytes, total leukocytes, lymphocytes, and Heterophil-lymphocyte index showed significant differences in groups T1 and T2 compared to T0. While the NDV antibody titer showed a significant difference at T2 compared to T0, it was not significantly different at T1 and T3. The best results and performance was indicated in T2 (100 ppm), characterized by body weight (1,839 g), and feed conversion ratio (1.573). The hematological profile in the T2 group included hemoglobin (11.78 g/dL), total erythrocytes (2.82 $\times 10^6$ u/L), total leukocytes (52.67 $\times 10^3$ u/L]), and the NDV antibody titer (48 ± 10.20) in the fifth week. In conclusion, the optimum dose of NGL is 100 ppm, which significantly influences growth performance, hematological profile, and antibody titer against NDV.
Keywords: Antibody titer**,** Broiler chicken, Growth performance, Hematological profile, Natural Guard Liquid

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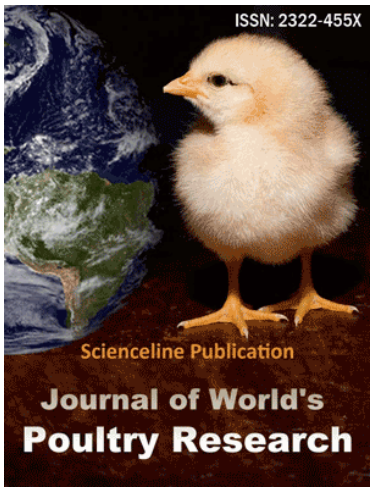

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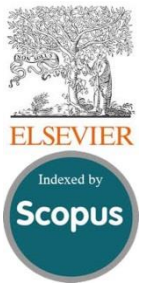

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Efficacy and Safety of an Inactivated Novel Variant Infectious Bursal Disease Virus in Broiler Chickens

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ABSTRACT

The infectious bursal disease virus (IBDV) is severe and highly contagious, causing high mortality and immunosuppression in chickens worldwide. A new novel variant, IBDV (nVarIBDV), has recently emerged in Asian countries, including Malaysia, highlighting the need to develop a new vaccine against this strain due to the inadequacy of existing commercial vaccines in protecting chickens from nVarIBDV infection. Therefore, the current study aimed to evaluate the efficacy and safety of inactivated nVarIBDV as a potential vaccine candidate in broiler chickens. A total of 65 one-day-old Arbo Acres broiler chickens were randomly divided into three groups (five animals in each group with four replications) before the challenge, namely A, B, and C. Groups A and B were immunized subcutaneously at day old with inactivated nVarIBDV (10^7 EID_{50}/0.2 ml), and Group B was boosted at day 14. Group C was an unimmunized control. The experimental animals were divided into three subgroups and were challenged with pathogenic nVarIBDV (105 EID50/1.0ml) on day 28 post-inoculation through ocular and oral routes. The challenge sub-groups were named ACH, BCH, and CCH, respectively. The live body weight, bursa weight, and blood samples of the chickens were recorded. Gross lesions were examined, and samples of the bursa of Fabricius were collected from all the groups for histological evaluation. All the chickens appeared healthy and normal throughout the trial. Body weight increased in all groups without significant differences. The bursa weight and the bursa-to-body weight ratio of the booster group (Group B) were significantly higher than the non-booster and control groups. Gross lesions were not observed in the investigated groups. The challenged control group had higher bursa lesion scoring than the vaccinated groups. The IBDV antibody titer of challenged chickens in ACH, BCH, and CCH groups was higher than those of unchallenged groups A, B, and C at 35 days post-inoculation. The IBDV antibody titer of challenged chickens in group B was higher than challenged chickens in groups A and C (ACH and CCH). In conclusion, the inactivated nVarIBDV demonstrated safety and efficacy, with the booster Group (B) showing elevated humoral immune responses compared to the non-booster group.

Keywords: Antibody, Chicken, Efficacy, Inactivated vaccine, Novel variant infectious bursal disease virus

INTRODUCTION

Infectious bursal disease virus (IBDV) belongs to the genus *Avibirnavirus* and family *Birnaviridae*, causing a severe, highly infectious disease in chickens (Delmas et al., 2019). The IBDV is classified into serotypes 1 (pathogenic in chicken) and 2 (non-pathogenic in chicken). Serotype 1 is made up of very virulent, classical, antigenic variants and artificially attenuated subtypes (Müller et al., 2003). The IBDV primarily affects young chickens, targeting the lymphoid organs, especially the bursa of Fabricius where it causes severe atrophy (Müller et al., 2003). However, the severity of the disease differs from strain to strain. However, all the strains have a heavy economic impact on the poultry industry worldwide. Some virus strains are highly virulent and may cause up to 20% or more mortality in chickens aged 3 weeks and even older, while other strains can cause a severe, prolonged immunosuppressive reaction in chickens infected at an early age (Eterradosi and Saif, 2013).

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The IBDV is a non-enveloped virus indicating an icosahedral symmetry with a diameter of about 55-65 nm. It has a double-stranded RNA genome with two segments A and genome B. The IBDV segments encode five viral proteins, namely VP1, VP2, VP3, VP4, and VP5 (Qin and Zheng, 2017). The VP2 and VP3 are the major structural proteins of IBDV, identified in western-blotting experiments with convalescent sera as important IBDV-derived antigens (Cheggag et al., 2020). In addition to these findings, recent advancements in classification methods based on VP1 and VP2 characteristics have been proposed (Wang et al., 2021). Based on the improved scheme, cIBDV, varIBDV, vvIBDV, and aIBDV are classified as Genotype A1B1 made up of serotypes A2aB1, A2bB1, and A2cB1 and A2B2 made up of A3B2 and A8B1.

A new variant called novel variant IBDV (nVarIBDV) which is genetically different from the earlier reported variant IBDV, has been circulating in China since 2017 (Wang et al., 2021). It was first reported in Malaysia in 2019 (Aliyu et al., 2021). Although this variant does not immediately result in mortality, it has high morbidity, causing severe atrophy of the bursa of Fabricius. This, in turn, results in immunosuppression, loss in production performance, and subsequently, severe economic losses (Fan et al., 2019; Babazadeh and Asasi, 2021). The nVarIBDV is classified under Genotype A2dB1 and was also reported in Japan (Myint et al., 2021) and South Korea (Thai et al., 2021). Recently, it was also described that the Chinese nVarIBDV and the early variant IBDV originally found in America belong to the same branch of variant IBDV although they are still divided to form two distinct sub-branches discrete from one another (Aliyu et al., 2021).

Given the adverse economic consequences of nVarIBDV on infected chickens, it is important to protect chicks against immunosuppression and production loss. However, nearly all commercial vaccines currently in use target vvIBDV, and do not mount a sufficient immune response against nVarIBDV (Wang et al., 2021). Thus, the development of vaccines that have the same antigenicity as the nVarIBDV circulating in Malaysia is essential for the prevention and control of nVarIBDV in the Malaysian poultry industry. A viral particle resembling a vaccine candidate named SHG19-VLP produced neutralizing antibodies, which provided 100% protection against the nVarIBDV (Wang et al., 2021). In another instance, an attenuated nVarIBDV strain termed Gt was used to develop a reassortment virus strain rGtVarVP2, which when used, completely protected chickens against nVarIBDV (Fan et al., 2020a). The aim of this study was to inactivate nVarIBDV and use it as a potential vaccine candidate to evaluate its safety, efficacy, viral load, and viral shedding on broiler chickens after challenge with a pathogenic field isolate of nVarIBDV.

MATERIALS AND METHODS

Ethical approval

The guidelines and ethics of the University Putra Malaysia (UPM) Institutional Animal Care and Use Committee (IACUC) on handling animals for experiments approved with reference number UPM/IACUC/ AUP-U014/2022 were followed in this study.

Virus

A nVarIBDV isolated from a 23-day-old broiler chicken from a commercial farm in Selangor, Malaysia, named UPM1432/2019 with accession number MT431217 (Aliyu et al., 2021), obtained from the Institute of Bioscience, UPM, and confirmed as a novel variant by PCR was used for this experiment.

Inactivation, preparation, and sterility test

This process was conducted in a Biosafety level 2 Virology laboratory in the Faculty of Veterinary Medicine, UPM, following standard biosafety and biosecurity measures for handling viruses (Artika and Ma'roef, 2017). In this regard, 6 ml of nVarIBDV was measured into a centrifuge tube, and 120 µl of Binary Ethylene Imine (BEI, Sigma-Aldrich, St. Louis, MO, USA) was also added and incubated at 37^{o}C. The mixture was vortexed every 30 minutes for 36 hours, after which 12 µl of sodium thiosulfate was added and mixed thoroughly by vortexing at 37^{o}C for an hour. The inactivated nVarIBDV isolate was then filtered through a 0.22 µm syringe filter and mixed with Montanide 71 VG adjuvant at a ratio of 30:70 (inactivated nVArIBDV: Montanide 71 VG) by vortexing for 2 hours and stored at 4^{o}C until use as vaccines for the study. Safety and sterility test was conducted by inoculating 0.1ml of the inactivated nVarIBDV with Montanide 71 VG into the specific-pathogen-free embryonated chicken egg through the chorioallantoic membrane route and incubated at 37^{o}C. The eggs were observed for mortality for 7 days (Habib et al., 2006).

Design and study animals

A total of 65 one-day-old Arbo Acres commercial broiler chicks with an average weight of 56.6 ± 1.57 were randomly divided into three groups (denoted as A, B, and

C, with five chicks in each group and four replications. On day 28 of the experiment, five chickens from each group were selected for the challenge and named ACH, BCH, and CCH (Okura et al., 2021). Commercial broiler feed and water were provided *ad libitum*. Light was constantly provided, and the temperature was kept at 24^{o}C throughout the trial. When they were day old, chickens from Groups A and B were inoculated (0.2 ml) with 10^7 EID_{50}/0.2 ml of inactivated nVarIBDV via the subcutaneous route. Chicks from Group C were not inoculated. Five chicks from Group C were sacrificed on the day of inoculation by cervical dislocation. At 14 days old, 0.2 ml of 10^7 EID_{50}/0.2 ml inactivated nVarIBDV were inoculated to chickens from Group B through the subcutaneous route. On days 14, 28, and 35, five chickens from each of Groups A, B, and C were humanely sacrificed through cervical dislocation. Throughout the 35-day trial period, all chickens were subjected to daily observation for clinical signs. When the chickens reached 28 days of age, those designated for the challenge in Groups A, B, and C were exposed to a pathogenic field strain of nVarIBDV, administered at 105 EID 50/1.0 ml through the ocular (0.2 ml) and oral (0.8 ml) routes (Mutinda et al., 2015). Subsequently, the chickens were monitored for 7 days post-challenge. In the case of chicken mortality, necropsy procedures were conducted, and for those that survived, euthanasia and necropsy were performed by a veterinarian.

Sample collection

For sampling, each chicken was placed on a digital scale, and its weight in grams was recorded. After sacrifice, the bursa from each chicken was removed aseptically and weighed on a digital scale. Live body weight and bursa weights were recorded for each sampled chicken. About 5 ml of blood samples were also collected for each chicken before sacrifice, and about 1 ml of serum was extracted for the detection of IBD antibodies using the ELISA technique (Orakpoghenor et al., 2020). Chickens (post-mortem) were examined for gross lesions in the bursa, spleen, muscles, proventriculus, and thymus, which were recorded accordingly for each chicken. Aseptically collected samples of the bursa and cloacal swabs were stored at -20^{o}C prior to the molecular detection of the challenge virus by RT-qPCR technique (Aliyu et al., 2021). Five bursa samples from each replicate in a group on each sampling day were fixed in 10% buffered formalin for histological examination and lesion scoring.

Enzyme-linked immunosorbent assay analysis

The serum was harvested within 24 hours from each sampled chicken, centrifuged at 240 × g for 5 minutes, and kept at -20^{o}C prior to use. A serum sample from each chicken was analyzed for IBDV antibodies using the ELISA technique with a commercial kit (BioCheck IBD ELISA, Hounslow, UK) according to the manufacturer's recommendation. The results were read at 405 nm with an ELISA reader (Dynatech MR7000, Rauw et al., 2009).

Copy number of nVarIBD challenge virus in the bursa and cloaca of challenged chickens

Quantitative real-time PCR was conducted targeting the VP2 protein of nVarIBDV with samples of bursa and cloaca swabs. RNA was extracted and purified using Kylt® RNA/DNA purification kit (SAN Group Biotech, Germany) according to the manufacturer's recommended procedures. The purified RNA was checked for purity and concentration with a spectrophotometer (Eppendorf, Germany) at a 260 nm wavelength. The purified RNA was used to conduct an RT-qPCR assay with specific nVarIBDV primers (F1432 – CCAACAAGGGAGTACACCGA and R1432 – CCAAATGCTCCTGCAATCTT) and probe (Probe2 – AGTACTTCATGGAGGTGGCCGACCTCAA) to quantify the viral genome copies in the samples (Aliyu et al., 2021).

Histopathology and lesion scoring

Bursa of Fabricius samples obtained from each chicken were first checked for gross lesions, and a portion was fixed in 10% buffered formalin solution for 48 hours (Zhao., 2015). After fixing, the bursa was processed into transparent glass slides, and the slides were stained using hematoxylin and eosin. The slide was allowed to air dry before examination under the light microscope (Leica ASP 300, Germany) for histopathological changes on a scale of 0 to 5, ranging from normal to severe (Elawad et al., 2020).

Statistical analysis

The data analysis was conducted using Analysis of Variance (ANOVA) in SPSS version 28.0 (Chicago, USA). To discern differences, the Turkey Honestly Significant Difference (HSD) posthoc test was applied at a significance level of 5% ($p < 0.05$).

RESULTS

Clinical signs

The findings indicated no abnormal clinical signs and no mortality in any of the chickens in the vaccinated groups and the non-vaccinated unchallenged group

throughout the 35 days of the trial. However, the non-vaccinated challenged group recorded mild depression and ruffled feathers 2 days post-challenge but recovered.

Body weight

The body weight of chickens in each group progressively increased until day 35 and was not significantly different across groups. There was no significant difference in the body weight of chickens between groups at days 14 and 35 post-inoculation (dpi; $p > 0.05$). However, at 28 dpi, the body weight of chickens in the non-booster group was significantly higher than those of the other groups ($p < 0.05$). At 35 dpi, corresponding to 7 days post-challenge, the challenged chickens in Group B had the highest body weight than Groups A and C, but they were not statistically significant ($p > 0.05$, Figure 1). The body weight of challenged chickens in Group B was significantly higher than their non-challenged counterpart ($p < 0.05$).

Bursa weight

No significant difference was observed in the bursa weight between different groups on days 14, 28, and 35 among the non-challenged chickens. However, bursa weight of the challenged chickens in Group B was significantly higher than the other groups at 35 dpi corresponding to 7-day post-challenge ($p < 0.05$, Figure 2).

Bursa to body weight ratio

There was no significant difference in the bursa-to-body weight ratio of the chickens among unchallenged throughout the trial ($p > 0.05$). However, the bursa-to-body weight ratio of challenged chickens in Group B was significantly higher than Groups A and C at day 35 post-inoculation or day 7 post-challenge ($p < 0.05$, Figure 3).

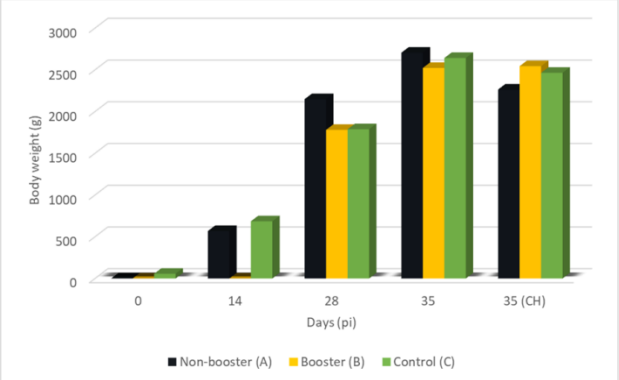

Figure 1. Body weight of Arbo Acres chickens inoculated with inactivated novel variant infectious bursal disease virus at day old for 35 days. CH: Challenged; pi: Post-inoculation

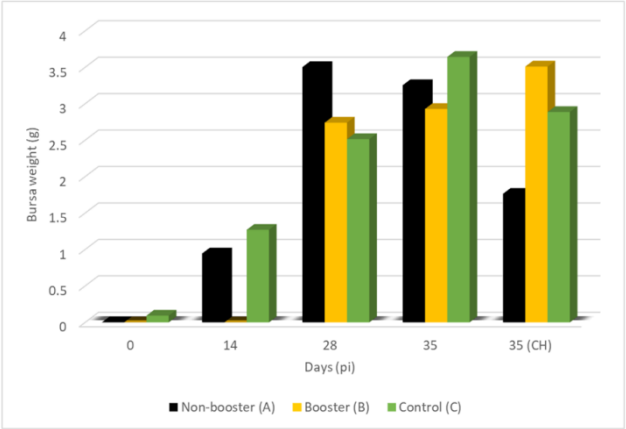

Figure 2. Bursa weight of Arbo Acres broiler chickens inoculated with inactivated novel variant infectious bursal disease virus at day old. CH: Challenged; pi: Post-inoculation

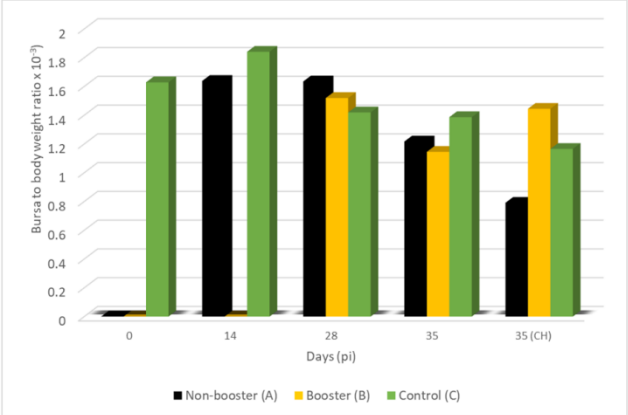

Figure 3. The ratio of bursa to body weight of Arbo Acres broiler chickens inoculated with inactivated novel variant Infectious Bursal Disease Virus at day old. CH: Challenged; pi: Post-inoculation

Gross lesions

Incubation time and 14 post-inoculation

The bursa of day-old chicks was normal, with no gross lesions on incubation time and 14 dpi (Figure 4).

Day 28 post-inoculation

The bursa of Fabricius from Groups A, B, and C was normal, with no gross lesions at 28 dpi (Figure 5).

Day 35 post-inoculation

The bursa samples from Groups A, B, and C were normal, with no gross lesions at 35 dpi (Figure 6).

Day 7 post-challenged

The bursa of Fabricius from Groups A, B, and C were normal, with no gross lesions (Figure 7) at day 35 pi among the challenged chickens in each group.

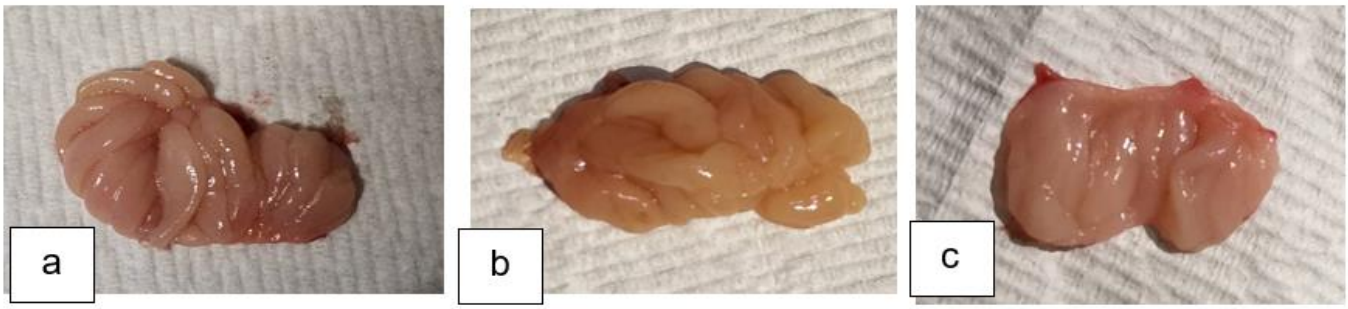

Figure 4. Normal bursa of Fabricius of Arbo Acres broiler chickens inoculated with inactivated novel variant infectious bursal disease virus at day old. Group C (a, 14 dpi), Group A (b, 14 dpi), and Group B (c, 14 dpi).

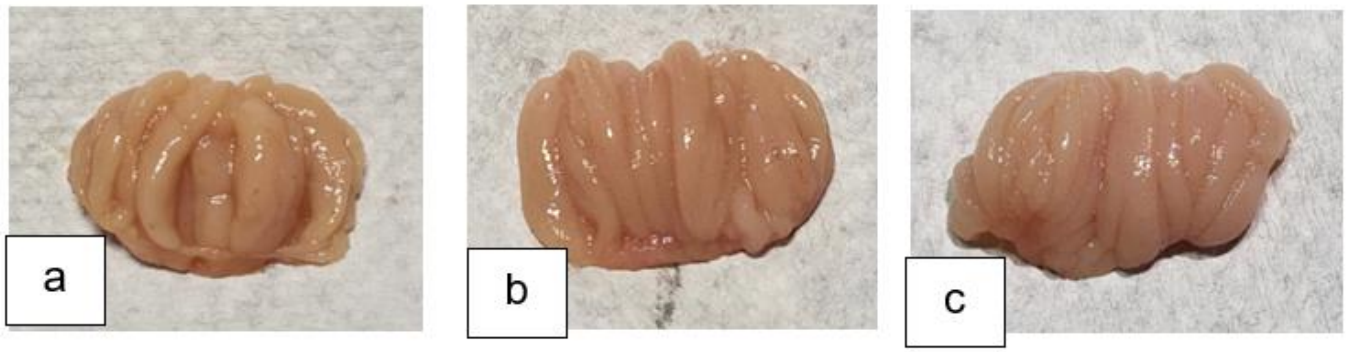

Figure 5. Normal bursa of Fabricius of Arbo Acres broiler chickens inoculated with inactivated novel variant infectious bursal disease virus at day old observed on 28 dpi. Group A (**a**), Group B (**b**), and Group C (**c**).

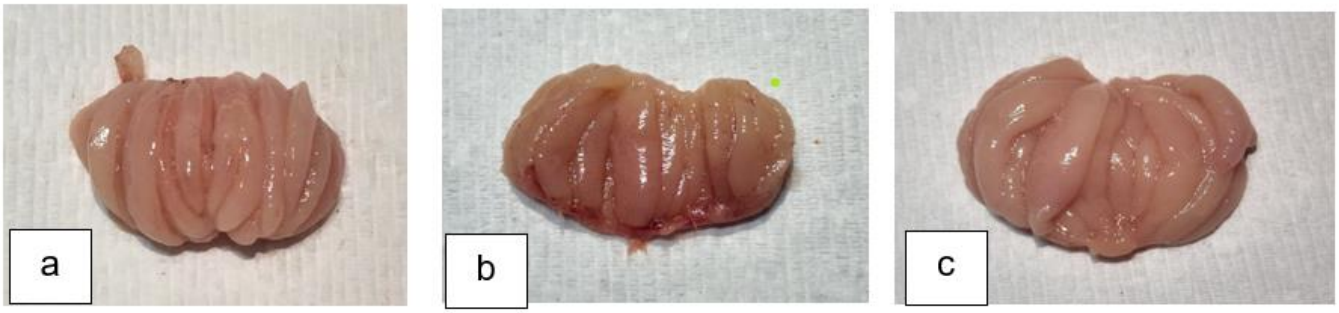

Figure 6. Normal bursa of Fabricius of Arbo Acres broiler chickens inoculated with inactivated novel variant infectious bursal disease virus at day old on 35 dpi. Group A (**a**), Group B (**b**), and Group C (**c**).

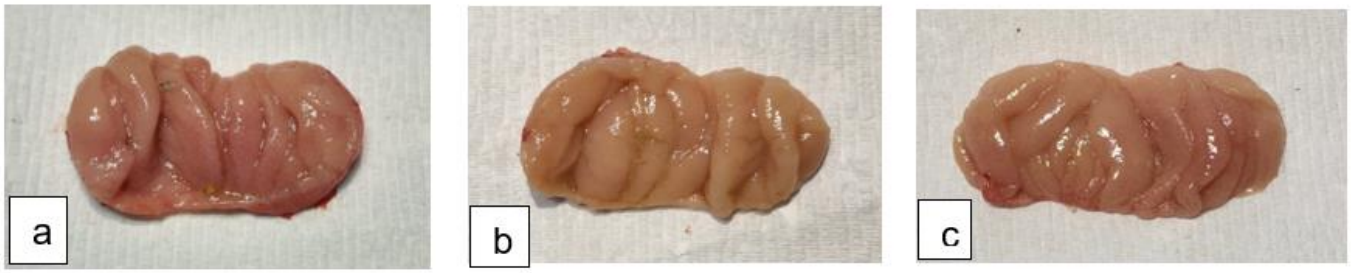

Figure 7. Normal bursa of Fabricius of challenged Arbo Acres broiler chickens inoculated with inactivated novel variant infectious bursal disease virus at day old on 35 dpi and 7-day post-challenge. Group A (**a**), Group B (**b**), and Group C (**c**).

Histological lesions

Incubation time and 14 post-inoculation

Mild degeneration, especially at the medullary region of the lymphoid follicles, was observed among the non-booster Group A chickens (white arrow). No necrosis and heterophils were present (Figure 8a). No histological lesions were observed from Groups A and C at 14 dpi (Figures 8b and 8c).

Days 28 and 35 post-inoculation(non-challenged chickens)

No histological lesions were observed for Group B. For Groups A (white arrow) and C (black arrow), mild degeneration, especially at the medullary region of the lymphoid follicles, was observed (Figure 9).

Day 35 post-inoculation (Challenged chickens)

For Groups A and B, there were mild degenerations, especially in the medullary region of the lymphoid follicles (white arrows). However, Group C chickens had mild to moderate degeneration, necrosis in the medulla, and infiltration of inflammatory cells (black arrow, Figure 10).

Bursa lesion score

The bursa lesion score was not significantly different throughout the trial for all the groups. The bursa lesion score of challenged Group C (CCH) was higher than Groups A (ACH) and B (BCH) but not statistically significant ($p > 0.05$, Figure 11).

Virus loading and shedding (*RT-qPCR)*

The virus copies in the bursa and cloacal swab samples were higher in challenged chickens in Group C compared to those in Groups A and B on 35 dpi or day 7 post-challenged (Figure 12).

Infectious bursal disease antibody titer

The IBD antibody titer of day-old chicks was 3546 ± 555.89 ELISA unit. The antibody titer of Group B was significantly higher ($p < 0.05$) than Groups A and C at 28 and 35 dpi (Figure 13).

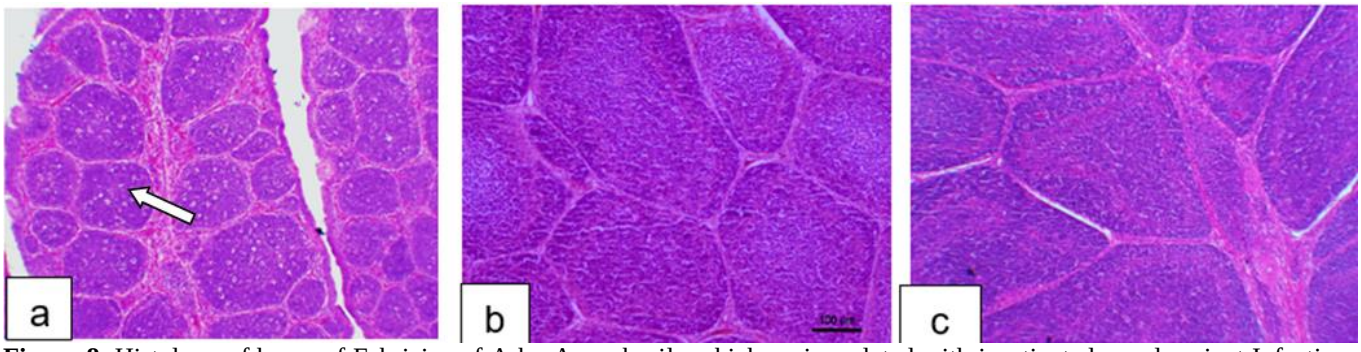

Figure 8. Histology of bursa of Fabricius of Arbo Acres broiler chickens inoculated with inactivated novel variant Infectious Bursal Disease Virus at day old on the day of inoculation and 14 post-inoculation (**a**), showing mild degeneration of the lymphoid follicles among Group A chickens (white arrow, Lesion scoring of 1); (**b**) and (**c**) on day 14 pi, showing normal bursa (Lesion scoring of 0). HE, Bar = 100µm.

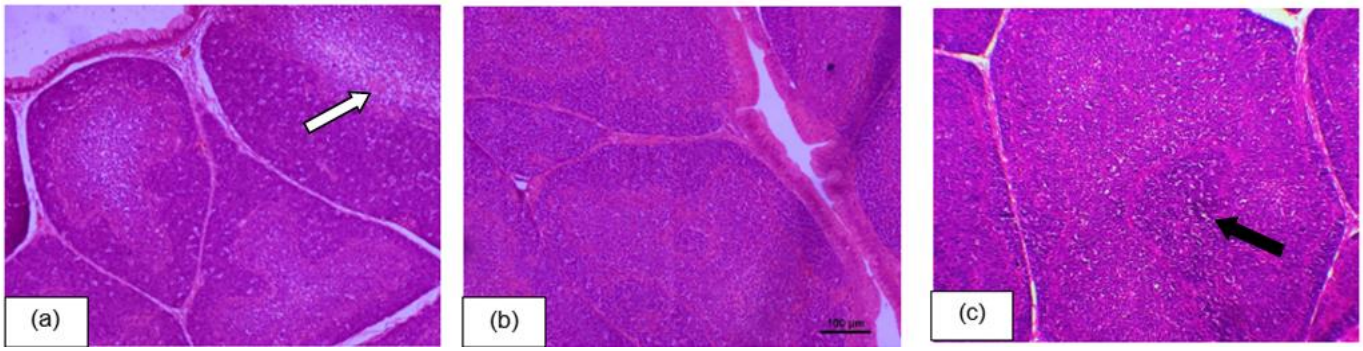

Figure 9. Histology of bursa of Fabricius of Arbo Acres broiler chickens inoculated with inactivated novel variant Infectious Bursal Disease Virus at day old on 28 dpi (CH) and day 35 post-inoculation. **a:** Mild degeneration (white arrow) Group A (Lesion scoring of 1), **b:** Group B (Lesion scoring of 0), and **c:** mild degeneration (white arrow) Group C (Lesion scoring of 1). HE, Bar = 100µm.

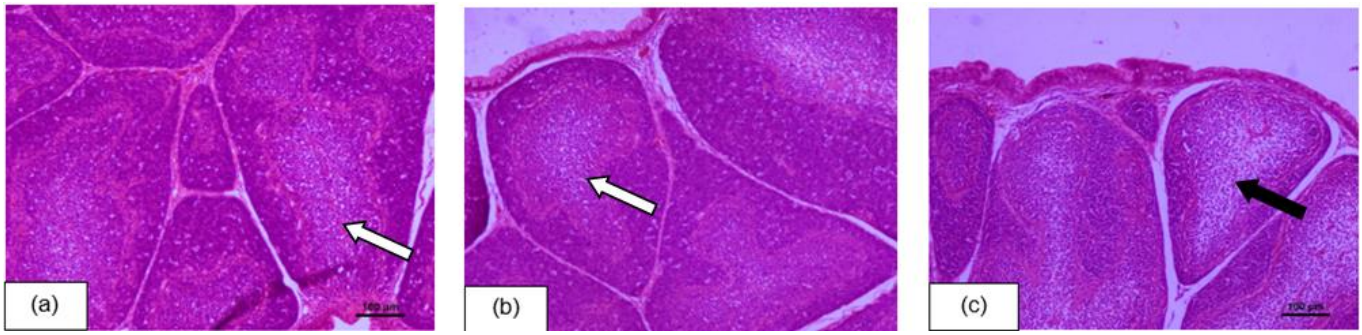

Figure10. Histology of bursa of Fabricius Arbo Acres broiler chickens inoculated with inactivated novel variant Infectious Bursal Disease Virus at day old on 35 dpi (CH) and day 7 post-challenge. **a:** Mild degeneration (white arrow), Group A (Lesion scoring 1), **b:** Mild degeneration (white arrow), Group B (Lesion scoring of 1), and **c:** Mild to moderate degeneration (black arrow), Group C (Lesion scoring of 2). HE, Bar = 100µm.

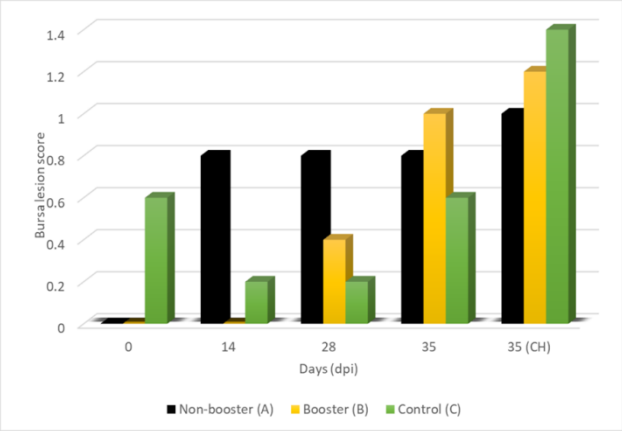

Figure 11. Bursa lesion score of Arbo Acres broiler chickens inoculated with inactivated novel variant infectious bursal disease virus at day old and monitored for 35 days . CH: Challenged; pi: Post-inoculation

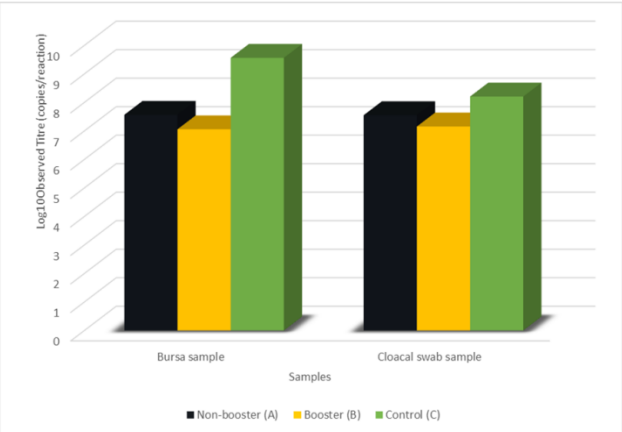

Figure 12. Virus load and shedding of Arbo Acres broiler chickens inoculated with inactivated novel variant infectious bursal disease virus at day old and challenged with the pathotype (35 dpi or day 7 post-challenged).

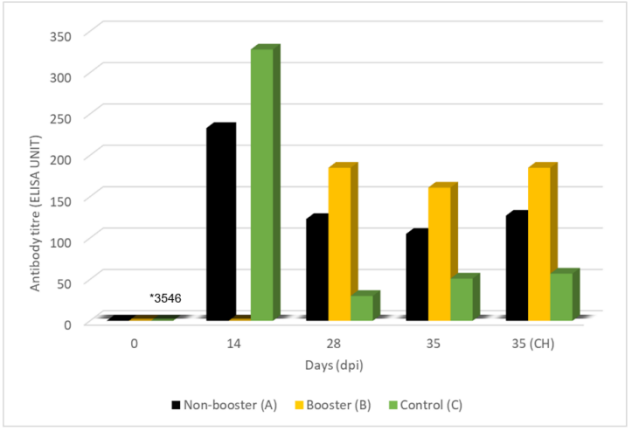

Figure 13. Infectious bursal disease virus antibody titer of Arbo Acres broiler chickens inoculated with inactivated novel variant Infectious Bursal Disease Virus at day old and monitored for 35 days. CH: Challenged; pi: post-inoculation

DISCUSSION

The novel variant IBDV isolate was successfully inactivated with binary ethylene imine (BEI), which does not interfere with the antigenicity of viruses (Delrue et al., 2012). Given that Montanide 71VG, employed as the adjuvant, has been documented to support the induction of long-lasting immunity (Tehrani et al., 2016), it is anticipated that the vaccine could be highly immunogenic and prove valuable in preventing IBD in chickens.

Throughout the trial, no abnormal clinical signs were observed. The body weight of chickens in all the vaccinated groups increased steadily until day 35, highlighting the safety of the inactivated nVarIBDV and the adjuvant on the broiler chickens. Since chickens

infected with the pathogenic nVarIBDV typically experience lower body weight (Fan et al., 2019), it is evident that the inactivated nVarIBDV did not impede the growth performance of the chickens as a pathogenic field isolate would have. Among the challenged chickens, booster Group B had the highest body weight than the other groups. The challenged chickens in the booster Group B had significantly higher body weight than their corresponding non-challenged chickens. This result indicated that the booster dose provided better protection for the chickens against challenges by maintaining better growth performance among the booster chickens.

The nVarIBDV causes lesions and severe atrophy in the bursa of Fabricius (Fan et al., 2020b; Myint et al., 2021; Thai et al., 2021). In this study, the bursa weight did not exhibit significant differences among all groups, except on day 35 among the challenged chickens, where the bursal weight of chickens in the booster Group B was significantly higher than that of Groups A and C. This suggests that the booster dose provided the most effective protection to the bursa against the nVarIBDV field isolate. The bursa-to-body weight ratio, which is a more accurate parameter to determine bursa atrophy caused by nVarIBDV was not significantly different for all the groups except on day 35 among challenged chickens. The bursa-to-body weight ratio challenged chickens in Group B was significantly higher than that of chickens in Groups A and C. This was similar to the report by Wang et al. (2020), in which the bursa-to-body weight index of the control chickens was significantly lower than that of the trial chickens vaccinated with live nVarIBDV vaccines. This shows that the inactivated nVarIBDV with Montanide 71VG adjuvant has the potential to protect chickens against pathogenic nVarIBDV.

According to a study by Fan et al. (2019), the bursa of chickens with nVarIBDV appeared to be atrophied, with hemorrhages, and yellowish with inflammatory exudation at 3-5 dpi. Similar gross lesions in the bursa of 24-38-day-old chickens were identified in studies conducted by Thai et al. (2021) and Myint et al. (2021) although congestion of the bursa appeared to be an additional finding. In this study, no gross lesions were present in the bursa of chickens inoculated with inactivated nVarIBDV at inoculation day, 14, 28, and 35 dpi. This indicates that the inactivated nVarIBDV with Montanide 71VG adjuvant does not cause gross pathological changes in the bursa and can be safely used. The bursa of the challenged chickens in all groups also showed no gross lesions. The absence of lesions in the challenged Group C might be attributed to the duration between the challenge virus inoculation and the bursa sampling, which may not have been sufficiently long enough to enable the obvious manifestation of clinical signs or lesions of nVarIBDV in the bursa of Group C chickens.

In the current study, no significant histopathological changes were recorded in chickens inoculated with inactivated nVarIBDV at 0, 14, 28, and 35 (non-challenged) dpi. This finding suggests that the inactivated nVarIBDV with Montanide 71VG did not cause histopathological changes in the bursa of chickens and is, therefore, safe to be used. The challenged chickens in Groups A and B showed no significant histopathological changes in the bursa. This is in line with the findings of Wang et al. (2021), where no microscopic lesions were observed in the vaccinated groups. However, chickens in Group C had mild to moderate degeneration and necrosis of lymphoid cells in the bursa follicles. Infiltration of inflammatory cells was also observed in the bursa of Group C-challenged chickens. Similar changes were previously reported (Fan et al., 2020b). Some histopathological changes that are typical of nVarIBDV include severe follicular lymphoid necrosis and depletion and multifocal follicular lymphoid infiltration. There may be minimal to no inflammatory response. Additionally, there could be reticular and macrophage infiltration in lymphoid follicles, cystic cavities in lymphoid follicles, proliferation of fibrous tissues, severe follicle atrophy, and infolding epithelium into damaged follicles in broilers aged 24-38 days infected with nVarIBDV (Fan et al., 2019; Myint et al., 2021; Thai et al., 2021). The results of this study suggest that the inactivated nVarIBDV elicited a sufficient humoral immune response, which prevented tissue damage in the bursa of the challenged chickens.

The histological lesions of the bursa were scored to provide a better understanding and statistical picture of the bursa lesions. The bursa lesion score was not significantly different throughout the trial for all groups. However, the bursa lesion score of challenged chickens in Group C was higher than Groups A and B although these findings were not statistically significant. This is consistent with the finding that nVarIBDV can cause lesions in the bursa of the Fabricus (Fan et al., 2020b). Together with the results of the above histological lesions, the current findings confirm the efficacy of inactivated nVarIBDV in providing an immunoprotective effect on infected chickens.

For both sample types, the virus copies of Group C were higher than Groups A and B. Given the lack of previous studies pertaining to evaluating virus loading and

shedding among inoculated chickens for nVarIBDV, it remains unclear to which degree the viral copies are attributed to the improved efficacy of the trial vaccine. It has been emphasized in previous reports that the evaluation of vaccine efficacy should address measuring the ability of the vaccine to limit the shedding of the pathogenic virus (Miller et al., 2009). This is crucial for preventing the dissemination of the virus in the environment and breaking the chain of transmission (Ugwu et al., 2022). However, the results of the RT-qPCR showed clear evidence of the ability of the inactivated nVarIBDV to elicit the production of sufficient neutralizing antibodies that reduce the viral load in the bursa and induced blocking immunity responsible for reduced virus shedding among the infected chickens.

The study showed that the IBDV antibody titer in Group B was significantly higher than in Groups A and C at 28 and 35 dpi, indicating a booster dose of inactivated nVarIBDV may be more desirable in the prevention of novel variant IBDV infection in chickens. The role of humoral immunity in protecting chickens against IBDV has been previously documented (Yang et al., 2020). Neutralizing antibodies are of utmost importance in preventing and controlling IBDV infection (Van Den Berg, 2000). The study revealed a significantly higher copy number of nVarIBDV challenge virus in the bursa of challenged control chickens than in the vaccinated chickens. This suggests that the inactivated nVarIBDV with Montanide 71VG induced the production of neutralizing antibodies, resulting in the effective clearance of the challenge virus from the bursa of vaccinated chickens. These findings are promising and position the inactivated nVarIBDV as a potential vaccine candidate. There is no significant difference in the antibody titer of the challenged and non-challenged groups. Two limitations may account for the observed results. Firstly, the shortened duration between the inoculation of the challenge virus and the sampling may not have allowed sufficient time for a robust immune response to develop. Ebrahimi et al. (2020) reported that the antibody titer of IBDV was higher at day 42 of the age of chickens in their trial compared to days 28 and 35. However, the titer recorded in this trial was comparable to that noted by Habib et al. (2006) in their trial with BEI-inactivated IBDV. Secondly, the limited understanding of the true pathogenicity and virulence status of nVarIBDV in existing studies may influence the extent of the immune response.

CONCLUSION

In conclusion, nVarIBDV inactivated with BEI and mixed with Montanide 71VG adjuvant was safe, immunogenic, and efficacious against pathogenic field strains of nVarIBDV in Arbo Acres broiler chickens in Malaysia. It is, however, recommended that the vaccine should be evaluated further with specific pathogen-free chickens to avoid maternal antibody interferences and that the duration of the trial should be increased especially after the challenge to better study the efficacy of inactivated nVarIBDV in chickens.

DECLARATIONS

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Availability of data and materials

The Data and materials are available upon demand.

Competing interests

The authors hereby declare that there is no competing interest.

Ethical considerations

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

Authors' contributions

Mohd Hair Bejo acquired the funds, conceptualized and supervised the work, and read the manuscript. Lathasha Gauthaman, Mazlina Mazlan, Norfitriah Mohamed Sohaimi, and Chidozie Clifford Ugwu conducted the experiments, collected and analyzed the data, and prepared the manuscript. All authors read and approved that last manuscript version.

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Effects of Palm (*Elaeis Guineensis*) Oil on Performance, Thermotolerance, and Welfare of Broiler Chickens in Heat Stress Condition

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ABSTRACT

Heat stress negatively affects the broiler chickens' productivity and well-being. This study was carried out to assess the effect of dietary palm oil inclusion on the growth performance, thermotolerance, biochemical parameters, and welfare of broiler chickens raised in tropical climates. A total of 500 broiler chickens aged 15 days were divided into four treatments, each consisting of five replicates with 25 chickens per replicate in a randomized design. The control group was fed a standard diet without palm oil (T), and the remaining diets contained palm oil at the inclusion levels of 1% (P_1), 2% (P_2), and 3% (P_3). During the 4 weeks of experimentation, daily temperature and relative humidity in the poultry house were measured by thermo-hygrometers, and growth performance was weekly recorded. At 45 days old, six broiler chickens were slaughtered with measurements taken for carcass compositions and intestinal length. At 42 days of age, blood samples were collected for the Triiodothyronine (T3) and Thyroxine (T4) hormones, biochemical profiles, and Heterophil: lymphocyte (H/L) ratio assessment at the Regional Center of Excellence on Avian Sciences. Gait abnormality and litter quality were assessed at 38 days of age. The results indicated that the incorporation of 1% palm oil improved the growth performance of chickens compared to other groups. Similarly, the concentrations of T4 and T3 were higher in the 1% palm oil group. Triglycerides and total protein concentrations were higher in the broiler chickens of the control group, compared to other treatment groups. The dropping weight and gait score decreased with the increasing rate of palm oil. The results suggest that palm oil can be a beneficial dietary supplement for broiler chickens, particularly under heat-stress conditions. The incorporation of 1% palm oil contributes to the improvement of growth performance and the well-being of broiler chickens in tropical climates. However, it is crucial to consider the appropriate level of palm oil inclusion, as higher levels may have adverse effects, such as increased mortality.

Keywords: Energy, Feeding strategies, Heat stress, Palm oil, Welfare

INTRODUCTION

In recent years, the substantial increase in global environmental temperatures, attributed to global warming, has inflicted significant damage on animal production, notably in broiler farming. This has led to elevated mortality rates and substantial economic losses (Sejian et al., 2018; Kpomasse et al., 2021). The challenging conditions pose a major threat to the broiler industry in hot and humid climates and jeopardize the sector's sustainability (Rahimi et al., 2021). A large proportion of broiler production industries are located in hot and humid areas (Daghir, 2008). The negative effects of heat stress include physiological impacts, metabolic disorders, and impairment of the functionality of the digestive system, which affects the chickens' welfare (Rostagno, 2020; Brugaletta et al., 2022). Apart from genetic and management strategies, nutritional approaches have also been suggested, such as incorporating oil into the diet (Attia et al., 2020).

Vegetable oils have been used to improve the performance of broilers under heat-stress conditions (Rafiei-Tari et al., 2021). Accordingly, the inclusion of vegetable oils in broiler diets enhances their performance and health status (Tari et al., 2020). The African oil palm (*Elaeis guineensis*), originating from West Africa, holds the status of the world's most significant palm species (Murphy et al., 2021). It is extensively cultivated and thrives in the humid tropics of Asia, Africa, and the Americas (Castellanos-Navarrete et al., 2021). Nonetheless, Rafiei-Tari et al. (2021) reported contradicting findings, indicating that adding palm oil had no positive effects on antioxidant activity and lipid attributes in Cobb 500 broiler chickens raised in a hot climate.

Little research has been carried out to improve the performance of broilers in the heat stress context. In addition, there is a need for evaluation of the adequate proportion of palm oil to be incorporated to improve heat-stressed broiler performance and welfare. The purpose of this experiment was to evaluate how adding palm (*Elaeis guineensis*) oil to the diet affected the broiler chickens' growth performance, thermotolerance, biochemical parameters, and overall welfare in tropical climates.

MATERIALS AND METHODS

Ethical approval

The care and handling of the animals were performed in strict accordance with the recommendations of the Guide for the Care and Use of Experimental Animals of the University of Lome, Togo. The protocol was approved by the Ethics of Animal Experimentation Committee of the same University. All efforts were made to minimize discomfort for the chickens (008/2021/BC-BPA/FDS-UL).

Study location

The experiment was carried out at the experimental unit of the Regional Center of Excellence on Avian Sciences (CERSA) in Badja, 41 km west of Lomé, Togo, located in a hot and humid climate. The average temperature was recorded at 28.85°C ± 0.62, while the average relative humidity was recorded at 71.62% ± 1.75. The study was conducted during the dry season in Togo from February to March 2023.

Study design

A total of 500 15-day-old Cobb 500 broiler chickens with 300 g of average weight acquired from the company "Le Poussin" were randomly divided into 4 treatments of 5 replicates of 25 chickens each using a completely randomized design (CRD). The dietary treatments included a control and a standard diet without palm oil (T), while the experimental groups were provided with diets containing 1%, 2%, and 3% palm oil inclusion, denoted as P1, P2, and P3, respectively. The trial lasted 28 days and the chickens were reared on a floor covered with wood shavings in an open poultry house with a density of 10 chickens/m2 and a lighting program of 23 hours of light and 1 hour of darkness. Water and feed were freely available. The chickens were vaccinated according to the following prophylaxis plan (Appendix 1).

Growth performance and carcass composition evaluation

In this trial, four thermo-hygrometers were used to measure the daily temperature and relative humidity in the poultry house. Growth performance parameters were measured during the trial to determine the weight gain and feed conversion ratio (FCR) according to Formula 1.

$$FCR = \frac{feed\ intake\ (g)}{weight\ gain\ (g)} \qquad \text{(Formula 1)}$$

Six chickens of identical weights from each treatment were humanely slaughtered and eviscerated at 42 days old to collect carcass data, such as belly fat, carcass yield, breast yield, thigh yield, the weights of empty gizzard, liver, heart, kidney, and intestines and length of intestines. Individual weights were represented as a percentage of body weight using Formula 2.

$$\text{Carcass yield (\%)} = \frac{\text{Carcass part (g)}}{\text{Body weight (g)}} \times 100 \qquad \text{(Formula 2)}$$

Biochemical profiles and physiological responses to heat stress

At 42 days of age, before slaughtering, approximately 3 mL of blood was collected from the brachial veins of 15 chickens per treatment, discharged into a dry tube without anticoagulant, and immediately centrifuged at 3000 rpm for 15 minutes to obtain serum. Then, the serum obtained was stored in a freezer at -20°C and further used for biochemical parameter analysis, such as total protein, total cholesterol, uric acid, glucose, and triglycerides, as well as physiological parameters, such as Triiodothyronine (T3) and Thyroxine (T4) and Heterophil: lymphocyte (H/L) ratio. Using automated COBAS® (Germany) systems, enzymatic procedures were used to evaluate the concentrations of glucose, triglycerides, total cholesterol, and total protein. The blood concentrations of total protein using the biuret method (Busher, 1990), total

cholesterol (Borner and Klose, 1977), serum triglycerides (Wahlefeld, 1974), glucose (Heinz and Beushausen, 1981), and uric acid (Walter, 1990), were measured. The samples were all run in the same essay to prevent variability across essays for every biochemical parameter. Thyroid hormone concentrations T3 and T4 were assessed by the Enzyme-Linked Fluorescence Assay (ELFA) method using Vidas Biomerieux kits (France). The ELFA method was performed on an in-house automated analyzer (Anderson et al., 2017). The H/L ratio was assessed by counting heterophils and lymphocyte cells using a Hemocytometer. The ratio was estimated by the Formula 3.

$$\text{H/L ratio (\%)} = \frac{\text{Total Heterophils}}{\text{Total Lymphocytes)}} \quad \text{(Formula 3)}$$

Welfare assessment

Gait score

The gait score was determined at 38 days old based on the notation adopted by Garner et al. (2002). In this regard, 0 was for a chicken that walks normally without ambiguity, 1 for a slight but unidentifiable gait impairment, 2 for a visible and identifiable anomaly with little effect on walking ability, 3 for an obvious anomaly affecting movement ability (the chickens are unable to stand for 15 minutes), 4 for a severe abnormality, and a score of 5 was not included since chickens with such gaits could walk and would have been culled previously. During the current study, gait scores 2 and 3 were chosen to represent moderate lameness, while scores 4 and 5 were utilized to represent severe lameness (Buijs et al., 2016). The prevalence of gait disorders was then determined using the Formula 4.

$$\text{Prevalence (\%)} = \frac{\text{Total no.of chickens with scores 3 and 4}}{\text{Number of live chickens at the time of assessment}} x\ 100\% \quad \text{(Formula 4)}$$

Litter quality assessment

The assessments were carried out on broiler chickens aged 42 days using the WQ (2009) procedure and Tuyttens et al. (2015). The litter score, litter depth, and dropping weight were all calculated. At the start of the experiment, the same amount of wood shavings (5 kg/m2) was put on the floor to guarantee that the whole floor area of each enclosure was covered. It was removed once every 4 weeks after weighing the litter and evaluating its depth and texture. The amount of droppings was calculated by subtracting the initial weight of the litter from the weight of the litter at the time of evaluation. The wood shavings were disinfected and spread on the floor of each cage at a density of 5kg per m^2. Each cage was separated into four zones for the different inspections to assess litter depth and texture. The average value of the four zones was the depth of the litter (measured using a metal ruler) and texture score (based on eye assessment) of the cage. Bouassi et al. (2016) used a five-point scale to assess litter texture including dry and friable litter, friable and slightly wet, friable but crusty in some places, crusty at the surface but friable by digging and designating a completely caked or wet litter.

Statistical analysis

The general linear model (GLM) procedure of GraphPad Prism 8 v.8.02. (GraphPad Software, San Diego, CA, USA) was used for statistical analysis. The growth performance parameters, slaughtering performance (carcass characteristics, abdominal fat weight, digestive organ weights, small intestine length, and weight), biochemical parameters, litter depth, and droppings weight were subjected to a one-way analysis of variance (ANOVA). When the difference was significant, further analyses were performed using Tukey's test (Benjamini and Braun, 2001). Mortality was analyzed with a $\chi 2$ test. The Kruskall-Wallis test followed by the Mann-Whitney U test was used for abnormal scores and litter quality which were ordinal variables. For each tested parameter, the difference was significant when the p-value was less than 5%. Data are presented as the mean ± standard deviation

RESULTS

Growth performance

As indicated in Table 2, daily feed intake was higher in P1, P2, and P3 chickens, compared to those of T ($p < 0.05$). The body weight and weight gain of chickens fed a diet containing palm oil especially those of P1 group were significantly higher ($p < 0.05$) than those of the control group (Table 2). The feed conversion ratio (FCR) of the broiler chickens in P1 was significantly lower than the P2, P3, and T groups ($p < 0.05$). Moreover, in the T and P3 groups, chickens recorded significantly higher mortality compared to those in the other treatment groups ($p < 0.05$).

Carcass' parts yield

The effect of palm oil on meat yield and abdominal fat is presented in Table 3. No significant differences were observed in thigh and breast weights between the control and other treatment groups ($p > 0.05$). However, the

carcass yield of chickens in treatment P1 was significantly higher than that of the other groups ($p < 0.05$). Additionally, abdominal fat was reduced in all treatment groups compared to the control group, howver this reduction was significantly recorded ($p < 0.05$) in broiler chickens of treatments P1, and P2 compared to the control group.

Internal organ weights

The weights of the kidney, bile, gizzard, heart, lung, and pancreas were not significanty different among all treatment groups ($p > 0.05$). However, Table 4 shows that the liver weight of P2 broilers was lower than that of P1 broilers and significantly higher than that of P3 broilers ($p < 0.05$).

The weight and length of different segments of the small intestine

The length of the small intestine was impacted by the dietary inclusion of palm oil (Table 5). The duodenum length of chickens in the P1 and P2 was significantly higher ($p < 0.05$), compared to that of the T group. The broiler chickens in the P1 group showed an increase in jejunum length ($p < 0.05$), compared to those of the other treatment groups, while P1 and P2 presented higher ileum length than T and P3 chickens ($p < 0.05$). No significant differences were indicated in duodenum, jejunum, and ileum weights among all the treatment groups ($p > 0.05$, Table 5).

Palm oil on gait score and litter quality

The effects of supplementing palm oil in the diet on gait score and litter quality are shown in Table 6. When palm oil was added to the diet, the dropping weight and litter depth decreased significantly, and also litter score significantly improved compared to those of the control group ($p < 0.05$). Compared to the control group, adding palm oil to broiler feed decreased significantly the prevalence of abnormal gait and its incidence ($p < 0.05$).

Blood biochemical profile

As indicated in Table 7, no difference was observed in glucose and cholesterol levels among all treatment groups ($p > 0.05$). Serum protein level was reduced by the inclusion of palm oil in the diet ($p < 0.05$). Similarly, serum uric acid level was reduced for chickens in P1, P2, and P3 compared to the control group ($p < 0.05$). The control group had the highest triglyceride level and it had a significant difference compared to P1 and P2 ($p < 0.05$).

Immune organ weights, thyroid hormone contents, and heterophil: Lymphocyte ratio

The impacts of adding palm oil to the diet on the thymus, spleen, and bursa of Fabricius weights, as well as the physiological reactions of broiler chickens, are shown in Table 8. The mean weights of the spleen and bursa of Fabricius did not differ significantly in all treatment groups ($p > 0.05$). However, the thymus weights of P1 and P3 chickens were higher than that of the chickens in the T group ($p < 0.05$). The level of T4 was not significantly different ($p > 0.05$) in T and P3 groups, but it was higher in P1 and P2 than in T and P3 broiler chickens ($p < 0.05$). Blood T3 content was higher in P1 and P2 than in T and P3 ($p < 0.05$). Lastly, the broiler chickens in the T group had a higher heterophil/lymphocyte (H/L) ratio than that of the other groups.

Table 1. Composition of experimental diets for broiler chickens during the dry season in Togo from February to March 2023

Experimental diets / Ingredients (kg)	T	P_1	P_2	P_3
Maize	53.6	46.3	35.9	25.5
Wheat	14.6	20.4	29.8	39.2
Soybean	19	19.5	19.5	19.5
Oyster shell	1.5	1.5	1.5	1.5
Salt	0.3	0.3	0.3	0.3
Broiler concentrate (5%)[1]	5	5	5	5
Dried brewers grains	6	6	6	6
Palm oil (%)	0	1	2	3
Total	100	100	100	100
Diet chemical composition				
Metabolizable energy (Kcal/kg)	3067.38	3102.66	3121.60	3140.55
Crude protein (%)	18.05	18.26	18.37	18.26
ME/CP	169.93	169.93	169.93	169.93

Chickens fed standard diets without palm oil (T) and broiler chickens fed diets containing palm oil inclusion: 1% (P_1), 2% (P_2), and 3% (P_3); ME/CP: Metabolizable energy (Kcal/kg) to crude protein ratio [1]:Composition Soybean meal, rapeseed meal, sunflower seed meal, corn gluten feed, vinasse, soybean oil, palm fatty acids, sodium chloride. Vitamin A: 12000 IU, Vitamin E, dl-a-tocophervl acetate: 20 mg, menadione: 2.3 mg, Vitamin D3: 2200 ICU. Riboflavin: 5.5mg, Calcium pantothenate: 12 mg, Nicotinı C acid: 50 mg, Choline: 250 mg. Vitamin B12: 10 ug Vitamin B6: 3mg, Thiamine: 3 mg, Folic acid: l mg, d-biotin: 0.05 mg. Trace mineral (mg/kg of diet): Mn: 80, Zn: 60, Fe: 35, Cu; 8, Selenium 0.1 mg

Table 2. Effects of dietary inclusion of palm oil on feed intake of 15 days old broiler chickens during the dry season in Togo from February to March 2023

Parameters \ Treatments	T + SD	P_1 +SD	P_2 + SD	P_3 + SD	P-value
Daily feed intake (g)	65.39 ± 2.46^{c}	86.17± 1.91^{a}	73.24 ± 3.31^{b}	73.05 ± 1.76^{b}	0.001
Body weight (g)	1583.00 ± 15.53^{d}	1835.00 ± 13.40^{a}	1697.00 ± 13.01^{c}	1757.00 ± 13.80^{b}	<0.001
Weight gain (g)	36.09 ± 1.46^{c}	53.91± 4.24^{a}	38.93 ± 4.05^{c}	41.85 ± 2.90^{b}	0.002
Feed conversion ratio	1.88 ± 0.05^{a}	1.62 ±0.06^{b}	1.80 ± 0.04^{a}	1.75 ± 0.16^{a}	0.076
Mortality (%)	4.00 ± 0.000^{a}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	4.00 ± 0.00^{a}	0.04

Chickens fed standard diets without palm oil (T) and broiler chickens fed diets containing palm oil inclusion at levels of 1% (P_1), 2% (P_2), and 3% (P_3). SD: Standard deviation. $^{a-c}$ Different superscript letters in the same row varied significantly differences ($p < 0.05$).

Table 3. Effects of dietary inclusion of palm oil on meat yield and abdominal fat of 15 days old broiler chickens during the dry season in Togo from February to March 2023

Parameters \ Treatments	T + SD	P_1 +SD	P_2 + SD	P_3 + SD	P-value
Carcass yield (%)	62.70 ± 0.80^{b}	75.20 ± 2.40^{a}	64.50 ± 2.57^{b}	62.72 ± 1.97^{b}	0.011
Thigh yield (%)	4.23 ± 0.04	4.30 ± 0.25	4.27 ± 0.15	4.55 ± 0 .23	0.123
Breast yield (%)	12.24 ± 0.91	12.93 ± 1.07	13.96 ± 0.13	14.73 ± 1.20	0.053
Abdominal fat (%)	1.60 ± 1.38^{a}	1.22 ± 0.27^{b}	1.24 ± 0.10^{b}	1.47 ± 0.28^{ab}	0.041

Chickens fed standard diets without palm oil (T) and broiler chickens fed diets containing palm oil inclusion at levels of 1% (P_1), 2% (P_2), and 3% (P_3), SD: Standard deviation. $^{a-c}$ Different superscript letters in the same row varied significantly differences ($p < 0.05$).

Table 4. Effects of dietary inclusion of palm oil on the physiological organs of 15 days old broiler chickens during the dry season in Togo from February to March 2023

Parameters \ Treatments	T + SD	P_1 +SD	P_2 + SD	P_3 + SD	P-value
Liver (%)	2.24 ± 0.21^{b}	2.50 ± 0.40^{a}	2.1 ± 0.11^{b}	1.94 ± 0.02^{c}	0.0245
Kidney (%)	0.42 ± 0.07	0.49 ± 0.32	0.43 ± 0.14	0.49 ± 0.05	0.132
Bile (%)	0.17 ± 0.09	0.19 ± 0.06	0.21 ± 0.13	0.21 ± 0.11	0.071
Gizzard (%)	3.19 ± 0.24	3.29 ± 0.34	3.86 ± 0.61	3.68 ± 0.68	0.057
Heart (%)	0.42 ± 0.07	0.46 ± 0.06	0.47 ± 0.06	0.46 ± 0.07	0.097
Lung (%)	0.50 ± 0.05	0.55 ± 0.07	0.60 ± 0.12	0.47 ± 0.04	0.510
Pancreas (%)	0.27 ± 0.06	0.31 ± 0.03	0.26 ± 0.02	0.26 ±0.02	0.062

Chickens fed standard diets without palm oil (T) and broiler chickens fed diets containing palm oil inclusion at levels of 1% (P_1), 2% (P_2), and 3% (P_3). SD: Standard deviation. $^{a-c}$ Different superscript letters in the same row varied significantly differences ($p < 0.05$).

Table 5. Effects of dietary inclusion of palm oil on small intestinal segments' weight and length of 15 days old broiler chickens during the dry season in Togo from February to March 2023

Parameters \ Treatments	T+ SD	P_1+ SD	P_2+ SD	P_3+ SD	P-value
Duodenum length (cm)	32.67 ± 1.45^{b}	36.00 ± 0.99^{a}	35.00 ± 0.97^{a}	34.67 ± 1.47^{ab}	0.045
Jejunum length (cm)	70.03 ± 2.30^{c}	83.00 ± 0.64^{a}	79.17 ± 1.05^{b}	73.33 ± 2.30^{c}	0.003
Ileum length (cm)	70.00 ± 2.88^{b}	75.67 ± 2.07^{a}	74.33 ± 1.46^{a}	71.03 ± 2.00^{b}	0.004
Duodenum weight (%)	1.15 ± 0.24	1.26 ± 0.42	1.19 ± 0.32	1.19 ± 0.18	0.083
Jejunum weight (%)	2.16 ± 0.26	3.06 ± 0.36	3.04 ± 1.00	2.52 ± 0.07	0.171
Ileum weight (%)	5.05 ± 0.39	5.36 ± 0.61	6.92 ± 1.12	5.19 ± 0.41	0.075

Chickens fed standard diets without palm oil (T) and broiler chickens fed diets containing palm oil inclusion at levels of 1% (P_1), 2% (P_2), and 3% (P_3). SD: Standard deviation. $^{a-c}$ Different superscript letters in the same row varied significantly differences ($p < 0.05$).

Table 6. Effects of dietary inclusion of palm oil on gait score and litter quality of 15 days old broiler chickens during the dry season in Togo from February to March 2023

Parameters \ Treatments	T+ SD	P_1+ SD	P_2+ SD	P_3+ SD	P-value
Droppings weight (g/chicken)	2.22 ± 0.08^{a}	1.86 ± 0.03^{b}	1.52 ± 0.02b^{c}	1.33 ± 0.12^{c}	0.005
Litter depth (cm)	2.30 ± 0.26^{a}	2.06 ± 0.31^{b}	1.76 ± 0.57^{c}	1.68 ± 0.73^{c}	0.004
Litter score	2.00 ± 0.000^{a}	1.4 ± 0.57^{b}	1.2 ± 0.333^{b}	1.3 ± 0.02^{b}	0.003
Abnormal gait prevalence (%)	6.62 ± 0.02^{a}	3.36 ± 0.13^{b}	3.34 ± 0.03^{b}	0.00 ± 0.000^{c}	<0.001

Chickens fed standard diets without palm oil (T) and broiler chickens fed diets containing palm oil inclusion at levels of 1% (P_1), 2% (P_2), and 3% (P_3). SD: Standard deviation. $^{a-c}$ Different superscript letters in the same row varied significantly differences ($p < 0.05$).

Table 7. Effects of dietary inclusion of palm oil on biochemical parameters of 15 days old broiler chickens during the dry season in Togo from February to March 2023

Parameters \ Treatments	T+ SD	P_1+ SD	P_2+ SD	P_3+ SD	P-value
Glucose (g/l)	2.37 ± 0.11	2.28 ± 0.06	2.30 ± 0.09	2.46 ± 0.01	0.068
Uric acid (g/l)	51.93 ± 1.78^{a}	34.37 ± 1.42^{b}	32.73 ± 1.56^{b}	29.19 ± 1.29^{b}	0.001
Triglycerides (g/l)	0.51 ± 0.03^{a}	0.41 ± 0.02^{b}	0.41 ± 0.04^{b}	0.46 ± 0.02^{ab}	0.016
Cholesterol (g/l)	1.38 ± 0.11	1.33 ± 0.09	1.37 ± 0.08	1.00 ± 0.04	0,124
Protein (g/l)	39.03 ± 1.13^{a}	36.54 ± 2.77^{b}	36.98 ± 1.80^{b}	31.24 ± 1.35^{c}	0.007

Chickens fed standard diets without palm oil (T) and broiler chickens fed diets containing palm oil inclusion at levels of 1% (P_1), 2% (P_2), and 3% (P_3). SD: Standard deviation. $^{a-c}$ Different superscript letters in the same row varied significantly differences ($p < 0.05$).

Table 8. Effects of dietary inclusion of palm oil on physiological parameters and immune organ weights of 15 days old broiler chickens during the dry season in Togo from February to March 2023

Parameters \ Treatments	T+ SD	P_1+ SD	P_2+ SD	P_3+ SD	P-value
T3	6.33 ± 0.28^{b}	8.22 ± 1.05^{a}	8.12 ± 0.24^{a}	6.51 ± 0.87^{b}	0.004
T4	8.60 ± 1.01^{b}	10.82 ± 1.01^{a}	10.35 ± 0.51^{a}	9.16 ± 0.85^{b}	0.024
H/L	1.82 ± 0.54^{a}	1.16 ± 0.12^{c}	1.17 ± 0.05^{c}	1.34 ± 0.19^{b}	0.030
Thymus (%)	0.24 ± 0.21^{b}	0.32 ± 0.02^{a}	0.29 ± 0.04^{b}	0.31 ± 0.02^{a}	0.035
Spleen (%)	0.17 ± 0.1	0.15 ± 0.02	0.18 ± 0.07	0.13 ± 0.05	0.052
Bursa of Fabricius (%)	0.07 ± 0.06	0.07 ± 0.03	0.12 ± 0.06	0.10 ± 0.08	0.078

Chickens fed standard diets without palm oil (T) and broiler chickens fed diets containing palm oil inclusion at levels of 1% (P_1), 2% (P_2), and 3% (P_3). SD: Standard deviation, T3: Triiodothyronine, T4: Thyroxine. $^{a-c}$ Different superscript letters in the same row varied significantly differences ($p < 0.05$).

Appendix 1. Vaccination program

Age (days)	Diseases	Vaccines
1	Marek's disease at the hatchery	PREVEXXION RN
3	Newcastle disease and infectious bronchitis	B_1H_{120}
6	Gumboro disease	Gumbo L
7	Newcastle disease and infectious bronchitis	B_1H_{120}
10	Gumboro disease	IBDL

DISCUSSION

The thermal challenge due to global warming is one of the most important environmental concerns in broiler production that negatively affects their optimum productivity, requiring mitigation strategies such as feeding strategies (Kpomasse et al., 2021; Olgun et al., 2021). The results showed that broiler chickens fed diets containing palm oil recorded a higher feed intake and consequently gained more weight than the control ones. In practice, under heat stress conditions, chickens reduce their feed intake to regulate and maintain their body temperature within the range of 40.6-41.0°C (Olgun et al., 2021). The incorporation of palm oil helped broiler chickens mitigate the effect of heat stress, resulting in improved feed consumption. Therefore, an increased feed intake of chickens led to the supply of more nutrients and, thus, an increase in body weight (Abdollahi et al., 2018).

The improved feed efficiency recorded for the 1% inclusion level of the palm oil suggests that low levels of dietary palm oil could be effective in improving the chickens' performance. This was evident as a higher mortality rate of chickens was recorded in the P3 group. In the same line, Wang et al. (2003) observed that the incorporation of 2% of fish oil in broiler diets could improve their performance. On the contrary, Jimenez-Moya et al. (2021) observed a positive impact on growth performance by including as much as 6% of palm oil in boiler chicken's diet. The differences observed can be explained by the environment, since in the present study, chickens were thermally challenged. Kang et al. (2001) reported the presence of polyunsaturated fatty acids in palm oil. This has been shown to improve both the immune status and nutrient digestibility in broilers and can also explain the low mortality of P1 and P2 chickens (López-Ferrer et al., 2001). Another compelling reason could be linked to an increase in palmitic acid in the palm oil used (44% of total fats) (Carta et al., 2017), which could affect some cells (Korbecki and Bajdak-Rusinek, 2019), including pancreatic cells, muscle cells, adipocytes

(Fat Cells), liver cells, and cardiovascular cells. Seifert et al. (2010) reported an adverse impact on mitochondria by increasing the generation of reactive oxygen species (ROS), which is harmful to cell development and chicken growth. Excessive ROS accumulation could be responsible for cell damage, death, or metabolism disturbance (Fedyaeva et al., 2014). These metabolic disturbances might also explain the lower feed intake and lower growth performance of P2 and P3 chickens.

The thigh, breast, kidney, bile, gizzard, heart, lung, and pancreas weights were not different among all the treatment groups in this present study. However, carcass weight increased in P1 broiler chickens. This could be linked to the increase in feed efficiency leading to reduced abdominal fat in these chickens. Since palmitic acid could impair myogenesis and negatively affect skeletal muscle (da Paixão et al., 2021), the inclusion of palm oil above a certain limit in thermally challenged chickens could potentially lead to a decline in muscle weight and growth performance in broiler chickens (Kpomasse et al., 2021). Furthermore, a diet high in fat may have increased bile secretion, which in turn may have caused liver hyperactivity and a subsequent drop in liver weight (Fouad and El-Senousey, 2014). Excessive fat deposition affects the consumers' acceptance of the meat (Schumacher et al., 2022). When the dietary energy is not fully used by the chickens, the liver converts the excess into fat (triglycerides) stored in adipose tissues, which leads to a loss of dietary energy (Hermier, 1997). This might explain the low blood triglyceride content recorded.

Regarding the different segments of the small intestine, the duodenum length of broiler chickens that received the palm oil was longer, compared to that of the control group, while 1% presented an increase in jejunum and ileum length in this present study. This suggests a morphological and histological alteration of the features of the gastrointestinal tract, which might affect partly the efficiency of the utilization of nutrients in broiler chickens (Swatson et al., 2002; Simon et al., 2019). An increasing length of intestinal segments leading to improved surface area available for nutrient absorption would have enhanced nutrient utilization in chickens (Ravindran et al., 2006).

The inclusion of palm oil in the diet decreased the weight of the droppings, and litter depth, with an improvement in litter score in the chickens that were given the oil palm. Also, abnormal gait and the prevalence of abnormal gait were considerably reduced by including palm oil in the broiler chickens' diets. Chickens that are thermally challenged produce a large amount of urine and wet droppings, which affect litter quality (Dayyani and Bakhtiari, 2013). Consequently, heat stress induces disturbances in bone metabolism, affecting the gait of broiler chickens (Dayyani and Bakhtiare, 2013). According to Dunlop et al. (2016), adding dietary palm oil to broiler chickens' diets under heat stress might have enhanced their metabolism by limiting disturbances and enhancing their welfare. However, an improvement in welfare status was not consistent with the higher mortality of P3 chickens. This supports the hypothesis developed concerning the 3% inclusion rate of dietary palm oil.

The incorporation of palm oil in the diet of broiler chickens did not affect glucose and cholesterol levels in this present study. However, serum protein and serum uric acid content were reduced by the inclusion of palm oil in the diet. The increase in blood protein content reflected a situation of inflammation, which is an infectious phenomenon stimulating gamma globulin production or dehydration caused by thermal challenge (Ansar and Ghosh, 2016). This occurs particularly in the liver and intestinal tracts (Quinteiro-Filho et al., 2012; Liu et al., 2022); Such infectious phenomena adversely affect the growth performance of broiler chickens (Remus et al., 2014). Protein degradation releases uric acid, which produces nitrogen, carbohydrates through gluconeogenesis, and lipids through lipogenesis, as well as carbon dioxide and energy (Gherghina et al., 2022). This might explain the relationship between protein and uric acid levels noticed in the present study.

The inclusion of palm oil did not affect the spleen or bursa of Fabricius. Nevertheless, thymus weight was higher and the heterophil/lymphocyte (H/L) ratio was lower in the experimental groups with increased blood T3 and T4 concentrations in the 1 and 2% palm oil diets. The thymus is a lymphoid organ involved in nonspecific (nonadaptive) and specific (adaptive) immune responses in poultry (Reese et al., 2006). More humoral antibody production is linked to a higher thymus weight (Igwe et al., 2020). The thymus produces the T lymphocytes that produce cellular antibodies or immunity (Davison et al., 2008). The inclusion of dietary palm oil in broiler chickens' diets could enhance immunity by fostering lymphocyte proliferation, consequently resulting in positive immune responses, including a transient decrease in the neutrophil/lymphocyte ratio and activation of leukocyte migration to infection sites (Dohms and Metz, 1991). Heat stress leads to a drop in Triiodothyronine and Thyroxin (Gonzalez-Rivas et al., 2019). The activity of antioxidant enzymes is regulated by thyroid hormones (Kpomasse et al., 2023). This implies that the improved

concentrations of the hormones T3 and T4 could be linked to the enhanced antioxidant status of broiler chickens. A heterophil to lymphocyte ratio (H/L ratio) in broiler chickens exposed to environmental challenges is the ratio of heterophils (a type of white blood cell involved in the immune system's reaction to stress and infection) to lymphocytes (another type of white blood cell involved in the immune system's response to specific pathogens). It serves as a stress resilience selection criterion (Al-Murrani et al., 2006; Gil et al., 2023). Thermal challenges may result in an increased H/L ratio (Bartlett and Smith, 2003; Mashaly et al., 2004). The body triggers stress responses during heat stress in broiler chickens, and these responses can change the immune system. A series of physiological reactions, including the release of stress hormones like corticosterone, are brought on by heat stress (Kyrou and Tsigos, 2009). Through affecting white blood cell production and function, this hormone can impact the immune system Gombart et al. (2020). In particular, heat stress tends to reduce the number of lymphocytes in the blood and increase the number of heterophils in circulation. This shift in the white blood cell count leads to an elevated H/L ratio. In the present study, results revealed that broiler chickens fed diets containing palm oil at levels of 1 and 2% showed a decreased H/L ratio compared to P1 and T treatments. Therefore, P1 broiler chickens expressed more health stress compared to P2 and P3 broiler chickens. This could be explained by a metabolic disruption caused by the increased consumption of palmitic acid, which makes up 44% of total fats in palm oil. This disruption might have impacted the type functions of certain cells (Carta et al., 2017). The higher H/L ratio in comparison to the broiler chickens in the T group indicates a change in the immune response toward a state that is less infection-fighting and more stress-related. It is a sign that the immune system of the chickens is stressed instead of actively fighting off infections (La Rosa et al., 2021)

CONCLUSION

The results of the study imply that broiler chickens' physiology and performance may be affected by feeding palm oil. Particularly under heat stress circumstances, it causes increased feed intake and weight gain. The low levels of palm oil inclusion (1%), which may boost the chickens' immune systems and nutritional digestibility, seem to be more beneficial for enhancing feed efficiency and growth performance. The effectiveness of palm oil, at higher concentrations (3%) can be linked to higher mortality rates. The findings also highlight the potential of palm oil to influence organ weights, gut morphology, and overall broiler chicken welfare. The specific effects of palm oil on internal organ physiology and gut function could be further investigated to optimize the inclusion of palm oil in broiler chicken diets.

DECLARATIONS

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Authors' contributions

Yarkoa Tchablémane Songuine Tchabltien, Pitala Wéré and Kpomasse Cocou Claude participated in the study conceptualization and performed the statistical analysis. Yarkoa Tchablémane, Parobali Tchilabalo, and Songuine Tchablitien carried out the design, methodology, and biochemical analysis. Karou Damintoti Simplice, and Pitala have contributed equally to this work. They conceived the study, participated in its design and administration, and helped draft the manuscript. All authors read and approved the final manuscript.

Availability of data and materials

There are no nucleic acid sequences, protein sequences, or atomic coordinates in the present study. The data are available upon request from the corresponding author.

Competing interests

The authors declare no conflict of interest.

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Ethical considerations

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

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Effects of Body Condition, Anatomical Measurement, and Age on the Cumulative Number of Individual Egg Production and Laying Pattern in First Laying Hens

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ABSTRACT

The individual egg production capacity of laying hens plays an outstanding role in achieving total production within a flock, which is affected by several internal and external factors. This study aimed to evaluate the effect of body condition, anatomical measurement, and age at the first laying (AFL) on the cumulative number of individual egg production (CNIEP) and laying pattern. Therefore, 172 Isa Brown laying chickens were investigated. Data on body condition and anatomical measurement were collected at the age of 16 weeks, while data on production was collected during 21 weeks of production (18-39 weeks). The obtained data were analyzed by Partial Least Square- Structural Equation Modeling (PLS-SEM) using smartPLS. The findings revealed that CNIEP could be predicted by body condition, anatomical measurement, and AFL. Compared to body condition and anatomical measurements, AFL was the most prominent factor in CNIEP. Body condition, anatomical measurement, AFL, and CNIEP had weak effects on the Isa Brown laying chickens' patterns.

Keywords: Anatomical measurement, Individual egg production, Laying pattern

INTRODUCTION

External characteristic properties of laying hens, such as the width of pubic bone and depth of the body, have been used as indicators to identify and select superior laying hens (Borrell and Torres, 2021). This practice has roots in the experiential knowledge gained during the domestication of these birds. As science and technology have advanced, numerous studies have been conducted to explore the correlation between anatomical and behavioral characteristics of egg production. Considering the egg production capacity, the laying hen typically falls into three categories, namely low, moderate, and superior productivity (Ajaero and Ezekwe, 2007, Ogbu et al., 2015; Preisinger, 2018). Body measurements on laying hens usually involve assessing the span of pubic bones, depth of body, width of cranium, and length of keel (Isaac and Obike, 2020). According to Latshaw and Bishop (2001), physical characteristics have large correlation ranging from 0.431 to 0.93 and that of pelvis width and body weight are factors naturally related to egg production.

The egg production in a flock is determined by the individual performance of each laying hen. Individual egg production capability of laying hen varies among the population (Sharifi et al., 2022) due to differences in the genetic potential of each individual. Although as much as 56% of the population can reach the production target, there is still a significant number of hens producing less than 100 or 150 eggs at the same time (Preisinger, 2018). This underscores the significant genetic variation in individual production capabilities among chickens. Individual assessment of laying performance is very important for discerning variations within the population. This enables selective breeding or culling so that the chickens with low production (poor layer) are not included in the population. The presence of inferior animals (low production) within the population can decrease overall production performance during rearing. This condition leads to economic losses in laying hen's business due to

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inefficient conversion of consumed feed to egg production.

The laying pattern of the chickens can be expressed in the form of clutches and days off. Superior laying hens certainly have a small number of clutches (NCs) in one rearing period, leading to a large number of eggs. There are different laying patterns in each population of laying hens. Recent studies have indicated that laying patterns in laying hens are regulated by genes located on chromosome 6 (Chen and Tixier-Boichard, 2003). Therefore, laying patterns can be used as a parameter in selecting and culling (Wolc et al., 2019). At present, there are a few studies explaining the relationship between this laying pattern from physical condition, age at first laying eggs, and anatomical measurements of the laying hen. This study aimed to deeply examine some variables, focusing based on anatomical measurements, body condition, and age at first laying in relation to individual egg production and laying pattern of laying hens. The primary objective of this study was to determine the capability of individual laying hens to produce eggs with regard to their physical conditions. Additionally, the study aimed to explore whether the physical conditions of individual chicken along with the production capacity of individual chickens also responded differently to individual laying patterns or not.

MATERIALS AND METHODS

Ethical approval

This research was conducted under the regulations of Animal Science faculty, Brawijaya University (Indonesia), in accordance with the recommendations in the Guide for the Care and Use of Animals (register number KEP.31/07/2022).

Study location

This research was performed at Pojok Village, Wates Sub-district, Kediri Regency, East Java Province Indonesia from February to May 2022. This region is 77 meters above sea level (MASL), -7.781 of latitude and 112.071 of longitude with a rainfall rate of 1860 mm per year and an average daily temperature was 27^{o}C (Indonesia Statistical Bureau, 2023).

Experimental chickens and their management

This research was conducted on 172 pullets (Isa Brown laying chickens) aged 18 weeks which were randomly selected at 16 weeks of age from a population of 300 chickens in a litter floor housing system, using Slovin's equation (Ryan, 2013). This research was conducted at an open house system in dimensions of 15 m x 4 m x 3.5 m (length x depth x height) with a battery aligned in six rows with 60 chickens per row (three rows face to face/V-shaped liked). The battery was arranged 1.5 m above the floor. This study was held from 16 to 39 weeks of age with lighting programs of 16L/8D (from 18 weeks of age until the age of 5% of laying [21 weeks]) and 14L/10D (from 5% of lay until the end of data collection/39 weeks of age). Laying hens had *ad libitum* access to water and their feeding regimen adhered to a feeding program prescribed from manual guidance of commercial laying hens for tropical countries (Hendrix Genetics, 2021). The commercial diet was from PT, Cargill Indonesia "Komplit Petelur Super" (Nutrient content is available in Table 1). During the rearing, the chickens were vaccinated against common diseases as a protocol from Medion company (Medion, 2018).

Table 1. Nutrient content of the prepared feed for the laying phase

Dry matter	88 %
Crude protein	16.5 – 18 %
Crude fat	7%
Metabolizable energy (ME)	2700-2970 Kcal/kg
Fiber	7%
Ash	14%
Calcium	3.25 -4.25 %
Phosphor	0.6 – 1 %
Aflatoxin (Max.)	50 ppb

ME: Metabolizable energy; Source of table: NRC, 1994

Body weight and anatomical measurement

Body weight and anatomical measurements (Width of pubic, size of the abdomen, Width of ischium, and depth of the body) were collected at the age of 16 weeks prior to the placement in battery cage. The body weight of the chicken was measured in grams (g) using a scale weight digital. Anatomical/body measurement was performed using a caliper in millimeters (mm).

Individual egg production parameters

Individual egg production parameters such as daily egg production and days off were recorded from the first laying until week 21 of the first production CYCLE (18 weeks to 39 weeks of rearing) using a Closed-Circuit Television (CCTV, China).

Statistical analysis

Data analysis on this research was performed using the Partial Least Square-Structural Equation Model (PLS-SEM) using SmartPLS (version 3.3.9). The assessment of model results was made as recommended by Hair et al. (2021) at a significant level of 0.05.

RESULTS AND DISCUSSION

The current study was performed to examine the relationship between the five latent variables, namely body condition at the age of 16 weeks, anatomical measurement at the age of 16 weeks, age at the first laying, cumulative number of individual egg production, and laying pattern to determine the effect of interaction between these variables. The summary result of the construct can be seen in Figure 1. Modeling from smartPLS comprising of body condition, anatomical measurement, age at the first laying, cumulative number or individual egg production, and laying pattern. On this five-structure model, it consists of one latent variable with a reflective measurement model (laying pattern) with two indicators and four latent variables with a formative measurement model (body condition, anatomical measurement, age on the first laying, and number of individual egg production) covering 8 indicators in total.

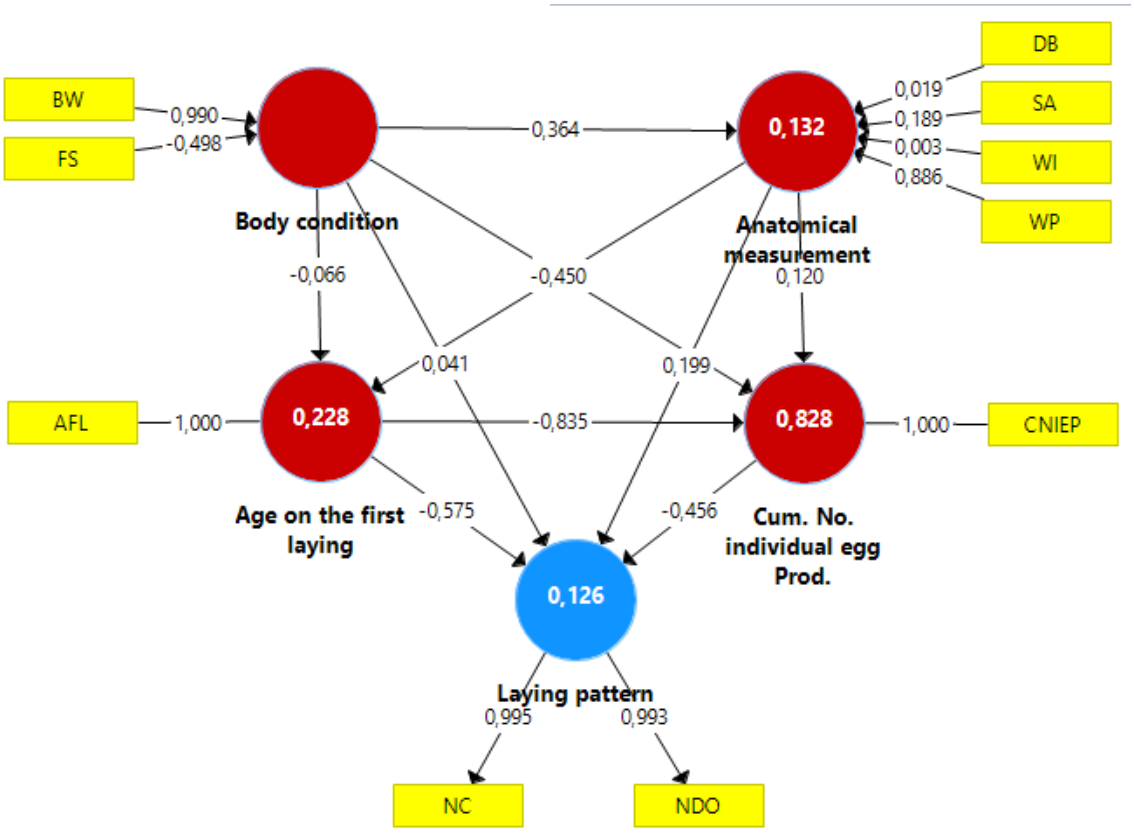

Figure 1. Modeling of the construct of body condition, anatomical measurement, age at the first laying, cumulative number or individual egg production, and laying pattern analyzed by SmartPLS model of laying hen. BW: Body weight, FS: Fleshing score, WP: Width of pubic, SA: Size of abdomen, WI: Width of ischium, DB: Depth of body, AFL: Age on the first laying, CNIEP: Cumulative number of individual egg production, NC: Number of clutches, NDO: Number of days off

Assessment of outer model (reflective and formative measurement models)

As a reflective measurement model, the laying pattern had two indicator variables, including NC and number of days off (NDO) for individual chickens throughout 148 days of production. The results indicated that NC and NDO had loading factors of 0.995 and 0.993, respectively (Table 2), meaning that NC and NDO had almost equal contributions to the construct. This finding aligns with Wolc et al.'s (2019) assertion that NC is a trait in egg-laying patterns controlled by the role of genes located on chromosome 6 from 28 to 29 Mb affecting parameters, such as NCs, maximum number of eggs in a clutch, and average clutch size (Bloom et al., 1993; Chen and Tixier-

Boichard, 2003; Roy et al., 2014). As reported by Ogbu et al. (2015), NCs are not the independent criterion and may not reveal much concerning individual laying performances. This is supported by studies indicating that better individual performance is associated with smaller NCs, longer clutch lengths, and fewer days open all indicative of high production and NCs were negatively correlated with a number of eggs (Gumulka et al., 2010; Ani and Nnamani, 2011; Wolc et al., 2019). The better individual performance is related to the smaller NCs with longer clutch lengths and fewer days open which indicate high production. However, Bednarczyk et al. (2000) mentioned that the NCs and NDO (Ogbu et al., 2015) could be used as one of the selection criteria describing the cyclic laying process. On the other hand, NDO indicates frequent NCs leading to less egg production (Ogbu et al., 2015).

In the assessment of formative measurement models, the fleshing score (FS) and width of the ischium (WI) were indicators that should be considered due to their very low loading weight (-0.498 and 0.003, respectively) with a non-significant p-value. The FS is a variable assessed subjectively using a predetermined score. It might be better to measure this variable using a measurement tool, to provide a measurement value that describes the size diversity of each individual in fleshing. On the other hand, WI is one of the variables with a low correlation with other variables in the latent variable. Based on the Variance Inflation Factor (VIF) value, there was no multicollinearity for all indicators. The width of the pubic was the best indicator to estimate the laying pattern (w = 0.886), compared to the size of the abdomen (w = 0.019), WI (w = 0.003), and the depth of the body (w = 0.189). The effects of the width of the pubic, size of the abdomen, and depth of the body with respect to the results of the bootstrap procedure were significant on the laying pattern variable ($p < 0.05$) but not the width of the ischium. Based on the result presented in Table 3, it was revealed that the construct of the laying pattern appeared to meet the criteria introduced by Hair et al. (2021). The values of average variance extracted (AVE), composite reliability (CR), Cronbach's alpha (CA), and Rho_A were full fill the requirements. Furthermore, NC and NDO indicators of the laying pattern were found to be significant based on the bootstrapping procedure ($p < 0.05$).

Table 2. Assessment of the laying pattern of laying hens for 21 weeks of observation (reflective measurement model) reared under tropical climate in Indonesia

	Reflective indicators	Convergent validity			Internal consistency reliability			Discriminant validity	t Value	p-value
		Loadings	Indicator reliability	AVE	Composite Reliability	Cronbach's Alpha	Rho_A			
		>0.5	>0.5	>0.5	0.70-0.95					
Laying pattern	NC	0.995	0.990	0.988	0.994	0.988	1.005	Yes	874	P < 0.05
	NDO	0.993	0.986						641	P < 0.05

NC: Number of clutches, NDO: Number of days off, AVE: Average variance extracted, Loading: Correlation value between physiological response, respiration rate, and rectal temperature, Cronbach's Alpha: A measure of internal consistency of a test, Rho_A: Measurement the strength of association between two variables, less than 0.05 show significant

Table 3. Assessments of body condition, and anatomical measurement of laying hen reared under tropical climate in Indonesia

	Formative indicator	Outer weight	T value	P value	95% bootstrap confidence interval	Outer VIF
Body condition	BW	0.990	2.621	P < 0.05	[-0.590,0,999]	1.056
	FS	-0.498	0.887	P > 0.05	[-0.603,0.643]	1.056
Anatomical measurement	WP	0.886	12.347	P < 0.05	[0.858,0.997]	1.343
	SA	0.019	5.514	P < 0.05	[0.376,0.820]	2.336
	WI	0.003	0.247	P > 0.05	[-0.241,0.315]	1.024
	DB	0.189	3.238	P < 0.05	[0.166,0.649]	1.914

BW: Body weight, FS: Fleshing score, WP: Width of pubic, SA: Size of the abdomen, WI: Width of ischium, DB: Depth of body, VIF: Variance inflation factor, ($p < 0.05$).

Table 4. Assessment of path coefficients of the structural model on all latent variables of laying hen reared under tropical climate in Indonesia

Constructs	Path coefficients	T value	p-value	95% bootstrap confidence interval	f^2 value (Effect size)
AFL → CNIEP	-0.835	27.164	p <0.05	[-0.889, -0.773]	3.119 (large)
AFL → LP	-0.195	3.080	p <0.05	[-0.363,-0.005]	0.071 (medium)
AM → AFL	-0.450	5.584	p <0.05	[-0.604,-0.280]	0.228 (medium)
AM → CNIEP	0.495	6.250	p <0.05	[0.326,0.634]	0.059 (medium)
AM → LP	0.232	2.450	p <0.05	[0.044,0.408]	0.030 (medium)
BC → AFL	-0.230	1.822	p <0.05	[-0.392, 0.197]	0.005 (small)
BC → AM	0.364	2.238	p <0.05	[-0.263, 0.543]	0.152 (medium)
BC → CNIEP	0.278	2.006	p <0.05	[-0.237, 0.430]	0.009 (small)
BC → LP	0.119	1.030	p <0.05	[-0.179, 0.305]	0.002 (small)
CNIEP → LP	-0.456	2.672	p <0.05	[-0.801, -0.152]	0.041 (medium)

AFL: Age on the first laying, CNIEP: Cumulative number of individual egg production, LP: Laying patter, AM: Anatomical measurement, BC: Body condition.

Assessment of structural model

From all the variables observed, the cumulative number of individual egg production (CNIEP) is one of the most important variables, indicating how well an individual chicken can produce eggs in the same timeframe. The expectation of raising laying hens is that all individuals can produce the same amount of each individual, but in reality, the ability of individuals in a population is very diverse. This diversity in individual abilities can be influenced by several factors, including the condition of the body at the pre-layer (Lacin et al., 2008), the anatomical size of each individual (Isaac and Obike, 2020), the age when they first laid eggs, lighting (Khalil et al., 2004), and other factors in terms of rearing management (Zaheer, 2015).

Based on the results presented in Table 4, the CNIEP is significantly influenced by body condition at 16 weeks, anatomical measurement, and age on the first egg, which is 82.2% (R^2). Of the three variables, age on the first laying (AFL) could play a significant role in withdrawing CNIEP, compared to body condition and anatomical measurements (γ = -0.835, p < 0.01). Alilo (2017) reported that the onset age of laying could affect the total egg production in its life cycle and selection of laying hen in the first part of the laying cycle could improve the production. Other researchers believe that age at the first egg is negatively correlated with the first three months of egg production and there is a positive and high correlation between egg production during the first three months and annual production (Khalil et al., 2004; Liu et al., 2018). Therefore, they suggest that selection can be performed at the beginning based on the first three months of production performance. Additionally, age at the first egg is an important trait in laying hens controlled hormonally and regulated by various genes and pathways (Xu et al., 2011; Tan et al., 2021).

According to the model, anatomical measurement (γ = 0.495, p < 0.01) showed up better contribution to CNIEP than body condition (γ = 0.278, p < 0.05). Present findings are in agreement with the study of Sherwood (1922) who found the correlation between external body characters and annual egg production in white leghorn fowls ranging from low to strong, both positive and negative. Sherwood (1992) found a correlation ranging from low to strong, both positive and negative between anatomical measurements and egg production. In the present study, the indicator of the width of the pubic had a very significant positive correlation as a bivariate correlation with CNIEP (r = 0.509, p < 0.01). In addition, the width of the pubic was the best indicator in representing the construct of anatomical measurement with the outer weight of 0.886 (Figure 1). This shows that the width of the public is the most dominant variable in explaining the construct in the latent variable (anatomical measurement). On the other hand, body condition had no effect on CNIEP and the effect size was 0.009. This means that body condition (body weight and fleshing score) does not have a real impact on the production ability of individual Isa Brown strain chickens. Consistent with the present study, Lacin et al. (2008) also investigated the impact of varying body weights in Lohman laying hens on egg production, finding no significant effect. These collective findings suggest that the performance of contemporary laying hen strains is no longer solely determined by differences in body weight. Instead, distinctions in egg production ability may be discerned based on other economic traits, such as anatomical size.

Body condition at 16 weeks and anatomical measurement accounted for 22.8 % of the total variability in AFL according to the model, and this was a weak effect (Figure 1). Anatomical measurement had a more key role in the age of the first laying (γ = -0.450, $p < 0.01$) since there was no effect of body condition on AFL egg (γ = -0.230, $p > 0.05$, $f^2 = 0.005$). Among the six indicators in body condition (two factors) and anatomical measurement (four indicators), only the width of the pubic bone and size of the abdomen demonstrated a significant negative correlation, with r values of -.443 and -.402, respectively ($p < 0.01$). On the other hand, body condition accounted for 13.2% of the total variability in anatomical measurement, showing the weakness of body condition in explaining the variance in anatomical measurement construct. Similarly, the findings indicated a weak correlation of body weight and fleshing score with all indicators in anatomical measurement (Table 4). Body condition, anatomical measurement, AFL, and CNIEP accounted for 12.6% of the total variability in laying pattern variables according to the model indicating a weak effect (Figure 1). This suggests that the laying pattern which includes the number of clutches and the number of days off is not much influenced by body condition (body weight and fleshing score), age at the first laying, and CNIEP. Among these three exogenous variables, CNIEP appeared to have more influence on laying pattern with a contribution of -0.456 (γ) than body condition (γ = -0.195), anatomical measurement (γ = 0.234), and AFL (γ = 0.119). Body condition did not affect the laying pattern, meanwhile, anatomical measurement and AFL had a positive and significant effect on the laying pattern which was very small ($p > 0.05$, $f^2 = 0.030$ for AM and $f^2 = 0.071$ for AFL).

CONCLUSION

Age on the first laying contributes significantly to the cumulative number of individual egg production and laying pattern of the laying hen. Anatomical measurement has a significant role in the first laying age, cumulative number of individual egg production, and laying pattern. Body condition at 16 weeks does not affect age on the first laying and the laying pattern but significantly contributes to anatomical measurement and cumulative number of individual egg production. It is suggested to manage the population to start producing eggs on time (18 weeks) by applying planned management practices. Since the result of current research indicated that the chickens whose production was late could reduce the total number of individual eggs production; therefore, it is highly recommended to take out the chicken that is late in production to maximize the profit.

DECLARATIONS

Availability of data and materials

The primary data of this study are authentic and available by request from the corresponding author via email.

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Authors' contributions

Heni Setyo Prayogi contributed to the conceptualization, investigation, data curation, writing, review, and editing of the manuscript. Suyadi, V.M. Ani Nugiartiningsih, and Osfar Sofjan were involved in every step of the research as well as manuscript revision based on reviewer feedback. All authors checked and approved the final version of the manuscript for publishing in the present journal.

Competing interests

The authors have declared that there are no competing interests in the results presented in this article

Ethical considerations

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

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Differential Gene Expression Profiling during Avian Immune Organ Development: Insights from Thymus and Bursa of Fabricius Transcriptome Analysis

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ABSTRACT

Pluripotent hematopoietic stem cells undergo maturation to regenerate blood cells. T-cell and B-cell maturation from common lymphoid progenitors (CLPs) is regulated by distinct transcription factors and signaling pathways. *Notch* signaling, *GATA3*, and *TCF1* drive T-cell fate, while *EBF1*, *PU.1*, *E2A*, *Pax5*, *TCF1*, and *Foxo1* regulate B-cell differentiation. Alterations in these factors can cause lineage deviations. Gene expression profiling of the chick thymus and bursa reveals dynamic expression patterns of transcription factors, cytokines, and signal molecules. *Notch1* and *Dll1* expression that increase during later stages indicate the ongoing role of *Notch* signaling in T-cell lineage maintenance. The current study aimed to identify differentially expressed genes during the development of the avian immune organs, focusing on the thymus and bursa using 24 Ross 308 avian breed. The mRNA libraries from these organs were analyzed using quantitative Real-time PCR analysis at six time points spanning the embryonic ages (days 15 and 18) and post-hatch age (days 3, 7, 14, and 28). The data for the gene expression indicated significant variations across different stages of immune organ development. Differential gene expression was observed between sorted T and B-cells, with *GATA3*, *CD3e*, *CD4*, and *Ptprc* showing higher expression in the T-cell population, and *Pax5* and *CD81* exhibiting higher expression in the B-cell population. Notably, *ENO1* and *IRF4* showed higher expression in T-cells at E15 and B-cells at E18. The study highlights the importance of regulatory factors and genes in maintaining cellular identity, furthers the understanding of avian immunology, and has the potential for improving poultry health and studying immune-related diseases in humans. These findings pave the way for further research on the role of biochemical components under important disease conditions in avian immunology and their potential applications for poultry health.

Keywords: Avian lymphocyte, B-cell maturation, Gene expression, Regulatory elements, T-cell

INTRODUCTION

Pluripotent hematopoietic stem cells (HSCs) undergo consecutive rounds of biochemical development and maturation during differentiation to regenerate and self-maintain all types of blood cells (Ogawa, 1993). These HSCs give rise to multipotent hematopoietic progenitors (MPPs), which then undergo lineage commitment, resulting in the formation of either common lymphoid progenitors (CLP) or common myeloid progenitors (CMP; Kondo et al., 1997; Akashi et al., 2000). The progression of CLPs toward a specific lymphoid lineage, either T-cell or B-cell, is highly regulated by a network of biochemical transcription factors that have been well established (Naito et al., 2011; Thompson and Zúñiga-Pflücker, 2011; Boller and Grosschedl, 2014; Rothenberg, 2014). As both B-cells and T-cells are initially generated from CLP, they have highly similar receptor structures, progression regulation, and pathways across different stages of development (Borst et al., 1996; Han et al., 2023).

Despite the similarities between T-cell and B-cell maturation, the differentiation and proliferation of precursor cells toward either a mature T-cell or B-cell are coupled to extensive and strictly regulated pathways

across different stages of development (Figure 1). This ensures the expansion of specific cell populations that have passed all the biochemical checkpoints required for expressing a specific repertoire of the properly selected receptor. For instance, in the case of T-cells, several biochemical transcription factors mediate the commitment of progenitor cells toward a T-cell fate (Busslinger, 2004; Rothenberg et al., 2010; Yang et al., 2010; Naito et al., 2011). The *Notch* signaling pathway is a crucial signal for the initiation of the T-cell generation pathway and differentiation in the early stages of T-cell development (Radtke et al., 1999; Sambandam et al., 2005). *GATA3* (a double zinc finger) and *TCF1* (T-cell factor 1) are important biochemical transcription factors required for determining T-cell fate and have been identified as downstream factors of *Notch1* in nearly all stages of early T cell progenitor lineage (Figure 1, Hozumi et al., 2008; Weber et al., 2011).

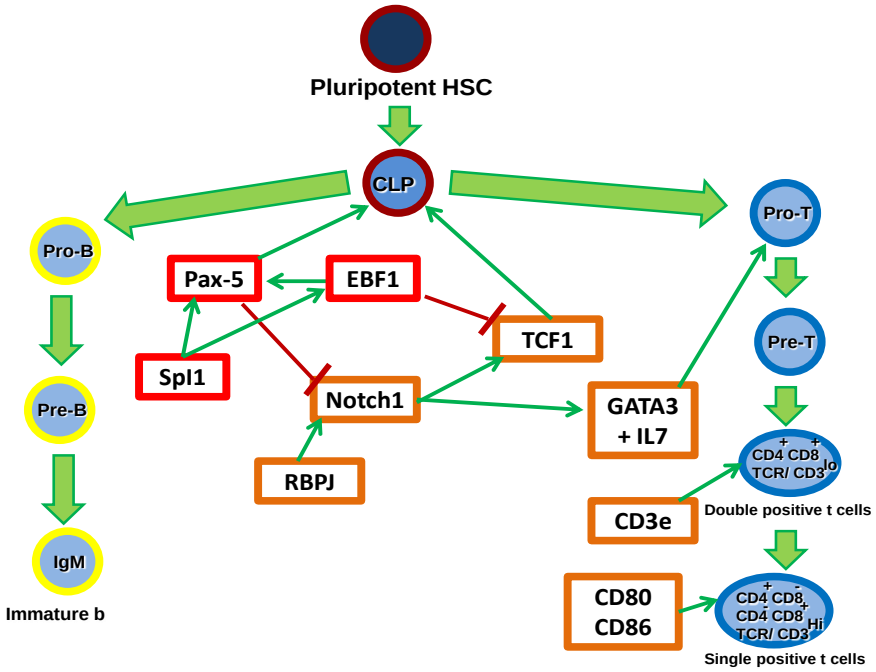

Figure 1. The pluripotent hematopoietic stem cells (HSCs) toward common lymphoid progenitor (CLP) and lymphocyte lineage commitment through the regulation of mRNA networks and pathway

In the case of B-cells, a network of transcription factors, including *EBF1*, *PU.1*, *E2A*, *Pax5*, *TCF1*, and *Foxo1*, regulates the differentiation of progenitor cells into pro-B-cells (Decker et al., 2009; Medvedovic et al., 2011; Boller and Grosschedl, 2014) as well as the rearrangement of immunoglobulin heavy chain loci in B-cells (Liu et al., 2003; Dengler et al., 2008). *Pax5* and *EBF1* play crucial roles in suppressing the T-cell lineage genes *Notch1* and *GATA3*, respectively, which are the key biochemical regulatory factors contributing to T-cell fate commitment (Delogu et al., 2006; Nechanitzky et al., 2013; Delpoux et al., 2021). Conditional knockout of *Pax5* in mature B-cells leads to their conversion into a T-cell-like state, indicating the importance of *Pax5* in maintaining B-cell identity (Cobaleda et al., 2007).

In pro-B-cells, *Pax5* deficiency can also lead to the deviation of B-cell lineage toward alternative lineage commitments, such as myeloid cell, dendritic cell, or even T-cell fates under the influence of other lineage gene signals (Nutt et al., 1999; Rolink et al., 1999; Mikkola et al., 2002). Moreover, *EBF1* is a key mediator of B lymphopoiesis (Lin and Grosschedl, 1995; Zandi et al., 2008). It has been shown to rescue B-cell fate commitment even in the absence of *E2A* or *Pax5* in knockout mice (Seet et al., 2004; Zandi et al., 2012). *EBF1* can also restore the activity of the Pu.1 transcription factor in Pu.1-deficient multipotent progenitor cells, further confirming its importance in the differentiation of progenitor cells into B-cells (Medina et al., 2004). Several studies have shown that a positive feedback loop exists between *Pax5* and *EBF1*, revealing the importance of *Pax5* in maintaining *EBF1* levels for the proper regulation of B-cell commitment of progenitor cells (Roessler et al., 2007; Decker et al., 2009; Haniuda et al., 2020).

A combination of transcription factor networks orchestrates gene expression in different types of cells to maintain their identity throughout the sequential signals of activation and repression. Consequently, determining an extensive view of gene expression will aid the elucidation of different genes' biochemical mechanisms and levels during immune cell development. Biochemical gene expression profiling was conducted to characterize avian immune lymphocyte development using the two primary immune tissues (the thymus and bursa of Fabricius) across different stages of chick development.

The current study aimed to comprehensively investigate the regulatory mechanisms and biochemical pathways governing the differentiation and maturation of pluripotent HSCs into various blood cell lineages and their subsequent commitment to T-cell and B-cell lineages. Through a profile analysis of the network of transcription factors involved in this process, to enhance the understanding of the molecular events that underlie avian immune cell development.

MATERIALS AND METHODS

Ethical approval

This study was performed and carried out following the Faculty of Agriculture, Cairo University, Committee of Animal and Poultry Production welfare treatment, and complied with relevant legislation of the Ministry of Agriculture in Egypt on animal ethics and welfare (Decree No. 27 (1967).

Animals

A total of 24 Ross 308 broiler chickens were used in this study from February to March 2020. All experiments were carried out following the Faculty of Agriculture Cairo University's intuitional approved protocols. The thymus and bursa of Fabricius were collected from the sacrifice of four chickens at each of the six crucial time points during the development of T- and B-cells maturation across two embryonic ages (days 15 and 18) and four post-hatches days (days 3, 7, 14, and 28), snap frozen in liquid nitrogen, and stored at -80°C until further analysis. T- and B-cells were further isolated from six Ross 308 embryos at 15 and 18 days of embryonic development to validate the gene expression in the sorted cells. The thymus and bursa of Fabricius were collected at two-time intervals, three at 15 embryonic days (E15) and the other three at 18 embryonic days (E18). Both the thymus and bursa of Fabricius were directly used for cell isolation to further assess the gene expression in the sorted cells compared to the whole organs for the Ross 308 avian breed thymus and burse.

RNA isolation and mRNA library construction

Total RNA was isolated from the 24 thymus and 24 bursa of Fabricius samples. The 6 (3 E15 and 3 E18) sorted B-cell, and the 6 (3 E15 and 3 E18) sorted T-cells samples were processed using TRI Reagent® RNA Isolation Reagent (Sigma, St. Louis, MO, USA) according to the manufacturer's instructions, then subjected to a DNase treatment using a TURBO DNA-free™ Kit (Life Technologies, USA). RNA quality was assessed with an RNA 6000 Nano Assay kit and the Model 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). An RNA integrity number (RIN) of greater than 9.0 was observed for all samples.

Semi-quantitative real-time PCR analysis for the selected mRNAs

First-strand complementary DNA (cDNA) was synthesized from 1 µg of DNaseI-treated total RNA from 24 thymus samples and 24 bursa of Fabricius samples as well as 12 samples of sorted T- and B-cells samples (6 samples from each cell type) using a Miscript II RT kit (Qiagen) following the manufacturer's instructions. Real-time PCR reactions were carried out in duplicate using the primer sets in Table 1 (Ye et al., 2012).

Table 1. Semi-quantitative real-time PCR primers for mRNA expression generated using Primer-BLAST developed at NCBI for thymus and bursa of Fabricius of broiler chickens

Gene Name	Primer Sequence
GATA3-F	TCTACTACAAGCTGCACAATA
GATA3-R	TCTCATTTTGAGACCGTAAG
Pax5-F	TCCTGTTTCCTTCCACCGAC
Pax5-R	AGCTGCCTGGAGATGTCGCA
ENO1-F	CGGAGCGGTGTTCAAGATGT
ENO1-R	CCCAAGTACAGGTCAGCCAG
Cd3e-F	ATCCACCCCATAGCCCTTCT
Cd3e-R	TGAAACGGCACCAGCAAATG
Cxcr4-F	CCCTTGCGTTCTTCCATTGC
Cxcr4-R	AACCACTTGTCCACAGGACC
Cd4-F	TGGAACCTGGATGTGTCACG
Cd4-R	AACATGAGCTTCCTCCACGG
IRF4-F	ATCCCCCTACCTGGAAGACC
IRF4-R	CGGGGCAAATTCTCTCCAGT
Ptprc-F	CTTCTCTGCTGGAGGCGAAA
Ptprc-R	CAAAGGTGGAGACCACTCCC
Cd81-F	AGCTGGAATCTGGGGGTTTG
Cd81-R	TCCATCTCCTCGGGACACAT

For mRNA expression analysis, each reaction contained 10 ng of cDNA, 500 nM of the forward primer, and 1× iQ SYBR green supermix (Bio-Rad, Hercules, CA, USA). PCR was performed using the cycling conditions of 95°C for 15 minutes followed by 40 cycles of (95°C for 10 seconds and 60°C for 20 seconds) using a MyiQ Real-Time PCR detection System (Bio-Rad, USA). Melting curve analysis was assessed to determine amplification specificity for each mRNA. Normalization was conducted using the expression of the GAPDH housekeeping gene (Huitorel and Pantaloni. 1985). The threshold cycle (Ct) values were transformed to a relative expression in arbitrary units by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Isolation of T- and B-cells

T and B lymphocytes were purified from the whole thymus and bursa of Fabricius, respectively, from three embryos at two embryonic ages (E15 and E18). Mouse anti-chicken CD3 and mouse anti-chicken Bu-1 antibodies (Southern Biotech, USA) were used for sorting T-cells and B-cells, respectively. Tissues were mechanically disrupted, passed through a 70µm nylon mesh cell strainer (BD Biosciences, USA), and washed three times with phosphate-buffered saline (PBS). The isolated cells were mixed with 1µg/ml anti-CD3-conjugated phycoerythrin (PE) and anti-Bu1a-conjugated Fluorescein Isothiocyanate (FITC), respectively, and incubated for 15 minutes at room temperature. An Easysep positive selection kit (Stemcell Technologies, USA) was used to isolate T-cells and B-cells according to the manufacturer's protocol. Briefly, 100µl/ml of the Easysep fluorescein isothiocyanate (FITC) or phycoerythrin (PE) positive selection cocktail was added to antibody-bound cells and incubated for 15 minutes at room temperature. Then, 50 µl/ml of the magnetic nanoparticles' beads were mixed with the cells and incubated for an additional 10 minutes. The tube was placed on the Easysep magnet for 5 minutes. Negative cells were aspirated, and the remaining cells were washed three times with a washing buffer and resuspended in a resuspension buffer (Stemcell Technologies, USA) with 1% FBS. A group of unsorted cells was treated with 1µg/ml of the isotype negative control IgG1 conjugated with either FITC or PE to set the gates accordingly.

Flow cytometric analysis

Phenotyping of the CD3+ or Bu1a+ cells was detected and sorted using a Beckman Coulter flow cytometry machine (USA), and Cell Lab Quanta SC analysis was utilized to assess the sorted cell population using an electronic volume of 2.60 and 7.20 side scatter value.

Statistical analysis

Relative fold change in expression and significant differences across development stages and between tissues were determined using Student's t-test (SPSS version 26, USA).

RESULTS

Functional and pathway analysis for the selected mRNAs

To provide a further assessment of the potential roles of these differentially expressed mRNAs across development in the avian immune organs, functional analysis using the DAVID 6.7 algorithm (Huang et al., 2008) was performed (Table 2). CD81, PrKcd, ENO1, IFNAR1, and IRF4 were identified as potential genes involved in B-cell proliferation, while CD4, CD3e, Cxcr4, and IL2ra were identified as T-cell differentiation and proliferation genes. Following a study by Franceschini et al. (2013), a group of these genes known to be involved in the development, proliferation, and/or differentiation of either T-cells or B-cells was selected for further analysis using the STRING v9.1 database (Figure 6). This analysis revealed a direct interaction between *EBF1* and *Pax5*, with both being actively regulated by PU.1 (SPI1), a member of the ETS family of transcription factors. SPI1 could also repress GATA3 expression. In turn, IRF4 could directly affect the expression of SPI1. Cxcr4, Ptprc, CD44, and CD3e were found to interact with CD4 which can activate their expression.

Table 2. mRNA functional annotation (DAVID) of the selected gene expressed in thymus and bursa of Fabricius development of broiler chickens

Functional classification	Gene symbol	Gene description
B-Cell Proliferation	CD81	Target Of Antiproliferative Antibody
	Prkcd	Protein kinase C, delta
	ENO1	Enolase 1, Alpha
	IFNAR1	interferon (alpha, beta, and omega) receptor 1
	SOCS3	Suppressor Of Cytokine Signaling 3
	IRF-4	Interferon Regulatory Factor 4
T-Cell Differentiation	CD4	membrane glycoprotein of T lymphocytes
	Nos2	Nitric Oxide Synthase 2, Inducible
	CD3e	CD3e molecule, epsilon (CD3-TCR complex)
T-Cell Proliferation	Cxcr4	Chemokine (C-X-C motif) receptor 4
	Il2ra	Interleukin 2 Receptor, Alpha

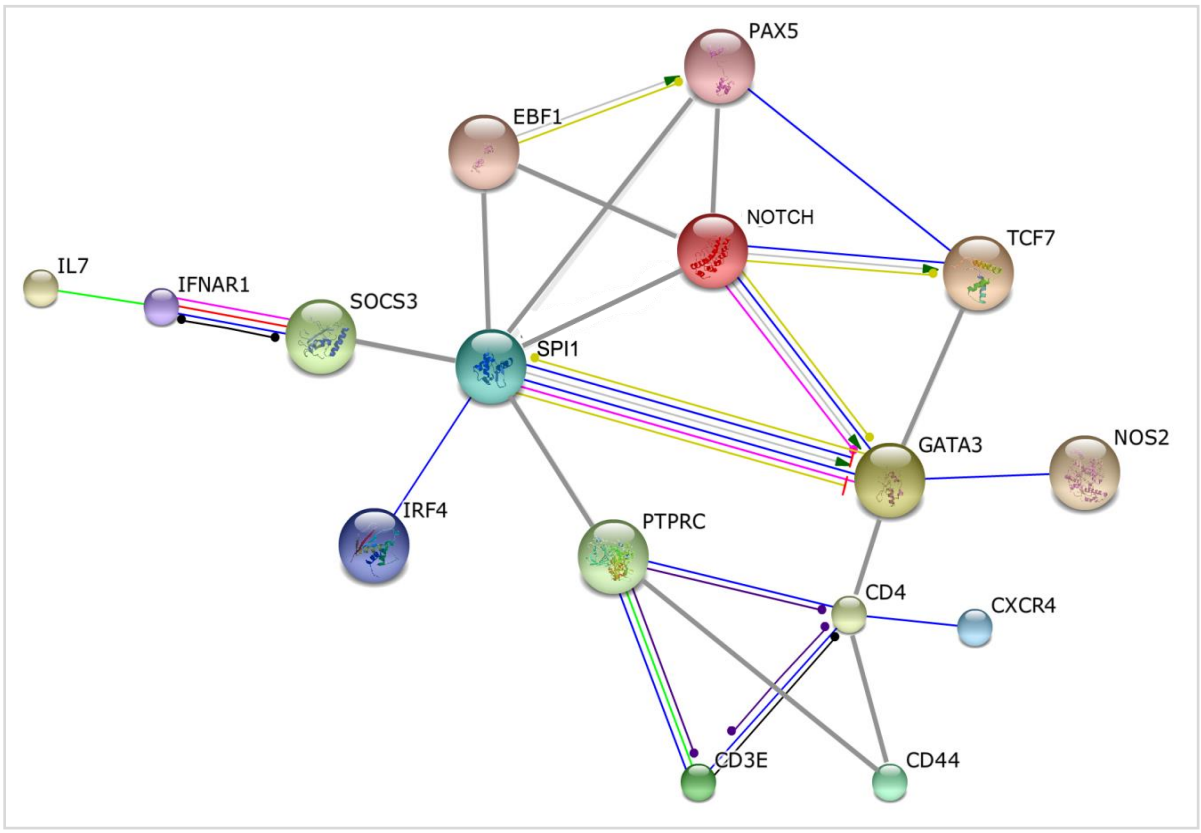

Figure 2. Pathway analysis of genes influencing the differentiation and proliferation of T-cells and B-cells in broiler chickens

Sorting B-cells and T-cells

T-cells and B-cells were purified from the thymus and bursa of Fabricius, respectively, using the EasySep system (Stemcell Technologies, USA) for subsequent gene expression analysis. In the sorted B cells, approximately 81.27% and 83.58% of the B-cell population expressing the Bu-1a surface marker were successfully recovered from the E15 and E18 in the bursa of Fabricius tissues, respectively (Figure 3). Similarly, approximately 77.45% and 74.36% of the cells expressing *CD3e* surface marker were successfully harvested from the E15 and E18 in thymus tissue, respectively (Figure 4).

Differences in gene expression in purified T-cell and B-cell populations

The expression of a selected set of differentially expressed genes was further assessed in sorted T-cells and B-cells, using bio rad real-time PCR (USA, RT-PCR) analysis (Figure 5). The expression of *GATA3*, *CD3e*, *CD4*, and *Ptprc* was high in the T-cell population at both E15 and E18, compared to B-cells at the same time points. Both *Pax5* and *CD81* were expressed more in B-cells, compared to T-cells. *ENO1* and *IRF4* were expressed more with a ~2 fold and a ~5 fold increase, respectively, at E15 in T-cells compared to B-cells, while at E18, their expression increased with ~3 and ~7 fold changes in B-cells, compared to T-cells. The semi-quantitative real-time PCR analysis was conducted. Ross 308 broiler chickens were used to study the thymus and the bursa of Fabricius across two embryonic ages (E15 and E18) and four post-hatch ages (D3, D7, D14, and D28). The results of functional gene category annotations revealed dynamic expression patterns of crucial biochemical transcription factors, cytokines, chemokines, cell surface markers, and signal molecules during chicken thymus and bursa of Fabricius development, reflecting the dynamic changes occurring in cell populations. Notably, the expression of the *Notch1* receptor and its ligand Delta-like1 (*Dll1*) showed an increasing trend in both tissues at later stages, suggesting the continuous requirement of the *Notch* signaling pathway for maintaining the T-cell lineage during chick development. The observed changes in the expression of various biochemical genes during different stages of avian immune development will provide insights into the underlying molecular processes and regulatory networks involved in immune cell maturation and differentiation.

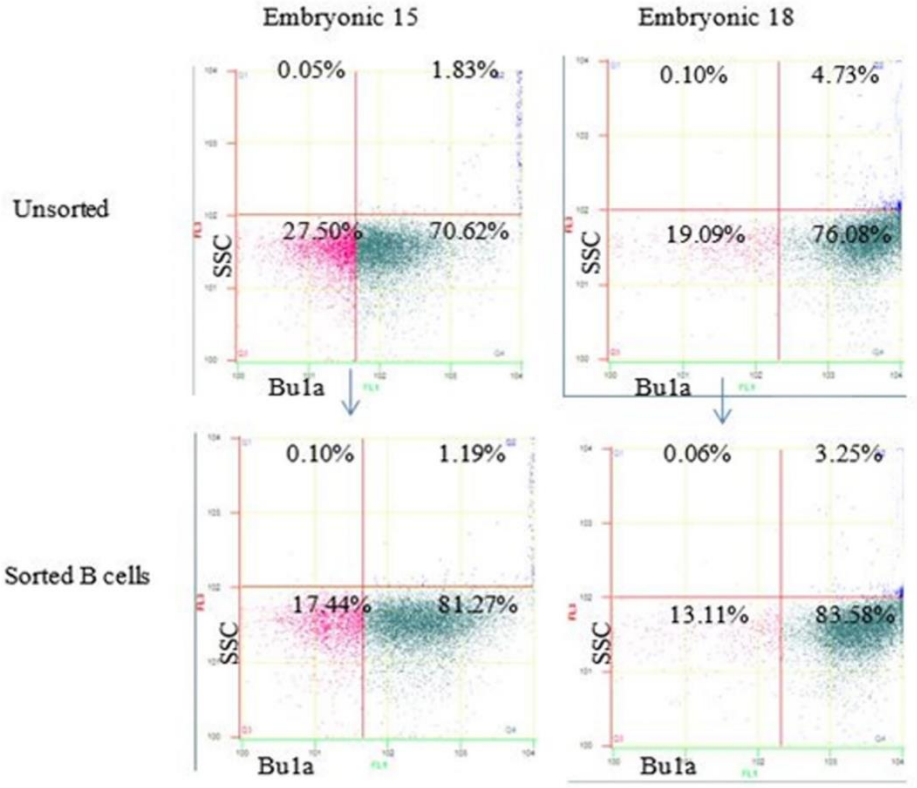

Figure 3. Flow cytometry analysis of sorted B-cells from whole bursa of Fabricius of broiler chickens using stem cell technology, magnetic beads and Bu-1a conjugated FITC antibody at two embryonic stages (E15 and E18)

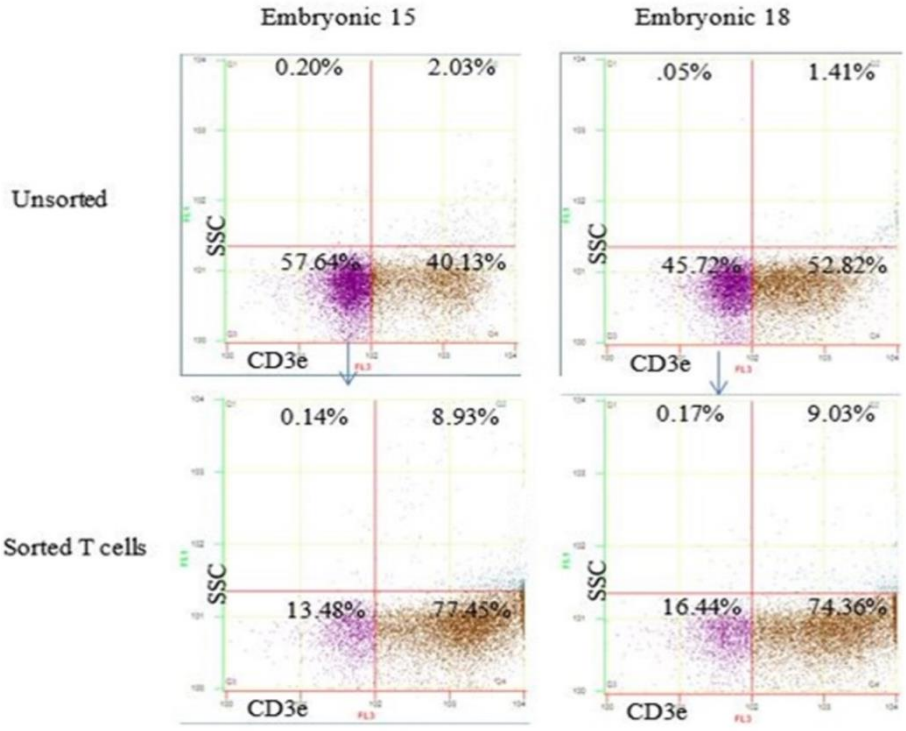

Figure 4. Flow cytometry analysis of sorted T-cells from the whole thymus of broiler chickens using stem cell technology magnetic beads and CD3 conjugated PE antibody at two embryonic stages (E15 and E18)

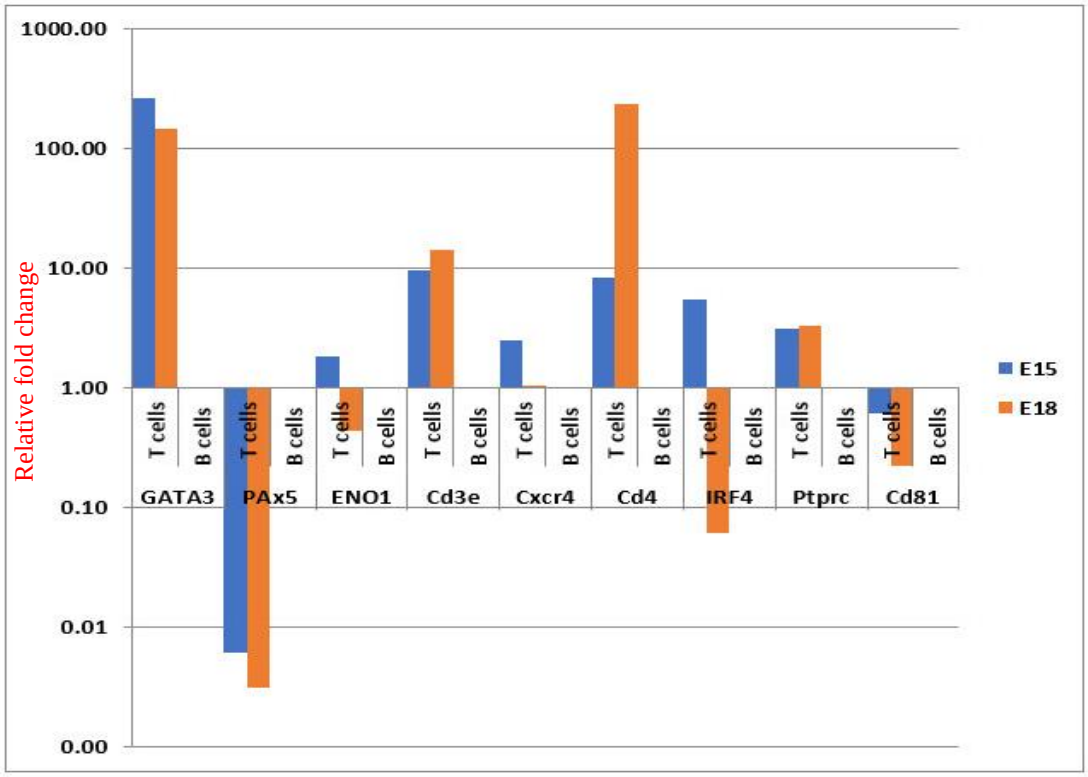

Figure 5. Relative fold change in expression of *GATA3, Pax5, ENO1, CD3e, Cxcr4, CD4, IRF4, Ptprc*, and *CD81* in sorted T-cells from thymus of broiler chickens relative to their expression in sorted B-cells lymphocytes from bursa of Fabricius of broiler chickens at two embryonic stages (E15 and E18) using quantitative real-time PCR

DISCUSSION

Semi-quantitative real-time PCR analysis was used to identify global expression patterns across developmental stages in the key immune organs of avian, the thymus, and the bursa of Fabricius, which represent major repertoires of T- and B-cells, respectively. Changes in gene expression during lymphocyte and immune organ development were profiled from embryonic stages (E15 and E18) to post-hatched stages (D3, D7, D14, and D28). *CD3e* and *GATA3* **(**Figure 6**)** were highly expressed in both thymus and sorted T-cells (Figure 5), compared to the bursa of Fabricius and sorted B-cells. Other genes, such as *Pax5* and *EBF1* (
Figure 7), were highly expressed in the bursa of Fabricius and sorted B-cells (
Figure 5), compared to their expression in the thymus or sorted T-cells. T-cells development is influenced by *Notch* signaling, which acts as a positive regulator of *GATA-3* and *TCF-1* expression to enhance T-cell proliferation and survival (Sambandam et al., 2005; Hozumi et al., 2008). *GATA-3* expression levels significantly decreased by D28 of development, compared to E15 (Figure 6). *GATA-3* exerts a dose-dependent effect on T-cell lineages, as high levels of *GATA-3* expression can suppress T-cell differentiation (Scripture-Adams et al., 2014). This suppression occurs by inhibiting *IL7r* and *TCH-1*, especially when *Notch* signaling is blocked (Rothenberg and Scripture-Adams, 2008; Scripture-Adams et al., 2014). *GATA-3* can also alter precursor cell fate, leading to mast cell differentiation (Taghon et al., 2007).

The functional and pathway analysis (Table 1 and Figure 6) suggest that interaction between T- and B-cell lineage genes may help maintain specific cell fates. Pu.1 (SPI1) is a known member of the ETS family of transcription factors and has a dose-dependent synergistic effect on B and T lymphocytes binding from CLPs (Anderson et al., 2002; Back et al., 2005; Nutt et al., 2005; Carotta et al., 2014). The B-cell maturation requires high levels of PU.1 (SPI1) to maintain B-cell development, whereas, in T-cells, PU.1 (SPI1) expression increases *GATA-3* levels during the early stages of T-cell development to maintain the CLPs commitment to T-cell fate (Rothenberg and Scripture-Adams, 2008; Real and Rothenberg, 2013). *EBF1* and *Pax5*, two key regulators of B-cell development, are critical for mediating and

maintaining B-cell fate under restricted conditions via *IL-7R* signaling during the early stages of CLP commitment (Åhsberg et al., 2013). A positive feedback interaction between *EBF1* and *Pax5* (Figure 7) blocks B-cell lineage differentiation and development and maintains B-cell identity (Decker et al., 2009; Lin et al., 2010; Mansson et al., 2012).

In the present study, the expression of *EBF1* and *Pax5* (Figure 7) gradually increased, reaching their highest expression in the bursa of Fabricius (B-lymphocyte repertoire) at D28 and D14, respectively. The role of *Pax5* in the commitment of hematopoietic cells-to B-cell fate is elucidated in a study using *Pax5*-deficient pro B-cells, which cannot fully commit to B-cells. However, these cells can maintain the same level of B-lineage gene expression if they are kept in a rich media with *IL-7* (Kikuchi et al., 2008). These pro-B-cells with knocked-out *Pax5* can switch to myeloid cell and dendritic cell fates upon removal of *IL-7* (Dias et al., 2005; Kikuchi et al., 2008) and if they are introduced to Notch signals they can deviate toward a T-cell fate (Nutt et al., 1999; Rolink et al., 1999). These studies illustrate the effect of *Pax5* in repressing *Notch* signaling to shut down T-cell lineage genes and maintain B-cell fates (Souabni et al., 2002). Furthermore, knockout *Pax5* in B-cell precursors can inhibit T-cell development completely in the presence of activated *EBF1* (Pongubala et al., 2008). While *EBF1-deficient* pro-B-cells with normal expression of *Pax5* can shift toward the T-cell pathway (Banerjee et al., 2013; Nechanitzky et al., 2013; Turner et al., 2020).

A study showed that *EBF1* blocks T-cell lineage genes by directly binding to *TCF-1* as well as two sites upstream of the transcriptional start site of *GATA-3*, leading to the repression of those genes critical in the determination of T-cell fate (Banerjee et al., 2013). Together, these studies indicate the vital roles of both *EBF1* and *Pax5* in determining B-cell precursor fate by acting as negative regulators for T-cell lineage genes to prevent the progenitor cells from deviating toward the T-cell pathway (Nutt et al., 1999; Rolink et al., 1999; Zandi et al., 2008; Nechanitzky et al., 2013).In this study, the expression of *EBF1* was significantly higher at E15 (Figure 7) in the thymus, compared to the other five stages of development (E18, D3, D7, D14, and D28), suggesting it may also contribute to adjusting the level of *GATA-3* in the thymus.

The interaction between the network of transcription factors outlined above contributes to determining immune cell fate. However, the diverse combinations of transcription factors and their dose-dependent effect leading to the activation or repression of certain cell types make it more important to profile their expression during different stages of development in immune tissues and in purified immune cell populations (Figure 5). More genome-wide studies need to be conducted to clarify these factors' functional mechanisms and their involvement during development. Moreover, the expression profile data can reveal the global effect of these factors and determine the relationship between them and other external signals. These results enrich the understanding of the expression of different key regulatory elements across the developmental stages in two of the primary avian immune organs (thymus and bursa of Fabricius) as well as sorted T- and B-cells. The findings further the understanding of the general gene expression profiles during vertebrate immune development and reveal a clearer picture of cell fate commitments during avian immune cell development.

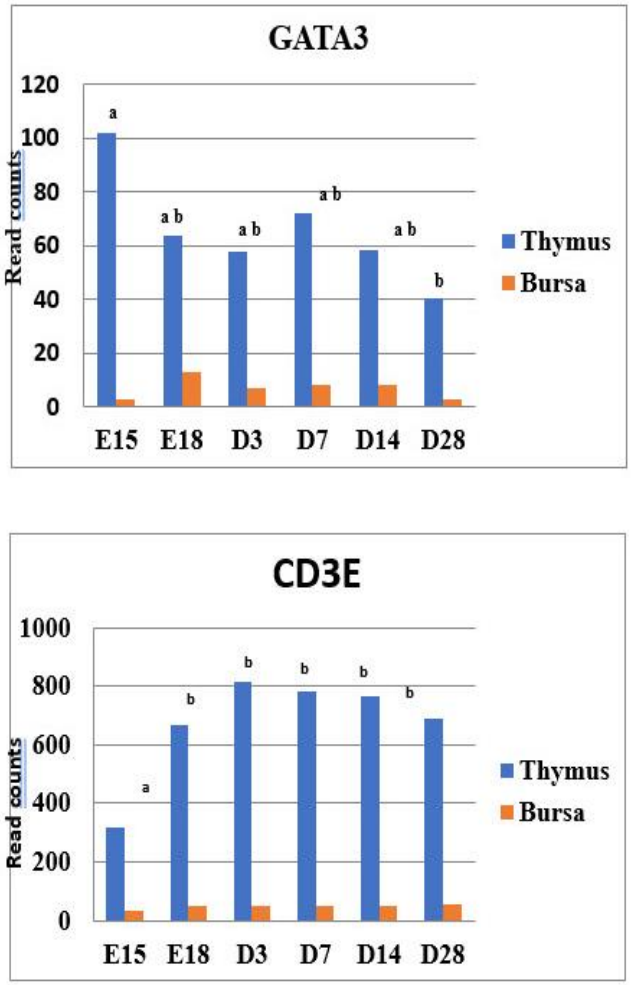

Figure 6. Expression of T-cell lineage genes; GATA3, CD3E identified in the bursa of Fabricius and thymus of broiler chickens throughout development, during the

embryonic stage (E15 and E 18) and the post-hatch days (D3, D7, D14, and D28).

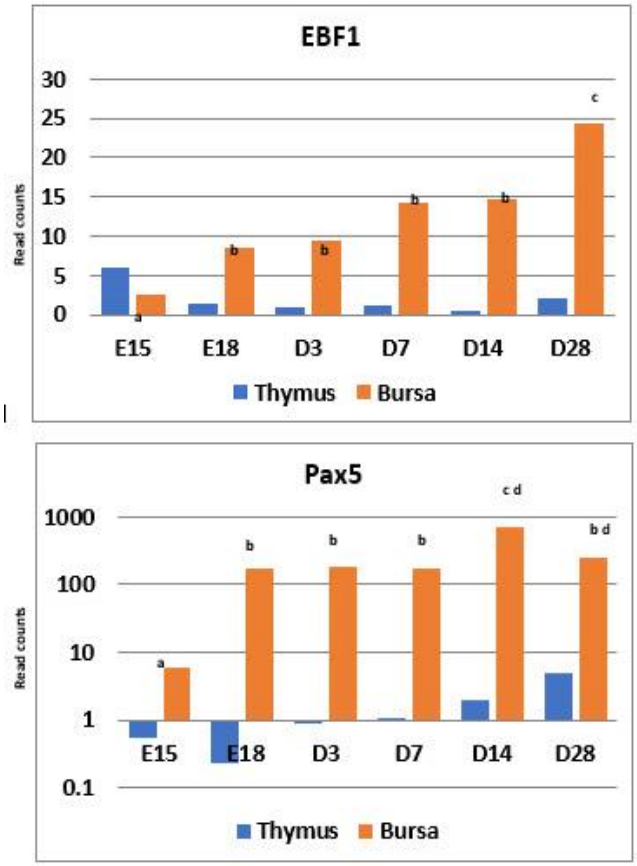

Figure 7. Expression of B-cell lineage genes; *Pax5* and *EBF1* identified in the bursa of Fabricius and thymus of broiler chickens throughout development, during the embryonic stage (E15 and E 18) and the post-hatch days (D3, D7, D14, and D28).

CONCLUSION

The results indicated that regulatory elements (*EBF1, Pax5, GATA3,* and *TCF1*) play critical roles in the development of the avian immune system, exhibiting dynamic expression patterns throughout the maturation stages. These conserved functions suggest their importance in controlling the T- and B-cell development across species. The results also highlighted the role of regulatory elements in avian lymphocyte maturation and lineage coupling and their importance in biochemical regulation. This knowledge has implications for the study of human immune-related diseases and the development of therapeutic strategies. Further research efforts will improve the understanding of the mechanisms by which these factors control lineage linkage in chickens and interact with other transcriptional regulators. Further studies are required to understand the transcriptional regulatory networks and post-transcriptional regulation of these genes to build a solid understanding of the lymphocyte developmental programs in birds.

DECLARATIONS

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Availability of data and materials

The datasets generated during and/or analyzed during the current study are available in the supplementary material file submitted and from the corresponding author on reasonable request.

Authors' contributions

The data curation was conducted by M. N. Goher. and M. A. El-Menawey while the statistical analysis was carried out by F. K. R. Stino, and M. N. Goher. The investigation was carried out by M. N. Goher, Reda E. A. Moghaieb, F. K. R. Stino. The methodology was performed by M. N. Goher, Reda E. A. Moghaieb, M. A. El-Menawey, and F. K. R. Stino. The project administration and validation were carried out by F. K. R. Stino and M. N. Goher. The whole visualization and writing were drafted by M. N. Goher. The writing, review, and editing of the manuscript were done by M. N. Goher, Reda E. A. Moghaieb, M. A. El-Menawey, and F. K. R. Stino. All authors have read and agreed to the published version of the manuscript.

Competing interests

The authors declare that there is no conflict of interest in the outcome of this research.

Ethical considerations

Ethical issues (including plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy) were checked by the authors before the submission.

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Production Performance and Profitability of Small-scale Commercial Poultry Farms in Arsi and East-Showa Zones, Central Ethiopia

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ABSTRACT

Poultry farming and the demand for chicken meat and eggs are growing due to their quality protein, essential nutrients, affordable price, and low investment cost. The current study was designed to investigate the productivity and profitability of small-scale commercial poultry enterprises. Data were collected from 221 poultry farms using a semi-structured questionnaire. The results were presented using descriptive statistics. Farm performances were measured by total-factor-productivity (TFP), benefit-cost-ratio (BCR), and net-profit-margin (NPM). The majority of the poultry farm owners were male (69.68%) and married (77.4%). Around 55.2% of poultry farmers aged 31-45 years. Group-owned farms had 4.59 ± 1.77 individual members. On average, a batch of layer chickens was kept for 17.0 ± 3.87 and 18.12 ± 4.25 months on individual and group-owned farms, respectively. Moreover, the mean selling ages of pullet and broiler chickens were 2.55 ± 0.71 and 2.18 ± 0.51 months, respectively. The flock size of layer chickens in sole-proprietor farms (562 ± 724) was significantly lower than in partnership farms (1165 ± 877). The average numbers of produced pullets by sole-proprietorship and partnership farms were 3177 ± 2360 and 3137 ± 1826, while the mean numbers of broilers produced were 2257 ± 1875 and 3269 ± 1669, respectively. The average egg and broiler weights in sole-proprietorship farms were significantly higher compared to group-owned farms. Annual egg production rates in individual and group-owned farms were 76.5% and 70.4%, respectively. The cost of feed, chicken, housing, labor, and medication were the top five production expenditures, while feed cost shares 60.4%. Egg producers had NPM, BCR, and TFP indices of 38.99%, 1.03, and 2.03, respectively, with notable differences by ownership types. The NPM, BCR, and TFP indices for broiler and pullet farms, respectively, were 42.78%, 0.93, and 1.93 for broilers and 35.21%, 0.92, and 1.67 for pullets. The results indicated that poultry firms performed optimally, regardless of ownership type. To further improve the efficiency and profitability of poultry enterprises, farmers need technical, finance, and management skills and input supply chains.

Keywords: Broiler, Layer, Poultry, Production, Profitability, Pullet

INTRODUCTION

Livestock makes an essential contribution to global calorie and protein supplies. According to some projections, average global animal protein consumption is expected to increase by 14% by 2030, compared to the base period average of 2018-2020, mainly due to income and population growth. By 2030, it is anticipated that the availability of protein from beef, pork, poultry, and sheep meat will increase by 5.9%, 13.1%, 17.8%, and 15.7%, respectively (OECD/FAO,2021). Globally meat consumption has been shifting toward poultry sources. Meat from poultry accounts for about 33% of global meat consumption and is expected to grow by 2 to 3% per year in the world (Teshome et al., 2019). This is due to poultry's cheaper cost, compared to other meats in low-income developing nations, while white meat consumption has increased in high-income nations due to its simplicity of preparation as a healthy food option (OECD/FAO, 2021). In 2030, poultry meat is anticipated to account for 41% of all the protein from meat sources globally, an increase of 2% from the baseline (OECD/FAO, 2021). In Ethiopia, chicken is the dominant type of poultry kept under the traditional production system with a population

of around 57 million chickens, of which 78.85% are indigenous, 12.02% are hybrid, and 9.11% are exotic breeds (CSA, 2021). The national mean annual egg production of Ethiopia was about 369 million in the year 2020 (CSA, 2021). In the year 2016, Ethiopia's poultry meat production reached 13,000 tonnes, constituting 2%, 0.2%, and 0.01% of the total poultry meat production in East Africa, Africa, and the world, respectively. Additionally, Ethiopia's share in egg production rates, when compared to East Africa, Africa, and the world, stood at 11%, 1.7%, and 0.07%, respectively (FAO, 2019).

The traditional family poultry production system is the dominant production system in Ethiopia and has served households as a source of income, nutrients, employment, and as a means of empowering women (Habte et al., 2017). To transform such a production system, the country has promoted small, medium, and large-scale commercial poultry production that involves unemployed youth, women, and entrepreneurs by providing some financial support to make use of the sector for wider job opportunities, food security, and economic values through the promotion of micro and small enterprises (MSE) (Ayele, 2018; Endris and Kassegn, 2022). Such agribusiness interventions contribute to broad economic growth, generating new employment, alleviating poverty, enhancing competition and entrepreneurship, empowering women, and strengthening nutritional status in most countries (Agyapong, 2010; Khatun et al., 2016; Tarfasa et al., 2016). In Ethiopia, the sector contributes 3.4% to the gross domestic product (GDP) and accounts for 90% of the workforce (Gebrehiwot and Wolday, 2006), while in Kenya MSEs contribute 40% to the GDP and account for 80% of the workforce; (Mwarari and Ngugi, 2013; Muriithi, 2017). Despite such initiatives, in Ethiopia, among commercial poultry farms, the number of large-scale commercial poultry farms with flock sizes of > 10000 chickens was small, compared to the country's potential (Woldegiorgiss et al., 2017). The combined number of large-scale and medium-scale commercial poultry farms in Ethiopia was reported as nearly 15 farms (Vernooij et al., 2012). On the other hand, the flock size of small-scale and medium-scale commercial poultry farms in Ethiopia was reported to be < 1000 and between 1000 and 10,000 chickens, respectively, owned either as sole proprietorships or partnerships (Tirfie, 2021).

In Ethiopia, a significant number of micro-and small-scale enterprises are engaged in poultry enterprises, mainly in egg, broiler, and pullet production. However, the majority of these small-scale enterprises who engaged in poultry businesses were not competent and sustainable in their production and productivity; they failed to thrive and meet expectations. Many SMEs in Africa confront a variety of obstacles, including a lack of electricity, finance, weak management skills and competencies, insufficient information, and corruption (Muriithi, 2017). To make such small-scale poultry enterprises the engine of economic growth and a means of poverty alleviation and unemployment reduction, it is very essential to know what factors dictate performance, growth, investment, and sustainability. Therefore, the current study was designed to investigate the poultry production practices and profitability of individual and group-owned small-scale commercial poultry farms in Ethiopia.

MATERIALS AND METHODS

The research was carried out in the Arsi and East Showa zones of the Oromia regional state, which is located in central Ethiopia. Tiyo, Dodota, and Hetosa districts in Arsi zone and Adama, Bishoftu, and Boset districts in East Showa zone were selected for the study. The study areas were an ideal location for poultry farming, due to their market opportunity, proximity to Addis Ababa, favorable weather conditions, and access to road and extension services.

Study participants

Small-scale commercial poultry farms owned by groups and individuals were included in the research. First, a list of poultry farms in the six study districts was collected from the respective study districts' livestock and fishery offices. Then, the farms were segregated into sole-proprietorship and partnership based on ownership type. A total of 221 poultry farms that were actively running their businesses and raising improved commercial breeds of layer and broiler chickens were sampled; of these, 122 were owned by individuals (sole-proprietorship) and 99 were owned by groups (partnership) either in the form of micro-and small-scale enterprises or cooperatives. Finally, the farms were regrouped into three production farms depending on the type of chicken reared (layer, broiler, and pullet). As a result, 132 layers, 56 pullets, and 33 broilers chicken-producing farms were sampled. Accordingly, 221 farm managers and/or owners were selected as research participants based on their willingness.

Methods of data collection

A descriptive research design was employed in this study using cross-sectional survey techniques to collect accurate and comprehensive data on the characteristics,

production, productivity, and profitability of commercial poultry farms. The data was collected using a semi-structured questionnaire prepared for this purpose after pre-testing the tool for its suitability for the selected farms. Pre-scheduled one-to-one interview was administered with farm owners or managers at the farm gate after a comprehensive discussion with the owners on the purpose, objectives of the study, and confidentiality of the information and data. The interview was conducted after obtaining informed consent from the participant farmers. Then one respondent from each poultry farm either owner or manager was interviewed based on their availability. Accordingly, data regarding; socio-economics, farm characteristics, farm management practices, feeding and feed resources, disease management, mortality, housing, production, productivity, cost of production, marketing, finance, and farm performances were collected through the interview process.

Farm budget model

The farm budget model adopted for this study was the costs and returns analysis. Indicators, such as net farm income, percentage profit margin, and benefit-cost ratio, were analyzed (Onuwa, 2022).

$$\text{Net farm income (NFI)} = \text{GFI} - \text{TC}$$

Where, GFI is Gross farm income and TC denotes Total cost.

Total cost (TC) is mathematically expressed as

$$\text{TC} = \text{TVC} + \text{TFC}$$

Where, TVC is the total variable cost which includes feed, medication, labor, chicken, litter costs, and cost of chicken loss, and TFC signifies the total fixed cost which includes the cost of housing rent. To further validate the profitability of this enterprise, profitability ratios, such as percentage of profit margin/net profit margin (PPM/NPM) and benefit-cost ratio (BCR), were analyzed using the following mathematical equations.

$$\text{PPM} = \frac{\text{NFI}}{\text{TR}} \times 100$$

Where, PPM defines the percent profit ratio, NFI is net farm income, and TR determines Total revenue.

$$\text{BCR} = \frac{\text{NFI}}{\text{TC}}$$

Where, BCR refers to the benefit-cost ratio, and TC is the total cost.

Total factor productivity

Total factor productivity is a method of calculating agricultural productivity by comparing an index of agricultural inputs to an index of outputs (Bamidele et al., 2008;Onuwa, 2022), as indicated in the following equation.

$$\text{TFP/TVC} = \text{Y}/\text{TVC} = \text{Y}/\sum \text{PiXi}$$

Where, Y is the quantity of output, TFP denotes total factor productivity, TVC refers to the total variable cost, Pi is the unit price of the i^{th} variable input, and Xi signifies the quantity of i^{th} variable input. The interpretations of the TFP index are given as < 1.0 for Sub-optimal, 1.0-1.09 for Optimal, and ≥ 1.10 for super-optimal according to (Bamidele et al., 2008).

Data analysis

Descriptive statistics, t-test, total-factor-productivity (TFP), BCR, and NPM were used to analyze the primary data. Descriptive statistics, such as mean, standard deviations, percentages, frequency counts, and graphs were used to present farm characteristics and husbandry practices. The TFP analysis was used for broiler, layer, and pullet-producing farm enterprises, to independently estimate agricultural productivity by comparing an index of agricultural inputs to an index of outputs. To analyze the costs, returns, and profitability of chicken production enterprises in the area, the farm budget technique (costs and returns analysis) was utilized. A t-test using SPSS version 26 was used to determine whether the difference between the means of individual-owned farms and group-owned farms is statistically significant or not at the 5% significance level.

RESULTS

Socio-economic characteristics

Evidence in Table 1 demonstrated that men (69.68%) made up the majority of the poultry farms' managers. The data showed that 46.7% of privately owned poultry farms and 33.3% of collectively held poultry farms had managers with lower-level educations. Regarding farm managers' previous poultry production experience, 18% of privately owned farms and 21.2% of farms owned by partnerships had no prior experience.

Farm characteristics

Among the total farms considered in this study, 59.7% were egg-producing farms, while 25.3% and 14.9% were pullet and broiler-producing farms, respectively. The age of the farms varies substantially depending on ownership type; the mean ages of the farms were 4.08 ± 1.55 years for sole-proprietor farms and 3.61 ± 1.55 years for farms owned in partnership. The study indicated that the members of partnership-based poultry enterprises range from 3-12 people with a mean value of 4.59 ± 1.77. Farm managers of sole-proprietorship farms significantly spent less time than farm managers of partnership farms

on a daily basis in terms of hours. The number of production cycles, age of production of different groups of chicken (layers, pullets, and broilers), daily labor consumption for layers, pullets, and broilers, and time spent on the farm by managers are presented in Table 2.

Flock sizes of the farms

According to the study, farms owned in partnerships often have larger flock sizes than farms operated as privately held businesses. The annual flock size of the farms was significantly different with the nature of their business ($p < 0.05$), where egg-producing farms had less flock size than broiler and pullet-producing farms. The study showed that group-owned farms had a significantly higher number of layers compared to privately owned farms ($p < 0.05$).

Table 1. Socio-economic profile of respondents in selected districts of Arsi and East-Showa zones in 2022 (n=221)

Criteria	Category	Individual-owned farms		Group-owned farms		Total	
		Number	Percentage	Number	Percentage	Number	Percentage
Sex of respondent	Male	85	69.7	69	69.7	154	69.68
	Female	37	30.3	30	30.3	67	30.32
Age of the respondent	15-30	23	18.9	29	29.3	52	23.5
	31-45	77	63.1	45	45.5	122	55.2
	46-60	22	18.0	25	25.3	47	21.3
Marital status of respondents	Married	91	74.6	80	80.8	171	77.4
	Unmarried	25	20.5	14	35.9	39	17.6
	Divorced	6	4.9	5	5.1	11	5.0
Previous chicken production experience	≤ 1 year	47	38.5	33	33.3	80	36.2
	2-3 years	44	36.1	35	35.4	79	35.7
	Above 3 years	9	7.4	10	10.1	19	8.6
	No experiences	22	18.0	21	21.2	43	19.5
Educational level of the respondents	Illiterate	14	11.5	12	12.1	26	11.8
	Grade 1-8	57	46.7	33	33.3	90	40.7
	Grade 9-12	34	27.9	40	40.4	74	33.5
	Diploma and above	17	13.9	14	14.1	31	14.0

Table 2. Farm characteristics by type of ownership in selected districts of Arsi and East-Showa zones in 2022

Variables	Measurements	Individual-owned farms	Group-owned farms	Total farms	P-value
Age of layers for one production phase (month)	Mean ± SD	17.0 ± 3.87[a]	18.12 ± 4.25[a]	17.43 ± 4.04	0.12
	Min-Max	10 - 26	12 - 24	10 - 26	
Production cycle of pullets per year	Mean ± SD	3.24 ± 0.50[a]	3.13 ± 0.76[a]	3.20 ± 0.62	0.51
	Min-Max	2 - 4	2 - 6	2 - 6	
Age of pullet (month)	Mean ± SD	2.53 ± 0.76[a]	2.58 ± 0.64[a]	2.55 ± 0.71	0.77
	Min-Max	1.5 - 4	1.5 - 4	1.5 - 4	
Production cycle of broilers per year	Mean ± SD	2.71 ± 0.49[a]	2.88 ± 0.91[a]	2.85 ± 0.83	0.64
	Min-Max	2 - 3	2 - 5	2 - 5	
Age of broiler (month)	Mean ± SD	2.14 ± 0.48[a]	2.19 ± 0.53[a]	2.18 ± 0.51	0.83
	Min-Max	1.5 - 3	1.5 - 3	1.5 - 3	
Age of poultry enterprises in years	Mean ± SD	4.08 ± 1.55[a]	3.61 ± 1.55[b]	3.87 ± 1.57	0.03
	Min-Max	2 - 8	1 - 7	1 - 8	
Number of labors /days for layer	Mean ± SD	1.31 ± 0.54[a]	1.52 ± 0.84[a]	1.39 ± 0.67	0.08
	Min-Max	1 - 3	1 - 6	1 - 6	
Number of labors /days for broiler	Mean ± SD	1.71 ± 0.95[a]	1.65 ± 0.85[a]	1.67 ± 0.85	0.87
	Min-Max	1 - 3	1 - 4	1 - 4	
Number of labors /days for pullet	Mean ± SD	1.65 ± 0.96[a]	1.48 ± 0.51[a]	1.59 ± 0.80	0.39
	Min-Max	1 - 6	1 - 2	1 - 6	
Time spent on the farm by managers in hours per day	Mean ± SD	2.92 ± 1.78[a]	4.55 ± 2.60[b]	3.65 ± 2.33	0.01
	Min-Max	0 - 8	1 - 12	0 - 12	

Means with [a, b] different superscript in the same raw indicate significantly at $p < 0.05$; SD: Standard deviation, Min: Minimum, Max: Maximum

Table 3. Flock sizes of different farms by chicken types in selected districts of Arsi and East-Showa zones in 2022

Variables	Measurements	Individual-owned farms	Group-owned farms	Total farms	P-value
Number of layers per year	Mean ± SD	561.88 ± 723.94[a]	1165.22 ± 876.83[b]	790.42 ± 835.43	0.01
	Min-Max	100-3000	100-4000	100-4000	
Number of broilers produced per year	Mean ± SD	2257.1 ± 1875.2[a]	3268.9 ± 1669.4[a]	3054.3 ± 1735.8	0.18
	Min-Max	1000-5000	1000-6000	1000-6000	
Number of pullets produced per year	Mean ± SD	3176.7 ± 2360.2[a]	3136.9 ± 1825.6[a]	3160.4 ± 2138.8	0.95
	Min-Max	900-9000	1200-6000	900-9000	
Weight of pullets (kg)	Mean ± SD	1.52 ± 0.17	1.47 ± 0.23	1.49 ± 0.21	0.69
	Min-Max	1 - 1.7	1.2 - 2	1 - 2	
Weight of broilers (kg)	Mean ± SD	2.55 ± 0.33[a]	2.23 ± 0.27[b]	2.28 ± 0.30	0.04
	Min-Max	2.2 - 3	2 - 3	2 - 3	
Egg production per year	Mean ± SD	154,802[a] ± 23411	295,307[b] ± 30839	208024 ± 19512	0.01
	Min-Max	20000-800000	27000-820000	20000-820000	
Egg weight (gm)	Mean ± SD	60.21 ± 8.72[a]	54.00 ± 10.04[b]	58.27 ± 9.50	0.03
	Min-Max	45 -75	40-75	40-75	

Means with [a, b] different superscript in the same raw indicate significantly at $p < 0.05$; SD: Standard deviation, Min: Minimum, Max: Maximum

Management practices of farms

The study revealed that, at the farm gate, the mean weights for broilers, pullets, and eggs were 2.28 ± 0.30 kg, 1.49 ± 0.21 kg, and 58.27 ± 9.50 gm, respectively. Notably, there were significant differences ($p < 0.05$) in the weights of eggs and broilers between individual and group-owned farms (Table 3). The mean weight of pullets in individual-owned farms (1.52 ± 0.17 kg) and group-owned farms (1.47 ± 0.23 kg) was not statistically different between the two groups ($p > 0.05$).

According to the survey, the most common forms of financing for most poultry farms were credit from a public institute (39%) and self-finance (28%), as shown in Figure 1. The findings revealed that there were unexpected deaths and culling of hens for various reasons, such as disease and fear of disease outbreaks in farms. In the case of unanticipated chicken culling, there were many intervention measures to handle the situation. The most important of which was the selling of hens for meat purposes (81%) at reduced prices (Figure 2).

The study indicated that the major sources of chicken for small-scale poultry businesses in the area were large-scale commercial poultry-producing companies (Alema, Ethio-chicken, Genesis, Golden, and ELFORA) farms (68%), as indicated in Figure 3. The contribution and share of each large commercial farm in supplying day-old chickens and pullets to chicken farms are presented in Figure 4.

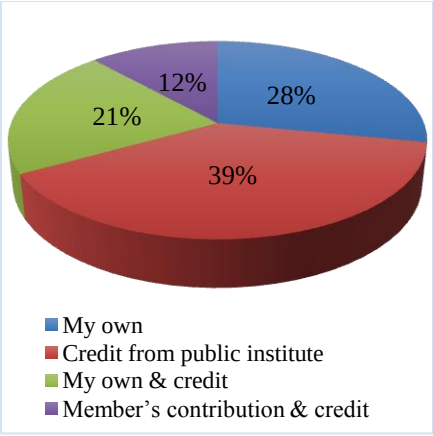


Figure 1. Initial finance sources of the farms in Arsi and East-Showa zones in 2022 during establishment.

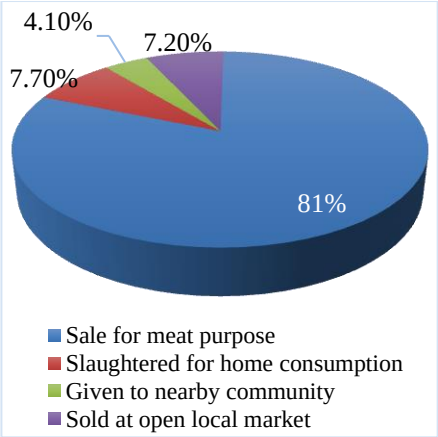


Figure 2. Actions taken on chickens culled unexpectedly by farm owners in Arsi and East-Showa zones in 2022

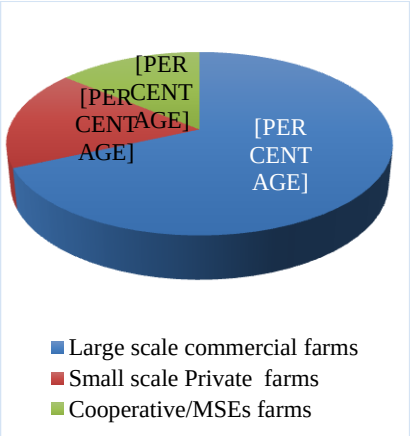
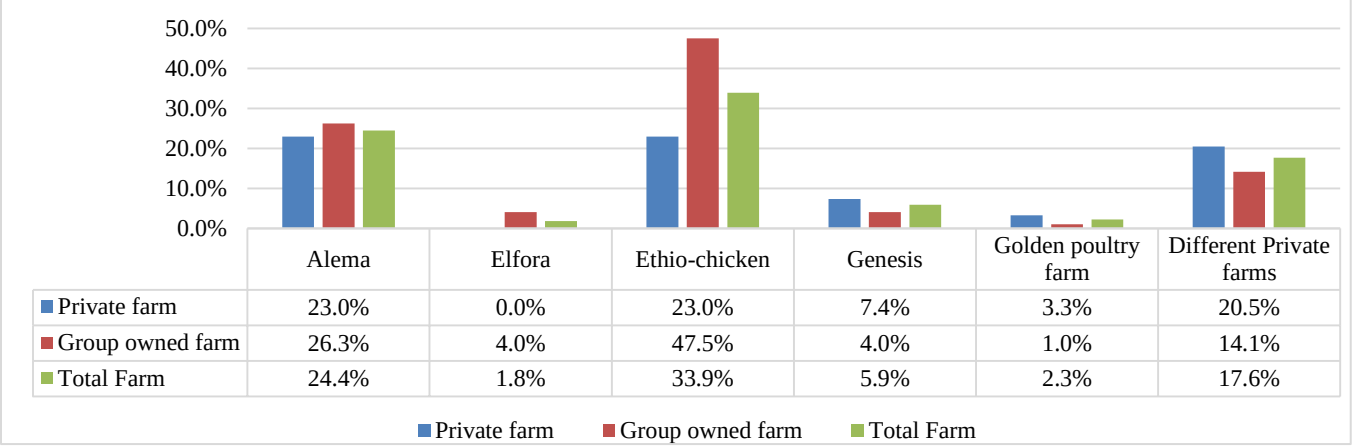


Figure 3. Type of farms that serve as a source of chickens for small-scale farms in Arsi and East-Showa zones in 2022



	Alema	Elfora	Ethio-chicken	Genesis	Golden poultry farm	Different Private farms
Private farm	23.0%	0.0%	23.0%	7.4%	3.3%	20.5%
Group owned farm	26.3%	4.0%	47.5%	4.0%	1.0%	14.1%
Total Farm	24.4%	1.8%	33.9%	5.9%	2.3%	17.6%

Figure 4. Large commercial farms that supply chickens for small poultry enterprises

Housing system and working premises

The current study found that the most common kind of poultry house was deep litter housing. Regardless of the kind of ownership, a negligible portion of farms grew chicken in cage systems, whereas 97.5% of farms owned as sole proprietorships and 93.9% of farms held as partnerships have been rearing their chicken in deep litter houses. Most farms (76%) used straw, and 19.9% utilized wooden shivering (sawdust) as a bedding material. Formalin (14%), Barakina/Sodium hypochlorite (23.5%) and both (60.6%) were used as disinfectants at the farm gate occasionally. According to the report, a sizable portion of farm owners do not have their own working spaces. Around 23% of sole-proprietorship and 56.6% of partnership farms have no own working space; instead, they utilize rented homes. The density of layer chickens per m^2 area differed significantly between individual and group-owned farms ($p < 0.05$); however, there was no significant difference in the density of pullet and broiler chicks based on ownership type ($p > 0.05$).

Feeding system and feed cost

The majority (97.05%) of respondents reported substantial feed price fluctuation throughout months and production cycles in the study region, independent of supplier type or market niche. The study confirmed that the major sources of feed for all types of farms were feed processing industries, retailing agents, both agents and industries, and homed-made feed resources as shown in Figure 5. Regarding the feeding frequency per day in each farm, the majority (75.10%) of the farms fed their chicken three times per day, though there was slight variability between the farms depending on the type of ownership as presented in Figure 6. The result indicated that daily feed provided in group and individual-owned farms was 90.86 ± 10.37 and 96.23 ± 15.56 grams per day per laying chicken, respectively, with a significant difference between the two farm ownership types ($p < 0.05$). The amount of feed, daily feed supply, and price of feeds in the study area are presented in Table 4.

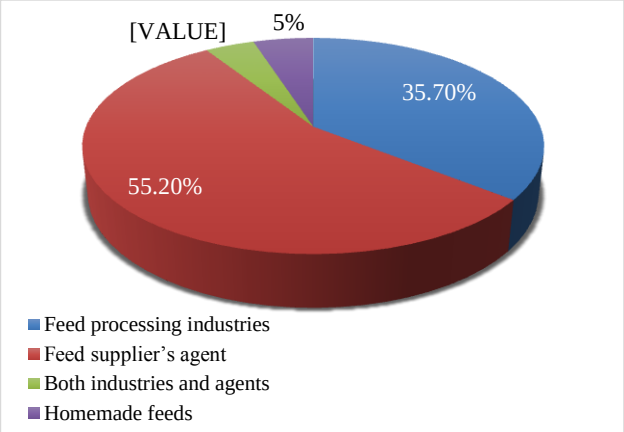


Figure 5. Sources of commercial feeds for farms in Arsi and East-Showa zones in 2022

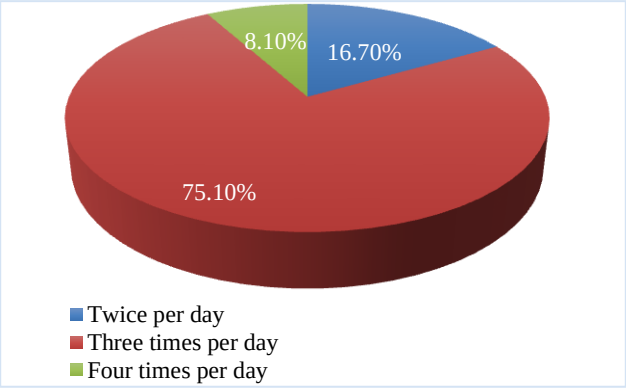


Figure 6. Frequency of feeding in each farm per day in Arsi and East-Showa zones in 2022

Table 4. Price of layer, broiler, and pullet feed and daily feed supply of farms in Arsi and East-Showa zones in 2022

Variables	Measurements	Individual-owned farms	Group-owned farms	Total farm business	P-value
Price of layer feed per quintal (Birr)	Mean ± SD	2697.99 ± 424.3[a]	2689.4 ± 429.6[a]	2694.73 ± 424.7	0.91
	Min-Max	1850 - 4000	1800 - 4000	1800 - 4000	
Price of starter feed per quintal (Birr)	Mean ± SD	2468.18 ± 441.38[a]	2418.7 ± 436.8[a]	2447.86 ± 436.21	0.68
	Min-Max	1750 - 3050	1750 - 3200	1750 - 3200	
Price of broiler feed per quintal (Birr)	Mean ± SD	2242.86 ± 544.23[a]	2530.31 ± 446.16[a]	2469.33 ± 474.65	0.16
	Min-Max	1500 - 3200	1800 - 3200	1500 - 3200	
Daily feed supply for pullets (gm)	Mean ± SD	60.78 ± 12.90[a]	59.09 ± 17.63[a]	60.09 ± 14.87	0.69
	Min-Max	40 - 85	30 - 100	30 - 100	
Daily feed supply for broilers (gm)	Mean ± SD	113.75 ± 15.98[a]	124.46 ± 35.70[a]	121.94 ± 32.27	0.42
	Min-Max	90 - 140	70 - 200	70 - 200	
Daily feed supply for layers (gm)	Mean ± SD	96.23 ± 15.56[a]	90.86 ± 10.37[b]	94.21 ± 14.04	0.03
	Min-Max	70 - 130	70 - 120	70 - 130	

Means with [a, b] different superscript in the same raw indicate significantly at $p < 0.05$; SD: Standard deviation, Min: Minimum, Max: Maximum

Production performances

According to the results of the current study, sole-proprietorship layer farms' egg productivity was 76.5%, producing close to 154,802 eggs annually, whereas egg productivity of group-owned farms was 70.4% with an annual egg output of 295,307 eggs, which is lower than that of individual-owned farms as evaluated by the hen day production index. The annual physical productions of eggs, pullets, and broilers by privately owned and group-owned farms are presented in Table 3. The study found that farm ownership type did not significantly affect the annual pullet and broiler chickens produced by the enterprises ($p < 0.05$).

Costs of production

In this study, both variable and fixed expenses were taken into account to gauge how well the farms were performing. Costs of feed, chicken, litter, labor, medicine, and chicken losses were taken as variable costs. The housing rental cost, which includes working facilities and land resources, was considered a fixed cost. The annual mean values of measurements of variable and fixed costs were presented in Table 5 based on the types of farms and kind of ownership. The average prices of layer, pullet, and broiler feed per quintal during the study period in the study area were 2694.73 ± 424.7 (1800-4000), 2447.86 ± 436.21 (1750-3200), and 2469.33 ± 474.65 (1500-3200) Ethiopian birr (EBR), respectively. Similarly, the mean daily feed supplies for layers, pullets, and broilers at the mid-age of the chickens in the area were 94.21 ± 14.04, 60.09 ± 14.87, and 121.94 ± 32.27 g, respectively. The study showed that high feed costs and price fluctuations over seasons were the major bottlenecks for chicken enterprises in the area. The total costs of production of different farm enterprises such as layer, broiler, and pullet were 781,828 ± 51,686; 664,374 ± 73,275, and 377,432 ± 28,490 EBR per year, respectively. The total cost of production in egg-producing farms was statistically different between individual and group-owned farms ($p < 0.05$), costing 635,343 ± 61,111 and 1,022,063 ± 82,673 EBR, respectively, due to the higher flock size in group-owned farms.

Profitability measurements

Gross income, total cost, and net income estimation for each farm business were used as the metrics by which the profitability of the farms was assessed. The gross revenue and net income in layer farms were statistically different ($p < 0.05$) between individual-owned farms (1,510,022 ± 234,955 and 874,679 ± 180,804) and group-owned farms (2,815,642 ± 317,386 and 1,793,579 ± 247,052) EBR, respectively. The result indicated that group-owned farms gain higher gross and net income due to the larger flock sizes of the farms. However, there was no statistically significant difference between individual and group-owned farms in the gross revenue and net income in broiler and pullet-producing enterprises ($p > 0.05$).

The gross income and net income of broiler-producing farms were 1,228,388 ± 129,510 and 564,014 ± 80,228 EBR, whereas, for pullet-producing farms, the gross and net income were 668,741 ± 64,534 and 291,309 ± 38,945 EBR, respectively (Table 6). The total factor productivity (TFP) technique compares an index of agricultural inputs to an index of outputs to determine agricultural productivity. Between farms held as sole proprietorships and partnerships, there were statistically substantial differences in the TFP index, PPM, and BCR

of layer farms ($p < 0.05$). As indicated in Table 6, these productivity measuring variables were insignificantly different for farms producing broilers and pullets.

According to Bamidele et al. (2008), the TFP index value was interpreted as sub-optimal (< 1.0), optimal (1.0-1.09), and super-optimal (≥ 1.10). The study showed that 81.06%, 100%, 89.29%, and 86% of layer, broiler, pullet, and aggregate of all chicken farms, respectively, have TFP indices of ≥ 1.10, indicating that they are super-optimal farm enterprises in terms of productivity (Table 7).

Table 5. Mean annual costs of measurements of variable and fixed costs poultry farms in Arsi and East-Showa zones in 2022

Variables measured in Ethiopian Birr	Individual-owned farms		Group-owned farms		Total farm business		P-value
	Mean	SD	Mean	SD	Mean	SD	
Chicken cost of a layer farm	106375[a]	109982	186066[b]	123691	136561	121265	0.01
Chicken cost of a broiler farm	125429[a]	101459	170967[a]	93810	161307	95722	0.27
Chicken cost of a pullet farm	164748[a]	114827	156578[a]	88978	161393	104186	0.78
Feed cost of layer farm	470506[a]	440910	764976[b]	459378	582048	468718	0.00
Feed cost of a broiler farm	353571[a]	342812	459858[a]	331749	437313	331609	0.64
Feed cost of a pullet farm	149395[a]	107421	164066[a]	130065	155421	116338	0.65
Medication cost of a layer farm	2206[a]	1812	3318[b]	2858	2627	2319	0.01
Medication cost of a broiler farm	5071[a]	3735	6373[a]	4224	6097	4105	0.37
Medication cost of a pullet farm	6088[a]	5115	6152[a]	3641	6114	4531	0.98
Litter cost of a layer farm	913[a]	822	1284[b]	932	1054	881	0.02
Litter cost of a broiler farm	1278[a]	931	1404[a]	917	1371	908	0.73
Litter cost of a pullet farm	1247[a]	1116	1039[a]	832	1166	1012	0.45
Mortality cost of a layer farm	4031[a]	3566	4859[a]	3544	4344	3567	0.20
Mortality cost of a broiler farm	7243[a]	3535	10535[a]	8628	9836	7897	0.34
Mortality cost of a pullet farm	3236[a]	3506	4591[a]	4361	3793	3900	0.20
Labor cost of a layer farm	19273[a]	9685	23112[a]	15423	20727	12266	0.08
Labor cost of a broiler farm	9229[a]	5063	11950[a]	8468	11373	7880	0.43
Labor cost of a pullet farm	11900[a]	6753	13578[a]	6051	12589	6471	0.34
Rental cost of a layer farm	36687.8[a]	4462	38448.0[b]	5649.14	37354.55	4998.4	0.04
Rental cost of a broiler farm	37371.43[a]	6588.3	36507.69[a]	5958.32	36690.91	6000.22	0.74
Rental cost of a pullet farm	37345.45[a]	5838.51	36240.00[a]	5483.83	36891.43	5671.26	0.48
Total cost of a layer farm/year	635343[a]	61111	1022063[b]	82673	784716	51686	0.00
Total cost of a broiler farm/year	539414[a]	167822	698017[a]	81791	663988	73275	0.39
Total cost of a pullet farm/year	374077[a]	37620	382245[a]	44556	377367	28490	0.89

Means with different superscripts (a, b) in raw differ significantly at $p < 0.05$; SD: Standard deviation

Table 6. Gross revenue, net income, total factor productivity index, percent profit margin, and benefit-cost ratio of poultry farm enterprises in Arsi and East-Showa zones in 2022

Variables	Individual-owned farms		Group-owned farms		Total farms		P-value
	Mean	SD	Mean	SD	Mean	SD	
Gross income (egg)	1,510,022[a]	2,127604	2,815,642[b]	2,244,261	2,004,575	2255457	0.00
Gross income (broiler)	1,018,000[a]	930338	1,285,031[a]	696,444	1,228,388	743,982	0.41
Gross income (pullet)	669,455[a]	526,673	667,717[a]	423,767	668,741	482,928	0.99
Net income (layer)	874,679[a]	1637245	1,793,579[b]	1746925	1,222,747	1731806	0.00
Net income (broiler)	478,586[a]	534,487	587,013[a]	448,018	564,014	460,872	0.59
Net income (pullet)	295,378[a]	320,777	285,472[a]	250,211	291,309	291,435	0.90
PPM Egg producers	30.0[a]	26.23	53.75[b]	18.22	38.99	26.14	0.00
BCR of Egg producers	0.75[a]	0.96	1.48[b]	0.94	1.03	1.01	0.00
TFP index of layer farm	1.75[a]	0.96	2.48[b]	0.94	2.03	1.01	0.00
PPM of broiler producers	42.32[a]	12.98	42.91[a]	18.28	42.78	17.11	0.94
BCR of broiler producers	0.82[a]	0.45	0.96[a]	0.74	0.93	0.68	0.63
TFP index of broiler farm	1.82[a]	0.45	1.96[a]	0.74	1.93	0.68	0.63
PPM of pullet producers	35.17[a]	16.51	35.26[a]	21.09	35.21	18.34	0.99
BCR of pullet producers	0.64[a]	0.40	0.71[a]	0.55	0.66	0.46	0.57
TFP index of pullet farm	1.64[a]	0.40	1.71[a]	0.55	1.67	0.46	0.57
PPM all Chicken farms	32.09[a]	23.51	46.61[b]	20.27	38.59	23.22	0.00
BCR of all chicken farms	0.72[a]	0.81	1.17[b]	0.87	0.92	0.87	0.00
TFP index of all farms	1.72[a]	0.82	2.17[b]	0.87	1.92	0.87	0.00

TFP: Total factor productivity, PPM: Percent profit margin (ratio, net profit margin), and BCR: Benefit-cost ratio; Means with different superscripts (a, b) in raw differ significantly at $p < 0.05$; SD: Standard deviation.

Table 7. Total factor productivity index for all kinds of poultry farm enterprises in Arsi and East-Showa zones in 2022

Item	Category	Individual-owned farms		Group-owned farms		Total	
		Number	Percentage	Number	Percentage	Number	Percentage
TFP index of layer farm	< 1	10	12.20	1	2.0	11	8.33
	[1-1.09]	14	17.07	0	0.0	14	10.61
	≥ 1.10	58	70.73	49	98.0	107	81.06
TFP index of broiler farms	≥ 1.10	7	100.00	26	100.00	33	100.00
TFP index of pullet farm	< 1	0	0.00	1	4.35	1	1.8
	[1-1.09]	3	9.09	2	8.70	5	8.93
	≥ 1.10	30	90.91	20	86.96	50	89.29
TFP index of all farms	< 1	10	8.2	2	2.0	12	5.4
	[1-1.09]	17	13.9	2	2.0	19	8.6
	≥ 1.10	95	77.9	95	96.0	190	86.0

TFP: Total factor productivity, Number of observations

DISCUSSION

Socioeconomic factors

Numerous factors, including technical, farm management, institutional, socio-economic, climatic, and regulatory considerations, affect the productivity and profitability of agricultural enterprises (Kahan, 2013). These factors are very integrated in their effect, and the impact of some may be greater than others depending on the location, ownership, farm type, and other factors (Bamidele et al., 2008). The productivity of agricultural companies was impacted by education, experiences, technical know-how, and skill, which had an impact on technological adoption and management effectiveness (Bamidele et al., 2008; Ukoha et al., 2010). In Ethiopia, both individual and group-owned poultry farms are very common, where group poultry farming has recently developed in the form of cooperatives and micro-and small-scale enterprises to exploit the urban market (Ibrahim and Goshu, 2020). Group farming may be able to provide farmers with economies of scale, a reliable labor force, more investable capital and expertise, increased bargaining power in input and output markets, and improved integration with governmental and non-governmental organizations that provide technological assistance, training, and advice (Agarwal, 2018). The farm owners were all dominated by men which might be related to the financial capability of the males rather than the female counterparts and the socio-economic and cultural practices of the community that empower the males. A study conducted in Ethiopia reported that 86% of poultry producers were male farmers (Gemechu and Abiy, 2019).

The results of the current study showed that, in contrast to group-owned farms, those who are self-sufficient in terms of their means of subsistence tend to operate as private farmers. While the majority of those without jobs engaged in group farming. A higher proportion of individual farmers and employees engaged in poultry farming as sole-proprietorship, whereas the majority of unemployed communities engaged in partiner farming which is attributed to lack of start-up capital and other resource limitations. This is consistent with findings reported by Agarwal (2018) and Agarwal and Dorin (2019), who stated group farming helps to mobilize and pool resources to enhance the performances of agricultural enterprises.

Farm characteristics and husbandry practices

Regardless of the kind of ownership, the study found that layer-producing farms were the most common poultry enterprises in the study area when compared to pullet and broiler farms attributed to poor awareness of broiler farming, lack of broilers DOC supply, and poor preference of the communities to meat from improved breeds of broiler chickens compared to the indigenous breeds. A similar finding was reported elsewhere in Ethiopia, where layer farms were dominating (Amare and Tesfaye, 2020). It was reported that only 3% of the commercial farms in Ethiopia were broiler producers, while 74% were engaged in layer production (Woldegiorgiss et al., 2017). Poor market access, low product demand, limited supply of day-old pullets and broilers, expensive and high-standard management, and infrastructure requirements for pullet and broiler husbandry practices could all be contributing factors (Woldegiorgiss et al., 2017). The current study indicated that poultry farmers lack basic knowledge of poultry husbandry practices and the required inputs across the value chains. The other reasons for having a small number of broiler farms in Ethiopia were, that the market was concentrated in central Ethiopia around Mojo, Bishoftu, and Addis Ababa, and there were poor extension services in promoting broiler production across the

country by concerned offices, institutions, and extension workers. Amare and Tesfaye (2020) reported that a shortage of day-old chicken supply, high prices of feed, and a lack of land constrained small-and medium poultry production in Ethiopia.

The current study showed that the average age of layers, pullets, and broilers per single production cycle were 17.43 ± 4.04, 2.55 ± 0.71, and 2.18 ± 0.51 months, respectively, regardless of significant variability between sole-proprietor and partnership farms ($p > 0.05$). The age of layer chickens in the current study is in contrast to the report by Woldegiorgiss et al. (2017), who reported that 35% of the commercial layer producers in Ethiopia keep layers for 2 years. The result of the current study for the age of layers is inconsistent with the recommendations set by different breeders' guides (72-84 weeks) (Lohmann, 2021). A study in Cosovo southeast Europe reported that the age of layer chickens ranges from 13.5-15.5 months with egg productivity of 79.3-80.4% (Ymeri et al., 2017). As layer chickens get older, their feed conversion efficiency and egg quality decrease which affects the profitability of the enterprises (Woldegiorgiss et al., 2017). In the case of broiler-producing enterprises, the age of broiler chickens was longer than the average economically feasible age recommended as 38 to 47 days (Brown et al., 2009; Aviagen, 2018; Compassion in World Farming, 2019), incurring additional costs. The longer length of production implies the profitability of poultry enterprises (Brown et al., 2009; Aviagen, 2018). The longer age of broiler chickens in the current study might be because of poor quality husbandry practices implemented by farm operators in the study area. Regarding layer farms in the current study, the average annual egg productivity per hen and mean weight of a broiler chicken were 275 eggs and 2.55 kg in individual-owned farms and 253 eggs and 2.23 kg in group-owned farms. Global statistics showed that commercial layer breeds lay nearly 300 eggs per bird per year, while broiler chickens reach a body weight of 2 - 2.5 kg by 45 days of age (Habte et al., 2017).

The study found that the annual frequencies of production cycles for firms producing pullets and broilers were 3.20 ± 0.62 and 2.85 ± 0.83 times, respectively. A study conducted in India reported a mean annual number of production cycles per year of 6.27 and 5.32 in contract and non-contract broiler farms, respectively, and a production length of 40.5 days in contract farming and 45.40 days in non-contract broiler farms (Singh et al., 2018). The results indicated that increasing the frequency of the production cycle per year is important to maximize the profitability of farm enterprises. However, poultry farmers are concerned about the seasonal shift in weather conditions over a year, which results in a severe cold environment, a high prevalence of diseases, expensive feed, and a scarcity of feed resources. Poultry enterprises were challenged by disease and parasites, uncertainty and dynamic weather conditions, a low capital base, inefficient financial management practices, housing, and marketing constraints in general, which increased the farmers' fear (Folajinmi and Peter, 2020).

The flock sizes of privately owned and group-owned layer farms were 561.88 ± 723.94 and 1165.22 ± 876.83 chickens, where there was a significantly larger flock size in group-owned farms. Contrary to the current findings, a study conducted in Bishoftu area of Ethiopia reported a farm flock size of 2134 pullets and 2381 layers, in an intensive commercial farm (Ebsa et al., 2019). The flock size in the current study in individual-owned farms was lower because private farmers started their business with minimal start-up capital and on a small plot of land without substantial emphasis on flock size. In contrast, group-owned farms pay particular attention to determining the optimal flock size for their establishments. Accordingly, partnership farms mobilize their startup capital either from their members or through loans from financial institutions, and they attempt to have a larger farm size to begin their business with a larger flock size. The mean weight of eggs in private farms (60.21 ± 8.72 gm) was statistically higher than that of group-owned farms (54.00 ± 10.04 gm), which could be attributed to the more stringent management practices implemented and close follow-up made by individual farmers than group-owned farms. In agreement with the current finding, an average egg weight of 60 grams was reported from hens producing 250 eggs per year in Ethiopia (Mengesha et al., 2022). Management, nutrition, genetics, age, body weight, and lighting factors determine the weight of eggs produced in poultry farms.

The study indicated that the mean weights of broiler chickens produced by individual-owned farms and group-owned farms were 2.55 ± 0.33 and 2.23 ± 0.27 kg, respectively. The difference was statistically significant between the two ownership types, attributed to the higher commitment made in the management of the chickens and better competing determination by individual farmers than those farms owned in groups. Flock size and management intensity affect the productivity of the farms, by improving the efficiency of input costs (Kawsar et al., 2018; Khan and Afzal, 2018).

The majority of group-owned farms (56.6%) and a sizeable fraction of individually owned farms (23%) did

not have their own working premises; instead, they operated their poultry farming in rented farmsteads or housing. In the present study, a lack of land resources for poultry farmers has limited the use of existing potential and discouraged farmers' motivation and engagement in chicken farming. The limited availability of land inhibited the potential of chicken enterprises (Bao Truong et al., 2021). The density of layers per m^2 area in private farms and group-owned farms was 8.52 ± 3.53 and 10.45 ± 4.11, respectively. In contrast, the number of pullets and broilers per unit area at mid-age was 13.74 ± 5.32 and 9.41 ± 3.11, respectively. The result implies overstocking and overpopulation per unit area of land as a result of scarcity of land resources and an unpredictable land supply system. The density of the three groups of chickens in the current study was greater than the standard recommendations set by different guidelines. Reports in Ethiopia indicated that, though there are few feed processing plants around urban areas such as Addis Ababa and Bishoftu, the feed is often poor in quality, expensive in price, and not easily accessible for farmers at nearby as they are located at a distance from the farms (Sambo et al., 2015; Habte et al., 2017). The result implies that the daily feed provided for different classes of chickens was below the standard requirements of the chicken, which is attributed to a high feed price, seasonal fluctuations in feed costs, and a limited number of suppliers. Such a trend of feeding would affect farm productivity and gradually lead to the failure of farm enterprises. Rising costs of raw materials and a lack of raw resources are the reasons for the rising trend in feed prices.

Farm production and productivity

According to the results of the present study, sole-proprietorship layer farms' egg productivity and egg production were 76.5% and 154,802 eggs annually, whereas the productivity and production of group-owned farms were 70.4% and 295,307 eggs, as evaluated by the hen day production index. The productivity of an individual-owned farm was higher than that of a group-owned farm, which might be because of better management as the farm is private. A sole proprietorship is a business owned by a single person, who has complete control over the business, makes all important decisions, is responsible for all day-to-day activities, and uses personal skills to manage business affairs (Senevirathne, 2019; Santoso, 2020). However, the result confirmed that the physical eggs produced from group-owned farms were higher because of the larger number of chickens reared in group-owned farms.

The study findings showed that, among the production expenses, the cost of feed, chickens (day-old chickens and pullets), housing rent, labor, and medicine were the top five expenditures in that order, with feed cost being the highest. This is probably due to the absence of adequate commercial feed milling industries, sufficient commercial hatcheries that supply day-old chickens (DOCs), and limited access to all inputs at nearby in the study areas. A shortage of feed processing industries and a shortage of supply were reported as the main causes of high feed costs in Vietnam (Bao Truong et al., 2021). The study found that the cost of feed accounted for 60.4% (ranging from 41.2-74.2%) of total expenses, making it the primary source of financial concern. In relation to the overall farm costs, the feed cost shares in Pakistan (58.1 – 63.6%), Indonesia (70%), and Vietnam (49.5%) were nearly comparable to the results of the current study (Afzal and Khan, 2017; Coyne et al., 2020; Bao et al., 2021).

Gross and net revenues were statistically higher ($p < 0.05$) in group-owned farms than in individual-owned farms because of the larger flock size. The net profit of the chicken enterprises is maximized with increasing flock size, which is in line with the finding, which stated that financial profit generated from chicken farms generally increases with flock size (Bao Truong et al., 2021). According to Bamidele et al. (2008), TFP index values were interpreted as sub-optimal (< 1), optimal (1.0 –1.09), and super-optimal (≥ 1.10). Accordingly, the study revealed that 81.06%, 100%, and 89.29% of layer, broiler, and pullet farms, respectively, laid in a range of super-optimal (≥ 1.10) TFP index ratings, with only layer farms showing substantial variations by ownership type. This implied that most of the small-scale poultry (layer, broiler, and pullet) enterprises performed at a higher productivity index and have promising profitability.

The benefit-cost ratio (BCR) is a profitability metric used in cost-benefit analysis to assess the viability of cash flows produced by an asset or project. A BCR greater than or equal to one indicates that, when a project benefits are discounted at the opportunity cost of capital, they outweigh project costs, and its value reflects the efficiency of the project and indicates that the project evaluated is economically advantageous (Delp, 1977; Kahraman et al., 2000). In addition, the profitability ratio, known as NPM, compares net income to sales. It calculates the amount of net income or profit as a proportion of sales, describing how much of each dollar in sales a firm receives turns into profit. It is the ratio of net profits to revenues for a company or business segment, illustrating that a firm with a high NPM can generate high profits (Nariswari and

Nugraha, 2020). The higher the value of NPM, the better the profitability of the enterprise. Furthermore, the NPM ratio measures the efficiency of production, management, and tax administration (Setiadi et al., 2018). In light of this, the current study found that the NPM and BCR of farms that produced eggs varied significantly between the two ownership types ($p < 0.05$), with the values for individual-owned farms being 30% and 0.75 and for group-owned farms being 53.75% and 1.48. Such a result was indifferent to the study, which reported an absence of profitability differences between sole proprietors and partnerships in broiler farms (Khan and Afzal, 2018). However, under the two ownership types, the ratios indicated that the farms were generating high profits although there was a significant difference in their efficiency in earning profit. Group-owned farms were more efficient as their BCR was greater than one.

In the case of broiler and pullet-producing farms, both NPR and BCR were not statistically different by ownership type. The NPMs of broiler and pullet-producing farmers were 42.78% and 35.21%, respectively. Khan and Afzal (2018) reported that the profitability of broiler farms 'was not significantly different between sole-proprietorship and partnership, but the difference was significant between small and large flock-size farms. The BCR of broiler and pullet-producing farms were reported as 0.93 and 0.92, respectively. These values, being close to one, indicate that farmers derived substantial benefits from their respective poultry businesses. The results revealed that such firms were economically advantageous, but they should focus on improving production efficiency and cost management to boost the BCR above one. Such firms should focus on increasing flocks' size so that they maximize profitability with constant input costs. Smaller flock sizes in poultry farms and high mortality rates of chickens were important variables that determined the profitability and financial losses of farm enterprises (Bao Truong et al., 2021). When the profitability of the three enterprises in terms of NPM was compared, the broiler, layer, and pullet farms ranked first, second, and third, respectively. However, in terms of efficiency, layer, broiler, and pullet farms rank first, second, and third in their order. In order to guarantee ongoing efficiency and success in their businesses, farmers often need technical, marketing, and financial management knowledge and skills.

CONCLUSION

The current study confirmed the instability of small-scale commercial chicken production in the study area, where a large number of poultry farmers freeze their businesses after one or two production cycles due to dynamics in input supply and the high cost of inputs. The profitability of poultry farms was maximized in farms that had a larger flock size, regardless of the type of ownership. In terms of weight and egg productivity, sole-proprietor farms produce higher quality products such as eggs, pullets, and broilers than partnership farms. Majority of the chicken farms are profitable when evaluated in terms of total factor productivity, benefit-cost-ratio, and net-profit-margin. Lack of chicken supplies, high feed costs, lack of finance, high disease prevalence, and seasonal fluctuation of demand for poultry products are the determinants of productivity and profitability of small-scale commercial chicken enterprises in the study areas. To promote small-scale commercial poultry production and sustain its productivity and profitability, there should be an integrated strategy among government authorities, entrepreneurs, producers' associations, large commercial poultry-producing farms, feed milling industries, and financial institutions so that they contribute to better performances. Technological options and different intervention strategies should be studied under different farming systems in order to alleviate the major bottlenecks of the industry and maximize productivity and profitability.

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Authors' contributions

This research was conducted by the contribution of all the authors. Dereje Tsegaye designed, conceptualized, collected, and analyzed the data, and drafted the manuscript. Berhan Tamir validated the methodology and protocol, supervised the data collection, and edited the manuscript. Getachew Gebru validated the methodology, supervised the data collection and analysis, and revised the manuscript. All authors read and approved the final version of the manuscript for publication in the present journal.

Ethical considerations

All the authors in the current research have checked ethical issues like (plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double

publication and/or submission, and redundancy).

Availability of data and materials

The data is available with the corresponding author and it can be available based on requests.

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Growth Performance in Laying Ducks Fed Protein Diets Supplemented by Fresh Black Soldier Fly Larva

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ABSTRACT

Live black soldier fly (BSF) maggots serve as an alternative feed for ducks and other poultry, boasting a protein composition similar to fish meal. The current study aimed to evaluate the effect of live BSF maggot supplementation as a protein source on increasing duck daily production, feed conversion ratio, and egg quality. A total of 120 female Alabio ducks aged 7 months were randomly divided into 24 cages with four treatments and six replications. Each cage consisted of five ducks. Performance parameters such as daily egg production, feed conversion ratio, and egg mass production were evaluated, along with the quality of duck eggs, including egg weight, yolk weight, yolk crude protein, and albumen crude protein. The results revealed that protein levels did not significantly affect feed consumption during the laying phase for ducks. However, the comparison of duck day production between treatment groups indicated that ducks receiving lower protein levels (13.43%) exhibited lower production, compared to those with higher protein levels (18.29%). Furthermore, feed protein content had a notable impact on egg weight, yolk weight, and albumen crude protein. The study demonstrated a significant increase in egg yolk weight, while the percentages of egg yolk weight and eggshell weight showed no significant differences. In conclusion, this research suggests that supplementing duck diets with live BSF maggots can enhance egg quality and performance parameters.

Keywords: Black soldier fly, Duck, Egg quality, Maggot, Performance, Source of protein

INTRODUCTION

Between 2014 and 2017, there was a noticeable surge in global duck production. The countries experiencing the most significant increases in duck population during this period were China, Vietnam, Bangladesh, Indonesia, Russia, Myanmar, France, India, and Thailand (Kovitvadhi et al., 2019). Ducks play an important role in Asian countries, especially Indonesia, contributing significantly to the consumption of animal-origin protein, encompassing both meat and eggs (Hutahaean et al., 2022). Ducks exhibit resilience in challenging environments, immunity to diseases, and a capacity to adjust to locally available feed. Additionally, they have a symbiotic production system and are highly profitable (Adzitey and Adzitey, 2011). Feed is a crucial aspect of the livestock industry, accounting for up to 70% of total production expenses (Singh et al., 2009). As a result, it is necessary to explore various options to minimize feed costs. In the case of large-scale duck farms, the practice of utilizing commercial feed, imported feed ingredients, and other supplements is commonplace to reduce production expenses (Ismoyowati et al., 2018). However, it is not economically viable for small-scale duck farmers in rural areas. Therefore, it is necessary to seek alternative protein source feed ingredients that can overcome this problem while providing sustainability for large or small-scale farming businesses (Biasato et al., 2018). Live black soldier fly (BSF) maggot is a cheap protein source that can be easily procured and can be used as an alternative feed ingredient, which can reduce feed costs (El-Kaiaty et al., 2022; Mlaga et al., 2022). Maggot is not only a source of high protein (52.79%) but also contains fat (more than 35%), rich in minerals such as Calcium (5-8%) and

Phosphor 0.6%-1.5% (Makkar et al., 2014; Herawati et al., 2020). In addition, the use of BSF maggot in feed can provide benefits in terms of nutritional content and the environment. In comparison to traditional protein sources, insect farming proves to be more practical, with lower greenhouse gas and ammonia emissions, contributing to a reduced environmental impact (Makkar et al., 2014; De Marco et al., 2015). According to Gunawan et al. (2022), adding BSF larva feed increases the carcass weight of the duck, making BSF maggot a popular alternative protein source for poultry feed (Gariglio et al., 2021). Furthermore, BSF maggot is an insect with nutrient quality and an environmentally friendly production system (Hanboonsong et al., 2013; Makkar et al., 2014; Sánchez-Muros et al., 2014). The results of research by several researchers have indicated that insects also can be used as a food source of protein, which can contribute to solving food problems for humans and livestock as especially for poultry and various types of birds (Bukkens, 1997; Makkar et al., 2014; De Marco et al., 2015).

Several research findings by different investigators suggest that insects can serve as a valuable protein source, offering a potential solution to food shortages for both humans and livestock, especially for poultry and various types of birds (Bukkens, 1997; Makkar et al., 2014; De Marco et al., 2015). Furthermore, Gunawan et al. (2018) and Gariglio et al. (2019) indicated an increase in carcass weight when ducks were provided with live maggots and supplemented with defatted larva meal from BSF. This is related to the elevated levels of protein, amino acids, minerals, and vitamins found in the BSF (Spranghers et al., 2017). However, data on the benefits or potential of using live maggot substitution as a source of protein to increase the production and quality of duck eggs are still limited. The objective of the research was to explore the effect of supplementation of fresh BSF larva on the growth performance of laying ducks fed low and high-protein diets.

MATERIALS AND METHODS

The present study was performed and approved by the Animal Care and Use Committee of the Faculty of Agriculture. Kalimantan Islamic University with number 007/U.CC/FP/V/23.

Rearing black soldier fly live

As a source of feed protein, this study used live BSF maggot in the prepupal stage (age 2-3 weeks) obtained through cultivation with a mixture of palm kernel cake media, hatchery waste, and bakery factory waste. The BSF maggot breeding was carried out using closed cages made of plastic nets. The enclosure had dimensions of 3 meters in width, 2.5 meters in height, and 5 meters in length. The breeding process began by placing plastic basins containing prepupae inside a closed enclosure made of plastic mesh, with dimensions measuring 3 meters in width, 2.5 meters in height, and 5 meters in length. These prepupae were left undisturbed for 2-3 weeks to allow the maggots to undergo transformation into pupae, eventually developing into BSF flies. Following this phase, the BSF flies mated and laid eggs on the prepared substrate.

After an additional 2-3 weeks, the substrate containing the matured maggots was harvested, employing sunlight for drying purposes. Due to the maggots' sensitivity to light and their tendency to aggregate in one location, they were carefully gathered and weighed, constituting the feed for the ducks. The prepupae phase was harvested twice daily, in the morning and evening, resulting in a total weight of 2.4 kg. Each cage plot received a daily live maggot supplement of 40 g. The nutrient content of BSF maggot is detailed in Table 1.

Experimental period

This research was conducted at the teaching farm and livestock production laboratory within the Faculty of Agriculture at Uniska Banjarmasin, Indonesia. The ambient temperature during the study ranged from 27.5 to 31.5°C, with humidity levels fluctuating between 72% and 84%. A total of 120 female *Alabio* ducks, sourced from breeders with an average weight of 1.75±0.14 kg and aged 7 months, were randomly distributed into 24 cages. Each cage, constructed from bamboo and wire, measured 220 cm x 145 cm and housed 5 ducks. The ducks were procured from the local livestock market in Amuntai District, Inodnesia.

The cage floor was layered with husks and equipped with feed and drinking water facilities. The ducks were provided with a daily feed allowance of 150 g/head, featuring varied protein contents. Drinking water was available ad libitum. The ducks were kept for seven weeks, with one week dedicated to feed adaptation, and no vaccinations were administered during the research. Lighting in the cages was maintained for 24 hours, utilizing 12 hours of sunlight and 12 hours of electric light. The initial production rate at the study's outset was recorded at 43.93%. The details of the ingredients and nutritional formulas employed in the study can be found in Table 2.

Table 1. Nutrient content of dry maggot and fresh black soldier fly

Nutrient	Dry maggot	Fresh maggot
	---------- (%) ----------	
Water content	0	68.32
Ash	17.57	5.56
Crude protein	46.15	14.62
Crude fat	22.13	7.01
Nitrogen free Extract	10.97	3.47
Crude fiber	3.19	1.01
Calcium	1.49	0.47
Phosphor	0.85	0.26
Gross energy (Kcal/kg)	4,622	1,464

Table 2. Ingredient and basic formula of nutrition for the research

Ingredient of feed	Basic formula of nutrition	
	Low protein	High protein
	---------- (%) ----------	
Concentrate for duck	17.00	17.00
Rice bran	24.10	21.40
Corn	58.90	43.60
Fish meal	0	18.00
Total	100	100
Nutrient content[1]		
Dry matter	93.80	94.16
Ash	8.86	9.73
Crude protein	13.43	18.29
Crude fat	4.32	3.52
Carbohydrate	53.84	49.29
Crude fiber	12.69	16.39
Metabolizable energy (Kcal/kg)	3,079	3,020

[1]Analysis by Building Research and Standardization Industry.

Experimental design

The research design employed a completely randomized design with four treatments and six replications. Treatment P1 involved the supplementation without live maggots (0 g/day) with a low-protein diet (13.43%). In Treatment P2, live maggots were supplemented at 40 g/day alongside a low-protein diet (13.43%). Treatment P3 comprised supplementation without live maggots (0 g/day) with a high-protein diet (18.29%), while Treatment P4 involved the supplementation with live maggots (40 g/day) with a high-protein diet (18.29%). The study aimed to explore the effects of these treatments on the growth performance of laying ducks fed both low and high-protein diets. Each treatment was replicated six times to ensure the statistical robustness of the results, contributing to a comprehensive analysis of the study outcomes.

Measurement of parameters

Performance

Throughout the six weeks of observation, feed consumption was measured weekly. Duck day production was assessed daily for the entire 42-day observation period, calculated by dividing the daily egg production by the number of ducks present in each cage and multiplying the result by 100%. The feed conversion ratio (FCR) was determined by dividing the food consumption by the weight of the eggs produced. Additionally, the egg mass was obtained by multiplying the duck day production by the average egg weight.

Egg quality

The daily weighing of egg weights was conducted individually for each cage. To determine the yolk weight, a random egg sample was selected from each cage, and the yolk weight of the entire egg was calculated by dividing the yolk weight by the egg weight and then multiplying by 100%. The shell weight was obtained by isolating the eggshell and measuring its weight separately. Analysis of the crude protein content in both the yolk and albumen was performed using the Kjeldahl method, following the procedure outlined by Jamal et al. (2020).

Statistical analysis

The observational data underwent analysis of variance, and if the results indicated a significant effect ($p < 0.05$), further testing was conducted using an orthogonal contrast test with SPSS 24 for Windows (George and Mallery, 2016). To discern differences among the treatment groups, contrasts were organized based on contrast components. These components were designated as follows: contrast component 1 involved comparisons between P1, P2 versus P3, P4; contrast component 2 encompassed contrasts between P1, P3 versus P2, P4; and contrast component 3 focused on comparisons between P2 and P3. Statistical significance was set at p values less than 0.05 ($p < 0.05$).

RESULTS

Performance

The impact of different protein levels and live Black Soldier Fly (BSF) supplementation on duck performance, including feed consumption, duck day production, feed conversion ratio (FCR), and egg mass, is detailed in Table 3. The results of the analysis of variance revealed no significant differences ($p > 0.05$) among the treatments in

terms of feed consumption and feed conversion ratio for the layer-phase ducks. However, significant effects were observed in duck day production and egg mass. Further examination through the orthogonal contrast test revealed that in contrast component 1 (P1, P2 versus P3, P4), ducks receiving low protein levels (13.43%) exhibited significantly lower duck day production ($p < 0.05$), compared to those receiving high protein levels (18.29%). Conversely, contrast component 2 (P1, P3 versus P2, P4) did not show a significant effect on duck day production during the 6-week observation period ($p > 0.05$), indicating that the supplementation of 40 g/head/day live BSF had no significant impact on duck day production ($p > 0.05$). Nevertheless, contrast component 3 (P2 versus P3), while not statistically significant, hinted at a positive effect of adding live BSF maggots ($p > 0.05$). This implies that a low-protein diet supplemented with 40 g/head/day of BSF maggots could potentially match the performance of a high-protein diets, demonstrating a noteworthy finding in the study.

In the orthogonal contrast test number 1 (P1, P2 versus P3, P4), the egg mass production analysis indicated a significant difference ($P < 0.05$) between ducks subjected to low protein levels (13.43%) and those treated with high protein levels (18.29%). Contrast component 2 (P1, P3 versus P2, P4) did not reveal any significant impact on egg mass during the 6-week observation period, suggesting that the supplementation of 40 g/head/day live BSF had no significant effect on egg mass production. However, in contrast to component 3 (P2 versus P3), although not statistically significant, the addition of live BSF maggots demonstrated a notably positive effect. This implies that a low-protein diet, supplemented with 40 g/head/day of BSF maggots, has the potential to match the egg mass production of ducks fed a high-protein diet (P4). On the other hand, the feed conversion ratio (FCR) across all treatments did not yield significant results between diets with low and high protein content ($p > 0.05$). Additionally, feed consumption did not show any significant differences between diets with low and high protein content ($p > 0.05$).

Egg quality

The results of this study about egg qualities in the research are shown in Table 4. As can be seen, treatment with live BSF maggot and protein content diet (14.62%) had a significant effect on egg weight ($p < 0.05$). Furthermore, egg weight in the orthogonal contrast test number 1 (P1, P2 versus P3, P4) showed that ducks in the treatment group with low protein (P2) levels (13.43%) indicated lower egg weight results ($p < 0.05$) than high protein level treatment group (18.29%). In the orthogonal contrast test number 3 (P2 versus P3), it was observed that ducks in the treatment with low protein levels (13.43%) supplemented with live maggots exhibited similar egg weights to the high protein level treatment group (18.29%). The protein level of the diet had a significant effect ($p < 0.05$) on yolk weight, as did maggot supplementation. Orthogonal contrast test number 2 revealed a significant difference ($p < 0.05$) between P1, P3 versus P2, P4, resulting in increased yolk weight. However, the percentage of yolk weight and shell weight did not show a significant effect ($p > 0.05$) among P1, P3 versus P2, P4. Furthermore, the protein level of the diet significantly increased the crude protein of albumen (contrast number 1), and maggot supplementation, as indicated by orthogonal contrast test number 2, significantly elevated the crude protein of albumen ($p < 0.05$).

DISCUSSION

In Indonesia, ducks are typically raised for meat production, egg production, or dual purposes encompassing both. The predominant share of production costs in duck farming is attributed to feed expenses, with a particular emphasis on the raw materials serving as a protein source. Consequently, there is a crucial need to mitigate dependency on conventional protein source feed ingredients by exploring alternative, cost-effective by-products. A strategic approach involves combining commercial feed with live maggots to formulate optimal and efficient diets for duck layers. This innovative combination not only addresses economic considerations but also enhances the nutritional profile, reflecting a promising initiative to optimize duck farming practices in Indonesia. The feed intake of ducks in all treatments was comparable. This is likely due to the uniform metabolizable energy content in the provided diets (Table 2). One of the parameters observed in this research was feed intake. The results of feed intake showed that there was no difference between the low protein ratio treatment and the high protein ratio treatment.

These findings are consistent with the observations of Fouad et al. (2018b) who reported a positive effect on laying ducks when the feed energy ranged from 2,600 to 3,100 kcal AME/kg. Furthermore, a study by Wickramasuriya et al. (2016) demonstrated that an increase in energy levels from 2,600 to 3,300 kcal AME/kg in native Korean ducks led to enhanced productive performance. On the other hand, daily duck

egg production in the treatment group with low protein levels (13.43%) was lower than that in the treatment group with high protein levels (18.29%). This finding aligns with the results reported by Fouad and El-Senousey (2014), who stated that the production and reproduction performance of laying ducks was influenced by the adequacy of protein and energy. Providing live BSF maggots in the diet turned out to be of great benefit to duck egg weight. Giving live BFS maggots containing high protein has an impact on *Alabio* duck egg weight. However, giving live BFS maggots to the low protein content treatment (13.43%) also resulted in low egg weight results when compared to the high protein treatment group (18.29%).

The results of the study were in line with the results of research by Fouad et al. (2018a) As observations, the laying performance of ducks, encompassing egg production, egg weight, and (FCR), is impacted by the presence of feed with a sufficient protein content (Finke, 2013). The study results indicated that the treatment with live BSF maggot and the protein level of the diet affected egg weight. This was understandable because giving live maggots to the feed will add nutritional value in the form of feed protein it has an impact on increasing egg production and weight. Dong et al. (1997) reported that using local feed resources like live BSF maggot was efficient in feeding Muscovy ducks. Contrastingly, Finke (2013) emphasized the rich protein and amino acid content of live BSF larvae. Furthermore, various studies (Spranghers et al., 2017) have pointed out that insects, in general, possess high levels of essential amino acids and exhibit a superior amino acid profile compared to traditional protein sources (Tran et al., 2015). Regarding the current study, it was noted that the introduction of live BSF maggots had a significant impact on the weight of duck egg yolks, as indicated by other parameters. The results of this study were in line with the research of Gunawan, et al (2018) which reported that the provision of live BSF maggots at 10% of the amount of diet given can result in a higher percentage of carcass but produce a lower percentage of carcass pieces than 0% BSF maggot (without maggot).

Table 3. Performance parameters of local ducks were given different diets

Variable	Diet				SEM	P-value	Orthogonal contrast[1]		
	P1	P2	P3	P4			1	2	3
Feed consumption (g/head)	127.18	137.28	133.88	140.68	1.99	0.090	-	-	-
Duck day production (%)	53.01	61.19	64.36	62.78	1.61	0.048	*	ns	ns
Feed conversation ratio (FCR)	5.31	6.39	5.48	4.24	0.34	0.174	-	-	-
Egg mass (g/head/day)	31.52	37.17	41.64	40.75	1.41	0.034	*	ns	ns

P1: Diet Crude Protein 13.43%; P2: Diet Crude Protein 13.43% + 40 g maggot; P3: Diet Crude Protein 18.29%; P4: Diet Crude Protein 18.29% + 40 g maggot. ns = no significance ($p > 0.05$); * ($p < 0.05$). [1]Comparison of orthogonal contrast; 1: P1, P2 versus P3, P4 (Diet -1 -1 1 1); 2: P1, P3 versus P2, P4 (Diet -1 1 -1 1); 3: P2 versus P3 (Diet 0 -1 1 0).

Table 4. Egg quality parameters of local ducks were given different diets

Variable	Diet				SEM	P-value	Orthogonal contrast[1]		
	P1	P2	P3	P4			1	2	3
Egg weight (g)	59.30	62.84	64.31	64.71	0.77	0.045	*	ns	ns
yolk weight (g)	18.77	20.78	20.70	21.26	0.27	0.001	*	*	ns
Yolk weight of whole egg (%)	31.64	33.06	32.19	32.81	0.23	0.120	-	-	-
Shell weight (g)	6.12	6.59	6.66	6.93	0.11	0.079	-	-	-
Crude protein of yolk (%)	13.81	12.94	13.81	13.79	0.31	0.730	-	-	-
Crude protein of albumen (%)	11.48	13.02	12.55	13.54	0.20	0.000	*	*	ns

P1: Diet Crude Protein 13.43%; P2: Diet Crude Protein 13.43% + 40 g maggot; P3: Diet Crude Protein 18.29%; P4: Diet Crude Protein 18.29% + 40 g maggot. ns = no significance ($p > 0.05$); * ($p < 0.05$). [1]Comparison of orthogonal contrast; 1: P1, P2 versus P3, P4 (Diet -1 -1 1 1); 2: P1, P3 versus P2, P4 (Diet -1 1 -1 1); 3: P2 versus P3 (Diet 0 -1 1 0).

CONCLUSION

Based on the results of the study it can be concluded that giving live BSF maggot to laying ducks can improve performance parameters such as day egg production and FCR and can improve egg quality parameters.

DECLARATIONS

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Authors' contributions

Aam Gunawan served as the principle researcher of the project and conducted data analysis. Abd. Malik contributed to the design and drafting of the article. Fitri Noor Hayati and Afridolin Sonya Goa were responsible for data collection, and Ahmad Junaedi and Dwi Wahyu Candra oversaw the rearing of experimental livestock. All authors thoroughly reviewed and approved the final version of the manuscript for publication in the current journal.

Competing interests

All authors have 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, were checked by all authors.

Availability of data and materials

The primary data for this study is available from the corresponding author.

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Molecular Detection of Avian Poxvirus in Chickens and Pigeons of Diyala Province in Iraq

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ABSTRACT

Fowlpox is an infectious viral disease affecting domestic and wild birds. The current study was conducted using PCR-based molecular detection to determine the avian pox virus (APV) and its genetic sequence. A total of 40 pigeons and 40 chickens suspected of APV were obtained from different bird markets. Following euthanasia, tissue samples were taken from the vents, eyes, wings, and beaks for molecular analysis. DNA extraction was focused on the core protein gene region (pb4). PCR results confirmed the presence of APV in all samples, generating specific cDNA bands of 329 bp. Four GenBank accession codes (OR670580, OR670581, OR670582, and OR670583) represented pigeon pox samples, whereas two GenBank accession numbers (OR619724 and OR619725) represented samples of fowlpox. A significant similarity of 99% was found by genetic analysis between the reference target sequences and the sequenced samples. In viral samples of fowlpox and avipoxvirus, nucleic acid variants (205 C > G and 204 T > C) were found with missense and silent effects on particular proteins (p.101Leu>Val and p.108Pro). Phylogenetic analysis organized the samples into clades representing fowlpox and pigeon pox viruses, showing close relationships with strains from different geographical regions. This study unequivocally demonstrates the susceptibility of both domestic and wild birds to avian pox, highlighting the pivotal role of phylogenetic analysis and molecular detection in elucidating novel perspectives on the genetic landscape of these viruses.

Keywords: Fowl pox, Gene, Pigeon pox, Polymerase Chain Reaction

INTRODUCTION

Fowlpox, a viral disease that affects both domestic and wild birds (Williams et al., 2021), is caused by a wide range of DNA viruses within the Poxviridae family. It has significant rates of morbidity and mortality and causes mild to severe lesions (Zhao et al., 2014). The Poxviridae family consists of two subfamilies, namely Chordopoxvirinae, and Entomopoxvirinae (Weli and Tryland, 2011). The fowlpox virus (FPV) is a distinctive, large, oval-shaped enveloped virus with linear dsDNA genomes ranging from 250 to 365 kb. Characterized by a viral particle diameter of 270-350 nm (Weli and Tryland, 2011), FPV demonstrates rapid growth in cell cultures and on embryonated eggs' chorioallantoic membranes (Diallo et al., 2010). The core structure harbors genes shared by all poxviruses, crucial for fundamental replication processes, while terminally positioned genes encode proteins influencing host range limiting (Tulman et al., 2004). Nearly all types and varieties of chicken are prone to contracting FPV (Cui et al., 2023). Birds with large wattles and large combs seem more likely to acquire pox lesions due to the greater surface area of these parts (Delhon, 2022). According to Pattison et al. (2008), there are two forms of the illness, the more frequent being cutaneous (dry pox) and diphtheric/or pharyngeal (wet pox). On featherless parts of the body (comb, the area around the beak, the wattles, the eyelids, or even the legs and wings), the cutaneous form manifests as papules, nodules, or scabs. These lesions have the potential to progress into ulcers, leading to worsening conditions that can hinder the bird's mobility, ability to eat, and vision (Sultana et al., 2019). If the cutaneous form is predominant, mortality rates are generally low. This is attributed to the fact that the lesions induced by the virus in the cutaneous form are primarily confined to the skin. Consequently, there is a limited impact on vital organs or essential physiological functions crucial for survival.

(MacLachlan and Dubovi, 2017; Sultana et al., 2019). The development of white opaque nodules or yellowish patches on the mucous membranes of the oral cavity, tongue, esophagus, or upper trachea of the birds distinguishes the diphtheritic form, which is more severe and causes significant mortality and economic losses in affected flocks (Singh et al., 2003). According to Yeo et al. (2019), several vectors can spread the APV, such as biting arthropods like mites and mosquitoes, aerosols generated by infected birds, and intake of contaminated food or water. Due to its characteristic dry, crusty skin lesions, which are typically seen on the face, comb and wattle, and other unfeathered regions of the bird, fowl pox is recognized as a prevalent, chicken enzootic disease (Skinner and Laidlaw, 2009; Delhon, 2022). Pigeon pox, on the other hand, is a slower-spreading disease that affects both sexes, has a global distribution, and can be fatal. Vaccination with live chicken poxvirus or pigeon poxvirus is commonly used to prevent the disease (Tripathy and Reed, 2013). Despite the effectiveness and regular use of vaccines to minimize morbidity and mortality, there have been instances of infections observed in certain flocks that have received immunizations (Odoya et al., 2006). Sequencing and PCR-based amplification of conserved genomic areas are frequently used in confirmatory diagnosis (Manaroll et al., 2010). The objective of this study was to analyze specific traits of the virus from a virological perspective. The study aimed to detect FPV using molecular detection methods and analyze the identified strain through phylogenetic analysis in both chickens and pigeons. Additionally, the study seeks to determine the similarity between the identified strains in Diyala Governorate and reference strains available in the NCBI GenBank database.

MATERIALS AND METHODS

Ethical approval

The Scientific Ethical Committee of the College of Veterinary Medicine, University of Diyala, Iraq, approved this study (Approval No. Vet Medicine (188); September 2023 (A and A).

Samples collection

From October 2022 to March 2023, the study was carried out in the College of Veterinary Medicine at Diyala University, Iraq. A total of 40 suspected cases of fowl pox in Gallus gallus and forty Columba Livia Domestica of varying ages were gathered from bird shops around the Diyala governorate. Trained experts examined the pigeons and chickens, documenting clinical signs, such as erosions, crusts, or nodules on the vent area, and head skin (particularly the beak and eyelids). Additionally, individual case histories for each bird were meticulously collected (Figure 1). Samples were obtained immediately after euthanizing the pigeons and chickens, and tissue samples from affected regions (vents, eyes, wings, and base of the beak) were processed for molecular detection.

Figure 1. Naturally infected chicken and pigeon with avian poxvirus. **A**: Chicken, pock lesions appeared on the head and around the eye. **B**, **C**, and **D**: An adult pigeon infected with poxvirus in different areas (Nodular Lesion).

Tissue preparation

Tissue samples were taken from the vent, eyes, wings, cere, and base of the beak after being euthanized. Viral DNA was isolated from the tissue samples using the gSYNC™/ DNA extraction Kit (Canada), following the manufacturer's instructions. For the PCR procedure, a classical PCR premix solution containing Taq DNA polymerase, MgCl2, dNTP mixture, buffer, and 1.5 µl of the template was used. The PCR components were thoroughly mixed and subsequently centrifuged. Following this, a fresh reaction mixture was prepared while maintaining a low temperature on ice. The RT-PCR tubes were then transferred to a thermal cycler for subsequent processing.

The commercially available Bioin Gentech Vet PCRTM Avian Pox Virus kit (Chile) was employed for the amplification of the APV (P4b) core protein gene. Oligonucleotide primers designed specifically for APV detection, sourced from the micro-gene company (Korea), were utilized to generate a target band of 329 base pairs (refer to Table 1). The amplification process comprised 32 cycles, involving denaturation at 95°C for 30 seconds, annealing at 58°C for 30 seconds, and extension at 72°C for 30 seconds. A final extension step at 72°C for 7 minutes was incorporated into the protocol (Manarolla et al., 2010). The resulting PCR products were subjected to electrophoresis on a 1.5% agarose gel stained with Red safe Nucleic acid staining. Fragment sizes were determined using a 100 bp ladder (Intron, Korea).

To investigate genetic polymorphism in comparison to samples in the NCBI database, a comprehensive tree was constructed to determine the serotyping and phylogenetic distribution of the observed variants. A commercial forward sequencing method was employed to amplify the PCR products, and the resulting DNA sequences were compared with known sequences in the reference database. BioEdit Sequence Alignment Editor Software (Version 7.1) was utilized for the editing, alignment, and analysis of the sequencing data. Numerical positions were assigned to nucleic acid variations within the reference genome, and these were deposited in the NCBI-bankit database.

The amino acid sequences of the targeted P4b-encoded core proteins were extracted from the protein data library to convert the nucleic acid variants into amino acid residues. Using the Expasy online software, nucleic acid sequences were transformed into corresponding amino acid sequences. Amino acid sequence alignment was carried out to compare the altered sequences with their reference counterparts. For a comprehensive phylogenetic analysis, a specific tree was generated.

The NCBI-BLASTn server was utilized to compare the detected variations with homologous reference sequences. The neighbor-joining method was applied to construct an all-inclusive tree, visualized as a circular cladogram using the iTOL suite (Letunic and Bork, 2019).

Table 1. Gene name and oligonucleotide primers sequence designed

Gene name and SNP		Primers sequence 5′→3′	Guanine to cytosine (%)	Product size (bp)	Annealing temperature
SNP of selected gene(4b)	F	AATCTTAGAAAAGACGCAGATGCT	55%	329	58
	R	AAGTTTGTTGATTGAAACCTAGTCG	50%		

SNP: Single nucleotide polymorphisms, GC:

RESULTS

All samples from forty pigeons and poultry tested positive for APV. All the amplified cDNA showed identical mobility on 1.5% agarose gel. Positive samples generated a specific DNA band of 329bp (Figures 2, 3).

Sequencing results

Within this genomic locus, six positive samples were analyzed, comprising two samples for fowl pox virus (S2 and S3) and four samples for Pigeon pox virus (S1, S4, S5, and S6). The amplification targeted the P4b gene sequences of both fowl and pigeon pox viruses. Consequently, the variation observed in the P4b gene can serve as a marker for polymorphism in both viruses, suggesting its potential adaptability to genetic diversity.

To validate the identity of these PCR amplicons, sequencing reactions were conducted and subjected to an NCBI BLASTn analysis, revealing an exact match (Ye et al., 2012). The NCBI BLASTn engine revealed that there were approximately 99% sequence similarities between the sequenced sample and the targeted reference target sequences for the 329 bp amplicons of the fowlpox viruses. The precise locations and other features of the

retrieved PCR fragments were determined by comparing the observed nucleic acid sequences of the studied sample with the retrieved nucleic acid sequences (GenBank accession NoOR099892.1; Figure 4A). The NCBI BLASTn engine also revealed that there were almost 99% sequence similarities between the sequenced sample and the desired reference target sequences for the 329 bp amplicons of the pigeon pox viruses. Accurate locations and other details of the retrieved PCR fragments were detected (Figure 4B) by comparing the observed nucleic acid sequences of the studied sample with the retrieved nucleic acid sequences (GenBank acc. OP131515.1).

The features of the 329 bp amplicons' sequences were emphasized after placing them within the genomic sequences of the pigeon and fowlpox viruses. The overall length of the amplified amplicons was also ascertained (Table 2 A and B).

Regarding fowlpox viruses, two of the examined samples had a single nucleic acid variation (more precisely, a nucleic acid substitution) according to the alignment of the 329 bp local samples.

Upon comparison of the local samples with the most closely related reference nucleic acid sequences obtained from NCBI, notable variations were observed (Figure 5A). Similarly, in the case of pigeonpox viruses, two of the examined samples exhibited a single nucleic acid variation, specifically represented by a nucleic acid substitution during the alignment of the 329 bp sample. Distinctions were identified when contrasting the local samples with the most similar reference nucleic acid sequences acquired from NCBI (Figure 5B).

Concerning fowlpox viruses, the identified nucleic acid variant was 205 C>G. This difference observed in the currently observed nucleic acid sequences in the analyzed local samples was not positioned in the corresponding reference sequences of the viral P4b gene (GenBank acc. no. OR099892.1). Subsequent investigation demonstrated that the main protein was missense-impacted by this nucleic acid alteration. In particular, it caused the core protein sequence's position 101 to contain valine (Val) instead of leucine (Leu, Figure 6A). The identified nucleic acid variation for pigeons' pox viruses was 204 T > C. The matched reference sequences of the viral P4b gene (GenBank accession number: OP131515.1) did not contain this variation. It was discovered through analysis of its effects on the core protein that this variant is quiet and has no effect on the amino acid sequence. Proline (Pro) at position 108 of the core protein sequence served as an example of this synonymous variant (Figure 6. B). All the sequences were translated to proteins by using the Expasy translate suite.

However, these changes might have been triggered by the invader as a response to medications that target its core protein (Topalis et al., 2016). To get a distinct accession number in the NCBI database, the detected alterations were correspondingly put in the NCBI-banks database. Two GenBank accession numbers (OR619724 and OR619725) were acquired for this investigation to denote the S2 and S3 fowlpox virus samples, respectively. Additionally, four GenBank accession numbers representing the pigeon pox virus samples of S1, S4, S5, and S6 were obtained (OR670580, OR670581, OR670582, and OR670583, respectively).

Phylogenetic analysis

A phylogenetic tree was created in this study using nucleic acid variations from the amplified 329 bp P4b gene amplicons of the investigated viral particles. This provided insights into the phylogenetic distances between the local samples from chickens and pigeons and other avian pox virus sequences from NCBI. In this study, a phylogenetic tree was created by analyzing nucleic acid variations within the amplified 329 bp region of the P4b gene amplicons obtained from fowlpox and pigeonpox viruses. The tree included samples namely (S1-S6) and other relevant sequences from fowlpox and pigeonpox viruses (Figure 7). This tree provided visual representations of the viral sequences and their phylogenetic relationships. The tree included a total of 53 aligned nucleic acid sequences. It organized the sequences into two main clades representing fowlpox viruses and pigeonpox viruses, with two out-group clades consisting of other viral sequences. The inclusion of out-groups helps assess the variations among fowlpox viruses, pigeonpox viruses, and related sequences from other viral strains. A strong correlation was observed between the samples obtained from fowls (S2 and S3) and adjacent sequences within the fowlpox virus clade, which were obtained from different regions worldwide. The viral isolates were evenly distributed within the fowlpox virus clade, which contained the highest number of viral sequences (18 strains) at varying phylogenetic distances (Figure 7).

Within this main clade, both S1 and S2 samples have shown an extremely slight tilt with respect to the other neighbor positions. This pattern of the tiny altered positioning was due to the presence of only one genetic variation (205 C>G) in both samples. The generated phylogenetic tree provides confirmation of the sequencing reactions as it accurately reflects the neighbor-joining-

based positioning of the investigated sequences. Interestingly, the local samples S2 (OR619724) and S3 (OR619725) were found to be closely related to variable strains that have been isolated from different geographical locations worldwide. Most of these strains of fowl pox virus have been deposited from Iraq (Tikrit, Salah Al Deen), (NCBI MN915017.1, Egypt (NCBI MN708968.1), Iran (NCBI MG787229.1), Brazil (NCBI MK651856.1), India (NCBI MK370901.1), USA (NCBI MH175260.1) Turkey (KF722862.1), Tanzania (NCBI KF722863.1) and from China (NCBI KF875986.1).

On the other hand, the examined viral local isolates, namely S1, S4, S5, and S6 are closely related to several neighboring sequences within the avipox (pigeons) virus clade. These neighboring sequences have originated from distinct regions. The studied viral isolates occupy similar positions within the avipox virus clade. This clade comprises 14 sequences of varying strains. Within this major clade, the altered S4 and S6 local samples exhibit a very subtle deviation compared to the positions of their neighboring sequences.

However, it is worth noting that the examined S1, S4, S5, and S6 samples are closely associated with variable strains isolated from Australia (NCBI OP131512.1, OP131513.1, OP131514.1, and OP131515.1).

Apart from the fowlpox and avipox (pigeons) clades, two out-group clades were incorporated namely (S1 and S5). These clades were attributed to the canarypox virus with NCBI, LN 795883.1, LK 021649.1, LN795887.1, LN795890.1, LK021655.1 and LK021658., LK021648.1 from France GQ487567.1, MF102266.1 and MF102267.1 from Iran and the pigeon pox virus with NCBI OR099893.1 and OR099894.1 from Libya, MF102269.1, MF102270.1 and MF102271.1 from Iran and MF496043.1, MH365477.1, MH721412.1 and MH721417.1 from India, MH175237.1 from Canada.

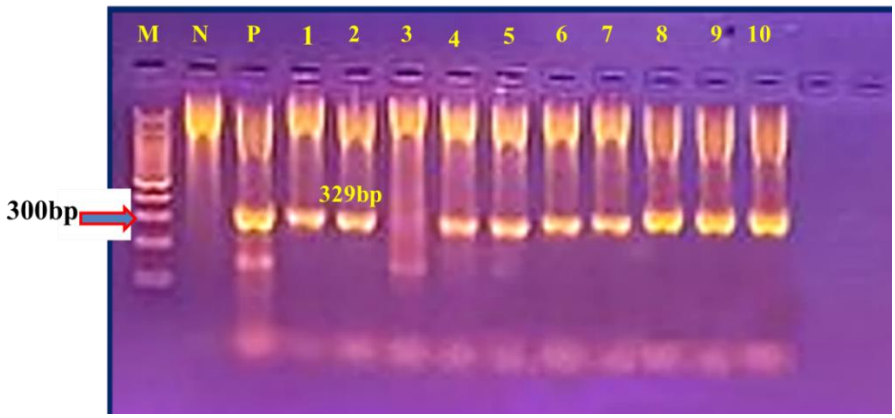


Figure 2. The P4b gene region was examined in tissue samples for the fowlpox virus using a conventional PCR method. Oligonucleotide primers were designed using NCBI to specifically amplify a 329 bp product size.

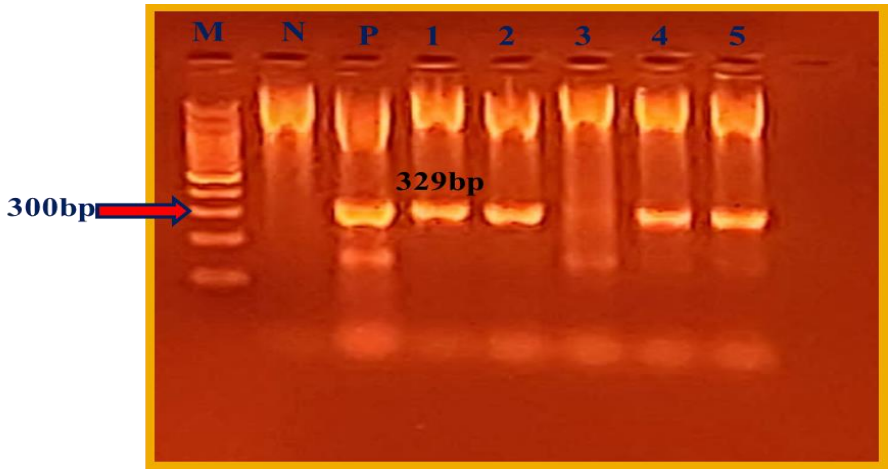


Figure 3. Tissue samples from pigeons and fowl suspected with avian pox virus were screened by PCR using designed oligonucleotide primers targeting the selected gene (p4b) to amplify a specific 329 bp band

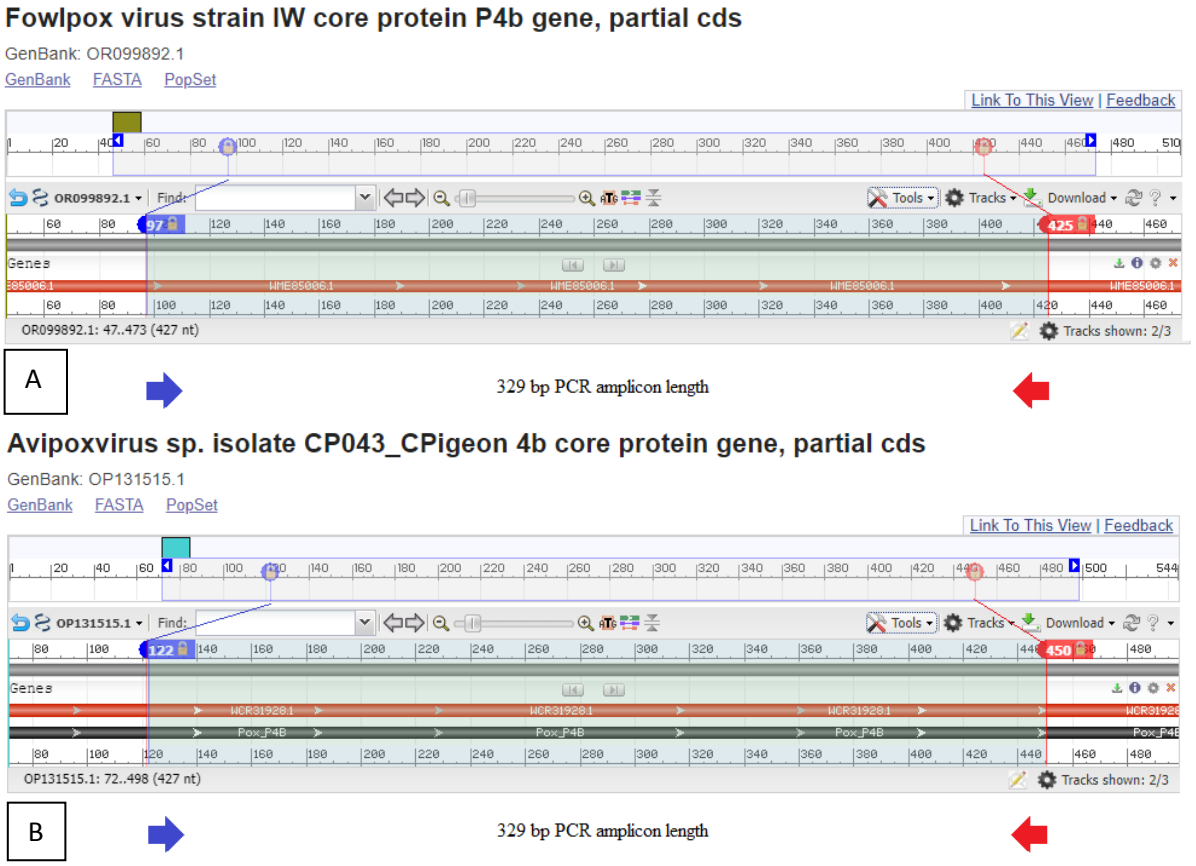


Figure 4. The retrieved 329 bp amplicon position partially encompassed a section of the P4b gene within fowlpox virus genomic sequences (GenBank acc. no. OR099892.1) and avipox virus genomic sequences (GenBank acc. no. OP131515.1) in branched A and B, respectively. The starting point of this amplicon is indicated by the blue arrow, while its endpoint is indicated by the red arrow

Table 2. The PCR amplicons, which measure 329 base pairs in length, were used to amplify a portion of the coding sequences of the P4b gene within the genomic sequences of fowlpox virus (GenBank acc. no. OR099892.1) and pigeon pox virus (GenBank acc. no. OP131515.1).

Amplicon	Reference locus sequences (5′ - 3′)	length
A) Fowlpox P4b gene sequences	*AATCTTAGAAAAGACGCAGATGCTATAGTAAGATATCTCATGGATAGAAAATGTGA TATAAATAACTTTACGATACAAGACCTTATTCGCGTTATGAGGGAATTAAATATTAT TAGAAATGAAAGACAAGAGTTATTCGAGTTACTATCTCATGTCAAAGGATCACTTTC TAGTAATAGCGTTTCGGTAAAAACGAGTCATCCTCTAATGGTTATTTATTCGCACTC AGATAATAAGATAGGAGAACAGTTAAAACTACTAGAAAATACATATGATCCATCCAG ATATCAAGCGTTGATAGATACGACTAGGTTTCAATCAACAAACTT**	329 bp
B) Avipox P4b gene sequences	*AATCTCAGAAAAGATGCAGATGCTATAGTAAGATATCTTATGGATAGAAAATGTGA CATAAATAACTTTACGATACAGGATCTTATTCGAGTTATGAGAGAATTAAATATTAT TAGGAATGAAAGACAAGAGTTATTCGAGTTACTATCTCACGTCAAAGGATCTCTTTC TAGTAATAGTGTTTCGGTCAAAACTAGTCATCCTCTAATGGTTATTTATTCACATTC AGATAACAAGATAGGAGAACAGTTAAAACTACTAGAAAATACTTACGATCCATCTAG ATATCAGGCTCTAATAGATACTACGAGGTTTCAATCTACAAACTT**	329 bp

A)


10 20 30 40 50 60 70 80 90 100
ref.
AATCTTAGAAAAGACGCAGATGCTATAGTAAGATATCTCATGGATAGAAAATGTGATATAAATAACTTTAC
GATACAAGACCTTATTCGCGTTATGAGGG
S2 .....
S3 .....
110 120 130 140 150 160 170 180 190 200
ref.
AATTAAATATTATTAGAAATGAAAGACAAGAGTTATTCGAGTTACTATCTCATGTCAAAGGATCACTTTCT
AGTAATAGCGTTTCGGTAAAAACGAGTCA
S2 .....
S3 .....
210 220 230 240 250 260 270 280 290 300
ref.
TCCTCTAATGGTTATTTATTCGCACTCAGATAATAAGATAGGAGAACAGTTAAAACTACTAGAAAATACAT
ATGATCCATCCAGATATCAAGCGTTGATA
S2 ...G.....
S3 ...G.....
310 320
ref. GATACGACTAGGTTTCAATCAACAAACTT
S2 .....
S3 .....

B)

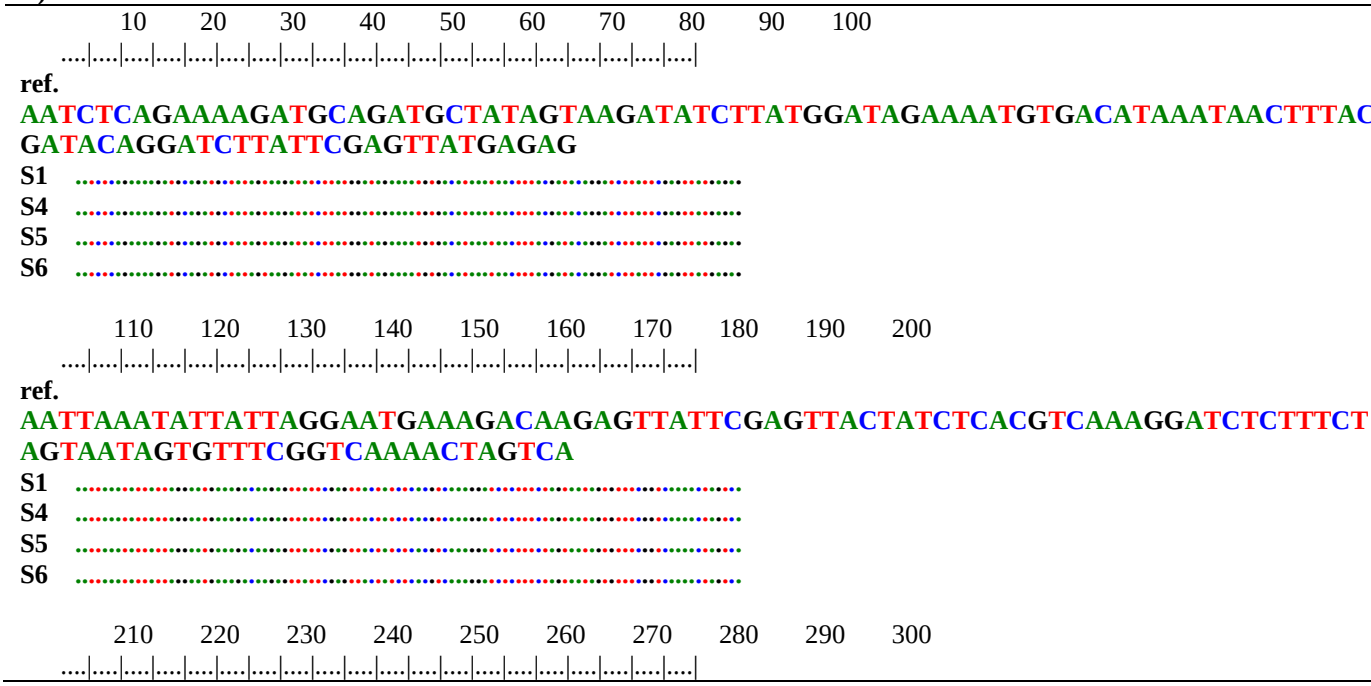
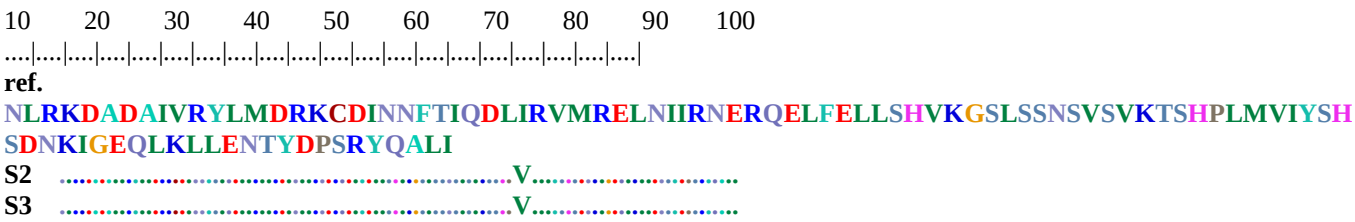

10 20 30 40 50 60 70 80 90 100
ref.
AATCTCAGAAAAGATGCAGATGCTATAGTAAGATATCTTATGGATAGAAAATGTGACATAAATAACTTTAC
GATACAGGATCTTATTCGAGTTATGAGAG
S1 .....
S4 .....
S5 .....
S6 .....
110 120 130 140 150 160 170 180 190 200
ref.
AATTAAATATTATTAGGAATGAAAGACAAGAGTTATTCGAGTTACTATCTCACGTCAAAGGATCTCTTTCT
AGTAATAGTGTTTCGGTCAAAACTAGTCA
S1 .....
S4 .....
S5 .....
S6 .....
210 220 230 240 250 260 270 280 290 300

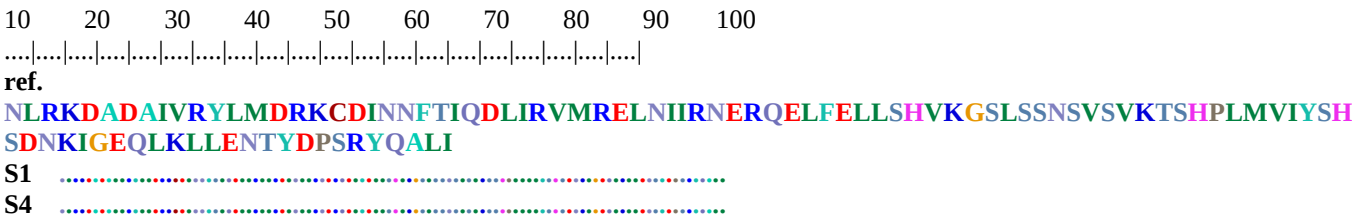
Figure 5. Nucleic acid sequences alignment of two samples with their corresponding reference sequences of P4b gene of fowlpox viruses in branch A, and avianpox viruses in branch B. The letter "S" followed by a number refers to the local sample number, whereas the sign "ref" refers to the NCBI referencing sequence.

A) sequences of amino acid residues of fowlpox virus P4b core protein



>WME85006.1 core protein P4b, partial [Fowlpox virus]
KPDICKGVSDSVKQKNTIINIDEITSTHDWQYNLRKDADAIVRYLMDRKCDINNFTIQDLIRVMRELNIIRNERQELFE
LLSHVKGSLSSNSVSVKTSHPLMVIYSHSDNKIGEQLKLLENTYDPSRYQALIDTTRFQSTNFVDMSTSSDMLFRFKD
QDSIGYVHPILVA

A) sequences of amino acid residues of avipox virus P4b core protein



ref. DTTRFQSTNF
S1 .....
S4 .....
S5 .....
S6 .....

>WCR31928.1 4b core protein, partial [Avipoxvirus sp.]
RLRSSSKPKPDICKGVSDSGKQKNTIINIDEITSTHDWQYNLRKDADAIVRYLMDRKCDINNFTIQDLIRVMRELNIIRN
ERQELFELLSHVKGSLSSNSVSVKTSHPLMVIYSHSDNKIGEQLKLLENTYDPSRYQALIDTTRFQSTNFVDMSTSSDM
LFRFKDQDSIGYVHPILVALFG

Figure 6. The amino acid residue alignment for variations in the P4b of fowlpox virus (branch A) and pigeonpox virus (branch B) samples. The positions of substitutions are highlighted within the 329 bp locus and the viral protein. Gray highlights indicate the amplified region. Missense and silent variants are highlighted in yellow and cyan, respectively, marking their specific positions on the amino acid residues in the chart.

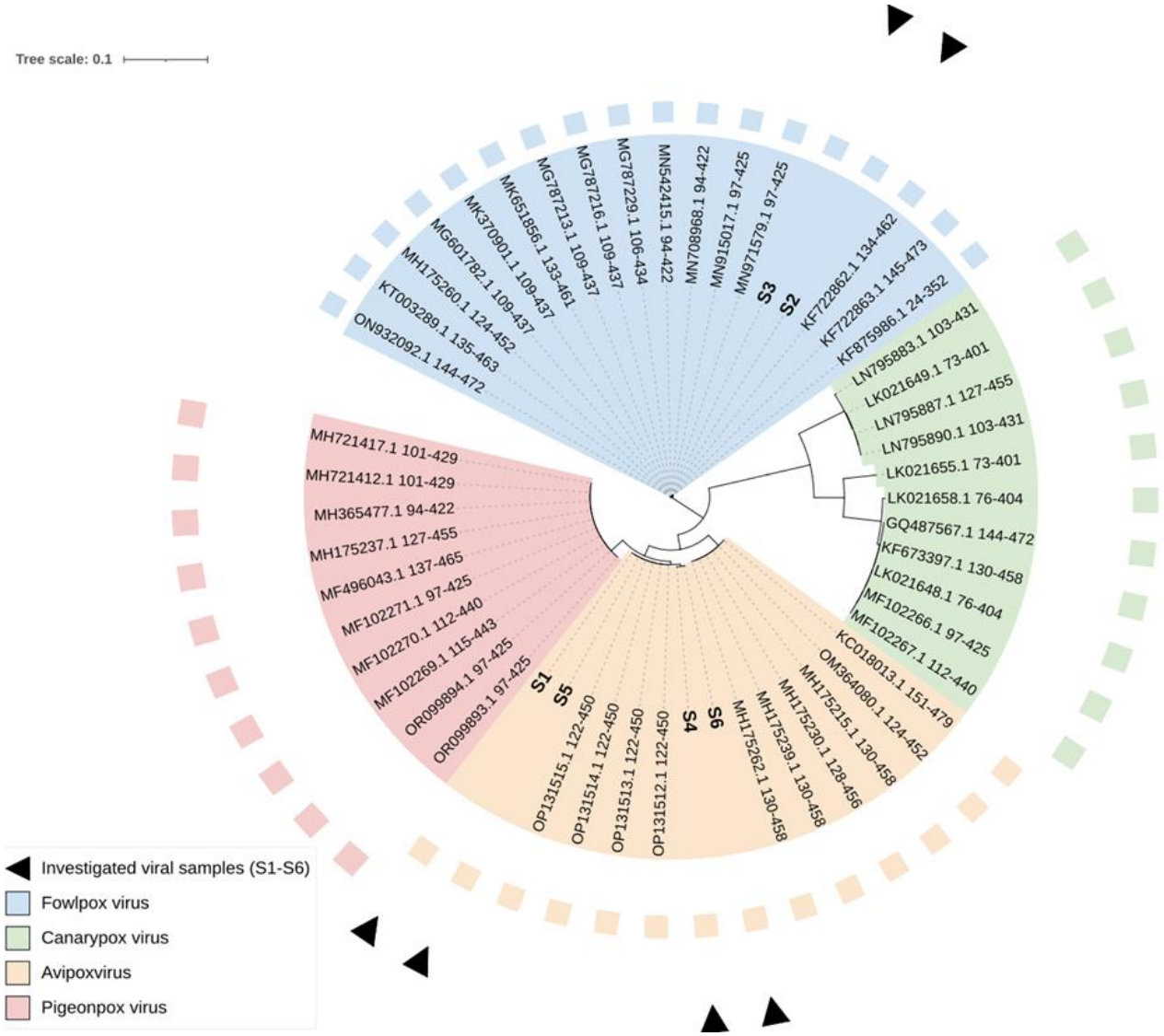


Figure 7. The circular cladogram phylogenetic tree illustrates the genetic variants of the P4b gene fragment in fowlpox and avipox viral samples. The analyzed viral variants are represented by black-colored triangles. The accompanying numbers correspond to the GenBank accession numbers of each respective species.

DISCUSSION

Avian pox is a highly contagious disease that affects domestic and wild birds worldwide. It is particularly prevalent in tropical and subtropical regions (Beukema et al., 2006).

The current study observed clinical signs in infected birds with the cutaneous form of the APV. These signs included an increase in small papules, pustules, or nodular lesions, which progressed to thick scabs resembling warts on featherless areas of the body. Initially, the lesions appeared as white or yellow patches and then converted into crusts that spread around the beak, legs, feet, combs, wattles, and other skin areas. Erosions, crusts, or nodules were also present in the vent area and on the skin of the head, specifically in the cere, beak, and eyelids of the bird (Figure 1). The present findings align with prior studies conducted by Mohan and Fernandez (2008) and Hibl et al. (2019), which highlight crusted and nodular lesions as characteristic features of the cutaneous form of fowl and pigeon pox virus. These manifestations were observed to be disseminated on the skin of the head and cranial cervical region. Notably, the majority of affected birds did not display additional clinical signs, and there was no notable increase in mortality within the examined flock. The primary focus of this study was on the core protein P4b shared by Fowlpox and avipox viruses. The researchers successfully amplified conserved regions within the gene responsible for encoding the P4b core proteins found in hen and pigeon virus strains. These regions are vital for the replication and structure of the viruses (Jarmin et al., 2006). The PCR assay, which was used in this investigation, proved to be a quick, incredibly sensitive, easy, and reliable diagnostic technique. The chosen oligonucleotide primers in the current study ensured a high sensitivity for APV particularly by targeting a highly conserved region of 4b. The P4b core protein gene of Apv was found to be present in all 40 samples taken from pigeons and hens in the current investigation using PCR analysis. The obtained result aligns with previous research by Fallavena et al. (2002) and Fagbohun, and Alaka (2021), demonstrating the practicality and effectiveness of PCR techniques in the identification of DNA viruses responsible for avipox and fowlpox.

In other circumstances, the intensity and diversity of the bird infection may be connected with the biological diversity of the P4b gene, which encodes the P4b core protein.

In this study, a particular PCR fragment was used to target the coding regions of the 4b gene in APV present in both pigeons and chickens. The amplified fragments were then subjected to direct sequencing to analyze the genetic variations in the local samples. All 40 samples obtained from pigeons and chickens tested positive for APV. Further examination was conducted on six selected samples, where two (S2 and S3) were associated with fowlpox virus, while the remaining four samples (S1, S4, S5, and S6) were linked to avipox virus specifically in pigeons. To amplify the P4b core protein gene sequences for the avipox and fowlpox viruses, respectively, these samples underwent a rigorous screening process. Since the P4b core protein gene may be able to adapt to varying genetic variety, as observed in various viral variations, it can therefore be utilized to explain the polymorphism of both viruses. Manarolla et al. (2010) study highlighted the avipoxvirus P4b core protein gene's molecular biological study. It was noted that this gene is frequently amplified for comparative genetic study and that it encodes a protein of 75.2 kDa. This study examined the 329 bp amplicons from the local viruses, and the findings revealed a striking degree of sequence similarity. The sequenced sample indicated over 99% sequence similarity with the corresponding nucleic acid sequences that were obtained from GenBank (OR099892.1 and OP131515.1), according to the NCBI BLASTn engine. Regarding the fowlpox and avipox viruses, the alignment results of 329bp revealed the presence of a single nucleic acid variation, represented by one nucleic acid substitution. This variation was observed in two fowl samples and two pigeon samples compared to reference sequences from NCBI. The present study identified a variant in fowlpox viruses (205 C>G) that caused a missense effect at position 101 (p.101Leu>Val) in the core protein sequences. However, a variant (204 T>C) in avipox viruses had a silent effect at position 108 (p.108Pro) in P4b. However, these alterations in the P4b may be a result of the invading organism adapting to drug treatments or implementing a preventative program using vaccinations for poultry and birds targeted against it (Topalis et al., 2016).

The construction of the phylogenetic tree relied on the nucleic acid variations identified in the amplified 329 bp region of the P4b gene amplicons from the investigated viral particles. Additionally, the study incorporated a phylogenetic analysis based on the nucleic acid variations in the P4b gene. The analyzed samples, along with other viral sequences, formed two distinct clades representing fowlpox viruses and pigeon pox viruses. The inclusion of out-group sequences expanded the scope of understanding regarding genetic diversity and evolutionary relationships

within the viral populations. These findings align with a prior local study conducted in Iraq by Hasan et al. (2021), which categorized f virus into two distinct clades. Clade A predominantly consisted of FPV strains from chickens (Gallus gallus), while the current study observed that pigeons were grouped within clade B. These results support previous research indicating the classification of fowlpox viruses into distinct clades, with chickens primarily belonging to clade A and pigeons grouped within clade B. In the main clade of the study, both the S1 and S2 samples showed a slight deviation compared to their neighboring positions. This deviation was attributed to a single genetic variation (205 C > G) present in both samples.

The positioning indicated that the 205 C > G variant had a minimal impact on causing a noticeable deviation from the original placement of other related viral samples within the main clade of the cladogram. The current study indicated that S2 and S3 were closely related to various strains of fowlpox virus isolated from different geographic locations worldwide. Many of these strains have been previously identified and deposited in GenBank from countries such as Iraq (Tikrit, Salah Al Deen), Egypt, Iran, Brazil, India, USA, Turkey, Tanzania, and China. The close positioning of the investigated S2 and S3 viral samples to strains from Asia, Africa, Europe, and the Americas suggests their international origins. This similarity could be attributed to factors, such as close borders and trade between neighboring countries, particularly Iran and Turkey. Moreover, the genetic similarity between local samples and globally distributed strains indicates the widespread presence of fowlpox virus variants in different regions, as noted in a study by Yeo et al. (2019). Furthermore, the observed similarity may arise from the importation of hens from infected countries to Iraq after completing their growth. Alternatively, it could indicate the presence of these isolates within Iraq due to their international distribution, as mentioned in the study by Jarmin et al. (2006).

In contrast, the analyzed viral isolates (S1, S4, S5, and S6) displayed a close relationship with adjacent sequences within the avipox virus clade, specifically those associated with pigeons. These neighboring sequences originated from various regions. The viral isolates occupied similar positions within this avipox virus clade, which included 14 sequences representing different strains. Among these sequences, the altered S4 and S6 local samples showed a slight deviation compared to the positions of their neighboring sequences. This deviation can be attributed to a single genetic variation (204 T > C) present in both of these local samples. The presence of this variation suggests that it has a limited impact on deviating from the original positions of other related viral samples within the main clade of the cladogram. The clustering of the majority of incorporated viral sequences further indicates a close phylogenetic distribution pattern among them. However, it is important to note that the examined S1, S4, S5, and S6 samples are closely associated with variable strains isolated from Australia (GenBank OP131512.1, OP131513.1).

Hence, the single nucleic acid variation identified in the local sample study indicates a minor deviation within the same viral clade, without imparting any notable evolutionary effects on the current viral positioning. The local samples S2 and S3, which are associated with the fowlpox virus, belong to the first clade, while samples S1, S4, S5, and S6, which are specific to avipox viruses that infect pigeons, form the second clade.

Although insignificant differences were observed in the current study, two outgroup clades were incorporated. These outgroup clades, namely S1 and S5, were attributed to the canarypox virus and the mixture of pigeon pox and mallard duck virus, respectively. Both outgroups were positioned in distinct phylogenetic positions, separate from the major clades of fowlpox and pigeons' viral sequences. The closest outgroup clade to the fowlpox virus was identified as the canarypox virus clade, while the closest outgroup clade to the avipox virus was the pigeonpox virus clade**.** Interestingly, the fowlpox viral sequences were located in close proximity to the roots of the phylogenetic tree, indicating their ancestral positioning compared to the canarypox virus, avipoxvirus, and pigeonpox viral sequences, respectively. Furthermore, the positioning of the three incorporated outgroups away from the root indicated that they are descendants of the ancestral fowlpox viral sequences. The results of the current study align with previous research conducted by Lüschow et al. (2004) and Jarmin et al. (2006). These studies reported that upon sequencing the amplicons, the majority of the isolates, excluding the QP-241 poxvirus from the Japanese quail, formed distinct clusters within two main Clades of avian poxviruses (APVs) including Clade A (Fowlpox virus) and Clade B (Pigeonpox virus), based on their genetic similarities. It is worth noting that the Japanese quail (QP-241) exhibited unique characteristics that distinguished it from other avian poxviruses.

CONCLUSION

In conclusion, the data presented in this study indicated that the analyzed P4b gene sequences offer a precise

description of the targeted fowl and pigeon poxviruses, without any confusion from closely related viral outgroup sequences. The use of P4b gene sequences in this study provided additional evidence for the accurate identification of viral serotypes. These findings support the observed divergence among pathogenic viral sequences infecting birds from various sources worldwide. The comprehensive phylogenetic tree constructed based on the P4b gene serves as a valuable tool for effectively identifying viral serotypes. This highlights the capability of the currently employed P4b gene-specific primers to accurately depict the studied fowl and pigeon poxviruses and their phylogenetic positions. Overall, the effectiveness of these P4b gene-based fragments in identifying viral sequences has been demonstrated, underscoring the precision of the currently utilized P4b gene-specific primers in characterizing the investigated fowl and pigeon poxviruses and determining their phylogenetic relationships.

DECLARATIONS

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Availability of data and materials

The data and materials utilized in this study can be obtained by contacting the corresponding author directly or accessing them through a designated data repository.

Ethical considerations

Ethical issues (plagiarism, consent to publication, misconduct, data fabrication and/ or forgery, double publication and/ or submission and replication) were checked by the authors.

Authors' contributions

Aisha Faisal and Amer Al-Azzawi jointly proposed the hypothesis, designed the study, collected samples from various poultry farms, conducted the molecular work Both authors contributed to the preparation of the manuscript, and approved the final edition of the article.

Competing interests

The Authors declare that they have no competing interests.

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Effects of *Moringa oleifera* Leaf Meal on Local Guinea Fowl Breeder Hens' Performance, Egg Quality, and Blood Parameters

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ABSTRACT

Breeding local guinea fowl has the potential to address protein malnutrition and alleviate poverty in West African countries. The current study aimed to examine the effects of incorporating *Moringa oleifera* leaf meal into the diet on hematology and biochemical parameters as well as the productive performance of local guinea fowl breeders in Togo. Thus, 512 local guinea fowls (22 weeks of age with an average weight of 1176.7 ± 2.9 g), comprising 384 females and 128 males, were examined for 31 weeks. The fowls were randomly assigned to four dietary groups, namely M0, M1, M2, and M3 containing 0%, 0.5%, 1%, and 1.5% of *Moringa oleifera* leaf meal in diets, respectively. Each group had 4 replicates of 32 fowls. During the study, feed intake, body weight, egg-laying rate, and feed conversion ratio were weekly recorded. Blood samples for hematology and biochemical analysis were taken from 12 females (3/replicate) at 34 and 50 weeks of age. Eggs were collected for the quality evaluation. Results showed that feed intake was comparable across all groups during the rearing period (23 to 33 weeks of age). However, it significantly decreased in fowls of the M2 and M3 during the laying period (34 to 50 weeks of age). The live weight of fowls in M3 was significantly higher than other groups before the laying period. The egg production, yolk ratio, and shell ratio of the birds in the M1 were like that of M0 and higher than that of M2 and M3. The feed conversion ratio was not significantly different between groups during the laying period. However, the albumen ratio and haugh unit were improved by feeding *Moringa* leaves especially at level 1.5%. The level of white blood cells and lymphocytes decreased by feeding the *Moringa* leaves at 1%. Neutrophils and platelet levels were comparable across groups. Total proteins, albumin, and transaminases increased in *Moringa* groups (M1, M2, M3), especially in M3. It was concluded that the use of *Moringa oleifera* leaves at 0.5% improved egg-laying performance in local guinea fowl. Moreover, 1% and 1.5% of incorporation improved the quality of eggs.

Keywords: Blood parameter, Breeder, Guinea fowl, Egg quality, *Moringa oleifera*

INTRODUCTION

The rapid human population growth has led to an increase in meat consumption (Boko et al., 2012; Popkin et al., 2012). The breeding of local guinea fowls (*Numida meleagris*) is one of the animal production sectors that contributes to meeting the animal protein requirements of the population in West African countries (Konlan et al., 2011; Sodjedo et al., 2022). Local guinea fowl production is considered in West African countries, especially in Togo, as a sector for poverty reduction (Konlan et al., 2011; Sodjedo et al., 2022). It originates from Africa (Ikani and Dafwang, 2004), and guinea fowls have also a cultural significance (Koné et al., 2018). However, guinea fowl production still faces many difficulties such as low growth and availability of post-hatch juveniles and their high mortality rate during the start (Sanfo et al., 2008; Houndonougbo et al., 2017). Also, laying in local guinea

fowl is in cycles (about 75 to 110 eggs per year) and coincides with the rainy season (Konlan et al., 2011; Sodjedo et al., 2022). However, Sodjedo et al. (2022) reported that guinea fowl can lay throughout the year if they are fed adequate feed *ad libitum*. Thus, feed is one of the major factors in developing local guinea fowl breeding (Lombo et al., 2018).

Several studies have shown that the nutritional composition of feed consumed by chickens influences egg laying, egg quality, and hatchability (Teteh et al., 2016; Voemesse et al., 2019; N'nanle et al., 2020). The productivity of animals can be enhanced by utilizing natural feed additives derived from herbs, spices, or other plants. These additives exert positive effects on digestibility, nutrient absorption, and the control of parasites in the digestive tract (Sadr et al., 2022; Eftekhari Hasan Abad and Ghaniei, 2023; Nyembo et al., 2023). *Curcuma longa*, incorporated at the levels of 0.5% and 1% in layer mash has improved egg weight, shell thickness, and yolk ratio (Radwan et al., 2008). Houndonougbo et al. (2012) reported significant improvement in egg weight, albumen, and yolk ratio of guinea fowls at 21 and 32 weeks of age when incorporating dried leaves of *Manihot esculenta* into their layer mash.

Moringa oleifera is a leguminous plant and a feed additive used in animal production (Ahmed and Lohakare, 2021; Giang et al., 2023). Its leaves are rich in carbohydrates, lipids, proteins, minerals, salts, and vitamins (Kashyap et al., 2022; Ahmed et al., 2023; Yang et al., 2023a). *Moringa oleifera* is known to have antioxidant, anti-inflammatory, hypoglycemic, hypolipidemic, cholesterol-reducing, and hepatoprotective properties (Khalid et al., 2023; Kashyap et al., 2022; Ntshambiwa et al., 2023). The leaves contain also antinutritional substances such as tannins, saponins that limit their use (Khalid et al., 2023; Kashyap et al., 2022). In breeding, several studies have shown an improvement in the production performance of cows (Mendieta-Araica et al., 2011), small ruminants (Fadiyimu et al., 2010; Sultana et al., 2015), rabbits (Abubakar et al., 2015) with the use of *Moringa oleifera* leaves. In poultry, Voemesse et al. (2019) and Ahmed and Lohakare (2021) reported an increase in the production and weight of eggs when *Moringa* leaves are incorporated into the diet of laying hens. Yang et al. (2023b) have shown an improvement in feed conversion ratio (from hatching to 4 weeks of age), laying rate, average egg weight, and feed conversion rate in laying ducks fed with *Moringa* flavonoid meal diets. The leaves of *Moringa Oléifera* improved the performance (feed conversion ratio, laying rate, average egg weight) of Sasso breeder hens and the internal quality of hatching eggs (N'nanle et al., 2020). *Moringa oleifera* also improves broiler chicken growth performance (Body weight gain, feed conversion ratio), carcass characteristics, and cecal microbial structure (El-Tazi 2014; Zhang et al., 2023).

Despite numerous studies and scientific reports on the effects of *Moringa oleifera* leaves, research on the incorporation of *Moringa oleifera* leaf meal in enhancing the productive performance of local guinea fowl breeders remains limited. Therefore, the current study aimed to evaluate the effects of *Moringa oleifera* leaf meal on hematology and biochemical parameters as well as the productive performance of local guinea fowl breeders.

MATERIAL AND METHODS

Ethical approval

The animal care guidelines recommended by the Animal Ethics Committee of the University of Lome in Togo were followed (008/2021/BC-BPA/FDS-UL).

Study area

The Regional Center of Excellence in Poultry Sciences (CERSA) of the University of Lomé in Togo served as a framework for the study. The manipulations were conducted in the Poultry Production Techniques laboratories of the Regional Center of Excellence in Poultry Sciences and the laboratories of the Higher School of Biological and Feed Techniques (ESTBA) of the University of Lomé. The experimental management of the breeders lasted 31 weeks, from November 2021 to June 2022. The guinea fowl were provided by the Poultry Production Techniques laboratories of the Regional Center of Excellence in Poultry Sciences.

Preparation of *Moringa oleifera* leaves and diet formulation

Moringa oleifera leaves were collected from rural areas of Togo (in Togoville: 6°14'28.68"N and 1°29'06.43"E; in the prefecture of Vo), spread out on a clean surface and dried with natural ventilation. The dried leaves were milled into powder form before their incorporation into feed. The experimental diets were formulated to contain 0% (control), 0.5% (500g added to 100kg of diet), 1% (1000g added to 100kg of diet), and 1.5% (1500g added to 100kg of diet) of dry matter of *Moringa oleifera* leaves. All diets were iso-nitrogenous and iso-caloric. The compositions of the experimental diets are shown in Table 1.

Table 1. Composition (%) of experimental diets according to *Moringa oleifera* treatments during 22-31 weeks of age and 32-50 week of age (laying period) in guinea fowl

	Feed composition according to age and group							
	22-31 weeks of age				32-50 weeks of age			
Ingredient	**M0**	**M1**	**M2**	**M3**	**M0**	**M1**	**M2**	**M3**
Maize	54	53.5	53.5	53	59	59	59	59
Wheat bran	17	17	17	17	13	12.5	12	12
Roasted soybean	19	19	18.5	18.5	18	18	18	17.5
Laying concentrate	2	2	2	2	2	2	2	2
oyster shell	7	7	7	7	7	7	7	7
Methionine	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Lysine	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Moringa oleifera leaf	0	0.5	1	1.5	0	0.5	1	1.5
Total	100	100	100	100	100	100	100	100
Calculated analysis								
ME (Kcal/Kg)	2787	2791	2795	2799	2849	2860	2870	2873
Crude protein (%)	17.72	17.67	17.45	17.40	17.06	17.13	17.21	17.13
Calcium (%)	2.27	2.26	2.26	2.26	2.27	2.28	2.29	2.30
Phosphorus (%)	0.53	0.52	0.52	0.52	0.49	0.48	0.48	0.48
Methionine (%)	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78
Lysine (%)	1.07	1.07	1.05	1.05	1.03	1.03	1.03	1.03
Methionine + Cystine (%)	1.02	1.02	1.01	1.01	1	1	0.99	0.99

ME: Metabolizable **e**nergy; M0, M1, M2, M3: Treatments with 0%, 0.5%, 1%, and 1.5% *Moringa oleifera* leaves in diet, respectively. The study lasted 28 weeks.

Study design and animal management

For this study, a total of 384 female local guinea fowl breeders and 128 males, all aged 22 weeks, were randomly divided equally into four dietary treatments, each with four replicates. This distribution entailed 24 females and 8 males per replicate following a study by Sodjedo et al. (2022). In each replicate, including the control group (M0) and three other groups (M1, M2, M3), guinea fowls were fed accordingly. The control group was provided with a basal diet devoid of *Moringa oleifera* leaves. The other treatment groups received the same basal diet, with the addition of 0.5% (M1), 1% (M2), and 1.5% (M3) dry matter of Moringa oleifera leaves. After one week of acclimatization, the four experimental diets were randomly assigned and fed to the guinea fowls *ad libitum*. Guinea fowls were reared in an open henhouse, wood partitioned (2.7 m × 2 m) with a deep litter floor housing system at a density of 6 per square meter (Nahashon et al., 2006). Water was offered *ad libitum* throughout the experiment and natural light (dry season with 25°C on average and rainy season with 27.3°C on average) was used as the source of lighting. All treatments followed the same prophylaxis program, including anti-infection prevention with oxyfuran 4® (1g/l of water) for days 1 to 4, anti-Newcastle vaccine with Hitchner B1 for day 3, coccidiosis prevention with Amprolium 20%® (3g/5l of water) for days 12 to 16, deworming with Pipérazine citrate® (2g/l of water for 1 day) and anti-Newcastle vaccine with Cevac® for week 3, deworming with VSP® (¼ tablet for 1 day) for week 8, anti-infection prevention with oxyfuran 4® (1g/l of water for 5 days) for week 10, an anti-Newcastle vaccine with Ita-New for week 14, deworming with VSV® for week 17, coccidiosis prevention with Amprolium 20%® (3g/5l of water for 5 days) for week 22, and deworming with VSV® for week 26 (Hien et al., 2002; Lombo et al., 2011).

Data collection and calculated parameters

Throughout the experiment, the amount of feed offered to the breeders each day was measured. Feed intake (FI) was calculated weekly as the difference between feed offered and leftover for each replication. Feed conversion ratio (FCR) was calculated as the ratio of total feed consumed to total body weight gain (BWG) before laying (total FI/total BWG, Voemesse et al., 2018). During the laying period, eggs were collected daily, and the laying rate was registered weekly (sum of daily egg number × 100/sum of daily bird number). Feed conversion ratio was calculated with average egg laying rate, egg weight, and FI as grams of egg mass per gram of feed consumed (daily FI/[laying rate × average egg weight]) following N'nanle et al. (2020).

At the onset of the experiment and then every week before the egg-laying period, the fowls underwent weighing to ascertain BWG corresponding to their respective treatment groups. Body weight gain was computed by subtracting the initial weight from the successive body weight measurements during the experimental (final weight-initial weight). Additionally, daily weight gain (DWG) was calculated as the ratio of BWG to the duration of the

experimental period (in days). Mortalities recorded were utilized to calculate the mortality rate.

At 38, 42, 46, and 50 weeks of breeders' age, 32 eggs per treatment were weighed and broken delicately to collect shell, albumen, and yolk (N'nanle et al., 2020). These components were also weighed to determine their ratio (component weight × 100/absolute egg weight, N'nanle et al., 2020) and Haugh Unit (HU) with the following relationship, $HU = 100 \times \log (Hb - 1.7W^{0.37} + 7.6)$, where, Hb denotes the height of thick albumen and W is egg weight (N'nanle et al., 2020).

At the end of the experiment, abdominal fat and organs (heart, liver, kidney, intestine, and gizzard), randomly taken from 8 females per treatment were weighed to determine their ratio (abdominal fat or organs weight × 100/live weight, N'nanle et al., 2020). Fowls were stunned and slaughtered for the samples.

At 33 and 50 weeks of breeders' age, venous blood samples (wing vein, approximately 2 ml) were taken from 12 females (chosen at random) per treatment. These blood samples were collected in tubes containing Ethylene Diamine Tetra Acetic Acid (EDTA) for blood count and blood formula (NFS) and in dry tubes, without anticoagulant, for assay of biochemical parameters. For hematological parameters, the blood samples were used on Mindray BC-3000 Auto Hematology Analyzer, from Mindray Buiding, Keji 12th Road South, High-tech Industrial Park, Nanshan, Shenzhen 518057, P.R. China. For biochemical parameters, blood samples were centrifuged at 3000 rpm. Serum was collected for total protein, albumin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatinine, uric acid, urea, triglycerides, total cholesterol, and HDL-Cholesterol measuring. Blood parameters were determined by colorimetric method using Mindray BS Auto-biochemical analyzer from China.

Statistical analysis

All data collected were analyzed using ANOVA with GraphPad Prism 8.1 software followed by Tukey's test for comparisons between treatments. Data obtained were expressed as mean ± standard deviation of the mean and as a percentage. Results were statistically significant when $p < 0.05$.

RESULTS

Feed intake

The effect of *Moringa oleifera* leaves on FI before and during lay is presented in Table 2. According to the result of the study, there was no significant difference in FI across treatments before laying (23 to 33 weeks of age, $p > 0.05$). However, during the laying (34 to 50 weeks of age), guinea fowls in M2 and M3 had significantly lower FI, compared to those in other groups ($p < 0.05$).

Daily weight gain

Before egg-laying, the daily weight gain was comparable between treatments M0, M1, and M2, but significantly lower than that of the M3 treatment, as indicated in Table 3 ($p < 0.05$).

Egg laying rate and egg weight

Figure 1 shows the effect of *Moringa oleifera* leaves on the improvement of the laying rate according to the age of guinea fowls. Egg laying rates were similar among treatments from week 34 to week 39 of breeders' age ($p > 0.05$). However, starting from week 40, the laying rate of groups M0 and M1 exceeded that of other groups, except for week 49 when laying rates remained similar between treatments ($p < 0.05$).

The average laying rate of groups M0 and M1 was higher than that of groups M2 and M3, as shown in Table 3 ($p < 0.05$).

The average egg weight increased with breeders' age in each group and there was no significant difference between groups ($p > 0.05$) although M3 had numerically the highest egg weight (Table 3).

Feed conversion ratio

The FCR, before and during the laying period, is shown in Table 2. Before laying, FCR values of groups M0, M1, and M2 were not significantly different ($p > 0.05$) but significantly higher than that of group M3 ($p < 0.05$). During laying, FCR values of all treatments were insignificantly different ($p > 0.05$). However, M2 and M3 groups recorded numerically the highest FCR.

Mortality rate

The mortality rate was relatively low in all groups. Group M0 recorded a higher rate of mortality ($p < 0.05$) and there was no significant difference between the M1 and M2 groups ($p > 0.05$). The M3 treatment recorded the lowest mortality rate (Table 3).

Average weight of organs and abdominal fat

Concerning gizzard weight the difference between treatments was insignificant ($p > 0.05$). The weight of the liver and the intestine of the guinea fowls of group M3 was significantly higher than that of the M0, M1, and M2

groups ($p < 0.05$). For these same parameters, the difference was not significant between M1 and M2 groups ($p > 0.05$). The M0 group recorded the lowest values ($p < 0.05$) of liver and intestine weight. The absolute heart weight values of groups M2 and M3 were higher than those of M0 and M1 ($p < 0.05$). Abdominal fat and carcass weight were higher in groups M0 and M1 than in groups M2 and M3, although the difference was not significant in the fat abdominal area ($p > 0.05$), as shown in Table 4.

Egg components and haugh unit

Table 5 shows the yolk ratio albumen ratio and shell ratio. Concerning the yolk ratio and shell ratio the difference was insignificant between all groups. The albumin ratio of groups M3 and M2 was significantly higher than that of M0 and M1 groups, especially at 38 and 50 weeks of age ($p < 0.05$).

Concerning the Haugh Unit, there was no significant difference across all groups at week 38 ($p > 0.05$). However, at 42, 46, and 50 weeks of age, the Haugh Unit of eggs from M3 breeders was significantly higher compared to the other groups, while the group M0 recorded the lowest values, as shown in Figure 2 ($p < 0.05$).

Hematological parameters

The level of white blood cells (WBC) and lymphocytes detected in guinea fowls of M0 and M1 treatments were higher than that of M2 and M3 treatments ($p < 0.05$) at 34 and 50 weeks of age. Regarding the red blood cell (RBC) count, there were insignificant differences between treatments ($p > 0.05$). At 34 weeks of age, blood platelets and neutrophils showed no significant differences among all treatments ($p > 0.05$). However, at 50 weeks of age, blood platelet levels decreased with the incorporation of Moringa oleifera leaves in the M2 and M3 groups (Table 6).

Biochemical parameters

Serum parameter concentrations are summarized in Table 7. Total protein level at 50 weeks of age was significantly higher in treatments M1, M2, and M3, compared to the control group ($p < 0.05$). At 34 weeks of age, the levels of alanine aminotransferase and uric acid were significantly higher in groups M1, M2, and M3, compared to the control ($p < 0.05$). At 50 weeks of age, the levels of albumin, aspartate aminotransferase, alanine aminotransferase, and uric acid were significantly higher in groups M1, M2, and M3, compared to the control ($p < 0.05$). Triglyceride levels were lower in M1, M2, and M3 compared to M0 at 34 weeks of age ($p < 0.05$). At 50 weeks of age, triglyceride decreased in M2 and M3, compared to M0 and M1 ($p < 0.05$). Total cholesterol level decreased in M1, M2, and M3, compared to M0 at 34 weeks of age. There was no significant difference between treatments concerning total cholesterol and HDL-CH levels at 50 weeks of age ($p > 0.05$).

Table 2. Feed intake and feed conversion ratio according to *Moringa oleifera* treatments and age of guinea fowls

Age \ Groups	Parameters	M0	M1	M2	M3	P-value
23 - 33	FI	60.3 ± 1.8	60.58 ± 1.07	59.36 ± 0.97	61.37 ± 1.4	0.7752
	FCR	13.08 ± 0.46^{a}	13.05 ± 0.23^{a}	14.44 ± 0.37^{a}	11.07 ± 0.14^{b}	0.0085
34 - 50	FI	89.76 ± 1.02^{a}	89.57 ± 0.58^{a}	82.62 ± 0.75^{b}	84.04 ± 0.41^{b}	0.0044
	FCR	4.44 ± 0.18	4.48 ± 0.11	4.71 ± 0.2	4.55 ± 0.23	0.7536

M0, M1, M2, M3: Treatments having received respectively 0%, 0.5%, 1% and 1.5% *Moringa oleifera* leaves in the diet; FI: Feed intake FCR: Feed conversion ratio; ^{a,b,c} Within row, values not sharing the same letters are significantly different ($p < 0.05$).

Table 3. Daily weight gain (23-33 weeks of age), laying rate and egg weight (34-50 weeks of age), mortality rate (22-50 weeks of age) of guinea fowls according to *Moringa oleifera* treatments

Parameters \ Groups	M0	M1	M2	M3	P-value
DWG (g)	4.20 ± 0.08^{b}	4.23 ± 0.11^{b}	4.04 ± 0.04^{b}	4.88 ± 0.13^{a}	0.0123
Laying rate (%)	50.53 ± 2.02^{a}	49.94 ± 1.88^{a}	43.04 ± 1.74^{b}	44.62 ± 1.58^{b}	0.0188
Egg weight (g)	39.14 ±0.40	39.26 ± 0.43	39.26 ± 0.41	39.75 ± 0.48	0.9143
Mortality rate (%)	15 ± 0.78^{a}	13.33 ± 0.57^{ab}	13.33 ± 0.48^{ab}	11.67 ± 0.24^{b}	0.0473

M0, M1, M2, M3: Treatments having received respectively 0%, 0.5%, 1%, and 1.5% *Moringa oleifera* leaves in the diet; DWG: Daily weight gain; ^{a,b,c} Within row, values not sharing the same letters are significantly different ($p < 0.05$).

Table 4. Average weight and ratio of organs and abdominal fat of guinea fowls at 50 weeks of age according to *Moringa oleifera* treatments

Parameters	Groups	M0	M1	M2	M3	p-value
Average weight (g)	Heart	6.67 ± 0.24^{b}	6.45 ± 0.26^{b}	7.4 ± 0.24^{ab}	8 ± 0.2^{a}	0.0023
	Liver	27.33 ± 0.33^{c}	31.33 ± 1.2^{b}	31.67 ± 0.88^{b}	38.67 ± 0.33^{a}	<0.0001
	Kidney	6 ± 0.41^{ab}	7.25 ± 0.25^{a}	5.88 ± 0.31^{b}	6.5 ± 0.29^{ab}	0.0403
	Gizzard	27 ± 1.23	27 ± 1.78	27.5 ±1.85	27.75 ±1.11	0.9796
	Intestine	100.75 ± 0.75^{c}	111 ± 1.08^{b}	119.75 ± 1.25^{b}	141 ± 4.45^{a}	<0.0001
	MPS	73.5 ± 2.22^{c}	92 ± 2.16^{a}	80.25 ± 0.63^{bc}	82 ± 2.04^{b}	<0.0001
	Abdomina fat	11.50 ± 0.65	11.75 ± 0.48	10.25 ± 0.48	10.5 ± 0.46	0.1678
	Carcass	1386.75 ± 34.4^{ab}	1465.25 ± 9.6^{a}	1247.5 ± 19.4^{c}	1300.5 ± 31.9^{bc}	0.0003
Ratio (%)	Heart	0.4 ± 0.007^{b}	0.37 ± 0.004^{b}	0.48 ± 0.009^{a}	0.51± 0.006^{a}	0.0033
	Liver	1.64 ± 0.1^{b}	1.82 ± 0.114^{b}	2.07 ± 0.13^{ab}	2.48 ± 0.1^{a}	0.0002
	Kidney	0.36 ± 0.015	0.42 ± 0.011	0.38 ± 0.007	0.42 ± 0.013	0.0923
	Gizzard	1.62 ± 0.03^{ab}	1.57 ± 0.02^{b}	1.8 ± 0.045^{a}	1.78 ± 0.053^{ab}	0.0496
	Intestine	6.03 ± 0.12^{c}	6.44 ± 0.15^{c}	7.82 ± 0.18^{b}	9.05 ± 0.19^{a}	<0.0001
	MPS	4.40 ± 0.11	5.34 ± 0.24	5.24 ± 0.19	5.27 ± 0.27	0.1251
	Abdominal fat	0.69 ± 0.012	0.68 ± 0.025	0.67 ± 0.015	0.67 ± 0.013	0.1977
	Carcass	83.01 ± 0.83^{ab}	85.02 ± 1.01^{a}	81.51 ± 0.92^{c}	83.51 ± 0.81^{bc}	0.0007

M0, M1, M2, M3: Treatments having received respectively 0%, 0.5%, 1%, and 1.5% *Moringa oleifera* leaves in their diet; MPS: Superficial pectoral muscle; ^{a,b,c} Within row, values not sharing the same letters are significantly different ($p < 0.05$).

Table 5. Egg components ratio of guinea fowls according to *Moringa oleifera* treatments at different ages

Parameters	Groups / Age (weeks)	M0	M1	M2	M3	p-value
Yolk ratio	38	34.12 ± 0.1	34.30 ± 1.05	36.08 ± 1.36	32.02 ± 1.01	0.1747
	42	31.54 ± 0.87	32.06 ± 1.1	32.35 ± 1.1	32.93 ± 0.5	0.7658
	46	29.95 ± 1.08	31.12 ± 1.85	29.60 ± 1.2	29.54 ± 0.44	0.7947
	50	31.06 ± 0.97	33.12 ± 2.02	30.48 ± 1.08	30.80 ± 2.2	0.6927
Albumen ratio	38	51.44 ± 0.22^{bc}	50.40 ± 0.2^{c}	52.42 ± 0.12^{ab}	52.99 ± 0.17^{a}	0.0020
	42	51.53 ± 0.5	51.45 ± 0.21	51.97 ± 0.18	52.28 ± 0.38	0.3931
	46	50.59 ± 0.19	51.99 ± 0.56	52.06 ± 1.09	52.78 ± 0.08	0.1376
	50	50.42 ± 0.21^{c}	50.94 ± 0.18^{bc}	51.80 ± 0.14^{b}	53.20 ± 0.16^{a}	0.0016
Shell ratio	38	14.46 ± 0.29	13.89 ± 0.23	14.20 ± 1.01	14.67 ± 0.17	0.7719
	42	16.38 ± 0.17	16.02 ± 0.84	16.75 ± 0.21	17.49 ± 0.33	0.8667
	46	18.60 ± 0.70	19.24 ± 0.98	17.41 ± 1.02	17.68 ± 0.79	0.3690
	50	17.66 ± 0.12	16.46 ± 0.67	17.12 ± 0.88	16.60 ± 1.01	0.9327

M0, M1, M2, M3: Treatments having received respectively 0%, 0.5%, 1%, and 1.5% *Moringa oleifera* leaves in the diet, ^{a,b,c} Within row, values not sharing the same letters are significantly different ($p < 0.05$).

Table 6. Hematology parameters of guinea fowls according to *Moringa oleifera* treatments at different ages

Age (weeks)	Groups / Blood parameters	M0	M1	M2	M3	p-value
34	WBC (10^{3}/µL)	44.85 ± 0.57^{b}	62.80 ± 2.81^{a}	30.88 ± 0.89^{c}	41.04 ± 3.44^{b}	<0.0001
	RBC (10^{6}/µL)	2.55 ± 0.16	2.34 ± 0.30	2.19 ± 0.13	2.23 ± 0.13	0.5795
	Hemoglobin (g/dl)	11.17 ± 0.49^{a}	9.17 ± 1.37^{b}	9.20 ± 0.36^{b}	9.13 ± 1.04^{b}	0.0347
	Hematocrit (%)	41.57 ± 3.08^{a}	38.13 ± 5.11^{ab}	37.70 ± 2.4^{b}	37.43 ± 3.74^{b}	0.0184
	PLT (10^{3}/µL)	1.67 ± 0.33	1.33 ± 0.33	2.33 ± 0.67	2.33 ± 0.33	0.3437
	Neutrophil (10^{3}/µL)	15.93 ± 3.02	33.54 ± 11.4	20.49 ± 1.8	37.60 ± 18.8	0.5022
	Lymphocyte (10^{3}/µL)	14.06 ± 0.3^{b}	23.39 ± 4.5^{a}	9.29 ± 1.28^{c}	12.28 ± 4.25^{b}	0.0125
	Monocyte (10^{3}/µL)	1.59 ± 1.2	0.45 ± 0.1	0.54 ± 0.07	0.66 ± 0.39	0.6171
	Basophil (10^{3}/µL)	7.04 ± 2.2	5.48 ± 0.5	7.23 ± 0.5	6.15 ± 0.6	0.7224
50	WBC (10^{3}/µL)	92.01 ± 4.2^{a}	64.53 ± 5.78^{b}	35.80 ± 2.9^{c}	61.97 ± 6.8^{b}	<0.0001
	RBC (10^{6}/µL)	2.43 ± 0.08	2.35 ± 0.27	2.54 ± 0.26	2.59 ± 0.3	0.8988
	Hemoglobin (g/dl)	9.40 ± 0.46	9.43 ±1.7	9.77 ± 1.5	11 ± 1.6	0.8267
	Hematocrit (%)	38.73 ± 0.74^{b}	38.93 ± 7.8^{b}	42.13 ± 5.8^{ab}	45.63 ± 5.9^{a}	0.0028
	PLT (10^{3}/µL)	1.67 ± 0.33	1 ± 0.58	1 ± 0.58	0.67 ± 0.33	0.5319
	Neutrophil (10^{3}/µL)	59.73 ± 19.62^{a}	40.34 ± 26.3^{b}	15.76 ± 1.6^{c}	36.28 ± 9.9^{b}	0.0028
	Lymphocyte (10^{3}/µL)	25.16 ± 6.35^{a}	17.81 ± 8.7^{b}	11.41 ± 2.34^{c}	14.14 ± 1.06^{bc}	<0.0001
	Monocyte (10^{3}/µL)	0.30 ± 0.03	0.25 ± 0.02	0.30 ± 0.1	0.28 ± 0.04	0.9130
	Basophil (10^{3}/µL)	6.82±1.6	6.17±1.4	7.33±1.3	7.93±1.7	0.8562

M0, M1, M2, M3: Treatments having received respectively 0%, 0.5%, 1%, and 1.5% *Moringa oleifera* leaves in the diet; WBC: White blood cell; RBC: Red blood cell; PLT: Blood platelets; ^{a,b,c} Within row, values not sharing the same letters are significantly different ($p < 0.05$).

Table 7. Biochemical parameters of guinea fowls according to *Moringa oleifera* treatment at different ages

Age (weeks)	Groups / Blood parameters	M0	M1	M2	M3	p-value
34	Total protein (g/L)	30.9 ± 0.33^{a}	23.7 ± 0.48^{b}	25.85 ± 0.14^{b}	25.95 ± 1.14^{b}	<0.0001
	Albumin (g/L)	7 ± 0.29^{b}	7.1 ± 0.36^{b}	7.2 ± 0.12^{b}	9.13 ± 0.26^{a}	0.0003
	AST (U/L)	251.5 ± 2.87^{c}	251.25 ± 3.04^{c}	271.25 ± 1.7^{b}	300.5 ± 3.86^{a}	<0.0001
	ALT (U/L)	9 ± 0.41^{c}	12 ± 0.41^{ab}	11.75 ± 0.32^{b}	13.5 ± 0.35^{a}	<0.0001
	Creatinine (mg/dl)	0.14 ± 0.04	0.21 ± 0.07	0.11 ± 0.03	0.14 ± 0.04	0.5290
	Uric Acid (mg/dl)	6.4 ± 0.24^{c}	7.5 ± 0.38^{b}	6.95 ± 0.27^{bc}	8.75 ± 0.36^{a}	0.0249
	Urea (g/L)	0.03 ± 0.007	0.03 ± 0.004	0.03 ± 0.007	0.03 ± 0.004	0.9780
	Triglycerides (g/L)	5.86 ± 0.13^{a}	3.52 ± 0.1^{c}	5.30 ± 0.11^{b}	2.95 ± 0.13^{d}	<0.0001
	Cholesterol (g/L)	1.34 ± 0.12^{a}	0.85 ± 0.04^{b}	0.99 ± 0.04^{b}	1.07 ± 0.04^{b}	0.0034
	HDL-CH (g/L)	0.37 ±0.04	0.45 ± 0.02	0.34 ± 0.04	0.45 ± 0.03	0.1068
50	Total protein (g/L)	27.53 ± 1.9^{b}	41.35 ± 1^{a}	37.93 ± 0.55^{a}	39.08 ± 0.62^{a}	<0.0001
	Albumin (g/L)	5.38 ± 0.19^{b}	6.73 ± 0.18^{a}	7.28 ± 0.28^{a}	7.23 ± 0.16^{a}	<0.0001
	AST (U/L)	252 ± 4.04^{c}	333.5 ± 10^{b}	381.25 ± 4.42^{a}	356.75 ± 5.2^{ab}	<0.0001
	ALT (U/L)	4.5 ± 0.29^{c}	8 ± 0.2^{a}	6 ± 0.41^{b}	7.5 ± 0.29^{a}	<0.0001
	Creatinine (mg/dl)	0.18 ± 0.01^{b}	0.25 ± 0.02^{ab}	0.25 ± 0.03^{ab}	0.30 ± 0.02^{a}	0.0118
	Uric Acid (mg/dl)	5.45 ± 0.22^{c}	7.49 ± 0.35^{b}	6.59 ± 0.18^{b}	9.38 ± 0.26^{a}	<0.0001
	Urea (g/L)	0.03 ±0.007	0.03 ± 0.005	0.04 ± 0.008	0.04 ± 0.005	0.3362
	Triglycerides (g/L)	5.37 ± 0.13^{b}	7.20 ± 0.3^{a}	4.44 ± 0.43^{bc}	4.12 ± 0.09^{c}	<0.0001
	Cholesterol (g/L)	1.15 ± 0.05	1.31 ± 0.08	1.20 ± 0.19	1.16 ± 0.13	0.8726
	HDL-CH (g/L)	0.35 ± 0.06	0.34 ± 0.08	0.35 ± 0.1	0.44 ± 0.1	0.8548

M0, M1, M2, M3: Treatments having received respectively 0%, 0.5%, 1%, and 1.5% *Moringa oleifera* leaves in the diet; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; HDL-CH: High density lipoproteins-cholesterol; ^{a,b,c} Within a row, values not sharing the same letters are significantly different ($p < 0.05$).

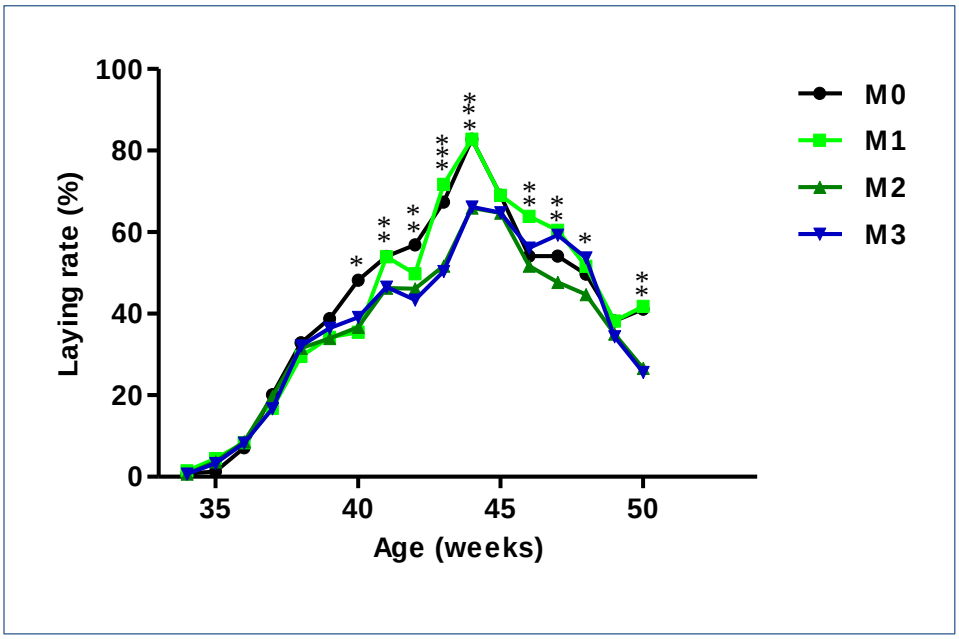

Figure1. Laying rate according to age (34-50 weeks of age) and *Moringa oleifera* treatment (* show significant difference at 0.05). M0, M1, M2, M3: Treatments having received 0%, 0.5%, 1% and 1.5% *Moringa oleifera* leaves in diet respectively; at each age, significant differences are indicated by*($p < 0,05$); ** ($p < 0,01$); *** ($p < 0.001$).

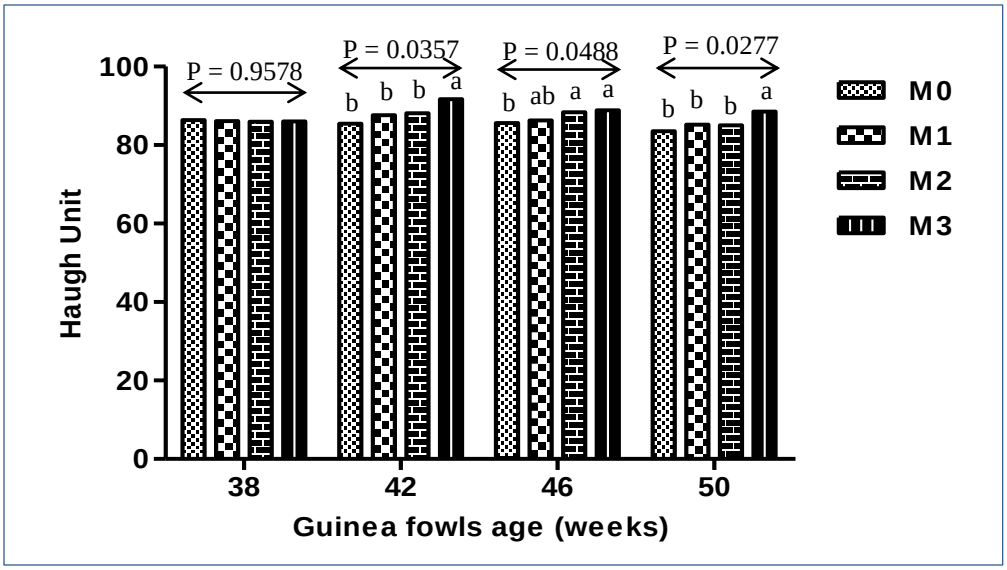

Figure 2. Haugh Unit according to different ages (38, 42, 46, and 50 week of age) and *Moringa oleifera* treatment. M0, M1, M2, M3: Treatments having received respectively 0%, 0.5%, 1%, and 1.5% *Moringa oleifera* leaves in diet; [a,b,c] Within a row, values not sharing the same letters are significantly different ($p < 0.05$).

DISCUSSION

The present study demonstrated clearly that the incorporation of *Moringa oleifera* leaves in the local guinea fowl breeder diet affected productive performance, hematological as well as biochemical values, and egg quality. Before the laying period, the similarity in FI is comparable to that reported by Voemesse et al. (2018) in laying chickens during juvenile growth and Teteh et al. (2016) in ISA brown chicks (layer-type). Sanchez et al., (2006) estimated that *Moringa oleifera* leaves did not contain any factors that could limit feed consumption. However, a decrease in feed consumption with 10% of *Moringa oleifera* leaves in one-week-old chicks was obtained by Ashong and Brown (2011). This difference could be explained by the level of incorporation of *Moringa oleifera* leaves, which reached a maximum of 1.5% in this experiment. Additionally, it is important to consider the poultry species used in this study, which was guinea fowl, as opposed to laying chickens or ISA brown chicks examined in the studies referenced. In the laying period, the decrease in feed consumption in M2 and M3 was also reported by N'nanle et al. (2020) on Sasso breeding hens and Voemesse et al. (2019) on laying hens. This result could be explained by the cumulative effect of *Moringa oleifera* leaves with the age of animals, which would increase the level of components such as saponins, tannins, and fibers (Gupa et al., 1989; Kakengi et al., 2007). According to Francis et al. (2001), the presence of these substances creates a feeling of satiety in animals and slows down the progression of feed in the digestive tract. This situation could lead to a decrease in feed consumption. Furthermore, the higher weight of intestines observed in the *Moringa oleifera* groups could be attributed to a slowing down of feed progression in the digestive tract.

The reduction of FCR in group M3 is consistent with the increase in weight gain of fowls in the same group. The same results were reported by Sarker et al. (2017) and Teteh et al (2013) in broilers and by Teteh (2016) and Voemesse et al. (2018) in laying hens. The high BWG in M3 treatment could be due to the richness and quality of the nutrients contained in *Moringa* leaves (Teteh et al., 2016; Manzo et al., 2016). Likewise, *Moringa oleifera* leaves have antimicrobial (Djakalia et al., 2011; Divya et al., 2014; Ahmed et al., 2023), immunomodulatory (Tété-Bénissan et al., 2013), and antioxidant (Santos et al., 2012; Wiwit et al., 2016) properties that can improve animal health and nutrients uptake. Thus, by reducing intestinal microflora (*Escherichia coli* and *Staphylococcus aureus*), *Moringa oleifera* leaves reduce gut competition for available nutrients (Voemesse et al., 2019; Ahmed et al., 2023). This mechanism contributes to the higher concentration of proteins, such as albumin in blood which is known as one of the main serum proteins serving as an amino acid source for the synthesis of tissue proteins in the growth period of birds (Yaman et al., 2000). All these factors would have contributed to the better BWG obtained by the M3 group. Furthermore, the hematological results at the start of the lay show a significant decrease in

white blood cell counts and lymphocytes with *Moringa oleifera* leaves levels in the diet. However it shows that a high white blood cell count is associated with an infection or the presence of foreign bodies or antigens in the circulating system (Ahamefule et al., 2008). The decrease in these immune cells is therefore synonymous with improving animal health.

The higher laying rate in M0 and M1 treatments confirms the results of Teteh et al. (2016) and Voemesse et al. (2019) in laying hens with the difference that the best level of incorporation of *Moringa oleifera* leaves for laying is 0.5% in the present study against 1% in Teteh et al. (2016) and Voemesse et al. (2019). The same results were reported by N'nanle et al. (2020) in Sasso breeders with 1% of *Moringa oleifera* leaves. This difference would be linked to the species of poultry used for the experiment. The lowest laying performance of M2 and M3 could be related to the high level of *Moringa oleifera* leaves which might increase estrogens level, because of phytoestrogens contained in the leaves (Zade and Dabhadkar 2014). Indeed, Zade and Dabhadkar (2014) obtained in rats a decrease of FSH and LH, associated with phytoestrogens, thus leading to an increase in the duration of the ovarian cycle of rats. In addition, Titi et al. (2013) showed that sterols contained in *Moringa oleifera* leaves are used as precursors for estrogen synthesis. These results confirm the reports of Musa-Azara et al. (2014) who claimed that the intake of some amount of estrogenic substances in *Moringa* leaves inhibits the secretion of LH and FSH and reduces endogenous estrogen and progesterone levels. This reduction may play a role in the low number of follicles and consequently a decrease in the laying rate.

The improvement in egg weight with the level of *Moringa oleifera* leaves is in agreement with that of N'nanle et al. (2020) in Sasso breeders and Teteh et al. (2016) in layers. According to the study of Suk and Park (2001), heavy eggs have proportionally more albumen and less yolk. However, the results of this study showed that the proportion of albumen was higher in the eggs from M2 and M3 treatments compared to M0 and M1. Thus, the amino acids profile of *Moringa oleifera* leaves notably the sulfurous amino acids which are essential for protein synthesis improve egg weight (Bunchasak, 2009). In addition, the phytochemical analysis of *Moringa* leaves revealed a significant presence of selenium (Tarmizi et al., 2023). This component could also justify the high egg weight of M3 treatment according to Attia et al. (2010) who indicated that the use of organic selenium sources improves egg weight.

The increase in albumen ratio, positively correlated with Haugh Unit would be due to the concentration of amino acids, vitamins, mineral salts, and other compounds contained in *Moringa oleifera* leaves (Yang et al., 2006; Moyo et al., 2012; Kashyap et al., 2022). Brought to the breeding hens these components would stimulate the synthesis of albumin by the liver, thus causing the increased serum albumen level and consequently the increase of albumen ratio (N'nanle et al. 2020).

The similar yolk ratio between treatments could be explained by the yolk formation process according to the results of Nys et al. (2011). These authors claimed that during the formation of yolk from embryogenesis, the lipoproteins are transported from the liver to the ovaries where they are deposited in oocytes to form follicles. Thus, nutritional problems related to the feeding of breeders have little influence on the yolk ratio. The maximum rate of 1.5% of *Moringa oleifera* leaves used in this experiment would also be insufficient to reduce the proportion of egg yolk. The same is true for shell ratio which was not influenced by *Moringa* leaves. The anti-nutritional factors such as phytates and oxalates, found in *Moringa oleifera* leaves, which have a less chelating effect on minerals such as calcium (Foidl et al., 2001), making phosphorus and calcium unavailable could explain these results. Moreover, hens have still the capacity to move calcium from bones compensating feed calcium intake for shell calcification (Wright et al., 1990).

The total serum proteins which include the proteins of the nutritional, immune, enzymatic, and inflammatory state (Tété-Bénissan et al., 2013) were low in *Moringa oleifera* leaves groups compared to the control at 34 weeks of age. At 50 weeks of age, the results showed that total proteins were higher in *Moringa oleifera* leaves groups. The increase in albumin level at 34 weeks of age with *Moringa oleifera* leaves would testify to nutritional improvement, especially with increased uric acid and similarity of creatinine. Indeed, uric acid and urea are indicators of an increase in protein catabolism (Donsbough et al., 2010). Thus, the decrease in total proteins at 34 weeks would be related to the decrease in proteins of immunity and inflammation, which, according to Tennant and Center (1997) and Tete-Benissan et al. (2013), would be a consequence of the improvement of animal health.

Results showed that AST and ALT levels were significantly higher in *Moringa oleifera* leaves treatments at 50 weeks of age. Indeed, the liver, involved in metabolic and detoxification, can present lesions, responsible for an increase in gamma-glutamyltransferase

and transaminases (AST and ALT). These lesions can be caused by infections, diet intoxication, large liver, and poisoning (Nkosi et al., 2005; Mega et al., 2021). Thus, the secondary metabolites of diet or products resulting from their metabolism can cause liver damage, especially with age, that can lead to the release of transaminases (Mega et al., 2021). Thus, the results of this study showed that liver and heart weight were higher in groups fed *Moringa oleifera* leaves, compared to the control. The results also noted an increase in creatinine levels with *Moringa oleifera* leaves, at 50 weeks of age, which could also show the effect of these toxins on the kidneys. The nephrons could be damaged by toxins found in *Moringa oleifera* leaves, such as phenols, especially with prolonged use. This could potentially lead to the accumulation of products like creatinine in the blood (Lauriola et al., 2023). These observations are in agreement with Hetland et al. (2003) results which stipulate that prolonged exposure of oat hulls and wood shaving in broilers and layers diet exacerbates the effect of antinutritional factors. The high levels of AST, ALT, and creatinine could explain the high levels of total protein in groups fed *Moringa oleifera* leaves at 50 weeks of age.

Concerning serum lipids parameters, the slight decrease in total cholesterol at 34 weeks of age would be linked to the decrease in LDL-cholesterol because of the similarity of HDL-CH levels between groups. The slight reduction in cholesterol levels could be explained by the low level of *Moringa oleifera* leaves. Indeed, phytosterols and stanols contained in *Moringa* leaves only compete with cholesterol in the formation of micelles necessary for cholesterol absorption if they are present in sufficient quantity in the gut (Serfaty-Lacrosnière et al., 2001). The significant decrease in triglycerides at 34 weeks of age would be due to the presence of some secondary metabolites such as saponins in *Moringa oleifera* leaves (Nweze and Nwafor, 2014). Indeed, the use of feed additives relatively rich in saponins reduced serum triglycerides (Afrose et al., 2010). Furthermore, Francis et al. (2002) showed that saponins are known to form insoluble salts with lipids.

The decrease of white blood cells and lymphocytes with *Moringa oleifera* leaves is in agreement with the results obtained by Voemesse et al. (2019) in laying hens, and could be explain by the antimicrobial properties of *Moringa* leave (Ahmed et al., 2023). According to Ahamefule et al. (2008), the increase in WBCs is often associated with bacterial infections or the presence of foreign bodies. Authors found that *Moringa oleifera* leaves reduced gut microflora by inhibiting the growth of some pathogenic germs such as *Escherichia coli* and *Staphylococcus aureus* (Djakalia et al., 2011; Divya et al., 2014; Ahmed et al., 2023), thus causing a reduction in WBC and lymphocytes. This reduction confirms the phyto-therapeutic properties of *Moringa oleifera* leaves mentioned by Tété-Bénissan et al. (2013) and Leone et al. (2015).

Contrary to the decrease in red blood cell and hemoglobin levels with incorporation of *M. oleifera* leaves at 3% observed by Voemesse et al. (2019) in laying hens, the use of these leaves had no significant influence on RBC in guinea fowl, although a slight decrease in hemoglobin level was observed at 34 weeks of age. This difference would be due to the species of poultry used, which was guinea fowl in this study, and the low level of incorporation of the leaves which was 1.5% in this experiment and 3% in a study of Voemesse et al. (2019).

CONCLUSION

This study reveals that the incorporation of *Moringa oleifera* leaves into local guinea fowl breeders' feed improved growth (1.5% of incorporation) and egg production (0.5% of incorporation), probably by the richness and quality of the nutrients contained in *Moringa* leaves as shown by improved albumin levels. From the point of view of egg production, the use of 0.5% of *Moringa oleifera* leaves in the diet should be encouraged. But for growth (23 to 33 weeks of age in this study), egg weight, and egg quality, the best performance is obtained with dietary inclusion of 1% and 1.5% *Moringa oleifera* leaves. Therefore, the investigation is necessary to explore the hatchability of eggs from guinea fowl breeders fed with a diet containing *Moringa oleifera* leaves and post-hatch performance.

DECLARATIONS

Authors' contributions

Komi Nukunu Patrik Atitso designed the protocol, performed the experiments, collected and analyzed data, and prepared the original draft, edited it, and finalized the manuscript. Kafui Amivi Tété-Benissan validated the protocol, supervised the data collection, and critically revised the manuscript. Oumbortime N'nanle and Kokou Voemesse validated the protocol and revised the manuscript. Lamboni Laré and Komi Attivi supervised the data collection and revised the manuscript. The authors confirmed the final version of the manuscript.

Availability of data and materials

All data presented in this study are available upon request from the corresponding author.

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Competing interests

The authors have declared that no competing interests exist.

Ethical considerations

This manuscript does not contain plagiarized sentences and has not been published or accepted for publication elsewhere or under editorial review elsewhere. The data are not fabricated or falsified.

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Dietary Protein Levels in the Small Intestine and Carcass Traits of Cross-Breed Chickens

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ABSTRACT

Protein is a source of nutrients that plays a significant role in biological processes. The current study aimed to evaluate the effects of feed with different protein levels on the pH and viscosity of the small intestine, ileum characteristics, and carcass traits of cross-breed chickens. A total of 160 cross-breed unsexed chicks aged 2 days were divided into three treatments (T1-T3), each consisting of 6 replications with 9 chicks per replication, consisting of a diet with protein level (T1; starter 18% and finisher 16%;), a diet with protein level (T2; starter 20% and finisher 18%), and a diet with protein level (T3; starter 22% and finisher 20%). Cross-breed chickens were crossed between Bangkok males and Lohmann laying hens. The variables were analyzed, including pH and viscosity of digesta, ileum characteristics consisting of total villous, height of villous, and depth of crypt. The carcass percentages consisting of the carcass, breast, thicks, wings, and back were measured. The research was analyzed using a completely randomized design. The results indicated that different protein levels in treatments were significantly different in total villous, height of villous, and depth of crypt but did not indicate a significant difference in pH and viscosity. Furthermore, the treatments have a significant difference in the carcass percentage and thick percentage but did not significantly affect the breast, wings, and back. It can be concluded that 22% crude protein in the starter and 20% crude protein in the finisher produced the greatest results in the intestinal characteristics and also in the carcass percentage of cross-breed chickens.

Keywords: Carcass, Cross-breed chicken, Feed, Intestinal characteristic, Protein

INTRODUCTION

Indonesian cross-breed local chickens have emerged, including Kampung Unggul Balitbangtan chicken (KUB) and Jowo Super chickens (joper). However, the cultivation of cross-breed chickens needs to be supported by appropriate feed. According to Sarjana et al. (2010), the nutrients that must be available in feed are protein, energy (carbohydrates and fat), minerals (calcium and phosphorus), vitamins, and water. Proteins and amino acids have a biological role in tissue biosynthesis in the body Alagawany et al. (2021). Protein is the most expensive nutrient in chicken feed, necessary for growth acceleration. Excessive protein in the feed reduces body fat accumulation, increases blood uric acid levels, and is excreted as nitrogen. Conversely, a lack of protein will increase fat accumulation in the body. This is because chickens convert excess energy into fat, whereas a lack of protein can reduce 6-7% of body weight per day. Singarimbun et al. (2013). Amino acids play a role in the metabolic processes of cells in the body Devignes et al. (2022). Essential amino acids such as lysine, methionine, threonine, and tryptophan are critical functions in the metabolic processes. Lysine has functions in muscle development, especially in chicken breast muscles.

Methionine in broiler chickens acts as a cysteine precursor, a source of sulfur, and an integral part of body protein (Kalbande et al., 2009; Ramadan et al., 2021). The availability of amino acids is more efficient in supplementing the lack of crude protein.

The healthy intestinal characteristics of cross-breed chickens can increase the maximum nutrient absorption process through improved villi height, number of villi, and crypt depth. According to Günther et al. (2012), proteins have an important role in cell formation, replacing dead cells and forming body tissue. Small intestinal epithelial cells are some of the cells and tissues formed by proteins. The wider surface area of villi maximizes nutrient absorption, leading to improved chicken performance and carcass quality. Protein intake in feed can be utilized in the process of forming muscle in chickens. As a result, the present study was initiated to evaluate the potential effects of different protein levels in intestinal characteristics and carcass traits of cross-breed chickens.

MATERIALS AND METHODS

Ethical approval

Ethical approval for the present experiment was given by the Ethic Clearance Commission under Universitas Brawijaya No. 118-KEP-UB-2022.

Experimental design

The present study was conducted between Juli to December 2021. This experiment used 162 Day Old Chicks (DOCs) unsexed cross-breed chickens using open-house colony systems. The cross-breed chicken was a cross between Bangkok Male and Lohmann laying hens produced by Berline Farm, Malang, East Java, Indonesia. The chicken was a new strain we called cross-breed chickens. The DOCs were vaccinated with ND-AI, Gentamicin, and ND-IB, and then in the first and second weeks, were vaccinated with the gumboro vaccine. The study was conducted for 60 days, divided into starter (0–30 days) and finisher (31–60 days) phases. The cages used were 18 open-house litter cages with length, width, and height dimensions, namely (1 m x 1 m x 2 m) every cage has nine cross-breed chickens. The composition of the basal diet was bran separator, soybean meal, yellow corn, Dried Distillers grains (DDGS), fish meal, copra meal, concentrate, coconut oil, salt, and methionine. The composition was formulated according to the poultry-balanced diet from the nutrient requirement of poultry NRC (1994). The composition of feed formulation and nutrient contents can be indicated in Table 1. During the experiment, chickens were given feed and drink *ad libitum*. The method was *in vivo*, using a completely randomized design with three treatments (T1-3) and six replications, where every replication consisted of nine chicks. The treatment provided includes feed with protein level (T1; starter 18% and finisher 16%), feed with protein level (T2; starter 20% and finisher 18%), and feed with protein level (T3; starter 22% and finisher 20%).

The variables measured in this study included pH, viscosity, intestinal characteristics, and carcass percentage.

Table 1. The feed composition and nutrient content of the diet in cross-breed chicken per treatment

Treatment* / Feed	T1		T2		T3	
	Starter (%)	Finisher (%)	Starter (%)	Finisher (%)	Starter (%)	Finisher (%)
Bran separator	20.57	18.33	14.69	12.33	9.91	6.45
Soybean meal	4.43	0.57	10.31	4.57	16.19	10.45
Yellow corn	38	45	38	45	37	45
Dried Distillers grains	10	10	10	10	10	10
Fish meal	10	8	10	10	10	10
Copra meal	5	5	5	5	5	5
Concentrate**	10	10	10	10	10	10
Coconut oil	0.43	1.64	0.44	1.55	0.55	1.56
Salt	0.19	0.12	0.1	0.1	0.21	0.1
Premix	1.3	1.34	1.46	1.45	0.98	1.44
Methionine	0.08	-	-	-	0.16	-
Nutrient Content***						
Dry Matter	87.64	90.88	87.97	90.33	86.80	91.21
Crude Protein	18.80	15.90	20.06	18.80	22.38	20.41
Extract Eter	6.07	6.85	5.48	6.61	5.01	6.43
Crude Fiber	6.18	5.42	5.04	5.55	5.82	5.11
EM (Kkal/g)	2814	2944	2834	2914	2822	2905
Lysin	1.08	1.10	1.18	0.92	1.00	1.06
Methionine	0.46	0.35	0.35	0.35	0.46	0.35

The composition was formulated according to the poultry-balanced diet NRC (1994). * T1: feed with protein level (starter 18% and finisher 16%), T2: feed with protein level (starter 20% and finisher 18%), T3: feed with protein level (starter 22% and finisher 20%). ** Nutrient composition of concentrate Crude Protein (min 20 %), extract eter (min 5 %), crude fiber (min 5 %), ash (max 8 %), Ca (0.8-1.1 %), P (min 0.5 %), enzyme phytase and aflatoxin max 50 µg/kg. ** Nutrient content was analyzed using proximate analysis

pH and viscosity

After 60 days of experiments, the cross-breed chickens were slaughtered by cutting using a knife in the jugular vein and carotid artery. Internal organs were removed from the body, especially the small intestine. Digesta obtained from the duodenum, jejenum, and ileum was taken and put into pot film. The digesta was used as a sample for the analysis of pH and viscosity. One gram of digesta was diluted with 10 mL of distilled water then immediately measured pH using a digital pH meter. Therefore, the mixture of 1 gram digesta and 10 mL distilled water was then centrifuged at 3000 rpm for five minutes. After that, the supernatant was taken and the viscosity was measured using a Brookfield viscometer. The measurement procedure for pH and viscosity was conducted following the method of Zhang et al. (2022).

Intestinal characteristics

Samples for intestinal characteristics analysis using an ileum. A 4-centimeter sample of the ileum was taken and then cleaned using distilled water. Ileum samples were placed in a pot film containing a 10% Neutral Buffered Formalin (NBF) solution to be made into preparations using a hematoxylin-eosin (HE) procedure Jamilah (2014). Measurement of the total number of villi, villi length, and crypt depth was measured using a light microscope (DIC Olympus BX51TF, Japan), 4x magnification connected to the Optilab application. Measurements of villi length and crypt depth were carried out using the Image Raster application.

Number of villi

The number of villi was counted by all the villi in each field of view with a four-time microscope magnification Emma et al. (2013).

Villi height

The height of the villi was carried out by initial measurement from the apical villi to the crypts of Lieberkuhn. Villous height was measured in 4 fields of view using a microscope magnification of 4 times Amri et al. (2022).

Depth of crypt

Crypt depth was seen in 4 different fields of view according to 12, 3, 6, and 9 clockwise directions. Crypt depth was calculated from the base to the base of the villi using 4x magnification Setiawan et al. (2018).

Carcass percentage

Variable measurements were carried out by taking samples from four chickens from each replication. The life bird chicken was weighed by the experimental chickens sampled in each experimental unit. Carcass weight was obtained by weighing the weight of the chicken after slaughter and subtracting the blood, feathers, head, feet, and internal organs except the lungs and spleen. The abbreviation of carcass percentage according to Formula 1 (Subekti et al., 2012).

(Formula 1)

$$\text{Carcass percentage} = \frac{\text{Carcass weight (g/bird)}}{\text{Live weight (g/bird)}} \times 100\%$$

Breast weight was obtained by weighing the chest area scapula to the sternum (g). The percentage of chest weight was calculated according to Formula 2 (Subekti et al., 2012).

(Formula 2)

$$\text{Breast percentage} = \frac{\text{Breast weight (g/bird)}}{\text{Carcass weight (g/bird)}} \times 100\%$$

The thigh weight was obtained by weighing the exact part in the area of the lower thigh joint to the knee (g). The percentage of thigh weight was calculated according to Formula 3 (Subekti et al., 2012).

(Formula 3)

$$\text{Thight percentage} = \frac{\text{Thick weight (g/bird)}}{\text{Carcass weight (g/bird)}} \times 100\%$$

The back weight was obtained by weighing the spine to the pelvic bone (g). Percentage back weight was calculated according to Formula 4 (Subekti et al., 2012).

(Formula 4)

$$\text{Back percentage} = \frac{\text{Back weight (g/bird)}}{\text{Carcass weight (g/bird)}} \times 100\%$$

The wing weight was obtained by weighing the joints between the upper arm and with scapula. Percentage wing weight was calculated according to Formula 5 (Subekti et al., 2012).

(Formula 5)

$$\text{Wings percentage} = \frac{\text{Wings weight (g/bird)}}{\text{Carcass weight (g/bird)}} \times 100\%$$

Statistical analysis

A statistical analysis was conducted using analysis of variance using SPSS version 27. An error was expressed as a standard deviation. In the end, probability values were subjected to the Duncan Multiple Range Test. The following formula was used.

(Formula 6)

$Y_{ij} = \mu + T_i + e_{ij}$

Where, Yij denotes the parameters observed, μ is the overall mean, Ti indicates the effect of different levels of protein, and eij signifies the amount of error number.

The treatment provided included a diet with a protein level (starter 18% and finisher 16%; T1), a diet with protein level (starter 20% and finisher 18%; T2), and a diet with protein level (starter 22% and finisher 20%; T3). One-way ANOVA was used to compare the means of pH, viscosity, total villous, height of villous, depth of crypt, carcass percentage, thicks percentage, wings percentage, and back percentage. The significance difference was established at ($p < 0.05$). All analysis was carried out in three replications and the significant difference was defined as the 5% level ($p < 0.05$). Finally, probability values were subjected to the Duncan Multiple Range Test (DMRT).

RESULTS

The results of the different levels of protein in the small intestine of cross-breed chickens including pH, viscosity, number of villi, length of villi, and depth of crypts were presented in Table 2.

pH and digesta viscosity

The results obtained that different levels of protein diet had no significant effect ($p > 0.05$) on the intestinal pH of cross-breed chicken. It can be seen that the pH value at T1 obtained a more acidic value than T2 and T3.

The statistical data presented in Table 2 indicated that the addition of protein at different levels to cross-breed chickens did not have a significant effect ($p > 0.05$) on the digesta viscosity. The results of this research indicated that there was a positive correlation between the level of protein in T3 and the resulting viscosity value.

Total, length and crypt depth villi in the small intestine

Table 2 indicated that different levels of protein had a significant effect on the number of villi cross-breed chickens in T1, compared to T2 and T3. ($p < 0.05$) The lowest number of villi was in treatment T1 (50.61 per transversal cut), while the most effective value was in treatment T3 (56.61 per transversal cut). An increase in the level of crude protein in the feed is followed by an increase in the number of villi. The results provided that the effect of different levels of protein on cross-breed chickens had a significant effect ($p < 0.05$) on the length of villi between T1 and T2 compared with T3. Where the lowest average value was T1 (467.86 μm) and the highest was T3 (506.89 μm).

The data revealed that the different levels of protein in the crypts of the intestines of cross-breed chickens had a significant difference ($p < 0.05$) between T1 and T3 (Figure 1; Table 2). The treatment with the lowest average value was T1 (124.27 μm), and the treatment with the highest average value was T3 (134.05 μm).

Carcass percentage

The results demonstrated that feeding cross-breed chickens with different protein levels had a significant effect on carcass weight ($p < 0.05$) in the T1, T2, and T3 (Table 3). The group with the highest treatment had the highest carcass weight, while the lowest weight was in the medium treatment group. The breast, wings, and back percentages of cross-breed chicken were not significantly different among the treatment groups ($p > 0.05$); however, the thick percentage significantly differed between T1 and T3 with the T2 ($p < 0.05$). In the thick percentage, medium crude protein (T2) decreases the thick percentage of cross-breed chicken. Although T3 had a higher percentage of carcass, breast, wings, thick, and back on cross-breed chicken, it yields different protein levels.

Table 2. Effect of different level protein diet on pH, viscosity, number of villi, length of villi, and depth of crypts in the finisher phase of cross-breed chickens

Variable / Treatment*	pH	Viscosity (cP)	number of villi (per transversal cut)	Length of villi (μm)	Crypts Depth (μm)
T1	6.22± 0.13	11.17 ± 1.17	50.61 ± 3.74^{a}	467.86 ± 29.26^{a}	124.27 ± 5.90^{a}
T2	6.39 ± 0.26	11.83 ± 3.19	55.50 ± 3.82^{b}	479.30 ± 12.22^{a}	129.19 ± 4.13^{ab}
T3	6.58 ± 0.69	12.50 ± 2.07	56.61 ± 2.76^{b}	506.89 ± 13.68^{b}	134.05 ± 7.54^{b}

Notes:* T1: feed with protein level (starter 18% and finisher 16%), T2: feed with protein level (starter 20% and finisher 18%), T3: feed with protein level (starter 22% and finisher 20%). Different superscript letters $^{(a,b)}$ in the column showed significant differences across the treatments ($p < 0.05$).

Table 3. Effect of different level protein diet on carcass percentage in the finisher phase of cross-breed chickens

Treatment*	Carcass (%)	Breast (%)	Thick (%)	Wings (%)	Back (%)
T1	56.344±0.82[b]	11.766±1.42	17.882±0.78[b]	8.976±0.37	14.606±1.47
T2	53.482±0.94[a]	11.846±1.08	15.956±0.81[a]	8.856±0.77	14.356±1.55
T3	58.872±2.73[c]	13.34±0.84	17.892±0.85[b]	9.522±0.57	16.182±1.06

Notes: *T1: feed with protein level (starter 18% and finisher 16%), T2: feed with protein level (starter 20% and finisher 18%), T3: feed with protein level (starter 22% and finisher 20%). Different superscript letters [(a,b)] in the column showed significant differences across the treatments ($p < 0.05$).

DISCUSSION

pH level in the small intestine was an effective way of determining the effectiveness of feed digestion. The findings indicated that the lowest or highest protein level in the feed did not affect the pH of the small intestine of cross-breed chickens. Increasing crude protein content was in line with increasing pH value in the small intestine, especially in the ileum. Based on the findings of Hafsah et al. (2021) and Zhang et al. (2022) the normal pH level of digestion in the small intestine of native chickens varies depending on the location. The pH level of the poultry in the duodenum was 5-6; in the jejunum was 6.5-7; and in the ileum was 7-7.5. The study indicated that the high levels of protein in T3, particularly 22% starter and 20% finisher, had an alkaline pH value. This finding did not follow the previous research. Simpson et al. (1976) stated that high protein levels increase H+ ions, leading to decreased pH levels in broiler chickens. According to Liu et al. (2010) an increase in H+ ions caused protein hydrolysis into amino acids. However, amino acids were mainly absorbed in the upper of the small intestine namely the duodenum and the jejunum in poultry as mentioned by Truong et al. (2017)Therefore, amino acids in the ileum were the same as those in the rest of the duodenum and jejunum, and protein hydrolysis into amino acids did not affect the pH level of the ileum.

The ability of crude protein level on feed can increase digesta viscosity of cross-breed chickens. This research indicated that 22 % crude protein in the starter phase and 20 % in the finisher phase improved the viscosity. According to previous research by Olfati et al. (2021), dissolved solid components, like amino acids and proteins, can increase viscosity in the small intestine of chickens. Dissolved proteins can contribute to an increase in viscosity (Damodaran, 2008; Saenphoom et al., 2013) indicating that when the digesta viscosity increases, there is also an increase in enzyme activity, amino acid content in the small intestine, protein digestibility, and metabolic energy in broiler chickens. The previous finding indicated that the viscosity of broiler chickens value ranged from 10.48% to 17.64% Amerah et al. (2008). The acquired viscosity value of cross-breed chickens in this research was lower when compared to the findings of earlier research. It has been observed that excessively high viscosity can reduce feed digestibility, while excessively low viscosity can accelerate digestion and limit nutrient absorption. According to a study by Khempaka et al. (2011) adding feed containing crude protein has been indicated to impact intestinal significantly in broiler chickens. The result of this study aligns with previous findings conducted by Khattab et al. (2021) who argue that increasing viscosity can slow down digestion, leading to a more efficient absorption process in ducks. However, previous research by Natsir (2016) stated that increased viscosity in the small intestine of broiler chickens can decrease digestive efficiency due to the slowing down of the diffusion rate of endogenous enzymes that react with nutrients.

Villus height and crypt depth were markers for gut health and can be used to assess intestinal characteristics. The higher protein level in cross-breed chicken indicated a higher villus height and crypt depth (Figure 1). Higher villi indicated an increase in the surface area available for nutrient absorption from the gut. An increase in height has been indicated to improve nutrient transport across the villus surface as reported by El Sabry and Yalcin (2023). Furthermore, intestine development can be monitored by measuring villus morphology and enzymatic activity, as well as determining the expression of genes involved in nutrient transport. A deeper crypt suggested a higher turnover of enterocytes, which in turn required more protein and energy. The crypt depth in the intestinal is related to the absorptive and secretory cells. The secretory cells are responsible for producing mucins, which are the primary component of the protective mucous layer in the intestines Nguyen et al. (2021). According to Devignes et al. (2022), protein plays a role in cell formation, forming

body tissue, and assisting in the growth of body organs, including small intestinal epithelial cells.

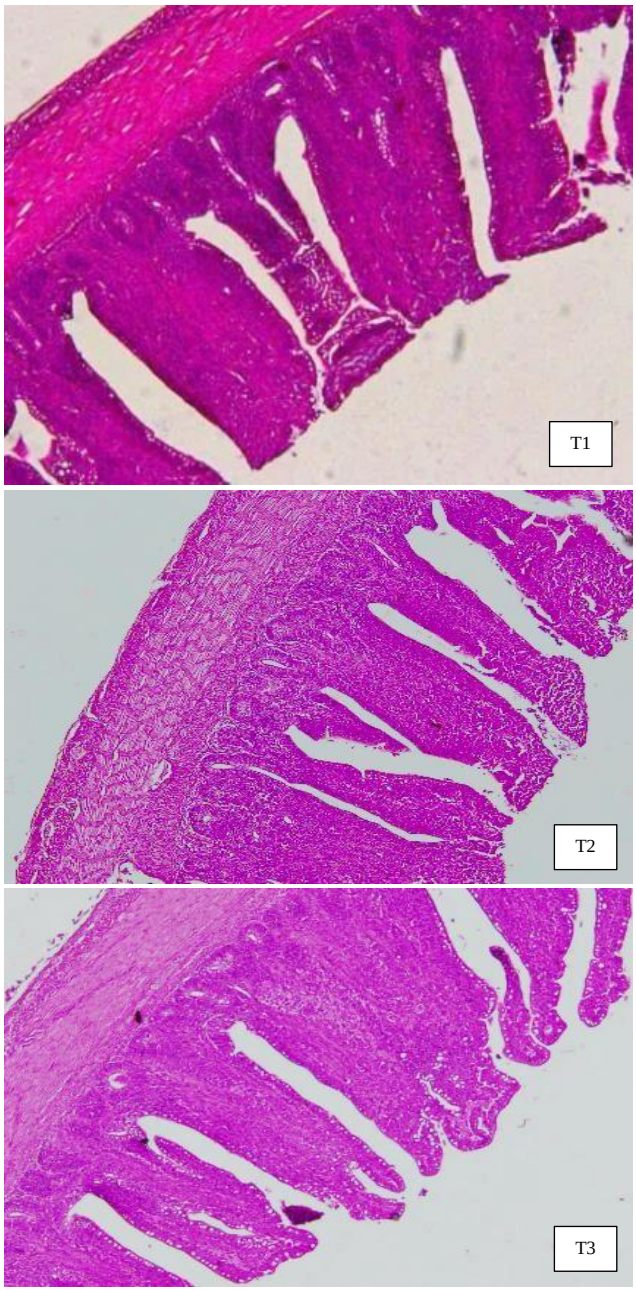

Figure 1. Ileum of cross-breed chickens in different treatment groups (T1, T2, and T3; 4x magnification).

Gu and Li (2004) reported that the growth and development of the small intestine, including the villi, in chickens occurs early in life and depends on nutrient intake at an early age which can be related to protein intake and villi development. The best result was obtained by using the T3 treatment (22% starter and 20% finisher) compared to other treatments on the cross-breed chicken. Protein feed was utilized by Lactic Acid Bacteria (LAB) as a source of nitrogen that forms the cell biomass. It is important to note that LAB in the gastrointestinal needs amino acids, vitamins, pirin, and pirimidin to grow (Nazzaro et al., 2012).

Feeding nutrients like protein and fat play a role in tissue formation and stimulate the proliferation of small intestine cells Wang et al. (2014). The larger protein content (T3) can increase the villi's size, making it longer and the intestinal lumen larger. Research by Nazzaro et al. (2012) reported that high protein levels resulted in more LAB colonies. Lactic acid bacteria require proteins based on nitrogen requirements. The nitrogen requirement for LAB was obtained from several sources including protein (organic nitrogen) in the feed. The growth of pathogenic microbes in the intestine can be suppressed by producing short-chain fatty acids (SCFA), lactic acid, and antimicrobial compounds of LAB. Conditions of the small intestine, such as high villi in the small intestine, represent an area for wider nutrient absorption. An increase in the height of the villi in the small intestine of broiler chickens was closely related to an increase in digestive and absorption functions due to the expansion of the absorption area which was an expression of a smooth nutrient transport system throughout the body and benefits the host (Tejeda and Kim, 2021).

The significant influence was because the crypt depth was closely related to the intestinal villi, where protein administration also had a significant influence on the number of villi and the length of the intestinal villi of cross-breed chickens. New epithelial cells and enterocytes were produced by the crypts, which then migrate to the villi (Bermudez-Brito et al., 2012). The T3 treatment (22% starter and 20% finisher) indicated the best result compared to the other treatment. It was because the results of protein hydrolysis in the form of nitrogen will be used by LAB as a nitrogen source. According to Marciniak et al. (2018), protein hydrolysis was influenced by the concentration of hydrolyzing ingredients, temperature and time of hydrolysis, and air pressure. Increasing the enzyme concentration will increase the volume of insoluble protein hydrolyzate into soluble nitrogen compounds. Nitrogen will be utilized by LAB as a source of nitrogen, which functions in cell biomass formation Ayivi et al. (2022). According to research by Mangisah et al. (2020), the addition of *Lactobacillus* sp. as a feed additive in ducks had a significantly different effect on the crypts' depth which greatly influenced the process of nutrient absorption in the small intestine in conditions that the absorption area was wider. The wider

villi improve nutrient absorption, making protein content more efficient in the growth of poultry (Tejeda and Kim, 2021). Protein is an important nutrient needed for the growth of cross-breed chickens. The finding of this experiment was aligned with the finding of Aftab et al. (2006), who reported that protein is the largest part of the carcass and is a very important element for muscle growth and development in chickens. The research study obtained the percentage of carcass cross-breed chickens affected by the different levels of dietary crude protein. This study implies that higher levels of crude protein, specifically at the starter phase (22%) and finisher phase (20%) for crossbred chickens, were necessary for optimal carcass and thigh percentage. The findings were not in agreement with Magala et al. (2012), which found no significant impact of dietary protein level (15-17%) on carcass yield in Ugandan cockerels. Carcass yield was influenced by various factors, such as genetics, feed, slaughtering conditions, live weight, and sex in broiler chickens (Havenstein et al., 2003; Kierończyk et al., 2017). In this study, protein deposition was characterized by carcass percentage which was determined by the rate of protein synthesis and protein degradation. A higher rate of protein synthesis or a lower rate of protein degradation can lead to increased quality of growth and protein deposition in the chicken Tesseraud et al. (2000). Chickens can adjust their feed intake to meet their protein requirements in the feed that has sufficient energy levels. These findings align with Kamran et al. (2008), who demonstrated that a low-protein diet with a better energy-to-protein ratio can increase energy consumption, which may be stored as fat after fulfilling energy requirements in broiler chicken. These results were consistent with Srilatha et al. (2018), who observed that broiler chickens fed lower protein levels (17.29%) at the finishing stage had more abdominal fat deposition. Tavaniello et al. (2022) stated that the order of percentage of broiler chicken carcass cuts from highest to lowest includes breast, upper thigh, lower thigh, wing, back, and front back. However, the research indicated that the carcass cut of cross-breed chickens from the highest to the lowest is a thicks, back, breast, and wings. The genetic nature of cross-breed chickens deposits the formation of meat on the thick and back. This finding indicated that the addition of a higher protein level diet of cross-breed chickens improved carcass cut compared to the lowest treatments. It disagreed with research before Eits et al. (2022) that there was no evidence that energy intake limited protein deposition at high amino acid intake. Limited amino acids did not depend on protein deposition in the carcass. However, this study finding proved that the higher protein also improves the carcass traits of cross-breed chickens.

CONCLUSION

The dietary addition of protein in the starter and finisher phases at levels of 22% and 20%, respectively, caused the best results for the intestinal characteristics and carcass percentage of cross-bred chickens. In the future, the expression gene analysis can evaluate the growth factor of cross-breed chickens.

DECLARATIONS

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Authors' contributions

Yuli Frita Nuningtyas contributed to doing the research, collecting data, data analysis, and preparing the manuscript. Osfar Sjofjan, Muhammad Halim Natsir, Aulanni'am, and Veronica Margareta Ani Nurgiartiningsih contributed to the research design, revised the manuscript, and supervised. Ahmad Furqon revised the manuscript grammatically. Suci Puji Lestari contributed to the research. All authors read and approved the final version of the manuscript and analyzed data in the present journal.

Competing interests

There is no potential conflict of interest relevant to the present article.

Ethical considerations

All ethical aspects, such as ethical issues, plagiarism, fabrication and falsification, double publication, and redundancy, have been thoroughly examined and addressed.

Availability of data and materials

All data generated or analyzed during the study are included in this article; supplementary information is available upon request.

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Identification of Extremely Virulent Infectious Bursal Disease Virus Via Molecular and Histological Methods in Broiler Chickens

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ABSTRACT

Infectious bursal disease (IBD) is caused by an RNA virus belonging to the Avibirnavirus genus within the Birnaviridae family. The global prevalence of infectious bursal disease virus (IBDV) is a significant concern, affecting birds of all ages. Birds infected with IBDV exhibit symptoms, such as depression, bleeding in the thighs and pectoral muscles, and enlargement of the bursa. This study aimed to identify predominant IBDV serotypes using molecular methods and to gain insights into the resulting pathological conditions in infected chickens. Additionally, the study investigated the viral sequence and the relationship between a local Diyala isolate and reference strains from the Genebank. In the current study, the IBDV was isolated from broiler chickens aged 2-3 weeks from 15 farms in the Diyala Governorate of Iraq. A total of 15 samples, each from a different farm, were collected. Necropsy samples were obtained from various organs of broiler chickens, including the bursa of Fabricius, lungs, liver, and kidneys. Specific primers targeting the VP2 gene were used for reverse transcription-polymerase chain reaction (RT-PCR) analysis. The RT-PCR analysis yielded a 727 bp fragment, confirming the presence of IBDV in 10 out of the 15 samples. One strain was assigned the accession number LC498531 in the NCBI database. Phylogenetic analysis using the neighbor-joining tree program revealed three distinct groups. All examined regional samples (S1) were situated within the constructed tree. Five samples formed a specific group, indicating a close relationship. Histological examination of the tissues showed visible alterations such as degeneration, necrosis, and infiltration of inflammatory cells, particularly heterophils, providing clear evidence of the disease. In conclusion, this study confirmed the presence of IBDV in broiler chickens from multiple farms in Iraq's Diyala Governorate, highlighting distinct clustering patterns in viral sequences. Moreover, the study confirmed the virus's presence using conventional RT-PCR, with histological examination supporting the findings.

Keywords: Broiler chicken, Bursal enlargement, Infectious bursal disease, RT-PCR

INTRODUCTION

The infectious bursal disease virus (IBDV), belonging to the genus Avibirnavirus within the Birnaviridae family, has an RNA genome. This virus is responsible for causing infectious bursal disease (IBD; Fauquet and Mayo, 2001). The IBDV has a single, icosahedral-symmetric capsid envelope with 32 capsomeres that are between 55 and 60 nm in diameter. The virus is non-enveloped and double-stranded (Fauquet and Mayo, 2001). The IBD is a highly transmissible disease that mainly strikes chickens leading to immunosuppression and increased susceptibility to secondary infections. This immunosuppression hampers effective immunization against other diseases, such as infectious bronchitis, Marek's disease, and Newcastle disease, therefore making chickens more susceptible to these opportunistic diseases (Allan et al., 1972; Lasher and Shane, 1994). While IBD is commonly observed in chickens worldwide, other bird species, such as ostriches, guinea fowl, turkeys, and ducks can be infected without showing apparent clinical symptoms. The presence of IBD poses a significant threat to the global poultry industry, leading to reduced profitability and hindering its expansion (Dye et al., 2019; Zhang et al., 2022). The disease was first identified in a broiler flock in Sussex, USA, and later named "Gumboro" following an outbreak

reported in the Gumboro region of southern Delaware, USA. Infected birds exhibit a distinctive lesion termed "avian nephrosis" that primarily affects the kidneys (Rauf et al., 2011; Dey et al., 2019). Remarkably, antigenic variant strains can evade cross-neutralization antisera against classical strains. In contrast, extremely virulent strains can infect birds even in the presence of high levels of previously protective maternally derived antibodies (MDA) against classical strains. (Jackwood and Sommer-Wagner, 2007). In the case of IBD, two distinct periods of occurrence exist, dependent on the age of the chickens (Dey et al., 2019). During the first period, the disease is noticed in birds younger than 3 weeks old, and infected chickens show few clinical signs but are grossly characterized by bursa atrophy, which causes severe immunosuppression. the clinical form of IBD affects poultry aged between 3 and 6 weeks. Affected chickens show signs, such as ruffled feathers, watery droppings, urate accumulation in their kidneys and urinary tubules, loss of appetite, depression, trembling, extreme prostration, and ultimately death (Lukert and Saif, 2003; Ingrao et al., 2013). The IBDV is categorized into two serotypes, namely serotype I and serotype II. Serotype I is pathogenic to broiler and layer chickens, while serotype II is considered non-pathogenic or of low virulence (Al-Sheikhly et al., 1978; Wang et al., 2009). Serotype I is the predominant worldwide serotype associated with severe clinical disease, and consequently, all vaccines are developed based on this serotype (Dey et al., 2019). Serotype I viruses can be divided into four subtypes based on changes in antigenicity and virulence, namely classic strains, antigenically variable strains, extremely virulent strains, and attenuated IBDV (Cao et al., 1998). The IBDV genome consists of five proteins known as VP1-VP5, with two segments, A and B. Segment A contains the genetic information for two structural proteins, VP2 and VP3, along with two non-structural proteins, VP4 and VP5 (Ferrero et al., 2015). According to Raja et al. (2016) and Deorao et al. (2021), segment B encodes the non-structural protein VP1, which is a representation of viral transcripts. A host-protective virus with at least three neutralizing epitopes is thought to have VP2 as its primary antigen (Coulibaly et al., 2005). A limited scope of minor amino acid variation is observed in the variable region of the VP2 gene, and this variation may be the cause of the development of antigenic variants in all IBDV strains.

The highly variable region (HVR) plays a critical role in stimulating the production of antibodies that neutralize viruses due to the presence of different epitopes stimulating their production (Lukert and Saif, 2003). One of the major challenges facing chicken farmers in Iraq, particularly in the Diyala Governorate located in eastern Iraq, is infectious bursal disease caused by the virus. However, little is known about the histological alterations accompanying the disease or the molecular evidence of IBDV in this region. The aim of this study was to examine the molecular evidence and histological changes linked to IBDV in broiler flocks from selected farms in the Diyala Governorate of Iraq.

MATERIALS and METHODS

Ethical approval

The research received approval from the Scientific Ethics Council, and the study's ethical number, Vet Medicine 301 August 2019 A, A, R, and K, was validated at the University of Diyala/College of Veterinary Medicine in Iraq.

Study area

This study was conducted in the molecular biology and virology labs of the College of Veterinary Medicine at the University of Diyala in Iraq.

Molecular detection

Reverse transcriptase polymerase chain reaction (RT-PCR) was employed to track the molecular detection of IBDV. Fifteen tissue samples were collected, each comprising the liver, spleen, kidney, and bursa of Fabricius from broiler chickens diagnosed with severe IBDV infections during necropsies at the age of 2-3 weeks. Tissue samples (one from each bird) were extracted for viral RNA from the kidneys, spleen, liver, and bursa of Fabricius using an extraction kit for tissue (Kylt® RNA / DNA Purification kit, Germany) according to the manufacturer's instructions. Sterile plastic containers were utilized to store the samples, which were then placed in Falcon tubes and kept at -20°C in a deep freezer until required. Infected chickens with IBDV indicated a severe infection and high mortality rate. During necropsy, the bursa of Fabricius showed inflammation with varying degrees of hemorrhage, edema, and follicles filled with gelatinous exudates. Ecchymotic hemorrhages were also observed on the thigh and breast muscles (See Figures 1 and 2).

As previously described by Meir et al. (2001), a set of publishing oligonucleotide primers selected from a highly conserved region was purchased from Microgen, Korea, and used for the detection of IBDV. A commercially available kit (Promega, USA) and PCR reaction mixtures prepared in a 25 Eppendorf tube were used to amplify a

727-bp fragment targeting specific genes within the VP2 region. These primers included IBDV-F (5-CAGGTGGGGTAACAACAATCA-3) and IBDV-R (5-CGGCAGGTGGGAACAATG-3) using Access RT-PCR System and RNasin® Ribonuclease Inhibitor kit (Promega®, Madison, USA). After a quick spin for a few seconds, the RT-PCR tubes were placed in an Eppendorf thermal cycler (USA) and run through 40 cycles of the following temperature profile for product detection. The RT-PCR was run for 10 minutes at an RT reaction temperature of 50°C. After that, 40 cycles of denaturation at 95°C for 1 minute, annealing at 59°C for 40 seconds, and extension at 720°C for 1 minute were carried out consecutively, with the last extension lasting 10 minutes at 72°C. After RT-PCR, the products from the 15 samples were subjected to electrophoresis. Following the staining of the DNA with a red dye, the amplified fragments became visible on gel electrophoresis (Meir et al., 2001).

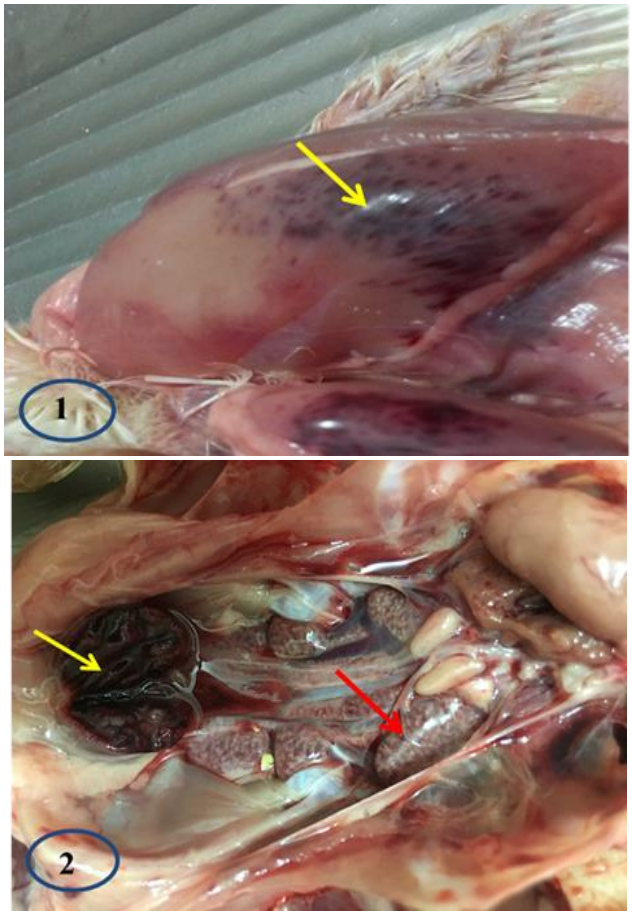

Figure 1. A boiler chicken suspected to infectious bursal disease virus. **1:** Ecchymotic hemorrhages on the thigh and breast muscles (yellow arrow). **2:** Congestion and bleeding on the bursal's serosal surface (yellow arrow), and kidneys swollen with urates (red arrow).

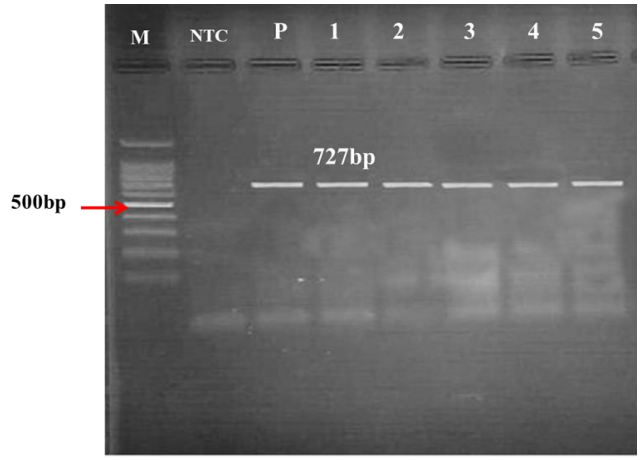

Figure2. Tissue samples of broiler chickens suspected to infectious bursal disease virus using RT-PCR and oligonucleotide primers (IBDV-F and IBDV-R) produced the target band of approximately 727bp stained by Red stain. **M**: DNA ladder (1000 bp); **NTC**: Non-template control; **P**: Positive control; **Lanes 1-5**: Positive samples from chickens infected with IBDV.

DNA sequencing of PCR amplicon

Following the guidelines provided by the sequencing company (Macrogen Inc., Geumchen, Seoul, South Korea), the 727 bp resolved PCR amplicons were commercially sequenced from both termini (forward and reverse). To ensure that annotation and variations were not the result of PCR or sequencing artifacts, only clear chromatographs obtained from ABI (Applied Biosystems) sequence files were analyzed. Virtual positions and other information of the retrieved PCR fragments were identified by comparing the observed DNA sequences of the viral samples with the retrieved DNA sequences of the viral database. Through a comparison of DNA chromatograms with the viral DNA sequences that were deposited using BioEdit versus 7.1 (DNASTAR, Madison), nucleic acid variations were made evident. Every variety found within the IBDV genes was annotated by SnapGene Viewer ver. 4.0.4 (Jasim et al., 2022).

Phylogenetic trees

In this study, a specific and comprehensive tree was constructed using the neighbor-joining method. Detected variations were then compared to nearby homologous reference sequences using the NCBI-BLASTn server (Zhang et al., 2000). Next, using the neighbor-joining technique, circular trees were constructed, including the variant that was observed. Using the iTOL suit, each generated form was annotated as a cladogram (Letunic and Bork, 2019). To determine the precise genotype of the

IBDV samples in the present study, multiple reference sequences representing each of the three IBDV genotypes were provided. Each phylogenetic genetic group's sequences in the tree were colored according to their classification.

Histopathology

Thirty Infected chickens with IBD were humanely euthanized with a high dose of ketamine (50mg/kg) and xylazine (5mg/kg; Kadhim et al., 2023). After that, an incision and dissection were made in abdominal regions, followed by tissue samples (6 mm thick) from different regions of the bursa of Fabricius, lungs, liver, and kidneys, which were removed and immediately immersed in the 10% fixative formalin solution for 24 hours at room temperature. Then, all tissue samples were processed by routine methods, embedded in paraffin wax, sectioned at 5 mm, and stained with routine staining (H&E). In the next step, photomicrographs were captured of each histological field (Aliyu et al., 2022).

RESULTS

Molecular detection

Fifteen extracted RNA samples from respective cases designated as Diyala isolates were screened by RT-PCR assay to detect IBDV. The oligonucleotide primer sequences were chosen from a highly conserved region essentially the hypervariable VP2 region. On a 2% agarose gel, all the amplified cDNA displayed the same mobility. Ten out of 15 samples (66.66%) were positive for IBDV. As seen in Figure 2, positive samples produced a distinct DNA band with a length of 727 bp.

One strain was registered in NCBI and received an accession number (LC498531). Following comparison with all reference strains available from GenBank.

Sequencing results

Following an NCBI blast analysis, the sequencing reactions accurately pinpointed the locations of the examined samples. Upon comparing the sequenced samples with the expected target, which encompassed the VP2 locus within the IBDV sequences, this method demonstrated the highest similarity. The precise locations and additional details of the retrieved PCR fragment were determined by comparing the observed DNA sequences of these viral samples with the reference sequences retrieved. With the GenBank accession number LC498531.1, the NCBI BLASTn engine revealed up to 98% homology with these predicted targets that covered designated regions of VP2 sequences (Figure 3).

After positioning the 727 bp amplicon sequences within the VP2 locus of IBDV sequences, detailed information was provided, including the positions of the forward and reverse primers within the targeted IBDV sequences (Table 1).

Alignment results of the 727 bp sample revealed the detection of 13 nucleic acid variations in comparison with referring sequences of the IBDV sequences (Figure 4). The results indicated the presence of 13 nucleic acid variants in the S1 local sample as compared to reference sequences of the IBDV (GenBank acc. no. LC498531.1). Investigated nucleic acid sequences were converted to their corresponding positions in the IBDV coat protein VP2. All nucleic acid sequences of investigated local samples were translated to their corresponding amino acid sequences using the Expasy translate suite. Amino acid alignment of these amino acid sequences with its reference sequences indicated that investigated 727 bp amplicons had consisted of 242 amino acid sequences in entire amino acid sequences in VP2 in IBDV (Figure 5). It was found that all detected variants had caused a silent effect on the VP2 protein.

A detailed description of identified variations in the investigated S1 local sample was presented in Table 2. Based on the nucleic acid sequences found in the examined sample, an inclusive phylogenetic tree was created in the current investigation. The S1 local sample of IBD virus sequences was included in this phylogenetic tree along with other relevant NCBI reference sequences. Due to the presence of several clades of the IBDV, a direct comparison between present samples with the previously known reference genotypes was conducted to find out the accurate positioning of the samples within the main clades of these viral particles. As a result, the viral isolate was directly compared to several representative reference samples coming from different viral variations inside the phylogenetic tree that was produced. It was possible to determine their actual evolutionary distances with greater accuracy as a result. In this extensive tree, 38 aligned nucleic acid sequences were present. This created three indicated the presence of several significant clades, and the viral sample under investigation was included in one of them. The clear phylogenetic information of this researched IBDV was disclosed, as deduced from the VP2-based tree.

The currently constructed tree was represented in two cladograms, which were made to generate a circular cladogram (Figure 6). In each generated form, a particular phylogenetic distribution of incorporated sequences was notified. The presence of the most common clades of IBDV was carried out to provide reference sequences for a more thorough analysis of the evolutionary relationships among the examined viral organisms. Using this neighbor-joining tree, three distinct phylogenetic groups were observed. Within one of the IBDV-VP2 clades, the investigated viral sample was incorporated. It was found

that all investigated samples of S1 were incorporated within a specific position of the constructed tree within a clade made of five samples. Within this clade, it should be noted that all these five samples were suited in the vicinity of variable reference sequences deposited from variable IBDV strains of India (GenBank KX223749.1), Tanzania (GenBank AB368970.1), Hungary (GenBank ON100651.1), and Egypt (GenBank KY610528.1) origins within this clade. However, no close phylogenetic positioning of the local sample toward these isolates was found. Due to the identified three nucleic acid substitutions, the obvious deviation was observed for the S1 sample compared to these reference samples. This data indicated the significant effects of the identified variants in inducing clear phylogenetic alterations within the generated tree. To indicate the ability of such genetic sequences to explain different IBDV mutations utilizing this genetic fragment, an inclusive tree has been created. This further demonstrates the effectiveness of the VP2-specific primers currently in use in locating the phylogenetic clustering of the virus isolates causing IBD that is being studied.

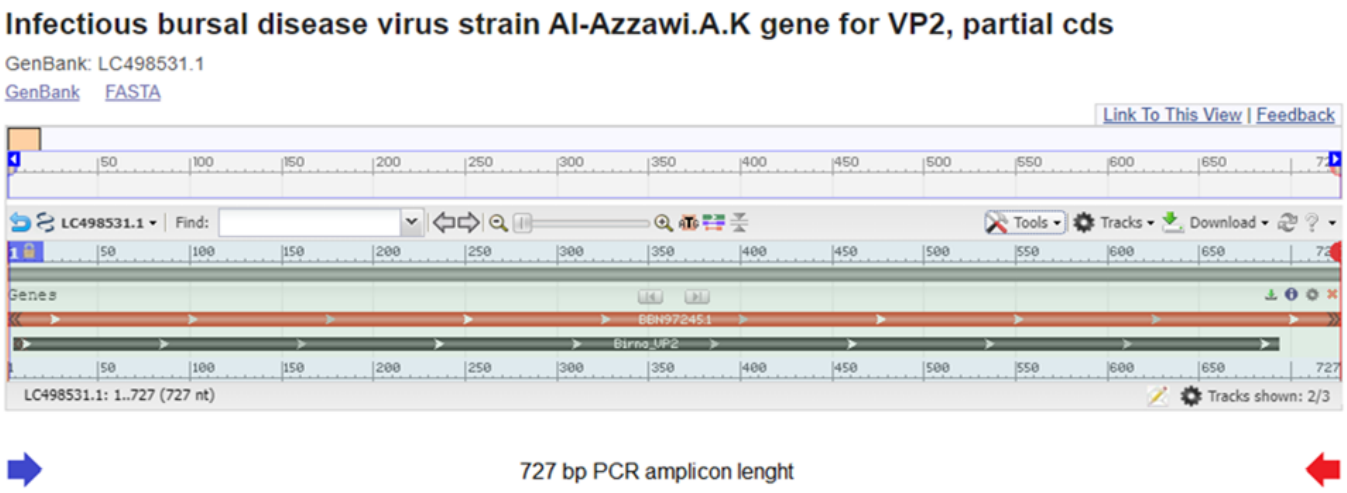

Figure 3. Precise locations of the VP2 locus-covering PCR amplicons within the genomic sequences of the infectious bursal disease virus in broiler chickens concerning the reference strains (GenBank accession number: LC498531.1). The red arrows point to the ends of these amplicons, while the blue arrows point to their beginnings.

Table 1. The length and location of the 727 bp PCR amplicons used to amplify the VP2 locus within the genomic sequences of infectious bursal disease virus in broiler chickens. The reference strains of infectious bursal disease virus (GenBank acc. no. LC498531.1)

Targeted gene	Sequences (5′-3′)	Length
VP2 fragment of infectious bursal disease virus	CCAGGTGGGGGTACAATCACACTGTTCTCAGCTAATATCGATGCTATCACGAGCCTCAGCA TCGGGGGAGAACTTGTGTTTCAAACAAGCGTCCAAGGCCTTATACTGGGTGCTACCATCTA CCTTATAGGCTTTGATGGGACCGCAGTAATCACCAGAGCTGTGGCCGCAGACAATGGGCT AACGGCCGGCACTGACAACCTCATGCCATTCAATATTGTGATACCAACCAGCGAGATAAC CCAGCCAATCACATCCATCAAACTGGAGATAGTTACCTCCAAAAGTGGTGGTCAGGCGGG GGATCAGATGTCATGGTCAGCAAGTGGGAGCTTAGCAGTGACGATTCACGGTGGCAACTA TCCAGGAGCCCTCCGTCCCGTCACACTAGTAGCCTACGAAAGAGTGGCAACAGGATCTGT CGTAACGGTCGCCGGGGTGAGCAACTTCGAGCTGATTCCCAATCCTGAACTAGCAAAGAA CCTGGTCACAGAATATGGCCGATTTGACCCAGGAGCCATGAACTACACAAAATTGATACT GAGTGAGAGGGACCGTCTTGGCATCAAGACCGTATGGCCAACAAGGGAGTACACAGACTT TCGCGAGTACTTCATGGAGGTGGCCGACCTCAACTCTCCCCTGAAGATTGCAGGAGCATTT GGCTTCAAAGACATAATCCGGGCCCTAAGGAGGATAGCTGTGCCGGTGGTCTCTACATTGT CCC	727 bp

```
          10       20       30       40       50       60       70       80       90       100
    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ref.
GCAGGTGGGGTAACAATCACACTGTTCTCAGCTAATATCGATGCCATCACAAGCCTCAGCATCGGGGGAGAACTCG
TGTTTCAAACAAGCGTCCAAGGCC
S1  ..........................................................................................

          110      120      130      140      150      160      170      180      190      200
    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ref.
TTATACTGGGTGCTACCATCTACCTTATAGGCTTTGATGGGACCGCAGTAATCACCAGAGCTGTGGCCGCAGACAA
TGGGCTAACGGCCGGCACTGACAA
S1  ..........................................T..G.....................................................

          210      220      230      240      250      260      270      280      290      300
    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ref.
CCTCATGCCATTCAATATTGTGATACCAACCAGCGAGATAACCCAGCCAATCACATCCATCAAACTGGAGATAGTT
ACCTCCAAAAGTGGTGGTCAGGCG
S1  ...T.....................T.....................................G.....................

          310      320      330      340      350      360      370      380      390      400
    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ref.
GGGGATCAGATGTCATGGTCAGCAAGTGGGAGCTTAGCAGTGACGATTCACGGTGGCAACTATCCAGGAGCCCTC
CGTCCCGTCACACTAGTAGCCTACG
S1  ....................................C.............C.....................G.....................

          410      420      430      440      450      460      470      480      490      500
    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ref.
AAAGAGTGGCAACAGGATCTGTCGTAACGGTCGCCGGGGTGAGCAACTTCGAGCTGATTCCCAATCCTGAACTAG
CAAAGAACCTGGTCACAGAATATGG
S1  ..........................T.....................................C..A.....................................C..

          510      520      530      540      550      560      570      580      590      600
    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ref.
CCGATTTGACCCAGGAGCCATGAACTACACAAAATTGATACTGAGTGAGAGGGACCGTCTTGGCATCAAGACCGTA
TGGCCAACAAGGGAGTACACAGAC
S1  ..........................................................................................T...

          610      620      630      640      650      660      670      680      690      700
    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ref.
TTTCGCGAGTACTTCATGGAGGTGGCCGACCTCAACTCTCCCCTGAAGATTGCAGGAGCATTTGGCTTCAAAGACA
TAATCCGGGCCCTAAGGAGGATAG
S1  ..........................................................................................

          710      720
    ....|....|....|....|....|.
ref.  CTGTGCCGGTGGTCTCTACACTGTTC
S1  ..........................
```

Figure 4. DNA sequence alignment of one viral sample with its corresponding reference sequences of the PV2 locus within the infectious bursal disease virus in broiler chicken genomic sequences in comparison with reference strains of infectious bursal disease virus (GenBank acc. no. LC498531.1). The symbol "ref" refers to the NCBI reference sequences, while "S" refers to the sample code.

Translated PCR amplicon

10 20 30 40 50 60 70 80 90 100

....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|

ref.

AGGVTITLFSANIDAITSLSIGGELVFQTSVQGLILGATIYLIGFDGTAVITRAVAADNGLTAGTDNLMPFNIVIPTSEITQPI TSIKLEIVTSKSGGQA

S1 .....

110 120 130 140 150 160 170 180 190 200

....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|

ref.

GDQMSWSASGSLAVTIHGGNYPGALRPVTLVAYERVATGSVVTVAGVSNFELIPNPELAKNLVTEYGRFDPGAMNYTKL ILSERDRLGIKTVWPTREYTD

S1 .....

210 220 230 240

....|....|....|....|....|....|....|....|..

ref. FREYFMEVADLNSPLKIAGAFGFKDIIRALRRIAVPVVSTLF

S1 .....

The entire protein

>AAF85953.1 polyprotein [Infectious bursal disease virus]

MTNLQDQTQQIVPFIRSLLMPTTGPASIPDDTLEKHTLRSETSTYNLTVGDTGSGLIVFFPGFPGSIVGAHYTLQSNGNYKFDQMLLTAQNLPASY
NYCRLVSRSLTVRSSTLPGGVYALNGTINAVTFQGSLSELTDVSYNGLMSATANINDKIGNVLVGEGVTVLSLPTSYDLGYVRLGDPIPAIGLDPK
MVATCDSSDRPRVYTITAADDYQFSSQYQAGGVTITLFSANIDAITSLSIGGELVFQTSVQGLILGATIYLIGFDGTAVITRAVAADNGLTAGTDNL
MPFNIVIPTSEITQPITSIKLEIVTSKSGGQAGDQMSWSASGSLAVTIHGGNYPGALRPVTLVAYERVATGSVVTVAGVSNFELIPNPELAKNLVTE
YGRFDPGAMNYTKLILSERDRLGIKTVWPTREYTDFREYFMEVADLNSPLKIAGAFGFKDIIRALRRIAVPVVSTLFPPAAPLAHAIGEGVDYLLG
DEAQAASGTARAASGKARAASGRIRQLTLAADKGYEVVANLFQVPQNPVVDGILASPGILRGAHNLDCVLREGATLFPVVITTVEDAMTPKALN
SKMFAVIEGVREDLQPPSQRGSFIRTLSGHRVYGYAPDGVLPLETGRDYTVVPIDDVWDDSIMLSKDPIPPIVGNSGNLAIAYMDVFRPKVPIHVA
MTGALNAYGEIENVSFRSTKLATAHRLGLKLAGPGAFDVNTGSNWATFIKRFPHNPRDWDRLPYLNLPYLPPNAGRQYDLAMAASEFKETPELE
SAVRAMEAAANVDPLFQSALSVFMWLEENGIVTDMANFALSDPNAHRMRNFLANAPQAGSKSQRAKYGTAGYGVEARGPTPEEAQREKDTRI
SKKMETMGIYFATPEWVALNGHRGPSPGQLKYWQNTREIPDPNEDYLDYVHAEKSRLASEEQILRAATSIYGAPGQAEPPQAFIDEVAKVYEINH
GRGPNQEQMKDLLLTAMEMKHRNPRRAPPKPKPKPNAPTQRPPGRLGRWIRAVSDEDLE

Figure 5. Amino acid residues alignment of detected variations of the VP2 protein within investigated infected broiler chickens with infectious bursal disease virus. Grey highlights refer to the amplified region of the encoded protein, blue highlights refer to the positions of silent variants detected.

Table 2. The details of the identified variants in the investigated sequences of the VP2 gene within infectious bursal disease virus in broiler chickens

No.	Variant	Position in PCR amplicon	position in the reference GenBank	Position in protein	Consequence/position of the variant
1.	C>T	144	144	Thr269	Silent (p.Thr269=)
2.	A>G	147	147	Ala270	Silent (p.Ala270=)
3.	C>T	204	204	Leu289	Silent (p.Leu289=)
4.	A>T	225	225	Ile296	Silent (p.Ile296=)
5.	T>G	276	276	Val313	Silent (p.Val313=)
6.	T>C	334	334	Leu333	Silent (p.Leu333=)
7.	T>C	348	348	Ile337	Silent (p.Ile337=)
8.	A>G	369	369	Gly344	Silent (p.Gly344=)
9.	A>T	426	426	Val363	Silent (p.Val363=)
10.	T>C	459	459	Ile374	Silent (p.Ile374=)
11.	C>A	462	462	Pro375	Silent (p.Pro375=)
12.	T>C	498	498	Tyr387	Silent (p.Tyr387=)
13.	A>T	597	597	Thr420	Silent (p.Thr420=)

The letter "S #" refers to the VIRAL sample code. Identified variants were named according to the international nomenclature system. P: Protein

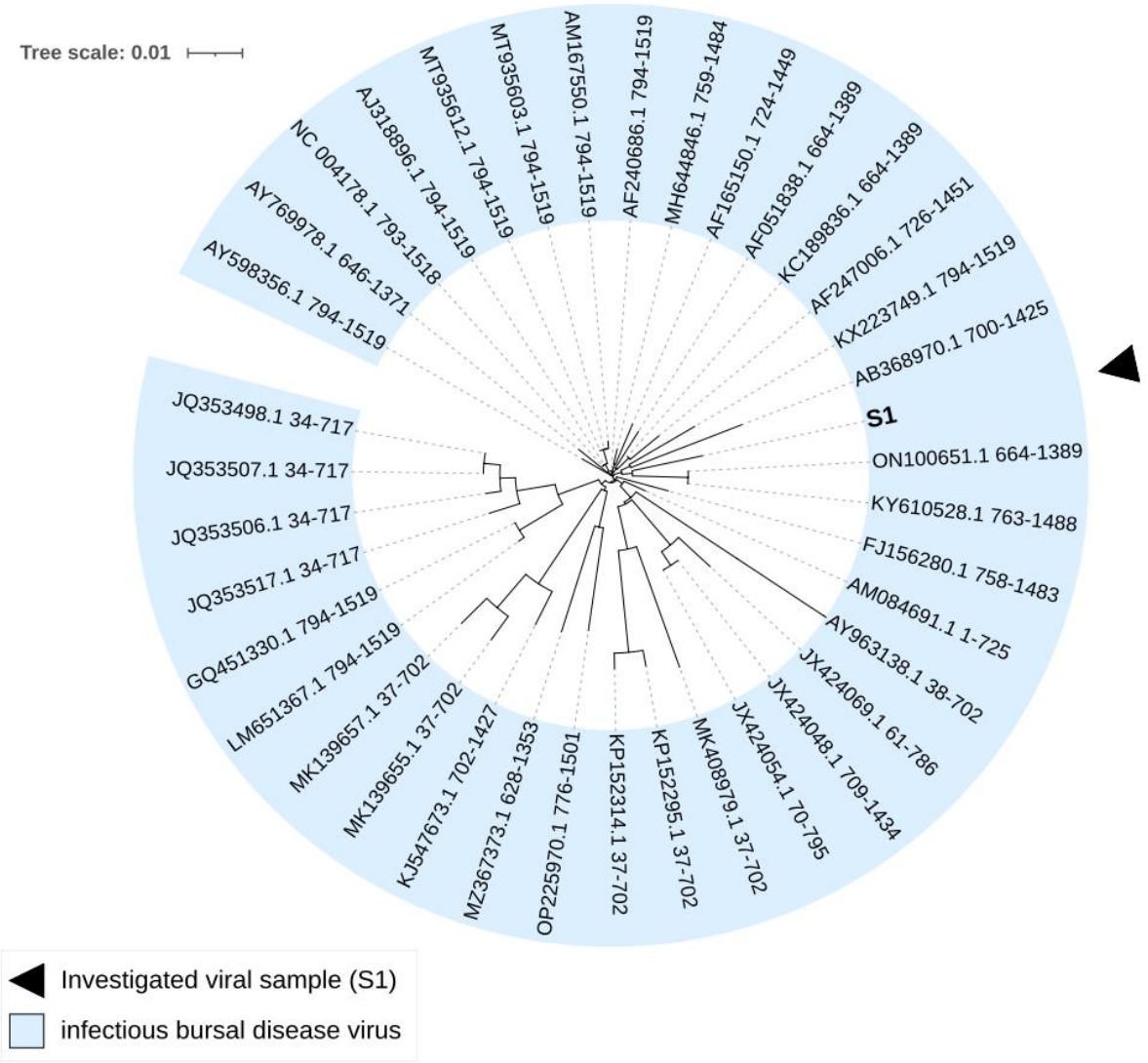

Figure 6. The entire circular phylogenetic tree indicated that genetic variations of the PV2 locus in a single infectious bursal disease virus isolate in broiler chickens. The left-hand portion of the tree's scale represents the degree of phylogenetic positions among the viral organisms classified in the tree. The code of the examined samples was indicated by the letter "S".

Histopathological results

Bursa of Fabricius

The histopathological lesions were observed in the bursa of Fabricius. These pathological changes indicated an obvious degeneration of the bursal epithelium associated with hemorrhage, and cystic cavities were observed, particularly in the follicular and epithelium of the bursa of Fabricius (Figure 7). The epithelial bursa showed thickening, and the cell hyperplasia encompassed some cystic structures. Follicles contained an abundance of inflammatory cells, mainly heterophils that were observed within follicles, along with macrophages in the inter-follicular spaces (Figure 8). Clear coagulative necrosis was detected in the interior follicular bursa, developing in areas occupied by eosinophilic materials. It also appeared that the cortical follicular regions contained a low density of heterophilic cells, compared to many cells located in the medullary follicular regions associated with macrophages and eosinophilic homogenous substances (Figure 8).

Lungs

The pathological lesions in the lung tissues displayed moderate to severe inflammation, combined with significant congestion and edema. Additionally, there was substantial fluid accumulation. In parenchymal cells of the lung tissues, both multifocal necrosis and mononuclear inflammatory cellular infiltration were seen (Figure 8). Several of the air capillaries exhibited thicker walls and/or parenchymal degeneration, both of which were associated with pronounced edema and congestion.

Kidney

Infected chickens with IBD had clear histological changes in their kidneys, such as bleeding and severe congestion in the subcapsular region, as well as edema caused by the breakdown of the epithelial cells that line tubules and ducts in some places (Figure 9). The epithelium tissue of lining tubules and ducts of the kidney also contained this protein. There was also apparent hyperplasia in some of the renal epithelium and localized mononuclear infiltration of cells. The proximal and distal convoluted tubules and ducts indicated eosinophilic casts. Cortical kidney tissue demonstrated signs of nephritis and glomeruli degeneration (Figure 9).

Liver

In the liver, the pathological changes indicated massive degeneration and severe inflammation, along with infiltration with mononuclear inflammatory cells as can be seen in Figure 10. There were many cystic cavities observed in the liver parenchyma. These cavities were detected diffusely in superficial and deep parenchymal tissues associated with severe congestion and hemorrhage (Figure 10). There were also clear periportal aggregations of heterophils, commonly perivascular.

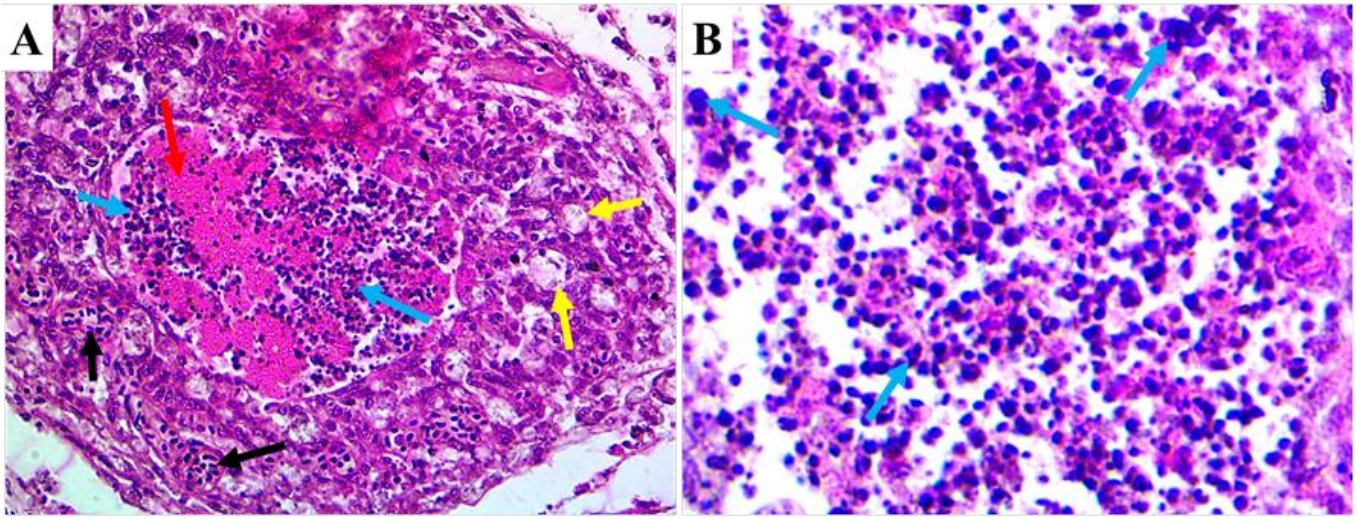

Figure 7. Histological changes of the bursa of Fabricius in chickens infected with infectious bursal disease virus. **A** and **B** indicate a clear degeneration and necrosis in the follicular bursa. Black arrows indicated severe congestion, red arrow showed eosinophilic homogenous material in the medullary region, yellow arrows indicated cystic cavities, whereas traquaze arrows present mononuclear inflammatory cells mainly heterophils and macrophage (**A** at 40X magnification, **B** at 60X magnification).

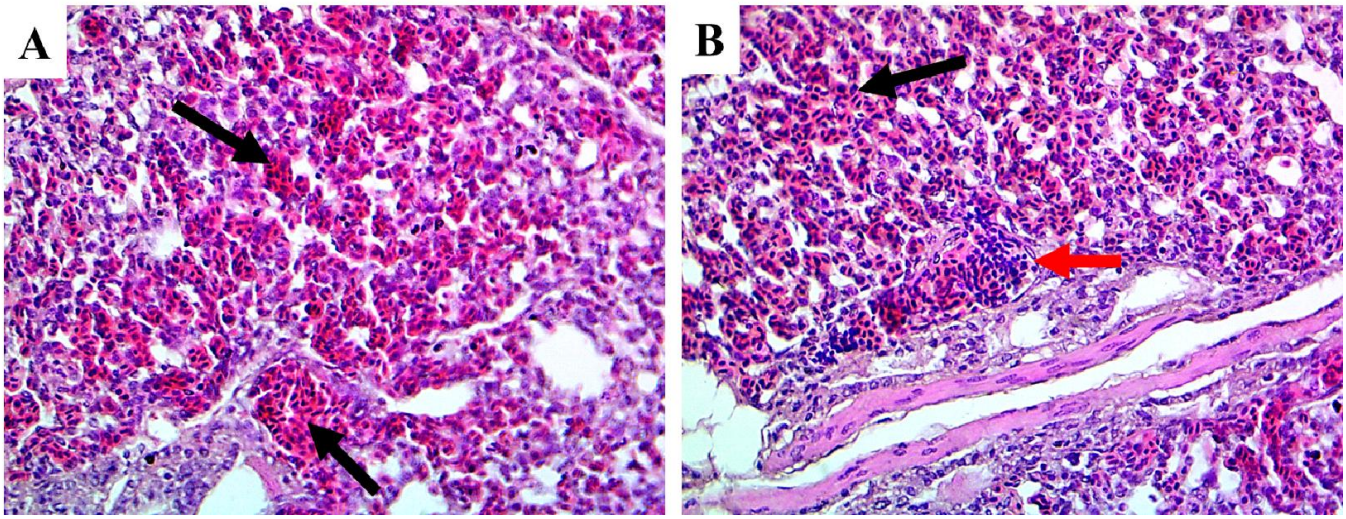

Figure 8. Pathological changes in infected broiler chickens with infectious bursal disease virus. **A** and **B** indicate severe inflammation of the lung parenchyma associated with severe congestion (black arrows) and infiltration with inflammatory cells represented by a red arrow (40x magnification).

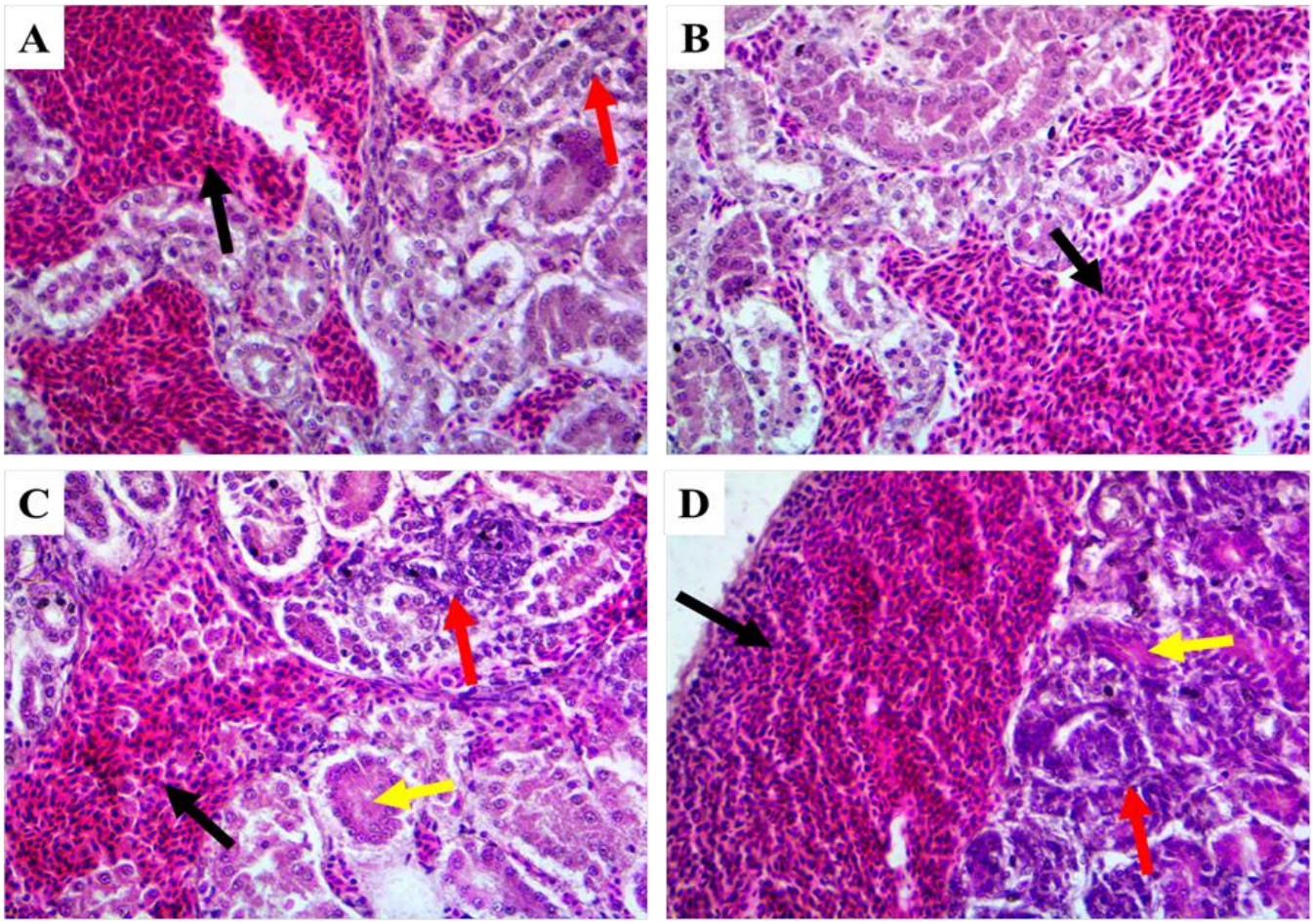

Figure 9. Histological changes in the kidney of broiler chickens infected with infectious bursal disease virus. **A**, **B**, and **C** show the pathological changes presented in the medullary region accompanied by severe hemorrhage (black arrows), eosinophilic casts (yellow arrows), and severe congestion was observed in the subcapsular area appeared clearly (**D**), red arrows indicated multifocal necrosis and severe degeneration and necrosis in the renal epithelium (40x magnification).

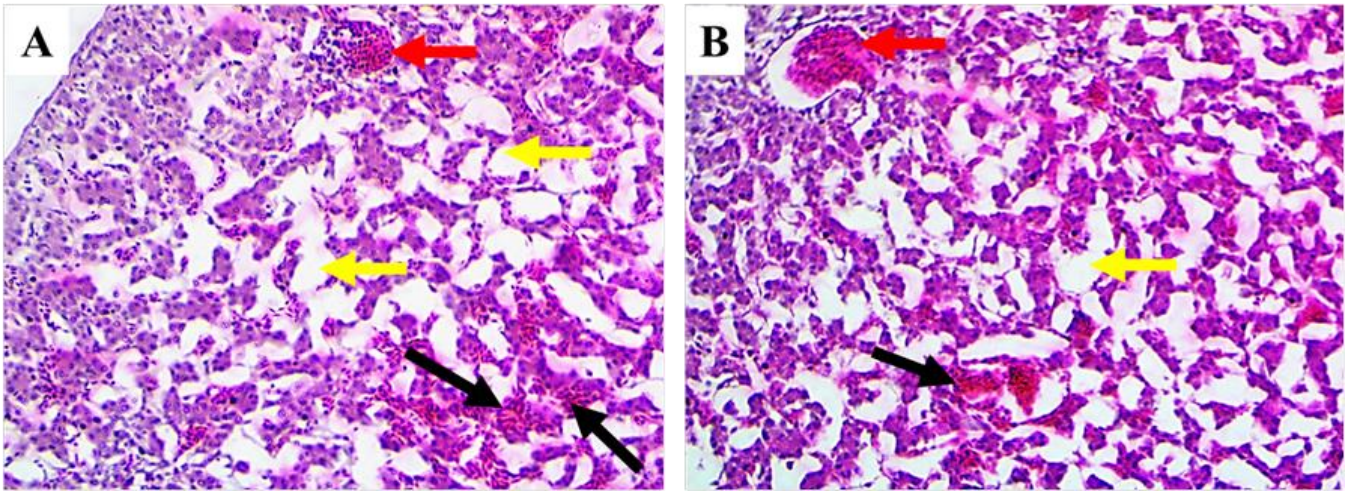

Figure 10. Histopathological lesions in the liver of broiler chickens infected with infectious bursal disease virus. **A** and **B**: Pathological changed appeared as a massive degeneration and necrosis in hepatocytes accompanied by severe infiltration and congestion (black arrows), and many mononuclear inflammatory cells (red arrows), whereas yellow arrows represented the development of many cystic cavities observed diffused in the superficial and deep parenchymal tissues (40× magnification).

DISCUSSION

Due to improper vaccination administration and insufficient vaccine evaluation, the IBDV remains a significant threat to the global chicken industry. Despite the possibility of infection in guinea pigs, ostriches, turkeys, ducks, and ducklings, the clinical condition only affects chickens (Müller et al., 2012; Al-Zuhariy et al., 2016). In chickens younger than 3 weeks of age, the disease typically presents as less severe or subclinical, whereas birds between 3 and 6 weeks of age often experience severe acute disease with significant mortality rates (Müller et al., 2012). The present study demonstrated that vvIBDV remains a substantial financial threat to the poultry sector across various regions within the Diyala Governorate, in addition to the other areas not specifically addressed in this research. High mortality rates were observed in all broiler flocks despite the widespread use of vaccines against the disease.

Using oligonucleotide primers selected from the highly conserved region PCR was widely used to successfully amplify the IBDV VP2 gene region, followed by sequence analysis and phylogenetic tree comparisons (Mawgod et al., 2014; Techera et al., 2019).

It has been essential to use the molecular characterization of the very virulent IBDV to examine global trends in the prevalence, evolution, and field status of the virus to effectively control IBD in broilers (Cheggag et al., 2020). The IBDV high genetic and antigenic diversity, the VP2 protein is widely used for phylogenetic genotyping and grouping of IBDV strains, either by using its entire nucleotide sequence or the sequence of its hypervariable region (VP2-HRV; Jackwood et al., 2018). This offered a helpful molecular replacement for dividing apart IBDV strains (Van den Berg, 2000). Using the method outlined by Meir et al. (2001), amplification of this gene was reduced. The VP2 gene was amplified using primers (IBDf and IBDr), which produced the anticipated DNA product of 727 bp in length. These findings were in line with those made public by other researchers (Moody et al., 2000; Zierenberg et al., 2001; Mawgod et al., 2014). These studies suggested that the major capsid protein of IBDV, VP2, harbors crucial immunodominant epitopes necessary for eliciting neutralizing antibodies against the virus. The IBDV isolates were classified into various antigenic subtypes and genotypes with the aid of antigenic and molecular analysis of this portion of the protein. The IBDV consists of two segments (A and B) according to Li et al. (2015) and Cheggag et al. (2020). In nature, viruses with fragmented genomes often exchange genetic material when they infect the same cell concurrently. Contrasting the local sample (GenBank acc. No. LC498531.1) with reference sequences of the IBDV revealed the presence of 13 nucleic acid variants.

The VP2 coat protein of the IBDV was translated to contain the sequences of the investigated nucleic acids at the appropriate places. Amino acid alignment of these amino acid sequences with its reference sequences indicated that the investigated 727 bp amplicons consisted of 242 amino acid sequences in the entire amino acid sequences in the VP2 in IBDV. It was found that all the detected variants had caused a silent effect in the VP2 protein. The majority of naturally occurring IBDV strains exhibit intermediate virulence levels, falling between strains of the classic pathotype and extremely virulent variants.

Due to the rapid mutation rate of RNA viruses and the significant selection pressure induced by widespread bird immunization, viruses may acquire distinct characteristics enabling them to thrive within immune populations (Dennehy, 2017). Changes in the virulence of circulating IBDV strains were caused by these mutations, which also caused antigenic variation. Consequently, it was imperative to promptly detect and describe newly discovered IBDV isolates, comparing them to previously documented viruses (van den Berg, 2000). Due to the IBDV's multiple clades being present in this study, a direct comparison between the local sample and the previously established reference genotypes was made to ascertain the precise positioning of the sample within the major clades of these viral particles. A thorough phylogenetic tree was constructed using the observed nucleic acid sequences present in the examined sample. Incredibly helpful genetic distances between all samples being studied were displayed on the neighbor-joining comprehensive tree that was being built. Most notably, the presence of a specific phylogenetic positioning in the locally examined sample may suggest that the VP2 fragment had a specific function in the evolution of IBDV sequences to attack broiler chickens (Zhang et al., 2000).

The current study revealed that the local IBDV sample under investigation, which was identified as S1, was incorporated within a specific position of the constructed tree within a clade formed of five samples of a group of very virulent IBDV strains. Within this clade, it deserved to be noted that all these five samples were suited in the vicinity of variable reference sequences deposited from variable IBDV strains of India (GenBank KX223749.1), Tanzania (GenBank AB368970.1), Hungary (GenBank ON100651.1), and Egypt (GenBank KY610528.1) origins within this clade. However, no close

phylogenetic positioning of the present sample toward these isolates was found. A different investigation found that the local isolate Diyala/VP2/MW883071 from the same region of the current study and the reference isolate (MF142560.1) were the most comparable to each other (Al-Azzawi et al., 2021). This local isolate, Diyala/VP2/MW883071, had a sequence alignment with their corresponding partial VP2 strains from NCBI 710-Jordan (MF142560.1), incorporated within a specific position in one cluster with the group of very virulent IBDV strains from the USA, primarily vvIBDV pathotype or vvIBDV reassortment strains, with an estimated 99.2% similarity between them (Al-Azzawi et al., 2021). The findings of Samy et al. (2020), who discovered that IBDV was present in nine out of ten samples collected during an IBD outbreak in the summer of 2015, were supported by these findings. The bulk of the Egyptian isolates belonged to the vvIBDV-related clade and were grouped with the antigenically changed vvIBDV strains, per phylogenetic analysis based on incomplete VP2 sequences. Additionally, based on the nucleotide sequences of HVR VP2, the current results were in agreement with those of Zierenberg et al. (2001). This region of the protein was subjected to antigenic and molecular investigation, which assisted in grouping the IBDV isolates into various antigenic subtypes.

Histopathological analysis of lymphoid tissue from infected chickens with IBD revealed that acute necrosis in the follicular bursa was linked to a decrease in lymphoid cells as well as severe interfollicular congestion and hemorrhage. Aliyu et al. (2022) demonstrated similar alterations in highly pathogenic IBDVs in chickens. Certain viral receptors on B cells, which were mostly present in the lymphoid follicles, may be responsible for the extensive damage seen in the bursa lymphoid follicles (Li et al., 2023). The findings of this study supported the argument posited by other researchers (Withers et al., 2005; Hasan and Ali, 2015) that B cells serve as the main target of IBD infection. Although similar pathological abnormalities in the bursal follicles have been observed before with Withers et al. (2005), the severity of these changes was greater. Additionally, bursal atrophy was primarily characterized by the degeneration of follicles of lymphoid tissue and the subsequent disintegration and deterioration of B cells (Li et al., 2023). The induction of alterations in the bursa's cytokine concentrations by the IBDV has been observed, suggesting that this phenomenon leads to the promotion of inflammatory response and disruption inside the tissue microenvironment (Huang et al., 2021). These changes serve as a strategic mechanism employed by the virus to diminish the activity of B lymphocytes, therefore evading or suppressing the immunological responses mounted by the host (Huang et al., 2021; Li et al., 2023). Non-lymphoid tissues (liver, lungs, and kidneys) indicated the same severe inflammation and multifocal necrosis as well as mononuclear inflammatory cells in the present histological results. Some studies have shown that the ability of IBD to induce such histological damage in non-lymphoid tissues could be interpreted as a potential property of hyper-virulent IBD which was consistent with the current published results (Silva et al., 2015; Damairia et al., 2023). However, the generalized pyknosis and tubular necrosis of the epithelial cells, along with severe necrosis and hemorrhage in the liver observed in the present study, appear to be more pathogenic than already demonstrated for the non-lymphoid organs in IBD infection (Al-Zuhariy et al., 2016; Yasmin et al., 2016). Hence, these extensive lesions in non-lymphoid organs could directly contribute to the elevated mortality rates observed during gross inspections, potentially leading to a widespread outbreak within the flock.

CONCLUSION

In this study, IBDV was identified and characterized in broiler chickens across multiple farms in the Diyala Governorate of Iraq. Employing molecular techniques such as RT-PCR targeting the VP2 gene, IBDV was detected in 10 out of 15 samples, with one strain assigned an accession number in the NCBI database. Phylogenetic analysis unveiled three distinct groups, with regional samples (S1) forming a closely related cluster of five samples, indicative of the local circulation of IBDV strains in the study area. Histopathological examination of the bursa of Fabricius, lungs, liver, and kidneys revealed significant pathological changes, including degeneration, necrosis, and infiltration of inflammatory cells, particularly heterophils. These findings offer compelling evidence of the disease and correlate with the observed clinical symptoms in affected chickens, which include depression, bleeding, and bursa enlargement.

DECLARATIONS

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Availability of data and materials

For access to the data and materials used in this study, interested individuals can reach out to the corresponding author directly. Instead, the data may also be available through a designated data source.

Ethical considerations

Ethical considerations, such as plagiarism, consent for publication, misconduct, data fabrication and/or forgery, double publication and/or submission, and replication, were thoroughly addressed by all authors.

Authors' contributions

The research team for this study comprised Ammar T. Nasser, Amer Al-Azzawi, and Karim Al-Ajeeli, who contributed to the molecular biology aspects of the investigation. Ramzi Al-Agele provided expertise in histology and histopathology. The manuscript was collaboratively written by Ammar T. Nasser, Amer Al-Azzawi, and Ramzi Al-Agele, consolidating their collective insights from the molecular and histological analyses. All authors checked the last edition of the article before publication.

Competing interests

The authors declare that they have no conflict of interest.

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Effects of Natural Guard Liquid (an Essential Oil-Based Product) on Growth Performance, Hematological Profile, and Antibody Response to Newcastle Disease Virus in Broiler Chickens

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ABSTRACT

Natural guard liquid (NGL) is an immunomodulator consisting of an essential oil blend (lavender oil, eucalyptus oil, and pine oil) that can improve the immunity (IgG) of animals. The objective of this research was to assess the effectiveness of a mixture of NGL comprising of essential oil (eucalyptus, lavender, and pine oil) on growth performance, hematological profile, and antibody titer response to the Newcastle disease virus (NDV) in Lohman strain broiler chickens. A total of 400-day-old unsexed broiler chickens with an average weight of 42.48 ± 2.08 g were randomly distributed into four groups, each comprising two replications with 50 chicks. The control group, T0, received no essential oil, while other treatment groups, T1, T2, and T3, were administered NGL at 80, 100, and 200 ppm, respectively. The mixture was administered in drinking water for 30 days. The animals had received vaccinations at the Hatchery, including active NDV, inactive NDV, and Gumboro. Regular recordings were made for feed consumption, water intake, mortality, and body weight. Blood samples for routine hematological examination (hemoglobin, erythrocytes, packed cell volume, erythrocyte index, leucocytes, and differential leucocytes) and immune parameter (NDV antibody titer) assessment were collected at the onset as well as after 15 and 30 days of treatment. The hemoglobin levels, erythrocytes, total leukocytes, lymphocytes, and Heterophil-lymphocyte index showed significant differences in groups T1 and T2 compared to T0. While the NDV antibody titer showed a significant difference at T2 compared to T0, it was not significantly different at T1 and T3. The best results and performance was indicated in T2 (100 ppm), characterized by body weight (1,839 g), and feed conversion ratio (1.573). The hematological profile in the T2 group included hemoglobin (11.78 g/dL), total erythrocytes (2.82 $\times 10^6$ u/L), total leukocytes (52.67 $\times 10^3$ u/L]), and the NDV antibody titer (48 ± 10.20) in the fifth week. In conclusion, the optimum dose of NGL is 100 ppm, which significantly influences growth performance, hematological profile, and antibody titer against NDV.

Keywords: Antibody titer**,** Broiler chicken, Growth performance, Hematological profile, Natural Guard Liquid

INTRODUCTION

Achieving success in the poultry industry is dependent on precise decision-making in the selection of genetic resources, nutrition, management, and medication programs. Global pressure in the last decade has called for the prompt discontinuation of antibiotics as growth promoters (AGP) because of the rise in antibiotic resistance and the presence of antibiotic residues in meat, eggs, and dairy products (Jabbar and Sajjad-Ur-Rehman, 2013; Qamar et al., 2023). The ban on AGP challenges

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intestinal integrity diminishes optimal nutrient absorption and adversely affects growth in chicken broilers (Shokri et al., 2017). Consequences of abstaining from AGP include disruptions in avian gastrointestinal health, a 5-10% reduction in egg production, a 12-18% rise in disease incidents and mortality rates, and a decline in feed efficiency (Mund et al., 2017). The resultant decrease in productivity and the simultaneous increase in production costs have compelled stakeholders in the poultry industry to explore alternative replacements for AGP. Some reported alternatives include prebiotics, probiotics, synbiotics, enzymes, acidifiers, Phytogenics compounds, and essential oils (Gopi et al., 2014; Mirzaei et al., 2022).

Essential oils are highly valued for safety reasons, being categorized as "Generally Recognized as Safe" (GRAS), a designation supported by the FEMA and FDA associations in the USA and extensively used in the feed and food industry (Hallagan et al., 2020). Characterized to be aromatic, volatile compounds with a pleasant aroma derived from plants, the oil has diverse pharmacological effects, including antimicrobial, antioxidant, anti-inflammatory, antifungal, anthelmintic, immunomodulatory, and antistress (Gopi et al., 2014; Amer and Aly, 2019). Other studies have indicated that essential oil is analgesic, antipyretic, cholagogue, choleretic, cholecystokinetic, and gastrointestinal stimulant (Spiridonov, 2012; Wang et al., 2016). The usage of essential oil as feed additives for AGP substitutes has increased dramatically in recent years (Omonijo et al., 2018). The makeup of active substances, functional groups, and the cooperative relationships between their constituent parts determine the mechanism of action. Essential oils are grouped into two compounds, terpenes, and phenylpropanes, each with various derivatives. The structures of the functional groups can lead to varied efficacy for similar active compounds originating from different plants (Jugreet et al., 2020). However, the appropriate selection and composition of essential oil combinations can synergistically produce optimal positive results in reducing antibiotic usage (Langeveld et al., 2014).

Researchers have explored the impacts of essential oil usage on the performance of various livestock, such as chickens, quails, pigs, and others, showing a range of positive outcomes (Hussein et al., 2019; Su et al., 2020). Strong antimicrobial properties of oregano oil, thyme, and cinnamon, enhanced with carvacrol, thymol, and cinnamaldehyde, improve the health and growth performance of broiler chickens (Du et al., 2016; Zhang et al., 2021). Essential oils' antibacterial properties are vital for preserving intestinal health and the proper balance of normal flora, promoting growth, feed efficiency, and nutrient absorption (Shokri et al., 2017). The use of singular essential oil compounds has also shown favorable effects on poultry. For instance, eucalyptus oil, administered through drinking water, holds the potential to enhance growth performance, carcass traits, and humoral immune response and acts as an anti-stress agent (Mashayekhi et al., 2018). This particular group of essential oils primarily contains active monoterpenes (Caputo et al., 2020), having bio-insecticidal, antiviral, antibacterial, anti-inflammatory, and antioxidant activities (Limam et al., 2020). On the other hand, pine oil has been reported to positively impact breast muscle growth, immune status, and antioxidant levels in broiler chickens (Ramay and Yalçın, 2020), enhance egg production, and maintain intestinal microbial balance (Guo et al., 2022). Another review mentioned that lavender oil at 600 mg/kg of feed enhances broiler growth and maintains intestinal microbial balance, intestinal tract integrity, and antioxidant performance (Barbarestani et al., 2020). These medicinal compounds are subjected to dynamic interactions within the animal's body, affecting various physiological functions of internal organs (Sadgrove et al., 2021). Thus, the purpose of this study was to evaluate the effectiveness of the Natural Guard Liquid (NGL) a product comprising a mixture of eucalyptus, pine, and lavender essential oils in broiler chickens. The observed parameters included growth performance and health status. Performance data comprised feed and water consumption, body weight, average daily growth, and feed efficiency (Sudira et al., 2021). The health status of the chickens was evaluated based on mortality and survival rates, hematological profiles, and antibody titer response to the Newcastle Disease Virus (NDV). Hematological profiles serve as supportive laboratory diagnostics to determine the health status of animals (Haile and Chanie, 2014).

MATERIALS AND METHODS

Ethical approval

The Animal Ethics Commission of the Faculty of Veterinary Medicine, Udayana University, Indonesia, granted ethical permission for all procedures involving experimental animals. This consent is documented by approval letter B/95/UN14.2.9/PT.01.04/2023.

Experimental animals and research design

This study conducted a completely randomized design with 400 day-old unsexed broiler chicks of the Lohman strain, weighing 42.48 ± 2.08 g. The chicks were sourced

from a commercial hatchery under the brand CP-707 (PT. Charoen Pokphand Jaya Farm, Denpasar, Indonesia). Day old chicks (DOCs) were randomly assigned to four treatment groups, including a control group without essential oil and three groups treated including T1, T2, and T3 with NGL essential oil through drinking water at doses of 80, 100, and 200 ppm, respectively. Each group consisted of 100 chicks which were further subdivided into two replications, each containing 50 DOC. Natural Guard Liquid treatment commenced for 30 days, starting on day 3 to day 33. Daily observations, including feed consumption, water intake, mortality, and medication, were recorded. Blood samples for routine hematological examination and immune parameter assessment were collected upon the arrival of DOC at the farm, at 18 days of age (after 15 days of treatment), and at 33 days of age (after 30 days of treatment). The treatment duration of 15 and 30 days aimed to assess NGL efficacy during the starter and grower-finisher phases. Concurrently, body weight data were collected by weighing the chickens every week at 7, 14, 21, 28 days of age and the data were recorded in the morning before feeding.

NGL formulation (PT. Rhea Natural Sciences, Jakarta, Indonesia) comprised 2.0 mg/kg lavender oil, 0.5 mg/kg eucalyptus oil, and 3.5 mg/kg pine oil. The DOC was administered a triple vaccination at the hatchery, consisting of Gumboro Vaccine (Nobilis Gumboro 228E), inactive NDV (Nobilis ND Broiler), and active NDV (Nobilis ND Clone 30), all produced by Intervet International B.V., Boxmeer, Holland. The commercial broiler feed, for the starter and grower-finisher period used by the Hi-Pro-Vite brand (PT. Charoen Pokphand, Tbk., Surabaya, Indonesia), has the nutritional composition as in Table 1.

Table 1. Chemical composition of Hi-Pro-Vite broiler diet at starter and grower-finisher phases

Nutrient content	Starter feed	Grower-finisher
Water content	13 %	13 %
Crude protein	21-23 %	19-21 %
Crude fat	5 %	5 %
Crude fiber	5 %	5 %
Ash	7 %	7 %
Calcium	0.8 -1.1 %	0.9-1.2 %
Phospor	0.5 %	0.6 %
Aflatoxin	50 µg/Kg	50 µg/Kg
Energy metabolism	2900-3000	3000-3100 Kcal/Kg

The research was conducted in February-March 2023 at Armada Farm in Dausa Village, Kintamani Subdistrict, Bangli Regency, Bali, Indonesia. The region, situated at an altitude of 700-800 masl, experienced temperatures ranging from 20-27°C (night) to 24-30°C (day) with humidity 70-80%. Broiler chickens were reared and housed in an open pen system with a size of 8x40 meters, with controlled temperature and humidity using artificial brooding. During the brooding period, the pen temperature was maintained at 33-34°C using Gas Infrared Heaters (Gasolec B.V, Bodergraven, Netherlands), gradually decreasing by 1-2°C every 3 days until the chickens reached two weeks old, and then left at natural temperature. Subsequently, the natural temperature and humidity conditions were followed through curtain and lighting management. The lighting program was set at 23 hours of light and 1 hour of darkness, with nighttime light intensity at 20 lux during the first week and 10 lux in the subsequent weeks. The experimental pens were equipped with facilities ensuring *ad libitum* access to feed and water (Sudira et al., 2021).

Growth performance

The performance parameters observed for broilers included total feed intake (g), body weight (g), weekly body weight gain (g), average daily gain (ADG, g), and feed conversion ratio (FCR). Total feed intake (FI) was recorded daily and calculated periodically. Weekly body weight measurements were used to compute cumulative body weight, ADG, and FCR. The number of sick, dead, and culled chickens was documented to calculate morbidity, mortality, and survival rates. FCR was determined by dividing the total feed consumption by the total carcass weight at harvest (MorbosEspina and Bestil, 2016; Gharechopogh et al., 2021).

Hematological profile

Blood samples were randomly selected from each pen replication, three chickens each. A 5 ml syringe with a 22G needle was used to take 3 ml of blood aseptically from the brachial vein. Subsequently, 1 ml of blood was transferred to a vacutainer tube with EDTA anticoagulant for the hematological profile investigation, and 2 ml to a vacutainer tube without anticoagulant for serum antibody examination. Transported within three hours to the Hematology Laboratory, Veterinary Teaching Hospital, Udayana University, Indonesia, the samples were stored in a cool box with ice packs at 4 °C.

A complete blood examination comprised total erythrocytes, hemoglobin, packed cell volume (PCV) or

hematocrit, and total leukocytes using the LICARE 3-Part Vet Auto Hematology Analyzer (Licare Biomedical Limited, China). Erythrocyte indices include mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC). The differential leukocyte count was performed by adopting a Giemsa-stained blood smear and counted under a microscope at 100x magnification using the straight-edge method (Buta et al., 2019). The differential leukocytes included heterophils, lymphocytes, eosinophils, monocytes, and the heterophil-lymphocyte ratio (Haile and Chanie, 2014; Buta et al., 2019).

Antibody titer against Newcastle disease virus

Specific Pathogen-free hens were used to generate a 1% erythrocyte suspension, prepared according to the procedures applied at the Veterinary Virology Laboratory, Udayana University, Indonesia. Approximately 2 ml of broiler chicken blood was drawn from the brachial vein and deposited in EDTA tubes. Following that, 5 ml of pH 7.2 Phosphate Buffered Saline (PBS) was added to the blood samples, which were centrifuged for 10 minutes at 2500 rpm. After discarding the supernatant, PBS was added before repeating the centrifugation process three times. The erythrocyte sediment was measured for concentration using a microhematocrit to determine the packed cell volume (PCV). The PCV was then diluted with PBS to a 1% concentration and ready for hemagglutination inhibition (HI) testing.

Hemagglutination inhibition testing adhered to the procedures of the Veterinary Virology Laboratory at Udayana University and the OIE (2018), and the test began with the addition of 0.025 ml of PBS to each microplate well. The first well got 0.025 ml of serum, which was serially diluted. Subsequently, 0.02 ml of 4 HAU antigen was added to each well, and the plate was kept at room temperature for 30 minutes. Each well received approximately 0.025 ml of 1% erythrocytes, and the plate was kept at room temperature for 40 minutes. Reading the HI titer comprised tilting the microplate 45º and observing the presence or absence of erythrocyte agglutination (tear-shaped). The HI antibody titer was determined by the highest serum dilution still capable of inhibiting 1% erythrocyte agglutination (OIE, 2018).

Statistical analysis

Research data were subjected to ANOVA tests and presented as mean ± SD, including feed intake, body weight, ADG, FCR, hematological profiles, and NDV antibody titers. If there is a significant difference ($p < 0.05$) between treatments, the Duncan test will be continued. The analysis was performed using IBM SPSS Statistics 26 for Windows software.

RESULTS

Growth performance

Table 2 provides a summary of growth performance data, while detailed weekly broiler performance was outlined in Tables 3, 4, and 5, along with Graph 1. Higher survival rates or lower mortality were observed in NGL treatment groups compared to control. Statistical analysis revealed that NGL supplementation in groups T1 and T2 significantly enhanced cumulative body weight, ADG, FI, and FCR when compared to group T0, but not significantly different when compared with T3 ($p < 0.05$). In the first week, there were no significant differences in growth performance between the treatment groups (Tables 3, 4, and 5, $p > 0.05$). Although group T3 outperformed the T0 group in terms of performance achievement, the difference was not statistically significant ($p > 0.05$).

Hematological profile

Table 6 presents the effect of NGL administration on the hematological profile of broiler chickens. Hemoglobin levels showed a profound increase in all NGL groups ($p < 0.05$) after 15 days of treatment compared to T0. After 30 days, a significant effect of increased hemoglobin was observed only in group T2, which did not differ from groups T1 and T3. Total erythrocytes had no effect ($p > 0.05$) after 15 days of NGL treatment, but increased significantly after 30 days in groups T1, T2, and T3 when compared to T0 ($p < 0.05$). The PCV and erythrocyte indices revealed no significant differences in all groups after 15 and 30 days of treatment ($p > 0.05$). Total leukocytes, heterophils, eosinophils, monocytes, and the heterophil-lymphocyte index were substantially lower in NGL groups compared to the control, except for monocytes, which showed no difference at 15 days ($p < 0.05$). Meanwhile, lymphocytes significantly increased in all NGL groups compared to the control group after both 15 and 30 days of treatment ($p < 0.05$).

Antibody titer against Newcastle disease virus

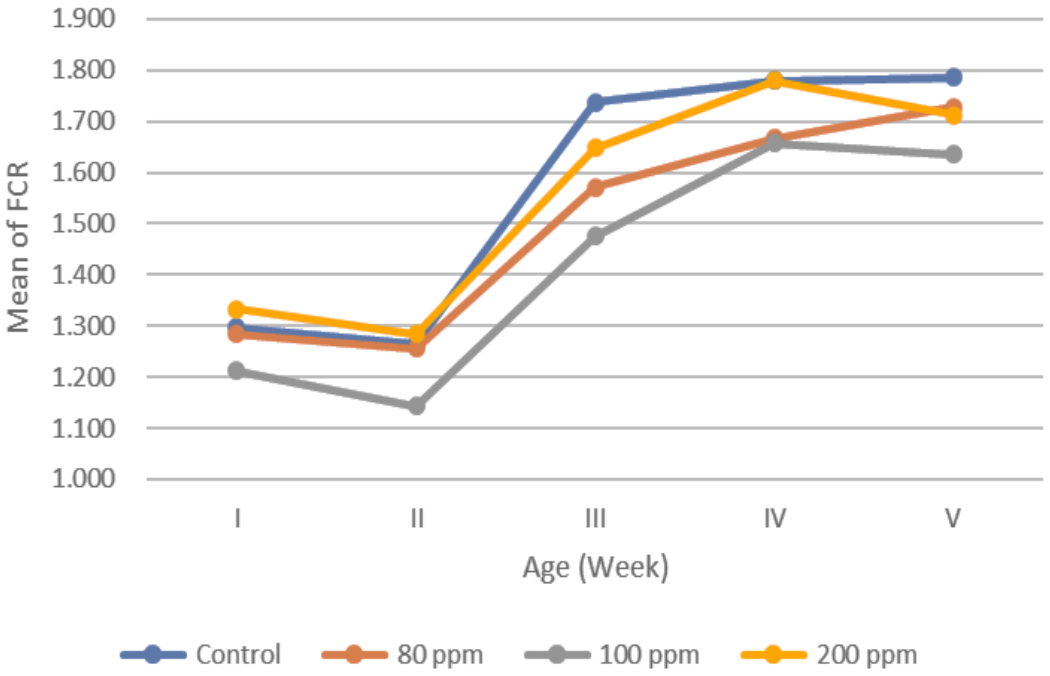
Table 7 presented the results of the Hemagglutination Inhibition test, showing antibody titer against the NDV at the time of DOC and after NGL supplementation. At the time of DOC, the titer appeared not significantly different among all groups ($p > 0.05$). Antibody titer measures against the NDV were significantly higher in group T2

compared to T0 after 15 days of treatment, with no substantial difference from groups T1 and T3 ($p < 0.05$). After 30 days of treatment, the NDV antibody titer measures were higher in group T2, compared to groups T0 and T3, with no significant difference from group T1 ($p < 0.05$).

Table 2. Performance of 33 days old broiler chickens given natural guard liquid for 30 days experiment (Mean ± SD)

Parameter \ Group treatment	T0	T1	T2	T3	P-value
Survival rate (%)	94.39	97.00	97.20	95.00	-
Depletion (%)	5.61	3.00	2.80	5.00	-
Cumulative feed intake (kg)	3.04±0.06^{a}	2.92±0.06^{b}	2.88±0.09^{b}	2.97±0.07^{ab}	0.026
Weight at 33 days (g)	1,704±69^{a}	1,807±73^{bc}	1,839±60^{c}	1,759±89^{ab}	0.001
Weekly weight gain (g)	332±13.7^{a}	353±14.5^{bc}	359±11.9^{c}	343±14.6^{ab}	0.001
Average daily gain (g/day)	51.65±2.08^{a}	54.74±2.20^{b}	55.72±1.81^{c}	53.29±2.21^{ab}	0.001
Feed Conversion Ratio	1.784±0.07^{a}	1.620±0,08^{b}	1.573±0,07^{b}	1.694±0,08^{ab}	0.013

T0: Control; T1: 80 ppm; T2: 100 ppm; T3: 200 ppm; ^{abc} Different superscript letters towards the row showed significant differences ($p < 0.05$).



Graph 1. The mean of feed conversion ratio of broiler chicken after 30 days of treatment with various doses of Natural Guard Liquid

Table 3. Cumulative feed intake (g) of 33 days old broiler chicken after 30 days of treatment with natural guard liquid (Mean ± SD)

Group	Week-1	Week-2	Week-3	Week-4	Week-5 (days 33)
T0	164±3.23	388±5.23^{a}	693±7.44^{a}	908±5.19^{a}	864±9.41^{a}
T1	170±3.48	363±5.98^{ab}	673±5.57^{b}	852±4.08^{b}	827±11.57^{b}
T2	162±3.25	357±5.27^{b}	640±6.72^{c}	856±5.39^{b}	820±10.09^{b}
T3	172±3.38	386±5.85^{a}	687±7.34^{ab}	883±5.17^{ab}	801±9.99^{b}

T0: Control; T1: 80 ppm; T2: 100 ppm; T3: 200 ppm; ^{abc} Different superscript letters towards the column showed significant differences ($p < 0.05$).

Table 4. Cumulative weekly body weight (g) of 33 days old broiler chicken after 30 days treatment of natural guard liquid (Mean ± SD)

Group	Week-1	Week-2	Week-3	Week-4	Week-5 (days 33)
T0	169±14.61	431±47.48^{a}	830±81^{a}	1,370±85^{a}	1,704±69^{a}
T1	175±19.92	484±42.71^{b}	917±93^{bc}	1,424±79^{b}	1,807±73^{bc}
T2	176±14.87	486±56.17^{b}	922±96^{c}	1,438±82^{b}	1,839±60^{c}
T3	171±14.34	471±57.95^{ab}	888±80^{ab}	1,394±97^{ab}	1,759±89^{ab}

T0: Control; T1: 80 ppm; T2: 100 ppm; T3: 200 ppm; ^{abc} Different superscript letters towards the column showed significant differences ($p < 0.05$).

Table 5. The average daily gain (g) of 33 days old broiler chicken after 30 days of treatment with natural guard liquid (Mean ± SD)

Group	Week-1	Week-2	Week-3	Week-4	Week-5 (days 33)
T0	18.01±2.22	40.97±6.41^{a}	57.01±14.41^{a}	69.11±17.34^{a}	72.94±33.08^{a}
T1	18.90±1.89	44.14±6.34^{b}	61.21±12.85^{b}	73.00±20.14^{b}	76.57±29.64^{bc}
T2	19.06±2.17	44.58±7.43^{b}	61.96±13.74^{b}	73.79±24.20^{b}	80.24±27.34^{c}
T3	18.43±1.90	43.90±6.86^{ab}	59.50±10.21^{ab}	70.91±14.92^{ab}	74.88±26.67^{ab}

T0: Control; T1: 80 ppm; T2: 100 ppm; T3: 200 ppm; ^{abc} Different superscript letters towards the column showed significant differences ($p < 0.05$).

Table 6. Hematological profile of 33 days old broiler chickens given natural guard liquid through drinking water for 30 days experiment (Mean ± SD)

Parameter	Time	T0	T1	T2	T3	P-value
Hemoglobin (g/dL)	15 days	8.08±0.86^{a}	9.78±1.08^{b}	9.85±1.16^{b}	9.43±0.99^{b}	0.026
	30 days	10.20±1.08^{a}	11.36±0.93^{ab}	11.78±0.95^{b}	11.16±1.11^{ab}	0.043
Erythrocyte (x10^{6} u/L)	15 days	2.19±0.34	2.36±0.21	2.32±0.43	2.25±0.22	0.790
	30 days	2.00±0.16^{a}	2.80±0.12^{b}	2.82±0.26^{b}	2.68±0.69^{b}	0.004
PCV (%)	15 days	24.19±1.83	26.82±2.20	26.68±1.16	25.60±1.73	0.065
	30 days	25.38±1.39	29.60±4.68	28.81±2.60	26.92±5.02	0.294
MCV fL)	15 days	113±21.54	114±11.12	119±12.82	115±15.58	0.951
	30 days	127±16.83	106±15.16	101±18.09	108±8.36	0.222
MCH (Pg)	15 days	37.50±6.74	41.75±7.02	44.88±7.67	42.30±6.62	0.665
	30 days	41.29±7.74	40.63±3.29	42.10±5.19	44.28±6.12	0.137
MCHC (%)	15 days	33.47±3.35	36.56±3.68	36.93±4.12	36.81±2.05	0.264
	30 days	40.31±5.30	39.29±7.24	42.72±5.72	42.75±9.02	0.772
Leucocyte (x10^{3} u/L)	15 days	105.03±34.69^{c}	79.07±14.58^{a}	77.33±11.08^{a}	86.37±16.59^{b}	0.042
	30 days	77.17±15.87^{b}	52.50±4.50^{a}	52.67±10.93^{a}	62.17±8.61^{a}	0.002
Heterophile (%)	15 days	22.99±2.04^{b}	16.33±2.42^{a}	15.50±4.08^{a}	18.32±3.54^{a}	0.009
	30 days	26.17±4.99^{c}	18.01±1.67^{a}	18.33±1.86^{a}	23.16±3.06^{b}	0.000
Lymphocyte (%)	15 days	62.50±3.98^{a}	71.83±2.56^{b}	74.99±5.39^{b}	69.83±5.56^{b}	0.000
	30 days	58.67±5.04^{a}	73.38±3.14^{c}	74.50±2.07^{c}	63.39±2.43^{b}	0.000
Eosinophil (%)	15 days	4.33±1.41^{b}	2.78±1.32^{a}	2.17±1.16^{a}	2.50±1.22^{a}	0.042
	30 days	7.16±2.13^{b}	4.10±1.41^{a}	3.33±1.21^{a}	4.61±3.44^{a}	0.034
Monocyte (%)	15 days	10.04±5.05	8.05±1.78	7.33±3.01	8.45±3.88	0.173
	30 days	8.12±3.03^{b}	4.50±1.04^{a}	3.83±1.17^{a}	7.83±2.78^{b}	0.005
Ratio H/L	15 days	0.36±0.03^{b}	0.23±0.04^{a}	0.21±0.04^{a}	0.26±0.06^{a}	0.000
	30 days	0.45±0.11^{b}	0.25±0.03^{a}	0.24±0.02^{a}	0.36±0.04^{b}	0.000

T0: Control; T1: 80 ppm; T2: 100 ppm; T3: 200 ppm; PCV: Packed cell volume/hematocrit; MCV: Mean corpuscular volume; MCH: Mean Corpuscular Hemoglobin; MCHC: Mean corpuscular hemoglobin concentration, and H /L: Ratio of heterophile/lympocyte. ^{abc} Different superscript letters towards the row showed significant differences ($p < 0.05$).

Table 7. Mean antibody titers response against the Newcastle disease virus before and after natural guard liquid treatment through drinking water (Mean ± SD)

Titer HI log 2 / Group	DOC	15 days experiment	30 days experiment
T0	512±280	16±0[a]	6±4.89[a]
T1	469.33±299.27	46±0[ab]	16±10.28[ab]
T2	426.66±310.03	136±13.14[b]	48±10.20[b]
T3	448±321.27	56±9.79[ab]	6±4.89[a]
P-value	0.962	0.033	0.048

T0: Control; T1: 80 ppm; T2: 100 ppm; T3: 200 ppm; DOC: Day-old chick; HI: Hemagglutinin inhibition; [abc] Different superscript letters towards the column showed significant differences ($p < 0.05$).

DISCUSSION

The essential oils can be utilized instead of AGP since they have pharmacological effects including antibacterial, antioxidant, digestive stimulant, immunomodulatory, antifungal, antiparasitic, antiviral, and bioinsecticidal. Natural Guard Liquid, sourced from *Eucalyptus spp.*, *Lavandula spp.*, and *Pinus spp.*, comprised medicinal active ingredients. Eucalyptus oil contains oxygenated monoterpene compounds, consisting of geranyl acetate, 1,8-cineole, and trans-sabinene hydrate acetate, as well as monoterpene hydrocarbons including γ-terpinene, terpinolene, and α-pinene (Caputo et al., 2020). Lavender oil comprised linalool, α-terpineol, 1,8-cineole, camphor, terpinen-4-ol (3.08%), lavandulyl-acetate, and α-terpineol. Meanwhile, pine oil featured α,β-pinene, camphene, borneol, and phellandrene (Amer and Aly, 2019). This research showed that the achieved performance was increased because the combination of eucalyptus, lavender, and pine oil worked synergistically to produce a positive pharmacological effect on feed consumption, growth, and health.

Table 2 presented, NGL at doses of 80 and 100 ppm, the mortality rate was 2.61% and 2.81% lower, compared to the control with a 5.61% mortality rate. The most favorable outcome was observed in NGL treatment at 100 ppm, resulting in a mortality rate of 2.80% or a survival rate of 97.20%. Siaga et al. stated that high-quality broiler farms should achieve a mortality standard below 5% (Siaga et al., 2017). The mortality rate in this research was very low, suggesting that chickens treated with NGL essential oil maintained a healthy condition, positively impacting FCR and performance index. These findings were corroborated by other reviews, which demonstrated that thymol and carvacrol-containing diets have antibacterial and anti-inflammatory effects (Du et al., 2016), enhancing the integrity of the digestive system and lowering mortality in broiler chickens (Du and Guo, 2021). Similarly, black seed extract administration (*Nigella sativa*) reduced the mortality rate in broiler chickens due to infectious diseases (Kusmiyati et al., 2022). Mortality, influenced by environmental factors, stocking density, and management practices, was often more prevalent in the first stocking week and toward harvesting, attributed to factors such as heat stress (Torrey et al., 2021).

The commendable feed efficiency (Graph 1) and growth (Tables 3, 4, and 5) were attributed to the active ingredients in NGL influencing physiological functions, metabolism, and the digestive system, while also suppressing subclinical infections in the chicken gastrointestinal tract (Fu et al., 2013). The multifaceted activities of NGL essential oil, acting as an antimicrobial, anti-inflammatory, antioxidant, digestive system stimulant, and immunomodulator, contributed to these results (Amer and Aly, 2019). For instance, linalool, linalyl, and lavandulyl acetate disrupted bacterial membranes, leading to the release of cellular material. Terpenoids and phenylpropanoids, due to their lipophilicity, penetrated bacterial membranes, causing damage to bacterial cells (Adaszyńska-Skwirzyńska and Szczerbińska, 2017). Previous research showed that the addition of eucalyptus leaf powder at 1-3 g/kg of feed reduced chicken feed intake (Farhadi et al., 2017), and a dose of 0.5% in the basal diet significantly affected FCR and body weight gain (Mashayekhi et al., 2018). Lavender oil, which contains the primary active components linalool, linalyl, and lavandulyl acetate, decreased the amount of *Escherichia coli* in the ileum and increased the length of the jejunal villi in broiler chickens (Barbarestani et al., 2020). Supplementing lavender oil at a dose of 500 mg/kg of feed in quails increased the length of jejunal and ileal villi and the quantity of *Lactobacillus spp* (Özbilgin et al., 2023). Pine essential oil supplementation at 100 mg/kg enhanced microbial composition and production performance in laying hens

(Guo et al., 2022). The inhibitory properties against pathogenic bacteria contributed to balancing the microbial population in the poultry intestine (Barbarestani et al., 2020).

Essential oil, when supplemented in both feed and drinking water, stimulates the digestive system (Gopi et al., 2014). The oil enhanced choleretic activity, promoting the secretion of bile and pancreatic juice (Wang et al., 2016), as well as cholekinetic activity, facilitating bile flow from the liver to the intestines (Spiridonov, 2012). Bile and pancreatic juice, rich in endogenous enzymes such as amylase, lipase, and protease, played a critical part in the hydrolysis of complicated bonds in nutrients, thereby increasing digestibility. Proteolytic activity maximized protein digestibility, reducing digesta viscosity, and facilitating broader transport of digesta into crypts through the villi of the small intestine. The cholekinetic activity of essential oil reduced intestinal peristaltic movement, ensuring optimal nutrient absorption (Alagawany and Abd El-Hack, 2020).

As shown in Table 6, red blood cells and hemoglobin increased, while PCV and the erythrocyte index remained within the normal range without significant differences from the control group. The standard range for broilers was reported as 2.2-4.5 (x106 cells/µL) for total red blood cells, 8.0-13.6 (g/dL) for hemoglobin, and 22-45% for PCV (Buta et al., 2019). Erythrocytes and hemoglobin play a crucial role in oxygen transport, carbon dioxide removal, nutrient transport, hormone circulation, metabolite movement, heat regulation, and immune product carriage. The significant increase observed in this research suggested a potential role of NGL bioantioxidants in influencing erythropoiesis and capturing free radicals in erythrocytes. The increased number of cells enhanced nutrient and substance transport, supporting the results in broiler chickens receiving essential oil formulations (Farhadi et al., 2017; Heydarian et al., 2020).

The total leukocytes and leukocyte differentials in NGL-treated broilers were significantly lower than the control, except for lymphocytes, which had higher values (Table 5). Elevated leukocyte counts have been linked to infectious agents, such as mycobacteria, viruses, fungi, and parasites. It was observed that broilers were highly susceptible to infections and subclinical inflammation (Torrey et al., 2021). The active compounds in NGL, acting as preventatives against subclinical infections, contributed to lower leukocyte counts. Similar results were observed in broilers treated with eucalyptus oil, lavender, clove, cinnamon, and *Aloe vera*. Moreover, the heterophil-to-lymphocyte (H/L) index was significantly lower in NGL group than in the control. That ratio represented an indicator of chickens adapting to environmental stressors, both internal and external. Studies have suggested that the essential oil in NGL mixtures contained antioxidants functioning as anti-stress agents (Wu et al., 2015; Limam et al., 2020).

Table 7 presented the protective maternal antibody titer against the NDV and the homogeneity in DOC. Antibody titer was considered protective when there was inhibition at a serum dilution of 1:16 (2^4 or $\log_2$ 4) (OIE, 2018). Maternal antibodies decreased and became non-protective by 10 days (HI titer < $\log_2$ 4, Gharaibeh and Mahmoud, 2013). By day 18, the titer decreased but remained protective in all groups, with only NGL 80 and 100 ppm groups maintaining protective titer by day 33. The most robust immune response statistically was observed at the 100 ppm NGL dose, maintaining high and protective levels into the fifth week. The antibody formation process comprised antigens overcoming non-specific defenses (Dahlia et al., 2019), leading to B lymphocyte-mediated humoral immune responses and plasma cell development (ShresthaSadeyen and Iqbal, 2018). In the control group, the consistent decline in antibody titer, still detectable until the fifth week, suggested no trigger by field infections. Chickens relied on maternal antibodies and triple hatchery vaccination for immunity, with essential oil NGL at 80 and 100 ppm influencing the immune system simulation by slowing down the decline in the titer and maintaining protection against the NDV in the fifth week. This proved that NGL essential oil possessed immunomodulatory capabilities, prolonging protective antibody titer compared to the control group. Researchers state essential oil immunomodulation acted to be an immunostimulator and immunosuppressant (Gopi et al., 2014), immunoregulator (Gandhi et al., 2020), immunostimulation and immunorestorative (Ogbue et al., 20220). Essential oil, applied as feed additives in poultry and piglet farming, enhanced immunity. The analysis results were supported by several publications showing increased immune responses in chickens with dietary administration of eucalyptus oil at 250 mg/kg (Farhadi et al., 2017), the addition of 0.5% eucalyptus leaf powder to basal feed (Mashayekhi et al., 2018), and pine extract at 100 mg/kg (Guo et al., 2022).

CONCLUSION

In conclusion, the supplementation of Natural Guard Liquid essential oil through drinking water improved growth performance and had a positive impact on the hematological profile and NDV antibody titer. The most

significant improvements were observed at a dose of 100 ppm, positioning natural guard liquid as an alternative reference for feed additives in poultry production. Therefore, more studies need to evaluate NGL on immune response against other infectious diseases.

DECLARATIONS

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Availability of data and materials

The author confirms that the data supplied are currently available for justifiable reasons.

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Authors' contributions

I Made Merdana, Ida Bagus Komang Ardana, Yousef Haig Setrak Babikian, and Rubiyanto Widodo Haliman designed the research. Luh Made Sudimartini, Kadek Nanda Maharanthi, Ni Luh Eka Setiasih and I Ketut Sumadi carried out the research, data collection, and laboratory examinations. Haig Yousef Babikian evaluated the molecular biology. Theng In Yen, Kristina, and Hendi Yanto Effendy traced the essential oil and conducted data analysis. Hendri Lainman as trial coordinator and organizer. All authors participated in writing and revising the manuscript and approved the final edition of the manuscript.

Competing interests

There were no conflicts of interest among the writers of this paper.

Ethical considerations

The authors have checked the ethical issues, including plagiarism, consent to publish, misconduct, double publication and/or submission, and redundancy.

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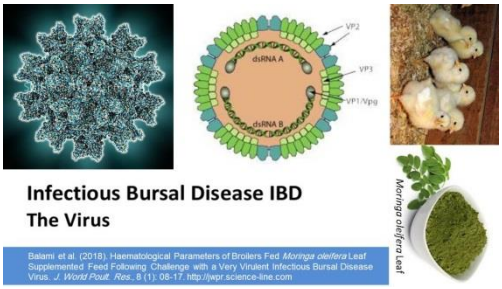

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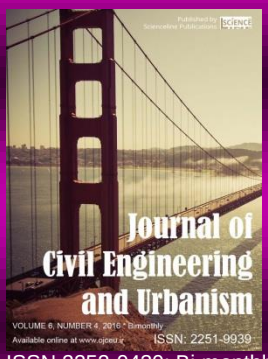

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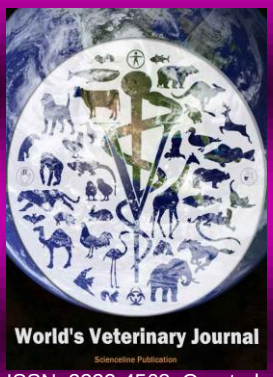

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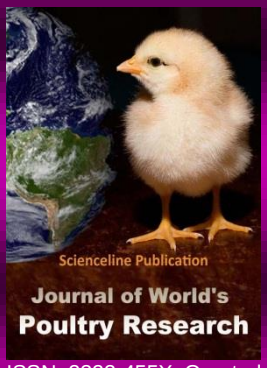

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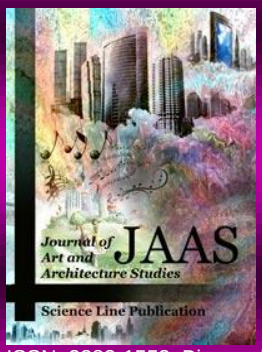

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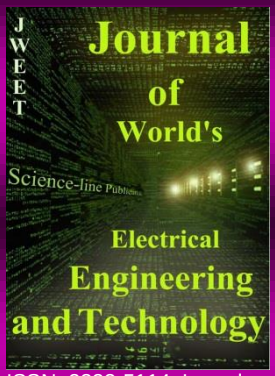

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