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BOOKLET

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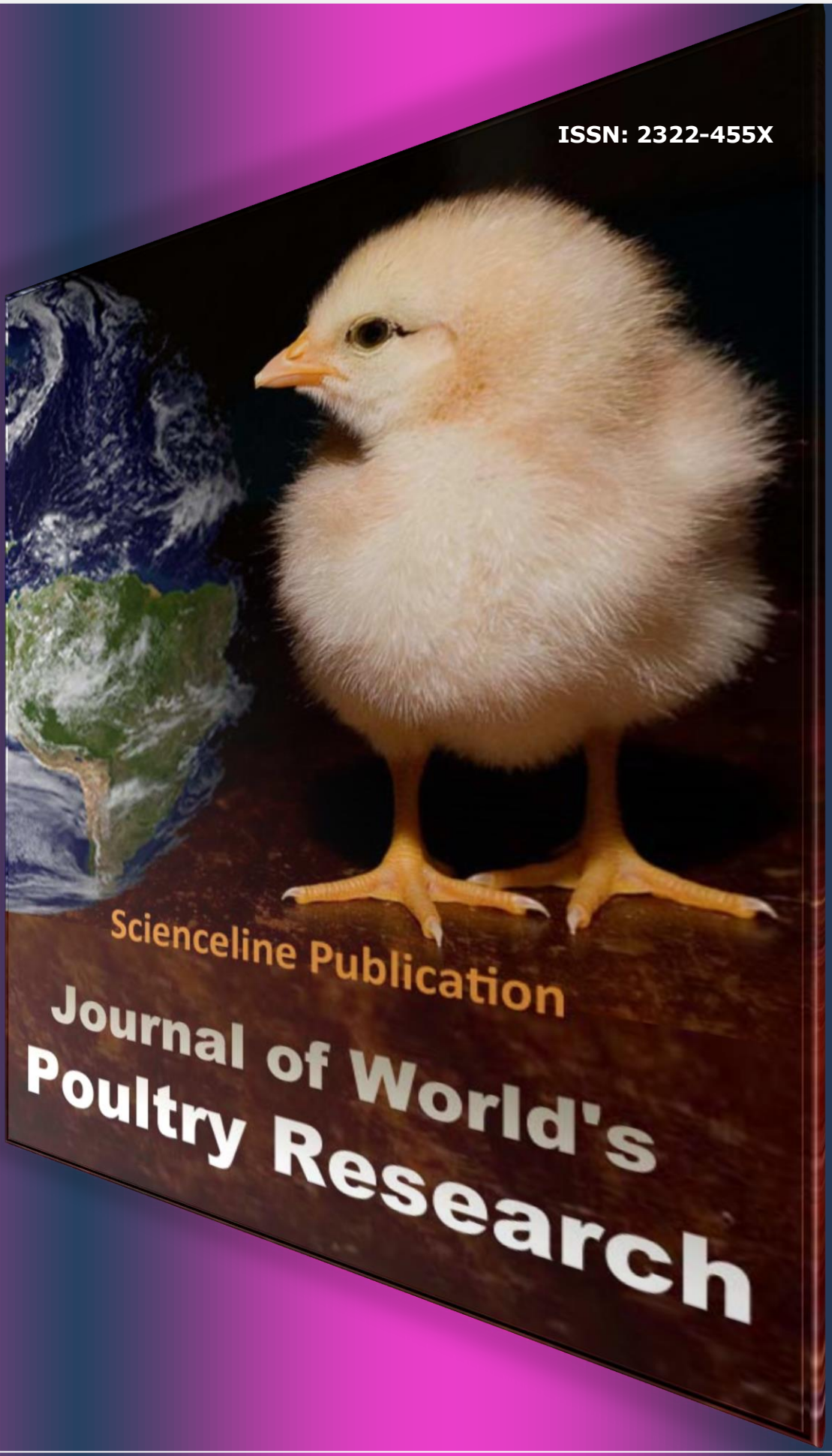

Volume 13, Issue 2, June 2023

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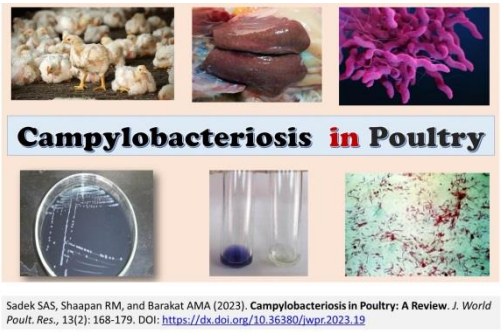
Review

Campylobacteriosis in Poultry: A Review

Sadek SAS, Shaapan RM, and Barakat AMA.

J. World Poult. Res. 13(2): 168-179, 2023; pii: S2322455X2300019-13

DOI: https://dx.doi.org/10.36380/jwpr.2023.19



ABSTRACT: *Campylobacter* is common in poultry, including layer and broiler chickens, geese, ducks, and turkeys. This review aimed to emphasize the prevalence of campylobacteriosis, recent poultry diagnoses, and strict prevention measures. *Campylobacter* species colonize the intestines of poultry and waterfowl but are generally nonpathogenic in poultry. However, they are the most common bacterial cause of sporadic human enteritis in both developed and developing countries. The main species responsible for campylobacteriosis is *Campylobacter jejuni*, followed by *Campylobacter coli*. A number of other *Campylobacter* species, such as *Campylobacter lari*, *fetus*, *upsaliensis*, and *hyointestinalis* are rarely associated with campylobacteriosis. *Campylobacter hepaticus* is the species linked to spotty liver disease in layers and breeder chickens, and it may be the etiological agent of the disease previously known as avian vibrionic hepatitis. The most prevalent infection source for *Campylobacter* is environmental contamination from poultry droppings. However, some *Campylobacter* species can be transmitted vertically, either on the surface of eggs or via trans-ovarian transmission in addition to consumption of contaminated feed or water. Due to the non-specific clinical signs such as diarrhea and weight loss, diagnosing campylobacteriosis in poultry requires culture or polymerase chain reaction tests. Little is known about the available vaccine or effective antibiotic treatment due to the rapid development of antibiotic resistance. Therefore, strict biosecurity measures play a crucial role in preventing *Campylobacter* infection in commercial poultry. These measures include decontaminating housing between flocks, preventing the entry of rodents, wild birds, and animals, and eradicating insects. To control campylobacteriosis and reduce infection risks, it is important to implement efficient on-farm biosecurity measures, conduct regular inspections of workers at meat processing plants and poultry farms, and ensure thorough preparation of chicken meat and eggs before consumption. These measures are vital in minimizing the *Campylobacter* transmission from both broiler and laying chickens, thereby reducing the risk of foodborne diseases caused by contaminated food.

Keywords: Campylobacteriosis, *Campylobacter jejuni*, Control, Diagnosis, Epidemiology, Poultry

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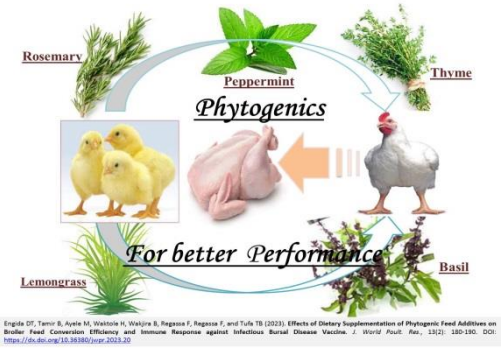
Research Paper

Effects of Dietary Supplementation of Phytogenic Feed Additives on Broiler Feed Conversion Efficiency and Immune Response against Infectious Bursal Disease Vaccine

Engida DT, Tamir B, Ayele M, Waktole H, Wakjira B, Regassa F, Regassa F, and Tufa TB.

J. World Poult. Res. 13(2): 180-190, 2023; pii: S2322455X2300020-13

DOI: https://dx.doi.org/10.36380/jwpr.2023.20



ABSTRACT: The ban on antibiotic growth promoters in livestock feeding has encouraged the utilization of phytogenic feed additives. These phytogenics recently attracted much attention and are generally recognized as residue-free ideal feed additives in animal Production. The current study was conducted to investigate the effects of the phytogenic herbs on feed intake, feed conversion ratio, and immune responses associated with the infectious bursal disease (IBD) vaccine in broiler chickens. For this study, 360 day-old broiler chicks were randomly assigned to six feeding trials, each with three replicates containing 20 chicks. The control group (T1) was only fed a basal diet alone, while the treatment groups were given the basal diet supplemented with 1% of basil (T2), lemongrass (T3), peppermint (T4), rosemary (T5), and thyme (T6) leaves powder, respectively. Body weight, feed intake, and feed conversion ratio were recorded. All chicks were vaccinated against IBD on days 7 and 19. A serology test was conducted to check the antibody titer against the IBD vaccine. The findings of this study showed that chickens in group T2 had

significantly consumed more feed, followed by T1 and T6. During the overall study period, chickens in group T4 had significantly better feed conversion efficiency, followed by T3 and T6. Chickens in groups T5 and T6 showed a more pronounced antibody titer against the IBD vaccine at days 21 and 42 of the experiment. Therefore, these findings indicated that supplementation of basil leaf powder improved feed intake. Moreover, peppermint and lemongrass leaf powder improved the feed conversion ratio. In addition, supplementation of rosemary and thyme enhances the immune status of broiler chickens and could be considered a natural growth promoter feed additive. Therefore, further studies should be done to discover their beneficial effects to use as alternative feed additives in broiler chickens.
Keywords: Body weight, Broiler chicken, Feed conversion, Feed intake, Immune response, Infectious bursa disease

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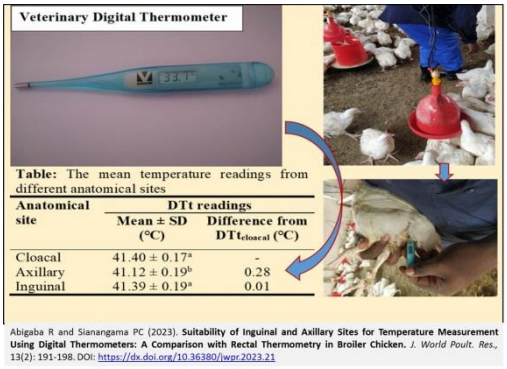
Research Paper

Suitability of Inguinal and Axillary Sites for Temperature Measurement Using Digital Thermometers: A Comparison with Rectal Thermometry in Broiler Chickens

Abigaba R and Sianangama PC.

J. World Poult. Res. 13(2): 191-198, 2023; pii: S2322455X2300021-13

DOI: https://dx.doi.org/10.36380/jwpr.2023.21



ABSTRACT: Core body temperature is one of the physiological parameters that must be assessed during the monitoring of the thermic and or health status of broiler chickens. In this regard, cloacal thermometry is a standard method used for temperature measurement although it has many drawbacks. This study was conducted to explore the suitability of other anatomical sites for temperature measurement using a digital thermometer. This was a single-factor experiment that considered the anatomical site as the main factor with three levels (treatments), including cloacal ($DTt_{cloacal}$), axillary ($DTt_{axillary}$), and inguinal ($DTt_{inguinal}$) sites. Out of 84 broiler chickens, a total of 28 chickens were randomly selected for temperature measurement. The temperature was measured for each anatomical site, and the readings were analyzed using appropriate statistics. The cloacal site had the highest mean temperature (41.40 ± 0.17℃), while the lowest mean value was observed for the axillary site (41.12 ± 0.19℃). There was no significant difference between the mean cloacal and inguinal temperatures. The cloacal and inguinal temperature readings were significantly correlated. The results for the cloacal and inguinal temperature measurements revealed a non-significant bias. The agreement interval between these two methods was sufficiently lower than the maximum acceptable difference between the anatomical sites. Both cloacal and inguinal temperature measurements had similar median points. The results indicated an underestimation of the temperature readings for the axillary site compared to those of the other sites. In conclusion, this study has revealed that the application of a digital thermometer using the inguinal site gives temperature readings that are similar to those of the conventional cloacal method.

Keywords: Axillary site, Broiler chicken, Cloacal site, Digital thermometer, Inguinal site, Temperature

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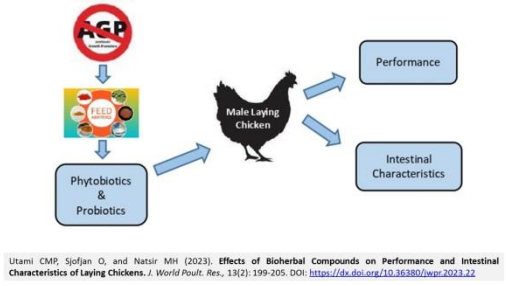
Research Paper

Effects of Bioherbal Compounds on Performance and Intestinal Characteristics of Laying Chickens

Utami CMP, Sjofjan O, and Natsir MH.

J. World Poult. Res. 13(2): 199-205, 2023; pii: S2322455X2300022-13

DOI: https://dx.doi.org/10.36380/jwpr.2023.22



ABSTRACT: Since the European Union banned the use of antibiotic growth promoters in poultry feed in 2006 (EC Regulation No 1831/2003), alternative feed additives have been investigated. The purpose of this study was to evaluate the effect of a bioherbal combination of probiotics and phytobiotics as a feed additive in drinking water on the performance and intestinal characteristics of male laying chickens. The study was performed on 200 male laying chickens for 60 days. This research method was a field experiment with a completely randomized design, consisting of four treatments and five replications. The treatments were T0 (drinking water without bioherbal, control), T1 (control + bioherbal code 1 M), T2 (control + bioherbal code 2 H), and T3 (control + bioherbal combination of 1M and 2H). The investigated parameters

included growth performance and intestinal profile of the male laying chickens. The addition of bioherbal increased the number of villi in the intestines of the male laying chickens; however, there was no significant difference among other parameters. It can be concluded that the addition of bioherbal code 2H as a feed additive with a composition of herbal leaves can improve the performance and intestinal characteristics of male laying chickens.

Keywords: Intestinal Characteristic, Male Layings, Performance Production, Phytobiotic, Probiotic

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

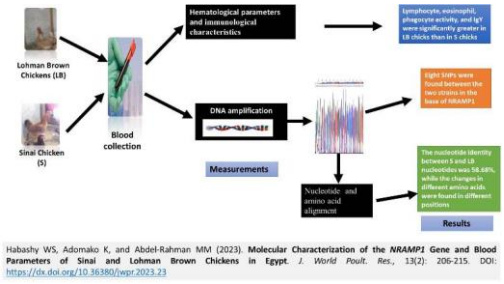
Research Paper

Molecular Characterization of the *NRAMP1* Gene and Blood Parameters of Sinai and Lohman Brown Chickens in Egypt

Habashy WS, Adomako K, and Abdel-Rahman MM.

J. World Poult. Res. 13(2): 206-215, 2023; pii: S2322455X2300023-13

DOI: https://dx.doi.org/10.36380/jwpr.2023.23



ABSTRACT: In almost all animal species, natural resistance-associated macrophage proteins (*NRAMPs*) have been linked to disease resistance. It plays a crucial part in innate immunity and can affect adaptive immunity as well. The aim of this study was to investigate some immunological traits and molecular genetics in the native breed of chickens, named Sinai (S) and a commercial strain of Lohman Brown (LB). The *NRAMP1* gene was reported to be associated with a defense mechanism against infection by bacteria and viruses. A total of 144 female day-old chicks, including 72 from the commercial layer strain (LB) and 72 from the Egyptian native chicken strain (S), were used in this study. At 38 days of age, blood samples were taken randomly from 8 chickens of each group for serum antibodies against the New Castle disease virus, avian influenza virus, and infectious bursal disease virus analysis. Additionally, genomic DNA was extracted from 20 blood samples at 38 days of age. Polymerase chain reaction (PCR) analyses were conducted on the DNA samples, followed by sequencing of the PCR products to identify single nucleotide polymorphisms (SNPs) in the *NRAMP1* gene in the two strains of chickens. The findings indicated that lymphocyte, eosinophil, phagocyte activity, and IgY were significantly greater in LB chicks than in S chicks. Sinai chickens, on the other hand, achieved dominance in Newcastle titter. Eight SNPs were found in *NRAMP1* of the two strains. The nucleotide identity between S and LB nucleotides was 58.68%, while the changes in different amino acids were found in different positions. Multiple SNPs in the *NRAMP1* gene have been discovered in Sinai and LB, suggesting that this gene can be used as a genetic marker for the selection of high-producing indigenous hybrids with the ability to resist pathogenic diseases in poultry.

Keywords: Disease resistance, Lohman Brown, Sinai, Gene, Single Nucleotide Polymorphism

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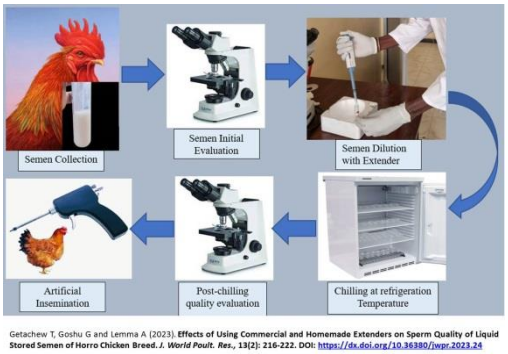
Research Paper

Effects of Using Commercial and Homemade Extenders on Sperm Quality of Liquid Stored Semen of Horro Chicken Breed

Getachew T, Goshu G and Lemma A.

J. World Poult. Res. 13(2): 216-222, 2023; pii: S2322455X2300024-13

DOI: https://dx.doi.org/10.36380/jwpr.2023.24



ABSTRACT: This study aimed to evaluate the suitability of homemade tris-egg yolk-based and Commercial Beltsville poultry extenders for short-term storage of semen from the Ethiopian Indigenous Horro chicken breed at refrigeration temperature. A total of 30 Horro roosters with an average age of 40 weeks were used to collect semen. The treatments (T) in the sperm quality experiment were control (semen without extender added), semen extended with homemade extender (E1), and semen extended with commercial Beltsville Poultry Semen Extender (E2). Changes in spermatozoa motility, *in vitro* viability, and morphology were evaluated in fresh semen and semen diluted as 1:4 (v/v semen to extender) and stored for 4, 8, 12, and 24 hours at 4°C. During semen storage, there was a decrease in mass motility, an increase in morphologically abnormal spermatozoa with a high incidence of the bent tail, and an increase in dead spermatozoa. The commercial Beltsville poultry extender was found to be the most suitable extender

regarding mass motility and *in vitro* viability of stored spermatozoa, but there was no significant difference in sperm abnormalities across all extenders. The results showed locally prepared tris-egg yolk-based extender could be a suitable extender for short-term storage of chicken sperm regarding the sperm quality attributes.

Keywords: Horro, *In vitro* viability, Motility, Morphology, Semen, Sperm

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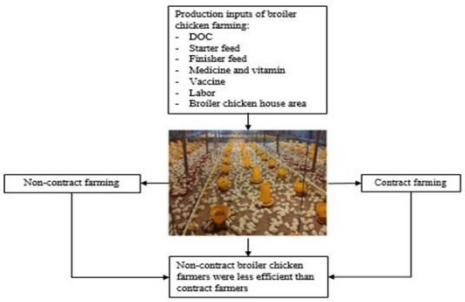
Research Paper

Comparative Analysis of Contract Farming Effect on Technical Efficiency of Broiler Chicken Farms in Indonesia

Junaidi E, Jamhari, and Masyhuri.

J. World Poult. Res. 13(2): 223-232, 2023; pii: S2322455X2300025-13

DOI: https://dx.doi.org/10.36380/jwpr.2023.25



Junaidi E, Jamhari, and Masyhuri (2023). **Comparative Analysis of Contract Farming Effect on Technical Efficiency of Broiler Chicken Farms in Indonesia.** *J. World Poult. Res.*, 13(2): 223-232. DOI: https://dx.doi.org/10.36380/jwpr.2023.25

ABSTRACT: The development of broiler chicken farms in Indonesia has taken two forms, namely non-contract and contract farming. This study aimed to compare the technical efficiency levels of production in these two types of farming in Banten Province, Indonesia. Data were collected randomly from 180 broiler chicken farmers, consisting of 103 non-contract and 77 contract farmers. The study used the stochastic frontier production function to meet its objectives. The results showed that non-contract broiler chicken farmers were less efficient in their production than those under contract. The mean technical efficiency of the production factor for non-contract broiler chicken farmers was 0.689, ranging from 0.339 to 0.996. On the contrary, broiler chicken farmers under contract had a higher mean efficiency value of 0.893, ranging from 0.638 to 0.988. Moreover, the type of input supplier had a significant positive effect on technical inefficiency in non-contract farms. Non-contract farmers who purchased their production needs from a poultry shop showed higher technical efficiency compared to those who used distributors. This research sheds light on the efficiency of broiler chicken farms, both non-contract and contract, enabling all stakeholders, including the government, to devise appropriate policies for the development of broiler chicken farming. The study provided valuable insights into the technical efficiency levels of broiler chicken farming in Indonesia, which can help farmers identify areas that need improvement and develop strategies to increase productivity and profitability.

Keywords: Broiler farm, Contract farming, Input suppliers, Stochastic frontier, Technical efficiency

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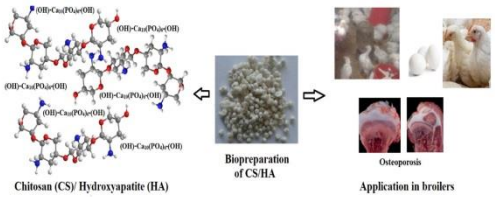
Research Paper

Chitosan Hydroxyapatite: Physic-chemical Properties and its Effect on the Growth and Development of Broiler Chickens

Vokhidova NR, Ergashev KX, Ibragimov D, Rashidova SSh.

J. World Poult. Res. 13(2): 233-243, 2023; pii: S2322455X2300026-13

DOI: https://dx.doi.org/10.36380/jwpr.2023.26



Vokhidova NR, Ergashev KX, Ibragimov D, Rashidova SSh. (2023). **Chitosan hydroxyapatite: physic-chemical properties and its effect on the growth and development of broiler chickens.** *J. World Poult. Res.*, 13(2): 233-243. DOI: https://dx.doi.org/10.36380/jwpr.2023.26

ABSTRACT: The current study aimed to obtain a calcium-containing, biocompatible drug based on chitosan *Bombyx mori*. Composites of Chitosan (CS) *Bombyx mori* with hydroxyapatite (HA) in the ratio of CS/HA = 50:50 mass percentage were synthesized *in situ* conditions at Ca/P = 1.67 mol% with intensive stirring for one hour at a speed of 1400 rpm and a temperature of 40 ± 2ºC. It was revealed that the components form an intermediate complex through –N-Ca, O-Ca, O (glycosidic bond)–Ca, H–O-bonds interacted by electrostatic forces. Atomic force microscopy studies indicated particles in the 100-50 nm size range on the polymer matrix surface. The polymer matrix prevented the growth of HA crystals and particle agglomeration. It was also determined that the CS/HA composite was non-toxic, and the LD_{50} was more than 5000 mg/kg. The composites were introduced into the chickens' diet in groups for 30 days at 25 to 40 mg/kg doses. The findings indicated an increased survival rate of chickens by 100%, improved the morphological parameters of the blood, and enhanced the contents of calcium, phosphorus, and hemoglobin. The addition of CS/HA=50:50 mass percentage contributed to an increase in the number of erythrocytes in the blood of broilers and hemoglobin by 11-12%. It should be noted that CS/HA did not adversely affect other morphological parameters of chicken blood. Therefore, CS/HA is recommended for the prevention of osteoporosis and osteomalacia in broiler chickens.

Keywords: Broiler chicken, Composites of chitosan Bombyx mori, Hydroxyapatite, *In situ*, Osteoporosis

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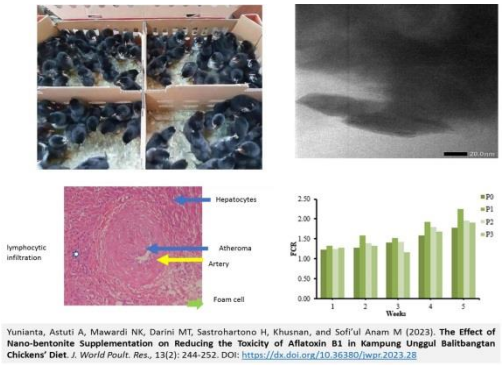
Research Paper

The Effect of Nano-bentonite Supplementation on Reducing the Toxicity of Aflatoxin B1 in Kampung Unggul Balitbangtan Chickens' Diet

Yunianta, Astuti A, Mawardi NK, Darini MT, Sastrohartono H, Khusnan, and Sofi'ul Anam M.

J. World Poult. Res. 13(2): 244-252, 2023; pii: S2322455X2300027-13

DOI: https://dx.doi.org/10.36380/jwpr.2023.27



ABSTRACT: *Aspergillus flavus* and *Aspergillus parasiticus* are fungi that produce toxic secondary metabolites known as aflatoxins. These toxins can contaminate various food and feed products, including grains and nuts, before or after they are harvested. This contamination is most commonly found in tropical countries. Many studies have demonstrated that clay additions can reduce animal aflatoxin toxicity. The objective of this research was to study how the usage of Pacitan's local bentonite, located in East Java, Indonesia, could potentially decrease the harmful effects of aflatoxin B1 in native chicken species. The Masking Gel Calcification method was used to create bentonite nanoparticles at the Center for Ceramics in Bandung, West Java, Indonesia. The *in vivo* study was conducted at a native chicken farm in Bantul, Yogyakarta, Indonesia, with 1200 unsexed Kampung Unggul Balitbangtan (KUB) chickens. Kampung Unggul Balitbangtan chickens were divided into 4 treatments and 6 replications, each containing 50 chickens. The diets in the treatments were named as T0 (the control group in which chickens were fed basal diet, without aflatoxin B1), T1 (T0 + 200 µg/kg aflatoxin B1), T2 (T0 + 200 µg/kg aflatoxin B1 + 1 g/kg Factory Feed with standard factory absorbent), and T3 (T0 + 200 µg/kg aflatoxin B1 + 1 g/kg nano bentonite). *Aspergillus flavus* isolates from PAU Universitas Gadjah Mada were created using crude aflatoxin (FNC 2262). This study found a significant difference in KUB chicken performance, specifically in feed conversion ratio (FCR). Compared to T0, the findings indicated that T1 had the highest FCR value, followed by T2 and T3. It can be concluded that nanoparticle bentonite has a looser structure because of decreased packing density with the lowest FCR. Based on hematology analysis, it can suppress aflatoxin B1 toxicity in KUB chickens.

Keywords: Aflatoxin B1, *Aspergillus flavus*, Bentonite, Feed conversion ratio, Kampung Unggul Balitbangtan chicken

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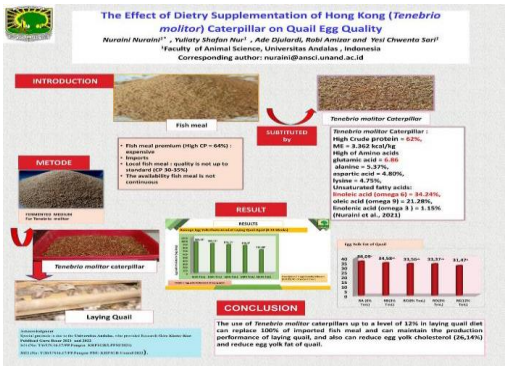
Research Paper

The Effect of Dietary Supplementation of Hong Kong Caterpillar (*Tenebrio molitor*) on Quail Egg Quality

Nuraini N, Nur YSh, Djulardi A, Amizar R, and Sari YCh.

J. World Poult. Res. 13(2): 253-260, 2023; pii: S2322455X2300028-13

DOI: https://dx.doi.org/10.36380/jwpr.2023.28

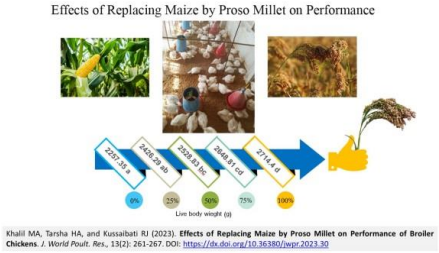


ABSTRACT: The Hong Kong caterpillar (HC) is an alternative source of animal protein for feed. This research aimed to study the effect of using Hong Kong caterpillars in the quail diet on egg quality. A total of 200 quail aged 8-14 weeks, weighing 110 ± 10 g, were used in the study, with 40% production. This study used a completely randomized design with five treatments and four replications. The laying quail diets were formulated with varying levels of HC, including 0% HC for group A, 3% HC for B, 6% HC for C, 9% HC for D, and 12% HC for E. The egg quality parameters measured were egg yolk fat, egg yolk cholesterol, egg white protein, and eggshell thickness. The results indicated that including 12% HC in the quail diet significantly reduced egg yolk cholesterol and egg yolk fat. However, eggshell thickness and egg white protein remained unaffected. Consequently, it can be concluded that Hong Kong caterpillars can be used in quail diets up to a maximum of 12% to reduce egg yolk cholesterol and fat while maintaining eggshell thickness and egg white protein levels.
Keywords: Egg quality, Fish meal, Hong Kong caterpillar, Quail

Research Paper

Effects of Replacing Maize by Proso Millet on Performance of Broiler Chickens

Khalil MA, Tarsha HA, and Kussaibati RJ.
J. World Poult. Res. 13(2): 261-267, 2023; pii: S2322455X2300029-13
DOI: https://dx.doi.org/10.36380/jwpr.2023.29



ABSTRACT: The continual rise in the cost of poultry feed ingredients, the fluctuations in price and the comparatively insufficient maize supply have prompted a search for less expensive alternatives. This research study was carried out to investigate the impact of a partial or total replacement of maize with proso millet on performance parameters of broiler chickens, including live body weight, feed conversion ratio, mortality rate and carcass yield. An experiment was carried out using 160 one-day-old broiler chicks of a commercial breed. The chicks were randomly assigned to 5 groups of 32. They consumed different isoprotein and isocaloric diets in which maize was replaced by proso millet at 0, 25, 50, 75, or 100% inclusion rates as T1, T2, T3, T4 and T5. Results showed that all broiler chickens fed on diets containing different rates of millet instead of maize significantly improved live body weight, feed conversion ratio, and carcass yield for females and males compared to T1. Additionally, it was observed that there was a significant decrease in the relative weight of the liver for females and males compared to T1. The use of millet in diets did not negatively affect the broilers' health, and the mortality rate was low throughout the experiment. These results confirmed that maize could be replaced by proso millet in broiler chicken diets up to 100%.
Keywords: Body weight, Broiler chickens, Feed conversion ratio, Maize, Proso millet

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Research Paper

Effects of *Cyperus alternifolius*, *Echinochloa pyramidalis*, *Typha angustifolia*, and *Imperata cylindrica* on Growth Performance, Feed Digestibility, Gut Microbiota, Haemato-biochemical and Immunity Parameters in Broiler Chickens

Nyembo KC, Ciza AP, Tchoffo H, Amani MI, Tchouan DG, Tchakounté FM, Edie NLW, Tindo TR, Taboumda E, and Kana JR.
J. World Poult. Res. 13(2): 268-279, 2023; pii: S2322455X2300030-13
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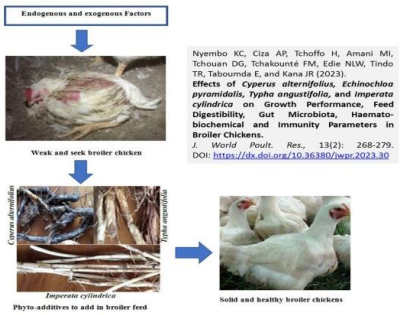

ABSTRACT: The rhizomes of Cyperus (C.) alternifolius, Echinochloa (E.) pyramidalis, Typha (T.) angustifolia, and *Imperata (I.) cylindrica* are rich in secondary metabolites and have diverse pharmacological activities. The present study was designed to evaluate the effects of dietary *C. alternifolius, E. pyramidalis, T. angustifolia*, and *I. cylindrical* rhizomes on the performance of broiler chickens. A total of 384 day-old chicks were randomly assigned to six treatment groups (each treatment replicated four times). The first group received a basal diet (negative control), and the second group received a basal diet with 1 gr of antibiotic (Doxicycline, positive control). Other groups received a basal diet with 2 gr of each phyto-additives/kg feed. The results revealed that treatments had no significant effects on feed intake and carcass yield in chickens. The *C. alternifolius* and *T. angustifolia* significantly increased live weight and weight gain, and decreased feed conversion ratio, compared to negative control. The addition of *C. alternifolius, T. angustifolia,* and *I. cylindrica* to broilers' diet significantly increased the apparent digestibility of dry matter and crude protein, compared to the negative control. Compared to the negative control, the lactic acid bacteria count significantly increased with the incorporation of *T. angustifolia* and *I. cylindrica*. The granulocytes count and globulins concentration were not affected by the different treatments. However, the lymphocyte count was significantly decreased with the diet containing *E. pyramidalis* compared to the negative and positive controls, and the diets containing *C. alternifolius* and *T. angustifolia*. The spleen and bursa weights and volumes significantly increased in all groups of chickens fed on phyto-additives, compared to the negative control. Except for haematocrit, which significantly increased with *C. alternifolius* and *T. angustifolia* in the treatments compared to the negative control, the feed additives did not significantly affect the hematological parameters. Compared to the negative control, *T. angustifolia* and *I. cylindrica* significantly increased HDL-cholesterol concentration in broiler chickens' serum, while all treatment groups were comparable for all the other biochemical parameters. Incorporating 2 g of *C. alternifolius* and *T. angustifolia* in broiler chickens' feed improves feed digestibility, enhances the population of lactic acid bacteria in the gut, and causes subsequent improvement in growth performance.
Keywords: Broiler chicken, Digestibility, Growth performance, Gut microbiota, Immunity, Phyto-additive
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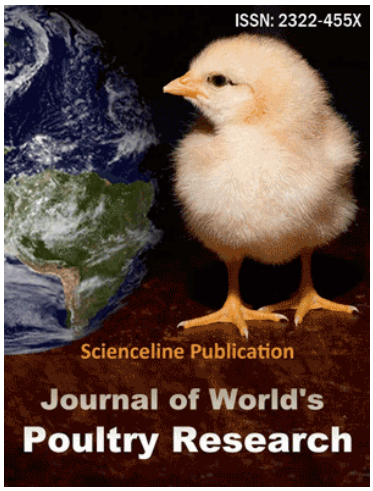

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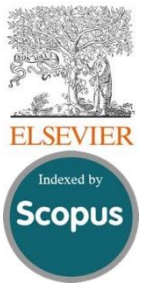

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Campylobacteriosis in Poultry: A Review

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ABSTRACT

Campylobacter is common in poultry, including layer and broiler chickens, geese, ducks, and turkeys. This review aimed to emphasize the prevalence of campylobacteriosis, recent poultry diagnoses, and strict prevention measures. *Campylobacter* species colonize the intestines of poultry and waterfowl but are generally nonpathogenic in poultry. However, they are the most common bacterial cause of sporadic human enteritis in both developed and developing countries. The main species responsible for campylobacteriosis is *Campylobacter jejuni*, followed by *Campylobacter coli*. A number of other *Campylobacter* species, such as *Campylobacter lari, fetus, upsaliensis*, and *hyointestinalis* are rarely associated with campylobacteriosis. *Campylobacter hepaticus* is the species linked to spotty liver disease in layers and breeder chickens, and it may be the etiological agent of the disease previously known as avian vibrionic hepatitis. The most prevalent infection source for *Campylobacter* is environmental contamination from poultry droppings. However, some *Campylobacter* species can be transmitted vertically, either on the surface of eggs or via trans-ovarian transmission in addition to consumption of contaminated feed or water. Due to the non-specific clinical signs such as diarrhea and weight loss, diagnosing campylobacteriosis in poultry requires culture or polymerase chain reaction tests. Little is known about the available vaccine or effective antibiotic treatment due to the rapid development of antibiotic resistance. Therefore, strict biosecurity measures play a crucial role in preventing *Campylobacter* infection in commercial poultry. These measures include decontaminating housing between flocks, preventing the entry of rodents, wild birds, and animals, and eradicating insects. To control campylobacteriosis and reduce infection risks, it is important to implement efficient on-farm biosecurity measures, conduct regular inspections of workers at meat processing plants and poultry farms, and ensure thorough preparation of chicken meat and eggs before consumption. These measures are vital in minimizing the *Campylobacter* transmission from both broiler and laying chickens, thereby reducing the risk of foodborne diseases caused by contaminated food.

Keywords: Campylobacteriosis, *Campylobacter jejuni*, Control, Diagnosis, Epidemiology, Poultry

INTRODUCTION

The world faces a significant challenge in terms of inadequate nutrition, especially for individuals who rely on animal-based food sources. The poultry industry plays a crucial role in the economies of developed countries by providing meat and animal protein to meet people's dietary needs. One important strategy to tackle the protein shortage, particularly in middle-income nations, is to increase chicken production (Barakat et al., 2012).

Avian campylobacteriosis is a serious bacterial infection that affects both farmed and wild birds. This disease is primarily caused by bacteria belonging to the *Campylobacter* genus, with *Campylobacter jejuni* (*C. jejuni*) and *Campylobacter coli* (*C. coli*) being the most common species involved (Malik et al., 2021). Gram-negative slender or spirally curved rods characterize *Campylobacter* species. When two or more bacterial cells are grouped together, they resemble a seagull. Most species have a corkscrew-like motion due to the presence of a single flagellum at one or both ends of the bacterium (On et al., 2017). *Campylobacter* species can be found in the gastrointestinal and/or genital tracts of various animal species, either as harmless commensals or as pathogens (Tshipamba et al., 2021; Hafez, 2022). The clinical effects of *Campylobacter* infection can vary significantly in humans and animals (Ranjbar and Babazadeh, 2017; Wu et al., 2022).

The prevalence of *Campylobacter* species in poultry, particularly in broiler flocks close to slaughter age, could be as high as 100% (Asmai et al., 2020). Despite being widely colonized, *Campylobacter* is largely commensal in

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birds, meaning it exists without causing harm to its host. However, it plays a major role in causing foodborne gastroenteritis in humans, with contaminated poultry meat being the primary source of exposure (Sahin et al., 2015). Studies have shown that *Campylobacter* can quickly spread from one bird to an entire flock within a week through the fecal-oral route (Stern et al., 2001). Once inside the birds, it primarily colonizes the ceca, which has the highest concentration of the bacterium, and to a lesser extent, the liver, spleen, deep muscles, thymus, and bursa of Fabricius (Awad et al., 2015). Therefore, the idea behind the prevention methods used on the farms is to reduce the likelihood that the bacteria will ever enter the flock. However, these preventive measures have largely been unsuccessful (Hermans et al., 2011), leading to a call for further research into the ecology of *Campylobacter* and methods to control its spread (Kretzschmar, 2020). Understanding the epidemiology and diagnostics of *Campylobacter* infections is crucial for implementing effective control measures. Consequently, this review aimed to highlight the campylobacteriosis epidemiology, recent diagnosis in poultry, natural and chemical treatment, and strict preventive measures and infection control in humans.

ETIOLOGY

Campylobacteriosis is a bacterial infection caused by species within the *Campylobacter* genus. These bacteria belong to the kingdom of bacteria, Phylum Proteobacteria, class Epsilonproteobacteria, order Campylobacterales, and family *Campylobacteraceae*. Recent studies have identified four main genera within this family, which include *Campylobacter*, *Arcobacter*, *Sulfurospirillum*, and *Dehalospirilum*. This family is made up of motile Gram-negative, microaerophilic or microaerobic, and non-saccharolytic bacteria (On et al., 2017). Individual species may be free-living, commensal, pathogenic, motile, or aflagellate and capable of colonizing the mouth, intestinal, stomach, or reproductive tracts of people, large production animals, such as sheep, cattle, and deer, birds, and reptiles with the temperature of 25-42°C (Lastovica, 2016). The genus *Campylobacter* was initially known to have 16 species (Foster et al., 2004) although some researchers have identified 20 species and subspecies in this genus (Fernández et al., 2008). Several studies recently claimed that the genus *Campylobacter* contains 23 species along with 6 subspecies (García-Sánchez et al., 2018**)** or even 39 species (Parte, 2018). *Campylobacter* is a group of bacteria that belong to the Gram-negative category and have a distinctive shape resembling small spirally curved rods (0.2-0.8 µm × 0.5-5 µm). When two or more bacterial cells are grouped together, they form an S or V shape that resembles a seagull (Ngulukun, 2017). The majority of the species move in a corkscrew pattern owing to a single polar flagellum at one or both ends of the bacterium (Figures 1a and b). *Campylobacter gracilis, hominis, ureolyticus,* and *blaseri* which are non-motile, and *Campylobacter showae*, which has multiple flagella, are the exceptions (Gilbert et al., 2018). *Campylobacter jejuni is* the most commonly isolated species from the confirmed cases of poultry or avian campylobacteriosis, and the remaining related to the other non-*jejuni* species, mainly *C. coli* (Indykiewicz et al., 2021). There are other minor species within the genus *Campylobacter* including *Campylobacter lari, fetus,* and *upsaliensis* that have been reported to cause infection in both humans and poultry (Facciolà et al., 2017).

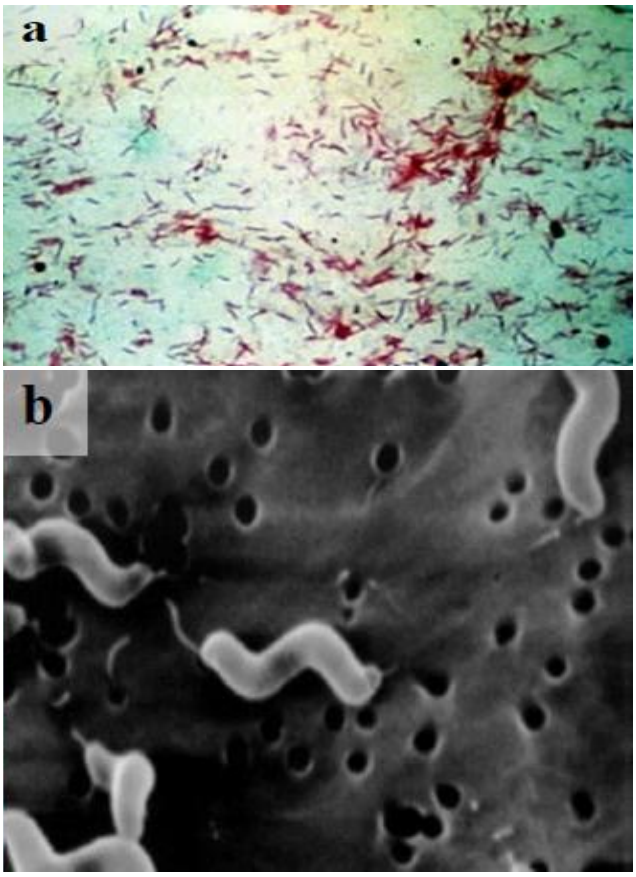

Figure 1. *Campylobacter* bacteria. **a:** Gram-negative after staining with Gram's stain, **b:** Motile flagellated under an electron microscope. Source: On et al. (2017).

HISTORY

The genus *Campylobacter* was initially proposed by Sebald and Véron (1963), which set them apart from the true *Vibrio* species. However, difficulties in the culture

and characterization of the causative organism kept it from being recognized as the major cause of disease until the 1970s. In 1906, two veterinarians in Great Britain identified large numbers of peculiar organisms in the mucus inside the uterus of pregnant sheep. These organisms were later recognized as *Campylobacter*, although their definition was not well-established at that time (Zilbauer et al., 2008). Initially, *Campylobacter* species were believed to cause diarrhea in animals and birds, and they were attributed to the Vibrio fetus, which is now known as the *Campylobacter fetus*. Veterinarians later discovered that *Campylobacter* species were responsible for many cases of septic abortion in cattle and sheep (Igwaran and Okoh, 2019). *Campylobacter* species are of great significance due to their increasing association with animal illnesses. Furthermore, the involvement of domestic and wild birds in the epidemiology of campylobacteriosis (Malik et al., 2021) contributes to its global prevalence, with sporadic occurrences reported (Upadhyay et al., 2019).

TAXONOMY

The term *Campylobacter* originates from the Greek words "kampylos," meaning "curved," and "baktron," meaning "rod." This name accurately describes the genus Campylobacter, as its members are spiral or curved rods (Linden, 2022). The taxonomic structure of the genus *Campylobacter* has changed dramatically since its inception, and some aspects of the current genus taxonomy are still debatable and require further investigation (Debruyne et al., 2008).

GROWTH AND SURVIVAL CHARACTERISTICS

Campylobacter species are non-spore forming, fastidious bacteria and mostly microaerophilic. They grow best in low-oxygen environments with 5% oxygen, 10% carbon dioxide, and 85% nitrogen (Malik et al., 2014). The survival of *Campylobacter* depends on species and other environmental conditions, including temperature, humidity, light, oxygen, or nutrient (Al-Qadiri et al., 2015). *Campylobacter* species can grow best at 37°C but not below 32°C (Figure 2). The high optimum growth temperature (42°C) distinguishes the thermophilic *C. jejuni* from most other *Campylobacter* species (Hakeem and Lu, 2021). This growth temperature is due to the absence of cold shock protein genes which play a role in low-temperature adaptation (Keto-Timonen et al., 2016). *Campylobacter jejuni* can survive for up to 6 days in chicken droppings after excretion, making them a potential source of transmission to the environment, particularly when manure is used as a fertilizer (Coorey et al., 2018).

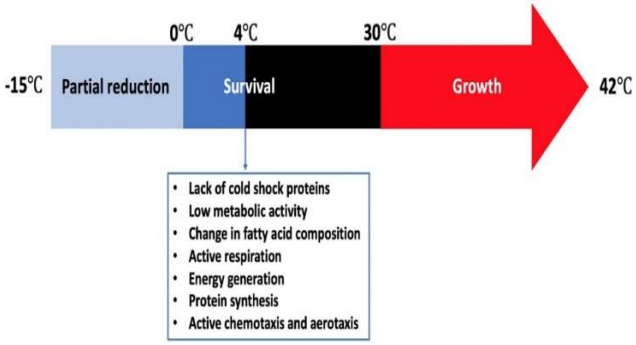

Figure 2. *Campylobacter* temperature range for survival and its reaction to stress at 4°C. Source: Hakeem and Lu (2021).

VIRULENCE FACTORS

The ability of *Campylobacter*, particularly *C. jejuni*, to adapt to unfavorable conditions and the host immune response appears to be one of the most important factors in successful gut colonization. Microorganisms go towards the intestinal environment during fecal-oral transmission under the effect of chemoattractants in order to colonize the intestinal tract of chickens (chemotaxis, Underwood et al., 2015). The proximal digestive tract also contains some proteins with antimicrobial properties, such as beta-defensin gallinacin-6 (van Dijk et al., 2007). Virulence factors determine the pathogenicity of *Campylobacter* species, and many studies have been conducted on the virulence characteristics of *C. jejuni* (Frasao et al., 2017). The virulence factors in the genus *Campylobacter*, in particular pathogenic species, such as *C. jejuni*, are multifactorial in nature, and the capacity of these bacteria to endure and withstand any physiological stress also adds to their pathogenicity (Casabonne et al., 2016).

Chemotaxis

Campylobacter jejuni adapts to various niches by using a procedure called chemotaxis, which mediates directional motility towards or away from chemical stimuli (chemo effectors/ligands that can be attractants or repellents) in the environment. The chemotaxis system comprises the methyl-accepting-domain-containing Transducer-like proteins (Tlps) and core signal transduction proteins. Chemotaxis proteins in the cytoplasm receive a signal from ligands binding to Tlps, and these proteins then start a signal transduction cascade that results in directional flagellar movement. Transducer-like

proteins make it easier for *C. jejuni* to engage in substrate-specific chemotaxis, which is crucial for the pathogen's ability to adapt, develop its pathobiology, and colonize the chicken gastrointestinal tract (Figure 3, Chandrashekhar et al., 2017).

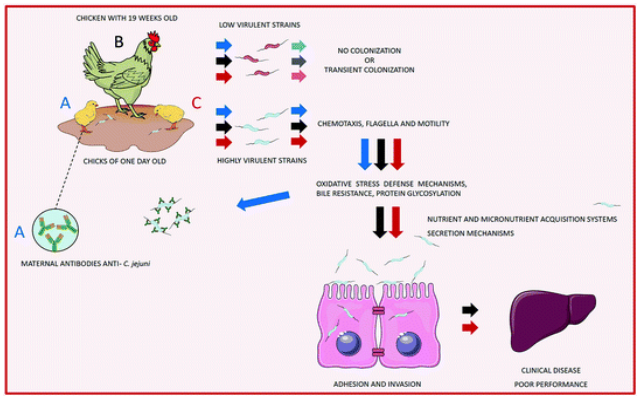

Figure 3. *Campylobacter* infection in the digestive system of early chicks can result in the destruction of bacteria (**A:** Blue arrows). Safe chickens against *Campylobacter jejuni* colonization (**B:** Black arrows). Chickens that are both unprotected (**C:** Red arrows). Source: Fonseca et al. (2016).

Flagellar motility

Motility is an essential factor for *Campylobacter* survival during a diversity of conditions that come along in the gastrointestinal tract. Due to flagella-driven motility, *Campylobacter* species can locate their appropriate habitat within the host. Two heavily glycosylated structural flagellins (FlaA and FlaB) are produced by the human foodborne pathogen *C. jejuni* (Radomska et al., 2017).

Oxygen tension and oxidative stress defense

Campylobacter can withstand a variety of unfavorable environmental factors in order to enter the gastrointestinal tract, including pH changes, oxygen restriction in the cecum, oxidative stress, increased osmotic pressure, and the presence of digestive fluids, including bile salts. The expression of genes involved in oxidative stress resistance is modulated by the peroxide resistance regulator and the *Campylobacter* oxidative stress regulator (Kim et al., 2015).

Bile resistance

For successful colonization, *C. jejuni* also needs to possess bile salt resistance. The detergent-like bile acids, such as cholates and bacteria, are killed by deoxycholates, which rupture the lipid bilayer of the cell membrane and cause the proteins in the bacterial cytoplasm to unfold and aggregate (Cremers et al., 2014).

Adhesion

Campylobacter jejuni has a number of adhesins, both individually and collectively, that can influence or mediate bacterial adherence to different cell structures and in different hosts. The adhesin that has received the most research is *Campylobacter* adhesion protein to fibronectin (CadF), a 37 kDa protein that binds to the ligand fibronectin found on epithelial cells and encoded by the gene CadF (Bolton, 2015).

Invasion

Campylobacter species have the ability to secret the invasion antigens (Cia), for example, CiaB, CiaC, CiaI, that are fundamental virulence factors by which the bacteria can invade the epithelial cells and colonize the host gastrointestinal tract in addition to the intracellular survival (Casabonne et al., 2016).

Cytolethal distending toxin

A toxin known as cytolethal distending toxin generates DNA damage that prevents cell division and kick-starts apoptosis because it exhibits DNase-like activity. This toxin causes diarrhea through its parasitical behavior with the destruction of the intestinal crypts (Carvalho et al., 2013).

EPIDEMIOLOGY

Prevalence

The European Food Safety Authority (EFSA) reports in 2019 confirmed 220,682 human cases in the European Union, with an average notification rate of 59.7 per 100,000 people (EFSA, 2021). Of the 429 meat samples from broiler chickens, 141 (32.9%) had *Campylobacter* species. In total, 3 (1.8%), 49 (36.6%), and 89 (66.9%) of the broiler chicken meat samples from Estonia, Latvia, and Lithuania tested positive for *Campylobacter* species (Tedersoo et al., 2022). *Campylobacter* has been isolated from various wild bird species worldwide, such as crows, pigeons, gulls, geese, and others. It has been found in different regions across the globe, including Africa, America, Europe, Australia, the Middle East, and Asia. These findings highlight the widespread distribution of the bacterium among wild bird populations (Antilles et al., 2021). The oldest common hosts for *Campylobacter* are the avian species due to their high body temperatures (Nur-Aziera-Aina et al., 2020; Babazadeh and Ranjbar, 2022).

Campylobacter jejuni bacteria are common commensals found in poultry and spread incredibly fast in avian flocks (Jokinen et al., 2011). Contact with a single *Campylobacter*-infected chicken for only three days is enough to infect the entire flock. Chickens show prolonged intestinal colonization at high levels with few or no symptoms or pathology (Singh and Mallick, 2019). The prevalence of *C. jejuni* in Egyptian farmed chicken intestine and liver was found to be 40.4% and 37.5% in the same manner (Elshraway et al., 2018). *Campylobacter jejuni* was isolated from chicken cloacal swabs at a rate of 15% and detected in the intestinal content of layers (17.5%) and broilers (20%, Ghoneim et al., 2020).

Transmission

The main methods of transmission for the infection are contaminated food and water, as well as direct contact with infected poultry or animals. Following an initial infection, campylobacteriosis can spread quickly within the flock (Facciolà et al., 2017). The young chicks did not become colonized until they were aged two-four weeks old, most likely because of maternally derived antibodies (Hermans et al., 2011). *Campylobacter* species transmission is made easier by various survival mechanisms. These include a variety of stress adaptation mechanisms, such as the ability to withstand oxygen exposure and desiccation, the development of biofilms and the enhancement of the viable but nonculturable state (Bolton, 2015).

Pathogenesis

Campylobacter infections are frequently linked to oral infections. The bacteria typically grow in abundance in the final third of the jejunum, ceca, and cloacae (Bolton, 2015). The first step in the pathogenesis of a *Campylobacter* infection is intestinal mucosa colonization, which is followed by adherence. The *Campylobacters* adhere, invade the epithelial cells, and then pass through the lamina propria to eventually reach the connective tissue beneath. Although the precise mechanism is unknown, it is possible that both paracellular and transcellular pathways are taken by the bacterial cells (Bolton, 2015). Cytolethal distending toxin is primarily responsible for cellular damage and death through cell cycle arrest (Facciolà et al., 2017).

CLINICAL SIGNS

The primary clinical symptoms of campylobacteriosis in chickens are diarrhoea and mucous-tinted droppings, which typically appear after 6 hours of infection. These clinical symptoms are typically exacerbated when other immunosuppressive agents are present. Infected poultry with *Campylobacter* species has also indicated a significant decrease in body weight and production (Umaraw et al., 2017).

Gross lesions

The principal symptoms of *C. jejuni* infection in chickens include considerable expansion of the distal intestine loops, a buildup of mucus and water in the intestinal lumen, as well as reddish or yellowish mottling of the liver parenchyma (Figure 4, Awad et al., 2015).

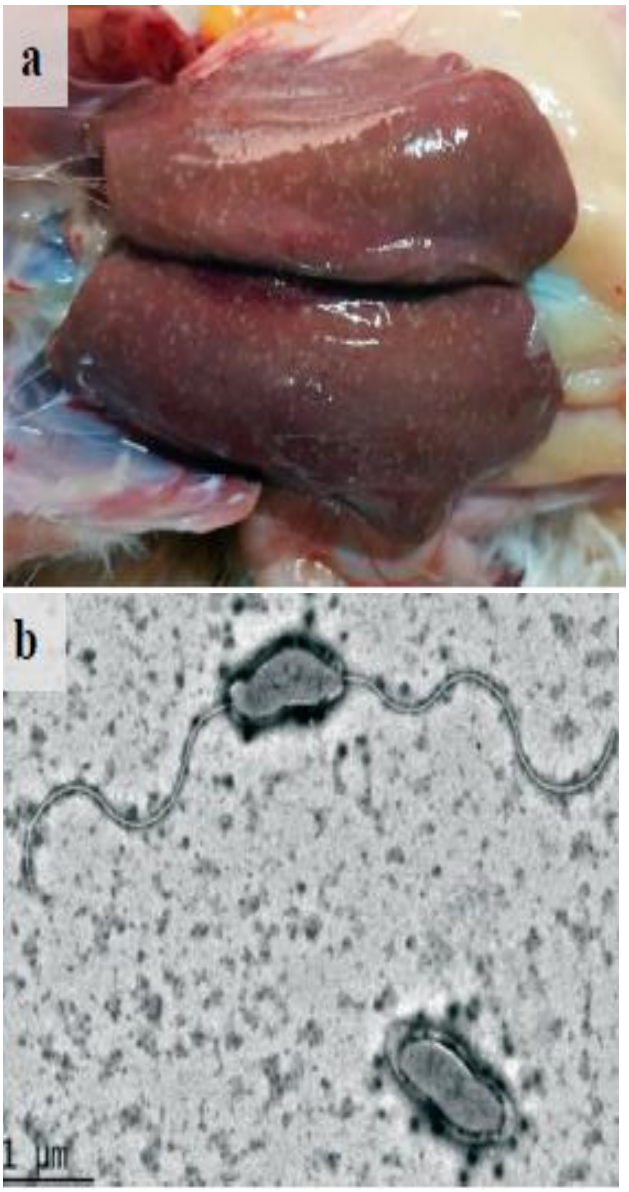

Figure 4. Liver of a chicken affected by spotty liver disease. **a:** Typical 1-2 mm lesions, transmission electron micrograph of *Campylobacter Hepaticus*, **b:** Bipolar flagella present on the top cell. Source: Moore et al. (2019).

DIAGNOSIS

Effective and quick diagnosis of *Campylobacter* species infection in avian hosts is essential for both individual care and farm-level disease management. Additionally, effective detection aids in the appropriate monitoring and surveillance of *Campylobacter* infection, which may present a risk to human health due to zoonotic transmission (Hassanain et al., 2018).

Isolation and identification

Enriching the sample in the proper broth, such as Bolton broth, followed by isolation by plating on niche medium, such as modified charcoal cefoperazone deoxycholate media, are the traditional steps in *campylobacter* species identification (mCCDA), for which a variety of commercial media are available (Figure 5a, Bolton, 2015). Bacterial isolation is usually followed by a variety of biochemical tests in the research lab, including oxidase and catalase production, urease expression, nitrate and nitrite reduction, H_2S production, and indoxyl acetate and hippurate hydrolysis (Figure 5b, Gharst et al., 2013).

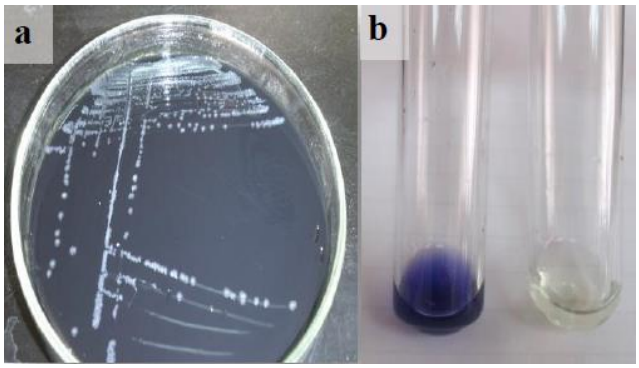

Figure 5. Isolation of *Campylobacter* on mCCDA medium showing trailing along the streak lines (**a**) and identification by hippurate hydrolysis (**b**), positive (purple), and negative (colorless). Source: Bolton (2015)

Immunological tests

Enzyme-linked immunosorbent assays (ELISA), quantitative immunofluorescence, and flow cytometry are among the enzyme immunoassays used for the diagnosis of *Campylobacter*, but ELISA dominates these immunological methods for targeting multiple specific antigens on the surface of microorganisms (Hassanain et al., 2013; Ricke et al., 2019). To identify pathogen-specific epitopes, both monoclonal and polyclonal antibodies can be produced. Additionally, antibodies can be altered, which frequently entails conjugating different detection systems, such as horseradish peroxidase, to increase the sensitivity and specificity of various target epitopes' detection (Shaapan et al., 2021). It is important to note that although immune-based detection techniques have some sensitivity with *Campylobacter* species (Figure 6), they produce false positive results. This has been observed in comparisons of commercial kits with conventional microbiological and molecular techniques (Gharst et al., 2013; Perdoncini et al., 2022).

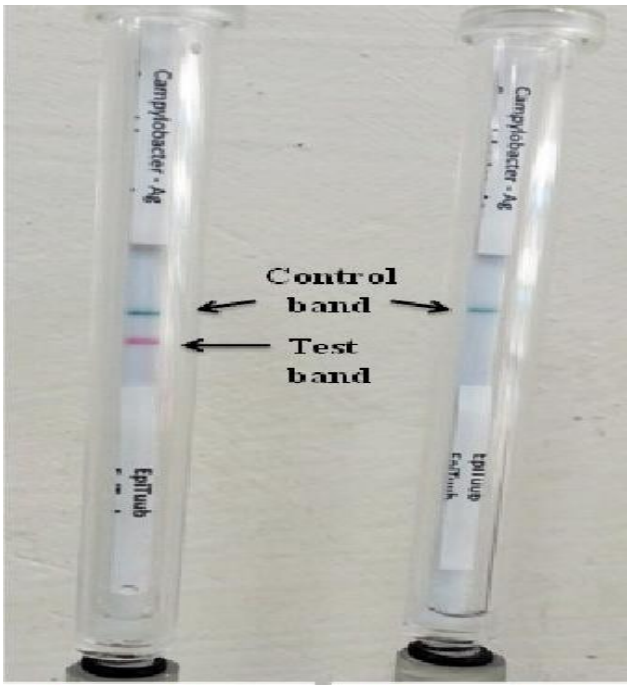

Figure 6. *Campylobacter* positive (colored test band) and negative samples by rapid immunochromatography test. Source: Gharst et al. (2013)

Molecular diagnosis

By using nucleic acid-based technologies, different and highly specific DNA or RNA sequences are discovered. These sequences can then be sequenced, amplified, and seen on a gel or else distinguished for identification, quantitative determination, and molecular typing (Ghatak et al., 2020). Polymerase chain reaction (PCR) and DNA sequencing can allow for the simple, quick, and precise identification of *Campylobacter* species while also revealing its epidemiological characteristics (Figure 7). Also, they allow researchers to generate data that can be communicated via web-based databases and used for phylogenetic studies (Negahdari et al., 2016). Quantitative PCR or real-time PCR are the two names for this technology, which are both referred to as qPCR (Ghoneim et al., 2020).

Differential diagnosis

The clinical signs of avian campylobacteriosis are similar to those of other enteric pathogens like *Salmonella*, *Shigella*, *Escherichia coli* (*E. coli*) 0157:H7, *Shiga* toxin-produced by *E. coli*, *Clostridium difficile*, *Yersinia*, *Entamoeba histolytica*, *coccidia*, and *Rota virus* (Nieder et al., 2018).

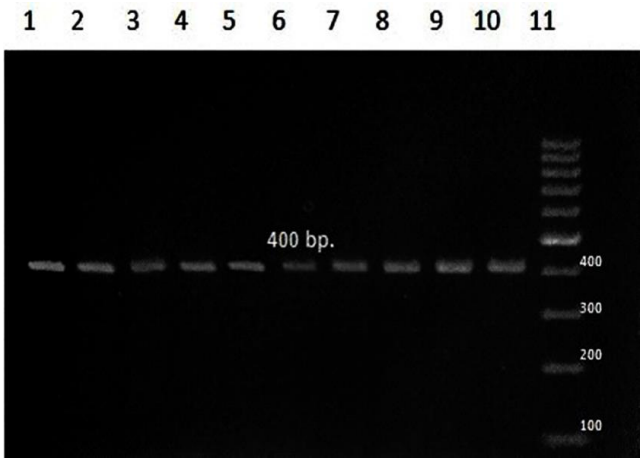

Figure 7. DNA ladder (100 bp.); Lanes (1-10): positive *Campylobacter jejuni* isolates showing specific bands at 400 bp. and *cadF* gene in *Campylobacter jejuni* isolates: Lane (11). Source: Ghoneim et al. (2020).

TREATMENT

Chemical additives, both natural and synthetic, have been tested *in vitro* and *in vivo* to verify their anti-*Campylobacter* effects. In such a study, caprylic acid at a dose of 0.175% (v/v) was administrated in the drinking water of one-day-old chicks for 6 days. The results indicated that the concentration of a mixture containing 5 strains of *C. jejuni* decreased by 3 log CFU/g by day 6 (Gracia et al., 2016). The administration of a ferric tyrosine complex at a concentration of 0.05 g/kg in broiler feed for 42 days indicated a 2-log CFU/g reduction in chickens naturally colonized with *Campylobacter* (Khattak et al., 2018). Cold plasma, ultraviolet light irradiation, high-intensity light pulses, pulsed electric fields, and ultrasound are examples of a number of novel technologies that have been investigated for their ability to inactivate Campylobacter on chicken meat (Soro et al., 2020). Many recent laboratory-scale experiments showed that the approved antimicrobials, such as acidified sodium, chlorite, cetylpyridinium, chlorine, chlorine dioxide, peroxyacetic acid, and trisodium phosphate, could reduce Campylobacter in chicken meat up to 5 log (Hakeem and Lu, 2021).

Potential pre- and postharvest interventions

Preharvest strategies include the successful oral application of phages to reduce *C. jejuni* colonization in birds and phages against *C. jejuni* as an alternative feed additive. Thus, the majority of preharvest intervention strategies of *Campylobacter* are focused on the reduction or removal of the microorganism from the ceca (Deng et al., 2020). Postharvest application of lytic phages could selectively target *Campylobacter* populations without interfering with the remaining microbiota. Phage treatment can be used to inactivate *Campylobacter* attached to food contact surfaces or grown as biofilms. *Campylobacter* bacteriophages isolated from retail poultry have been used in some post-slaughter experiments (Olson et al., 2022).

Biosecurity measures

Poultry are reservoirs of *Campylobacter* species although the birds are generally asymptomatic. *Campylobacter* is an important zoonotic pathogen, underscoring the importance of implementing suitable food safety practices and disease management methods among small flock keepers. These measures are crucial for preventing and controlling the transmission of *Campylobacter* species to humans, which can occur through direct contact with infected poultry or by consuming contaminated poultry meat (Schweitzer et al., 2021). Contaminated feed, water, and fomites, as well as wild birds, rodents, and insects, are sources of *Campylobacter* species in poultry and improper handling of contaminated food and consumption of undercooked food, in particular poultry products, and direct contact with livestock and pets, are major risk factors for *C. jejuni* and *C. coli* infections in humans (Abd El-Hack et al., 2021).

PREVENTION AND CONTROL

Strict hygiene routines and sanitary management of farm facilities and husbandry operations are the first steps in controlling infection in birds, especially poultry. Farm machinery needs to be cleaned up, especially hatcheries. An efficient method to stop the spread of infection also involves chemically treating litter. Incorporating fatty acids and bacteriocins, plant-derived substances, and the use of bacteriophage are some of the more recent methods being used to reduce colonization although further research is necessary before deciding whether they will be

effective (Facciolà et al., 2017). Some cutting-edge methods for preventing campylobacteriosis in poultry include a DNA prime/protein boost protocol for *C. jejuni* vaccination, an inventive *in ovo* vaccination in broilers using bacterin and subunit vaccine, and the use of reverse vaccinology to find potential novel targets for vaccination (Figure 8, Hassanain et al., 2018). Early colonization of the gastrointestinal tract (GIT) by probiotics may serve as an inhibitor to the growth of foodborne pathogens. Probiotics are thus a promising feed additive for lowering and eradicating Campylobacter colonization in the GIT of chicken (Deng et al., 2020).

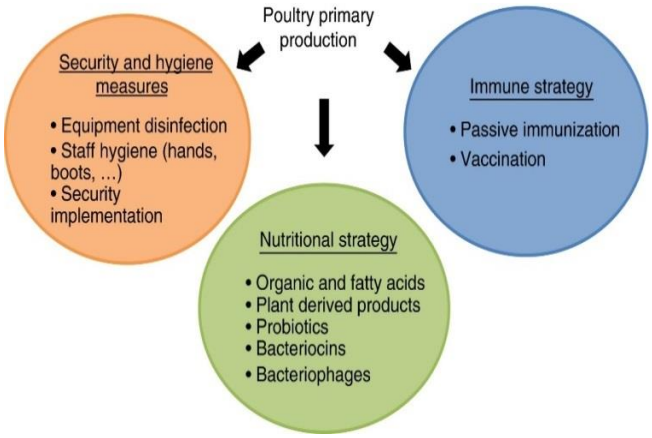

Figure 8. Control measures performed at the primary production stage to prevent human campylobacteriosis infections and the intestinal colonization of broiler chickens with *Campylobacter* (Source: Meunier et al., 2016).

PUBLIC HEALTH SIGNIFICANCE

Chickens pose the largest reservoir and the highest risk for human diseases caused by *Campylobacter* (Hermans et al., 2012). While the primary route of human infection is through oral ingestion, there is also evidence of occupational transmission of the disease. In terms of poultry meat serving as a source of human campylobacteriosis, reported rates of occupational infection by *Campylobacter* species among employees can range from 57% to 83% (Skarp et al., 2016). Genetic analysis of *Campylobacter* isolates from both humans and wild poultry has revealed frequent overlaps in clonal complexes (Sequence 5 Types, ST), indicating a potential risk of human infection from wild fowl (Wei et al., 2019). This highlights the importance of considering wild fowl as a possible source of Campylobacter transmission to humans.

ANTIMICROBIAL RESISTANCE

Antimicrobials are used for prophylaxis, treatment, or as growth promoters in food animals, and antimicrobial resistance (AMR) is a major public health threat worldwide. *Campylobacter* isolates were more resistant to tetracyclines, macrolides, ketolides, and lincosamides (Dramé et al., 2020). Among the high contamination levels of broilers (71.4%) in Morocco, five *Campylobacter* strains were analyzed, namely erythromycin (92.8%), ampicillin (95.2%), ciprofloxacin (85.7%), tetracycline (92.8%), and gentamycin (7.1%). This finding raises concerns about the effectiveness of such antibiotics for the treatment of animal diseases (Asmai et al., 2020).

VACCINATION

Vaccination is considered a promising intervention measure for reducing Campylobacter in poultry. As part of this approach, two vaccine candidates have been extensively studied and evaluated. These candidates involve a novel vaccination strategy that combines the *in ovo* vaccination route with a newly formulated DNA vaccine. The aim was to control *Campylobacter* in broiler chickens effectively. This innovative approach holds the potential to enhance the efficacy of Campylobacter vaccines in poultry (Liu et al., 2019). *Campylobacter jejuni* vaccination trials may reflect the antigen, challenge strain, vaccine administration, and adjuvant. Refinement of glycoconjugate vaccines by increasing glycosylation levels or using highly immunogenic protein carriers could improve their efficacy (Vohra et al., 2020). In a proof-of-concept study aiming to develop live-attenuated *C. jejuni* vaccines, researchers focused on oxidative stress defense mutants. They found that pre-colonizing chickens with a mutant lacking the *ahpC* gene resulted in a significant reduction in the level of *C. jejuni* and an increase in body weights among the chickens. This discovery highlights the potential of targeting the *ahpC* gene for constructing live-attenuated *C. jejuni* vaccines specifically designed for chickens (Jeon et al., 2022).

CONCLUSION

The most common manifestation of campylobacteriosis in poultry is a digestive disease, leading to diarrhea and weight loss. The rapid diagnosis is made based on the observation of symptoms or the gross lesions after

slaughter, but it can also be supported by causative agent isolation and culture. Treatment is difficult to apply in poultry. The strict hygiene and sanitary management of farm facilities and husbandry operations are the first steps in controlling infection in poultry, especially poultry. Feeding and watering equipment must be thoroughly cleaned and disinfected. Prevent crowding in the poultry house, and chemical treatment of the litter. Incorporating fatty acids and bacteriocins, plant-derived substances, and the use of bacteriophage are some of the more recent methods being used to reduce colonization. Thus, to prevent foodborne illness from contaminated food, it is advised to boil chicken meat and eggs thoroughly before consumption, implement effective on-farm biosecurity measures, and conduct routine employee checks at meat processing facilities and on chicken farms.

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Authors' contributions

Sabry A. S. Sadek conceptualized this study, surveyed the literature, drafted, and revised the manuscript. Ashraf M. Barakat was responsible for data acquisition and manuscript revision, while Raafat M. Shaapan revised, edited, and suggested changes to the manuscript. All authors have read and approved the final version of the manuscript for publication in the present journal.

Competing interests

The authors have declared that no competing interest exists.

Ethical consideration

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

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Effects of Dietary Supplementation of Phytogenic Feed Additives on Broiler Feed Conversion Efficiency and Immune Response against Infectious Bursal Disease Vaccine

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ABSTRACT

The ban on antibiotic growth promoters in livestock feeding has encouraged the utilization of phytogenic feed additives. These phytogenics recently attracted much attention and are generally recognized as residue-free ideal feed additives in animal Production. The current study was conducted to investigate the effects of the phytogenic herbs on feed intake, feed conversion ratio, and immune responses associated with the infectious bursal disease (IBD) vaccine in broiler chickens. For this study, 360 day-old broiler chicks were randomly assigned to six feeding trials, each with three replicates containing 20 chicks. The control group (T1) was only fed a basal diet alone, while the treatment groups were given the basal diet supplemented with 1% of basil (T2), lemongrass (T3), peppermint (T4), rosemary (T5), and thyme (T6) leaves powder, respectively. Body weight, feed intake, and feed conversion ratio were recorded. All chicks were vaccinated against IBD on days 7 and 19. A serology test was conducted to check the antibody titer against the IBD vaccine. The findings of this study showed that chickens in group T2 had significantly consumed more feed, followed by T1 and T6. During the overall study period, chickens in group T4 had significantly better feed conversion efficiency, followed by T3 and T6. Chickens in groups T5 and T6 showed a more pronounced antibody titer against the IBD vaccine at days 21 and 42 of the experiment. Therefore, these findings indicated that supplementation of basil leaf powder improved feed intake. Moreover, peppermint and lemongrass leaf powder improved the feed conversion ratio. In addition, supplementation of rosemary and thyme enhances the immune status of broiler chickens and could be considered a natural growth promoter feed additive. Therefore, further studies should be done to discover their beneficial effects to use as alternative feed additives in broiler chickens.

Keywords: Body weight, Broiler chicken, Feed conversion, Feed intake, Immune response, Infectious bursa disease

INTRODUCTION

Poultry production is a fast growing industry in the meat-producing agriculture sector, with the demand for protein sources to meet the growing population worldwide (Bogale and Engida, 2022; El-Sabrout et al., 2022). In order to ensure this demand for animal-origin food, efforts are made by the producers to develop new strategies for animal production (El-Sabrout et al., 2022). Poultry producers widely used antibiotic growth promoters (AGPs) for the last decades because of their useful effect on production performance and the health condition of the chickens (Barreto et al., 2008; Mirzaei et al., 2022). This long-term utilization of antibiotics as a growth promoter in chickens led to reduced beneficial endogenous bacteria, the development drug resistant pathogenic microorganisms, and resulting in the accumulation of antibiotic residues in the environment

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and animal consumable products, which is a threat to consumer health (Barreto et al., 2008; Kurniawati et al., 2021; Mirzaei et al., 2022). These conditions force the world to ban the utilization of AGPs as feed additives on food-producing animals and motivated the search for alternative resources which are safer to use as feed additives (Singh and Yadav, 2020; Petričević et al., 2021).

The international animal feed industry faced the challenge of increased feed costs, the prohibition of the utilization of AGPs, and consumers' awareness of food safety (Osman et al., 2010; Madhupriya et al., 2018). Therefore, poultry nutritionist develops an interest in natural feed additives to use as an alternative additive to replace the AGPs with natural products (Upadhaya and Kim, 2017; El-Saadany et al., 2022; El-Sabrout et al., 2022). Among abundant natural feed additives that are available for chicken feed, phytogenic feed additives (PFAs) are widely advocated and received consumer acceptability (Saeed et al., 2018; Odunowo and Olumide, 2019; Abd El-Ghany, 2020; Kurniawati et al., 2021). PFAs are natural heterogeneous groups, less toxic, residue-free, and ideal non-antibiotic growth promoters derived from plants, herbs, fruit, spices, and their essential oil used as feed additives in meat animal production (Upadhaya and Kim, 2017; Madhupriya et al., 2018; Abd El-Ghany, 2020).

Phytogenics and their extract are supplemented to animal ratio to stimulate their appetite and improve nutrient digestibility, growth enhancer, improve gastrointestinal morphology (by stimulating the development of the gastrointestinal macro-architecture) and physiological functions, have antioxidant properties, help to treat certain diseases and immune modulator activities in chickens have been reported by different researchers (Upadhaya and Kim, 2017; Madhupriya et al., 2018; Babazadeh and Asasi, 2021). Furthermore PFAs active ingredients improve chicken performance by inducing digestive enzyme secretion, leading to enhance feed digestion and nutrient absorption (Odunowo and Olumide, 2019) and improving the feed conversion ratio in poultry (Huang and Lee, 2018). Currently, many scholars have revealed that supplementation of PFAs in animal feed enhances the immune response and can guard the gastrointestinal tract against external stressors. Their phytochemicals are considered the most promising feed additives due to their positive effect on regulating some viral vaccines' immune response in broiler chickens (Upadhaya and Kim, 2017; Huang and Lee, 2018; Abdelli et al., 2021).

One of the means advisable to enhance the productivity of chickens is through improving their feeds for optimum utilization as well as by enhancing their well-being, especially through feed obtained from phytogenics. The PFAs are comparatively young classes of poultry feed additives that have attracted the attention of poultry nutritionists in recent years (Abd El-Hack and Alagawany, 2015; Engida et al., 2023). Phytogenics have a beneficial role in disease prevention and control strategies to enhance the immune response against disease and for superior prevention of disease challenges (Gado et al., 2019). Broiler chickens supplementing with phytogenic essential oil significantly improve *infectious bursal disease (IBD)* antibody titer (Nahed et al., 2020). In this regard, it is necessary to extend the outlook on PFAs to promote their use as an alternative source to improve production performance in chickens. The PFAs evaluated in this research are available in almost all areas of the country, which are affordable or freely available and easy to process and utilize. Therefore, this study aimed to evaluate the utilization of basil, lemongrass, peppermint, rosemary, and thyme leaves powder as PFAs on the performance and immune response of broiler chickens regarding IBD vaccination.

MATERIALS AND METHODS

Ethical approval

All procedures related to animal handling, blood collection, and their routine manipulations were carried out according to animal care guidelines protocols approved by the Institutional Review Board of the College of Veterinary Medicine and Agriculture (CVMA, Ethiopia) animals ethics committee with approval number VM/ERC/01/13/12/2020.

Study areas

The experiment was conducted in the CVMA poultry house of Addis Ababa University, Bishoftu, Ethiopia, from January 1 to February 18, 2020. The area is located 47 km southeast of Addis Ababa at an altitude of 1900 m above sea level in the central highland of the country, a latitude of 8.44` North and a longitude of 38.57` East. The average annual rainfall is 686.9 mm with an average minimum and maximum temperature of 10.9^{o}C and 27^{o}C, respectively, and the average relative humidity is 60.0%.

Preparation of experimental herb powders

The experimental herbs included basil (*Ocimum basilicum*), lemongrass (*Cymbopogon schoenanthus)*,

peppermint (*Mentha piperita)*, rosemary (*Rosmarinus officinalis*), and thyme (*Thymus vulgaris)* green leaves were purchased from Green mark herbs private limited company (PLC), found in Hawassa, Ethiopia. The herbs were washed, and the leaves were detached from the stem, spread out on a plastic sheet, and allowed to dry at room temperature in the physiology laboratory of CVMA. The dried leaves were prepared in powder form and stored in plastic material until used.

Experimental design and animals management

A total of 378 day-old broiler chicks of Cobb-500 strain were purchased from Bishoftu Alema farms, (Ethiopia). Blood collection was performed from 18 randomly selected chicks on day one of their age before vaccination by incising the jugular vein and transferring it into the non-heparinized tubes (Legese et al., 2022). The remaining chicks (n = 360) were weighed and randomly assigned into six different treatment groups with three replicates based on a completely random design. Chicks were reared in a wire-meshed wood partitioned deep litter floor housing system (1.20 m × 1.80 m) for 49 days of the experimental period. Electric power was used as a source of heat and light according to the recommendation of the broiler management guide (Anonymous, 2018). All experimental chickens were vaccinated against IBD. The chicks were fed commercial broiler feed (1-10 days for starter, 11-30 days for grower, and 31-49 days for finisher) throughout the study, as per the recommendation by the feed supplier (Alema Koudijs Feed PLC, Ethiopia). Chicks of the control group were fed only broiler commercial feed (basal diet), whereas the other treatment groups were fed a basal diet plus 1% of one of the five herbs prepared in powder form as treatment herbs. The prepared treatment herb was homogeneously mixed with the broiler diet manually (Nielsen, 2010).

All diets were provided in mash form. Water and weighed feed were provided *ad libitium* to the chicks throughout the experiment. The standard bio-security protocol was employed throughout the experiment (USDA, 2014; SAPA, 2022). The study protocol for the starter, grower, and finisher broiler diet during the experiment included T1 as the control group receiving the basal diet, T2 receiving the basal diet + 10g of basil leave powder per kg of basal diet, T3 receiving the basal diet + 10g of lemongrass leave powder per kg of basal diet, T4 receiving the basal diet+ 10g of peppermint leave powder per kg of basal diet, T5 receiving basal diet + 10g of rosemary leave powder per kg of basal diet, and T6 receiving basal diet + 10g of thyme leave powder per kg of basal diet.

Nutrient composition of basal diets and treatment herbs

Nutrient compositions of basal diet and herbal powder of the treatments were analyzed from their representative sample at two different laboratories, namely Animal Product, Veterinary Drug and Feed Quality Assessment Center, and the Ethiopian Institute of Agriculture Laboratory (Table 1). Samples were analyzed for crude protein (CP), dry matter (DM), crude fiber (CF), Ether extract (EE), phosphorus (P) and total ash (Ash). Nitrogen was analyzed using the Kjeldahl method, and CP was calculated by multiplying nitrogen content by 6.25. The metabolizable energy (ME) value of the basal diet and experimental herb samples were calculated indirectly from the EE, CF, and ash using the following formula adopted from the equation proposed by Wiseman (1987).

$$\text{ME (Kcal/kg DM)} = 3951 + 54.4\,\text{EE} - 88.7\,\text{CF} - 40.8\,\text{Ash}$$

Data collection

Body weight gain

At the beginning of the experiment and then every week and at the end of each phase (starter, grower and finisher) all chicks were weighed to determine the body weight gain (BWG) of chicks respective to treatment groups. BWG was determined by subtracting initial weight from successive body weight (BW) for each replication during the experimental time (Engida et al., 2023).

Feed intakes and feed conversion ratios

Feed intake (FI) was calculated as the difference between feed offered and feed leftover for each replication. Feed conversion ratio (FCR) was calculated as the ratio of total consumed feed (gm) to total BW gain (gm, El-Ghousein and Al-Beitawi, 2009). At the same time, cumulative FI was computed at the end of the experiment.

Immune response measurement

Blood collection was performed from 18 randomly selected chicks on hatching before vaccination to harvest serum samples to have baseline immune data (Legese et al., 2022). All the chickens were vaccinated against IBD (Gumboro) with the recommended dose and as per the schedule of the product on days 7 and 19 (CEVAC® IBD L, France). Then, 3 ml of blood samples were collected into a plain vacutainer tube using a disposable 3 mL syringe with a

22-gauge needle from the wing vein on the 21 and 42 days of age to measure post-vaccination antibody titer from 3 randomly selected chicks. The collected blood sample was kept undisturbed to clot in the laboratory for 30 minutes, then centrifuged at 1500 rpm for 10 minutes for separation of serum, and the serum sample was harvested into labeled cryovials and kept at -20°C until serology was conducted at the National Veterinary Institute laboratory (Ethiopia). The indirect ELISA test (ID screen® IBD indirect, ID.vet, France) was performed according to the directions provided by the manufacturer with the kit for the detection of antibody titer developed against IBD. It is a quantitative test for the determination of IBD-specific antibodies in chicken serum. The antibody titer was computed to $\log_{10}$ as per the instruction of the manufacturer provided with the kit (Tesfaye et al., 2017). The test was valid if the mean optical density value of the positive control was greater than 0.250 and the ratio of the mean value of the positive and negative controls was greater than 3 (Tesfaye et al., 2017).

Statistical analysis

All data pertaining to measured parameters were analyzed using the R tools (R project, 2020). Treatment means were compared by one-way ANOVA.

Significant differences among the treatments' effects were separately analyzed with Duncan's multiple comparison test. Significance mean differences between treatments were considered at $p < 0.05$. The following model was used to analyze the experiment where PFAs are the main effects (Gomez and Gomez, 1984).

$Yij = \mu + Ti + Eij$.

Where, Yij is an observation of chicken, μ denotes the overall mean of a response variable, Ti determines the effect due to treatment herbs, and Eij represents the error term.

RESULTS

Nutrient composition of basal diets and treatment herbs

As can be seen in Table 1, basil had a higher CP content compared to other herbs. Additionally, basil exhibited lower DM and EE content in comparison to Peppermint. On the other hand, lemongrass displayed higher ash and ME content than rosemary and basil. Peppermint had lower EE content but higher P content, with an optimum ME level. Rosemary stood out with the highest DM, EE, and CF composition among all the herbs, while displaying the lowest P and ME content. Thyme showcased the highest ME content and the lowest CF content when compared to the other herbs. Additionally, thyme had higher EE content compared to rosemary.

Table 1. Nutrient composition (%) of basal diets and treatment herbs

Composition / Sample	DM	CP	EE	CF	P	Ash	ME (kcal/kg DM)
Treatment herbs							
Basil	85.7	31.56	2.14	38.52	0.36	6.72	376.516
Lemongrass	88.17	14.9	4.6	19.52	0.45	14.21	1890.05
Peppermint	88.81	26.92	1.88	21.72	0.52	11.58	1654.24
Rosemary	90.22	12.45	7.62	42.95	0.17	6.76	280.055
Thyme	88.88	11.96	6.12	18.09	0.27	11.03	2229.32
Basal diet							
Starter diet	90.9	21.95	4.02	7.86	0.48	9.88	3069.4
Grower diet	90.79	20.51	4.85	7.62	0.39	8.95	3173.79
Finisher diet	91.08	19.0	5.63	7.25	0.32	9.42	3230.07

DM: Dry matter, CP: Crude protein, EE: Ether extract, CF: Crude Fiber, P: Phosphorus, Ash: Total ash, ME: Metabolizable energy

Feed intake

The effect of the treatment herbs on FI is presented in Table 2. According to the result of the present study, the inclusion of phytogenic herbs significantly affected FI. The chickens kept on treatment herbs had significantly ($p < 0.05$) higher FI, compared to the control group during the starter phase, and there was no significant difference ($p > 0.05$) in FI between treatment herbs. During the grower phase, there was a significant difference between treatments ($p < 0.05$). Significantly higher FI was seen in chickens in group T2 ($p < 0.05$), while significantly lower FI was seen in chickens in group T4 ($p < 0.05$). No significant

difference was seen in chickens fed on T1, T3, and T6 regarding FI. During the finisher phase, the highest FI was recorded in chickens kept on T1, whereas chickens fed on T4 had the lowest FI ($p < 0.05$). During the finisher phase, T1, T2, T3, and T6 did not differ significantly in terms of FI. There was a significant difference in FI ($p < 0.05$) between treatments during the overall study period. Chickens fed on T2 had the highest FI, whereas chickens kept on T4 had the lowest FI than all other treatments. There was no significant difference in FI ($p > 0.05$) among T1, T2, and T6 and among T3, T5, and T6.

Body weight gain

Body weight gain data of broiler chickens fed on treatment herbs is presented in Table 3. BWG was highly significant between treatments during the starter, grower, and finisher phases ($p < 0.05$). Significantly higher BWG was seen in chickens kept on T3 and T6 than in other treatments ($p < 0.05$), whereas chickens kept on T1, T4, and T5 developed a lower body weight during the starter phase. Similarly, chickens kept on T3 showed higher BWG, compared to other treatments during the grower phase ($p < 0.05$). No significant difference was seen in chickens fed on T1, T4, and T5 and had lower BWG than other treatments. During the finisher phase, chickens kept on T6 showed significantly higher BWG than other treatments ($p < 0.05$). On the other hand, chickens that consume T4 had lower BWG, followed by T1. There was no significant difference in BWG between T2 and T3 during the finisher phase ($p > 0.05$). During the overall study period, significantly higher BWG was achieved from chickens that consumed T3 and T6 than in all other treatments. However, lower BWG was seen in chickens fed on T1 and T4.

Table 2. The effect of inclusion of treatment herbs on feed intake of broiler chickens during the starter, grower, and finisher phases

Parameter \ Treatment		T1	T 2	T3	T4	T5	T6	SEM	P-value
FI (g/bird)	**Age (days)**								
Starter	1-10	288.21^{b}	308.13^{a}	308.37^{a}	309.46^{a}	312.71^{a}	308.79^{a}	2.26	0.003
Grower	11-30	1959.94^{b}	2059.58^{a}	1929.47^{bc}	1871.13^{d}	1899.41^{cd}	1984.31^{b}	16.07	0.000
Finisher	30-49	3593.12^{a}	3520.68^{ab}	3358.29^{ab}	3004.87^{c}	3297.82^{b}	3363.26^{ab}	53.34	0.004
Overall	1-49	5841.27^{ab}	5888.39^{a}	5596.14^{bc}	5185.47^{d}	5509.94^{c}	5656.37^{abc}	63.42	0.001

[a-e] Means within the same row bearing different superscripts are significantly different at $p < 0.05$, FI: Feed intake, SEM: Standard Error of Mean, g: Gram, T1: control (basal diet), T2: Basal diet + 1% basil leaf powder, T3: Basal diet +1% lemongrass leaf powder, T4: Basal diet + 1% peppermint leaf powder, T5: Basal diet + 1% rosemary leaf powder, T6: Basal diet + 1% thyme leaf powder

Table 3. The effect of treatment herbs on body weight gain of broiler chickens during the starter, grower and finisher phases

Parameter \ Treatment		T1	T 2	T3	T4	T5	T6	SEM	P-value
BWG (g)	**Age (days)**								
Initial BW	1	40.85	40.79	40.7	40.75	40.67	40.68	0.04	0.810
Starter	1-10	171.29^{c}	179.54^{b}	194.45^{a}	168.91^{c}	168.32^{c}	190.24^{a}	0.85	0.000
Grower	11-30	644.38^{c}	680.77^{b}	716.49^{a}	654.80^{c}	647.36^{c}	688.20^{b}	2.10	0.000
Finisher	30-49	1098.75^{d}	1131.02^{bc}	1137.91^{b}	1085.80^{e}	1120.87^{c}	1156.04^{a}	2.10	0.000
Overall	1-49	1922.84^{d}	1991.34^{b}	2033.97^{a}	1920.18^{d}	1947.65^{c}	2023.48^{a}	3.14	0.000

[a-e] Means within the same row bearing different superscripts are significantly different at $p < 0.05$, BWG: Body weight gain, BW: Body weight, g: gram, SEM: Standard Error of Mean, T1: Control (basal diet), T2: Basal diet + 1% basil leaf powder, T3: Basal diet +1% lemongrass leaf powder, T4: Basal diet + 1% peppermint leaf powder, T5: Basal diet + 1% rosemary leaf powder, T6: Basal diet + 1% thyme leaf powder

Feed conversion ratio

The mean FCR data are presented in Table 4. The obtained results indicated that the inclusion of phytogenic herbs significantly affected FCR during the starter, grower, finisher, and overall experimental period ($p < 0.05$). During the starter phase, FCR was found to be significantly better in chickens fed on T3 followed by T6 compared to other treatments ($p < 0.05$), whereas chickens kept in T4 and T5 had lower FCR. Significant FCR was seen between treatments during the grower phase ($p < 0.05$). Chickens kept on T3 had better FCR than all other treatments, and significantly lower FCR was recorded for chickens fed on T1 and T2 among the treatments ($p < 0.05$). During the finisher phase, significantly better FCR values were seen in chickens kept on T4 than in all other treatments, and significantly lower FCR was recorded in T1 and T2 chickens ($p < 0.05$). However, there was no significant difference among T2, T3, T5, and T6 in FCR regarding the finisher phase ($p < 0.05$). As to the current study finding, FCR was significantly affected by PFAs during the overall study periods ($p > 0.05$). Chickens fed T4 had significantly better FCR followed by T3 ($p < 0.05$). On the other hand, chickens kept on T1 had significantly low FCR.

Table 4. The effect of treatment herbs on a feed conversion ratio of broiler chickens during the starter, grower, and finisher phases

Parameter \ Treatment		T1	T 2	T3	T4	T5	T6	SEM	P-value
FCR	**Age (days)**								
Starter	1-10	1.68^{bc}	1.71^{b}	1.58^{d}	1.83^{a}	1.85^{a}	1.62^{dc}	0.025	0.000
Grower	10-30	3.04^{a}	3.02^{a}	2.69^{c}	2.85^{b}	2.93^{b}	2.88^{b}	0.029	0.000
Finisher	30-49	3.27^{a}	3.11^{ab}	2.95^{bc}	2.76^{c}	2.94^{bc}	2.91^{bc}	0.046	0.006
Overall	1-49	3.03^{a}	2.95^{ab}	2.75^{c}	2.70^{c}	2.83^{bc}	2.79^{c}	0.032	0.001

[a-e] Means within the same row bearing different superscripts are significantly different at $p < 0.05$, FCR: Feed conversion ratio, SEM: Standard Error of Mean, T1: Control (basal diet), T2: Basal diet + 1% basil leaf powder, T3: Basal diet +1% lemongrass leaf powder, T4: Basal diet + 1% peppermint leaf powder, T5: Basal diet + 1% rosemary leaf powder, T6: Basal diet + 1% thyme leaf powder

Immune response

The current study determined the effects of the experimental phytogenic herbs on broiler chickens' immunity, with a recommended vaccination program against IBD/Gumboro disease. During the study, the experimental herb that had the response of immune against the IBD vaccine was conducted via antibody titer from representative chickens' serum samples by a serological test. The effect of the experimental herbs on IBD (log_{10}) immune responses is illustrated in Figure 1. The mean IBD antibody titer from the serum samples of day-old chicks was (log_{10}) 3.60. The statistically significant difference means value antibody titers were observed in chickens fed different treatments ($p < 0.05$). On day 21, chicks fed on T5 and T6 as a supplement in their diet had developed the highest mean antibody titer of 3.853 and 3.567, respectively, and chickens fed on T2 and T3 had developed almost similar mean antibody titer values. In contrast, the lowest antibody titer was seen on chickens fed on T4 with 1.271 antibodies. Similarly, the mean antibody tillers value at the age of 42 days further increases in chickens fed the same treatment herb. Their mean antibody titer was 4.463 for T5 and 3.850 for T6, followed by chickens fed on T3 with a titer of 2.990. The lowest titer was recorded in chickens fed on T4, with a titer of 1.562 (Figure 1).

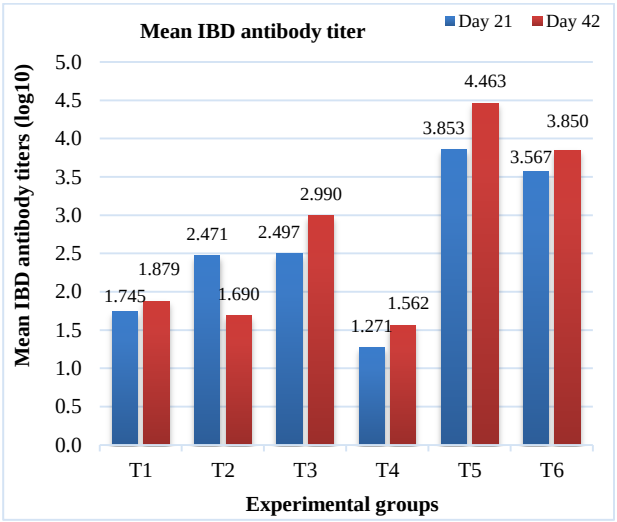

Figure 1. Mean antibody titer of broiler chickens treated with different herbs against IBD vaccine during days 21 and 42. T1: Control (basal diet), T2: Basal diet + 1% basil leaf powder, T3: Basal diet +1% lemongrass leaf powder, T4: Basal diet + 1% peppermint leaf powder, T5: Basal diet + 1% rosemary leaf powder, T6: Basal diet + 1% thyme leaf powder

DISCUSSION

Feed intake

The present study result showed that mean total feed consumption was lower in chickens that consumed peppermint and rosemary during the entire period. Considering the treatment herbs, the highest feed consumption data were recorded in chickens supplemented with basil and a control diet and as well as in a thyme leaf followed by lemongrass leaf powder at the end of the experiment. This result is supported by different scholars' findings that the utilization of PFAs in poultry feed improves feed intake and can stimulate digestion (Pournazari et al., 2017; Madhupriya et al., 2018). Similarly, the result also coincides with the finding of Gurbuz and Ismael (2016), who stated that the biological properties of basil leaf have an appetizing activity, improved feed intake, enhanced digestion process, and intestinal motility (Al-Kelabi et al., 2013). As a natural feed additive, thyme is known to improve feed palatability and stimulate appetite (Hassan and Awad, 2017). Similarly, Witkowska et al. (2019) also noted the highest feed intake in broiler chickens fed on thyme essential oil. Contrary to the present study, Parade et al. (2019) reported that broiler chickens supplemented with lemongrass leaf powder consume lower feed than the control group.

Body weight gain

Body weight gain of broiler chickens was improved by supplementing phytogenic herbs with their diet. The highest BWG observed in chickens fed on the treatment herbs may be due to the beneficial effect and properties of the treatment herbs. In the current study, chickens fed on peppermint had the lowest BWG, compared to the other treatment herb. Similarly, Khodadust et al. (2015) found that peppermint leaf extract did not improve body weight in broiler chickens. During the finisher phase, chickens fed on thyme leaf were recorded with the highest BWG, followed by lemongrass. This result coincides with El-Ghousein and Al-Beitawi (2009), who reported that supplementation of thyme in the basal diet of broiler chickens improved BW, BWG, and FCR. Considering the overall period, chickens fed on lemongrass recorded the highest BWG. The result of the current study agrees with Parade et al. (2019) and Mmereole (2010), who stated that the utilization of a lemongrass leaf powder improved BW and BWG in broiler chicken. Alagawany et al. (2021) and Shaheed (2021) also clarified that supplementation of lemongrass essential oil in the quail diet improved growth performance and boosts health status. A similar trend was also observed by Khattak et al. (2014) and Tiwari et al. (2018), where their experiment revealed that the inclusion of lemongrass essential oil in the broiler diet improved BWG and had positive effects on FCR. The retained heaviest BWG in chickens fed on lemongrass started during the starter and grower phase and continued up to the end of the experiment, which enabled the broilers to utilize their diets more efficiently and grow more. This growth trend was similar to Mmereole (2010), who found continued growth from the first week to the end of the experiment in broiler chickens that consumed lemongrass leaf powder. The higher BWG recorded in chickens fed on lemongrass leaf powder could be due to the active compounds found in the lemongrass improving feed digestion and digestion enzyme secretion in broiler chickens. These digestion physiological activities improved feed intake which was reflected in the weight gain of experimental chickens (Alagawany et al., 2021; Shaheed, 2021). Indeed, natural feed additives could improve the digestibility and absorption of dietary protein in the small intestine, which interns attributed to body weight gain, and chickens grew faster than those fed in the control group (Gurbuz and Ismael, 2016).

Feed conversion ratio

The current experiment indicated that the FCR value was significantly lower in chickens consumed on feed supplemented with lemongrass during the starter phase. Similarly, chickens fed on lemongrass, peppermint, and thyme had better feed conversion efficiency during the grower phase, and this trend continued up to the end of the experiment. This is an indication that the amount of feed consumed by chickens supplemented with these herbs was significantly lower to gain a unit of body weight than other treatments. Consistent with this result, Sadek et al. (2014) and El-Ghousein and Al-Beitawi (2009) reported that supplementing thyme leaf powder significantly improved mean FCR throughout the entire period. Similarly, Al-Kassie (2010) noted that supplementing peppermint in the diet stimulates growth performance and improved FCR in broiler chickens. Supplementing peppermint to broiler chicken improved average BWG with better FCR than control over the whole experimental period (Petričević et al., 2021). Other scholars have also argued that the supplementation of lemongrass to broiler chick improved FCR (Tiwari et al., 2018; Parade et al., 2019). Similarly, Alagawany et al. (2021) also reported that supplementation of lemongrass essential oil in the quail diet for up to five weeks improved FCR. Contrary to the present study, Sariözkan et al. (2016) stated that supplementation of

lemongrass to the quail diet did not affect FCR.

Antibody titer

Currently, improving production performance, boosting health status, and enhancing the immune system in the absence of antibiotics are the interest of livestock nutritionists. The PFAs improve and promote the immune response, reduce pathogenic microorganisms, balance intestinal microbial flora, improve disease resistance, promote feed digestibility, absorption, and nutrient availability, and strengthen the immune function (Zhu et al., 2014; Abdelli et al., 2021; Wakjira et al., 2021). Feeding PFAs improves nutrient availability for absorption and boosts the immune of the chicks during critical situation, when there is stress, and support the animal for better growth (Jameel et al., 2014). In the current experiment, the chickens fed on diets supplemented with thyme and rosemary showed a more pronounced antibody titer. This result is in line with the finding of Nahed et al. (2020), who found significantly increased IBD virus antibody titer at 28 days in broiler chicken supplemented with a mixture containing thyme essential oil in drinking water. Similarly, Jameel et al. (2014) indicated that the highest Newcastle antibody titers were for broiler chickens fed on PFAs, and they confirmed that the supplementing broiler fed with 1% thyme powder and also its mixture significantly improved and stimulated the immune system of broiler against infectious organisms. Huang and Lee (2018) also reported that chicken supplemented with PFAs has improved immune response and protective capacity. Similarly, two studies (Genena et al., 2008; Abo Ghanima et al., 2020) also confirmed that rosemary extracts have antioxidant, antiviral, antibacterial, and antifungal activities. Alhajj et al. (2015) and Mandey and Sompie (2021) also reported that broiler chickens supplemented with Chinese star anise as feed additives had a higher antibody titer against the IBD virus and the herb improved performance and could be used as a feed additive to boost the immune responsiveness. The improvement of antibody titer could be due to active compounds in the experimental herb having an immune-modulating effect (Jameel et al., 2014). The highest antibody titer on chickens supplemented with thyme and rosemary in the current study could possibly be due to the immunostimulant properties of essential oil in these plants. Thymol and carvacrol are active ingredients found in thyme and cineol, rosemarinic acid and rosmarole are in rosemary, which has antioxidative properties, antimicrobial activity against intestinal bacteria, promote growth, enhance the health status and improved the immune system of broiler chickens (Jameel et al., 2014; Yildirim et al., 2018). Generally, when antibody production was improved against the disease producing pathogens, the chickens expended less energy on non specific immune system and the growth and production were improved as a result of the more energy that is available (Jameel et al., 2014; Huang and Lee, 2018).

CONCLUSION

It is concluded all phytogenics tested can be used in Cobb 500 broiler chicks at 1 % of the diet. The findings of the present study indicated that supplementation of lemongrass as well as thyme leaf powder as PFAs improved the growth of the chickens. Feeding peppermint, lemongrass, and thyme as PFAs in the broilers' diet improved FCR, while supplementation of rosemary and thyme developed the highest antibody titer against the IBD vaccine. Supplementation of broilers with PFAs had an appetizing action which was attributed to growth and weight gain performance and boosted immune responsiveness, possibly due to its immunostimulant properties to reduce the risk of vaccine failures. These herbs possess potential beneficial effects and could serve as effective materials for the preparation of pure substances. They have the potential to be developed as alternative feed additives for broiler chickens. However, their effects have not been extensively studied in Ethiopia. Therefore, further research is necessary to explore their potential side effects on the body of broiler chickens.

DECLARATION

Competing interests

The authors have declared that no competing interest exists.

Availability of data and materials

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

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Authors' contributions

Tesfaye Engida D. conceptualized the idea and methodology, performed the experiments, collected all samples, analyzed, generated data, compiled information, and prepared the original and final manuscript. Berhan Tamir contributed to the conceptualization, methodology, validation, supervision, and review of the manuscript. Mihretu Ayele contributed to the methodology, performed the experiments, collected the samples, and laboratory analysis of bacteriological data, and reviewing the manuscript. Hika Waktole contributed to the conceptualization and methodology of the study and reviewed the manuscript. Berhane Wakjira contributed to the methodology and laboratory analysis. Fekadu Regassa contributed to the conceptualization and review of the manuscript. Fikru Regassa contributed to the conceptualization, methodology, validation, supervision, and review of the manuscript. Takele Beyene Tufa contributed to the conceptualization, methodology, validation, and supervision of the study, editing and reviewing the manuscript, project administration and funding acquisition. All authors approved the results of the study and the final version of the manuscript.

Ethical consideration

All Authors have checked the ethical issues, including plagiarism, consent to publish, misconduct, double submission, and redundancy.

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Suitability of Inguinal and Axillary Sites for Temperature Measurement Using Digital Thermometers: A Comparison with Rectal Thermometry in Broiler Chickens

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ABSTRACT

Core body temperature is one of the physiological parameters that must be assessed during the monitoring of the thermic and or health status of broiler chickens. In this regard, cloacal thermometry is a standard method used for temperature measurement although it has many drawbacks. This study was conducted to explore the suitability of other anatomical sites for temperature measurement using a digital thermometer. This was a single-factor experiment that considered the anatomical site as the main factor with three levels (treatments), including cloacal ($DTt_{cloacal}$), axillary ($DTt_{axillary}$), and inguinal ($DTt_{inguinal}$) sites. Out of 84 broiler chickens, a total of 28 chickens were randomly selected for temperature measurement. The temperature was measured for each anatomical site, and the readings were analyzed using appropriate statistics. The cloacal site had the highest mean temperature (41.40 ± 0.17℃), while the lowest mean value was observed for the axillary site (41.12 ± 0.19℃). There was no significant difference between the mean cloacal and inguinal temperatures. The cloacal and inguinal temperature readings were significantly correlated. The results for the cloacal and inguinal temperature measurements revealed a non-significant bias. The agreement interval between these two methods was sufficiently lower than the maximum acceptable difference between the anatomical sites. Both cloacal and inguinal temperature measurements had similar median points. The results indicated an underestimation of the temperature readings for the axillary site compared to those of the other sites. In conclusion, this study has revealed that the application of a digital thermometer using the inguinal site gives temperature readings that are similar to those of the conventional cloacal method.

Keywords: Axillary site, Broiler chicken, Cloacal site, Digital thermometer, Inguinal site, Temperature

INTRODUCTION

Chicken remains one of the fastest growing, most prolific, and thus most numerous livestock species worldwide (Paputungan et al., 2020; Lawal and Hanotte, 2021). Its adaptability to diverse environmental conditions and potential for breeding improvement provide a unique resource for addressing the persistent food security challenge (Lawal and Hanotte, 2021; Nawaz et al., 2021). Despite their adaptation, the realization of the full productive and reproductive potential of chicken has been undermined by many factors, including diseases, heat stress, and inter alia (Paputungan et al., 2020; Ahmed et al., 2022). This is particularly the case among the chickens that evolved to suit the temperate regions, and they are exotic to the sub-Saharan region (Omodewu and Tiamiyu, 2021). For example, broiler chickens (broilers) are highly susceptible to heat stress and diseases compared to their indigenous counterparts (Omodewu and Tiamiyu, 2021; Ahmed et al., 2022). That notwithstanding, this breed is widely preferred for rearing because of its productive and meat quality characteristics (Chang, 2007; Ahmed et al., 2022). Given this, the broiler industry must improve its production methods, particularly in the areas of health, environment, and animal welfare (Chang, 2007).

Among the crucial requirements, there is a need to implement timeous on-farm strategies to minimize the resulting undue economic losses attributed to the negative effects of heat stress and diseases, among others (Nawaz et al., 2021; Ahmed et al., 2022). These primarily undermine

the productive and reproductive performance in terms of egg and meat production, as well as the welfare and mortality rate of broiler chickens (Goel, 2020; Kim et al., 2021). Consequently, one strategy needed to minimize losses is the timely diagnosis of disease or febrile conditions in chickens through routine clinical examination (Sellier et al., 2014; He et al., 2022). The initial part of the clinical examination can employ many physiological parameters, such as body temperature, heart rate, respiratory rate, serum biochemistry, and reproductive performance (Abdisa, 2017; Omodewu and Tiamiyu, 2021). Under pathological or heat stress conditions, the temperature will change due to infection or emergence response (He et al., 2022). For example, some poultry diseases, such as avian influenza, fowl typhoid, and leg conditions, like bamboo foot, increase temperature. Accordingly, a timeous detection of this temperature increment facilitates disease diagnosis and prompt farm-decision making (He et al., 2022). Other than the health aspect, body temperature-taking is useful in ascertaining the thermoregulatory and reproductive status of broiler chickens (Sellier et al., 2014). However, the measurement of temperature is often bypassed due to the potential risks, such as stress and injury, to the chicken or handlers (Anderson et al., 2019).

There are many anatomical sites, including cloaca, tympanic membrane, axilla, eyes, wattles, forehead, inter alia, which have been studied and or used for temperature measurement in chickens (Anderson et al., 2019; Kim et al., 2021). Among these sites, the rectum or cloaca remains the conventional site that is widely used for measuring temperature in many species, including chicken (Pourjafar et al., 2012; Aluwong et al., 2017). Generally, the cloacal or rectal temperatures are closer (about 2℃) to the core body temperature compared to those of the other body sites, particularly the peripheral (shell) parts (Anderson et al., 2019). Similarly, many temperature sensors, such as mercury and digital thermometers, temperature loggers, and infrared devices, have been innovated or used; however, the mercury and digital thermometer types remain the standard measuring devices (Anderson et al., 2019; Bloch et al., 2020). Mercury and digital thermometers are applied per cloacal, and their readings are usually close to the core body temperature (Anderson et al., 2019).

Although mercury and digital thermometers are widely favored for their reliable accuracy, these devices can be a source of transmissible diseases or lead to rectal injury to the chicken (Pourjafar et al., 2012; Anderson et al., 2019; Kim et al., 2021). This is more so when these devices are applied per cloacal by an unskilled handler. Less invasive evaluation of body temperature will result in a complete physical examination and provide a more accurate and appropriate clinical decision about the chicken's status (Anderson et al., 2019). Thus, it is necessary to identify and validate the less-risky anatomical sites in chickens, whose temperatures are closely in agreement with the cloacal or the core body temperature. Currently, digital thermometers are the most common temperature devices that are utilized in veterinary clinics; hence they are readily available for veterinary use (Abdisa, 2017; Kahng and Brundage, 2019). Additionally, they are time-saving, accurate, and user-friendly to clinicians (Anderson et al., 2019; Kahng and Brundage, 2019). However, some clinicians and many poultry farmers can easily injure or propagate infections among the chicken, particularly when this device thermometer is inappropriately applied per cloaca. Hence, this study was undertaken to assess the suitability of inguinal and axillary sites for temperature measurement using a digital thermometer in broiler chickens.

MATERIALS AND METHODS

Ethical approval

This study was conducted with approval (No. 1595-2021) by the institutional committee on animal research, University of Zambia, Lusaka, Zambia. Furthermore, the feeding, housing, handling of the chickens, and experimentation were carried out in compliance with the guide for the care and use of agricultural animals in research and teaching (ASAS, 2020).

Study location

This study was conducted at the Poultry farm owned by the Department of Animal Science, School of Agricultural Sciences, University of Zambia, Lusaka, Zambia, in January 2023. Zambia is located at latitude S 14° 20' 0" and longitude E 28° 30' 0" (GeoNames geographical database Google Earth-January, 2023). Furthermore, this country lies in the tropics, specifically in the Southern-African region.

Experimental chicken and management

A total of 100 broiler chickens (one-day-old) of the strain Cobb 500 were procured for this study. The average weight of these chickens at the time of procurement was 37.30 g. These chickens were raised in the poultry unit that belonged to the Department of Animal Science. In terms of their management, all the chickens were

vaccinated against gumboro, Newcastle, and marek's diseases, following an established vaccination schedule at the field station. A 17 light and 7 dark photoperiods was subjected to the chicken, with the lighting provided from 05:00 to 22:00 hours. The ambient temperature was maintained at about 32℃, using infrared lamps, for 21 days. Physical observation and ambient temperature measurement with liquid-in-glass thermometers (Easy-Read®, Thomas Scientific, New Jersey, USA) were employed to monitor heat stress among the chicken. The ventilation in the poultry house was provided using a wire mesh, of which the control of temperature and humidity levels was not comparable with an automated system. With regard to feeding, three different diets were used, including broiler starter, broiler grower, and broiler finisher. The type of diet provided was dependent on the age of the broiler chicken. Additionally, all the feeds were sourced from Entrust stock feed Limited, Lusaka, Zambia. The stated (provided) nutrition information for the feeds (diets) used is presented in Table 1. All the diets were fortified with premix containing a range of vitamins, minerals, and amino acids at an undisclosed rate. In this case, the effect of individual components of the premix on the physiology or performance of the chicken may not be pinpointed. Nevertheless, all the chickens were allowed to feed *ad libitum*, with the feeders routinely refilled at 10:00 and 15:00 hours. Similarly, these chickens had unrestricted access to clean water for drinking. About 10% mortality rate was recorded; at the time of the experiment, a total of 84 chickens were physically healthy and available for the trial.

Table 1. Chemical nutritional characteristics of feed(s) provided to broiler chickens at 55 days of age

Nutrition composition	Starter (1-12 D)	Grower (13-23 D)	Finisher (24-55 D)
Crude protein (min %)	20.0	19.0	19.0
Moisture content (max %)	12.0	12.0	12.0
Crude fiber (max %)	5.0	5.0	5.0
Phosphorus (available %)	0.6	0.5	0.4
Calcium (max %)	1.0	1.2	0.85
Lysine (min %)	1.23	1.0	1.04
Total methionine (min %)	0.5	0.4	0.39
Metabolizable energy (kcal/kg)	2900	3100	3200

D: Days; min: Minimum; max: Maximum; %: Percentage

Experiment design

This was a cross-sectional study that employed a single-factor experimental design. The study considered anatomical site as the main factor, which had three-factor levels (as treatments), including inguinal, axillary, and cloacal locations of the chicken. The measurement of temperature was done when the chickens were 55 days of age, with an average weight of 2.80-3.21 kg. A total of 28 physically-healthy chickens were randomly selected for temperature measurement. For each chicken, temperature measurements, namely $DTt_{cloacal}$, $DTt_{axillary}$, and $DTt_{inguinal}$, for inguinal, axillary, and cloacal sites, respectively, were conducted, and $DTt_{cloacal}$ was considered as a control. The sampling strategy was based on the previous procedure (Anderson et al., 2019). The order for the measurement of sites was determined by means of a simple random assignment, which used folded papers bearing the names of each site. Additionally, the study followed all the precautions necessary to minimize the potential effects of psychogenic fever or hyperthermia. Temperature measurement was conducted in the morning (8:00-11:45 hours) and before feeding the chicken. A functional veterinary digital thermometer (DT; GB Kruuse digital thermometer, New Taipei City, Taiwan) was used to measure the temperature. The measuring range and the resolution of the DT were 30.0-43.9℃ and 0.1℃, respectively.

The measurement of temperature

Before the measurement of temperature, a chicken was physically restrained (Nash, 1976). The procedure for the DT application was based on the manufacturer's directions, with some additions depending on the site. With the chicken restrained, the $DTt_{cloacal}$ was obtained by inserting a sterile DT into the cloaca (approximately 2 cm), gently and at a slight angle dorsally, towards the cloacal wall. The device was left in position for 20-60 seconds until the degree sign stopped flashing, and an alarm went off before the $DTt_{cloacal}$ readings were recorded (℃). The DT was properly disinfected using isopropyl alcohol before temperature-taking for each chicken to prevent cross-contamination and/or disease transmission. A similar procedure was followed to obtain the $DTt_{axillary}$ readings. Here, the DT probe was snugly placed between the chicken's breast and biceps, approaching from the cranial aspect and aiming dorsally toward the shoulder joint. Similarly, the $DTt_{inguinal}$ was measured by snugly positioning the DT probe between the chicken's breast and thigh, aiming dorsally/deep into the hip joint. A double temperature measurement was performed for each site, and the average of the two measurements was recorded as a single datum. Furthermore, the same researchers took the temperature to minimize human errors and undue stress on the chicken. During the experiment, the temperature and

humidity ranged from 26.8 to 27℃ and 72 to 75%, respectively.

Data analysis

Data were analyzed in the Statistical Package for Social Scientists (SPSS® IBM 26 version, USA) using descriptive statistics, including the selected central tendency, dispersion, and distribution measures. The temperatures taken from different anatomical sites were analyzed by a One-way ANOVA test using the General Linear Model, a univariate analysis procedure. The following model was used to determine the main effect of the treatment factor;

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

Where, Y is the dependent variable representing a value of the measured parameter, u signifies the overall mean, A_i refers to the effect of the treatment factor (site) with three levels (i = cloacal, inguinal, and axillary sites), e_{ij} defines the random error term. Tukey's HSD (post-hoc) test was employed to obtain the mean treatment pair(s), where significant differences existed. The correlation between the sites (by DTt readings) was determined using a Pearson's correlation test. In all cases, significance was taken at a level of $p < 0.05$. The Bland-Altman plot was employed to analyze the agreement between the two temperature measurements that exhibited the highest correlation coefficient.

RESULTS

Mean temperature readings for different anatomical sites

The mean temperature readings taken by a digital thermometer (DTt), including $DTt_{cloacal}$, $DTt_{axillary}$, and $DTt_{inguinal}$, for the different anatomical sites, namely cloacal, axillary, and inguinal, respectively, are presented in Table 2. The mean $DTt_{cloacal}$ was the highest among the observed treatments, while the mean $DTt_{axillary}$ was the lowest. Analysis of variance revealed a main effect of the anatomical site of broiler chickens on the mean DTt readings, $F(2, 81) = 12.75$, $p < 0.05$, $\eta_p^2 = 0.339$. The post hoc analyses (Tukey's HSD test) indicated that the mean $DTt_{cloacal}$ was higher than that of the $DTt_{axillary}$ ($p < 0.05$), but did not differ significantly from the mean $DTt_{inguinal}$ ($p > 0.05$). Additionally, the mean $DTt_{inguinal}$ was significantly higher than that of the $DTt_{axillary}$ ($p < 0.05$).

Table 2. The mean temperature readings from different anatomical sites of broiler chickens at 55 days of age

Variable	DTt readings	
Anatomical site	**Mean ± SD (℃)**	**Difference from $DTt_{cloacal}$ (℃)**
Cloacal	41.40 ± 0.17^{a}	-
Axillary	41.12 ± 0.19^{b}	0.28
Inguinal	41.39 ± 0.19^{a}	0.01

DTt: Temperature readings by a digital thermometer, SD: Standard deviation, ℃: Degrees Celsius. ^{a,b}Different superscript letters within the same column indicate a significant difference ($p < 0.05$).

Correlation between temperature readings and different sites

Results from multiple correlation analyses of the temperature readings, including $DTt_{cloacal}$, $DTt_{axillary}$, and $DTt_{inguinal}$, are presented in Table 3. The $DTt_{cloacal}$ had a relationship with both the $DTt_{axillary}$ and $DTt_{inguinal}$ readings. The highest correlation was observed between the $DTt_{cloacal}$ and $DTt_{inguinal}$ ($r(28) = 0.90$, $p < 0.05$). Among the treatments, the correlation between the $DTt_{cloacal}$ and the $DTt_{axillary}$ readings was the lowest ($r(28) = 0.758$, $p < 0.05$).

Table 3. Correlation between temperature readings taken at different anatomical sites of broiler chickens at 55 days of age

	$DTt_{cloacal}$	**$DTt_{axillary}$**	**$DTt_{inguinal}$**
$DTt_{cloacal}$	1		
$DTt_{axillary}$	0.758**	1	
$DTt_{inguinal}$	0.900**	0.823**	1

DTt: Temperature readings by a digital thermometer, correlation coefficient 0.00-0.10: Negligible, 0.10-0.39: Weak, 0.4-0.69: Moderate, 0.7-0.89: Strong, 0.9-1.0: Very strong correlation, Correlation coefficient with an asterisk (**): statistical significance at $p < 0.05$

Comparison between the cloacal and inguinal temperature measurements

The Bland-Altman plot of the temperature readings (Figure 1) presented a relationship between the $DTt_{cloacal}$ and $DTt_{inguinal}$ measurement methods. This plot displays the points that were within the 95% confidence interval (CI) of the distributed data; the mean of $DTt_{cloacal}$ and $DTt_{inguinal}$ and the difference between the two measurements were used to generate the plot. The results revealed that, with a 95% CI, many paired DTt readings were not significantly different ($p > 0.05$).

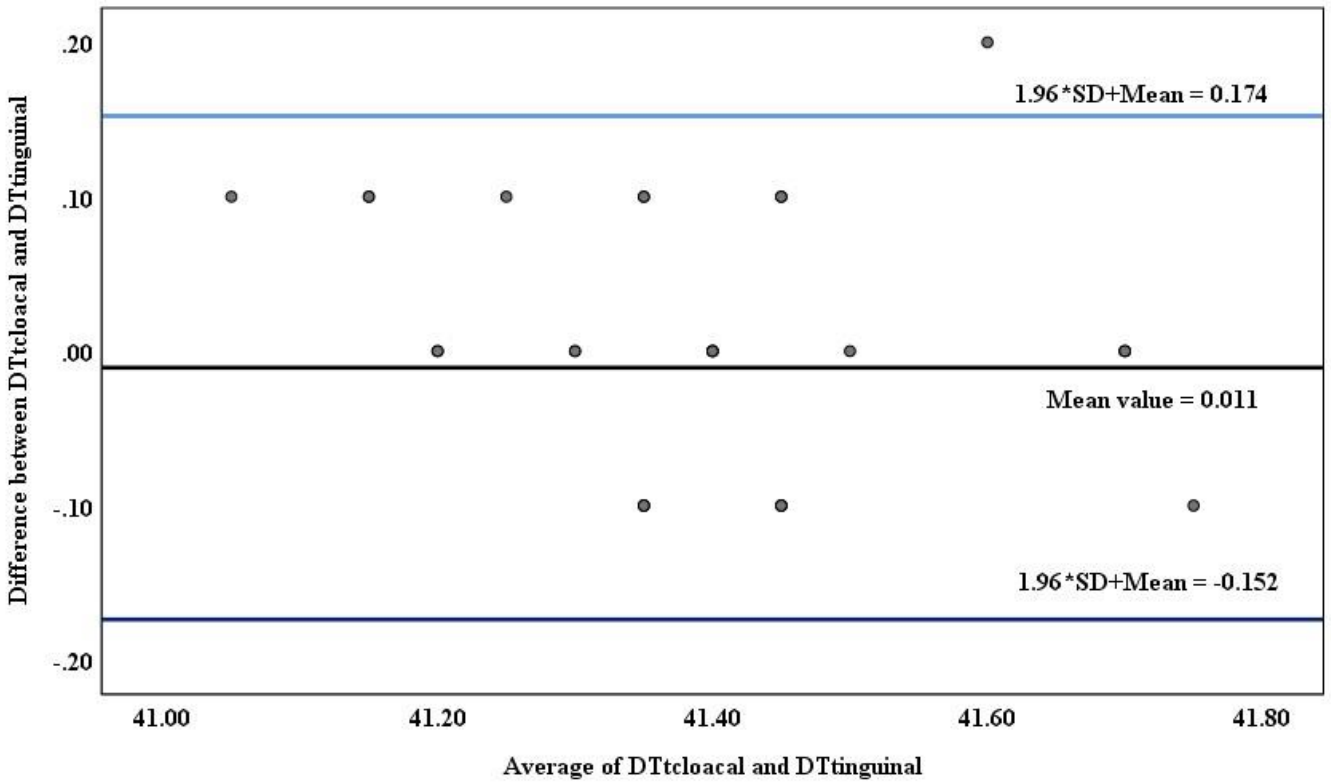

Figure 1. Bland-Altman plot visualizing the agreement between $DTt_{cloacal}$ and the $DTt_{inguinal}$ readings obtained from broiler chickens in Zambia

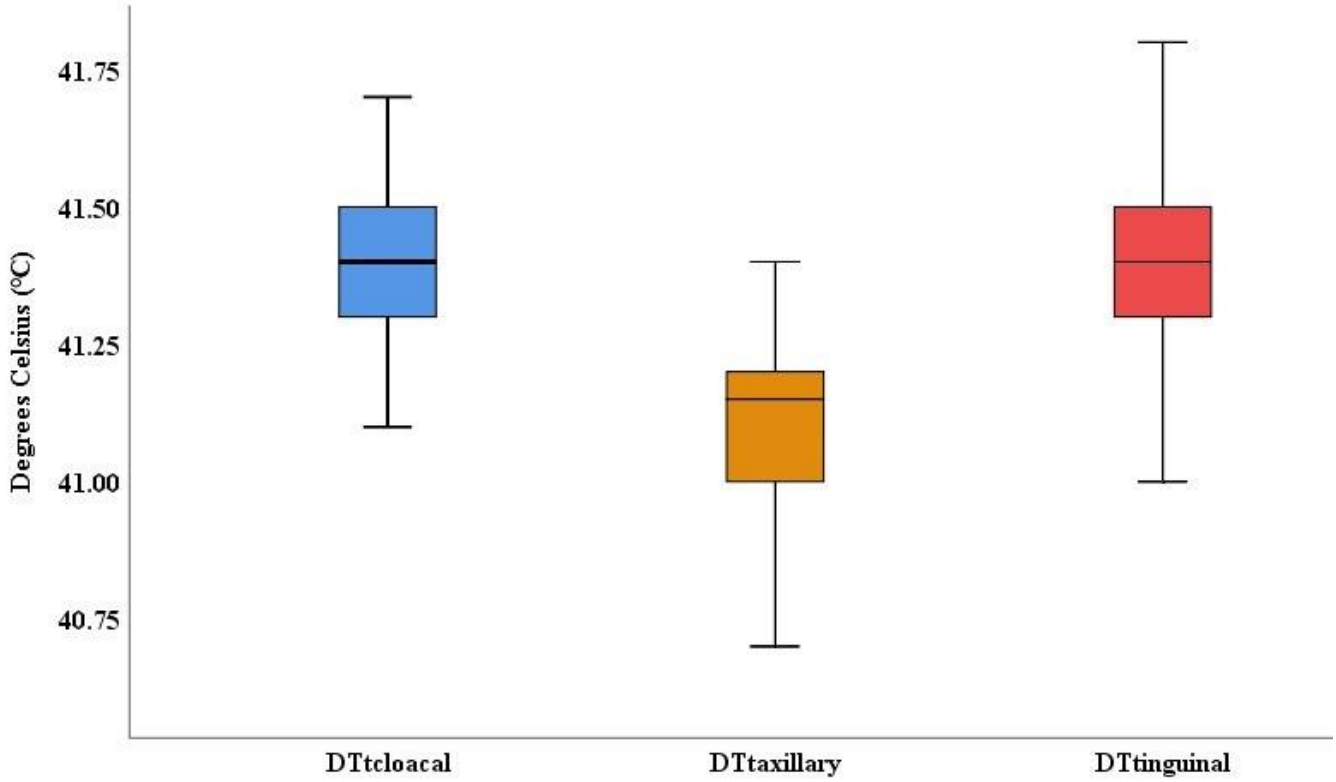

Figure 2. The Box-and-Whisker plot demonstrating the relative distribution of temperature readings for each anatomical site

The distribution of temperature readings for each anatomical site

The pictorial presentation of the relative dispersion and distribution of the DTt readings for each anatomical site, including the cloaca, axilla, and inguinal, is shown in Figure 2. The $DTt_{inguinal}$ range was generally larger than that of the $DTt_{cloacal}$. The $DTt_{cloacal}$ and $DTt_{inguinal}$ generally had similar medians, with most DTt readings tightly clustered around the median. Generally, the analysis showed an underestimation of the values ($DTt_{axillary}$ readings) for the axillary site compared to those of the other sites. There was considerable skewing to the lower temperature limits for the case of $DTt_{axillary}$ readings.

DISCUSSION

Cloacal temperature taking has been conventionally regarded as the "standard/gold method" for core body temperature assessment in chickens because of its physiological accuracy and repeatability (Anderson et al., 2019; Reis et al., 2022). Nevertheless, some drawbacks associated with cloacal temperature taking, such as injuries and disease transmission among chickens, have recently compelled researchers to search for less invasive and safer temperature sensors (Anderson et al., 2019; Kim et al., 2021). Temperature measurement is often bypassed for fear of the potential risks associated with cloacal thermometry (Anderson et al., 2019). So far, many advanced temperature sensors have been studied and or used, including infrared thermometers, infrared cameras, inter alia (Sellier et al., 2014; Bloch et al., 2020). However, many of these are expensive for an average chicken farmer or generally not user-friendly, and some are not as accurate as the case is for cloacal-based devices (Cândido et al., 2018; Bloch et al., 2020). Cognizant of this, the current study found a non-invasive temperature measurement method or site whose readings agree with the conventional cloacal temperature.

Furthermore, the observed main effect of the measurement site on the mean temperature value supports an earlier study that confirmed the dependency of a thermometer's readings on the point of skin used (Abioja et al., 2019; Reis et al., 2022). In addition, the absence of a significant difference between the cloacal and inguinal temperatures indicated the proximity of the latter to the core body temperature in broiler chickens. This disagrees with the earlier study findings that reported significantly lower temperatures of most skin points, compared with the cloaca (Abioja et al., 2019; Reis et al., 2022). This may be attributed to the type of thermometer used and the proximity of the inguinal site to the liver and other innards that are metabolically very active. Additionally, the disposition of the thigh and breast parts that make up the inguinal site, as well as the feathers around this site, perhaps minimized the excessive heat loss (Saeed et al., 2019).

With regard to the axillary site, the current study findings varied from the previous study that showed similar mean cloacal and axillary temperatures (Abioja et al., 2019). This disparity may be attributed to the type of thermometers used. Nevertheless, the current mean temperature values for both the axillary and inguinal sites were within the reported normal chicken body temperature range of 40.6-43.0℃ (Reece, 2009). This may be attributed to the fact that these sites are mostly kept covered, given their anatomical predisposition, with less heat loss to the environment. On the other hand, the similarity between the observed temperatures and the reported normal range points to the fact that the production performance of the study chicken was not critically affected by disease or environmental conditions, which agrees with the findings of an earlier study conducted in Brazil (Reis et al., 2022).

The correlation between the cloacal and inguinal temperature in the current study revealed the potential use of the latter for core body temperature assessment using digital thermometers. From a previous report, the cloacal temperature was found to correlate well with the core body temperature (Candido et al., 2020). The observed positive and very strong correlation between the cloacal and inguinal temperatures qualifies the inguinal site for the digital thermometer application in broiler chickens. Given the observed non-significant bias between the cloacal and inguinal temperature measurements ($p > 0.05$), the current results of the Bland-Altman analysis confirmed an agreement between these two temperature measurement methods. Moreover, the agreement interval is sufficiently narrow, compared to the maximum acceptable difference (± 0.2℃) for temperatures between two anatomical sites (Fulbrook, 1993). On the other hand, although the current correlation between the cloacal and axillary temperatures was significantly strong ($p < 0.05$), which finding is consistent with the previous reports, their observed mean difference renders the axillary site inappropriate for the digital thermometer application (Abioja et al., 2019).

The observed similar median points and close distribution of readings within the lower and upper quartiles for both cloacal and inguinal temperatures attest to observations on their means and correlation coefficient.

These observations largely indicate the potential and reliability of the inguinal temperature measurement method for broiler chickens' thermal status monitoring. Moreover, inguinal thermometry is devoid of the reported rectal thermometry drawbacks, namely invasiveness, disease transmission, and potential temperature variations attributed to fecal masses, muscle tone, digestion, inter alia (Pourjafar et al., 2012; Kim et al., 2021). On the other hand, the findings of the current study on the axillary temperature agree with the report of a recent study which indicated lower skin temperature readings compared to cloacal temperature (Reis et al., 2022).

The current findings reveal the potential of inguinal thermometry for monitoring the temperature or health of broiler chickens, more so among smallholder farmers. Moreover, early identification of the health status of chickens contributes to the timeous decision-making in smallholder or commercial poultry farms (Ahmed et al., 2022; Reis et al., 2022). Temperature estimation is used as a physiological marker for chickens' thermic status during disease, reproductive status, and heat stress assessments (He et al., 2022; Reis et al., 2022). For example, disease infections in chickens cause abnormal temperature, avian influenza, fowl typhoid, inter alia, and fever (He et al., 2022). Hence, this clinical symptom helps in the evaluation or identification of the sick chicken. It is noteworthy that changes in environmental or farm temperature can also influence the body temperature of broiler chickens (Ahmed et al., 2022). The high temperature increases susceptibility to diseases and compromises the productivity of the chicken (Ahmed et al., 2022). The application of inguinal thermometry, which is generally safer and more user-friendly than the cloacal temperature measurement method (Anderson et al., 2019), will likely benefit the poultry subsector. Nevertheless, whether the inguinal thermometry gives similar results among younger broilers or other chicken breeds remains unclear.

CONCLUSION

A timeous body temperature taking in chickens, including broilers, is crucial as part of their initial clinical examination to confirm disease status, heat stress, and productive and/or reproductive status. This study has revealed that applying a digital thermometer using the inguinal site gives as accurate temperature readings as the case with the conventional cloacal method/site. Hence the inguinal temperature is recommended, as a reliable physiological marker, for the body temperature assessment in broiler chickens. The inguinal site is non-invasive and user-friendly to clinicians and smallholder chicken farmers. The limitations of the current study included the use of one breed, small sample size, and one age (adult) group of the chicken. Thus, future studies intended to replicate the current research must consider these factors for the generalization of the findings.

DECLARATION

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Authors' contributions

Pharaoh C. Sianangama designed and supervised the study and reviewed the manuscript, and Rubaijaniza Abigaba conceived and designed the study, collected and analyzed data, and wrote the manuscript. Both authors approved the final manuscript for publication.

Conflict of interests

The authors declare no conflict of interest.

Ethical consideration

The authors declare that this manuscript is original and has not been submitted elsewhere for possible publication. The authors also declare that the data used/presented in this manuscript has not been fabricated.

Consent to publish

Both authors informed their consent before the study conduction.

Availability of data and materials

The authors will provide data of the present study in case of reasonable request.

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Effects of Bioherbal Compounds on Performance and Intestinal Characteristics of Laying Chickens

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ABSTRACT

Since the European Union banned the use of antibiotic growth promoters in poultry feed in 2006 (EC Regulation No 1831/2003), alternative feed additives have been investigated. The purpose of this study was to evaluate the effect of a bioherbal combination of probiotics and phytobiotics as a feed additive in drinking water on the performance and intestinal characteristics of male laying chickens. The study was performed on 200 male laying chickens for 60 days. This research method was a field experiment with a completely randomized design, consisting of four treatments and five replications. The treatments were T0 (drinking water without bioherbal, control), T1 (control + bioherbal code 1 M), T2 (control + bioherbal code 2 H), and T3 (control + bioherbal combination of 1M and 2H). The investigated parameters included growth performance and intestinal profile of the male laying chickens. The addition of bioherbal increased the number of villi in the intestines of the male laying chickens; however, there was no significant difference among other parameters. It can be concluded that the addition of bioherbal code 2H as a feed additive with a composition of herbal leaves can improve the performance and intestinal characteristics of male laying chickens.

Keywords: Intestinal Characteristic, Male Layings, Performance Production, Phytobiotic, Probiotic

INTRODUCTION

Farmers strive to achieve high production results from male laying animals to increase their income. One effective way to achieve this is by regulating feed management, as feed constitutes the majority of production costs, accounting for around 60-70% of the total production costs (Ardiansyah et al., 2022). However, providing feed for animals also contributes significantly to land and water use, as well as greenhouse gas emissions (Adli, 2021).

To increase feed efficiency and reduce production costs, many farmers turn to feed additives. Antibiotics are commonly used as feed additives to suppress pathogenic digestive bacteria, leading to improved intestinal's health. However, the use of antibiotics as feed additives has been associated with many negative effects on both livestock and humans who consume them. Consequently, the Indonesian government banned the use of antibiotics as feed additives on January 1, 2018, as stipulated in the Minister of Agriculture Regulation Number 14/2017 Article 16, which applies to both finished products and raw ingredients for veterinary drugs that are mixed into feed (Adli et al., 2023; Shoilikin et al., 2023).

With the prohibition of using Antibiotics Growth Promoters (AGP), many researchers have been finding substitutes for antibiotics as additives, such as natural additives derived from herbals. Derived from natural ingredients, alternative feed additives (such as probiotics, prebiotics, enzymes, organic acids, and phytobiotics) are becoming increasingly popular in the livestock industry, particularly in poultry (Sami and Fitriani, 2019; Mirzaei et al., 2022). Bioherbal is an additive combination of probiotics and phytobiotics, where the synergistic of both are expected to provide a more optimal effect on increasing the productivity and health of livestock (Adli et al., 2023).

Probiotics are additives derived from live microorganisms that can increase growth and feed efficiency without leaving residues since the process of absorbing probiotics into the body of livestock does not occur (Mirzaei et al., 2022). Probiotics can be applied to

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feed, medicine, and feed supplements in powder or liquid form, while phytobiotics are additives in the Natural Growth Promoter (NGP) group derived from herbs, spices, or other plants because they contain antimicrobial substances and antioxidants (Kurniawati et al., 2021). Some herbal plants often used as additives include aromatic ginger, curcuma, cinnamon, ginger, turmeric, kalmegh, moringa, papaya, and betel leaves (Saeed et al., 2018; Ardiansyah et al., 2022; Li et al., 2023).

Based on the above, this research was conducted to evaluate the effects of adding bioherbal, a feed additive combination of probiotics and phytobiotics, both rhizomes and leaves biopharmaceuticals, on performance and intestinal characteristics in male layings to achieve maximum productivity.

MATERIALS AND METHODS

Ethical approval

Ethical approval for the study was given by the AnimalCare and Use Committee, University of Brawijaya, No.118-KEP-UB-2022 on August 21, 2022.

Research materials

In this study, 200 male ISA brown laying chickens were growing for 60 days. Male ISA brown laying chickens were chosen due to regulations the Indonesian government was not allowed to sacrifice the female laying chickens. Open house cages, commercial starters, finisher feeds, bioherbal codes 1M and 2H, vaccines, medicines, and cage equipment were used as essential components of a well-managed poultry operation. The bioherbal compositions of 1M and 2H are presented in Table 1.

Table 1. Bioherbal compositions of 1M and 2H

1M	Consisted	2H	Consisted
Aromatic ginger	1ml	Kalmegh leaves	2ml
Curcuma	1ml	Betel leaves	2ml
Ginger	1ml	Moringa leaves	2ml
Turmeric	1ml	Papaya leaves	2ml
Actinocetes	1.2×10^6 cfu/ml	Actinocetes	2.9×10^6 cfu/ml
Lactic Acid Bacteria	1.2×10^6 cfu/ml	Lactic Acid Bacteria	2.9×10^6 cfu/ml
Photosynthetic Bacteria	1.2×10^6 cfu/ml	Photosynthetic bacteria	2.9×10^6 cfu/ml
Yeast	1.2×10^6 cfu/ml	Yeast	2.9×10^6 cfu/ml
Fermented Fungi	1.2×10^6 cfu/ml	Fermented fungi	2.9×10^6 cfu/ml

Methods

The *in vivo* field experiment using a completely randomized design was employed to evaluate the effectiveness of bioherbal combinations of probiotics and phytobiotics as additives in drinking water. The experiment included two codes, namely codes 1M and 2H and was composed of four treatments, each with five replications. Thus, there were a total of 20 experimental units, with each replication consisting of 10 chickens.

In this study, four different treatments were given to evaluate the effectiveness of bioherbal combinations as additives in poultry production. The first treatment, T0, served as a control and involved providing drinking water without any bioherbal additives. The second treatment, T1, involved adding Bioherbal Code 1M to the control group. The third treatment, T2, involved adding Bioherbal Code 2H to the control group. Finally, the fourth treatment, T3, involved adding both Bioherbal Code 1M and 2H to the control group, alternating between the two every week. By comparing the results from each of these treatments, the study aimed to determine the impact of bioherbal additives on the health and productivity of poultry.

Research variables

The variables measured in this study included performance and intestinal characteristics.

Performance

Feed consumption

Feed consumption can be determined by weighing the feed difference between the feed given and the remaining feed (Putri and Bintari, 2021).

Feed consumption = Σ feed given (g) - Σ remained feed (g)

Body weight gain.

Body weight gain for male layings can be determined by weighing the chickens by calculating the difference between the final body weight and the initial body weight.

Body weight gain = Final body weight (g) - Initial body weight (g)

Feed conversion ratio

Feed conversion ratio (FCR) expressed between feed offered and total body weight produced (Ardiansyah, et al., 2022).

FER = Σ feed consumption (g) / body weight gain (g)

Income over feed cost

Income over feed cost (IOFC) was expressed from total income by differences between amount of laying chicken harvested and total cost of feed at whole periods

(Fitro et al., 2015).

IOFC = (Body weight x chicken price (alive)) - (Σ feed consumption x feed cost)

Intestinal characteristics

Number of villi, length of villi, surface area of villi, and depth of crypts

The prepared villi were analyzed under a DIC Olympus BX51TF light microscope (Japan) connected to the Optilab application. Measurements of the number of villi, villi length, villi surface area, and depth of the crypt were carried out using the Image Raster application, which was adjusted for magnification at the time of observation.

Statistical analysis

For the statistical analysis, analysis of variance (ANOVA) using a general linear model (GLM) was carried out in SAS OnDemand for Academics (ODA, Cary, NC, USA). The results were presented as standard error mean (SEM). Moreover, probability values were calculated using the least significant different testing. The following model was used by following Adli et al. (2022) and Ardiansyah et al. (2022).

$$Yij = \mu + Ti + eij$$

Where, Yij signifies the parameters observed, μ is the overall mean, Ti denotes the effect level of date seed flour, and eij determines the amount of error number. Furthermore, the probability values were calculated using the least significant difference of $p < 0.05$.

RESULTS AND DISCUSSION

Tables 2 and 3 show the results of observations and statistical analysis on performance and intestinal characteristics of male layings, including feed consumption, body weight gain, feed egg, income over feed cost, number of villi, length of villi, the surface area of villi and depth of crypt with four treatments and five replications.

Table 2. Effect of adding bioherbals on feed consumption, body weight gain, FER, IOFC, of 60 days male laying chicken during research

Parameters	T_0	T_1	T_2	T_3	SEM
FI (g/bird)	2362.51	2378.51	2344.00	2363.24	12.33
BWG (g/bird)	842.20	832.00	873.19	852.12	11.30
FER	2.69	2.74	2.58	2.66	0.12
IOFC (IDR/head/day)	4391.13	3592.06	5302.04	4493.82	89.77

FI: Feed intake, FER: Feed egg ratio, FBW: Final body weight, IOFC: Income over feed cost. [a,b,c,d] Means with different superscripts in the row differ significantly ($p < 0.05$). T_0= Drinking water without bioherbal (control); T_1= Control + bioherbal code 1M, T_2= Control + bioherbal code 2H, T_3= Control + bioherbal code 1M and 2H, with alternate every week

Feed consumption

Table 2 presents feed consumption data from a field experiment by giving control drinking water and adding bioherbal. The results of data analysis showed that the addition of bioherbal to drinking water had no significant effect on the whole parameters of growth performance, including feed intake, body weight gain, feed egg ratio, and income over feed cost ($p > 0.05$). This suggests that the bioherbal was not optimally absorbed by the laying chickens, and its content did not significantly impact feed intake ($p > 0.05$). Similar findings were reported by Tini et al. (2020), who indicated that giving herbal supplements (ginger, turmeric, and curcuma) to quails did not have a significant effect on feed intake, possibly due to suboptimal absorption of the herbal ingredients.

The highest value of feed intake was found in T1 with a total of 2378.75 gram/bird when 1M bioherbal was added. In contrast, the lowest value was 2344.00 gram/bird in T2 with the addition of 2H bioherbal. The content of essential oils and curcumin contained in 1M bioherbal can increase the appetite of chickens because these ingredients play a role in the rapid emptying of stomach contents, leading to higher feed consumption (Tini et al., 2020). palatability, environment, and livestock behavior are also important factors that influence feed consumption (Avianti et al., 2020). Palatability can be affected by the ingredients contained in bioherbal, which give a different taste, one of which is the bitter taste caused by turmeric, while the behavior of livestock is related to the feeding system (Ardiansyah et al., 2022). *Ad libitum* system can make it easier for livestock to consume feed every time. Ultimately, these related factors influenced the treatment of the laying chickens.

Body weight gain

Table 2 presents the results of a field experiment on body weight gain by providing controlled drinking water and adding bioherbal. The data analysis showed that the addition of bioherbal to the drinking water did not have a significant effect ($p > 0.05$) on the body weight gain of male layings. The average values from lowest to highest were T1 (832.00), T0 (842.20), T3 (852.15), and T1 (873.19) gram/bird. The highest body weight value of 873.19 g/bird was observed in T2 chickens with the addition of 2H bioherbal, while the lowest value of 831.00 g/bird was observed in T1 with the addition of 1M bioherbal. These findings are consistent with the results of a study conducted by Sukmaningsih and Rahardjo (2019), which reported that the use of probiotics and herbs in drinking water did not successfully increase the feed intake and body weight gain of broiler chickens.

Body weight gain is influenced by the amount of feed consumed. The more feed consumed, the more rapidly the body weight will increase. The feed consumed must meet the nutritional needs of livestock to be converted into the desired product. The increase in feed consumption was consistent with the increase in body weight in each treatment. The addition of 2H bioherbal to T2 showed that a small amount of feed consumption was able to produce maximum body weight gain, presumably because the bioherbal content was able to improve feed quality, allowing the body to utilize it better for increasing body weight. Daud et al. (2017) reported that the physiological function of livestock affects body weight gain because the feed consumed will preferably be used for the formation of body tissues. Several factors that affect livestock body weight gain are breed, feed, rearing management, and environmental conditions (Sholikin et al., 2023).

Feed conversion ratio

Table 2 displays the results of FCR based on a field experiment involving the provision of control drinking water and the addition of bioherbal. The data analysis indicated that the addition of bioherbal to the drinking water did not have a significant effect on the FCR ($p > 0.05$), with an average result from lowest to highest of T2 (2.58), T3 (2.66), T0 (2.69), and T1 (2.74). This lack of significant effect may be due to the relationship between feed intake and body weight gain, which did not show a significant effect and is used to calculate the feed egg ratio. Similar to the research conducted by Sukmaningsih and Rahardjo (2019), the addition of a combination of probiotics and herbs did not have a significant effect on the feed egg ratio of broiler chickens. However, the addition of bioherbal did affect the feed egg ratio according to the treatment given. The optimal digestive tract ecosystem of livestock can be achieved through the use of certain leaves contained in the 2H bioherbal, which play a role in increasing body weight. For instance, the active compounds found in Moringa leaves, such as essential oils, flavonoids, and antioxidants, can increase productivity by promoting the optimal functioning of organs. Additionally, fragrant papaya leaves can increase consumption and body weight gain, which can affect the feed egg ratio (Sami and Fitriani, 2019). The feed egg ratio can be influenced by genetics, feed and water quality, type of livestock, and rearing management (Daud et al., 2017).

Income over feed cost

Table 2 presents the income over feed cost (IOFC) of male layings based on a field experiment with control drinking water and the addition of bioherbals. The data analysis showed that the addition of bioherbals to drinking water had no significant effect ($p>0.05$) on IOFC, with an average from lowest to highest of T1 (3592.06), T0 (4391.13), T3 (4493.82), and T2 (5302.04) IDR/bird. This is allegedly due to the lack of optimal absorption of bioherbals, as evidenced by the non-significant effect of feed consumption in the statistical analysis. IOFC increased in each treatment, with the addition of 2H bioherbals to T2 resulting in the highest IOFC of 5302 IDR/bird compared to other treatments. The probiotics in the bioherbals provide benefits in terms of increased digestibility, nutrients, and feed efficiency, resulting in the high body weight of chickens produced. The IOFC is influenced by the body weight of chickens, feed intake, and the cost of feed during the rearing period, as well as the selling price of chickens at harvest. The cost of feed and the conditional selling price of chickens can result in unstable income. The IOFC value is proportional to the efficiency of feed use, where a more efficient IOFC value will be higher.

Number of villi

Table 3 displays the results of the number of villi observed in the laboratory based on a field experiment that gave control drinking water and the addition of bioherbals. The data analysis revealed that the addition of bioherbals to drinking water had a significant effect ($p <0.05$) on the number of villi, with an average value ranging from lowest to highest: T1 (51.00), T0 (53.33), T3 (57.40), and T1 (61.60). The increase in villi number is attributed to the

probiotics and herbs containing essential oils in the bioherbals, which reduce the growth of pathogenic bacteria and stimulate optimal villi growth. Moreover, the production of lactic acid bacteria in the bioherbals affects villi density (Sjofjan et al., 2020). This finding is consistent with the research conducted by Sjofjan et al. (2020), which states that lactic acid bacteria produced by probiotics and turmeric can increase the number of villi in broiler chickens. Probiotics undergo a fermentation process, which produces short-chain fatty acids that help expand the area of nutrient absorption and multiply intestinal epithelial cells. Furthermore, they protect the villi from damage caused by pathogenic bacteria, leading to optimal growth and functioning of the digestive tract walls (Elisa et al., 2017). The highest number of villi was observed in T2, which received the addition of 2H bioherbals compared to other treatments. This result may be attributed to the chlorophyll content in the bioherbals' leafy herbs. Chlorophyll has high antioxidant levels, which help counteract free radicals (Jumadin et al., 2016).

Table 3. Effects of adding bioherbals on number of villi, length of villi, surface area of villi, and depth of crypth in 60 days male laying chicken

Parameters	T_0	T_1	T_2	T_3	SEM
Number of villi (tranversal cut)	53.33[a]	51.00[a]	61.60[ab]	57.40[a]	4.15
Length of villi (µm)	526.32	544.93	578.93	542.51	23.44
Surface Area of Villi (mm^2)	0.60	0.61	0.622	0.64	0.03
Depth of Crypth (µm)	137.16	143.39	155.42	153.79	12.33

[a,b,c,d] Means with different superscripts in the row differ significant ($p < 0.05$). T_0= Drinking water without bioherbal (control); T_1= Control + bioherbal code 1M, T_2= Control + bioherbal code 2H, T_3= Control + bioherbal code 1M and 2H, with alternate every week

Length of villi

Table 3 presents the results of villi length based on a field experiment involving male layings given control drinking water and the addition of bioherbals, which were observed in the laboratory. Data analysis revealed that the addition of bioherbals to drinking water had no significant effect ($p > 0.05$) on villi length, with an average range from lowest to highest of T0 (526.32), T3 (542.51), T1 (544.93), and T2 (578.93) µm. This could be due to suboptimal production of lactic acid bacteria in the intestine. This finding is consistent with Rahmah's (2013) research, which reported that adding herbals up to a level of 1.5% to feed did not improve broiler production performance due to the suboptimal effect of bioactive substances in the herbals. Lactic acid bacteria (LAB) in the digestive tract compete with pathogenic bacteria for feed nutrition. When the number of harmful pathogenic bacteria is high, a small amount of LAB will disrupt nutrient absorption, affecting the condition of the digestive tract.

Surface area of villi

Table 3 presents the results of the surface area of villi based on a field experiment that involved providing control drinking water and the addition of bioherbals, which were observed in the laboratory. The data analysis showed that the addition of bioherbals to drinking water did not have a significant effect ($p > 0.05$) on the surface area of the villi. The average value of surface area from lowest to highest was T0 (0.60), T1 (0.61), T2 (0.62), and T3 (0.64) mm2. This is likely to be correlated with the villi length value, which also showed no significant effect. The surface area of villi can be determined by measuring the apical width, basal width, and length of villi. Villi with a broad surface have the potential to absorb optimal feed nutrients. The condition of the digestive tract has a positive impact on livestock productivity. A larger intestine size enables more nutrient absorption, resulting in more efficient feed consumption (Lisnahan et al., 2019).

Depth of crypt

Table 3 presents the results of the depth of crypts in male layings based on a field experiment involving control drinking water and the addition of bioherbals, which were observed in the laboratory. The data analysis results indicated that the addition of bioherbals to drinking water had no significant effect ($p > 0.05$) on the depth of crypts, with an average value ranging from lowest to highest as follows: T0 (137.16), T1 (143.39), T3 (153.79), and T2 (155.42) µm. This condition is presumed to be caused by insufficient dosage of bioherbal administration. The administration of additives in accordance with the livestock's needs is expected to have a positive effect on productivity. In line with the research conducted by Ramadhan et al. (2022),

the addition of herbs, such as turmeric and probiotics, did not have a significant effect on the depth of crypts.

The depth of crypts is closely related to the growth of villi and the absorption of nutrients from the ration. Additionally, Lisnahan et al. (2019) reported that a higher depth of crypt leads to increased nutrient digestion and absorption, which has a positive impact on the growth of organs in native chickens. Deep crypts in the intestine help absorb more nutrients, leading to optimal absorption in livestock organs and achieving maximum livestock growth.

CONCLUSION

It can be concluded that the addition of 2H bioherbal gave the best result in improving the growth performance and intestinal profile of male laying chicken. Further studies are suggested to investigate using bioherbal mixed into the feed of laying chicken.

DECLARATIONS

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Authors' contributions

Chafifah Mienati Permata Utami contributed to collecting data, analysis of proximate, data analysis and preparing original draft manuscript. Osfar Sjofjan contributed to the research design, supervision, and revised the draft of the manuscript, Muhammad Halim Natsir contributed to the research design and supervision. Danung Nur Adli contributed to revising the original draft and analyses of the data.

Competing interests

No potential conflict of interest relevant to this article was reported.

Ethical consideration

All authors have checked the ethical issues, plagiarism, fabrication and/or falsification, double publication, and redundancy.

Availability of data and materials

This article includes all data generated or analyzed during this research.

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Molecular Characterization of the *NRAMP1* Gene and Blood Parameters of Sinai and Lohman Brown Chickens in Egypt

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ABSTRACT

In almost all animal species, natural resistance-associated macrophage proteins (*NRAMPs*) have been linked to disease resistance. It plays a crucial part in innate immunity and can affect adaptive immunity as well. The aim of this study was to investigate some immunological traits and molecular genetics in the native breed of chickens, named Sinai (S) and a commercial strain of Lohman Brown (LB). The *NRAMP1* gene was reported to be associated with a defense mechanism against infection by bacteria and viruses. A total of 144 female day-old chicks, including 72 from the commercial layer strain (LB) and 72 from the Egyptian native chicken strain (S), were used in this study. At 38 days of age, blood samples were taken randomly from 8 chickens of each group for serum antibodies against the New Castle disease virus, avian influenza virus, and infectious bursal disease virus analysis. Additionally, genomic DNA was extracted from 20 blood samples at 38 days of age. Polymerase chain reaction (PCR) analyses were conducted on the DNA samples, followed by sequencing of the PCR products to identify single nucleotide polymorphisms (SNPs) in the *NRAMP1* gene in the two strains of chickens. The findings indicated that lymphocyte, eosinophil, phagocyte activity, and IgY were significantly greater in LB chicks than in S chicks. Sinai chickens, on the other hand, achieved dominance in Newcastle titter. Eight SNPs were found in *NRAMP1* of the two strains. The nucleotide identity between S and LB nucleotides was 58.68%, while the changes in different amino acids were found in different positions. Multiple SNPs in the *NRAMP1* gene have been discovered in Sinai and LB, suggesting that this gene can be used as a genetic marker for the selection of high-producing indigenous hybrids with the ability to resist pathogenic diseases in poultry.

Keywords: Disease resistance, Lohman Brown, Sinai, Gene, Single Nucleotide Polymorphism

INTRODUCTION

Poultry plays a major role in the rural economy as a source of animal protein and as a contributor to income since its products are relatively inexpensive and widely available. In an attempt to increase the productivity of poultry, numerous breeding programs have been developed, and the current breeding programs that enhance poultry resistance to diseases have grown in popularity as an alternate strategy for increasing productivity while lowering production costs (FAO, 2013). The importance of native chickens for the rural economy and backyard farming is immense in some countries (Agarwal et al., 2020). Local chickens are important in contributing to the food security of rural households across developing countries. Indigenous chickens in Africa account for 80% of all chickens (Goodger et al., 2002).

The Sinai chicken breed was raised in the desert areas of the governorates of North and South Sinai (Gebriel et al., 2018). Local chicken farms often face low disease resistance (Pagala et al., 2013). The Sinai breed comes from the natural mating of some foreign breeds with local Egyptian chickens. In Egypt, this breed is used as egg-type chicken. Numerous studies have revealed that genetic factors significantly determine disease vulnerability and resistance in chickens (Saleh et al., 2020; 2021). Lohman Brown (LB) is a

commercial chicken breed that was created in Germany. It is known for its good egg-laying ability, high production rate, and the ability to adapt to a different environment.

Natural resistance-associated macrophage protein 1 (*NRAMP1*) is a protein that plays a crucial role in eliminating iron ions from macrophages. It is abundantly present in cells and exhibits upregulation following pathogen infections in various animal species (Soe-Lin et al., 2009; Tohidi et al., 2013). Numerous animal species have the functional candidate *NRAMP1* gene, which exhibits disease-resistance action (Lamont et al., 2002). The *NRAMP1* gene is linked to the characteristics of chicken immunity (Hu et al., 2011). On chicken chromosome 7, a homolog of *NRAMP1* has been located. It has a promoter region, 15 exons, 14 introns, and flanking regions that total 5760 bp in length (Desmond et al., 2019). Advances in molecular technology have created a new horizon for the genetic improvement of traits in poultry (Salah et al., 2021).

A single nucleotide polymorphism (SNP) is the most common type of genetic variation in the genome (Eichler et al., 2007). Two types of SNPs are found in coding sequences, synonymous (cause no changes to an amino acid) and nonsynonymous. Nonsynonymous SNPs are interesting because they might influence how proteins are expressed. Conversely, synonymous SNPs most likely have no impact on gene expression. For mapping investigations, synonymous and nonsynonymous SNPs are excellent genetic markers (Emara and Kim, 2003). According to Ardiyana et al. (2020), Newcastle disease antibody titers in SenSi-1 Agrinak chickens were substantially correlated with the TC genotype of the *NRAMP-1* gene. The present study aimed to screen SNPs within the *NRAMP1* gene of indigenous and exotic chicken strains. Additionally, immunological investigations between the two strains were also conducted.

MATERIALS AND METHODS

Ethical approval

Animal and Poultry Production Scientific and Ethics Committee, Faculty of Agriculture, Damanhour University, Egypt (DUFA-2020-11) authorized all experimental techniques.

Study animals

The study started in December 2020 until December 2021 at the Animal and Poultry Research Farm, El-Bostan, and genetic engineering laboratory, Damanhour University, Egypt. A total of 144 female day-old chicks, including 72 from the commercial layer strain (Lohman Brown) and 72 Egyptian native chickens from the Sinai strain, were used in this study. Chicks were wing banded at hatching and randomly divided into two groups with similar initial body weights in battery brooders (35 cm [L] × 25 cm [W] × 30 cm [H]) from day 1 to day 38 of age. There were 12 replicates per strain, with 6 chickens per replicate. Hitchiner B1 (LAPROVET, France) + Gumboro (Zoetis, USA), Influenza H5 N2 (Zoetis, USA), Colon30 (LAPROVET, France), Gumboro 123 (Zoetis, USA), Colon79 (LAPROVET, France), and Lasota (LAPROVET, France) vaccinations were given on days 7, 9, 10, 16, 20 and 30, respectively. A 23:1 light/dark cycle was used from day 2 until day 38 of the experiment, with the ambient temperature and relative humidity being 32.4 ± 4°C and 46.9 ± 6%, respectively, and distributed to battery brooders. Until 3 weeks of age, all chicks were fed a commercial diet containing 23% crude protein and 3040 kcal/kg of ME and were fed *ad libitum*. They were fed a diet with 21% crude protein and 3102 kcal/kg until they were 6 weeks old.

At 38 days of age, approximately 3 ml blood samples were taken randomly from the wing vein of 8 chickens from each strain. Two sterile centrifuge tubes, one with and one without EDTA as an anticoagulant, were used to collect the blood samples. A tube with EDTA was used to measure blood hematology, including white blood cell count (WBC), differentiation of WBC, red blood cell count (RBC), hemoglobin, packed cell volume (PCV), phagocytic activity (PA), and phagocytic index (PI). The tubes were centrifuged for 20 minutes at 3,000 rpm to clearly separate the serum from the plasma, and then the serum and plasma were kept at -20°C. Plasma total protein (g/100 ml) was measured according to Weichselbaum (1946). The albumin concentration (g/100 ml) was determined according to the method of Dumas et al. (1997). The globulin concentration (g/100 ml) was estimated by subtraction of the albumin concentration from the plasma total protein value according to Coles (1986). Serum immunoglobulin (IgY, IgM, and IgA) was determined as described previously by ELnaggar et al. (2016). Blood hematology (Table 2) was performed as indicated by Attia et al. (2014). According to Kawahara et al. (1991), phagocytic activity and phagocytic index were also determined. The hemagglutination inhibition (HI) test was used to detect serum antibodies against Newcastle disease virus (NDV) and avian influenza virus (AIV), according to King and Seal (1998) and Takátsy (1955), respectively. The antibody titer for the infectious bursal disease virus (IBDV) was determined using an infectious bursal disease virus antibody test kit (HIBD-001, China).

DNA amplification

A total of 20 chickens at 38 days old from each strain had blood drawn into sterile, EDTA-treated collecting tubes. Whole blood genomic DNA purification mini kit (Thermo Scientific, Gene JET, Germany) was used to isolate genomic DNA from whole blood. DNA samples were analyzed on a spectrophotometer (T80, UK) to determine their concentration and purity. For PCR experiments, approximately 100 ng of genomic DNA from the pool of each strain was used as a PCR template. 20 ul of distilled water, 10 ul of PCR master mix (Fast gene taq ready mix, lot No. LS 27-192708, Germany), and 1 ul of each primer (F, R) were used for the amplification. The PCR reactions were subjected to amplification using a thermal cycler (Bio-Rad, USA) under the following cycling conditions, an initial denaturation step at 95°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 66 °C for 30 seconds, and extension at 72 °C for 30 seconds. The ultimate elongation was executed at a temperature of 72 °C for 5 minutes. The electrophoretic analysis of the PCR product was conducted on a 1.5% agarose gel at 100 volts for a period of 35 minutes. The visualization of the product was accomplished through the utilization of a gel documentation system manufactured by Bio-Rad (Gel Doc EZ System, USA). Table 1 indicates the National Center for Biotechnology Information (NCBI) accession number, forward and reverse primers, as well as amplicon sizes utilized in the present study. Primers were designed using Primer-BLAST from the database sequence of GenBank accession. This primer was obtained from Invitrogen, Thermofisher Scientific, UK.

Table 1. The primer sequence used for amplification of the coding region of *NRAMP1* gene

Gene name	NCBI	Amplification size	Sequence
NRAMP1	AY072001	421 bp	Forward 5`CAATGAGACGGTGTCTGTGG3`
			Reverse 5` CCCAGAAGAAATCTCCCTGC3`

NCBI: National Center for Biotechnology Information; *NRAMP1*: Natural resistance-associated macrophage protein 1

PCR sequencing and processing

The PCR products were purified using a PCR purification kit (QIAquick, Qiagen, Germany). The sequence was carried out using a DNA automated sequencer. Many amplicons from the *NRAMP*1 gene were chosen and sequenced in one way (forward primer). The sequence results were analyzed using Chromas 1.45 software. The obtained sequences were aligned using Clustal Omega (Sievers et al., 2011). The amino acid translation was performed using EXPASY-Translate tools (Gasteiger et al., 2003).

Statistical analysis

The statistical analysis was performed using the general linear model (GLM) using SAS (2016). The statistical model used in this study was according to Formula 1.

$$Y_{ij}= \mu+S_i+e_{ij} \quad \text{(Formula 1)}$$

Where, Y_{ij} is the observation, μ denotes the general mean, S_i signifies the fixed effect of the strain *(i)*, and e_{ij} determines the random error. Tukey's test was utilized to evaluate the statistical significance between means at a significance level of p less than 0.05.

RESULTS

The data in Table 2 show the effect of strain on blood hematology in chicks. The results showed that the percentage of lymphocytes, the percentage of eosinophils, the percentage of heterophils, the ratio of heterophils to lymphocytes, and the phagocyte activity were all significantly affected by strain. The results showed that the lymphocyte ($p < 0.05$), eosinophil ($p<0.05$), and phagocyte activities ($p < 0.05$) of LB chicks were significantly greater than those of Sinai chicks. On the other hand, the heterophil-to-lymphocyte ratio of Sinai chicks was higher than those of LB chicks ($p < 0.05$). Table 3 shows the difference in blood biochemistry between the two strains. Sinai chicks had higher albumin and hemagglutination inhibition antibody against Newcastle disease virus (HINDV) compared to the Lohman strain. The opposite trend was observed for the immunoglobulin yolk (IgY) concentration, where the LB chicks had a significantly higher IgY concentration than the indigenous chicks. Meanwhile, there was no statistically significant difference between strains in hemagglutination inhibition antibody against avian influenza disease virus and hemagglutination inhibition antibody for infectious bursal disease virus.

***NRAMP1* gene polymorphism**

The *NRAMP1* gene fragment size after amplification was 421 bp. The *NRAMP1* gene was discovered to be nonpolymorphic across all chicken strains, and only one allele form was found. Sequence alignment of the *NRAMP1* gene between the Sinai and LB strains revealed the presence of repeated SNPs at various positions (Figure 1). The results reveal 58.68% identity for nucleotides between Sinai and LB chicks by Clustal Omega and 99% by NCBI in this gene, as shown in Table 4.

In the current study, eight SNPs were identified in *NRAMP1*. The translation of chicken *NRAMP1* sequencing in Sinai and LB is illustrated in Figure 2. At position 1bp nucleotide transition (A ↔ C), 6 bp (A ↔ G), 10 bp (T ↔ G), 11 bp (G ↔ A), and 12 bp (A ↔ G) were observed. Moreover, at position 13 bp, 14 bp (T ↔ G), and 162 bp (T ↔ C) were seen.

Present results revealed that the percent identity for amino acids were 88.30%. A change in amino acid number 84-85 from arginine to glycine (84-85 R > G) and amino acid number 86 from threonine to glycine (86 T > G), amino acid number 87 from serine to glutamic acid (87 R > G), amino acid number 88 from histidine to alanine (88 H > A), and amino acid number 89 from histidine to glycine (89 H > G). Moreover, a change in amino acid number 90 from aspartic acid to lysine (90 D > K), amino acid 91 from alanine to tryptophan (91 A > W), amino acid 92 from alanine to histidine (92 A > H), amino acid 93 from arginine to valine (93 A > V), and amino acid number 94 from alanine to arginine (94 A > R) are illustrated in Figure 3.

Table 2. Hematological parameters of Sinai and Lohman Brown chicks at 38 days of age in Egypt

Variable	Lohman Brown	Sinai	p-value	SEM
WBC (10^3/mm3)	19.50	20.57	0.107	0.454
Lymphocyte (%)	42.43[a]	40.50[b]	0.009	0.483
Monocyte (%)	11.79	11.21	0.189	0.299
Basophils (%)	0.714	0.357	0.061	0.226
Eosinophils (%)	11.57[a]	10.28[b]	0.002	0.258
Heterophils (%)	33.50[b]	37.64[a]	0.0001	0.608
Heterophil to lymphocyte ratio	0.789[b]	0.929[a]	0.0009	0.029
Phagocytic Activity	20.86[a]	19.71[b]	0.020	0.325
Phagocytic Index	1.730	1.650	0.285	0.051
RBC (10^6/mm3)	1.490	1.520	0.665	0.046
Hemoglobin (g/dl)	10.78	10.93	0.626	0.205
PCV (%)	32.00	32.21	0.796	0.580
MCH (pg)	73.25	72.65	0.876	2.690
MCHC (g/dl)	33.73	33.94	0.718	0.414
MCV (mm3)	217.7	214.1	0.758	8.230

SEM: Standard error of the mean, WBC: White blood cell, RBC: Red blood cell, PCV: Packed cell volume, MCH: Mean corpuscular hemoglobin, MCHC: Mean corpuscular hemoglobin concentration, MCV: Mean corpuscular volume. [ab] Means with different letters in a row differ significantly ($p < 0.05$).

Table 3. Effect of chicken strain on blood immunological characteristics in Egypt

Traits	Lohman Brown	Sinai	p-value	SEM
Total protein	5.58	5.74	0.187	0.082
Albumin	3.31[b]	3.53[a]	0.003	0.049
Globulin	2.28	2.21	0.6145	0.099
IgM	234.0	236.5	0.1181	1.09
IgY	977.1[a]	968.2[b]	0.002	1.79
HINDV	3.71[b]	4.21[a]	0.035	0.025
HIIBD	3.07	2.64	0.209	0.055
HIAI	1.50	1.71	0.452	0.039

SEM: Standard error of the mean, IgA: Immunoglobulin A, IgM: Immunoglobulin M, IgY: Immunoglobulin Y, HINDV: Hemagglutination inhibition antibody against Newcastle disease virus, HIIBD: Hemagglutination inhibition antibody against infectious bursa disease virus, HIAI: Hemagglutination inhibition antibody against avian influenza virus [ab] Means with different letters in a row differ significantly ($p < 0.05$).

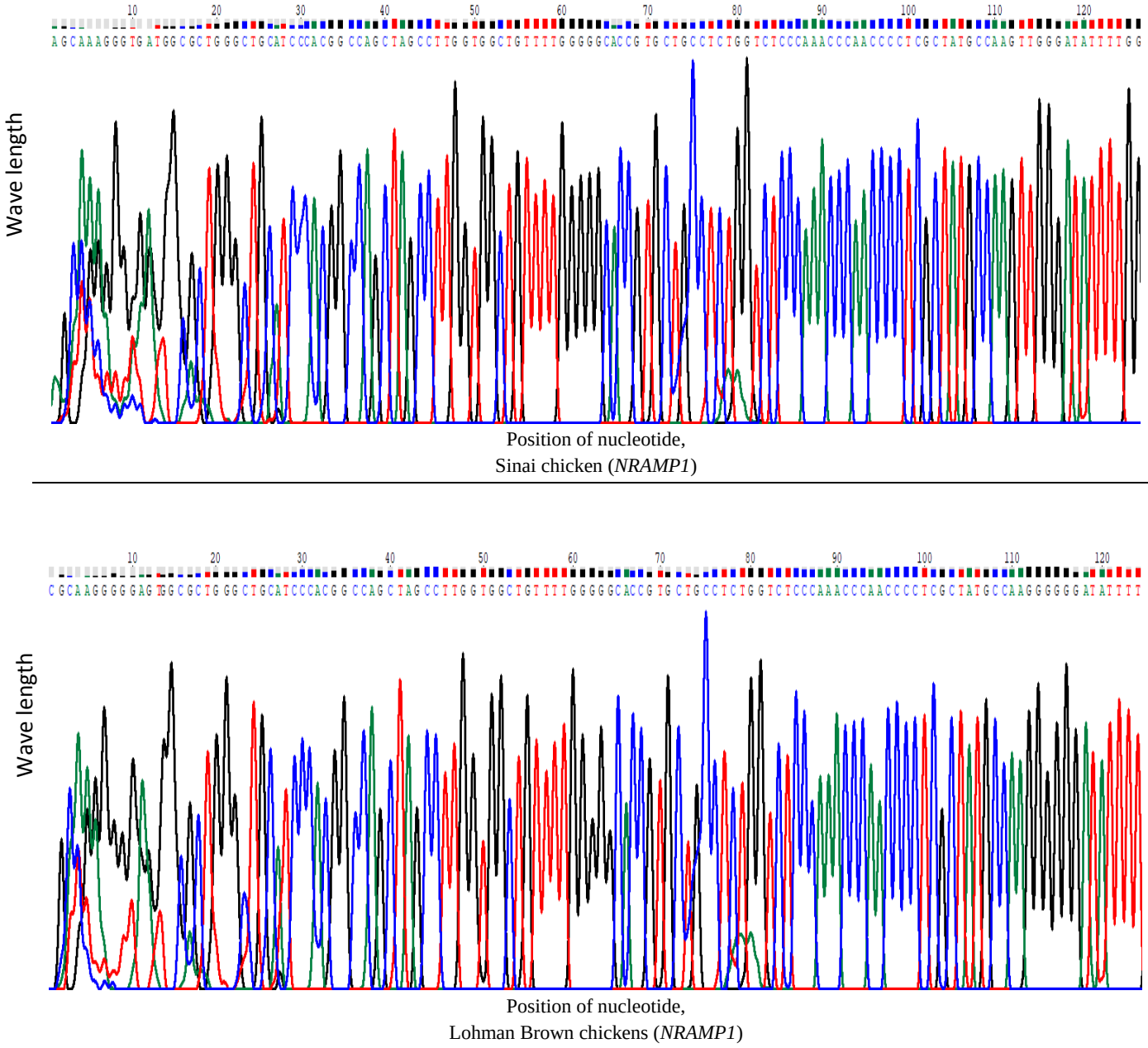

Figure 1. *NRAMP1* gene sequences collected from local Egyptian strain (Sinai) and Lohman Brown chickens in Egypt

Table 4. Percent identity matrix for nucleotide and amino acids in Siani and Lohman Brown chicks in Egypt

Strain	Nucleotide	Amino acids
sp|P69905|*NRAMP1*_Sinai	100.00 58.68	100.00 88.30
sp|P01942|*NRAMP1*_Lohman	58.68 100.00	88.30 100.00

Figure 2. Nucleotide alignment (bp) of *NRAMP1* for Sinai and Lohman Brown strains in Egypt

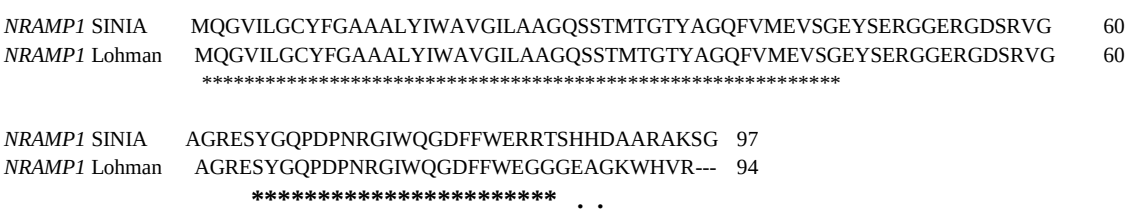

Figure 3. Amino acids alignment (bp) of *NRAMP1* for Sinai and Lohman strains in Egypt

DISCUSSION

In this study, commercial chicks (LB) had a higher lymphocyte and eosinophil percentage than the local chicks. This finding contrasts with the results of El-Safty (2012), who reported that local Libyan chicks had a higher lymphocyte percentage and lower heterophil percentage. According to Ferrante et al. (2016), heterophils increase while lymphocytes decrease when chickens are stressed. Therefore, the ratio of the two parameters is an indicator of stress and infection response. Heterophil and lymphocyte responses to stress have a genetic component (Minias, 2019), and their ratio has been employed as selection criteria for infection resistance in chickens (Thiam et al., 2021). The Egyptian native chicks (Sinai) had a significantly higher H/L ratio than the LB strain. This finding revealed that the Sinai chicks experienced higher stress levels than the LB strains under specific environmental conditions in Egypt. This could be attributed to the origins of the Sinai chicks, which resulted from the natural mating of certain foreign breeds with Egyptian indigenous chickens.

In terms of disease resistance, phagocytic activity is crucial. Chickens with a stronger phagocytic potential and nitrite generation from macrophages may be more resistant to bacterial, viral, and parasite diseases (Galal et al., 2007). It is worth noting that the genetic differences between LB and Egyptian native chicks may have resulted in differing phagocytic responses. Several studies have found that chicken phagocytic activity is genetically controlled (Kundu et al., 2015). The IgY is a maternal antibody passed from progeny through blood serum supplied to egg yolk (Murai, 2013). Both environment (maintenance management, disease exposure) and genetics influence chicken antibody production (Al-Habib et al., 2020). Wibawan et al. (2010) reported that exposure to a

bacterial disease increased IgY concentrations. Moreover, the concentration of IgY in crossbred chickens was higher than in local chickens (Setyawati et al., 2019; Al-Habib et al., 2020). According to the findings of this study, LB chicks had higher IgY concentrations than Sinai chicks under the same maintenance management. The LB chicks are hybrid chicks, and heterosis has a beneficial influence on the immunological response, as indicated by the quick increase in IgY concentration (Al-Habib et al., 2020). Genetics influence the production of Sinai's antibody, as this chicken strain has not been subjected to any artificial selection.

Newcastle disease is a viral disease produced by virulent strains of NDV that affect chickens worldwide (Oberländer et al., 2020). Depending on the viral strain and the host's sensitivity, the virus has a morbidity and death rate of up to 100% (Swayne and Boulianne, 2020). Furthermore, the performance of diseased flocks significantly declines, and infected animals' eggs become thin-shelled. Infections with lentogenic strains of the virus cause modest respiratory symptoms in chickens; however, infections with velogenic strains cause mucous membrane inflammation and central neurological problems in chickens, including torticollis and opisthotonos (Swayne and Boulianne, 2020). The Newcastle disease affects poultry all over the world and specific antibody titers can be used to assess the immunological response. Antibody titers are affected by various factors, including health, the severity of viral infection, and the period of infection (Oberländer et al., 2020). Antibodies are formed 6-10 days after Newcastle infection and reach their peak at 3-4 weeks, after which they decline for up to 3-4 months and are no longer detectable after a year (Anamu and Rohi, 2005).

The local Egyptian strain has a high potential for developing disease-resistant chickens (Kolstad and Abdou, 2000). The genetic potential of disease resistance in local chickens can still be developed using the antibody titer indicator as a selection marker. The selection program can be effectively conducted by implementing optimal vaccination strategies, enabling the identification of the chicken's response to diseases, and facilitating the selection of chickens with superior genetic traits. The superiority of local chickens that are more susceptible to disease agents provides advantages and facilitates genetic improvement in the quality of local Egyptian chickens.

Bioinformatics indicators for variation in allelic frequency at a given locus are provided by genetic markers. Due to the presence of molecular markers in poultry, it is now possible to conduct in-depth investigations and evaluations of genetic diversity and to identify genes impacting economically significant features (Khalil et al., 2021). The *NRAMP1* gene fragment amplified in both native and exotic chicken types were nonpolymorphic, indicating the presence of a single homozygous allele, and the same outcomes were found by Fulton et al. (2014).

The promoter region of the *NRAMP1* gene contains one major and two minor transcription initiation sites, a classical TATAA component, and consensus sequences for the binding of the myeloid-specific PU1 factor, many lipopolysaccharides (NF-IL6 and NF-ardB), and interferon-inducible response components (Marquet et al., 2000). Different genotypes of this gene have been reported to impact the antimicrobial function mediated by microglial cells (Mazzolla et al., 2002). Due to the genetic variability in the *NRAMP1* gene, Girard-Santosuosso et al. (2002) demonstrated that various chicken populations have varying heritability of susceptibility to infection. Tge SNP markers have already been used to identify disease resistance genes in chickens. Additionally, it can be used as an alternative for microsatellite markers in various study areas involving chickens (Emara and Kim, 2003; Malek and Lamont, 2003).

Polymorphism in the *NRAMP1* gene has been linked to a *Salmonella enteritidis* (SE) response in various strains of chickens (Lamont et al., 2002) although some line broiler sires with specific alleles of the *NRAMP1* gene demonstrated enhanced resistance to the infection. Muhsinin et al. (2016) found that genetic heterogeneity in the resistance to *Sallmonell purllorum* in Indonesian native chickens could be linked to a polymorphism in *NRAMP1* in chickens. They also mentioned several SNP variations between local and foreign strains, as well as some amino acid changes, that could have a substantial impact on disease resistance in these chickens. In poultry, different SNPs have been linked to various traits. These findings suggest that SNPs have an important role in gene expression and, as a result, the expression of the protein or phenotype (Khoa et al., 2013; Aboukila et al., 2021).

The observed genetic similarities between indigenous chickens and hybrid broiler sequences may indicate the presence of similar capacities to resist pathogenic infections distinct from laying hens, suggesting that the presence of this gene and nucleotide variability may be implicated as major genetic factors in congenital diseases. Tohidi et al. (2013) and Liu et al. (2003) reported associations between SNP polymorphisms in *NRAMP1* and pathogen challenge responses in Leghorn chicks,

showing that either *NRAMP1* or related genes control the *Salmonella enteritidis* response traits.

In the present study, the sequencing of the *NRAMP1* gene in chickens was applied to two strains of chickens. In the *NRAMP1* gene, several SNPs were found in chickens. Desmond et al. (2019) discovered six SNPs in *NRAMP1* that were linked to amino acid changes in Nigerian and foreign chicken breeds. In this study, eight SNPs in *NRAMP1* were associated with amino acid changes.

CONCLUSION

Significant immunological differences between LB and Sinai strains, including albumin, IgY and HINDV, lymphocyte, and phagocyte activity, were discovered in this investigation. The alterations suggest that LB hens have a more evident cellular immune system as well as a general stronger innate and humoral immune response. On the other hand, Sinai (local chickens) exhibited a higher level of dominance in terms of Newcastle antibody titer. As a result, more consideration should be given to Sinai native chickens and the use of various crossing and selection procedures to generate egg-type native chicken lines to improve their productive features while preserving and benefiting from their remarkable Newcastle disease resistance. In addition, multiple SNPs in the *NRAMP1* gene have been discovered in Sinai, a native chicken, and LB, suggesting that this gene might be useful as a genetic marker for selecting high-producing indigenous hybrids with the ability to resist pathogenic diseases in poultry. In addition, substantial research is needed in various poultry species to examine the effect of polymorphisms on gene expression and the molecular mechanism induced by polymorphisms within this gene.

DECLARATIONS

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Availability of data and materials

Data will be available on request.

Authors' contributions

Walid Habashy (ORCID ID: 0000-0002-2009-5145) performed conceptualization and study design, methodology, formal analysis, and data curation. Walid Habashy and Manal Abdel-Rahman performed writing original draft preparation, Walid Habashy and Kwaku Adomako performed writing reviewing and editing. The manuscript has been read and approved by all authors.

Competing interests

The authors state that there is no conflict of interest with any financial, personal, or other relationships with other people or organizations that are relevant the material discussed in the manuscript.

Ethical consideration

The authors have examined ethical issues, such as consent to publish, misbehavior, data fabrication and/or falsification, duplicate publication and/or submission, and redundancy.

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Effects of Using Commercial and Homemade Extenders on Sperm Quality of Liquid Stored Semen of Horro Chicken Breed

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ABSTRACT

This study aimed to evaluate the suitability of homemade tris-egg yolk-based and Commercial Beltsville poultry extenders for short-term storage of semen from the Ethiopian Indigenous Horro chicken breed at refrigeration temperature. A total of 30 Horro roosters with an average age of 40 weeks were used to collect semen. The treatments (T) in the sperm quality experiment were control (semen without extender added), semen extended with homemade extender (E1), and semen extended with commercial Beltsville Poultry Semen Extender (E2). Changes in spermatozoa motility, *in vitro* viability, and morphology were evaluated in fresh semen and semen diluted as 1:4 (v/v semen to extender) and stored for 4, 8, 12, and 24 hours at 4°C. During semen storage, there was a decrease in mass motility, an increase in morphologically abnormal spermatozoa with a high incidence of the bent tail, and an increase in dead spermatozoa. The commercial Beltsville poultry extender was found to be the most suitable extender regarding mass motility and *in vitro* viability of stored spermatozoa, but there was no significant difference in sperm abnormalities across all extenders. The results showed locally prepared tris-egg yolk-based extender could be a suitable extender for short-term storage of chicken sperm regarding the sperm quality attributes.

Keywords: Horro, *In vitro* viability, Motility, Morphology, Semen, Sperm

INTRODUCTION

Growing demand for poultry products and the high rearing cost of breeder stock necessitates the development of modern solutions to increase production efficiency at reducing costs. Artificial insemination is one of the solutions that significantly lowers the cost of rearing by decreasing the number of males in the flock (Łukaszewicz et al., 2020). Artificial insemination was the first great biotechnology applied to improve the reproduction of farm animals. It has an impact worldwide on many species of farm animals and endangered species (Foote, 2002). Artificial insemination technology laid the foundation for developing other reproductive technologies, such as cryopreservation and sexing of sperm, estrous cycle regulation, embryo harvesting, freezing, culture and transfer, and cloning. Artificial insemination in poultry grew significantly during the last few decades after the development of semen collection through abdominal massage (Siudzin´ska and Łukaszewicz, 2008). Artificial Insemination in poultry reproduction has caused investigators to become interested in studying the semen characteristics of different poultry breeds (Haunshi, et al., 2010).

One of the advantages of AI application in poultry is the efficient use of males. This, in turn, decreases the cost of breeding directly by reducing the number of roosters (Benoff et al., 1981). The increasing importance of AI in poultry reproduction has caused investigators to be interested in developing the proper conditions for liquid (short-term) semen storage (Lake, 1983). The possibility of dilution and storage of poultry semen would enable poultry breeders to use superior males and inseminate many females even on distant farms (Reddy, 1995). The most common procedure for short-term storage of semen requires suspending sperm in an extended to retain their viability *in vitro* (Reddy, 1995). A comparison of diluted and undiluted stored semen showed

that applying extenders is necessary to sustain good-quality sperm (Bilgili et al., 1987, Łukaszewicz et al., 2020). Studies have indicated that diluted poultry semen could be stored for up to 24 hours without impairing its viability and fertilizing ability (Soler et al., 2016; Silyukova et al., 2022). According to Gerzilov (2010), many factors could affect the quality of stored semen, such as the types of diluents, packaging, and cooling rates. The aim of this study was to determine the effect of two types of extenders on the qualitative characteristics of spermatozoa during short-term storage.

MATERIAL AND METHODS

Ethical approval

The present study followed institutional guidelines for humane animal treatment and complied with relevant legislation from Addis Ababa University College of Veterinary Medicine, Ethiopia.

Roosters' management

For the purpose of semen collection, thirty adult Horro roosters with an average age of 40 weeks were purchased from Debrezeit Agricultural Research Center, Bishoftu, Ethiopia. All experimental animals were managed at the poultry farm of the Debre Zeit Agricultural Research Center. The roosters were kept separately from the hens and trained for semen collection by abdominal massage technique for 2 weeks. The roosters were kept in a deep litter system and fed with a breeder ration containing 17% CP and 2800 Kcal/Kg energy (Table 1). Feed was provided twice a day with an amount of 110 gm/rooster/day, and water was provided *ad libitum*. All experimental chickens were dewormed and vaccinated for major diseases, including Newcastle, Marek's, Gumboro, fowl pox, and fowl typhoid (Table 2). The roosters were acclimatized for two weeks before sample collection.

Table 1. Breeder ration formula used during the experiment

Serial number	Feed ingredient	Inclusion rate (%)
1	Corn	52
2	Soy cake	10
3	Meat and bone meal	6
4	Wheat bran	15
5	Noug cake	9
6	Limestone	6
7	Breeder premix	0.5
8	Lysine	0.1
9	Methionine	0.1
10	Molasses	1
11	Salt	0.3

Breeder premix: Industrial, well-balanced premix that ensures fertile, hatching eggs and ultimately strong chicks. It contains vitamins and minerals.

Table 2. Vaccination schedule of Horro chickens in the present experiment

Age	Vaccine	Administration route
Day-1	Marek	Subcutaneous (neck)
Day-2	Newcastle disease	Eye drop
Day-7	Gumboro	Drinking water
Day-14	Newcastle (Lasota)	Drinking water
Day-18	Gumboro	Drinking water
Week-6	Newcastle (Lasota)	Drinking water
Week-8	Fowl typhoid	Injection
Week-9	Deworming	Drinking water
Week-10	Fowl pox	Wing stab
Week-14	Fowl typhoid	Injection

The vaccines originated from the National Veterinary Institute, Bishoftu, Ethiopia

Table 3. Contents of the homemade extender

Contents	Amount
Tris (base)	2.42 gm
Citric acid	1.48 gm
Fructose	4 gm
Egg-yolk	20 % v/v
Gentamicin	25 mg
Double distilled water	100 ml

pH was adjusted to 6.8

Extender preparation

The homemade extender used in this study was tris-egg-yolk-based. Semen diluents were prepared by mixing tris (base), citric acid, fructose, and egg yolk, into which an antibiotic was added. The ingredients of the extender were purchased from a local supplier. The composition of diluents is presented in Table 1. The second extender (E2) was the Beltsville commercial extender (P2-7450, continental, Delavan, WI, USA), a standard extender for the preservation of avian semen. Its composition was sodium glutamate (8.67 g/l), dipotassium phosphate (7.59 g/l), sodium acetate (3.2 g/l), fructose (5 g/l), potassium citrate (0.64 g/l), n-tris (hydroxymethyl) methyl 1-2 amino ethane sulfonic acid (TES; 3.2 g/l), monopotassium phosphate (0.7 g/l) and magnesium chloride (0.34 g/l). Osmolarity and pH were set at 310 mOsmol/kg and 7.1, respectively.

Semen collection and initial evaluation

Semen was collected using the Quinn and Burrows abdominal massage technique developed in 1936. The semen was collected with a sterile tube. Two ejaculates were collected from each rooster. The ejaculate volume varies from rooster to rooster, which averages 0.3 ml. The roosters were trained for semen collection following the two weeks of acclimatization. After collection, the semen was maintained in a water bath at 37°C and subjected to on-site pre-freeze evaluation, including volume, color, pH, sperm concentration

(bill/ml), motility (%), morphological abnormality (percentage of abnormal sperms) and live percent. Qualifying ejaculates having > 60% motility, > 70% live percent, and < 30% morphological abnormality were pooled to get sufficient semen for replication and further processing (Getachew et al., 2015).

Semen processing for liquid storage assessment

After pre-freeze evaluation for semen quality attributes, qualifying semen was pooled to get 15 ml of semen aliquots. The semen aliquots were divided into three groups with 5ml each and diluted at 37°C within 10 minutes with two pre-warmed extenders (homemade extender (E1), and commercial Beltsville extender (E2) at 1:4 ratio (v/v)). A 5 ml third un-extended semen portion was set as a control. Each treatment had 5 replications. Semen was diluted immediately after initial evaluation and stored at 4°C for 4, 8, 12, and 24 hours. All the semen quality assays were performed at 4, 8, 12, and 24 hours of storage (Silyukova et al., 2022).

Semen quality assays for liquid-stored semen

Semen was first evaluated for volume (ml), color, texture, and pH. The concentration (mil/ml and billion) was measured using a hemocytometer (Counting chamber, Muhwa, China), while motility (%), viability (%), and morphology (%) were evaluated under the light microscope (MSC-P200). An eosin-nigrosin stain was used to evaluate morphology at X1000 magnification under oil immersion. A total of 200 spermatozoa were counted to determine the percentage of abnormal sperms (Siudzin´ska and Łukaszewicz, 2008).

Statistical analysis

The data collected during the study period were subjected to Analysis of Variance (ANOVA) using STATA software (version 12). Means values were compared using LSD. A significance level of 5% was used to determine statistical significance when F-test was found significant ($p < 0.05$). Factorial 3*4 completely randomized design was utilized to evaluate the effect of storage time and types of extenders.

Table 4. Treatments and experimental layout for fresh semen quality assessment of Horro Breed

Type of extender \ Time of storage	4 hours	8 hours	12 hours	24 hours
0 (Control)	0 (H4)	0 (H8)	0 (H12)	0 (H24)
E1	E1 (H4)	E1 (H8)	E1 (H12)	E1 (H24)
E2	E2 (H4)	E2 (H8)	E2 (H12)	E2 (H24)

E1: Extender 1; E2: Extender 2; H: Hour

RESULTS

Fresh semen characteristics

A summary of the results of semen characteristics addressed in this study is presented in Table 5. The effect of semen extenders and storage time on sperm quality is presented in Table 6.

Effect of semen extenders and storage time on sperm quality

There were significant differences in sperm motility and *in vitro* viability across the interactions of storage time and a group of extenders ($p < 0.05$). No significant difference was observed in sperm morphological abnormalities across the interactions of storage time and extenders ($p < 0.05$). Significantly highest sperm motility and *in vitro* viability rate was observed in semen extended using a commercial extender at 4 hours of storage ($p < 0.05$). The percentages of live sperms in treatments were observed at 83.6% and 82.6% for commercial extender extended semen and locally prepared extender extended semen, respectively. Semen extended with E2 and E1 extenders was observed consistently higher motility, compared to the control extender, irrespective of storage time.

Effect of semen extenders on sperm quality

There were significant differences ($p < 0.05$) in sperm motility, morphological abnormalities, and *in vitro* viability between the control and the two extenders. However, there was no significant difference ($p < 0.05$) in all semen quality parameters between the commercial and locally prepared extenders. Significantly highest sperm motility, morphological abnormalities, and *in vitro* viability rates were observed in semen extended samples using commercial and locally prepared extenders when compared to the control treatment ($p < 0.05$).

Effect of interaction of semen extenders and storage time on sperm quality

There were significant differences in progressive sperm motility across all groups of treatments ($p < 0.05$). There were significant differences observed in sperm morphological abnormalities between all groups except the 12 and 24 hours of storage ($p < 0.05$). There were also significant differences ($p < 0.05$) in *in vitro* sperm viability across the durations of storage except for between 8 and 12 hours of storage. Significantly highest sperm motility, lower morphological abnormalities, and higher *in vitro* viability rates were observed in semen stored for 4 hours compared to other groups ($p < 0.05$).

Table 5. General semen characteristics of the Horro chicken breed

Semen characteristics	Mean semen characteristics
Ejaculate volume (ml)	0.36
Color	Milky white
Texture	Moderate viscous
Sperm total concentration/ml	$5.5X10^9$
Sperm count/ejaculate	$1.98X10^9$
Ph	7.2

Table 6. Effect of interaction of semen extenders and storage time on sperm quality of Horro chicken

Mean ± SE sperm parameters / Factor	Progressive motility (%)	Abnormality (%)	Viability (%)
Extender (storage time Significance) at $p < 0.05$	***	NS	***
Control (4 hours)	77 ± 2.54^a	15.4 ± 1.81	73.2 ± 1.39^b
Control (8 hours)	59 ± 1.87^b	16.2 ± 1.80	55.8 ± 1.66^c
Control (12 hours)	42 ± 1.22^c	25.4 ± 2.78	49 ± 1.22^c
Control (24 hours)	21 ± 1.00^d	27.2 ± 1.59	11.4 ± 1.21^d
E2 (4 hours)	87 ± 1.22^a	10.4 ± 0.51	83.6 ± 1.63^a
E2 (8 hours)	79 ± 1.00^a	15 ± 1.82	77.8 ± 1.28^a
E2 (12 hours)	50 ± 2.74^b	17.8 ± 1.62	68.4 ± 1.50^b
E2 (24 hours)	46 ± 1.87c	23 ± 2.30	51 ± 1.14^c
E1 (4 hours)	84 ± 1.00^a	12.2 ± 1.39	82.6 ± 1.36^a
E1 (8 hours)	72 ± 1.22^a	16.6 ± 1.57	73.8 ± 1.93^b
E1 (12 hours)	49 ± 1.87^b	19.8 ± 2.08	66.4 ± 1.50^b
E1 (24 hours)	45 ± 1.58^c	25.4 ± 1.21	46.4 ± 1.44^c

E1: Extender 1; E2: Extender 2; NS: Non-significant; SE: standard error; [abcd] Different letters within the same row show significant differences among the groups ($p < 0.05$).

Table 7. Effect of semen extenders on sperm quality of Horro chicken

Mean ± SE sperm parameters / Factor	Progressive motility (%)	Abnormality (%)	Viability (%)
Control	49.75 ± 4.82^b	21.05 ± 1.54^b	47.35 ± 5.21^b
E1	65.25 ± 4.22^a	16.55 ± 1.30^a	70.2 ± 2.90^a
E2	62.75 ± 3.71^a	18.5 ± 1.32^a	67.3 ± 3.15^a

E1: Extender-1; E2: Extender-2; SE: Standard error; [abc] Different letters within the same row show significant differences among the groups ($p < 0.05$).

Table 8. Effect of storage time on sperm quality of Horro chickens

Mean ± SE sperm parameters / Factor	Progressive motility (%)	Abnormality (%)	Viability (%)
4 hours	82.67 ± 1.45^a	12.67 ± 0.91^a	79.8 ± 1.48^a
8 hours	70 ± 2.34^b	15.93 ± 0.95^b	69.13 ± 2.70^b
12 hours	47 ± 1.44^c	21 ± 1.46^c	61.26 ± 2.45^b
24 hours	37.33 ± 3.19^d	25.2 ± 1.05^c	36.26 ± 4.77^c

SE: Standard error; [abcd] Different letters within the same row show significant differences among the groups ($p < 0.05$).

DISCUSSION

Semen color depend on the species of chicken used; however, generally, present findings of milky white semen were in agreement with previous reports by Peters et al. (2008) and Mussa et al. (2023). The color of domestic fowl semen varies from a dense opaque suspension to a watery fluid secreted by various reproductive glands, from a relatively high sperm density or degrees of clear to milky white, with declining sperm numbers (Hafez and Hafez, 2000). According to Yadav et al. (2019), the color of semen may depend on the species of chicken used, but generally semen should be creamy which indicates a high sperm concentration which is in agreement with the current study. Color could also serve as an indicator of contamination (Yadav et al., 2019).

According to Peters et al. (2008), the average ejaculate volume of semen from chicken using the

abdominal massage technique was 0.01 ml to 0.35 ml in Giriraja, Frizzled feathered chicken. Bah et al. (2001) also reported an ejaculate volume of 0.28 ml in Nigerian local cocks. Cole and Cupps (1977) reported ejaculate volume within the range of 0.1 ml to 1.5 ml per ejaculation. On the other hand, Hafez and Hafez (2000) indicated that the average sperm volume collected from white leghorn varies from 0.2 to 0.5 ml. These studies are in agreement with the result found in this study on Ethipian Horro Chicken which is 0.36 ml/ejaculate.

The average sperm concentration in the present study was $5.5X10^9$/ml. Results from Antalan et al. (2015); AL-Saeedi et al. (2019) showed that, the concentration of ranging 3.4 to $6.8X10^9$/ml in Lohmann Brown cocks. According to Gordon (2005) reported the average sperm concentration of poultry semen was $5000X10^6$ sperm/ml. On the other hand, the sperm concentration recorded from the present study was within the range of a report by Hafez and Hafez (2000), which is $3000\text{-}7000X10^6$ spermatozoa/ml. The average pH of the semen collected was slightly alkaline and ranges from 7.2-7.5. Alkalinity of the poultry semen is due to the accessory sex gland fluid is generally alkaline as reported by Bah et al. (2001) and Peters et al. (2008). Results from Hafez and Hafez (2000), Gordon (2005), Antalan et al. (2015), and AL-Saeedi et al. (2019) are all within the range of the current study.

The results from the present study demonstrated the effect of a Glycerolized tris-egg-yolk-based extender on the Ethiopian indigenous Horro chicken breed's semen sperm motility, morphology, and *in vitro* viability. Results in this study showed that semen stored in a Glycerolized tris-egg-yolk-based extender has sperm motility that is fit for insemination. The current result of sperm motility agrees with a similar study by AL- Saeedi et al. (2019) which utilized a Tris-based extender for short-term storage of Lohmann brown breeders. As reported by Ponglowhapan et al. (2004) motility is an important indicator of sugar utilization by spermatozoa as sugars serve as an external energy source essential for maintaining motility. This study demonstrated that semen extended with a commercial extender and stored at 4 hours produced higher sperm motility (87 ± 1.22 %). In this study, the overall average sperm motility was 59.25%, which was in general agreement with 42-80% reported by Hafez and Hafez (2000).

In this study, extending semen with a commercial extender and storing it for 4 hours yielded the least sperm abnormalities (10.4 ± 0.51%). Whereas, the average sperm morphological abnormality semen stored using a Glycerolized tris-egg-yolk-based extender was 18.5%. The number of live sperm with abnormalities in fresh cockerel semen varied from 6 to 9 percent (Tselutin et al., 1999), which was lower than the results of this study. However, Tuncer et al. (2006) reported that the number of abnormal sperm in cockerel semen varied from 9.2 to 11.23%, which is in agreement with sperm abnormalities recorded in semen extended using a commercial extender.

A commercial extender at 4 hours of storage was the best combination (83.6 ± 1.63%) for better *in vitro* sperm viability as compared to other treatments. Sperm *in vitro* viability recorded using LPE at 4 hours of storage was slightly lower than that of commercial extenders (82.6 ± 1.36%). The LPE improved the longevity of sperm in this study as Bearden et al. (2004) reported "presence of fructose will not greatly change the metabolic rate, however, will extend the life span of the sperm". According to the report by Gebriel et al. (2009), 81.79% of sperm *in vitro* viability was recorded at 6 hours of storage, which was a similar to results of the present study. In this study, the percentage of dead sperm increased by 36.2% over 24 hours of storage for semen extended with LPE and which was positively correlated with the storage time. In general, the results of sperm quality attributes observed in this study are comparable to several studies (Lukaszewicz et al., 2008; AL- Saeedi et al., 2019).

CONCLUSION

In this study, tris-egg-yolk-based LPE yielded comparable results in all sperm quality attributes when compared with commercial Beltsville Poultry Semen extender. Semen stored for more than 12 hours at refrigeration temperature showed significantly lower sperm quality. Semen stored using a commercial extender for 4 hours was recorded with a higher level of sperm quality. Further studies are recommended to explore the possible ways to store poultry semen for 24 hours at refrigeration temperature without decreased sperm quality significantly.

DECLARATION

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Authors' contributions

Tarekegn Getachew, Gebeyehu Goshu and Alemayehu Lemma designed the experiments and

Tarekegn Getachew performed the experiments. Tarekegn Getachew derived the models and analyzed the data. Gebeyehu Goshu and Alemayehu Lemma assisted with standardizing data collection and data analysis. Tarekegn Getachew wrote the manuscript in consultation with Gebeyehu Goshu and Alemayehu Lemma. All authors read and approved the final version of the manuscript for publishing in the present journal.

Competing interests

The authors have declared that no competing interest exists.

Ethical consideration

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

Availability of data and materials

The data that support the findings of this study are available from the corresponding author, T. Getachew, upon reasonable request.

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Comparative Analysis of Contract Farming Effect on Technical Efficiency of Broiler Chicken Farms in Indonesia

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ABSTRACT

The development of broiler chicken farms in Indonesia has taken two forms, namely non-contract and contract farming. This study aimed to compare the technical efficiency levels of production in these two types of farming in Banten Province, Indonesia. Data were collected randomly from 180 broiler chicken farmers, consisting of 103 non-contract and 77 contract farmers. The study used the stochastic frontier production function to meet its objectives. The results showed that non-contract broiler chicken farmers were less efficient in their production than those under contract. The mean technical efficiency of the production factor for non-contract broiler chicken farmers was 0.689, ranging from 0.339 to 0.996. On the contrary, broiler chicken farmers under contract had a higher mean efficiency value of 0.893, ranging from 0.638 to 0.988. Moreover, the type of input supplier had a significant positive effect on technical inefficiency in non-contract farms. Non-contract farmers who purchased their production needs from a poultry shop showed higher technical efficiency compared to those who used distributors. This research sheds light on the efficiency of broiler chicken farms, both non-contract and contract, enabling all stakeholders, including the government, to devise appropriate policies for the development of broiler chicken farming. The study provided valuable insights into the technical efficiency levels of broiler chicken farming in Indonesia, which can help farmers identify areas that need improvement and develop strategies to increase productivity and profitability.

Keywords: Broiler farm, Contract farming, Input suppliers, Stochastic frontier, Technical efficiency

INTRODUCTION

Contract farming has been proposed as an instrument to enhance farmer welfare. Contract farming is assessed to solve farmers' classic problems, such as capital limitation, technology mastery limitation, low productivity, and marketing problems. Many studies showed evidence that contract farming could increase farmer income and welfare (Otsuka et al., 2016; Ton et al., 2017; Bellemare and Bloem, 2018) as well as their productivity (Reardon et al., 2009; Bellemare, 2010; Mishra et al., 2022). Most empirical studies about contract farming have indicated a positive and significant effect on farmer income (Otsuka et al., 2016). Contract farming might also increase farmer opportunities to adopt technology and invest more in technology (Mao et al., 2019).

Smallholder farming is a high-risk and vulnerable sector, susceptible to fluctuations in commercial broiler

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chicken farming. Smallholder farmers are considered the weakest and most vulnerable group in the commercial broiler chicken business, compared to integrators and semi-integrators (Pambudy, 2013). This vulnerability is due to several factors, including limited knowledge and financial capacity to implement business management, intensive technology, and high biosecurity, smallholder farmers' heavy reliance on obtaining quality inputs, particularly day-old chicks, feed, and medicines; and their exposure to price fluctuations as price takers for both input and live chicken sale prices. These risk factors significantly affect the sustainability of smallholder farming businesses (Pambudy, 2013).

In the risky commercial broiler chicken farming environment, farmers are motivated to manage their farming activities efficiently. Efficient farming activities are closely related to decision-makers' ability to allocate resources efficiently to achieve maximum results (Ellis, 1993). One way to enhance efficiency and expand the scale of commercial broiler chicken farming is through contract farming, which presents a greater likelihood of increasing the scale of smallholder farming businesses (Wakhidati et al., 2018). According to Key and Runsten (1999), contract farming offers several advantages, including facilitating access to markets, credit, and technology, better risk management, and improved job opportunities for farmers. At the same time, core companies can benefit from reduced investment costs and can concentrate on gaining entry into modern and global markets.

The implementation of contract farming in broiler chicken agribusiness has existed for the last three decades alongside non-contract broiler chicken farms. Non-contract broiler chicken farmers are independent farmers who purchase production factors (inputs) and freely sell their output in the market. Production factor is an economic concept that refers to the inputs needed to produce goods and services. The purchase of production factors (inputs) is usually through production agencies, such as distributors, poultry shops, or other non-contract farmers. A contract farmer is a farmer who partners with other parties as a core company, such as an integrator or poultry shop, based on an agreement. In practice, broiler chicken contract farmers can be classified into three types, including profit-sharing contract farmers, makloon" contract farmers, and forward contract farmers. The first group involves smallholder farmers who buy inputs from the core company and sell the output to the core company or other parties with a core company permit, and the sale result is divided according to the agreement. "Makloon" contract farmers are paid by the core company according to the number of day-old chicks (DOC) when starting the production, which is known as the management fee system. Forward contract farmers conduct production according to the contract agreement with the core company and sell the output to the core company with a fixed price stated in the contract (Amam et al., 2019; Indrawan et al., 2020).

Broiler chicken farming is mostly carried out by smallholder broiler chicken farmers in Indonesia. Smallholder farming in Ethiopia generally faces production efficiency problems (Yami et al., 2013; Mezgebo et al., 2021). Production efficiency is an important factor for broiler chicken farmers to increase their productivity, and it depends on how production factors are allocated. The present study aimed to compare the technical efficiency levels of non-contract and contract broiler chicken farming in Banten Province, Indonesia, to shed light on the potential benefits and drawbacks of each approach.

MATERIALS AND METHODS

Ethical approval

This research has been proposed and approved to be carried out by the Faculty of Agriculture, Universitas Gadjah Mada, Yogyakarta, Indonesia.

Study design

This study was carried out in Banten Province, Indonesia, using a multistage sampling method. The research location was determined purposively based on the areas that are known for broiler chicken production. The selected regencies were in Tangerang Regency and Serang. The sub-districts in Tangerang Regency were Pakuhaji, Teluknaga, Kemiri, Rajeg, and Cisoka (BPS-Statistics Tangerang Regency, 2014), and Serang Regency was Kopo, Jawilan, Cikande, Pamarayan, and Cinangka (BPS-Statistics Serang Regency, 2014).

The data collection for this study was carried out between May and September of 2021, and the sample population included 324 smallholder broiler chicken farmers, comprising 185 non-contract farmers and 139 contract farmers, from 10 sub-districts. The sample size of 180 farmers was determined using the Slovin technique, and the sample was allocated proportionately to both non-contract and contract farmers, with 103 non-contract farmers and 77 contract farmers being selected. Respondents were randomly selected from each sub-district based on the number of farmers. The data were

collected through direct interviews using a questionnaire and only one production period was considered for this cross-sectional study.

The data collected for broiler chicken farming pertained to both production factors (input) and production (output). Production data was measured in kilograms and represented the quantity of live birds harvested during a production period. Production factor data (input) included the number of day-old chicks (DOC), amount of starter and finisher feed, medicine and vitamins, vaccine, number of workers, and the broiler house area, all measured during a production period, and respectively expressed in head, kilograms, grams, milliliters, days, and square meters. Additionally, data was collected on the selling price of live birds per kg and the price of each input. Socio-economic data was also collected, including information on the farmers' age, education, experience, type of job, and input suppliers for non-contract farmers. For contract farmers, data was gathered on the type of integrator company they partnered with, whether it was an integrator or a poultry shop, along with details on the contract form (written or oral) and the contract type (forward contract or profit sharing/management fee).

Statistical analysis

This study employed stochastic frontier analysis using the Cobb-Douglas production function, which was transformed into natural logarithm form and estimated using Maximum Likelihood Estimation (MLE). The Frontier version 4.1 software was used for data processing. Stochastic frontier Cobb-Douglas production function for non-contract broiler chicken farmers and contract farmers were followed as Formula 1:

$$LnY = \alpha_0 + \alpha_1 LnX_1 + \alpha_2 LnX_2 + \alpha_3 LnX_3 + \alpha_4 LnX_4 + \alpha_5 LnX_5 + \alpha_6 LnX_6 + \alpha_7 LnX_7 + (v_i - u_1)$$

(Formula 1)

Where Y, is the output of broiler chicken produced in one period (kg), X_1 denotes the number of day-old chicks in one period (head), X_2 determines the number of starter feed in one period (kg), X_3 signifies the number of finisher feed in one period (kg), X_4 refers to the number of medicines and vitamins in one period (g), X_5 tabulates the number of vaccines in one period (ml), X_6 is the number of labor in one period (days), X_7 stands for the broiler chicken house area (m^2), α_0 presents constant, $\alpha_1 - \alpha_7$ are estimated parameters, $(v_i - u_1)$ is error term (v_i stands for disturbing effect and u_1 determines inefficiency effect). The model is used to estimate the technical efficiency levels of broiler chicken farmers, with the aim of identifying factors that contribute to differences in technical efficiency levels between contract and non-contract farmers.

The analysis of regression coefficients was used to assess the impact of input variables (x) on production. A positive regression coefficient indicates a positive effect of the variable x on production, while a negative regression coefficient implies a negative effect of x on production. The p-value reflects the probability that the observed effect of the variable x on production is due to chance. A p-value less than the significance levels that were $p < 0.05$ indicated that the impact of x on production was statistically significant, and $p < 0.01$ was highly significant.

Analysis of technical efficiency (TE) of broiler chicken farms used the method developed by Coelli et al. (1998). It was obtained from the ratio of the observation output of farmer-ith (Y_i) to the frontier output (Y_i^*), which was followed as Formula 2:

$$TE_i = \frac{Y_i}{Y_i^*} = \frac{Y_i}{exp(X_i\beta)} = \frac{\exp(X,\beta,-u_i)}{exp(X_i\beta)} = \exp(-u_i)$$

(Formula 2)

Where, TE_i denotes technical efficiency of farmer-ith, Y_i is output observed of farmer-ith, Y_i^* signifies output frontier estimated, $\exp(-u_i)$ is expected mean of inefficiency effect (u_i). Evaluation of technical efficiency value using the Criteria of 0 and 1. A technical efficiency value of 1 indicates that the farmer produces an optimal output and uses inputs efficiently. Conversely, a value of 0 suggests that the farmer is not achieving optimal output. For values of technical efficiency less than 1, it implies that the farmer has room for improvement in their production process. A comparative analysis was performed to determine the technical efficiency values of contract and non-contract farming. A two-sample mean-comparison test was employed to conduct the analysis, and the statistical software Stata version 12 was used.

Furthermore, an analysis of technical inefficiency effects and the factors that influence technical inefficiency was carried out using the technical inefficiency effect model from Coelli et al. (1998). The technical inefficiency effect model for non-contract and contract farms were followed as Formula 3:

$$u_i = \beta_0 + \beta_1 Z_1 + \beta_2 Z_2 + \beta_3 Z_3 + \beta_4 Z_4 + \beta_5 Z_5$$

(Formula 3)

And the technical inefficiency effect model was followed as Formula 4:

$$u_i = \beta_0 + \beta_1 Z_1 + \beta_2 Z_2 + \beta_3 Z_3 + \beta_4 Z_4 + \beta_6 Z_6 + \beta_7 Z_7 + \beta_8 Z_8$$

(Formula 4)

Where u_i is the technical inefficiency effect, Z_1 determines age of the farmer (years), Z_2 presents education level (years), Z_3 denotes the farming experience (years), Z_4 is dummy variable of job type (1 = main job, 0 = side job), Z_5 is a dummy variable of input suppliers, for non-contract farms only (1 = integrator, 0 = poultry shop/other farmers), Z_6 is dummy variable of core company type (1 = integrator, 0 = poultry shop/other farmer), $Z_7$is dummy variable of contract form (1= written, 0 = oral), Z_8 presents dummy variable of contract type (1 = forward contract, 0 = profit sharing/management fee), β_0 signifies constant, $\beta_1 - \beta_8$ is an estimated parameter. Dummy variable is a binary variable that takes the values 0 or 1 to indicate the absence or presence of some categorical effect that may be expected to shift the outcome. The coefficients estimated for each explanatory variable would indicate the direction and strength of their impact on technical inefficiency. A statistically significant coefficient indicates evidence suggesting that the explanatory variable has a non-zero effect on technical inefficiency.

RESULTS AND DISCUSSION

Average cost production

The average production cost of broiler chicken farms for both non-contract and contract farms is shown in Table 1. The majority of broiler chicken production costs for both types of farmers was the cost of feed, which was more than 60% of the total production cost. The cost of feed for contract farmers was higher than for non-contract farmers. However, other variable production costs for contract farmers were lower, except for fuel, electricity, and litter.

Table 1. Average production cost of non-contract and contract broiler chicken farms in Banten Province, Indonesia

Variables	Unit	Non-contract farms (n=103)		Contract farms (n=77)	
		Total cost	Percentage	Total cost	Percentage
DOC	IDR	24,542,143.69	30.47	93,268,571.43	26.10
Starter Feed	IDR	26,784,500.00	33.26	72,823,431.82	20.38
Finisher Feed	IDR	23,361,116.50	29.01	169,012,116.88	47.29
Medicine and vitamin	IDR	636,533.98	0.79	1,095,137.66	0.31
Vaccine	IDR	665,756.10	0.83	572,796.43	0.16
Labor	IDR	2,019,478.65	2.51	6,009,713.94	1.68
Fuel, electricity, litter	IDR	1,840,744.34	2.29	11,326,466.91	3.17
Depreciation of broiler chicken house	IDR	475,383.94	0.59	1,988,538.45	0.56
Depreciation of equipment	IDR	208,371.87	0.26	1,283,700.16	0.36
Total cost	IDR	80,534,029.07	100.00	357,380,473.68	100.00

Source: Primary data analysis (2021), n: Number of samples, DOC: Day old chick

Descriptive statistics of production and inputs

Table 2 presents the descriptive statistics of the variables used to analyze stochastic frontier Cobb-Douglas production. The results showed that contract farmers employed significantly higher amounts of production inputs than non-contract farmers, which resulted in higher levels of output for contract farmers (19,440.77 kg) than non-contract farmers (3,929.92 kg). On average, both groups of farmers were above 40 years of age, had 9 years of education, and possessed extensive experience in broiler chicken farming (average of 9 and 11 years for non-contract and contract farmers, respectively). Additionally, broiler chicken farming was the main occupation for both groups of farmers. Most non-contract farmers obtained their production inputs from distributors, while the majority of contract farmers cooperated with integrators and operated under written contracts with a profit-sharing/management fee. These findings provide valuable insights into the differences in production practices between contract and non-contract broiler chicken farmers and underscore the importance of examining the impact of these differences on technical efficiency levels.

Table 2. Descriptive statistics of the variables used in Stochastic Frontier Cobb-Douglas production function for non-contract and contract broiler chicken farms in Banten Province, Indonesia

Variables	Unit	Non-contract farms (n = 103)		Contract farms (n = 77)	
		Mean	SD	Mean	SD
Production	kg	3929.92	7940.64	19440.77	50226.27
DOC	Heads	3165.70	6377.21	11832.47	24614.03
Starter Feed	kg	3330.26	4980.87	8545.70	16657.57
Finisher Feed	kg	3020.29	9981.15	20323.61	62946.18
Medicine and vitamin (g)	g	980.49	1907.36	3586.88	8451.14
Vaccine	ml	210.63	636.63	1515.71	6428.12
Labor	Days	21.19	33.83	81.45	201.82
Broiler chicken house area	m^2	347.58	533.32	1023.23	1730.94
Age	Years	41.79	10.10	45.94	10.65
Education	Years	9.08	3.75	9.77	3.20
Experience	Years	9.93	5.96	11.19	6.48
Type of Job (1 = main job, 0 = side job)	Dummy	0.60	0.49	0.69	0.47
Input suppliers (1 = distributor, 0 = poultry shop)	Dummy	0.43	0.50	-	-
Core company (1 = integrator, 0 = poultry shop/other farmer)	Dummy	-	-	0.58	0.49
Contract form (1 = written, 0 = oral))	Dummy	-	-	0.60	0.49
Contract type (1 = forward contract, 0 = profit sharing/management fee)	Dummy	-	-	0.43	0.50

Source: Primary data analysis (2021), n: Number of samples, DOC: Day old chick, SD: Standard deviation

The estimation of the stochastic frontier production function

The production function consists of two types of variables, namely independent and dependent variables. The study included several independent variables, namely the number of DOC, starter feed, finisher feed, medicine and vitamins, vaccines, labor, and broiler chicken house area. The dependent variable was the output of the broiler chicken produced. The MLE results of the stochastic frontier Cobb-Douglas production function are presented in Table 3.

The findings revealed that DOC was a significant input for non-contract and contract farmers ($p < 0.01$) and estimated elasticities of 0.9061% and 1.0170%, respectively, indicating its significant impact on broiler chicken production. Similarly, starter and the finisher feeds were significant ($p < 0.05$) for non-contract farmers, with elasticities of 0.0576 Kg and 0.0176 Kg, respectively. For contract farmers, finisher feed and vaccines were significant with elasticities of 0.0183% and 0.0163%, respectively ($p < 0.05$). Increasing the use of these inputs has a positive and significant impact on broiler chicken production.

For non-contract and contract farmers, DOC costs contributed 30.62% and 26.12% to production costs, respectively. The results of this study were in line with the research by Harianto et al. (2019) and Ullah et al. (2019). Ullah (2019) found the estimated coefficient of the amount of DOC was statistically significant. Harianto et al. (2019) also stated that DOC was the major driver for broiler chicken production.

The study also highlighted the significance of starter feed and finisher feed for non-contract farmers ($p < 0.05$). The use of these inputs could lead to a significant increase in production. Previous studies have also reported similar findings, demonstrating the positive effect of feed on production (Udoh and Etim, 2009; Pramita et al., 2018; Wantasen et al., 2021). The present study provides further evidence supporting the importance of feed as a significant input in the production process for both non-contract and contract farmers. The findings underscore the need for farmers to exercise meticulous control over their feed inputs to maximize their production output. In the other hand, starter feed and finisher feed were not significant input for contract farmers ($p > 0.05$). This is due to the fact that feed usage in contract-based broiler chicken farming is determined by the core company.

The feed was categorized into starter and finisher with regard to the type. Starter feed is the type of feed given to the broiler chicken at the age of 2-4 weeks. In contrast, finisher feed is given to the broiler chicken at 4-6 weeks. Production cost for these types of feeds accounted for more than 60% of the total cost of production. The same result was shown by Adeyonu and Odozi (2022),

indicating feeding is the primary factor responsible for elevating the broiler chicken production costs by approximately 75% of the variable cost.

Vaccination is a commonly used method by farmers to enhance the immunity of broiler chickens and reduce their susceptibility to diseases. The current results indicated that vaccines significantly and positively affected the production of contract farmers. These positive and significant coefficients suggested that increasing the use of vaccines could lead to higher broiler chicken production ($p < 0.05$). This finding is consistent with previous research by Harianto et al. (2019), indicating the positive effect of vaccines on broiler chicken production. In contrast, the study revealed that the coefficient for the use of vaccines was negative and statistically non-significant for non-contract farmers. This suggests that the quantity of vaccines used by non-contract farmers was consistent across all farmers, as observed in previous research by Ullah et al. (2019).

This study was designed to estimate the value of the gamma parameter (ɣ) in non-contract and contract broiler chicken farms. The estimated value of ɣ in non-contract farms was 0.9878, which was statistically significant ($p < 0.01$). This indicates that 99.99% of the residual variation in the model was due to technical inefficiencies that farmers can control, while the remaining 0.01% was due to stochastic effects (v_i). The high value of ɣ implies that non-contract farmers had a high degree of control over their production processes, and that any inefficiencies can be mitigated by improvements in technical efficiency. On the other hand, the estimated value of ɣ in contract broiler chicken farms was 0.3777%, which was not significant at $p < 0.01$ but statistically significant at $p < 0.05$. This implies that 37.77% of the residual variation in the model was due to technical inefficiencies that farmers can control, while the remaining 62.23% was due to stochastic effects (v_i). The lower value of ɣ in contract farms compared to non-contract farms suggests that farmers in contract farms have less control over their production processes, and are more vulnerable to external factors that affect production efficiency. Overall, the estimated values of ɣ in both non-contract and contract broiler chicken farms suggest that there is room for improvement in the technical efficiency of broiler chicken production.

The Likelihood Ratio test values for non-contract and contract farms were 19.1553 and 27.3582, respectively. Both values were greater than the critical value of χ^2 at $\alpha = 0.01$, as presented in Kodde and Palm's table (1986), which was 17.755 and 20.972, respectively. The LR test values were highly significant at $p < 0.01$, suggesting that technical efficiency and technical inefficiency factors significantly impact broiler chicken production. This implies that the combined inefficiency variables in the inefficiency effect model contribute to technical inefficiency in the broiler chicken production process.

Table 3. Estimates of the stochastic frontier production function of non-contract and contract broiler chicken farms in Banten Province, Indonesia

Variables	Unit	Non-contract farms (n=103)		Contract farms (n=77)	
		Coefficient	T-ratio	Coefficient	T-ratio
Constant	Kg	0.6949**	2.5383	-0.0234	-0.1132
DOC	Heads	0.9061***	19.1075	1.0170***	17.2030
Starter Feed	Kg	0.0576**	2.2752	0.0347	1.0040
Finisher Feed	Kg	0.0176**	2.1055	0.0183	1.7578
Medicine and vitamin	g	-0.0117	1.1938	-0.0149	-0.6845
Vaccine	ml	-0.0030	-0.3947	0.0163**	2.0693
Labor	Days	0.0503	1.1690	-0.0110	-0.3078
Broiler chicken house Area	m^2	0.0056	0.1520	-0.0067	-0.1359
Sigma-squared		0.0327***	7.2688	0.0362***	3.9777
Gamma		0.9999***	71.2554	0.3777**	2.2774
Log likelihood function		33.7219		28.7530	
LR test		19.1553***		27.3582***	

Source: Primary data analysis (2021), ** $p < 0.05$, *** $p < 0.01$, n: Number of samples, LR test: Likelihood ratio test

The value of the technical efficiency of broiler chicken farmers is presented in Table 4. The technical efficiency value of non-contracted broiler chicken farmers ranges from 0.339 to 0.996, with a mean value of 0.689. The average efficiency value means that the average non-contracted broiler chicken farmer could only achieve a production of 68.9% of the production input that has been used. This implies that the opportunity for non-contracted broiler chicken farmers to increase output is still very large (31.1%) in case they want to be technically efficient and achieve frontier output. Meanwhile, the mean efficiency of contract farms was 0.893, with the lowest value of 0.638 and the highest of 0.988. The average efficiency value showed that contract broiler chicken farmers achieved production of 89.3% of all production inputs used. The implication is that contract broiler chicken farmers still have the opportunity to increase output by 10.7% to be technically efficient and achieve frontier output.

Based on the distribution of technical efficiency value, 56.31% of non-contract farmers with an efficiency value below 0.70, 33.98% of non-contract farmers had technical efficiency of 0.70 to 0.89, and 9.71% of non-contract farmers had technical efficiency above 0.90. Meanwhile, the technical efficiency value of contract farmers below 0.70 was 2.60% of contract farmers, between 0.70 and 0.89 was 42.86%, and the majority of broiler chicken farmers (54.55%) had a technical efficiency above 0.90. The results showed that farmers under contract had greater technical efficiency than non-contract farmers in Banten province. The comparative test results confirmed a significant difference in the level of technical efficiency between non-contract and contract farmers.

Non-contract farmers could be classified as low efficiency because the average value of technical efficiency was only 0.689. It means that efficiency is still low and can still be increased. The low value of mean technical efficiency (TE = 0.6803) was also found in non-contract farming in Nigeria (Adebisi et al., 2020). Meanwhile, contract farmers were categorized as highly efficient because they have a mean technical efficiency value of 0.893 (close to 1). The average value of TE was lower than the average value of TE found in the other studies that are higher than 0.90 (Ullah et al., 2019; Bana et al., 2021; Zimunya and Dube, 2021).

Several studies have indicated that contract farming can increase efficiency (Begum et al., 2012, Suwarta, 2012; Harianto et al., 2019). Begum et al. (2012) found that contract farming had a positive and significant effect on the technical efficiency of poultry farms. Suwarta (2012) investigated the efficiency of broiler chicken farms under the core company integrator and independent broiler chicken farms in Sleman Regency. The study results indicated that farmers under contract were more technically efficient than non-contract farmers. Furthermore, the technical efficiency of broiler chicken farms under contract with the core company integrator was higher than those under contract with an independent broiler chicken farm as the core. Harianto et al. (2019) revealed that broiler chicken farms under written formal and detailed contracts had better technical efficiency than those under non-formal and unwritten contracts. Moreover, contract farming could increase the productivity of broiler chicken farms, as contract farmers have better production performance than non-contract farmers (Bahari et al., 2012; Majid and Hassan, 2014; Saptana et al., 2017). However, a study by Bana et al. (2021) compared contract and non-contract broiler chicken farms in Kupang, East Nusa Tenggara, Indonesia. The result indicated that although both types of farming were technically efficient, contract farming was less efficient than non-contract farming.

The results of the comparative test of technical efficiency levels between contract and non-contract broiler chicken farmers are presented in Table 5. The $p < 0.01$ indicated a significant difference in technical efficiency levels between contract and non-contract farmers. These results indicated a significant difference in the level of technical efficiency between contract and non-contract farmers, with contract farmers exhibiting higher levels of technical efficiency. The finding of this study supported the argument that contract farming could improve the technical efficiency levels of broiler chicken farmers. This may be due to the support and guidance provided by the corporations, which enables contract farmers to access better inputs, such as feed and vaccines, and to implement more efficient farming practices. Non-contract farmers, on the other hand, may face challenges in accessing these resources and may lack the necessary knowledge and skills to optimize their production processes.

Table 6 presents the estimated technical inefficiency effect model of broiler chicken production. This study has identified a significant variable impacting technical inefficiency among non-contracted and contracted farmers. For non-contracted farmers, input suppliers were significant ($p < 0.01$). The input supplier variable has a positive coefficient value, indicating that buying inputs from an integrator through a distributor will cause

technical inefficiency to increase for non-contract farmers. However, purchasing inputs from a poultry shop can improve technical efficiency for non-contract farmers. These findings are significant for non-contract broiler chicken farmers. The results focus on the importance of carefully selecting input suppliers and utilizing experience to enhance technical efficiency and improve production outcomes.

Experience was a significant variable for contracted farmers ($p < 0.01$). Experience was the variable that impacts technical inefficiency in contracted farmers. The positive coefficient sign for contracted farmers indicates that more experienced farmers will increase technical inefficiency or become less efficient. This shows that technical inefficiency will increase in line with the increase in experience. The observed positive effect of experience on technical inefficiency can be attributed to the fact that contract farmers work according to the instructions of the core company, limiting the role of experience in improving efficiency.

Compared to non-contract farms, the efficiency of contract farms can be attributed to the involvement of the core company in the production process. Although in the current study, the variables of core company type, contract form, and contract type were not significant ($p > 0.05$), the core company plays a role in providing inputs and offering technical guidance on production, enabling contract farmers to understand resource allocation better and allocate them more efficiently. This was found in studies conducted by Eaton and Sheperd (2001), Simmons (2002), Bellemare et al. (2013), and Cahyadi and Waibel (2016).

Table 4. Technical efficiency level of non-contract and contract broiler chicken farms in Banten Province, Indonesia

Level of technical efficiency	Non-contract farms (n=103)		Contract farms (n=77)	
	Total	Percentage	Total	Percentage
< 0.70	58.00	56.31	2.00	2.60
0.70 - 0.79	28.00	27.18	16.00	20.78
0.80 - 0.89	7.00	6.80	17.00	22.08
≥ 0.90	10.00	9.71	42.00	54.55
Total	103.00	100.00	77.00	100.00
Maximum	0.996		0.988	
Minimum	0.339		0.638	
Average	0.689		0.893	

Source: Primary data analysis (2021), n: Number of samples

Table 5. Comparative test results of technical efficiency level of non-contract and contract broiler chicken farms in Banten Province, Indonesia

Variables	Non-contract farms (n=103)		Contract farms (n=77)		T-ratio	Significant
	Mean	SD	Mean	SD		
Technical efficiency	0.6887	0.1321	0.8933	0.1003	-11.8114	0.0001***

Source: Primary data analysis (2021), *** $p < 0.01$, n: Number of samples

Table 6. Estimates of technical inefficiency of non-contract and contract broiler chicken farms in Banten Province, Indonesia

Variables	Units	Non-contract farms (n=103)		Contract farms (n=77)	
		Coefficient	T-ratio	Coefficient	T-ratio
Constant		0.3833	1.8677	0.1180	0.3257
Age	Years	0.0002	0.1011	-0.0031	-0.5584
Education	Years	0.0003	0.0584	-0.0030	-0.1952
Experience	Years	-0.0080	1.9634	0.0217***	2.4704
Type of Job (1 = main job, 0 = side job)	Dummy	0.0159	0.3942	-0.0287	-0.2565
Input Suppliers (1 = distributor, 0 = poultry shop)	Dummy	0.1529***	3.3857	-	-
Core company (1 = integrator, 0 = poultry shop/other farmer)	Dummy	-	-	-0.0872	-0.5141
Contract form (1 = written, 0 = oral)	Dummy	-	-	0.0526	0.3298
Contract type (1 = forward contract, 0 = profit sharing/management fee)	Dummy	-	-	-0.0900	1.7560

Source: Primary data analysis (2021), *** $p < 0.01$, n: Number of samples, T-ratio: T-value or T-statistic

CONCLUSION

Non-contract broiler chicken farms were less technically efficient than those under contract. The mean value of technical efficiency on non-contract broiler chicken farmers was 0.689 ranging from 0.339 to 0.996. Meanwhile, broiler chicken farmers under contract had a higher mean efficiency value of 0.893, with the lowest value of 0.638 and the highest of 0.988. The input suppliers' type had a positive and significant effect on technical inefficiency in non-contract farming, where buying input from a poultry shop increases technical efficiency compared to buying input from the distributor. Technical efficiency improvements in production need to be made by contract and non-contract farmers through improved production management. In addition, the availability of production inputs and ease of access to inputs can support farmers' technical efficiency improvements. The findings could have important implications for broiler chicken farmers as the study highlights the critical role of inputs, such as DOC and feed in the production process. Farmers should ensure that these inputs are readily available as their optimal utilization positively affects production outcomes. Government support through regulation is crucial to ensure the availability of inputs and ease of access for farmers. Future research is related to the input market structure, distribution of the input supply to farmers and strategy for selecting input suppliers and core companies.

DECLARATION

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Authors' contributions

Efri Junaidi contributed to data collecting, data analysis, analysis of the results, and preparing the manuscript. Jamhari and Masyhuri contributed to the design and supervision of the research, analysis of the results, and revised the manuscript. All authors have checked and approved the final version of the manuscript for publication in the present journal.

Competing interests

The authors confirm that there was no conflict of interest with any financial, personal, or other relationships with other people or organizations related to this paper.

Ethical consideration

Before publication in the present journal, the authors have checked the ethical issues, including plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy.

Availability of data and materials

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

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Chitosan Hydroxyapatite: Physic-chemical Properties and its Effect on the Growth and Development of Broiler Chickens

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ABSTRACT

The current study aimed to obtain a calcium-containing, biocompatible drug based on chitosan *Bombyx mori*. Composites of Chitosan (CS) *Bombyx mori* with hydroxyapatite (HA) in the ratio of CS/HA = 50:50 mass percentage were synthesized *in situ* conditions at Ca/P = 1.67 mol% with intensive stirring for one hour at a speed of 1400 rpm and a temperature of 40 ± 2ºC. It was revealed that the components form an intermediate complex through –N-Ca, O-Ca, O (glycosidic bond)–Ca, H–O-bonds interacted by electrostatic forces. Atomic force microscopy studies indicated particles in the 100-50 nm size range on the polymer matrix surface. The polymer matrix prevented the growth of HA crystals and particle agglomeration. It was also determined that the CS/HA composite was non-toxic, and the LD_{50} was more than 5000 mg/kg. The composites were introduced into the chickens' diet in groups for 30 days at 25 to 40 mg/kg doses. The findings indicated an increased survival rate of chickens by 100%, improved the morphological parameters of the blood, and enhanced the contents of calcium, phosphorus, and hemoglobin. The addition of CS/HA=50:50 mass percentage contributed to an increase in the number of erythrocytes in the blood of broilers and hemoglobin by 11-12%. It should be noted that CS/HA did not adversely affect other morphological parameters of chicken blood. Therefore, CS/HA is recommended for the prevention of osteoporosis and osteomalacia in broiler chickens.

Keywords: Broiler chicken, Composites of chitosan Bombyx mori, Hydroxyapatite, *In situ*, Osteoporosis

INTRODUCTION

Biopolymer chitosan is a linear polysaccharide consisting of linked residues of N-acetyl-2-amino-2-deoxy-D-glucose (45-5%) and 2-amino-2-deoxy-D-glucose β(1→4) (55-95%; Lv, 2016; Mittal et al., 2018). Chitin is contained in crustacean skeletons, insect cuticles, and fungal cell walls (Suneeta and Kishor, 2020; Jiménez-Gómez and Antonio, 2020). Accordingly, chitin contents obtained from these sources differ in morphological parameters and physical and mechanical properties (Sampaio et al., 2005; Paulino et al., 2006; Suneeta and Kishor, 2020; Jiménez-Gómez and Antonio, 2020). Due to protonated amino groups, CS can interact with DNA, proteins, lipids, charged organic substances or synthetic polymers. These properties of CS increase the possibilities of its use in combination with various inorganic and organic compounds, where they are successfully used in bone tissue engineering (Aguilar et al., 2019; Hassan et al., 2022).

Hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) is a member of the calcium phosphate class, with bioactivity and osteoconductivity close to natural bone with regard to its chemical composition (Mehdi et al., 2013; Sherif, 2019). Hybrid (nano) composites of hydroxyapatite (HA) can be derived based on biopolymers (Gašparič et al., 2017; Heshmatpour et al., 2018). There are several methods for synthesizing CS/HA composites, of which the most

common methods are *in situ* synthesis, coprecipitation, freezing, lyophilization, and biomimetic (Venkatesan and Kim, 2010; Li et al., 2013; Szatkowski et al., 2015).

Composites CS/HA has been introduced in traumatology, dentistry, and veterinary medicine, as well as in wastewater treatment and as an agent that absorbs toxins (Le et al., 2012; Rita et al., 2021). The use of chitosan for this purpose is of particular importance due to its good matrix formation and bioactive properties. According to Shakir et al. (2017) and Sharifianjazi et al. (2022), materials used to fill bone defects should have four main properties, including osteoconduction, osteoinduction, osseointegration and osteogenesis, which are characteristic of chitosan.

Undoubtedly, chitosan compounds with HA and other calcium phosphates are distinguished by high biodegradation, bioactivity, and antimicrobial properties in the correction of defects in bone tissue cells (Wasso et al., 2020; Walaa et al., 2020; Widnyana et al., 2021; Mohamed et al., 2022). Calcium phosphate is effectively used in nutrients to prevent osteoporosis and osteomalacia in chickens and cattle (Uhl, 2018).

Currently, oral administration of bioactive substances based on CS is successfully considered in veterinary practice (Adams and Hewison, 2010; van de Graaf et al., 2015). In modern poultry farming, several new diseases of the bone tissue in fast-growing chickens have increased. The effective action of nano-HA in treating such diseases can be obtained using *in ovo*, which has a positive effect on increasing body weight and improving the condition of the bones. However, when taken orally, the size and high degree of crystallinity of HA impede body absorption (Oshida, 2015; Matuszewski et al., 2020). In CS/HA composites, the CS macromolecule prevents the growth of HA crystals and controls the size of their particles, which contributes to the absorption of the drug through the cells.

Depending on the synthesis conditions, the chemical composition of calcium-phosphorus compounds changes. For example, at a ratio Ca/P > 1.67 mol, calcium phosphate is formed with a pH > 7. It also leads to the formation of insoluble unstable composites when interacting with water or physiological fluids (Ca/P < 1.67, Eliaz and Metoki, 2017).

In addition to HA-based materials, regenerating or completely resorbable materials have been developed. They are based on the use of a porous resorbed matrix carrying proteins and bone cells in tissue engineering. Calcium phosphates at Ca/P < 1.67 ratio include tricalcium phosphate (TCP-$Ca_3(PO_4)_2$), calcium pyrophosphate (CPP-$Ca_2P_2O_7$), calcium polyphosphate ($(Ca(PO_3)_2)_n$), carbonat hydroxyapatite (KHA, Na_2O-CaO-P_2O_5, Na_2O-CaO-P_2O_5-SiO_2, K_2O-CaO-P_2O_5; Georgiou and Knowles, 2001).

Calcium phosphate-based preparations, such as HA, tricalcium phosphate (TCP), and carbonate-substituted carbonate-hydroxyapatite (KHA), have effective bioactive properties, high protein adsorption and osteoblast formation in tissues where cells are involved in bone regeneration (Meyer et al., 2004).

The effect of a phosphorus source on the balance of calcium in the body and on the bones of broilers was studied by Kaveh et al. (2020). It has been established that the inclusion in the diet of chickens of an inorganic source of phosphorus-containing dicalcium phosphate (14% active phosphorus and 23% calcium) has a more effective effect on the growth rates and productivity of chickens than the addition of an organic source - roasted bone meal (14 ± 2% of available phosphorus and 32% calcium).

Chitosan is a natural polycation and is extracted from the exoskeletons of crabs, fungi, and insects (Swiatkiewicz et al., 2015). Chitosan and its derivatives have bacterial, fungicidal, insecticidal, biostimulating, immuno-modulating properties (Amar Cheba, 2020). Recently, there has been a trend in the use of chitosan and its modifications in veterinary practices (Kobayashi et al., 2002; Shi et al., 2005; Raphaël and Meimandipour, 2017). Due to polyelectrolyte interaction, chitosan interacts with lipase, including fat is absorbed in the small intestine, reducing the amount of cholesterol in the blood (Lokman et al., 2019). On the practical effect of 0.2-0.3% solution of chitosan on the value of eggs, it was established that chitosan-containing feed lowered the cholesterol of brioler chickens (Kobayashi et al., 2002; Nogueira et al., 2003; Swiatkiewicz et al., 2015). Unlike chitosan at a rate of 0.5 g/kg, a comparative study showed that chitin effectively stimulated the growth, quality of the carcass, state of the organs of broilers, compared to the chickens in the control group fed the standard that diet gained weight and had more fat accumulation (Lokman et al., 2019).

The effect of dietary chitosan on growth, productivity, and protein retention in broilers was investigated. Results indicated that broilers fed a diet containing 0.5 or 1.0 g/kg of chitosan contributed to increased growth and feed absorption (Shi et al., 2005).

Poultry farming is a dynamically developing animal husbandry field, which solves many agro-industrial problems (Gržinić et al., 2023). However, there is also a deterrent to the development of poultry farming, which is the availability of forage (Vincenzo et al., 2018). There are

many uses for new types of feed additives that are cost-effective and improve the diet balance (Gerber et al., 2015). In addition, they can improve the quality of products and physiological state of chickens (Hafez and Attia, 2020). In recent years, such feed additives mainly include natural feed bio-additives. These non-toxic and biocompatible dietary supplements have adsorption and ion exchange properties and cleanse the body of toxic substances (Abudabos et al., 2013).

Under experimental conditions, the current study aimed to investigate the effect of chitosan/HA on safety and weight gain, the morphological composition and leukocyte blood formula, and acute toxicity of broiler chickens. This study aimed to obtain chitosan-apatite composites with controlled particle sizes and study their physicochemical and bioactive properties.

MATERIALS AND METHODS

In this study, CS *Bombyx mori* with a molecular mass of 2×10^5 and a degree of deacetylation (DD) of 90%. To obtain HA, $CaCl_2$, and KH_2PO_4 (98.0%) were used (Sigma-Aldrich, USA).

Ethical approval

All procedures were approved by the Animal Care Committee of Veterinary Medicine, Animal Husbandry and Biotechnology, Samarkand, Uzbekistan. The principles of laboratory animal care were followed, and specific international rules and regulations were observed.

Morphology of the films Chitosan/Hydroxyapatite

The morphology of CS/HA was studied with an Atomic Force Microscopy (AFM) Agilent 5500 atomic force microscope (USA) at 22°C using silicon cantilevers with a stiffness of 9.5 N/m and a frequency of 145 kHz. The maximum scanning area on AFM was 25×25 μm^2 for X and 1 µm for Z.

Acute toxicity of Chitosan/Hydroxyapatite

Acute toxicity composites CS/HA was studied at the Scientific Center for Standardization of Medicines Samarkand, Uzbekistan on 24 white mice of mixed sex weighing 20 ± 1 g, divided into four groups. Chitosan hydroxyapatite was dissolved in 2% acetic acid to gain a gel state. It was then intragastrically administered to groups one to four once at doses of 1000 mg/kg, 2000 mg/kg, 4000 mg/kg, and 5000 mg/kg, respectively (Habriev, 2005). Experimental mice were under continuous hourly observation during the first day of the test. The general condition of mice and their behavior, the intensity and characteristics of motor activity, the presence of convulsions, coordination of movements, response to external stimuli and skeletal muscle tone, appetite, body weight, quantity, and the consistency of feces as the indicators of their functional state were taken into account. The clinical states of the animals, including the presence/absence of signs of poisoning, the time of their appearance, and the death of mice, were monitored during the study. All experimental animals were kept under standard conditions, on a general diet with free access to water and food. After the completion of the experiment, the average lethal doses (LD_{50}) were determined (Tisserand and Young, 2014).

Study design

Due to the fact that broiler chickens were kept in cramped quarters and under artificial lighting. Due to the inferiority of the diet, they were prone to hypovitaminosis, diseases, immunodeficiency, namely calcium, and phosphorus deficiency. Consequently, this negatively affected the increase in live weight and egg production of broiler chickens, leading to osteoporosis and osteomalacia in chickens in some cases. To improve the immune system of chickens and ensure a stable Ca-P balance in the body, the effect of additives of CS/HA on the physiological state of broiler chickens was studied. To do this, the test duration and the CS/HA powder dose added to their diet were controlled. Biologically, the active properties of CS/HA were studied in laboratory conditions for 100 one-day-old chickens of the breed Ross-308, adding 25 30, 35, and 40 mg/kg of composite powders to their diet. Then, the chickens were divided into 10 groups, and changes in their weight were recorded 30 days. During the 30-day experiment, the control group of chicken was fed a standard diet (Barekatain et al., 2021, Table 1).

Table 1. Standard diet for broiler chickens (Percentage of additives relative to 100 kg of feed)

Number	The composition of the diet	Volume
1	Soy flour	20 kg
2	Corn	32.6 kg
3	Wheat	25.6 kg
4	Sunflower flour	7.3 kg
5	Cotton flour	2 kg
6	Vegetable oil	2 liters
7	Methionine	51 g
8	Calcium orthophosphate	1780 g
9	Limestone	8.22 g
10	Salt	50 g

In the experimental groups (2-10), instead of vitamins and premixes, CS/HA powders were added to the diet for 15-25 days, and the results were obtained on day 30. The effectiveness of the composites used in the experiments was evaluated by the survival rate of chickens and their live weight gain.

Blood parameters

Hematological studies were performed on days 10, 20, and 30 of the experiment. Blood for the analysis of experimental and control chickens was taken from the axillary vein. Hemoglobin concentration was determined in the presence of acetone cyanohydrin by the hemoglobin-cyanide method on the KFK2 device (Whitehead et al., 2019). The number of erythrocytes, leukocytes, and platelets in 1 mm^3 of blood was counted in the Goryaev chamber after staining them based on Romanovsky-Giemsa and dye-methyl violet according to the method by Campbell (1988).

The leukocyte formula in blood smears was determined after double staining according to Papenheim with the Filipchenko three-field method (Alturkistani et al., 2015; Sohair et al., 2017; Sufiriyanto et al., 2018; Kolesnik et al., 2020). Preliminary laboratory experiments were conducted to determine the optimal dose of chitosan hydroxyapatite and the timing of their addition to chicken feed. The prepared diet did not include vitamins and minerals. From days 10 to 20, the chickens were fed the supplements of CS/HA were added in modules of 0, 25, 30, 35, and 40 mg per 1 kg of feed, respectively. After that, blood samples of experimental chickens were obtained and the content of Ca/P in the composition of their blood and blood serum was determined. Biochemical parameters of blood samples were determined on Automatic Chemical Analyzer CC-T180, indicating the hematological composition of blood. Acute toxicity of CS/HA preparations was determined under *in ovo* conditions (Yair and Uni, 2011; Shokraneh et al., 2020). During the experiment, the indicators of the effectiveness of drugs, the survival rate of chickens, and the average live weight of one head of chickens were determined according to the following formula.

$$P = \frac{W_t - W_0}{W_0} x100$$

Where, P is the percentage of live weight gain, Wt denotes the live weight of 1 chicken head at the end of the experiment, Wo determines live weight of 1 chicken at the beginning of the experiment, 100 is coefficient. The data from this study were transferred to SPSS software version 23 for statistical analysis and were compared using the Kruskal-Wallis test. The Mann-Whitney test was used to compare the groups. In this study, a p-value less than 0.05 was considered statistically significant.

Obtaining Chitosan/Hydroxyapatite composites with the variation of synthesis conditions

The CS/HA composites were obtained under *in situ* conditions using 2% acetic-water solution of CS, 1 M aqueous solutions of $CaCl_2$ and KH_2PO_4. First a solution containing Ca/P = 1.67 mol% was mixed with a solution of chitosan atmassratio CS/HA = 50:50 mass percentage and intensively mixed on a magnetic stirrer. Then, with dropwise addition 6% NaOH precipitates into the reaction mixture. The target product is CS/HA (Nikpour et al., 2012), where the precipitate in solution NaOH was kept for 8 hours. After that, the obtained samples of CS/HA were washed with distilled water up to pH = 7 and dry up freeze-dried to constant weight (Figure 1).

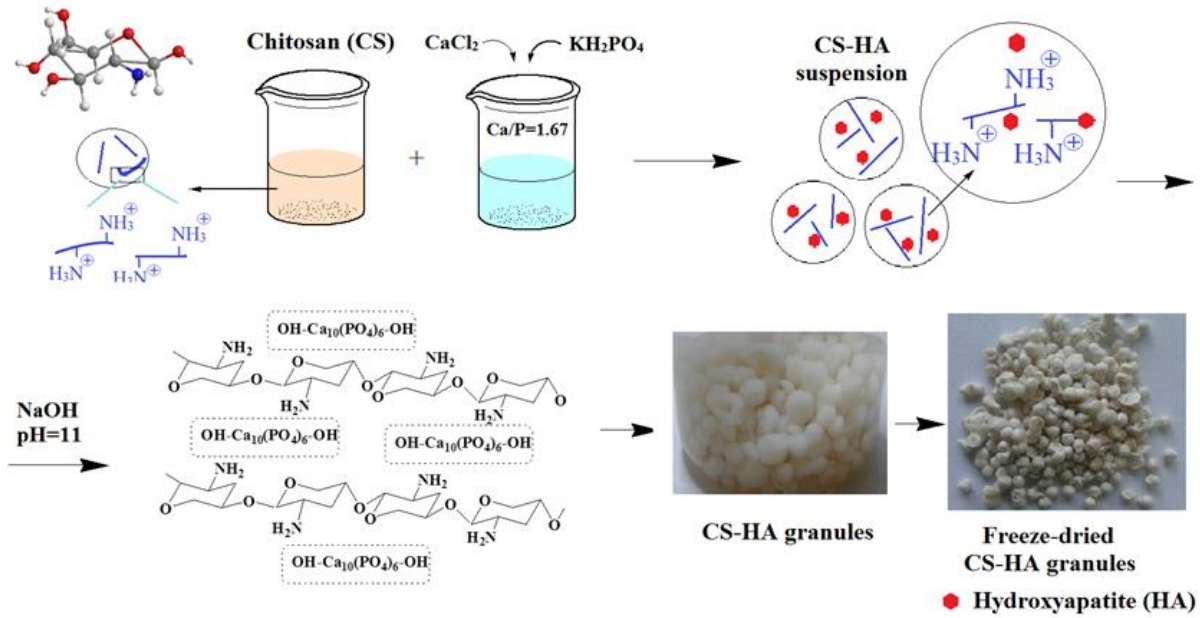

Figure 1. Preparation of Chitosan/Hydroxyapatite composites under *in situ* condition

RESULTS AND DISCUSSION

Experimental samples of hydroxyapatite chitosan *Bombyx mori* have been produced under laboratory conditions and identified by physicochemical research methods. The elemental composition and some characteristics of CS and its composites are determined with HA (Table 2).

The results indicated that with the introduction of 50 mass percentage HA into a chitosan macromolecule, its features of water absorption decreased approximately by three times. Accordingly, the degree of its solubility in acetic acid fell and formed a suspension. The content of calcium ions (Ca^{2+}) in the composite and the ash content of the preparation were 9.16% and 59.9%, respectively.

Under the chosen synthesis conditions, apatite nanoparticles stabilized by the polymer matrix were formed when the intermediate complex of chitosan with hydroxyapatite was stored at pH >7 for 8 hours. Hydroxyapatite nanoparticles stabilized by a chitosan macromolecule have a hexagonal structure corresponding to the interplanar distances of HA with hexagonal syngony (JCPDS-No.-00-09-0432; Vokhidova et al., 2022). Thus, a preliminary structure of the CS/HA composite was proposed based on experimental and theoretical research methods (Figure 2).

Table 2. Some physicochemical characteristics of Chitosan/Hydroxyapatite samples

Number	Samples	N_{total} (%)	Ca^{2+} (%)	Ash content (mass percentage)	Humidity (%)	Solubility in 2% CH_3COOH (%)
1	Chitosan	8.27	-	3.6	1.87	98
2	Chitosan/Hydroxyapatite = 50:50	4.10	9.2	59.9	0.65	Suspension

Ca^{2+}: Calcium

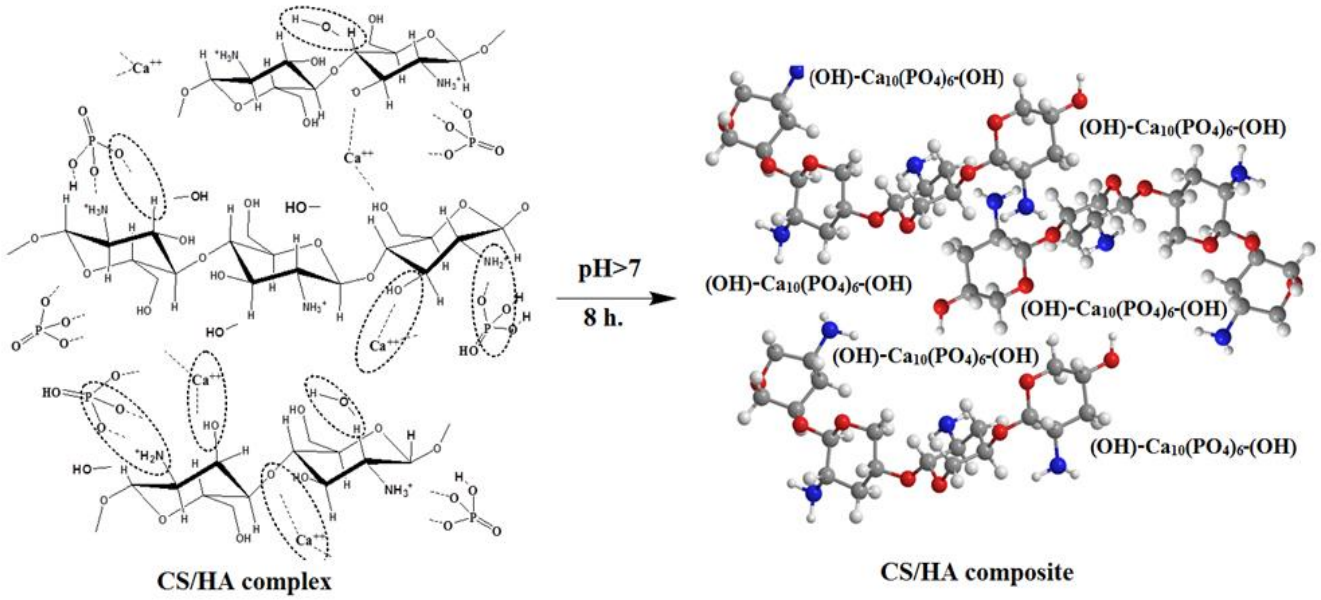

Figure 2. Preliminary structure of the resulting Chitosan/Hydroxyapatite composite (Source: Vokhidova et al., 2022).

Atomic force microscopy analysis

The morphology of the chitosan/HA films was studied by the AFM method. It was found that nanoparticles within the range of 100 to 450 nm were formed on the surface of the films of the studied composites (Figure 3).

The AFM micrographs showed that HA crystallites were formed on the surface of the films under *in situ* conditions. It was found that with a synthesis duration of up to 8 hours, polydisperse particles were formed in the 100-50 nm size range, where the average size was 280 nm

(25%). Moreover, the formed particles were unevenly distributed over the polymer matrix.

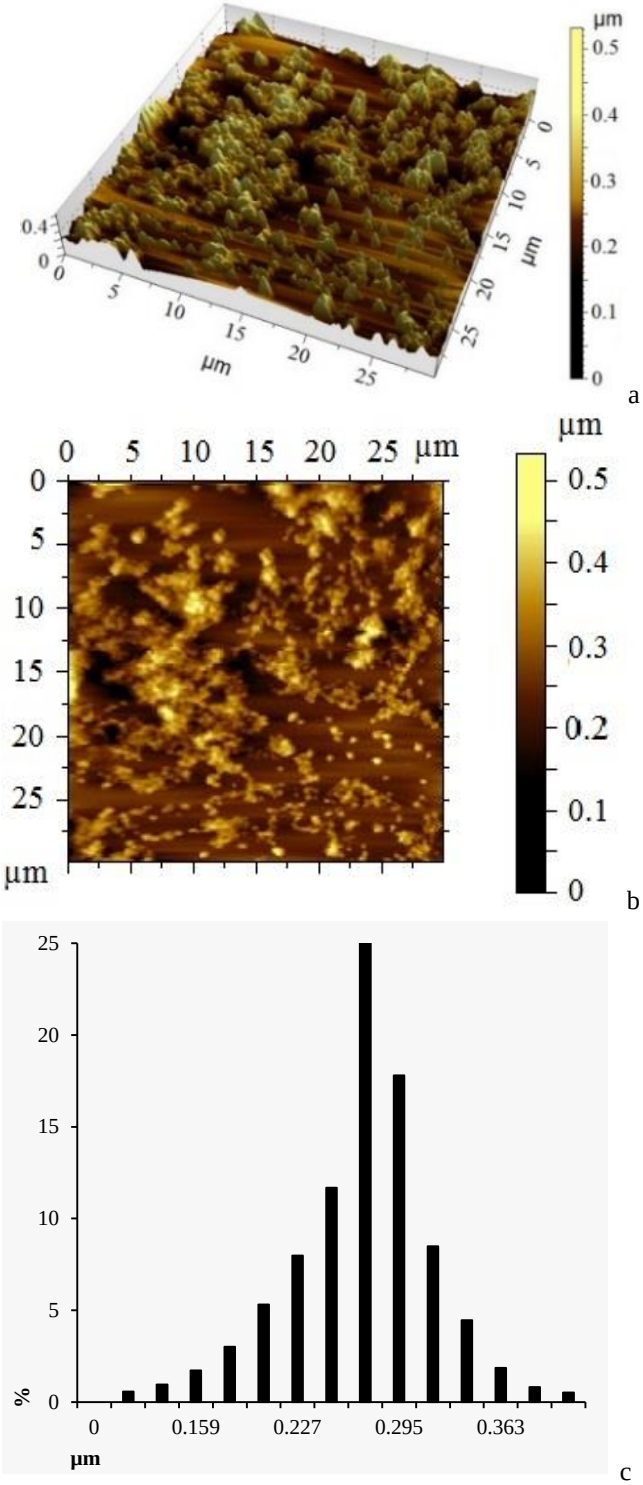

Figure 3. Phase (a) and topographic (b) atomic force microscopy images of Chitosan/Hydroxyapatite (50:50 mass percentage) and particle distribution histogram (c)

Acute toxicity of composite Chitosan/Hydroxyapatite

Composite acute toxicity study showed that after a single intragastric administration CS/HA = 50:50 mass % at doses of 1000 mg/kg, 2000 mg/kg, 4000 mg/kg, and 5000 mg/kg. The general condition of the mice remained stable, and no visible changes were observed in their behavior (Table 3).

During the observation of the experimental mice, it was revealed that the coordination of movements and the tone of the skeletal muscles were normal, and there were no convulsions. Mice responded to tactile, pain, sound, and light stimuli. The condition of the skin and hair was unchanged, and the color of the mucous membranes and the size of the pupil were without deviations. Mice in all groups indicated no loss of appetite, increase in water consumption, change in body weight. Moreover, the amount and consistency of feces were normal. Within 14 days, the death of mice was not observed. Thus, as a result of the experiments, it was found that the data obtained showed that LD_{50} CS/HA = 50:50 mass percentage was > 5000 mg/kg; the drug was non-toxic.

Table 3. Determination the acute toxicity of chitosan hydroxyapatite

Number of mice	Chitosan hydroxyapatite *Bombyx mori*			
	Weight (g)	Dose (mg/kg)	Way introductions	Fatal outcome
1	21	1000	intra-gastric	Not
2	19			Not
3	20			Not
4	21			Not
5	19			Not
6	20			Not
1	20	2000		Not
2	21			Not
3	19			Not
4	21			Not
5	21			Not
6	20			Not
1	20	4000		Not
2	20			Not
3	21			Not
4	19			Not
5	20			Not
6	20			Not
1	20	5000		Not
2	20			Not
3	19			Not
4	20			Not
5	19			Not
6	19			Not
LD_{50}		>5000 mg/kg		

Live body weight and survival rate

The results show that when 25-40 mg/kg of chitosan apatite was added to the feed composition, 100% safety was observed, and the weight gain was 71.5-75.5% (Table 4). Bioadditives chitosan/HA effectively influenced the morphological parameters and leukocyte blood count, contributing to a significant increase in erythrocytes and hemoglobin ($p \leq 0.05$). As a result of the study, the survival rate of chickens in the control group was 90%, and the survival rate of chickens in experimental groups (2-10), where CS/HA was added to the diet at a dose of 25-40 mg/kg, reached 100%.

During the test period, the live weight of one broiler from the control group increased from 310 to 1350 g. At the same time, the survival rate reached 90%, and the increase in the average live weight of chickens was 67.5%. In the experimental groups, the live weight increased from 311 ± 5 to 1200-1510 g, with an efficiency of 100%, and the average live weight of chickens increased to 75.5%. Therefore, 40 mg/kg of chitosan hydroxyapatite and 15 days were selected as the optimal dose and duration of CS/HA introduction into the chickens' diet. During the experiments, the live weight of the experimental chickens increased slightly by only 8%. This is probably due to the lack of vitamin and mineral supplements in the diet of chickens. However, it should be noted that the addition of chitosan hydroxyapatite, regardless of the dose, could lead to an increase in the immunity of chickens, and no clinical symptoms of infectious diseases were observed.

Table 4. The effect of chitosan *Bombyx mori* hydroxyapatite on the live weight gain of chickens and their safety during a 30-day study

No.	Groups	Dose of Chitosan/Hydroxyapatite (mg/kg)	Duration of adding Chitosan/Hydroxyapatite (day)	Average live weight of 1 chicken at the beginning of the experiment (g)	Live weight of 1 chicken after 30 days (g)	Survival rate (%)	Increase in average live weight of chickens (%)
1	Control	-	Standard ration	310	1350	90	67.5
2	Chitosan/Hydroxyapatite	25	10-20	310	1200	100	60.0
3		30		313	1250	100	62.5
4		35		309	1290	100	64.5
5		40		311	1350	100	67.5
6		25	15-25	313	1250	100	62.5
7		30		309	1340	100	67.0
8		35		312	1430	100	71.5
9		40		311	1510	100	75.5

Ca-P balance in blood serum

With an increase in the dose of the composite in the diet of chickens, the amount of calcium and phosphorus naturally increased. Calcium content in the control group chickens was 2.2 ± 0.415 mmol/l, while the content of Ca increased to 3.5 ± 0.354 mmol/l at the same time as in the study group fed dose of CS/HA 40 mg/kg. It should be noted that the content of phosphorus increased in proportion to the dose of CS/HA supplements. At a dose of CS/HA 40 mg/kg, the phosphorus content compared to the control sample increased by approximately 1.3 times (Table 5). In fact, calcium ions can increase intestinal pH and reduce the solubility and availability of minerals (Adedokun et al., 2004; Olukosi and Fru-Nji, 2014; Gautier et al., 2017).

It was found that minor additions of CS/HA to the standard diet of broiler chickens contributed to an increase in the amount of Ca and P in the blood serum, but had little effect on the amount of K^+ and Na^+. According to studies on the effect of CS/HA on the biochemical parameters of blood serum, the amount of Ca and P in the experimental groups was higher than in control chickens (Xu et al., 2021). This is due to the fact that Ca and P from the composition of CS/HA are effectively absorbed by the body of chickens. The data obtained in the current study are in good agreement with the results of Sharaviev (2015) as they investigated the probiotic Bacell-M and its effect on the biochemical parameters of the blood of chickens. Thus, CS/HA at a dose of 35-40 mg/kg in the diet of chickens increases their safety and weight gain in broiler chickens. There is no negative effect on the hematological parameters of their blood.

Table 5. The effect of different doses of Chitosan/Hydroxyapatite on the serum biochemical parameters in broiler chickens

Number	Dose (mg/kg)	Ca^{2+} (mmol/l)	P (mmol/l)	Na^{+} (mg/%)	K^{+} (mg/%)
1	Control	2.20 ± 0.42	1.4 ± 0.87	364 ± 11.8	22.0 ± 10.7
2	25	2.31 ± 0.38	2.0 ± 0.37	371 ± 20.5	22.0 ± 10.6
3	30	2.44 ± 0.43	1.7 ± 0.69	365 ± 20.2	22.3 ± 11.1
4	35	2.75 ± 0.29	2.2 ± 0.44	370 ± 20.3	22.4 ± 8.11
5	40	3.51 ± 0.35	2.3 ± 0.51	364 ± 20.1	22.8 ± 11.3

Ca^{2+}: Calcium, P: Phosphorus, Na^{+}: Sodium, K^{+}: Potassium

Morphological parameters and leukocyte count

The morphological blood parameters of broiler chickens were studied and the effect of CS/HA on the content of retinol in the liver and blood serum was investigated. Four groups of broiler chickens were selected for the study. The first group was the control and were fed a basal diet. For the first 10-20 days, the diet of the second group included basal diet and CS/HA powders at a dose of 25 mg/kg. During 20-30 days, the diet of the third group entailed CS/HA at a dose of 30 mg/kg. Finally, between days from 30 to 40, chitosan hydroxyapatite supplements were added at a dose of 35 mg/kg to the diet of chickens in the fourth group. After that, the leukocyte formula was determined in all groups of chickens on days 20, 30, and 40 (Tables 6 and 7).

Compared with the experimental control groups, erythrocytes, and hemoglobin increased by 7-8%, 9-10%, and 11-12%, in the blood of chickens in the second, third, and fourth groups, respectively. At the same time, the number of leukocytes and platelets in the blood of chickens in all experimental groups practically did not differ from that of the control group ($p < 0.05$, tables 6, 7). The results were in an agreement with the literature data (Abudabos et al., 2013). It was found that the addition of powders of the CS/HA = 50:50 mass percentage composite to the diet of broiler chickens, regardless of the period of administration, contributed to an increase in the number of erythrocytes and hemoglobin in the blood of chickens. It can be inferred that the composite based on chitosan and hydroxyapatite positively affected the morphological parameters of blood due to the synergistic effect. The inclusion of CS/HA in the diet of chickens stimulates the activity of hematopoietic organs.

Table 6. The influence of Chitosan/Hydroxyapatite on the number of erythrocytes (10^{-12} g/l) and leukocytes (10^{-9} g/l) in broiler chickens

Number	Dose of Chitosan/Hydroxyapatite, (mg/kg)	Erythrocytes (10^{-12} g/l)			Leukocytes (10^{-9} g/l)		
		Experiment period (day)					
		10	20	40	10	20	40
1	Control	2.62 ± 0.07	2.64 ± 0.10	2.68 ± 0.11	29.3 ± 1.09	29.7 ± 1.10	28.8 ± 1.11
2	25	2.83 ± 0.09	2.85 ± 0.12	2.89 ± 0.05	28.5 ± 1.20	28.5 ± 1.25	28.1 ± 2.30
3	30	2.61 ± 0.08	2.87 ± 0.13	2.94 ± 0.01	28.1 ± 1.12	28.2 ± 1.03	28.3 ± 2.77
4	35	2.60 ± 0.05	2.65 ± 0.10	3.00 ± 0.06	27.9 ± 1.21	28.5 ± 1.15	28.1 ± 1.55

Table 7. The effect of Chitosan/Hydroxyapatite supplements with different composite on platelets count (10^{-9} g/l) and hemoglobin (g/l) in broiler chickens

Number	Dose of Chitosan/Hydroxyapatite (mg/kg)	Platelets (10^{-9} g/l)			Hemoglobin (g/l)		
		Experiment period (day)					
		10	20	40	10	20	40
1	Control	37.2 ± 2.01	38.5 ± 2.03	37.5 ± 2.55	79.3 ± 1.11	85.5 ± 1.13	90.0 ± 1.12
2	25	35.6 ± 1.19	36.7 ± 1.17	37.0 ± 2.06	84.8 ± 1.17	91.4 ± 1.00	97.2 ± 0.06
3	30	36.4 ± 1.12	37.3 ± 1.20	37.1 ± 2.00	79.8 ± 0.09	93.2 ± 0.06	99.0 ± 0.09
4	35	36.7 ± 1.33	37.0 ± 1.35	36.8 ± 0.95	78.2 ± 1.15	85.8 ± 0.05	100.6 ± 1.2

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CONCLUSION

To conclude, the optimal conditions for the synthesis of the formation of chitosan hydroxyapatite Bombyx mori nanocomposites in the ratio of 50:50 mass percentage. It has been shown that chitosan apatite is a non-toxic preparation, and the LD_{50} of the CS/HA composite is 5000 mg/kg. In the current study, powders of CS/HA were introduced into the diet of broiler chickens, and their positive effect on the physiological state of chickens, as well as on the morphological and some biochemical parameters of their blood, were analyzed. It was found that the addition of CS/HA in a dose of 40 mg/kg to the diet of chickens could maintain the balance of Ca and P and was easily absorbed by their organism.

DECLARATION

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Authors' contributions

Noira R. Vokhidova, the head of project, conceived the presented idea and verified the analytical methods. Sayyora Sh. Rashidova supervised the project. Kandiyor Kh. Ergashev synthesised the samples of composites, prepared figures and tables. Davletbay I. Ibragimov conducted experiments on the bioactivity of the composite. All authors checked the statistical results and contributed to the final version of the manuscript.

Competing interests

There are no conflicts to declare.

Ethical considerations

The authors follow the required ethical standards for publishing all available data related to this study for the first time without copying any data from other published papers.

Availability of data and materials

The full data of the present study will be sent according to the reasonable request that will be sent to the corresponding author.

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The Effect of Nano-bentonite Supplementation on Reducing the Toxicity of Aflatoxin B1 in Kampung Unggul Balitbangtan Chickens' Diet

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ABSTRACT

Aspergillus flavus and *Aspergillus parasiticus* are fungi that produce toxic secondary metabolites known as aflatoxins. These toxins can contaminate various food and feed products, including grains and nuts, before or after they are harvested. This contamination is most commonly found in tropical countries. Many studies have demonstrated that clay additions can reduce animal aflatoxin toxicity. The objective of this research was to study how the usage of Pacitan's local bentonite, located in East Java, Indonesia, could potentially decrease the harmful effects of aflatoxin B1 in native chicken species. The Masking Gel Calcification method was used to create bentonite nanoparticles at the Center for Ceramics in Bandung, West Java, Indonesia. The *in vivo* study was conducted at a native chicken farm in Bantul, Yogyakarta, Indonesia, with 1200 unsexed Kampung Unggul Balitbangtan (KUB) chickens. Kampung Unggul Balitbangtan chickens were divided into 4 treatments and 6 replications, each containing 50 chickens. The diets in the treatments were named as T0 (the control group in which chickens were fed basal diet, without aflatoxin B1), T1 (T0 + 200 µg/kg aflatoxin B1), T2 (T0 + 200 µg/kg aflatoxin B1 + 1 g/kg Factory Feed with standard factory absorbent), and T3 (T0 + 200 µg/kg aflatoxin B1 + 1 g/kg nano bentonite). *Aspergillus flavus* isolates from PAU Universitas Gadjah Mada were created using crude aflatoxin (FNC 2262). This study found a significant difference in KUB chicken performance, specifically in feed conversion ratio (FCR). Compared to T0, the findings indicated that T1 had the highest FCR value, followed by T2 and T3. It can be concluded that nanoparticle bentonite has a looser structure because of decreased packing density with the lowest FCR. Based on hematology analysis, it can suppress aflatoxin B1 toxicity in KUB chickens.

Keywords: Aflatoxin B1, *Aspergillus flavus*, Bentonite, Feed conversion ratio, Kampung Unggul Balitbangtan chicken

INTRODUCTION

Indonesia is among the world's most populous countries, so plant and animal nourishment production is critical. Chicken is one of the livestock that can be used as a source of animal protein. This domesticated animal is frequently raised by Indonesians and plays a significant role in supplying animal protein to the community. Local chicken is one of the dozens of strains of chicken that have the potential to be produced in Indonesia (Depison et al., 2020). In 2019, there were 301,761,386 chickens in the local area, contributing 8.33%, or 292,329 tons, to the national production of poultry meat (Bakrie et al., 2021). The Kampung Unggul Balitbangtan (KUB) chicken is a locally adapted type with significant genetic variability. Additionally, the Minister of Agriculture issued a decree on the release of KUB chicken, which was produced over six generations in West Java and DKI Jakarta from a combination of indigenous breeds (Masito et al., 2022). Following this development, the animal feed industry is a

rapidly growing poultry farming industry in Indonesia, with more than 34 animal feed manufacturers established by 2020. As a result, poultry feed producers (feed mills) will work hard to improve feed quality, which results in improved livestock production and efficiency.

Currently, a great effort is being made to avoid contaminations, such as aflatoxin, in feed (Mgbeahuruike et al., 2018). Aflatoxin, a secondary toxic metabolite of *Aspergillus* species, especially aflatoxigenic *Aspergillus flavus*, and *parasitics*, is harmful to poultry health and production (Motbaynor et al., 2021). Aflatoxicosis is a prominent issue associated with tropical poultry production (Mgbeahuruike et al., 2018). Considering the severe economic losses and health issues that aflatoxins pose, this toxin is a serious concern in poultry production and public health (El-Nabarawy et al., 2020). Aflatoxin contamination reduces feed quality and animal efficiency through poor nutrient conversion or problems such as reproductive abnormalities (Alahlah et al., 2021). Aflatoxicosis in poultry also results in listlessness, anorexia, a slower growth rate, poor feed utilization, lower egg production, and increased mortality (Oguz, 2011). Thus, it is critical to control these toxins' economic and health risks (Oguz, 2011).

Of the four forms of aflatoxin molecules, B1, B2, G1, and G2, aflatoxin B1 is the most common and significant toxigenic hazard (Kumar et al., 2017). In chickens, the small intestine rapidly absorbs aflatoxin B1 into the mesenteric venous blood (Noreddine, 2020). Aflatoxin B1 is the most potent carcinogen and can cause many systemic side effects and interfere with normal organ and tissue function, resulting in inhibition of growth, swelling, immune suppression, and an increased risk of liver cancer in humans and animals (Wogan et al., 2012). Aflatoxin B1 contamination can be found in corn, peanuts, and animal feed, with many exceeding the threshold (Nuryono et al., 2010; Yunianta and Agus, 2013)

However, the aflatoxin risks can be avoided by using proper management measures to reduce mycotoxin contamination in agricultural products used as feed ingredients (during planting, harvesting, and storage, Kumar et al., 2021). Different approaches, whether physical, chemical, or biological, have been employed for elimination objectives. In the last decade, a binder or absorbent that can effectively prevent aflatoxicosis was developed, and it is now used in industry to reduce the effects of aflatoxins (Nazarizadeh and Pourreza, 2019). The issue is that, in Indonesia, the binder is still entirely imported from developed countries. Commercially, clay's aluminosilicate has been utilized as a feed additive to enhance the nutritional value of animal feed and as a binding agent for mycotoxins (Nadziakiewicza et al., 2019). Up to 2% of feed contains aluminum silicate as an anti-caking agent. The aluminosilicate group includes several members that fall under the phyllosilicate subclass, such as bentonite, montmorillonite, smectite, kaolinite, and illite (Kolosova and Stroka, 2011). On the other hand, zeolite and clinoptilolite are among the materials that do not belong to the phyllosilicate subclass (EFSA, 2009; Brezonik and Arnold, 2011).

According to the results of previous research, the administration of activated charcoal and bentonite clay produced better immunological and histopathological features than the control group (Ramandani, 2020). Much research has been done on this binder material, including by researchers with the mycotoxin team of Universitas Gadjah Mada, with natural bentonite obtained from Pacitan (East Java, Indonesia, Nuryono et al., 2012). Bentonite has been widely used as a feed additive to bind aflatoxins commercially, but feed mill industries in Indonesia generally use bentonite as an adsorbent imported from abroad. This study aimed to determine the effects of local bentonite from Pacitan, East Java, Indonesia, on the reduction of aflatoxin B1 toxicity in KUB chicken.

MATERIALS AND METHODS

Ethical approval

The current experiment was carried out at the poultry farm of PT. Sari Rosa Asih Feed Mill and CV Kurnia in Bantul, Yogyakarta, Indonesia. The Research Ethics Committee of the Faculty of Veterinary Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia, approved this study (0122/EC-FKH/Eks. /2022).

Analysis of active and non-active nano bentonite

The first step to produce nano bentonite was collecting bentonite samples in Pacitan, East Java, Indonesia. Using the masking gel calcification method, the bentonite samples were then transported to the Center for Ceramics in Bandung, West Java, Indonesia, for Bentonite Nano Processing. The results were divided into two groups for activation after they were obtained. The two outcomes were then characterized using transmission electron microscopy (TEM, JEM-1400, Japan).

Preparation of tested isolates

An inoculum of the fungus *Aspergillus flavus* from PAU Universitas Gadjah Mada in Yogyakarta, Indonesia,

was used to produce crude aflatoxin in the laboratory from corn, stored for 14 days with a moisture level of more than 18%. Aflatoxin crude was generated by the PAU-UGM Laboratory using the isolates FNCC 2262. The biological laboratory at Universitas Gadjah Mada, Yogyakarta, was used as the study's location. Corn with a moisture content ranging between 20-22% was stored for 14 days to produce naturally contaminated aflatoxin B1 corn as an additional measure. The Redascreen Aflatoxin kit (R-Biopharm AG, Germany) Enzyme-Linked Immunosorbent Assay (ELISA) and High-performance liquid chromatography (HPLC) were used to test the levels of aflatoxin B1 (Beyene et al., 2019).

Experimental design

The presented study was carried out for eight weeks at the Commanditaire Vennootschap Kurnia in Bantul, Yogyakarta, Indonesia. The trial ran from August until October 2022. In the study, the chickens were initially provided with crumb feeding during the first week, and then gradually transitioned to the research feed in the following week to help them adjust to the new diet. Performance calculations for the chickens began in the second week. The chickens were provided with *ad libitum* water and feed, and their feeding was controlled three times a day. The feed was in the form of crumbles, which were processed by PT. Sari Rosa Asih's industry partner. The KUB chickens in this experiment were vaccinated with Newcastle Disease (ND) vaccine at 4 and 21 days old, and with the Gomboro vaccine at 16 days old. The Medivac La sota vaccine (PT. Medion Farma Jaya, Indonesia) was used for the ND vaccination, and the Gomboro vaccine (PT. Medion Ardhika Bhakti, Indonesia) was registered through drinking water. After the trial, when the chickens were 8 (eight) weeks old with an average weight of 277 g, the performance of KUB chickens was evaluated cumulatively. Four treatments and six replications were applied to a total of 1200 (600 males and 600 females) at the age of 450-old KUB chickens randomly assigned to 24 bamboo cages with electric heating. The average temperatures were 32.5°C during the day (12.00 p.m.) and 30.8°C in the afternoon (5.00 p.m.), respectively. For the first two weeks, heating lamps were utilized both during the day and at night. However, lamps were only utilized for lighting, with two 25-watt bulbs given in separate places from 6 p.m. until 5.00 a.m. for even lighting. The humidity was not particularly measured; however, it was consistently above 70% due to the rainy season during the study. The wire and wooden box cages utilized were 90 cm long, 60 cm wide, and 50 cm high, and each contained 25 chickens. Outside the cage walls were feeding and drinking stations constructed of plastic, with two of each provided in each cage. The four treatment groups are displayed in Table 1.

Table 1. Treatment grouping for Kampung Unggul Balitbangtan chickens aged eight weeks

Group	Treatment
T0	Control (basal diet, without aflatoxin B1)
T1	T0 + 200 µg/kg aflatoxin B1
T2	T0 + 200 µg/kg aflatoxin B1 + 1 g/kg Factory Feed with standard factory absorbent
T3	T0 + 200 µg/kg aflatoxin B1 + 1 g/kg nano bentonite

Performance of the growth process and nutrient digestion

A basal feed with the composition of the ingredients shown in Table 2 was used for pretreatment in day-old chicks up until week 2 (NRC, 1994). The information that was logged weekly included body weight, feed intake, and feed conversion. Two chickens from each experimental group were chosen randomly and slaughtered at the experiment's conclusion for hematopathology and histopathology examinations.

Hematology analysis

A 5 mL blood sample was collected from chickens by wing vein puncture within 2 minutes of removing the hen from its cage. The heparinized blood was stored on ice. Blood samples were centrifuged at x 700 for 15 minutes at 20℃ (Cheng et al., 2001). Hemoglobin, leukocytes, Packed Cell Volume (PCV), Total Plasma Protein (TPP), and fibrinogen were analyzed. Blood smears were prepared by placing a drop of blood on a glass slide and fixing it with methanol.

Histopathology analysis

After the trial was completed, two chickens from each treatment were subjected to ether anesthesia and subsequently dissected to extract the liver, spleen, and duodenum. The samples were preserved with 10% formalin, dehydrated, embedded in paraffin, and sectioned into slices that are 5 µm thick, ensuring the preservation of their integrity. These thin tissue slices were then mounted on glass slides and stained with Hematoxylin and eosin, which highlighted cellular structures and enabled observation of tissue morphology. Finally, the stained slides were examined under a microscope (Motic, China) to identify any abnormalities or histopathological changes with 40x magnification. The assessment and evaluation of

these changes are based on established criteria and scoring systems, which help to provide a standardized and objective assessment of the tissues (Ross and Pawlina, 2010).

Table 2. The composition of the basal diet for a duration of 8 weeks

Feed ingredients	Kg	CP (%)	ME (Kcal/kg)	CFa (%)	CFi (%)	Ca (%)	P (%)	Met (%)	Lis (%)	Trp (%)
Corn	60	5.16	2.022	2.34	1.20	0.012	0.18	0.108	0.12	0.06
Soybeans	34	16.49	860.2	0.27	1.02	0.088	0.21	0.248	1.08	0.22
Tallow	2.5	-	175.25	2.50	-	-	-	-	-	-
Bisphosphonates	0.5	-	-	-	-	-	0.10	-	-	-
Lime	1	-	-	-	-	0.76	-	-	-	-
Multivitamins	0.35	-	-	-	-	-	-	-	-	-
Fine	1.5	-	-	-	-	-	-	-	-	-
Total	100	21.65	3057.5	5.11	2.22	0.94	0.49	0.356	1.20	0.28

Kg: Kilogram; CP: Crude protein; ME: Metabolizable energy; CFa: Crude fat; Cfi: Crude fiber; Ca: Calcium; P: Phosphorus; Met: Methionine; Lis: Lysine; and Trp: Tryptophan (Source: NRC, 1994).

Statistical analysis

Body weight, feed consumption, and feed conversion were all tracked every week. Data obtained were analyzed using SPSS software (version 22). In a Completely randomized design (CRD), the ANOVA was used, and Duncan's Multiple Range Test was followed to find the significant level. The chosen level of significance for all comparisons was $p < 0.05$.

RESULT AND DISCUSSION

In the previous investigations, bentonite was also utilized as an aflatoxin absorbent; however, the highest aflatoxin absorption was only 78% (Nuryono et al., 2012). This low absorption could be because the particles used in this study were micron-sized. In the present study, the morphology of bentonite-A and B was investigated using a transmission electron microscope. The findings are shown in Figure 1. According to Gong et al. (2016), bentonite-A and B micrographs show a structure made up of thin layers.

Figure 1 A depicts the inter-silicate layer on bentonite-A, enabling observation of the monolayer silicate layers. The dark shade, consistent throughout the sample, reveals the well-defined structure of the inter-silicate layer in bentonite-A. This structure's clarity allows for a comprehensive view of the bentonite-A layers, allowing for an in-depth examination of their properties. Figure 1 B, on the other hand, shows that the shade of the bentonite-B sample is less dark in some areas, especially in the red circle, suggesting a looser structure. This could be due to the inter-silicate layers' decreased packing density, which can cause variations in the degree of coloration. Other variables, such as differences in mineral composition or processing techniques, could also account for the looser structure of the bentonite-B sample.

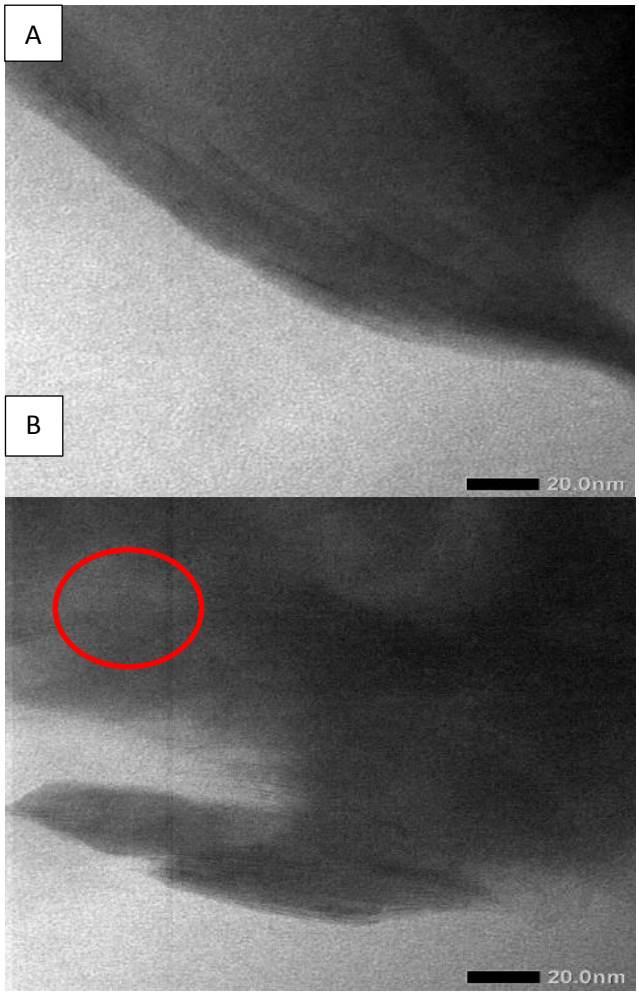

Figure 1. The morphological characteristics of natural (A) and activated (B) bentonite under transmission electron microscopy at a 20 nm spatial resolution

Table 3. HPLC and ELISA analysis of feed for Kampung Unggul Balitbangtan chickens aged 8 weeks

Treatment	HPLC (ppb)	ELISA (ppb)
T0	-	13.0
T1	236.0	208.0
T2	192.0	224.0
T3	178.0	218.0

HPLC: High-performance liquid chromatography; ELISA: Enzyme-Linked Immunosorbent Assay; T0: Control (basal diet, without aflatoxin B1); T1: T0 plus 200 µg/kg aflatoxin B1; T2: T0 plus 200 µg/kg aflatoxin B1 plus 1 g/kg factory feed with standard factory absorbent; T3: T0 plus 200 µg/kg aflatoxin B1 plus 1 g/kg nano bentonite

An analysis was carried out using HPLC and ELISA to determine the degree of aflatoxin B1 contamination in the animal feed utilized in this experiment. The fundamental goal of HPLC is to identify, quantify, and analyze the concentrations of active components in a sample; meanwhile, the basis of ELISA is the highly-sensitive and specific interactions between antigens and antibodies. Table 3 indicates that the highest level of contamination was observed in T1 during HPLC analysis, where the base diet was contaminated with 200 µg/kg of aflatoxin B1. However, during ELISA analysis, the highest contamination was detected in T2, where the base diet was contaminated with 200 µg/kg of aflatoxin B1, and 1 g/kg of standard factory absorbent was added.

The T3 treatment with a basal diet combined with 200 µg/kg aflatoxin B1 and 1 g/kg nano bentonite had the lowest value on HPLC analysis in the aflatoxin B1-contaminated treatment. Bentonite, a type of clay, is abundant in silica and aluminum minerals that contain OH groups. These OH groups can bind with the hydrogen substrate of aflatoxins, causing them to transform into H_2O and be eliminated from the chicken's body via the urinary system. The ability of bentonite to absorb toxins in the gastrointestinal tract is a widely recognized mechanism. It works by slowing down nutrient movement in the intestine, which results in slower digestion and increased feed digestibility. It is possible to improve the availability of lipase, phospholipase, and carbohydrase by employing bentonite, which is rich in silicon and aluminum. It is because bentonite enhances the binding of digestive enzymes or co-factors (Moosavi, 2017).

The results of research performance that considered feed intake, body weight, and feed conversion ratio are shown in Table 4. The feed consumption in this study ranged from 786.82 to 792.79 g (Figure 2). The statistical analysis of each treatment did not indicate any significant differences in the outcomes ($p < 0.05$). It is likely because the treatment feed was formulated using identical ingredients and had comparable levels of protein and energy, with a crude protein content of 19% and a metabolic energy level of 2,800 kcal/kg. As a result, it was possible to achieve similar feed consumption across all treatments without any notable disparities. According to research on feed consumption in KUB chickens of that age in different Indonesian locations, it ranged from 727 to 776 g, 600 to 700 g, and 450 to 500 g, and in the meantime, it reached 803 to 870 g (Sinurat, 2017; Yunianta et al., 2021). It demonstrates that feed consumption throughout the trial was within the usual range for commercial feed that is not contaminated with aflatoxin and those that have binders added to it.

Table 4 shows the subjects' body weight during the investigation. T0 had the highest weight (538.77 g), while T1 had the lowest (424.27 g). According to the statistical analysis, there was a substantial weight loss in the aflatoxin-contaminated meal for T0 compared to T1 ($p < 0.05$). Aflatoxin-free diet did not significantly differ from feed containing a commercial binder and nano bentonite, so commercial binder and nano binder may prevent weight loss brought on by aflatoxin contamination ($p < 0.05$). Figure 3 illustrates that the weight of KUB chickens in the T0 treatment group consistently increased over the course of the study, resulting in overall stable weight, while the T1 treatment group exhibited a delay in weight gain during the final week of the experiment. In the first and second weeks of the study, there was still no significant difference between the T1 treatment group and other treatments in terms of feed consumption ($p < 0.05$). This is due to aflatoxin not being utilized at T0 and its mixing without a binder at T1. The results show how aflatoxin contamination in the chicken's feed impacted the growth of muscle mass. The body weight of chickens can vary significantly, as evidenced by previous research (Kurniasih and Prakoso, 2019).

Table 4. Performance of Kampung Unggul Balitbangtan chickens aged 8 weeks

Mean ± SD Treatment	Body weight (g)	Feed consumption (g)	Feed/bodyweight ratio
T0	$538.77^{a} \pm 28.57$	$786.82^{a} \pm 13.14$	$1.78^{c} \pm 0.11$
T1	$424.27^{b} \pm 31.24$	$792.79^{a} \pm 45.38$	$2.24^{a} \pm 0.23^{c}$
T2	$485.78^{a} \pm 22.42^{a}$	$790.20^{a} \pm 16.99$	$1.95^{b} \pm 0.16^{b}$
T3	$485.65^{a} \pm 23.04^{a}$	$782.21^{a} \pm 15.80$	$1.92^{b} \pm 0.13^{c}$

[a-c] Means within each column with different superscripts are statistically different $p < 0.05$. SD: Standard Deviation; T0: Control (basal diet, without aflatoxin B1); T1: T0 plus 200 µg/kg aflatoxin B1; T2: T0 plus 200 µg/kg aflatoxin B1 plus 1 g/kg factory feed with standard factory absorbent; T3: T0 plus 200 µg/kg aflatoxin B1 plus 1 g/kg nano bentonite

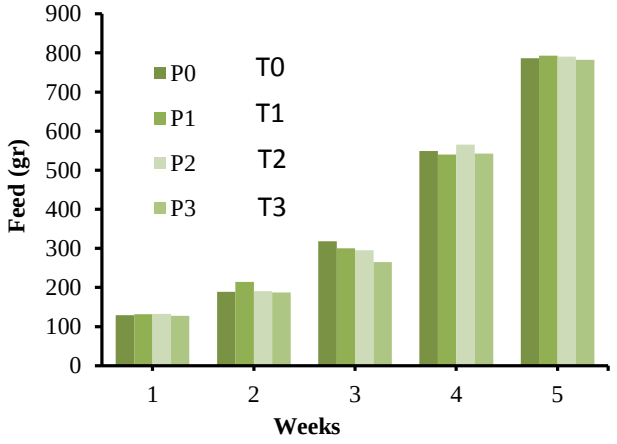

Figure 2. Feed consumption of Kampung Unggul Balitbangtan chickens for five weeks. T0: Control (basal diet, without aflatoxin B1), T1: T0 plus 200 µg/kg aflatoxin B1, T2: T0 plus 200 µg/kg aflatoxin B1 plus 1 g/kg factory feed with standard factory absorbent, T3: T0 plus 200 µg/kg aflatoxin B1 plus 1 g/kg nano bentonite.

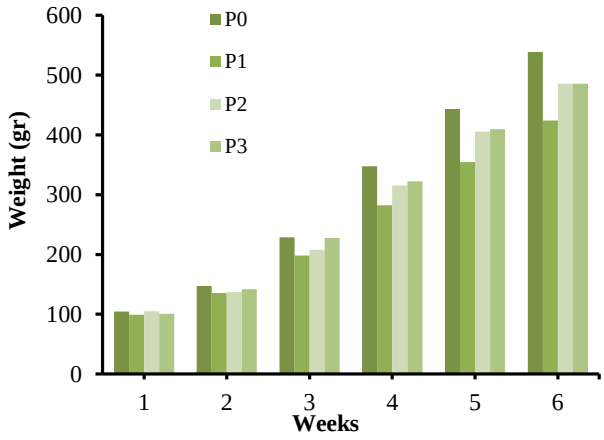

Figure 3. Weight gain of *Kampung Unggul Balitbangtan* chickens for 6 weeks. T0: Control (basal diet, without aflatoxin B1), T1: T0 plus 200 µg/kg aflatoxin B1, T2: T0 plus 200 µg/kg aflatoxin B1 plus 1 g/kg factory feed with standard factory absorbent, T3: T0 plus 200 µg/kg aflatoxin B1 plus 1 g/kg nano bentonite.

According to Table 4, group T1 demonstrated a significantly higher Feed Conversion Ratio (FCR) compared to the other groups ($p < 0.05$). This result confirmed that aflatoxin had affected the chicken's metabolism by disrupting the function of the intestines and liver (Zuidhof et al., 2014). Additionally, Figure 4 shows that among the treatment group infected with aflatoxin, T2 had the highest FCR, and T0 had the lowest. It is because the weight of the KUB Chicken organs engaged negatively impacts aflatoxin due to cellular abnormalities (Kurniasih and Prakoso, 2019). In this research, the aflatoxin-contaminated feed had a higher feed conversion ratio (FCR) than the control feed that was not contaminated with aflatoxin. Similarly, a hepatological examination of the liver revealed liver damage in the contaminated feed group, especially in the adsorbent-free group (T1). It suggests that aflatoxin-contaminated grain increases FCR. This negative impact, however, can be mitigated by using nano bentonite adsorbents or commercial adsorbents in the feed. This result is consistent with Yunianta and Agus (2013) research, which found that broiler chickens fed with aflatoxin-contaminated feed had liver damage, which can disrupt metabolism and reduce productivity.

The atheroma was observed in the tunica media, which is attributed to the damaging effects of aflatoxin on the endothelium. The intima also included foam cells. At the cellular level, increased vacuolation of aflatoxin-exposed hepatocytes allows for the accumulation of high levels of lipids, which is why foam cells form. Lymphocyte infiltration occurs when lymphocytes invade the hepatic portal vein and form clumps or follicles. The increased formation of globules of triglycerides and other lipid metabolites within the cytoplasm is known as hepatocellular fatty vacuolation (hepatocyte degeneration), which results in microscopic holes or blank areas in the liver. Necrosis will result from hepatocytes that have too much fat in them. Table 5 displays the impact of each treatment on the liver, spleen, and lungs.

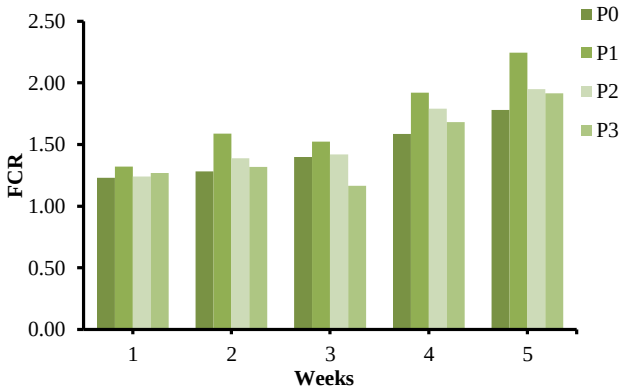

Figure 4. Feed conversion ratio chart of *Kampung Unggul Balitbangtan* chickens for five weeks. T0: Control (basal diet, without aflatoxin B1), T1: T0 plus 200 µg/kg aflatoxin B1, T2: T0 plus 200 µg/kg aflatoxin B1 plus 1 g/kg factory feed with standard factory absorbent, T3: T0 plus 200 µg/kg aflatoxin B1 plus 1 g/kg nano bentonite.

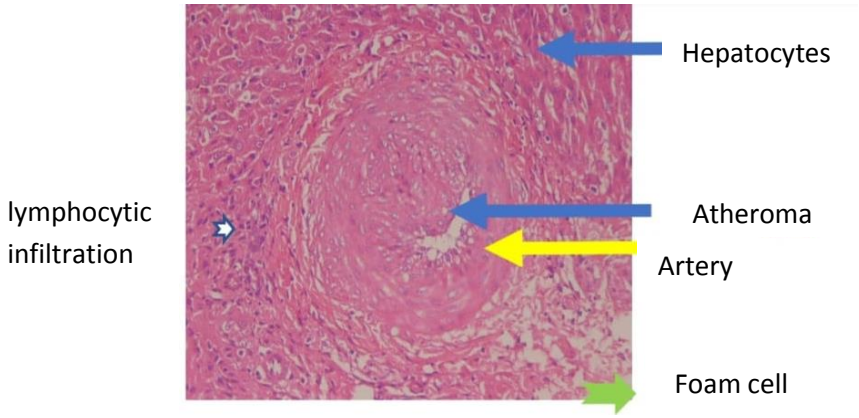

Figure 5. The hepatic tissue of Kampung Unggul Balitbangtan chicken received aflatoxin B1 and factory feed with standard factory absorbent. The triassic zone, Hematoxylin and eosin, 40x

Table 5. Effects of several treatments on the liver, spleen, and lung of Kampung Unggul Balitbangtan chicken for 8 weeks

Treatment	Liver	Spleen	Lung
T0	No pathological changes Mild lipidosis scale 2	There were no pathological changes	There were no pathological changes
T1	Multifocal necrosis Severe lipidosis scale 4 Mild lymphocytic infiltration	25% necrosis Lymphocyte	25% had pneumonia
T2	Bridging necrosis Severe lipidosis scale 4 Mild lymphocytic infiltration	25% necrosis Lymphocyte	25% had pneumonia
T3	No necrosis Mild lipidosis on a scale of 1 Multifocal and 50% parenchymal infiltration	25% necrosis Lymphocyte	25% had pneumonia

T0: Control (basal diet, without aflatoxin B1); T1: T0 plus 200 µg/kg aflatoxin B1; T2: T0 plus 200 µg/kg aflatoxin B1 plus 1 g/kg factory feed with standard factory absorbent; T3: T0 plus 200 µg/kg aflatoxin B1 plus 1 g/kg nano bentonite.

Table 6. Blood parameters of old Kampung Unggul Balitbangtan chickens aged 8 weeks after several treatments

Treatment	Erythrocyte (10^6/µL)	PCV (g/dL)	TPP (%)	Fibrinogen (cells/µL)	Leukocytes (cells/µL)
T0	3.47	27.50	4.47	0.60	10375
T1	0.99	23.00	4.30	0.90	19125
T2	1.16	27.25	4.80	0.45	16866
T3	1.36	31.25	4.25	0.30	17975

PCV: Packed cell volume; TPP: Total plasma protein; T0: Control (basal diet, without aflatoxin B1); T1: T0 plus 200 µg/kg aflatoxin B1; T2: T0 plus 200 µg/kg aflatoxin B1 plus 1 g/kg factory feed with standard factory absorbent; T3: T0 plus 200 µg/kg aflatoxin B1 plus 1 g/kg nano bentonite

Table 6 contains data on collected and analyzed blood samples of KUB chickens. As shown in Table 6, Aflatoxin B1 infection resulted in a decrease in erythrocyte production when compared to T0 (control, $p < 0.05$). Among the three groups that received Aflatoxin B1, group T3 had the highest erythrocyte count. Aplastic anemia is defined by low erythrocyte counts (Hsi-Tang et al., 1975). Mild anemia is usually present when the packed cell volume (PCV) of the blood being examined is low (Nwogor et al., 2015). The treatment of the T3 group indicates that this group had no anemia. This study's analysis of the total plasma protein (TPP) test did not show any significant differences between the different treatment groups ($p < 0.05$). It is possible due to the liver's minor but non-fatal damage, which enables near-normal albumin production. When activated by infection, the amount of fibrinogen in the blood increases (Roy et al., 2014). According to the data in Table 6, the fibrinogen in

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the T3 group's treatment had the lowest value. If the blood leukocyte count is high, it is likely that the leukocytes are actively fighting an internal infection or that the organism is experiencing a stressful period (James et al., 2019). Even though the T3 group's value in the leukocytes test is not the lowest, it is not far from it. It can be inferred that the nano-bentonite binder effectively bound the toxin, Aflatoxin B1, in the feed mill.

CONCLUSION

Based on the findings of this research, it is achievable to determine that using nano bentonite as an adsorbent in Kampung Unggul Balitbangtan chicken diets contaminated with aflatoxin can significantly reduce its toxicity. According to the hematological analysis, the binder was more effective in group T3 where they had the highest erythrocyte count and the lowest fibrinogen, resulting in a reduced feed conversion ratio value. To completely comprehend the efficacy of nano bentonite as an adsorbent for aflatoxin-contaminated Kampung Unggul Balitbangtan chicken diets, more research is recommended until the production stage of local chickens is reached.

DECLARATION

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Authors' contributions

Yunianta, Ari Astuti, and Hermantoro Sastrohartono conducted the experiment; Nanang Kusuma Mawardi, Maria Theresia Darini, Khusnan, and Mohammad Sofi'ul Anam conducted the analysis and data calculations; and, Yunianta, Ari Astuti, Nanang Kusuma Mawardi, and Maria Theresia Darini wrote and revised the manuscript. All authors agreed to the final version of this manuscript.

Competing interests

The author states that this manuscript has no personal or other conflicts of interest.

Ethical consideration

All authors have reviewed all essential ethical issues.

Availability of data and materials

The authors confirm that the data supporting the study's results are accessible upon reasonable request from the corresponding author.

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The Effect of Dietary Supplementation of Hong Kong Caterpillar (*Tenebrio molitor*) on Quail Egg Quality

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ABSTRACT

The Hong Kong caterpillar (HC) is an alternative source of animal protein for feed. This research aimed to study the effect of using Hong Kong caterpillars in the quail diet on egg quality. A total of 200 quail aged 8-14 weeks, weighing 110 ± 10 g, were used in the study, with 40% production. This study used a completely randomized design with five treatments and four replications. The laying quail diets were formulated with varying levels of HC, including 0% HC for group A, 3% HC for B, 6% HC for C, 9% HC for D, and 12% HC for E. The egg quality parameters measured were egg yolk fat, egg yolk cholesterol, egg white protein, and eggshell thickness. The results indicated that including 12% HC in the quail diet significantly reduced egg yolk cholesterol and egg yolk fat. However, eggshell thickness and egg white protein remained unaffected. Consequently, it can be concluded that Hong Kong caterpillars can be used in quail diets up to a maximum of 12% to reduce egg yolk cholesterol and fat while maintaining eggshell thickness and egg white protein levels.

Keywords: Egg quality, Fish meal, Hong Kong caterpillar, Quail

INTRODUCTION

Poultry farming is a promising business venture due to the growing demand for poultry eggs yearly. Eggs, including quail eggs, are a valuable source of animal protein that humans require and are relatively affordable compared to other animal protein sources, such as meat. Moreover, eggs are considered a complete source of nutrients, providing a balanced combination of essential amino acids, minerals, and vitamins (Saraswati and Tana, 2021).

Quail (*Coturnix coturnix japonica*) is cultivated for its eggs due to its high egg productivity, reaching 200-300 eggs/head/year (Amo et al., 2013). Quail eggs are foods with complete nutritional content that are helpful for the body, especially for children in their infancy. The nutritional content of quail eggs is 3-4 times greater than that of chicken eggs. Quail eggs have a nutrient composition that includes 13.30% crude protein, 11.99% crude fat, and 1993 kcal/kg metabolic energy (Thomas et al., 2016). In addition, they contain various amino acids, such as lysine 790 mg/100 g, valine 865 mg/100 g, and leucine 1139 mg/100 g, and fatty acids such as linoleic 2.58 g/100 g, oleic 8.84 g/100 g, palmitic 5.39 g/100 g, and stearate 2.03 g/100 g. However, quail eggs contain a high amount of egg yolk cholesterol (877.38 mg/100 g; Tunsaringkarn et al., 2013; Nuraini et al., 2020).

During 2016-2020, quail egg production increased from 23.575 tons to 24.205 tons (Statistics of Livestock and Animal Health, 2020). Quail eggs are in great demand among the public and producing high-quality quail eggs suitable for feed (Emeka Aba and Anayo Onah, 2015). Currently, fish meal is the primary source of animal protein used in artificial feed, as it has a high crude protein content of 64.27% (Praptiwi and Wahida, 2021). However, the availability of imported fish meals is still a challenge, and alternative sources of animal protein are needed (Nuraini et al., 2022). One alternative feed source of

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animal protein is the Hong Kong caterpillar (HC, *Tenebrio molitor*), which is the larvae of insects found in grain products with high nutritional content. The HC cultivation is relatively easy and has promising business opportunities due to increasing demand. Moreover, HC has a protein content based on the nutrient content of their culture media (Nuraini et al., 2022).

The nutritional content of HCs with a mixture of 50% concentrate culture medium and 50% tofu dregs fermented with microbes in Natura Organic Decomposers was 62.35% crude protein, 17.07% crude fat, 7.35% crude fiber, 0.23% calcium, 0.97% phosphorus, and 3998.31 kcal/kg metabolic energy (Nuraini et al., 2022). Hong Kong caterpillars contain high levels of amino acids, such as glutamic acid (6.86%), alanine (5.37%), aspartic acid (4.80%), lysine (4.75%), leucine (4.49%), valine (3.83%), glycine (3.40%), tyrosine (3.04%), and methionine (0.43%). They also contain high unsaturated fatty acid percentages, including 34.24% linoleic acid (omega-6), 21.28% oleic acid (omega-9), 2.45% stearic acid, 1.20% myristic acid, and 0.15% linolenic acid (omega-3), and saturated fatty acids, such as 10.04% palmitic acid (Nuraini et al., 2022). Hong Kong caterpillars contain 7.2% chitin in the larval phase, while it is 9.54% in the pupal phase, and 11.8% in the adult phase (Yu et al., 2021).

The use of *Tenebrio molitor* in the diet of laying hens as a replacement for meat bone meal up to 5% can lead to good production performance, including increased egg weight and improved egg quality with the production of omega-3 fatty acids (Rahmawati et al., 2022). Hong Kong caterpillars, as a source of animal protein, have a high crude protein content (62.35%), high lysine content (4.75%), and high linoleic acid content (34.24%, Nuraini et al., 2022). Hong Kong caterpillars have almost the same crude protein content as grade one fish meal, that is 64.27% (Praptiwi and Wahida, 2021), 5.0% amino acid lysine, and 8.30% linoleic fatty acid (Zahroh et al., 2015). This makes them a suitable replacement for imported fish meals in the diet of quail.

High linoleic acid in the diet can affect the cholesterol content of the egg yolk (Fukumitsu et al., 2013), as alpha-linoleic acid (ALA) reduces cholesterol synthesis (Fukumitsu et al., 2013). Cholesterol synthesis uses saturated fatty acids as raw materials, while unsaturated fatty acids are not used in cholesterol synthesis (Ye et al., 2011). Chitin compounds found in HC can also reduce the egg yolk fat content. Chitin is a high fiber content that binds with fat and cholesterol, inhibiting their absorption by the body (Ye et al., 2018). It has a positive impact on lowering egg yolk fat levels and is also more effective in fat absorption (Ambarwati, 2017). As more cholesterol is bound to chitin, the greater the reduction in the cholesterol content of quail eggs (Herdyastuti and Daniar, 2019).

Shell thickness is closely related to the levels of calcium and phosphorus in the diet. The calcium and phosphorus contents in HCs (0.19% and 0.82%, respectively), are lower than those in imported fish meal (3.10% calcium and 1.89% phosphorus; Nuraini et al., 2022). The effect of using HCs as a substitute for imported fish meal on egg yolk fat, egg yolk cholesterol, egg white protein, and eggshell thickness of quail eggs is still unknown. Therefore, the current study aimed to evaluate the effect of HCs as a replacement for imported fish meals on quail egg quality.

MATERIALS AND METHODS

Ethical approval

This research was approved by the Research Ethics Committee of the Faculty of Animal Science, Universitas Andalas Padang, Indonesia. Ethics study of experimental animals' guideline, according to law number 18 of the Republic of Indonesia (2009) about Animal livestock and animal husbandry.

Birds and housing

Quail were kept in a quail house at the Faculty of Animal Science, Universitas Andalas Sumatra Barat, Indonesia. The temperature of the quail house ranged 23-25°C with a humidity of 50-70%. Vaccination was administered to one-week-old quails by the breeders, and vaccination was carried out via drinking water when the quails reached 4 weeks old. The lighting on laying quail maintenance was given 15 hours a day.

The experimental birds used in this study were 200 quails (*Coturnix coturnix japonica*) in the 8-week-old layer phase (weight of 110-120 gram) with 40% egg production. Quail were reared for 6 weeks to evaluate the egg quality of the quail. The laying quails were purchased from quail breeders at the age of 3 weeks and reared up to 6 weeks of age with commercial rations. Following this, a two-week adaptation period to the treatment ration was conducted before commencing the actual treatment, which took place between the ages of 8 to 14 weeks. The experimental setup consisted of 20 wire cages, each measuring 60x50x50 cm and housing 10 quails per cage unit.

Hong Kong Caterpillar propagation

The culture medium for HCs consisted of Concentrate 126 production PT Charoen Phokp and tofu waste (1:1) that was sterilized in an autoclave, cooled to room temperature (27-30°C), inoculated with 1% Natura Organic Decomposer (1 ml of Natura Organic Decomposer with 100 ml of water and 2 g of sugar) and incubated for 4 days. After that, the fermented medium was dried and ground. Then, the medium was put into a biopon (up to 500 g), and 5 g of HCs were added and aged for 10 days. After 30 days, the HCs were harvested and sieved to separate them from the media. Finally, the HC was dried and ground into flour (Nuraini et al., 2022).

Treatment feed

The feed given to quail were self-mixed diets using feed ingredients, such as corn meal, soybean meal, imported fish meal, HC, fine bran, coconut oil, bone meal, and top mix. All the ingredients for the rations were finely ground and mixed into rations. The rations were given in mash form. The water was given *ad libitum*. The content of feed substances and metabolic energy of the ingredients for the treatment diets are shown in Table 1.

Research design

The current experimental method employed a completely randomized design with five treatments and four replications. The treatment diets used HCs in quail diets at percentages of 0% (A), 3% (B), 6% (C), 9% (D), and 12% HC (E). A total of 200 quails were divided into five groups (A, B, C, D, and E), with 40 quails per group. Each group was further subdivided into four replications denoted as A1, A2, A3, and A4, with each replication comprising of 10 quails. The diet was composed of iso-protein and iso-energy with a crude protein content of 20% and metabolic energy of 2800 kcal/kg. The composition of the feed ingredients of the treatment diets is shown in Table 2, and the contents of feed substances and metabolic energy of the treatment diets are presented in Table 3.

Measured parameters

The study measured several parameters related to egg quality. The fat content of the egg yolk was determined as a percentage using the Sokhlet method, according to the Association of Official Analytical Chemists (AOAC, 2016). The egg yolk cholesterol concentration was also measured and expressed as milligrams per 100 grams of egg using the Lieberman-Buchards method as described in a study by Sudarman et al. (2018). Additionally, the egg white protein content was measured as a percentage using the Kjeldahl method, which is a standard analytical procedure established by the AOAC (2016). Finally, the thickness of the eggshell was determined by breaking quail eggs and measuring the eggshell parts at the blunt end, middle, and pointed end using a scrub micrometer. The eggshell thickness was determined following the protocol described by Sudrajat et al. (2014).

Data analysis

All data obtained were statistically processed by SAS software (version 2003), and analysis of variance was done. Duncan's multiple range test was used to evaluate significant differences between treatments ($p < 0.05$).

Table 1. The feed ingredients and metabolic energy (kcal/kg) of the quail diet[a]

Nutrient Feed Ingredient (%)	Crude protein	Fat	Crude fiber	Ca	P	EM (kcal/ kg)[c]	Lysine	Methionine	Glutamate	Linoleate	Chi tin
Corn meal	9.58	2.66	3.50	0.38	0.19	3300	0.20	-	2.28	2.20	-
Soybean meal	43.76	2.49	4.50	0.63	0.36	2240	2.60	0.50	8.15	0.81	-
Import ted fish meal	64.00	2.85	3.90	3.10	1.89	2540	5.00	0.99	7.30	8.30	-
Hong Kong caterpillar[b]	62.35	14.96	6.18	0.19	0.82	3362	4.75	0.43	6.86	34.24	7.20
Fine bran	12.34	5.09	14.50	0.69	0.26	1630	0.77	0.29	1.92	3.57	-
Coco nut oil	-	100.00	-	-	-	8600	-	-	-	1.92	-
Vitamin B12 [d]	-	-	-	49.00	14.00	-	-	-	-	-	-
Top mix [d]	-	-	-	0.06	-	-	-	-	-	-	-

[a] Nuraini et al. (2020); [b] Nuraini et al. (2022) [c] Scott et al. (1982); [d] Top Mix production of PT.

Table 2. Composition of the feed ingredients in each treatment diet

Feed Ingredient (%) \ Treatment Ration	A	B	C	D	E
Corn meal	52.75	52.75	52.75	52.75	52.75
Soybean meal	13.00	13.25	13.25	13.50	13.75
Fish flour	12.00	9.00	6.00	3.00	0.00
Hong Kong caterpillar	0.00	3.00	6.00	9.00	12.00
Fine bran	14.00	14.00	13.75	13.75	13.75
Coconut oil	2.75	2.50	2.25	2.00	1.75
Vitamin B12	5.00	5.00	5.50	5.50	5.50
Top mix	0.50	0.50	0.50	0.50	0.50
Total	100.00	100.00	100.00	100.00	100.00

Table 3. Feed substances and metabolic energy (kcal/kg) of each treatment diet

Nutrient (%) \ Ration	A 0% HC	B 3% HC	C 6% HC	D 9% HC	E 12% HC
Crude protein	20.15	20.21	20.13	20.19	20.25
Fat	5.53	5.65	5.75	5.87	5.99
Crude fiber	3.96	4.08	4.16	4.28	4.40
Calcium	3.20	3.12	3.27	3.19	3.10
Phosphorus	1.11	1.08	1.12	1.09	1.05
Lysine	1.15	1.15	1.14	1.14	1.14
Methionine	0.40	0.40	0.39	0.39	0.38
Glutamate	3.40	3.40	3.39	3.40	3.41
Linoleate	2.81	3.69	4.35	5.13	5.91
Chitin	0.00	0.22	0.43	0.65	0.86
Metabolic energy	2.801	2.810	2.809	2.818	2.826

Description: Calculated based on Tables 1 and 2

RESULTS AND DISCUSSION

Egg quality

Egg quality is a collection of egg characteristics that affect consumer tastes. Egg quality refers to several standards that determine both internal and external quality. The quality of internal eggs carried out in this study was the content of cholesterol, fat and protein, and shell thickness for external egg quality. The previous study, according to Nuraini et al. (2022), *Tenebrio molitor* caterpillar could be used up to 12% in laying quail rations (substituted 100% fish meal) and maintain the same egg production as control. Nuraini et al. (2020) found the quail egg yolk fat 27.98- 29.58% and the egg yolk cholesterol 711.00-877.38 mg/100 g when fed fermented cacao pod. Jajić et al. (2020) found unsaturated fatty acid content of *Tenebrio molitor* larvae is very high, that is oleic acid (C18:1) in 40.83%, linoleic acid (C18:2, omega-6 fatty acid) with 29.80% and linolenic acid (C18:3) with 1.08%. Nuraini et al. (2022) found the fatty acid content of *Tenebrio molitor* was 34.24% linoleic acid and 21.28% oleic acid. The longer the carbon chain and the greater the number of double bonds in an unsaturated fatty acid, the greater its tendency to lower cholesterol levels in eggs (Shramko et al., 2020). Ait-Kaki et al. (2022) have reported the egg cholesterol of laying hens decreases if fed *Tenebrio molitor* larvae and Turmeric powder (Curcuma). According to Secci (2021), found the egg shell thickness of quail 29.52 µm when fed *Tenebrio molitor* larvae meal replaced 20% protein of soybean. Table 4 tabulates the data on the quality of quail eggs when the quails' diet was supplemented with HCs for 6 weeks.

Egg yolk fat

Table 4 shows that the egg yolk fat of quail eggs was affected by HCs in the diet and ranged 31.47-36.09%. Moreover, the use of HCs up to 12% in the diet had a significant effect on quail egg yolk fat compared to other treatment groups ($p < 0.05$). The test results showed that quail egg yolk fat in treatment E (12% HC) was significantly lower than that in treatments D, C, B, and A ($p < 0.05$).

Compared to treatments D, C, B, and A, the lower quail egg yolk fat in treatment E was associated with decreased quail egg yolk cholesterol. The study indicated that the concentration of cholesterol in quail egg yolk decreased by 26.14% in treatment E, compared to other groups. This reduction in cholesterol content also led to a decrease in the fat content of the quail egg yolk since

cholesterol is a lipid. Regarding the chemical structure of cholesterol, Yanagisawa et al. (2022) have reported that cholesterol is a complex lipid compound. The broader category of lipids includes consists of triglycerides (neutral fat), phospholipids, and cholesterol. According to Huff et al. (2022), fat is composed of various components, including cholesterol, pigments, and vitamins A, D, E, and K.

The chitin content also influenced the decrease in egg yolk fat in treatment E in the diet. The chitin content in treatment E was 0.86%. This amount is related to the chitin content of the HC, which is 7.2% in the larval stage (Yu et al., 2021). Chitin binds fat, and the bound fat is carried away in the feces (van den Broek and Boeriu, 2019). According to Zhen et al. (2022), chitin can bind fat (as well as cholesterol) to inhibit fat absorption by the body. The fat bound to chitin forms a compound that is not absorbed. The decrease in fat absorption is because chitin in the stomach is converted into a gel by gastric acid and then wraps cholesterol and fat molecules in gastric juice (Zhou et al., 2020).

Egg yolk cholesterol

The highest quail egg yolk cholesterol was recorded for treatment A (0% HC, 965.13 mg/100 g), and the lowest egg yolk cholesterol was seen in treatment E (12% HC, 712.88 mg/100 g). The analysis of variance showed that the use of HCs up to 12% in the quail diet had a significant effect on quail egg yolk cholesterol ($p < 0.05$), compared to another group. It was found that quail egg yolk cholesterol with treatment E was significantly lower than that with treatments D, C, B, and A ($p < 0.05$). Increasing the use of HCs could reduce the quail egg yolk cholesterol content. The high level of the unsaturated fatty acid linoleic acid caused the low cholesterol content in quail egg yolks with diet treatment E. The E treatment resulted in a linoleic fatty acid content of 5.91%, which was related to the high linoleic acid content in HCs. A recent study found that the fatty acid content of HCs was 34.24% linoleic acid and 21.28% oleic acid (Nuraini et al., 2022). Jajić et al. (2020) found unsaturated fatty acid content of Tenebrio molitor larvae is very high, that is oleic acid (C18:1) in 40.83%, linoleic acid (C18:2, omega-6 fatty acid) with 29.80% and linolenic acid (C18:3) with 1.08%. Linoleic unsaturated fatty acid is a fatty acid that contains double bonds (Coniglio et al., 2023). According to Shramko et al. (2020), the longer the carbon chain and the greater the number of double bonds in an unsaturated fatty acid, the greater its tendency to lower cholesterol levels. According to Azemi et al. (2023), linoleic acid is one of the foods shown to lower cholesterol levels in the blood. Research by Azemi (2023) showed that the linoleic acid content could also reduce cholesterol. The provision of a diet containing the unsaturated fatty acids linoleic acid and linolenic acid can lower cholesterol, triglyceride, and LDL levels and raise HDL levels (Dwiputra et al., 2015).

Furthermore, one factor that lowers blood cholesterol is replacing saturated fatty acids with polyunsaturated fatty acids (unsaturated fatty acids). A study by Fukumitsu et al. (2013) showed that alpha-linolenic acid (ALA) reduced cholesterol synthesis. According to Jajić et al. (2020), Tenebrio molitor has linoleic acid 28% and linolenic acid 1%. Cholesterol synthesis uses saturated fatty acids as raw materials, while unsaturated fatty acids are not used in cholesterol synthesis. Linolenic acid is an unsaturated fatty acid. Higher linolenic acid levels in the blood are not used for cholesterol synthesis, causing lower egg yolk cholesterol.

The chitin content had a significant effect on the reduction of the egg yolk cholesterol content in treatment E. The chitin content was 0.86% higher in treatment E than in the other treatments. The chitin content of HCs was reported to be 7.2% in the larval stage (Yu et al., 2021). According to Xu (2017), chitin has been shown to bind with bile acids and excrete them in the feces, resulting in lower cholesterol levels due to the requirement of bile acids to form cholesterol. Furthermore, feed containing chitin derived from shrimp heads can reduce duck egg cholesterol levels (Saty et al., 2014).

The quail egg yolk cholesterol level at 12 weeks using HCs in diets up to 12% was found to be 712.88 mg/100 g. This level is lower than that obtained by Nuraini et al. (2017), which measured quail egg yolk cholesterol levels at 20 weeks at 746.38 mg/100 g. The cholesterol content of quail egg yolks was also lower than that of quail egg yolks aged 7-13 weeks, which was reported to be 877.38 mg/100 g by Nuraini et al. (2020).

Egg white protein

In the present study, the use of HC (*Tenebrio molitor*) as a source of animal protein resulted in egg white protein levels in the range of 12.00-12.58%. The results of the analysis showed that HC had no significant effect on quail egg white protein ($p > 0.05$). The crude protein content in the diet from treatment A (0% HC) to treatment E (12% HC) ranged from 20.15%-20.25%, respectively. The protein content of the diet indicated that the protein quality of HCs was 62%, similar to that of fish meal grade one (64%). Using up to 12% HCs in the laying quail diet had a similar effect on quail egg white protein

and could reduce the use of fish meal by 100%. According to Omidiwura et al. (2016), protein is an essential nutrient in the diet since it influences egg production and quality (Ardiansyah, 2016).

The protein content of quail egg whites obtained in this study was within the normal range of 12-12.58%. The protein content of egg white with 12% HC in the quail diet was 12.24%. The protein content of quail egg whites was lower than that obtained in the study by Djaelani (2017), who obtained a quail egg protein percentage of 13.10%.

Egg shell thickness

Table 4 shows that the quail eggshell thickness ranged from 0.27 to 0.29 mm. The dietary supplementation of HCs up to 12% had no significant effect on quail eggshell thickness ($p > 0.05$). The difference stems from the fact that eggshell thickness is closely related to the calcium and phosphorus contents in the diet. The calcium and phosphorus contents in the diet for each treatment were almost the same, ranging from 3.10-3.27% to 1.05-1.12%, respectively. Calcium and phosphorus are needed to form eggshells, and their availability in the blood determines eggshell formation (Rathnayaka et al., 2020).

The present study showed that the shell thickness of quail eggs using 12% HCs in the diet was between 0.27 and 0.29 mm. The results of the thick quail eggshell were higher than those reported in the study by Rondonuwu et al. (2018), in which the quail shell thickness ranged from 0.18 to 0.19 mm. The results of the current study were not much different from the thickness of the quail egg shells obtained by Rudini et al. (2020), which was 0.26-0.27 mm. Based on the results of Ergun and Yamak (2017), a suitable eggshell thickness for quail eggs ranges from 0.2 to 0.35 mm. Agboola et al. (2017) found the quail eggshell thickness to be 0.30 mm when the given ration included protein 20% and energy metabolism 3000 kcal/kg.

Table 4. Egg yolk fat, egg yolk cholesterol, egg white protein, and eggshell thickness of quail eggs (mean ± standard error)

Treatment	Egg yolk fat (%)	Egg yolk cholesterol (mg/100 g)	Egg white protein (%)	Eggshell thickness (mm)
A (0% HC)	$36.09^{a} \pm 0.76$	$965.13^{a} \pm 5.26$	12.00 ± 0.03	0.28 ± 0.02
B (3% HC)	$34.58^{ab} \pm 0.70$	$909.51^{b} \pm 5.02$	12.02 ± 0.02	0.27 ± 0.01
C (6% HC)	$33.56^{ab} \pm 0.74$	$874.75^{c} \pm 4.96$	12.56 ± 0.01	0.29 ± 0.02
D (9% HC)	$33.37^{ab} \pm 0.80$	$845.40^{d} \pm 5.13$	12.43 ± 0.02	0.28 ± 0.01
E (12% HC)	$31.47^{b} \pm 0.79$	$712.88^{e} \pm 5.16$	12.58 ± 0.04	0.28 ± 0.02

[abcde] Different superscripts in the same column showed significant differences at level of $p < 0.05$.

CONCLUSION

In conclusion, the use of up to 12% HCs in the diet of laying quails can replace 100% of imported fish meal, reduce egg yolk fat and egg yok cholesterol, and maintain egg white protein and eggshell thickness. Under these conditions, the obtained egg yolk fat content was 31.47%, the egg yolk cholesterol content was 712.88 mg/100 g (a decrease of 26.14%), the egg white protein content was 12.58%, and the eggshell thickness was 0.28 mm. Therefore, future studies are suggested to investigate the dietary supplementation of HC in laying ducks to obtain the optimum level and to study the efficiency ratio to reduce egg yolk cholesterol of laying ducks.

DECLARATION

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Authors' contributions

Nuraini contributed to creating the research ideas, designing the experiments, analyzing the data, and writing this article. Ade Djulardi and Robi Amizar contributed to using HC in quail feed and reviewed the written paper. Yuliaty Shafan Nur and Yesi Chwenta Sari analyzed the data and revised the article. All authors checked and approved the final version of the manuscript for publication in the present journal.

Ethical consideration

The author has ensured that this article complies with the journal's ethical issues (including plagiarism, consent to publish, infringement, data falsification, double publication, and redundancy) for submission and publication.

Conflict of interests

No conflicts of interest come from all the authors.

Availability of data and materials

Materials and data are provided by the authors upon reasonable request.

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Effects of Replacing Maize by Proso Millet on Performance of Broiler Chickens

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ABSTRACT

The continual rise in the cost of poultry feed ingredients, the fluctuations in price and the comparatively insufficient maize supply have prompted a search for less expensive alternatives. This research study was carried out to investigate the impact of a partial or total replacement of maize with proso millet on performance parameters of broiler chickens, including live body weight, feed conversion ratio, mortality rate and carcass yield. An experiment was carried out using 160 one-day-old broiler chicks of a commercial breed. The chicks were randomly assigned to 5 groups of 32. They consumed different isoprotein and isocaloric diets in which maize was replaced by proso millet at 0, 25, 50, 75, or 100% inclusion rates as T1, T2, T3, T4 and T5. Results showed that all broiler chickens fed on diets containing different rates of millet instead of maize significantly improved live body weight, feed conversion ratio, and carcass yield for females and males compared to T1. Additionally, it was observed that there was a significant decrease in the relative weight of the liver for females and males compared to T1. The use of millet in diets did not negatively affect the broilers' health, and the mortality rate was low throughout the experiment. These results confirmed that maize could be replaced by proso millet in broiler chicken diets up to 100%.

Keywords: Body weight, Broiler chickens, Feed conversion ratio, Maize, Proso millet

INTRODUCTION

Most diets used globally to feed chickens are composed of maize and soybean meal. Maize has been recognized as a source of energy. However, the increasing demand and competition between humans and animals and the diversity of its industrial uses for biofuel production raised the prices of maize especially in the drier regions (Ranum et al., 2014). Consequently, the cost of producing poultry diets, which accounts for about 70% of the total production costs, increased (Dei, 2017). This encouraged researchers to study the possibility of replacing maize with cereals that are rich in energy and do not need large amounts of water for cultivation. It was found that millet grains can be used as a substitute for maize, and it could partially or completely replace maize in poultry diets (Baurhoo et al., 2011).

More than 20 varieties of millet have been cultivated around the world at different times (A Millet Atlas, 2006). The most common are proso, pearl, finger, kodo, foxtail, little, and barnyard millet varieties (Habiyaremye et al., 2017).

Millet is a fast-growing cereal plant that is widely grown in warm countries and regions with poor soils, periodic rainfall, and high temperature (Maidala and Abdullahi, 2016).

The most important characteristic of millet is the potential to cultivate it after the main crops such as wheat, barley and sunflower due to its short productive season and its high efficiency in resisting drought. (Nielsen and Vigil, 2017). In addition, proso millet is considered to be of a high nutritional value and its applications are varied between human and bird nutrition and industrial purposes (Das et al., 2019). It is one of the most important crops suitable for rain-fed agriculture as it has a shallow root system (90-120 cm) and a short productive season (60–90 days). This makes it an exceptional crop for lands with rainfed farming systems (Rajasekaran et al., 2023).

Through the research conducted on millet, it was found that the gross energy content of millet was similar to, or slightly better, than that of maize (4331, 4325 kcal/ kg respectively; Khalil et al., 2022). The true metabolisable energy (ME) corrected for nitrogen (TMEN) of maize is 3,350 Kcal/kg compared to 3,300 - 3,450 Kcal/kg for pearl millet (Cisse et al., 2017). The protein concentration of millet is higher than that of maize, but has similar apparent digestible amino acid coefficients (Vasan et al., 2008). For proso millet, the category on which this research was conducted, protein content may vary from 12.4 to 17%, with a high content of amino acids, especially sulfur amino acids (methionine and cysteine; Das et al., 2019). Moreover, millet is also considered rich in fat, and contains a higher percentage of fibers compared to other cereals, polyphenols and other nutraceutical compounds (Habiyaremye et al., 2017). Millet also contains a lot of bioactive compounds that have a beneficial effect on health, such as phenolics and dietary fiber together with micronutrients (carotenoids and tocopherols). They have antioxidant properties which are important to reduce the harmful effects of oxidation (Liang and Liang, 2019). The inclusion of millet grains in animal feed has gained momentum in recent years. It has been demonstrated that millets have the potential to be used as an alternative source of energy in poultry diets (Hassan et al., 2021). Therefore, this research aimed to study the possibility of replacing maize with proso millet (*Panicum miliaceum L*) in broiler chicken diets and its effect on production performance.

MATERIALS AND METHODS

Ethical approval

This research was carried out as a part of PhD researches in poultry nutrition at the Faculty of Veterinary Medicine, department of animal production after the approval of the Ethics Committee of the Faculty of Veterinary Medicine, Hama University, Syria, under the registration number 540, on 3/17/2021 in compliance with all local animal welfare legislation.

Experimental design

A total of 160 unsexed one-day-old Hubbard broiler chickens (39.25 ± 0.75 g at hatch) were used in this study. The chickens purchased from Al-Masri hatchery in Damascus city, Syria. Broiler chickens were randomly divided into five treatment groups with 4 replicates of 8 chicks each. The experimental period was divided into 3 phases: starter (days 1-10), grower (days 11-24), and finisher (days 25-42).

The diets used were formulated to meet the nutritional specifications recommended by the breed producer according to the management guide (Hubbard, 2007). All diets were offered as mash form-and formulated to be isoprotein and isocaloric by adjusting oil and soybean meal content to compensate for lower metabolizable energy (ME) and relatively higher protein content of millet, compared to maize. Millet replaced maize in the diets at 0%, 25%, 50%, 75%, and 100% inclusion rates as T1, T2, T3, T4 and T5 respectively. The composition and analysis of the experimental diets are shown in Table 1.

Housing and management

Broiler chickens were raised in open-sided housing conditions with litter floors. Feed and water were offered *ad-libitum* throughout the trial period.House temperatures (indoors) started at 33 °C and thereafter reduced by 0.5 °C per day until 24 °C was attained on day 19. Continuous lighting was provided for 24 hours in the first three days and then 22 hours of lighting and 2 hours of darkness pattern was adopted for the rest of the experimental period.

Vaccination schedule

The broiler chickens were vaccinated with Spain's HIPRAVIAR vaccinations as follows: on day 7 Newcastle disease plus Infectious bronchitis (B1, H120) by eye drops through intraocular route, on day 14 Infectious bursal disease (CH/80) by drinking water, on day 21 and 35 Newcastle disease (Clone 30) by eye drops.

Experimental procedures

The experiment lasted for 6 weeks. Live body weight (LBW), feed consumption and feed conversion ratio (FCR) were estimated every week (WK). Mortality rate was daily recorded. Feed conversion was calculated based on the relationship between feed intake and weight gain.

Carcass yield

At day 42 of age, the feed was removed for 6 hours before slaughter in order to ensure emptiness of the digestive tract. Six male and six female broilers were randomly taken from each group-and carcass yield was estimated. Prior to slaughter, the LBW of each bird was recorded and the percentage weight of the carcass relative to LBW was calculated. In addition, the liver, breast and thigh meat weights were calculated.

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) using the SPSS statistical package (IBM SPSS Statistics 25.0). The significance of difference between means was determined by the method of Least Significance Difference (LSD). Statistical significance was accepted when $p < 0.05$.

Table 1. Ingredient composition of broiler chicken's diets for a period of 42 days

Item	Starter (days 0-10)					Grower (days 11-24)					Finisher (days 25-42)				
	Diet 1	**Diet 2**	**Diet 3**	**Diet 4**	**Diet 5**	**Diet 1**	**Diet 2**	**Diet 3**	**Diet 4**	**Diet 5**	**Diet 1**	**Diet 2**	**Diet 3**	**Diet 4**	**Diet 5**
Maize (%)	57.6	43	28.5	14.9	0	62.5	46.6	31.55	15.5	0	66.5	50.5	33.2	16.5	0
Proso millet (%)	0	14.5	29	42.7	57.6	0	16.1	31.4	47.5	63.5	0	15.9	33.2	50.5	67.2
Soybean meal (46%)	37.4	36.95	36.45	36	35.4	32	31.4	30.9	30.3	29.6	28.9	28.35	27.8	27.1	26.5
Sun flower oil (%)	0.3	0.81	1.27	1.68	2.16	0.8	1.25	1.66	2.17	2.52	0.53	1.08	1.65	2	2.48
Dicalcium phosphate (%)	2.4	2.4	2.4	2.4	2.4	2.2	2.2	2.2	2.2	2.2	1.89	1.9	1.9	1.9	1.9
Limestone (%)	0.89	0.93	0.98	0.94	1.03	1.12	1.09	0.99	0.99	0.81	0.97	1.06	1.06	0.83	0.77
L-Lysine (70%)	0.29	0.3	0.3	0.3	0.31	0.29	0.29	0.29	0.3	0.33	0.22	0.24	0.24	0.24	0.24
DL-Methionine (99%)	0.31	0.29	0.28	0.26	0.25	0.28	0.26	0.24	0.23	0.21	0.23	0.21	0.19	0.17	0.15
L-Thrionine, (100%)	0.06	0.07	0.07	0.07	0.1	0.06	0.06	0.05	0.06	0.08	0.01	0.01	0.01	0.01	0.01
Choline chloride (60%)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Salt (%)	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Sodium bicarbonate (%)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Minerals[a] + Vitamins[b] (%)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Chemical analyses															
Crude protein (%)	22.7	22.7	22.7	22.7	22.7	20.6	20.6	20.6	20.6	20.6	19.5	19.5	19.5	19.5	19.5
ME, Kcal/Kg	2928	2930	2929	2930	2929	3000	2999	2999	3000	2999	3024	3024	3026	3025	3025
Total Lysine (%)	1.44	1.45	1.44	1.44	1.45	1.29	1.29	1.3	1.3	1.3	1.16	1.17	1.17	1.17	1.2
Total Methionine (%)	0.67	0.65	0.64	0.6	0.6	0.61	0.59	0.58	0.6	0.6	0.54	0.53	0.51	0.5	0.5
Total (Met+Cys) (%)	1.08	1.08	1.08	1.08	1.08	0.99	0.99	0.99	0.99	0.99	0.9	0.9	0.9	0.9	0.9
Calcium (%)	1.06	1.08	1.1	1.1	1.1	1.09	1.07	1.03	1	1	0.95	0.98	0.98	0.9	0.9
Available Phosphorus (%)	0.47	0.47	0.5	0.5	0.5	0.44	0.44	0.44	0.44	0.44	0.39	0.39	0.4	0.4	0.4

[a]Trace mineral premix provides the following in grams per ton of diets: CU: 16, I: 1.28, FE: 21, MN: 120.9, SE: 0.3, ZN: 112.5. [b]Vitamin premix provides the following in grams per ton of diets: A: 12, D3: 10, E: 175, K: 3.25, B1: 3.5, B2: 8.8, B3: 65, B5: 20, B6: 4.4, B7: 0.22, B9: 2.2, B12: 0.017.

RESULTS

The effect of the inclusion of millet in experimental diets on live body weight (LBW) in the different ratios is presented in Table 2. The result showed that the LBW at the end of the experiment was greater ($P < 0.05$) for T5 as compared to T1, T2, T3 but, similar to T4. Live body weight for groups fed diets of T2, T3, T4 and T5 was higher (7.5, 12, 17 and 20 %, respectively) as compared to those fed with control basal diet T1.

Mortality rate during the experiment is shown in Table 2. During the trial period, there was no observable sign of morbidity, but mortality occurred fortuitously within the first week of chicks' life. At the end of the experiment, cumulative mortality was 6.25, 0, 6.25, 6.25 and 6.25% for T1, T2, T3, T4 and T5 respectively. This may be due to stress or mechanical injury during handling and transportation, and there was no significant difference in mortality percentage among the treatments.

Feed conversion ratio was significantly ($P < 0.05$) improved from the first week until the end of the experiment. Overall FCR at six weeks of age, and the value was higher for T1 and T2 than T3, T4 and T5 (Table 3). A significant ($P < 0.05$) improvement in FCR was recorded in the groups fed with diets T2, T3, T4 and T5 as compared to the control group T1. In general, the highest level of millet showed the best FCR compared to other groups.

The results concerning carcass yield and liver weight, expressed as a percentage of the live body weight, are demonstrated in Table 4. The results showed that the carcass yield of broiler chickens fed with diets T4 and T5 was significantly higher ($p < 0.05$) than T1 in males, and a significant ($P < 0.05$) improvement was recorded in T5 as compared to T1 in females, with no difference between the other groups. Similarly, for the breast and thigh meat, T5 recorded the highest value for the breast meat, and T4 recorded the highest value for the thigh meat with significant difference ($p < 0.05$) compared to T1 in females. However, in males the highest value for the breast meat was recorded in T2, and the highest value for thigh meat was recorded in T4 with a significant difference ($p < 0.05$) compared to T1. It was also noted that the percentage of liver decreased linearly and significantly ($p < 0.05$) with increasing inclusion rates of millet.

Table 2. Live body weight of Hubbard broiler chickens (g) ± SEM fed on diets containing different levels of millet

Treatments / Age (week)	T1	T2	T3	T4	T5
1	158.55 ^{a} ± 3.31	156.45 ^{a} ± 2.5	160.33 ^{ac} ± 20	167.66 ^{bc} ±1.86	168.39 ^{b} ± 2.66
2	383.39 ^{a} ± 6.9	396.1 ^{ac} ± 6.93	405.97 ^{bc} ± 9.05	411.78 ^{bc} ± 4.5	395.71 ^{ac} ± 5.57
3	757.23 ^{a} ± 13.15	779.48 ^{ac} ± 16.37	808.62 ^{bc} ± 15.32	819.97 ^{b} ± 9.59	822 ^{b} ± 12.44
4	1193.1 ^{a} ± 23.91	1234.45 ^{ac} ± 27.51	1281.59 ^{bc} ± 26.22	1355.63 ^{d} ± 16.13	1386.48 ^{d} ± 20.54
5	1737.97 ^{a} ± 31.93	1799.23 ^{a} ± 60.9	1867.62 ^{ab} ± 55.69	1948.63 ^{b} ± 35.81	2001.1 ^{b} ± 51.5
6	2257.35 ^{a} ± 38.57	2426.29 ^{ab} ± 79.22	2528.83 ^{bc} ± 75.33	2648.81 ^{cd} ± 55	2714.4 ^{d} ± 67.85
Mortality (%)	6.25 ± 3.6	0	6.25 ± 3.6	6.25 ± 3.6	6.25 ± 3.6

^{abcd}Means within each row with the different superscripts are significantly different ($p < 0.05$). T1: inclusion rate of millet 0% (control), T2: inclusion rate of millet 25%, T3: inclusion rate of millet 50%, T4: inclusion rate of millet 75%, T5: inclusion rate of millet 100%. SEM: Standard Error of Mean

Table 3. Feed conversion ratios in Hubbard broiler chickens ± SEM fed on diets containing different levels of proso millet.

Treatments / Age (week)	T1	T2	T3	T4	T5
1	1.00 ^{a}	1.00 ^{a} ± 0.01	0.98 ^{b}	0.95 ^{c}	0.90 ^{d}
2	1.45 ^{a}	1.39 ^{b} ± 0.01	1.35 ^{c}	1.35 ^{c} ± 0.01	1.38 ^{b}
3	1.62 ^{a}	1.58 ^{b} ± 0.01	1.46 ^{c}	1.39 ^{d}	1.36 ^{e}
4	1.85 ^{a}	1.83 ^{b} ± 0.01	1.78 ^{c} ± 0.01	1.59 ^{d}	1.54 ^{e} ± 0.01
5	1.92 ^{a} ± 0.01	1.88 ^{b} ± 0.01	1.88 ^{b} ± 0.01	1.87 ^{b}	1.87 ^{b}
6	1.98 ^{a} ± 0.01	1.95 ^{b} ± 0.01	1.95 ^{b}	1.92 ^{c} ± 0.01	1.89 ^{d}
Cumulative	1.74 ^{a} ± 0.018	1.72 ^{ab} ± 0.011	1.69 ^{b} ± 0.006	1.63 ^{c} ± 0.01	1.61 ^{c} ± 0.004

^{abcde}Means within each row with the different superscripts are significantly different ($p < 0.05$). T1: inclusion rate of millet 0% (control), T2: inclusion rate of millet 25%, T3: inclusion rate of millet 50%, T4: inclusion rate of millet 75%, T5: inclusion rate of millet 100%. SEM: Standard Error of Mean

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Table 4. Carcass meat yield and liver of Hubbard broilers chickens (%) ± SEM fed on diets containing different levels of proso millet

Items / Treatments	[1]Carcass (%)		[1]Breast meat (%)		[1]Thigh meat (%)		[1] Liver (%)	
	Female	Male	Female	Male	female	Male	female	Male
T1	75.72 [a] ± 0.74	75.16 [a] ± 0.44	24.21 [a] ± 0.91	23.42 [a] ± 0.39	17 [a] ± 0.24	16.92 [a] ± 0.23	2.26 [a] ± 0.14	2.10 [a] ± 0.15
T2	75.74 [a] ± 0.39	76.1 [ab] ± 0.66	24.36 [ab] ± 1.23	25.64 [b] ± 0.93	18.1 [b] ± 0.54	17.72 [ab] ± 0.21	1.89 [b] ± 0.07	1.81 [b] ± 0.55
T3	76.57 [ab]±0.58	76.31 [ab] ± 0.38	26 [ab] ± 1.01	25.6 [b] ± 0.41	17.37 [ab] ±0.35	18.04 [ab] ± 0.52	1.80 [b] ± 0.08	1.91 [ab] ± 0.93
T4	76.8 [ab] ± 0.34	76.91 [b] ± 0.62	24.93 [ab] ± 1	24.89 [ab]±0.43	18.33 [b] ± 0.17	18.7 [b] ± 0.5	1.80 [b] ± 0.05	1.8 [b] ± 0.05
T5	77.34 [b] ± 0.52	76.65 [b] ± 0.13	27.06 [b] ± 0.33	25.46 [b] ± 0.38	18.16 [b] ± 0.38	17.89 [ab] ± 0.46	1.76 [b] ± 0.08	1.86 [ab] ± 0.06

[ab]Means within each column with different superscripts are statistically different ($p < 0.05$). T1: inclusion rate of millet 0% (control), T2: inclusion rate of millet 25%, T3: inclusion rate of millet 50%, T4: inclusion rate of millet 75%, T5: inclusion rate of millet 100%. SEM: Standard Error of the Mean

DISCUSSION

Broiler chicken's performance was found to be enhanced when the replacement ratio of maize with proso millet was increased. The higher crude protein digestibility and the greater oil content, either in treatment diets or in millet compared to that of the control diet or maize, could be the reasons for improvement the performance (Khalil et al., 2022).

Since the millet grains are slightly lower in (ME) in comparison with maize, the addition of sun-flower oil to compensate this shortage was required to maintain the diet's isocaloric, which enhanced the broilers' appetite (Adil et al., 2020). It was found that broiler chickens preferred diets containing higher levels of oil (Bueno et al., 2015). Furthermore, both oil and fat increase the duration of the nutrients remaining in the intestines. That provides a longer time for the enzymes to work. and remain in the digest for more time in contact with the intestinal villi, which leads to improving absorption and reflection of it on FCR (Boroojeni et al., 2011). In addition, significant improvement ($p < 0.05$) can result in the presence of the highest level of amino acid balance, since the synthesis of protein requires an adequate amount of amino acids, the presence of which in millet grains is greater than that found in yellow corn (Manaah and Alkassar, 2021).

The results of this study are similar to what was found by (Baurhoo et al., 2011) who confirmed that replacing maize with millet in broiler diets improved live body weight and feed conversion ratio. In addition, a previous study indicated that the diets based on proso millet recorded the highest value in weight gain and feed consumption without affecting the feed conversion ratio compared to the diets based on yellow maize (Ibitoye et al., 2012). The results of this study follow several studies which also show that broiler chickens fed on diets based on millet and formulated to maintain isocaloric and isoprotein have better performance parameters than those of corn-based diets (Hidalgo et al., 2004; Garcia et al., 2005). It could be stated that the higher nutrient content and low concentrations of anti-nutrients kept the production responses of broiler chickens effective, without causing any adverse effects (Boroojeni et al., 2011). The liver is considered the main site of detoxification and nutrient metabolism; thus, it is suggested that the liver size is dependent on the amount of work it does (Zaefarian et al., 2019). The current study suggests that millet-based diets decreased liver sizes significantly ($p < 0.05$), probably because millet has a low incidence of mycotoxins compared to other cereals such as wheat and maize (Manaah and Alkassar, 2021). Meanwhile, (Rao et al., 2004) show that the total replacement of yellow maize by millet did not influence the relative weight of the liver of broiler chickens at day 42 of age.

The observed improvement in the carcass yield ($p < 0.05$) may be explained by the higher content of amino acids in millet compared to maize, especially sulfur amino acids which are essential for optimum muscle accretion (Tjetjoo et al., 2022). The results of this study are similar to those found in a previous study that showed the percentage of carcass yield was not different between groups of broiler chickens fed on diets containing up to 50% millet, and it was even better than those fed diets without millet (Baurhoo et al., 2011). Likewise, (Rao et al., 2003) reported that breast and thigh muscle were significantly influenced by replacing maize with millet.

CONCLUSION

According to the findings of the present study, proso millet can be considered an exceptional alternative to completely replace of maize in broiler chicken diets without causing any adverse effects on performance, especially in arid and semiarid areas that suffer from water scarcity and where maize cannot be raised. Attending to millet, making more progress in the genetic improvement and the selection of suitable strains, as well as being more productive in applying it in ruminant rations as a rich source of fiber, are recommendations for future studies.

DECLARATIONS

Ethical consideration

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

Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Authors' contributions

Conceived and designed the experiments, and the supervision by Riad Kussaibati Prof. Dr. Department of Animal Production, Faculty of Veterinary Medicine, Hama University, Hama, Syria. It was also supervised by Hasan Tarsha Ass. Prof. Dr. Department of Animal Production, Faculty of Veterinary Medicine, Hama University, Hama, Syria. the experiment was performed, the data was analyzed and the paper was written by Melad Khalil. Practical works and analysis were performed at the faculty of agriculture, Damascus University, Damascus, Syria. All authors read and approved the final version of the manuscript for publication in the present journal.

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Availability of data and materials

The raw and analyzed data of this study will be presented to anyone who asks the corresponding author.

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Effects of *Cyperus alternifolius, Echinochloa pyramidalis, Typha angustifolia,* and *Imperata cylindrica* on Growth Performance, Feed Digestibility, Gut Microbiota, Haemato-biochemical and Immunity Parameters in Broiler Chickens

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ABSTRACT

The rhizomes of *Cyperus (C.) alternifolius, Echinochloa (E.) pyramidalis, Typha (T.) angustifolia,* and *Imperata (I.) cylindrica* are rich in secondary metabolites and have diverse pharmacological activities. The present study was designed to evaluate the effects of dietary *C. alternifolius*, *E. pyramidalis, T. angustifolia,* and *I. cylindrical* rhizomes on the performance of broiler chickens. A total of 384 day-old chicks were randomly assigned to six treatment groups (each treatment replicated four times). The first group received a basal diet (negative control), and the second group received a basal diet with 1 gr of antibiotic (Doxicycline, positive control). Other groups received a basal diet with 2 gr of each phyto-additives/kg feed. The results revealed that treatments had no significant effects on feed intake and carcass yield in chickens. The *C. alternifolius* and *T. angustifolia* significantly increased live weight and weight gain, and decreased feed conversion ratio, compared to negative control. The addition of *C. alternifolius, T. angustifolia,* and *I. cylindrica* to broilers' diet significantly increased the apparent digestibility of dry matter and crude protein, compared to the negative control. Compared to the negative control, the lactic acid bacteria count significantly increased with the incorporation of *T. angustifolia* and *I. cylindrica*. The granulocytes count and globulins concentration were not affected by the different treatments. However, the lymphocyte count was significantly decreased with the diet containing *E. pyramidalis* compared to the negative and positive controls, and the diets containing *C. alternifolius* and *T. angustifolia.* The spleen and bursa weights and volumes significantly increased in all groups of chickens fed on phyto-additives, compared to the negative control. Except for haematocrit, which significantly increased with *C. alternifolius and T. angustifolia* in the treatments compared to the negative control, the feed additives did not significantly affect the hematological parameters. Compared to the negative control, *T. angustifolia* and *I. cylindrica* significantly increased HDL-cholesterol concentration in broiler chickens' serum, while all treatment groups were comparable for all the other biochemical parameters. Incorporating 2 g of *C. alternifolius* and *T. angustifolia* in broiler chickens' feed improves feed digestibility, enhances the population of lactic acid bacteria in the gut, and causes subsequent improvement in growth performance.

Keywords: Broiler chicken, Digestibility, Growth performance, Gut microbiota, Immunity, Phyto-additive

INTRODUCTION

The use of antibiotic growth promoters in poultry nutrition has been banned due to concerns about the accumulation of their residues in animal tissues and pathogens' resistance (Mirzaei et al., 2022). This ban could lead to

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increased morbidity and mortality, reduced growth performance, and decreased economic profitability for farmers (Chardon and Brugere, 2014). As an alternative solution, the use of medicinal plants with positive effects on the digestive tract health, immune system, and growth performance has been encouraged (Onu, 2010).

Medicinal plants used in traditional pharmacopoeia have preventive and curative properties in human and animal health (Shalukoma et al., 2015; Bashige et al., 2020). These plants are rich in phytochemicals compounds, such as alkaloids, flavonoids, steroids, terpenoids, and quinones possessing antibacterial, antioxidant, antiviral, antimycotic, antiparasitic, immune-modulatory properties and digestive tract stimulatory effects (Dieumou et al., 2009; Ruiz-Navajas et al., 2013). Several studies have reported the improvement of growth performance in poultry through modulation of gut flora, reduction of pathogenic bacteria counts, stimulation of digestive secretions, improvement of feed components digestibility, absorption of nutrients, and stimulation of the immune system (Malekizadeh et al., 2012; Gong et al., 2014; Kana et al., 2017). Some plants commonly used in the Democratic Republic of Congo with pharmacological properties include *Cyperus (C.) alternifolius, Echinochloa (E.) pyramidalis, Typha (T.) angustifolia* and *Imperata (I.) cylindrica*.

These plant rhizomes are rich in secondary metabolites, including alkaloids, terpenoids, steroids, flavonoids, and tannins with antimicrobial and antiparasitic potential (Varghese et al., 2009; Lalthanpuii et al., 2015; Bashige et al., 2020). According to Varghese et al. (2009), *T. angustifolius* extracts have antibacterial effects on *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*) and antifungal effects on *Aspergillus flavus*. Lalthanpuii et al. (2015) noted antibacterial and antiparasitic effects of *I. cylindrica* rhizome powder against Gram-negative bacteria, such as *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*, and Gram-positive bacteria of *Bacillus subtilis.* However, the extract of this plant is effective against intestinal parasites, such as the tapeworm *Raillietina echinobothrida* and the roundworm *Ascaridia galli*. Bashige et al. (2020) reported antimicrobial activities of *Cyperus alternifolius, E. pyramidalis, T. angustifolia,* and *I. cylindrica* rhizome powder on *Neisseria meningitidis, S. aureus, Candida albican, Streptococcus pneumoniae, Salmonella typhi, E. coli* and *Trichophyton rubrum.* These phyto-additives are available and considered in several African countries as herbs. They contain bioactive compounds that can balance the gut microbiota, improve digestion and nutrient absorption, stimulate the immune system, and enhance growth performance if administered as feed additives to livestock. The main objective of the present study was to evaluate the individual effects of powdered rhizomes of *C. alternifolius, E. pyramidalis, T. angustifolia* and *I. cylindrica* as feed additives on growth performance, feed digestibility, gut microbiota, haemato-biochemical and immunity parameters in broiler chickens.

MATERIAL AND METHODS

Ethical approval

This study was carried out in strict accordance with the recommendations of institutional guidelines for the care and use of laboratory animals. Chickens were humanly handled in respect of the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

Study area

The study was conducted at the Teaching and Research Farm of the University of Dschang, Cameroon, from February to April 2022. This farm is located at 5°26' North latitude, 10°26' East longitude at an average altitude of 1420 m. The average temperature was around 21°C, the average rainfall was 2000 mm, the average annual insolation was 1873 hours, and the average relative humidity was 76.8%.

Phyto-additives

The *C. alternifolius, E. pyramidalis, T. angustifolia* and *I. cylindrica* were harvested in the vicinity of the Centre de Recherche en Sciences Naturelles (CRSN) in Lwiro, 30 Km from the town of Bukavu in the Democratic Republic of Congo. The identification of the plants used as phyto-additives was confirmed at the CRSN/Lwiro herbarium. The rhizomes were separated from other parts of the plant, open-air dried in the shade, then grind (using a Nima mill brand, China) and the different powders obtained (1 kg each plant) were kept in hermetically sealed boxes and later used as feed additives. Phytochemical analysis of the powdered rhizomes of each plant was carried out in order to identify the bioactive compounds present (Table 1). The determination of the classes of compounds present in the different extracts was made according to the standard methods described by Harborne (1973). Flavonoids were determined by the Shinoda test, 0.1 g of extract was dissolved in 3 ml of methanol. The mixture was treated with 0.05 g of magnesium chips and 3 drops of concentrated HCl. Flavonoids were identified by the presence of stains (orange for flavones, red for

xanthones and pink for flavonols). Alkaloids were determined by the Meyer test, 0.1 g of extract was placed in a test tube in the presence of 3 ml of an aqueous hydrochloric acid solution (50% V/V). The mixture was treated with 3 drops of Meyer's reagent. The formation of a white or yellowish precipitate indicated the presence of alkaloids. Triterpenes and steroids were determined by the Liebermann-Burchard test. Then, 0.1 g of extract was dissolved in 3 ml of chloroform, 3 ml of acetic anhydride was added, and the mixture was cooled in ice for 3 minutes. Finally, one drop of concentrated sulfuric acid was added. The presence of triterpenes was confirmed by the appearance of a purplish red color and that of steroids by the successive appearance of blue, green, red or orange colors. Anthraquinones, 0.1 g of extract was dissolved in a 4 ml mixture of ether-chloroform (1:1 v/v). The solution was treated with 4 ml of 10% (W/V) sodium hydroxide. The quinones were identified by the presence of red coloration. For phenols, 0.1 g of extract was dissolved in 3 ml of ethanol. The mixture was treated with 3 drops of 10% (v/v) iron III chloride. The appearance of the violet-blue or greenish coloration indicated the presence of phenols. For tannins, 0.1 g of extract was boiled for 5 minutes in a tube containing 5 ml of water. The mixture was treated with 5 ml of 2% NaCl (W/V) and 5 ml of 1% gelatine (W/V) after cooling. The appearance of a precipitate confirmed the presence of tannins.

Table 1. Phytochemical composition of phyto-additives

Plant	Alkaloids	Phenols	Flavonoids	Sterols	Triterpenoids	Tannins	Anthraquinones
Cyperus alternifolius	-	+	+	+	+	-	-
Echinochloa pyramidalis	-	+	+	+	+	-	-
Typha angustifolia	-	+	+	+	+	-	-
Imperata cylindrica	-	+	+	+	+	-	-

Present: +; Absent: -

Chickens

A total of 384 one-day-old Cobb 500 chicks were randomly assigned to 6 experimental groups (treatments) following a completely randomized design. Each treatment had 4 replicates of 16 chicks (8 males and 8 females). As soon as the chicks arrived, multivitamins and minerals (5 g in 2 liters of water, INTROVITA+WS, Holland) were administered through drinking water for the first 3 days. They were then vaccinated against infectious bronchitis (H52, Holland) and Newcastle (Hitchner B1, Holland) disease on day 7 and against Gumboro (CEVAC^{R} TRANSMUNE IBD, Holland) disease on day 10 with a booster on day 18. Multivitamins and minerals (5 g in 2 liters of water) were administered through drinking water after each weighting session and vaccination of the chicks.

Experimental diets

Chicks in the control group received basal diet (Table 2), the positive control group received a basal diet with 1 g of Doxycycline® in /kg of diet (dry matter [DM]), and other groups received a basal diet supplemented with 2 g of each plant in /kg of diet (DM).

Table 2. Composition of the experimental diet at the starter and finisher phases

Ingredients (%)	Starter	Finisher
Maize	60	67
Cotton meal	5	5
Soybean meal	22	15
Fish meal	5	5
Wheat bran	2	2
Oyster shell meal	1	1
Premix 5%*	5	5
Total	100	100
Chemical composition		
Metabolizable energy (kcal/kg)	2977	3108
Crude Protein (%)	23.01	20.3
Energy/protein	129.4	153.1
Calcium (%)	1.05	1.03
Phosphorus (%)	0.6	0.6
Calcium/Phosphorus	1.75	1.72
Lysine (%)	1.4	1.2
Methionine (%)	0.5	0.45
Lysine/Methionine	2.8	2.7
Crude cellulose (%)	2.43	2.61

Premix 5%*: Vit A: 3 000 000 IU, Vit D3:600 000 IU, Vit E: 4 000 mg, Vit K: 500 mg, Vit B1: 200 mg, VitB2: 1000 mg, Vit B6: 4000 mg, Vitamin B12: 4 mg, Iron: 8000 mg, Cu: 2000 mg, Zn: 10 000 mg, Se: 20 mg, Mn: 14000 mg.

Data collection

Growth performance, feed digestibility, and microbial flora

Throughout the study period (1-49 days), growth parameters (feed intake, weight gain, live weight, and feed conversion ratio [FCR]) were collected weekly. At the end of the study, 5 female and 5 male chickens were randomly selected from each group. They were then fasted for 24 hours to evacuate all digestive tract contents, weighed, plucked, and eviscerated without anesthesia.

Carcass yield and relative weights of organs were calculated. Moreover, intestine length was measured using a measuring tape, and intestine density was calculated by dividing the intestine length by the intestine weight.

The apparent digestive utilization coefficients (aDUC) of feed components were evaluated for 6 broiler chickens , including 3 males and 3 females, per treatment for 3 consecutive days. The 6 chicks per treatment were kept in the digestibility cages, and tarps were placed underneath the cages after 3 days of adaptation period to collect faces from each replicate. Feed was weighed prior to feeding. Afterward, faces and feed refusals were collected and weighed daily for 3 days. Fecal samples were oven-dried at 60°C to constant weight for proximate analysis of DM and organic matter (OM) in accordance with AOAC (1990) processes. Neutral Detergent Fiber (NDF) was determined by Van Soest et al. (1991) method, and crude protein (CP) by the Kjedhal method. The apparent digestive utilization coefficients (aDUC) of DM, OM, CP, and NDF of the experimental diets were calculated.

At the end of the trial (49 days), fecal samples were collected from the cloaca of four chickens per treatment (two males and two females), using cloacal swabs and immediately used for the identification and quantification of lactic acid bacteria, *E. coli* and *Salmonella* in their respective specific culture media, for determining of Lactic acid bacteria, the culture medium used was lactobacilli M.R.S AGAR produced by Acumedia® (India) and ISO 9001 reference. The final pH was 7.5 ± 0.2 at 25°C. The preparation procedure consisted of dissolving 70 g of this medium in 1 liter of distilled water in an Erlenmeyer flask, then heating with frequent stirring until complete dissolution. This medium was autoclaved at 121°C for 15 minutes.

For *E. coli*, the culture medium used was Mac Conkey Agar manufactured by Liofilchem® (India, dignostic and reference ISO 610028). The final pH was 7.1 ± 0.2 at 25°C. The preparation procedure consisted of pouring 51.5 g of the suspension into 1 liter of distilled water, then heating the mixture until completely dissolved. Finally, it was autoclaved at 121°C for 15 minutes.

For *Salmonella*, the culture medium used was SS AGAR of reference ISO 610042 and produced by Liofilchem® (India) dignostic. The final pH was 7 ± 0.2 at 25°C. The preparation procedure consisted of pouring 52 g of the suspension into 1 liter of distilled water, then boiling until complete dissolution without autoclaving according to the manufacturer's prescription. The inoculum was prepared by decimal dilutions, which consisted of placing 9 ml of physiological water in tubes numbered at the base by the type of sample and the dilution number. The swab bearing the sample was then introduced into the first tube. The latter was shaken in order to homogenize the solution (S1), then 1 ml of S1 was taken with a micropipette and introduced into the second tube to complete the solution to 10 ml, thus obtaining the 10-2 dilution. After homogenizing this solution, the procedure was carried out up to the 10-8 dilution. 1 ml of the 10-6 and 10-8 dilutions of each sample was taken and introduced into a petri dish each (Afnor, 1991).

The previously prepared solution of each culture medium (MRS Agar, SS Agar, and Mac Conkey Agar) was introduced each time just after the introduction of the inoculum into the petri dish and homogenized.

Immune system and haemato-biochemical parameters

During carcass evaluation, lymphoid organs (bursa of Fabricius and spleen) of 6 chickens/treatment were collected and weighed. Their indices were calculated by the ratio of organ weight (g)/fasting live weight (g) multiplied by 100 according to Stice (2000). In the next step, 5 ml of blood samples were collected in tubes containing anticoagulant for the quantification of white blood cells, red blood cells, haemoglobin, haematocrit, blood platelets, mean cell volume, and packed cell volume (PCV) using Urtit 3000 plus haematimater (China) and blood without anticoagulant was used to measure alanine aminotransferase (ALT), aspartate aminotransferase, Urea, Creatinin, Trygliceride, Total cholesterol, high-density lipoproteins (HDL) and low-density lipoproteins (LDL)-Cholesterol according to kit manufacturers' instructions (Chronolab®, Barcelona, Spain). With regard to the immune system status, the immune cells quantified including granulocytes and lymphocytes, and the immune system proteins including

albumin and globulins were investigated according to the instruction of the Urtit 3000 Plus kit (China).

Statistical analysis

The statistical software Statistical Package for Social Sciences (SPSS version 20.0) was used for the analyses. All collected data were submitted to a one-way analysis of variance (ANOVA). Duncan's multiple range test was used to separate significant level at $p < 0.05$. The normality of data was tested by the Shapiro-Wilk test.

RESULTS

Growth performance

Table 3 summarizes the effects of different treatments on growth performance in broiler chickens. The different additives did not significantly affect feed intake in any of the study periods. During the starter phase (1-21 days), the addition of different additives in the diets did not have significant effects on the live weight and weight gain of chickens. Over the entire study period (1-49 days), live weight and weight gain recorded in broilers fed diets supplemented with *C. alternifolius*, *T. angustifolia*, and *I. cylindrica* were comparable to the result recorded with antibiotic, but significantly higher than the negative control diet ($p < 0.05$). During the finisher phase and throughout the study period, the addition of *C. alternifolius*, *T. angustifolia*, and *I. cylindrica* in feed significantly lowered FCR, compared to the control group ($p < 0.05$).

Feed digestibility

The apparent digestive utilization coefficients (aDUC) of DM induced by the supplementation of the diet with *T. angustifolia*, *I. cylindrical*, and antibiotic were significantly higher than that of the negative control diet ($p < 0.05$). The aDUC of CP significantly increased with *C. alternifolius* and *T. angustifolia* compared to that of negative control ($p < 0.05$, Table 4). The aDUC of organic matter and neutral detergent fiber were not significantly affected by the inclusion of *C. alternifolius*, *T. angustifolia*, and *I. cylindrical* in the diets.

Carcass characteristics

The different treatments did not significantly affect relative organ weights and carcass yields (Table 5). However, carcass yields tended to increase by supplementing diets with different additives.

Microbial flora

Table 6 shows that incorporating the different phyto-additives in the diet has no significant effect on *E. coli* and *Salmonella counts* in the gut of broiler chickens ($p > 0.05$). However, *E. coli* and Salmonella counts tended to decrease with the dietary supplementation of different phyto-additives compared to the negative control ($p > 0.05$). Lactobacilli counts in the gut of chickens significantly increased with the incorporation of *C. alternifolius, T. angustifolia, and antibiotic*, compared to the number recorded with the control diet ($p < 0.05$).

Effects of in-feed additive on the immune system

As can be seen in Table 7, the weights and volumes of spleen and bursa of Fabricius increased significantly with the incorporation of phyto-additives in the diet compared to the negative control ($p < 0.05$). With the exception of chickens fed on *I. cylindrical*, supplementing broiler's feed with *E. pyramidalis* rhizome significantly decreased blood lymphocytes, compared to other treatments ($p < 0.05$). Meanwhile, the phyto-supplements did not significantly affect granulocyte count, and the number of globulins in the blood of broiler chickens, compared to the controls ($p > 0.05$).

Hematological parameters

The supplementation of broiler's feed with rhizomes of *C. alternifolius* and *T. angustifolia* significantly increased blood hematocrit level, compared to the negative control ($p < 0.05$). Nevertheless, blood hematocrit level of chickens fed on diets supplemented with these two phyto-additives was comparable to that of chickens fed on an antibiotic as supplement. On the other hand, the phyto-additives did not significantly affect the other hematological parameters regardless of their type ($p > 0.05$, Table 8).

Biochemical parameters

All treatments induced comparable biochemical parameters values ($p > 0.05$), except HDL-cholesterol level which increased significantly with the incorporation of *T. angustifolia* and *I. cylindrica* in the diet, compared to the negative control and *Cyperusalternifolius* supplemented diet ($p < 0.05$, Table 9). However, the analysis of variance revealed no significant influence of phyto-additives on ALT, total cholesterol and LDL-cholesterol relative to controls. Inversely, total protein, albumin, and creatinine levels showed a slight increase with the addition of these phyto-additives in broiler fed, compared to the negative control ($p > 0.05$).

Table 3. Growth performances of broiler chickens fed with phyto-additives for 49 days

Study period (days)	Controls		Phyto-additives (2 g/kg feed)				p
	0-	0+	Ca	Ep	Ta	Ic	
Feed intake (g)							
01-21	1232.27 ± 71.49	1184.72 ± 52.05	1203.41 ± 84.46	1214.04 ± 66.85	1185.12 ± 94.02	1225.21 ± 75.29	0.231
22-49	5173.82 ± 98.23	5119.82 ± 73.19	5083.28 ± 84.15	5224.37 ± 94.18	5134.41 ± 118.88	5213.76 ± 85.74	0.088
01-49	6406.09 ± 102.66	6304.54 ± 97.95	6286.69 ± 97.03	6438.41±167.97	6319.53 ± 172.46	6438.97 ± 145.67	0.384
Live body weight (g)							
01-21	632.91 ± 53.52	646.48 ± 80.64	648.50 ± 77.44	627.15 ± 88.76	641.36 ± 60.87	653.97 ± 52.30	0.145
01-49	2785.41 ± 98.53^{c}	3044.51 ± 109.36^{a}	2941.72 ± 77.44^{ab}	2887.93 ± 131.96^{bc}	3034.46 ± 109.71^{a}	2935.86 ± 99.43^{ab}	0.004
Weight gain (g)							
01-21	594.48 ± 63.52	608.05 ± 70.64	610.07 ± 77.44	588.72 ± 98.76	602.93 ± 90.87	615.54 ± 62.30	0.145
22-49	2152.50 ± 107.08^{c}	2398.03 ± 129.71^{a}	2293.22 ± 88.82^{ab}	2260.78 ± 126.07^{bc}	2393.10 ± 106.71^{a}	2281.89 ± 95.21^{ab}	0.004
01-49	2746.98 ± 148.53^{c}	3006.08 ± 149.36^{a}	2903.29 ± 188.71^{ab}	2849.50 ± 131.96^{ab}	2996.03 ± 159.71^{a}	2897.43 ± 119.43^{ab}	0.004
Feed conversion ratio							
01-21	2.07 ± 0.05	1.95 ± 0.07	1.97 ± 0.06	2.06 ± 0.04	1.97 ± 0.11	1.99 ± 0.02	0.053
22-49	2.40 ± 0.03^{a}	2.14 ± 0.02^{c}	2.22 ± 0.10^{bc}	2.32 ± 0.11^{ab}	2.15 ± 0.02^{c}	2.29 ± 0.09^{b}	0.001
01-49	2.33 ± 0.05^{a}	2.09 ± 0.03^{d}	2.16 ± 0.07^{cd}	2.26 ± 0.06^{ab}	2.11 ± 0.03^{d}	2.22 ± 0.03^{bc}	0.001

^{a,b,c}: Means the same letter on the same row is not significantly different ($p > 0.05$); 0-: Diet without additive (R0-), 0+: 1 g Doxycycline®/kg diet, Ca: R0- + 2 g Cyperus alternifolius/kg diet, Ep: R0- + 2 g Echinochloa pyramidalis/kg diet, Ta: R0- + 2 g Typha angustifolia/kg diet, Ic: R0- + 2 g Imperata cylindrica/kg diet, p: P-value

Table 4. Effects of phyto-additives on apparent digestive utilization coefficients of feed components in broiler chickens

aDUC (%)	Controls		Phyto-additives (2 g/kg feed)				p
	0-	0+	Ca	Ep	Ta	Ic	
DM aDUC	92.45±0.94^{b}	95.89±1.90^{a}	95.65±2.61^{a}	93.95±2.84^{ab}	96.25±1.46^{a}	95.71±2.49^{a}	0.026
OM aDUC	94.24±2.75	95.47±2.74	95.73±2.61	93.86±3.25	94.61±4.23	95.34±3.66	0.274
CP aDUC	88.92±0.83^{b}	92.17±1.42^{a}	93.77±2.58^{a}	88.00±2.7^{b}	93.74±2.22^{a}	91.35±3.98^{ab}	0.001
NDF aDUC	92.91±3.05	94.24±3.25	93.65±2.47	93.16±1.44	93.79±2.72	93.88±2.18	0.957

^{a,b,c}: Means the same letter on the same row is not significantly different ($p > 0.05$); 0-: Ration without additive (R0-); 0+ : 1 g Doxycycline®/kg ration; Ca: R0- + 2 g Cyperus *alternifolius*/kg ration; Ep: R0- + 2 g *Echinochloa pyramidalis*/kg ration; Ta: R0- + 2 g *Typha angustifolia*/kg ration; Ic : R0- + 2 g *Imperata cylindrica*/kg ration; p: P-value; aDUC: Apparent digestive utilisation coefficient; DM: Dry matter; OM: Organic matter; CP: Crude protein; NDF: Neutral detergent fiber

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Table 5. The carcass yields and relative weights of broiler chickens' organs with respect to phyto-additives at the age of 49 days

Caracteristics (% LBW)	Control		Phyto-additives (2 g/kg feed)				p
	0-	0+	Ca	Ep	Ta	Ic	
Carcasse yeild	75.16 ± 2.89	80.10 ± 3.42	78.37 ± 2.80	76.08 ± 4.46	77.74 ± 3.42	77.69 ± 5.32	0.084
Head	2.08 ± 0.15	2.07 ± 0.27	2.19 ± 0.20	2.17 ± 0.18	2.07 ± 0.17	2.26 ± 0.37	0.357
Legs	3.28 ± 0.33	3.22 ± 0.49	3.52 ± 0.59	3.41 ± 0.53	3.26 ± 0.57	3.37 ± 0.62	0.815
Liver	1.74 ± 0.27	1.71 ± 0.36	1.82 ± 0.22	1.62 ± 0.17	1.66 ± 0.32	1.60 ± 0.25	0.497
Heart	0.47 ± 0.05	0.41 ± 0.07	0.46 ± 0.06	0.49 ± 0.06	0.46 ± 0.07	0.50 ± 0.10	0.087
Abdominal fat	1.63 ± 0.65	1.64 ± 0.54	1.69 ± 0.43	1.56 ± 0.47	1.44 ± 0.45	1.71 ± 0.36	0.856

[a,b,c]: Means the same letter on the same row is not significantly different (p > 0.05); LBW: Live Body Weight, 0-: Diet without additive (R0-), 0+: 1 g Doxycycline®/kg diet, Ca: R0- + 2 g Cyperus alternifolius/kg diet, Ep: R0- + 2 g Echinochloa pyramidalis/kg diet, Ta: R0- + 2 g Typha angustifolia/kg diet, Ic: R0- + 2 g Imperata cylindrica/kg diet, p: P-value

Table 6. The gut microbiota counts (Log_{10} CFU) with respect to the phyto-additives in broiler chickens at 49 days old

Bacteria count (Log_{10}CFU)	Control		Phyto-additives (2 g/kg feed)				p
	0-	0+	Ca	Ep	Ta	Ic	
Escherichia coli	2.25 ± 0.52	1.07 ± 0.33	1.34 ± 0.62	1.85 ± 0.36	1.56 ± 0.84	1.26 ± 0.26	0.056
Salmonella	2.11 ± 0.76	1.22 ± 0.22	1.49 ± 0.43	1.56 ± 0.78	1.27 ± 0.39	1.45 ± 0.50	0.279
Lactobacilli	0.85 ± 0.21^{b}	2.68 ± 0.96^{a}	1.79 ± 0.82^{ab}	0.86 ± 0.30^{b}	2.43 ± 0.67^{a}	1.79 ± 0.82^{ab}	0.001

[a,b,c]: Means the same letter on the same row is not significantly different (p > 0.05); LBW: Live Body Weight, 0-: Diet without additive (R0-), 0+: 1 g Doxycycline®/kg diet, Ca: R0- + 2 g Cyperus alternifolius/kg diet, Ep: R0- + 2 g Echinochloa pyramidalis/kg diet, Ta: R0- + 2 g Typha angustifolia/kg diet, Ic: R0- + 2 g Imperata cylindrica/kg diet, p: P-value

Table 7. The immune system parameters of 49 days old broiler chickens with respect to phyto-additives

Parameters	Control		Phyto-additives (2 g/kg feed)				p
	0-	0+	Ca	Ep	Ta	Ic	
Spleen weight (%LBW)	0.11 ± 0.05^{b}	0.24 ± 0.10^{a}	0.22 ± 0.05^{a}	0.23 ± 0.05^{a}	0.21 ± 0.05^{a}	0.20 ± 0.07^{a}	0.001
Spleen volume (ml)	3.60 ± 1.35^{b}	6.50 ± 0.97^{a}	6.30 ± 1.34^{a}	6.30 ± 1.25^{a}	6.60 ± 1.58^{a}	6.30 ± 1.70^{a}	0.001
BF weight (%LBW)	0.13 ± 0.02^{b}	0.23 ± 0.05^{a}	0.21 ± 0.05^{a}	0.22 ± 0.05^{a}	0.20 ± 0.05^{a}	0.20 ± 0.06^{a}	0.001
BF volume (ml)	3.10 ± 0.88^{b}	5.30 ± 1.16^{a}	5.60 ± 1.16^{a}	3.40 ± 0.84^{b}	5.20 ± 1.40^{a}	5.30 ± 1.42^{a}	0.001
Granulocytes (%)	3.30 ± 0.86	2.48 ± 0.80	2.80 ± 0.78	3.08 ± 0.62	3.10 ± 0.94	2.72 ± 0.71	0.519
Lymphocytes (%)	83.28 ± 3.1^{a}	79.32 ± 4.11^{ab}	79.73 ± 3.86^{ab}	74.07 ± 3.99^{c}	79.42 ± 3.06^{ab}	76.83 ± 4.77^{bc}	0.008
Globulins (g/dL)	3.43 ± 0.85	3.90 ± 1.00	3.73 ± 0.81	3.58 ± 0.99	3.94 ± 1.10	3.85 ± 0.71	0.352

[a,b,c]: Means the same letter on the same row is not significantly different (p > 0.05); 0-: Diet without additive (R0-), 0+: 1 g Doxycycline®/kg diet, Ca: R0- + 2 g Cyperus alternifolius/kg diet, Ep: R0- + 2 g Echinochloa pyramidalis/kg diet, Ta: R0- + 2 g Typha angustifolia/kg diet, Ic : R0- + 2 g Imperata cylindrica/kg diet, p: P-value, BF: Bursa of Fabricius, LBW: Live Body Weight

Table 8. Effects of phyto-additives on hematological parameters in 49 days old broiler chickens

Parameters	Control		Phyto-additives (2 g/kg feed)				p
	0-	0+	Ca	Ep	Ta	Ic	
WBC (10^9/ul)	177.78 ± 17.67	151.78 ± 24.06	159.52 ± 12.11	158.88 ± 22.89	153.15 ± 19.24	155.63 ± 21.62	0.271
RBC (10^{12}/ul)	3.04 ± 0.21	3.49 ± 0.23	3.47 ± 0.53	3.26 ± 0.42	3.14 ± 0.75	3.63 ± 0.62	0.302
HGB (g/dl)	13.45 ± 2.83	15.25 ± 3.84	15.97 ± 4.19	13.62 ± 3.34	15.23 ± 4.44	14.50 ± 3.32	0.116
HCT (%)	37.32 ± 2.68^{b}	41.85 ± 4.03^{ab}	43.23 ± 5.78^{a}	36.95 ± 3.53^{b}	43.67 ± 5.63^{a}	40.85 ± 3.9^{ab}	0.046
PLT (10^9/ul)	1.17 ± 0.41	2.33 ± 1.51	2.00 ± 0.89	1.67 ± 0.82	2.50 ± 1.38	2.67 ± 1.86	0.316
MCV (f/L)	123.00 ± 1.86	125.10 ± 2.20	124.98 ± 3.41	122.47 ± 2.83	123.57 ± 2.78	123.72 ± 2.34	0.449

[a,b,c]: Means the same letter on the same row is not significantly different (p > 0.05); 0-: Diet without additive (R0-), 0+: 1 g Doxycycline®/kg diet, Ca: R0- + 2 g Cyperus alternifolius/kg diet, Ep: R0- + 2 g Echinochloa pyramidalis/kg diet, Ta: R0- + 2 g Typha angustifolia/kg diet, Ic: R0- + 2 g Imperata cylindrica/kg diet; p: P-value, WBC: white blood cells, RBC: red blood cells, HGB: Haemoglobin, HCT: Haematocrit, PLT: Blood platelets, MCV: Mean cell volume

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Table 9. Effects of phyto-additives on biochemical parameters of 49 days old broiler chickens

Parameters	Control			Phyto-additives (2 g/kg feed)			p
	0-	0+	Ca	Ep	Ta	Ic	
AST (UI/L)	177.62 ± 24.56	184.00 ± 56.58	209.96 ± 68.14	152.01 ± 24.22	195.61 ± 35.34	197.94 ± 65.09	0.264
ALT (UI/L)	55.50 ± 10.04	66.38 ± 16.55	43.88 ± 6.29	48.00 ± 11.48	44.63 ± 6.84	46.38 ± 10.81	0.072
Urea (mg/dL)	3.46 ± 0.50	3.53 ± 0.70	3.44 ± 0.56	3.41 ± 0.86	3.79 ± 0.66	3.77 ± 0.62	0.748
Creatinine (mg/dL)	1.67 ± 0.38	1.91 ± 0.51	1.63 ± 0.39	1.68 ± 0.45	1.87 ± 0.40	1.76 ± 0.56	0.764
Total Protein (g/dL)	5.62 ± 0.38	6.40 ± 1.16	7.06 ± 1.16	6.09 ± 0.99	6.90 ± 1.59	6.94 ± 1.09	0.090
Albumin (g/dL)	3.90 ± 0.41	4.44 ± 0.67	4.42 ± 0.86	4.35 ± 1.10	4.59 ± 1.06	4.80 ± 1.24	0.535
Tryg (mg/dL)	83.22 ± 4.88	81.16 ± 6.11	83.10 ± 10.12	75.00 ± 4.54	84.36 ± 5.89	83.56 ± 6.48	0.071
Total Chol (mg/dL)	158.50 ± 8.52	147.88 ± 12.71	142.13 ± 12.92	143.75 ± 17.28	151.25 ± 16.12	149.38 ± 12.43	0.218
HDL- Chol (mg/dL)	76.28 ± 7.34^{b}	88.78 ± 10.66^{a}	76.87 ± 6.67^{b}	82.86 ± 13.39^{ab}	88.54 ± 10.66^{a}	88.93 ± 9.45^{a}	0.030
LDL-Chol (mg/dL)	67.13 ± 11.32	68.25 ± 14.69	62.24 ± 16.11	69.03 ± 9.62	62.53 ± 22.43	53.64 ± 12.39	0.338

^{a,b,c}: Means the same letter on the same row is not significantly different (p > 0.05); 0-: Diet without additive (R0-), 0+: 1 g Doxycycline®/kg diet, Ca: R0- + 2 g Cyperus alternifolius/kg diet; Ep: R0- + 2 g Echinochloa pyramidalis/kg diet, Ta: R0- + 2 g Typha angustifolia/kg diet, Ic: R0- + 2 g Imperata cylindrica/kg diet, p: P-value, AST: Aspartate amino transferase, ALT: Alanine amino transferase, Tryg: Tryglyceride, Total Chol: Total cholesterol, HDL-Chol: HDL-cholesterol, LDL-Chol: LDL- cholesterol

DISCUSSION

The addition of-phyto-additives in the broiler chicken feed had no significant effects on feed intake compared to the controls throughout the study period. This result is in contradiction with the findings of Durrani et al. (2008), who reported that feed intake in broilers decreased significantly with the incorporation of neem leaf powder (4%) in the drinking water. This decrease could be explained by the presence of salannin (1.3 mg/g), a triterpenoid contained in neem leaves that is able to inhibit feed intake in chickens by reducing their appetite (Singh, 2015). The variability of the results between these two studies could be explained by the difference between the phyto-additives used, the incorporation rates, the diets and the rearing conditions.

Throughout the study period, a significant increase in live weight and weight gain was observed in broilers fed diets supplemented with *C. alternifolius*, *T. angustifolia*, and *I. cylindrical*. Similar results were obtained by Zainali et al. (2009), who noted that supplementation of broiler feed with turmeric rhizome powder at a dose of 10g/kg significantly increased live weight and weight gain in broilers. In contrast, Rahmatnejad et al. (2009) reported that the addition of 2g/kg turmeric rhizome powder to broiler feed did not significantly affect live weight and weight gain in broilers. The increases in weight gain and live weight observed in the present study could be attributed to the anti-inflammatory and antioxidant activities induced by the phenolic compounds contained in these plants. According to Humphrey and Klasing (2004), the use of anti-inflammatory drugs could reduce inflammation which has an energetic cost for the animal to the detriment of its growth. Tchoffo et al. (2019) reported that substances with antioxidant properties could reduce the reactive oxygen species that attack the animal cell membrane and consequently increase the cell membrane thickness and animal weight.

The increase in live weight and weight gain could be linked to increased lactic acid bacteria in the digestive tract of chickens recorded in the present study. Lactobacilli regulate the intestinal flora by selectively eliminating pathogenic bacteria such as *Escherichia coli* and *Salmonella* by producing bacteriocins and hydrogen peroxides (Elaroussi et al., 2008). They compete with pathogenic bacteria for nutrients and occupation of attachment sites on the intestinal mucosa (Fooks and Gibson, 2002). This increase in the number of lactic acid bacteria and the decrease in pathogenic microbes in the digestive tract of chickens could explain their good health and improvement in growth.

The incorporation of *C. alternifolius*, *T. angustifolia*, and *I. cylindrica* in the diet significantly decreased feed conversion ratio in broilers. This decrease should be the result of an increase in weight gain in chickens fed these phyto-additives. The decrease in feed conversion in this study would be the logical consequence of a significant increase in weight gain and the trend of decreasing feed intake in chickens induced by diets containing this phyto-additive. Supplementing the broiler's diet with powdered rhizome of *T. angustifolia* and *I. cylindrical*, induced a significant increase in DM

digestibility compared to the negative control. Meanwhile, CP digestibility increased significantly with the incorporation of *C. alternifolius* and *T. angustifolia* in the diet, compared to the negative control. The increase in DM and CP digestibility induced by these phyto-additives could be due to the phenolic compounds, flavonoids, terpenoids, and sterols contained in these plants, which stimulated the secretion of enzymes that improve the digestibility of feed components, thus increasing their availability in the digestive tract and their susceptibility to be utilized by the chickens. Phenolic compounds increase the villi/crypt ratio in the gut, which would indirectly increase the surface area for nutrient absorption, thus improving their uptake, and consequently the growth performance of the animals (Kothadia et al., 2018). The results of the present study are contrary to those of Brenes and Roura (2010) reported that the incorporation of doses of grape seed extract (15, 30, and 60 g/kg) in the diet of broilers had no significant effect on protein digestibility. The digestibility of OM and fiber was not affected by the different treatments. This could be explained by the fact that the bioactive compounds in these different plants did not effectively stimulate the production of enzymes responsible for cutting the bonds of these feed components.

Although carcass yield tends to increase with the inclusion of these additives in the diet, no significant differences were recorded among the treatment groups. The increase in carcass yield is associated with an increase in live weight and weight gain in chickens fed these phyto-additives. In contrast, Nouzarian et al. (2011) reported that supplementation of broiler diets with turmeric rhizome powder (3.3, 6.6, and 10g/kg) significantly reduces abdominal fat weight compared to control. The result of the present work is in agreement with those of Ouedraogo et al. (2021), who noted that the incorporation of turmeric rhizome powder at a rate of 1.5% had no significant effects on carcass characteristics in broilers.

The incorporation of the different additives in feed did not have a significant effect on *E. coli* and *Salmonella* counts in the digestive tract of chickens. On the other hand, the addition of *C. alternifolius* and *T. angustifolia* in feed induced a significant increase in lactic acid bacteria counts in the broiler chickens' gut. This could be due to the antibacterial properties induced by the phenolic compounds and terpenoids contained in these plants, which significantly reduced the proliferation of pathogenic bacteria in the digestive tract, thus favoring the multiplication and colonization of the digestive tract by lactic acid bacteria. The latter plays the role of regulator for the intestinal flora by eliminating pathogenic bacteria, such as *Salmonella* spp. and *E. coli* through the production of bacteriocins and hydrogen peroxides (Elaroussi et al., 2008). They compete with pathogenic bacteria for nutrients and occupation of attachment sites on the intestinal mucosa. This increase in lactic acid bacteria count in the digestive tract of chickens explains the good health and improved growth performance observed.

The weights and volumes of the spleen and bursa of Fabricius significantly increased with the incorporation of the phyto-additives in the diet. The increase in weights and volumes of immune organs in chickens suggested that they participated in the increased production of immune cells by these organs, thereby increasing the immunological defense capacity in these animals. The increase in the defense capacity of chickens against external aggression induced by secondary metabolites of these phyto-additives would explain the good health status observed in chickens and the improvement in their growth performance. Supplementing feed with rhizome of *E. pyramidalis* significantly decreased Lymphocyte count in the blood of chickens. This decrease suggests that chickens are more susceptible to infection. This could explain the decrease in growth performance observed in chickens fed diet supplemented with this rhizome compared to other phyto-additives. However, the incorporation of supplements in feed did not significantly affect the granulocyte count and the number of globulins in the blood of broilers. These results are contradictory to those of Hassan and Awad (2017), who concluded that the addition of 5g thyme powder/kg feed, significantly increases the serum concentration of white blood cells and globulins in broiler chickens.

With the exception of the hematocrit level, which increased significantly with the incorporation of *C. alternifolius* and *T. angustifolia* in feed, all other hematological parameters studied were not affected by the different treatments. On the other hand, Toghyani et al. (2010) found that the inclusion of thyme powder in the broiler diet had no significant effect on hemoglobin levels, white blood cells, and red blood cell counts. The increase in hematocrit levels in the blood of chickens on diets containing *C. alternifolius* and *T. angustifolia* rhizome powder in the present work reflects good oxygen and nutrient transport in the blood, leading to accelerated growth of chickens.

The addition of different phyto-additives in the broiler's feed did not have significant effects on biochemical parameters, except HDL-cholesterol level, which significantly increased with the incorporation of *T. angustifolia* and *I. cylindrica* in the diet. Ali et al. (2007)

indicated that the supplementation of broiler feed with thyme powder could decrease HDL and total cholesterol levels in blood serum.

In the present study, the increase in high-density lipoproteins responsible for transporting cholesterol did not use in the target cells to the liver for their elimination, suggesting a considerable decrease in the risk of cardiovascular diseases, sometimes caused by excessive deposition of cholesterol in the arteries by low-density lipoproteins, leading to the sudden death of chickens. The results of the present work are similar to those of Oleforuh-Okoleh et al. (2015), who concluded that the incorporation of 50 ml aqueous extract of ginger, garlic, and ginger-garlic mixture per liter of drinking water has no significant effect on the serum urea concentration.

CONCLUSION

Supplementing feed with powdered rhizomes of *C. alternifolius* and *T. angustifia* at 2g/kg of feed improves feed digestibility, growth performance, and the immunity system, and increases lactic acid bacteria count in broiler's gut. *E. pyramidalis* and *I. cylindrica* induced poor weight gain and high feed conversion ratio, compared to the results obtained with the antibiotic (positive control). It would be useful to extract, isolate and quantify the major bioactive compounds present in each phyto-additive studied and assess their individual effects on the growth performance of broilers.

DECLARATIONS

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Authors' contributions

Nyembo Camile, Kana Jean Raphael and Ciza Pascaline conceived, designed the research and wrote the manuscript. Tchoffo Hervé, Amani Innocent, Tchakounte Mael, Tindo Romario, and Tabounda Evariste collected the data, carried out data analysis, and wrote the manuscript. Tchoun Gilchrist and Edie Wilfried revised the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare no conflict of interest.

Ethical consideration

The authors have ensured that this article respects the ethical issues of the journal, such as plagiarism, fabrication and/or falsification of data, permissions to publish and duplicate publication.

Availability of data and materials

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

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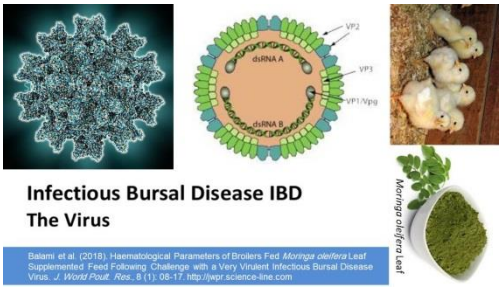

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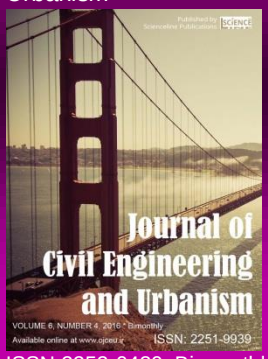

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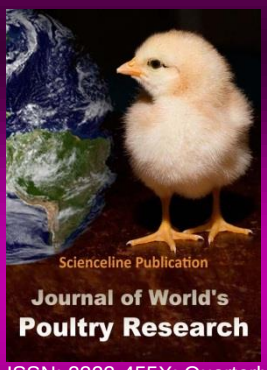

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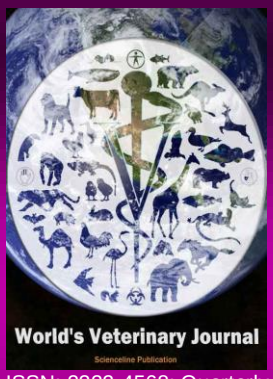

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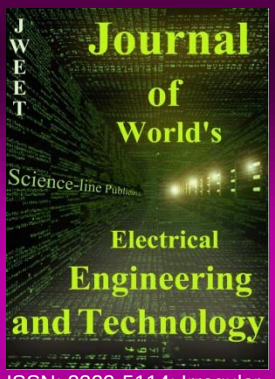

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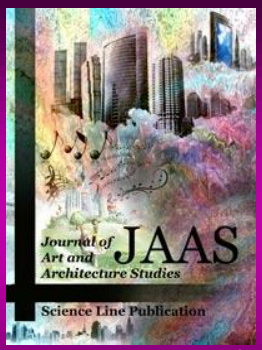

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