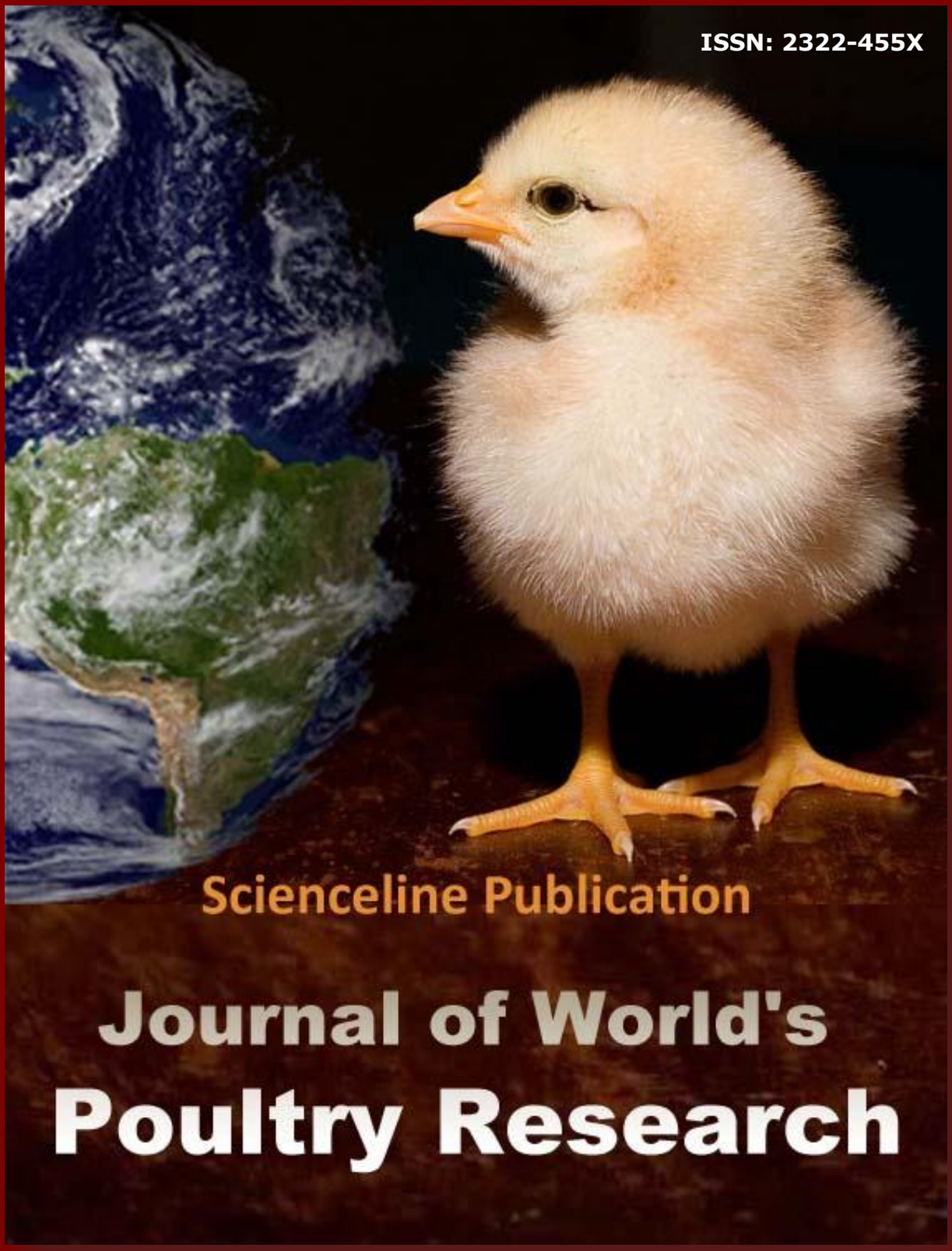
In the Name of God



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Journal of World's Poultry Research

J. World Poult. Res. 11 (1): 01-150; March 25, 2021

Editorial Team

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Volume 11 (1); March 25, 2021

Research Paper

Effects of Diet Containing Fermented Canola Meal on Performance, Blood Parameters and Gut Health of Broiler Chickens.

Elbaz AM.

J. World Poult. Res. 11(1): 01-07, 2021; pii: S2322455X2100001-11

DOI: https://dx.doi.org/10.36380/jwpr.2021.1

ABSTRACT: The current research aimed to study the effects of the fermented canola meal (*Lactobacillus*) diet on productive performance, blood parameters, and gut health of broiler chickens under high ambient temperature conditions. A total number of 320 (Ross-308) one-day-old broiler chickens were allocated randomly into four experimental groups for 42 days. Four experimental groups with four types of diet, including the control group (CON) received basal diet, and three other experimental groups were supplemented with 20% of the canola meal (CM), 20% fermented canola meal (FCM), and 20% canola meal with probiotic (PCM). The chickens that fed FCM presented improvement in live body weight, feed conversion ratio, and higher nutrient digestibility, compared to CM and PCM groups. Serum glucose, total protein, albumin, and aspartate aminotransferase (AST) of levels of chickens fed by FCM were higher than chickens fed CM and PCM, while there was a decrease in cholesterol. Fermented canola meal resulted in some noticeable beneficial changes in the cecum microflora communities through increasing the population of *Lactobacillus* spp. and decreasing the *Escherichia coli* and improved its morphology by increasing villus height. The results indicated that the fermentation of canola meal has enhanced performance, nutrient digestibility, and gut health, which allow using greater amounts of fermented canola meal as a replacement of soybeans meal in the broiler diet.
Keywords: Broiler, Canola meal, Fermentation, Gut health, Performance, Serum parameter**.**

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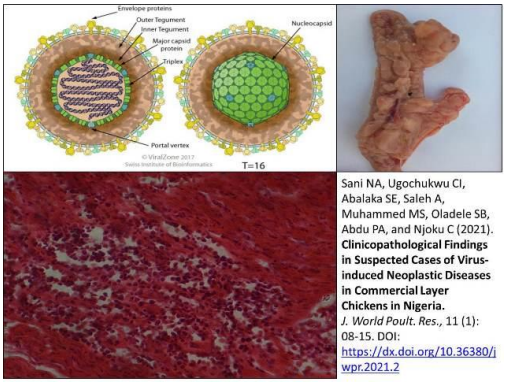
Research Paper

Clinicopathological Findings in Suspected Cases of Virus-induced Neoplastic Diseases in Commercial Layer Chickens in Nigeria.



Sani NA, Ugochukwu CI, Abalaka SE, Saleh A, Muhammed MS, Oladele SB, Abdu PA, and Njoku C.

J. World Poult. Res. 11(1): 08-15, 2021; pii: S2322455X2100002-11
DOI: https://dx.doi.org/10.36380/jwpr.2021.2

ABSTRACT: Avian neoplastic diseases, including Marek's disease (MD), avian leukosis (AL), and reticuloendotheliosis (RE), are of economic importance in the chicken industry. However, it is difficult to differentiate MD from AL and RE by clinical signs and postmortem examination. Therefore, the present study aimed to classify the avian neoplastic diseases affecting commercial layer chickens in Nigeria using clinical history, postmortem examination, and histopathological technique. Carcasses of commercial layer chickens from 7 and 20 poultry farms in Kaduna and Plateau States were studied, respectively, from February 2017 to March 2018. The age, morbidity, and mortality rates in each of the affected farms were determined. Detailed postmortem examinations were carried out on the carcasses from the affected farms, and organs observed to have neoplastic lesions were fixed in 10% neutral buffered formalin for histopathology. The age means of the affected layers were 20.6 weeks and 20.8 weeks in Kaduna and Plateau States, respectively. The average morbidity rates of neoplasm in the affected layers were 3.9% and 9.3% in Kaduna and Plateau States, respectively, while the average mortality rates were 8.6% and 8.5% in Kaduna and Plateau States, respectively. The clinical observation of affected chickens indicated that they were anorexic and emaciated. Generally, the neoplastic lesions were characterized by white to gray, multifocal, firm nodules of varying sizes on the affected organs. In Kaduna State, the neoplasms were commonly observed on the liver (85.7%), spleen (71.4%), heart (42.9%), and kidneys (42.9%), while in Plateau State, the affected organs included liver (50%), spleen (25%), proventriculus (25%) and lungs (25%). The histopathological changes in the affected tissues were similar and characterized predominantly by the infiltration of lymphocytes, lymphoblasts, and macrophages. The patterns of distribution of the pleomorphic neoplastic cells within the liver were multifocal and perivascular in most cases. Findings from the current study indicated that cases of neoplasms in commercial layer chickens in Kaduna and Plateau States, Nigeria, could be attributed to MD.
Keywords: Avian neoplastic diseases, Layer chickens, Pathology.

[Full text-PDF] [XML] [Crossref Metadata]

Research Paper

Effect of Pre-Slaughter Antacid Supplementation of Drinking Water on Carcass Yield and Meat Quality of Broiler Chickens.

Namted S, Srisuwan K, Bunchasak C, and Rakangthong C.

J. World Poult. Res. 11(1): 16-21, 2021; pii: S2322455X2100003-11
DOI: https://dx.doi.org/10.36380/jwpr.2021.3

ABSTRACT: Antacid is a mixture of sodium bicarbonate, bicarbonate, and citric acid, which can neutralize stomach acidity and may stabilize the pH of post-mortem carcass and meat. Therefore, the present study aimed to investigate the carcass and meat quality of broiler chickens by supplementing the antacid in drinking water. A total of 48 male broiler chickens (Ross 308) were divided into two groups that the first group was the control group (did not receive antacid supplementation in the drinking water) and the second group was supplemented with antacid in drinking water (0.10%) for three days pre-slaughter. It was found that the antacid supplementation increased the percentage of breast meat, while carcass yield, and thigh, drumstick, and wing were not significantly affected. The pH of breast meat 45 minutes and the drip loss at 24 hours post-slaughter was significantly higher. The shear-force of breast meat was reduced ($P < 0.05$) by antacid supplementation. For the color of the breast meat, there were no significant differences in lightness (L*), redness (a*), and yellowness (b*) between the two groups, but the total difference in the color of meat was slightly increased. It can be concluded that supplementing the drinking water with an antacid for three days before slaughter improves the carcass and meat quality of broiler chickens by maintaining the pH and water holding capacity of the meat.
Key words: Antacid, Broiler chickens, Carcass yield, Meat quality.

[Full text-PDF] [XML] [Crossref Metadata]

Research Paper

Effects of Acetaminophen and Vitamin Supplement on Feed intake, Body Weight, and Acute Pain Responses of Pullets Subjected to Beak-trimming.

Okoroafor ON, Okereke HN, and Udegbunam RI.

J. World Poult. Res. 11(1): 22-30, 2021; pii: S2322455X2100004-11
DOI: https://dx.doi.org/10.36380/jwpr.2021.4

ABSTRACT: The first experiment aimed to compare the effects of manual and electric beak-trimming on feed intake, body weight, and some biochemical parameters of eight-week-old pullets. A total of 20 pullets aged 3 weeks were assigned to two treatment groups; those in group A were manually debeaked while the samples in group B were electro debeaked. The findings indicated that 2 hours post-beak-trimming (PBT), the packed-cell volume of group A was significantly higher than that of group B. Plasma cortisol level of group A was significantly higher than that of group B at 2 and 6 hours PBT while total plasma protein level of group A was significantly lower than that of the group at 2 and 72 hours PBT. Furthermore, feed intake and body weight markedly decreased in the pullets debeaked with both methods until 72 hours PBT. The chickens' beak trimmed with both methods experienced intense pain of varying degrees lasting up to 72 hours. In the second experiment, 40 eight-week-old pullets were assigned to four groups; group A was the control, group B was treated with a vitamin supplement, group C was treated with acetaminophen, and group D was treated with vitamin supplement plus acetaminophen. After 24 hours, chickens were beak-trimmed using a manual cutter. The results revealed that 2 hours PBT, plasma cortisol level in groups B, C, and D were significantly lower than that of group A. Blood glucose was lowest in groups A and D at 6 and 24 hours PBT, respectively. It is concluded that the pre-treatment with vitamins and NSAIDs could reduce stress and pain in debeaked chickens.
Keywords: Anti-stress, Debeaking, Pain, Pullet**.**

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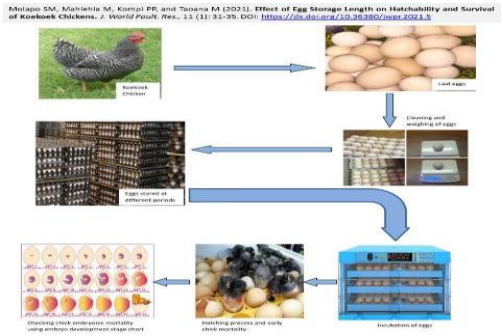
Research Paper

Effect of Egg Storage Length on Hatchability and Survival of Koekoek Chickens.



Molapo SM, Mahlehla M, Kompi PP, and Taoana M.

J. World Poult. Res. 11(1): 31-35, 2021; pii: S2322455X2100005-11
DOI: https://dx.doi.org/10.36380/jwpr.2021.5

ABSTRACT: Chicken production plays a major in the livelihood of rural people due to the provision of eggs and meat which are high sources of

protein. This calls for sustainable production of chickens through strategies aimed at improving the hatchability of eggs and survival of chickens. Therefore, the present study was conducted to determine the effect of egg storage length on egg hatchability and survival of the Koekoek chickens. A total number of 270 eggs were divided into three treatment groups, and the eggs of each group were stored for 3, 7, and 11 days before incubation. Each treatment consisted of three replicates. The General Linear Model procedure was used to analyze the data. The eggs that were stored for three days before incubation had a higher hatching percentage, compared to those that were stored for 7 and 11 days before incubation. Storing eggs for few days before incubation resulted in reduced embryonic mortality rate and lower mortality of chickens during the first seven days after hatching. Based on these results, is recommended that Koekoek chicken eggs should be stored for three days before incubation to maximize hatchability and survival of chickens before the age of seven days.
Keywords: Eggs, Storage, Embryo mortality, Hatchability, Koekoek chicken.

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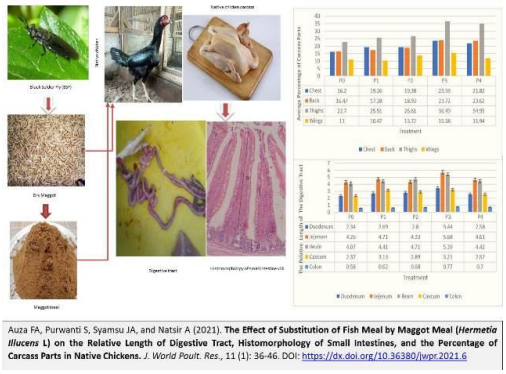
Research Paper

The Effect of Substitution of Fish Meal by Maggot Meal (*Hermetia Illucens* L) on the Relative Length of Digestive Tract, Histomorphology of Small Intestines, and the Percentage of Carcass Parts in Native Chickens.

Auza FA, Purwanti S, Syamsu JA, and Natsir A.

J. World Poult. Res. 11(1): 36-46, 2021; pii: S2322455X2100006-11
DOI: https://dx.doi.org/10.36380/jwpr.2021.6



ABSTRACT: The development of the digestive tract organs is closely related to the increased body weight growth in chickens. The present study aimed to determine the effect of using maggot meal as an antibacterial and protein source of fish meal substitution in diets on the relative length of the digestive tract organs, small intestine histomorphology, and the percentage of the native chicken carcass. A total of 140 one-day-old chickens were randomly assigned to one of the five treatments according to a completely randomized design with four replications for each treatment. The treatments included P0 (basal diet + 15% fish meal + 0% maggot meal), P1(basal diet + 11.25% fish meal + 3.75% maggot meal), P2 (basal diet + 7.5% fish meal + 7.5% maggot meal), P3(basal diet + 3.75% fish meal + 11.25% maggot meal), and P4 (basal diet + 0% fish meal + 15% maggot meal). The results showed that the use of maggot meal in P3 had a significant effect ($P < 0.05$) on the relative length, villi height, depth of duodenal crypt, jejunum and ileum, villi surface area, the density of jejunum and ileum villi, and percentage of thigh and wing weight. Besides, the treatment tended to increase the relative length of the caecum and colon, surface area of the duodenal villi, and chest weight percentage. However, the treatment did not affect the duodenal villi density and percentage of back weight in native chickens. The use of maggot meal up to 11.25% in diets can improve the relative length of intestinal, histomorphology of small intestine's villi, and the percentage of carcass parts of native chickens.
Keywords: Carcass parts, Digestive tract, Histomorphology, Maggot meal, Native chicken.

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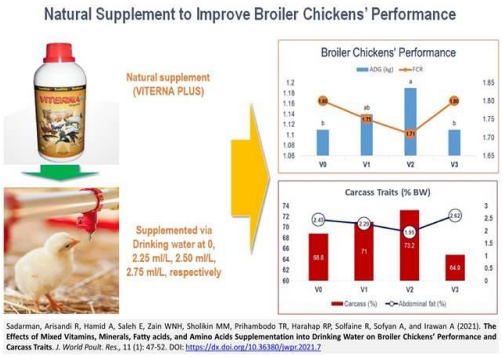
Research Paper

The Effects of Mixed Vitamins, Minerals, Fatty Acids and Amino Acids Supplementation into Drinking Water on Broiler Chickens' Performance and Carcass Traits.

Sadarman, Arisandi R, Hamid A, Saleh E, Zain WNH, Sholikin MM, Prihambodo TR, Harahap RP, Solfaine R, Sofyan A, and Irawan A.

J. World Poult. Res. 11(1): 47-52, 2021; pii: S2322455X2100007-11
DOI: https://dx.doi.org/10.36380/jwpr.2021.7



ABSTRACT: The present study was conducted to evaluate the effects of different levels of the feed supplement containing minerals, fatty acids, vitamins, and amino acids added to drinking water on broiler chickens' performance and carcass traits. A total of 100 one-day-old Cobb 707 (mean weight 46.7 g) were randomly assigned into four treatments, including control group (C), C + 2.25 ml/L Viterna Plus (V1), C + 2.50 ml/L Viterna Plus (V2), and C + 2.75 ml/L Viterna Plus (V3). Each treatment group contained 5 replicates of 5 birds in each (25 birds per treatment). Birds were maintained for 28 days. The results suggested that feed supplement at 2.50 ml/L could successfully improve final body weight, performance index, and carcass weight ($P < 0.05$). Concurrently, the treatment also reduced the percentage of abdominal fat ($P 0.05$). In conclusion, the incorporation of commercial feed supplement containing mixed of minerals, vitamins, and amino acids at 2.50 ml/L into drinking water improved the overall performance of the broiler chickens.
Keywords: Broiler chicken, Carcass, Feed supplement, Tropics, Viterna plus.

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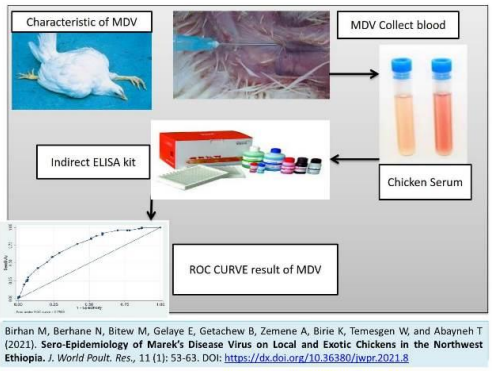
Research Paper

Sero-Epidemiology of Marek's Disease Virus on Local and Exotic Chickens in the Northwest Ethiopia.

Birhan M, Berhane N, Bitew M, Gelaye E, Getachew B, Zemene A, Birie K, Temesgen W, and Abayneh T.

J. World Poult. Res. 11(1): 53-63, 2021; pii: S2322455X2100008-11
DOI: https://dx.doi.org/10.36380/jwpr.2021.8



Birhan M, Berhane N, Bitew M, Gelaye E, Getachew B, Zemene A, Birie K, Temesgen W, and Abayneh T (2021). **Sero-Epidemiology of Marek's Disease Virus on Local and Exotic Chickens in the Northwest Ethiopia.** *J. World Poult. Res.*, 11 (1): 53-63. DOI: https://dx.doi.org/10.36380/jwpr.2021.8

ABSTRACT: Marek's disease (MD) is a lymphoproliferative and neuropathic disease of domestic fowl caused by alphaherpesviruses. The current cross-sectional study with a simple random sampling method was undertaken from January 2018 to May 2020. The current study aimed to estimate sero-epidemiology and assess potential risk factors, it is very important to determine MD sero-positivity. Moreover, to measure the association of host and environmental risk factors, the occurrences and spread of MD were identified in local and exotic chickens in Northwest Ethiopia. A total of 768 serum samples from 3 zones were collected and assayed for Marek's Disease Virus (MDV) antibodies using the indirect enzyme-linked immunosorbent assay test. A questionnaire survey was also conducted to gather information on the potential risk factors of MDV sero-positivity, as well as the status of occurrences and spread in the chicken flocks. In the present preliminary study, a high flock and chicken level of MDV was demonstrated, with an overall seroprevalence of 59.11%. The mixed-effect logistic regression analysis of the host potential risk factors showed that the odds of seropositive for MD was significantly higher in local chickens (OR: 1.70, 95% CI: 1.26-2.28) than exotic chickens, higher in chickens getting non-proper feed (OR: 0.26, 95% CI: 0.13-0.54) than getting proper feed, higher in vaccinated chickens (OR: 1.04, 95% CI: 0.76-1.43) than non-vaccinated chickens. Rearing chicken of different batches in one house was decreased the odds of occurrence of MD by 55% (95% CI: 0.38-0.80) than all-in-all-out, higher in the well-ventilated type farms decreased the odds of occurrence of MD by 60% (95% CI: 0.39-0.80) than in poor ones. Litter management when farmers used chickens as fertilizer has decreased the odds of occurrence of MD by 55% (95% CI: 0.01-0.47) and chickens were tossed into open sheds 40% (95% CI: 0.01-0.31), compared to buried chickens management. The study results indicated that the number of farms where farmers wearing no clothes and shoes (95% CI: 0.10-0.58) were significantly decreased the occurrence of MD by 24% than those where farmers were equipped with clothes and shoes. The study area was highest in West Gojjam (OR: 0.40, 95% CI: 0.27-0.58) and South Gondar (OR: 0.19, 95% CI: 0.13-0.28) compared to North Gondar zone. In conclusion, the present study revealed a high flock and chicken seroprevalence level of MDV among chicken flocks in northwest Ethiopia, suggesting that environmental dust/dander and farm management systems might be a source of this disease for chicken infection. Besides, the observed association of MD, sero-positivity with environmental dust/dander, and farm management systems may suggest the economic importance of the disease for chicken production. Therefore, it warrants control attention to reduce its economic and disease spread burden in the study areas. Further works on the economic impacts, virus isolation, and molecular characterization of the disease are suggested.
Keywords: Chicken, Marek's Disease, Northwest Ethiopia, Risk factors, Sero-epidemiology.

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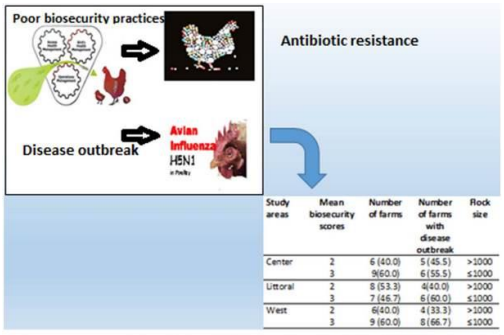
Research Paper

Biosecurity Practices and Characteristics of Poultry Farms in Three Regions of Cameroon.

Tatfo Keutchatang FDP, Bouelet Ntsama IS, Medoua Nama G, and Kansci G.

J. World Poult. Res. 11(1): 64-72, 2021; pii: S2322455X2100009-11
DOI: https://dx.doi.org/10.36380/jwpr.2021.9



Tatfo Keutchatang FDP, Isabelle Sandrine B N, Medoua Nama G, and Kansci G (2021). **Biosecurity Practices and Characteristics of Poultry Farms in Three Regions of Cameroon.** *J. World Poult. Res.*, 11 (1): 64-72. DOI: https://dx.doi.org/10.36380/jwpr.2021.9

ABSTRACT: The outbreak of diseases is the main factor affecting poultry production in Cameroon. The implementation of biosecurity measures in poultry farms is essential to reduce disease outbreaks. This study aimed to assess biosecurity practices in poultry farms in three regions of Cameroon. The study was carried out using a structured questionnaire on 90 randomly selected poultry farms. Most of the farmers were men (85%) with deep litter (77.8%), battery cage (2.2%), and both deep litter and battery cage (20.0%) housing systems. Amongst the farms surveyed, 9/30 (30.0%) in the Centre; 8/30 (26.7%) in the Littoral; and 13/30 (43.3%) in the West were aware of biosecurity measures. The biosecurity score (BS) of surveyed farms ranged between 2 and 3. The findings indicated that 39 farms (12 in the Centre, 14 in the Littoral, and 13 in the West) were at moderate risk, and 51 farms (18 in the Centre, 16 in the Littoral, and 17 in the West) were at high risk. Reasons for keeping chickens and the number of chickens per farm did not significantly influence BS, while the farm category could significantly affect it. The outbreak of diseases correlated with BS, showing a tendency of increase in the outbreak of diseases with increasing BS. This study underlines the fact that biosecurity practices in Cameroon have not been well implemented by chicken farmers. This leads to disease outbreaks, and consequently, important economic losses as well as massive use of drugs that may be unsafe for human consumption. Therefore, the effective monitoring of biosecurity in chicken farming should be encouraged by extension of training to the farmers to support the efficient production of chickens by respecting biosecurity that drastically reduces the risk of disease outbreaks and provides good quality chicken products for human consumption.
Keywords: Assessment, Biosecurity practices, Biosecurity scores, Cameroon, Poultry farms.
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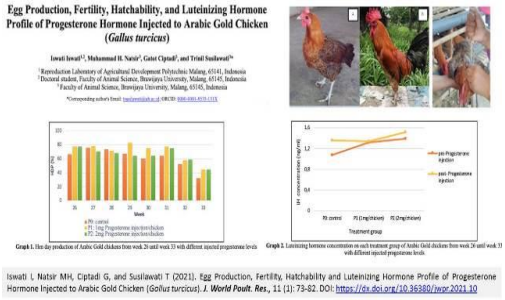
Research Paper

Egg Production, Fertility, Hatchability and Luteinizing Hormone Profile of Progesterone Hormone Injected to Arabic Gold Chicken (*Gallus turcicus*).



Iswati I, Natsir MH, Ciptadi G, and Susilawati T.

J. World Poult. Res. 11(1): 73-82, 2021; pii: S2322455X2100010-11
DOI: https://dx.doi.org/10.36380/jwpr.2021.10

ABSTRACT: The production and reproduction performance of chicken depends on their hormonal status, especially progesterone hormone, which has been known to correlate with egg production. The present study aimed to analyze the effect of progesterone hormone injection on Arabic Gold chickens (*Gallus turcicus*) regarding egg production and luteinizing hormone concentration in blood plasma. A total number of 60 Arabic Gold chickens aged 26-weeks were divided into three groups based on injected hormone concentration (P_0: control; P_1: 1 mg/chicken; P_2: 2 mg/chicken). The study was conducted using a completely randomized design and the obtained data were analyzed with a descriptive analysis for qualitative data and one-way analysis of variance followed with Duncan's Multiple Range Test as a post hoc test for the quantitative data. The results presented that progesterone hormone injection had a significant effect on hen day production two and six weeks after injection. The P_1 group was able to reach its peak production (82.9%) at week 29, while the P_2 group reached its peak at week 26 (78.9%). In addition, it was found that the P_2 group produced a soft-shelled egg and double egg yolk. Progesterone injection led to no significant effect on the egg weight, shape index, fertility, embryo viability, hatchability, and chick weight at hatch. The luteinizing hormone concentration was higher in P_2 (1.52 ng/ml), compared to P_0 (1.36 ng/ml) and P_1 (1.34 ng/ml) groups. It was concluded that progesterone hormone injection during the production phase of Arabic Gold chicken had a significant effect on egg production and caused varying egg production peak and luteinizing hormone concentration.
Keywords: Arabic Gold chicken, Egg quality, Hen day production, Luteinizing hormone, Progesterone.

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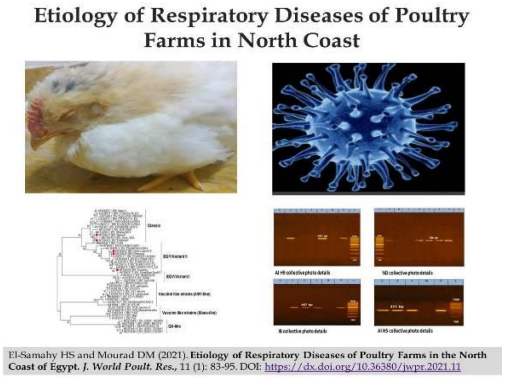
Research Paper

Etiology of Respiratory Diseases of Poultry Farms in the North Coast of Egypt.



El-Samahy HS and Mourad DM.

J. World Poult. Res. 11(1): 83-95, 2021; pii: S2322455X2100011-11
DOI: https://dx.doi.org/10.36380/jwpr.2021.11

ABSTRACT: The current study aimed to identify the respiratory problems in poultry farms located in the north coast of Egypt from October 2018 to November 2019. A total of 89 poultry flocks (79 broilers, 5 layers, 3 ducks, and 2 turkeys) were investigated for four major viral respiratory pathogens, namely avian influenza (AI) H9N2, AI H5 subtypes, Newcastle Disease (ND), and Infectious Bronchitis (IB) viruses. All 89 flocks were subjected to real-time PCR to investigate AI H9N2 virus. The samples of 31, 43, and 15 out of 89 flocks were selected for the investigation of ND, IB, and AI H5 subtypes viruses, respectively, using real-time PCR. Sample selection was performed according to the mortalities, clinical signs, and post mortem lesions. The positive findings indicated that 22 out of 89 flocks were positive for AI H9N2 virus (2 layers + 20 broilers), 32 out of 43 flocks were positive for IB virus (2 layers + 30 broilers), 24 out of 31 flocks were positive for ND virus (1 Duck + 1 layer+ 22 broilers) and 9 out of 15 flocks were positive for AI H5N8 virus (1 turkey + 1 duck + 7 broilers). Partial sequencing for selected isolates of six ND, five IB, four H9N2, and three H5N8 viruses was applied, then nucleotide sequences were accessed on GenBank. Six ND isolates belonged to genotype VII viruses circulating in Egypt. Two IB isolates were related to the classical strain circulating in Egypt, while the other three IB isolates belonged to EGY/Variant II. Four H9N2 AI isolates were related to G1-lineage of H9 viruses circulating in the Middle East and Egypt. Three H5N8 AI isolates belonged to the highly diverse clade 2.3.4.4.b viruses circulating in Egypt. It was concluded that ND and IB viruses isolated in this study were not related to their vaccinal strains. Also, AI H5N8 circulating alone in affected flocks while AI H9N2 circulating alone and/or mixed with either IB or ND viruses. Finally, there is a need to devise a complete strategy to control the isolated respiratory viruses on the north coast of Egypt.
Keywords: Poultry, Respiratory, RRT-PCR, Sequence, Viruses.

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Research Paper

Isolation and Identification of Newcastle Disease Virus from Ducks Sold at Traditional Livestock Market Center in Indonesia.

Azizah AN, Anwar Ch, and Rahardjo AP.

J. World Poult. Res. 11(1): 96-100, 2021; pii: S2322455X2100012-11
DOI: https://dx.doi.org/10.36380/jwpr.2021.12

ABSTRACT: Newcastle disease (ND) is one of the important infectious diseases in the poultry industry. The traditional poultry markets have great potential in ND transmission. The ducks infected by the ND virus rarely show clinical symptoms, thus they can potentially spread the disease to other fowls. The current study aimed to isolate and identify the ND virus from ducks in a traditional live bird market center in East Java, Indonesia. Cloacal swab samples were taken from 300 ducks. The study consisted of 100 pooled samples, each containing a cloacal swab sample obtained from 3 individual ducks. The samples were inoculated in specific antibody-negative embryonated chicken eggs for 8-10 days. Hemagglutination and hemagglutination inhibition tests were performed for confirmation and identification of ND virus. Based on the result of the current study, out of 100 pooled samples, there were three to nine ducks infected with the ND virus.
Keywords: Cloaca Swab, Duck, Livestock, Newcastle Disease

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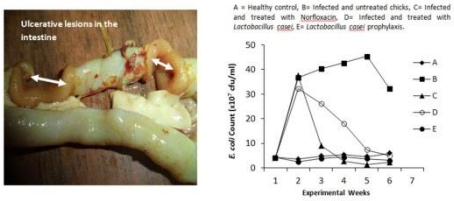
Research Paper

Control of Intestinal *E. coli* Infection in Broiler Chicks Using *Lactobacillus casei* Isolated from *Nono*.



Ikele OM, Ezeonu IM, and Umeh ChN.

J. World Poult. Res. 11(1): 101-109, 2021; pii: S2322455X2100013-11
DOI: https://dx.doi.org/10.36380/jwpr.2021.13

ABSTRACT: The current study aimed to evaluate whether the probiotic *Lactobacillus casei* could be effective in controlling chicken intestinal colibacillosis. Avian pathogenic *Escherichia coli* (*E. coli*.) and *Lactobacillus casei* (*L. casei)* isolates were obtained from *nono* (a sour milk product produced by Fulani tribe of Nigeria), and were used for the chicken infection and probiotic treatment, respectively. The experimental design was conducted on three-week-old broiler chicks, which were divided into five groups, namely A (healthy control), B (infected without treatment), C (infected and treated with antibiotic), D (infected and treated with *L. casei*), and E (initially given *L. casei* before infecting with *E. coli*). Groups C and D were treated using 15 g/L norfloxacin and 1.5 ml of $1.1x10^9$cfu/ml *L. casei,* respectively. Group E was given the oral infusion of 1.5 ml of 1.1×10^9 cfu/ml *L. casei* before infection with 1.5 ml of 1.3×10^7 cfu/ml avian pathogenic *E. coli*. Weight, hematological parameters, liver function, and fecal *E. coli* counts of the chicks were monitored and used to evaluate the level of protection elicited by the probiotic organism. There was weight gain in chicken groups, except for group B. There was a significant difference in the sodium, chlorine, and bicarbonate levels amongst the groups. The hematological profile revealed a significant difference in the hemoglobin, white blood cells, lymphocyte, and neutrophil counts of the chicken groups. Assessment of liver enzymes showed no significant difference amongst the chick groups except in group B. Similar results were obtained for the urea, creatinine, and C-reactive protein levels. The microbial tests revealed a decrease in the total *E. coli* count for groups C, D, and E. The results of the current study indicated that *L. casei* could be used as a probiotic in the control of chicken colibacillosis.
Keywords: Broiler, Colibacillosis, *Escherichia coli*, *Lactobacillus*, *Nono*, Probiotics

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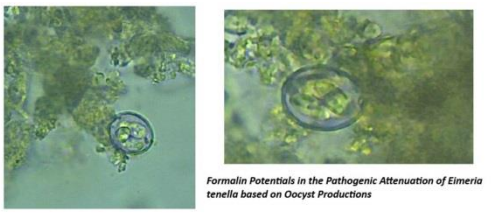
Research Paper

Formalin Potentials in the Pathogenic Attenuation of *Eimeria tenella* based on Oocyst Productions.



Anggraini RD, Luqman EM, and Budhy S.

J. World Poult. Res. 11(1): 110-115, 2021; pii: S2322455X2100014-11
DOI: https://dx.doi.org/10.36380/jwpr.2021.14

ABSTRACT: Coccidiosis is a disease found in poultry caused by parasitic protozoa, namely *Eimeria tenella* (*E. tenella*), which may lead to high rates of morbidity and mortality. To prevent coccidiosis, vaccination is required to inactivate and attenuate *E. tenella* protozoa. One of the compounds applied for attenuation is formaldehyde. Formaldehyde reduces the

pathogenicity of an organism by creating rigidity in its structure. As a result, the organism cannot inflict disease and has a higher impact on building antibodies although it is still alive. The current research was an experimental study aimed to determine the formalin potential in attenuation of *E. tenella* pathogenesis in terms of oocyst production. The present study was conducted using the completely randomized design method. A total number of 25 broiler chickens were applied and their feces were tested to observe oocysts production and clinical symptoms. The obtained data would be analyzed by the ANOVA statistical test. The treatment groups presented clinical symptoms of *E. tenella* infection. The number of oocysts in treatment group I fluctuated from the lowest number which was zero on day five and then increased by day six, seven, and eight and it has reached the peak with the most significant amount of 4,050,460 oocysts on day nine. The treatment group II with the same condition reached its peak with the highest number of 1,363,160 oocysts on day nine. The treatment group III peaked with the most significant number of 618,960 oocysts on day nine. In addition, the treatment IV group attained the apex with the highest number of 719,480 oocysts on day nine. Meanwhile, the treatment V group reached the highest number of 284,200 oocysts on day nine. The difference in formalin concentration affected the amount of *E. tenella* oocyst production of broiler chickens. Formalin soaking with a concentration of 1.2% was the most optimal concentration to attenuate *E. tenella.*
Keywords: Broiler chicken, *Eimeria tenella*, Formalin, Oocyst**.**

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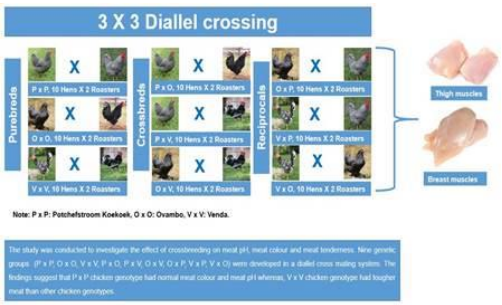
Research Paper

Diallel Analysis on Breast and Thigh Muscle Traits in the Cross of Three South African Indigenous Chicken Genotypes.

Tyasi TL, Ng'ambi JW, and Norris D.

J. World Poult. Res. 11(1): 116-122, 2021; pii: S2322455X2100015-11
DOI: https://dx.doi.org/10.36380/jwpr.2021.15



Tyasi TL, Ng'ambi JW, and Norris D (2021). **Diallel Analysis on Breast and Thigh Muscle Traits in the Cross of Three South African Indigenous Chicken Genotypes.** *J. World Poult. Res.*, 11 (1): 116-122. DOI: https://dx.doi.org/10.36380/jwpr.2021.15

ABSTRACT: The present study aimed to estimate carcass characteristics of pure and crossbred chickens produced from three parental populations. A 3 × 3 complete diallel mating system involving three indigenous breeds, namely Potchefstroom Koekoek (P), Venda (V), and Ovambo (O), was used to produce three purebred (P × P, V × V, O × O), three crossbreds (P × O, P × V, O × V) and three reciprocals (O × P, V × P, V × O). The nine genetic groups were reared from hatch to 10 weeks of age in an open house with deep litter. At 10 weeks of age, six chickens per genetic group were randomly selected for slaughter. After slaughtering the breast and thigh muscles samples for analysis of the carcass characteristics (Meat colour, meat pH, and Shear force). The results showed that the Potchefstroom Koekoek breed had higher values in all colour indicators, L* (lightness), a* (redness), and b* (yellowness), compared to the other chicken breeds. The Potchefstroom Koekoek and P × O breed had higher pH values ranging from 5.66 to 6 at two hours post-slaughter and from 5.54 to 6.38 at 24 hours post-slaughter. The pH declines in all the nine genetic groups after two to 24 hours, with the exception of the crossbred P × O, which increased from 6.06 to 6.38. In terms of shear force, the O × P had the highest shear value, ranging from 35.89N to 74.80N, compared to other genetic groups. Potchefstroom Koekoek had normal meat colour and pH, whereas the Venda breed had tougher meat than other genotypes. The results of the present study might be useful for local chicken farmers to improve carcass traits.
Keywords: Crossbred, Meat colour, Meat pH, Purebred, Shear force.

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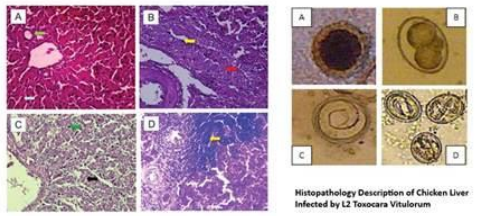
Research Paper

Histopathology Description of Chicken Liver Infected by L2 *Toxocara Vitulorum*.

Auliyah R, Kusnoto, and Hamid IS.

J. World Poult. Res. 11(1): 123-128, 2021; pii: S2322455X2100016-11
DOI: https://dx.doi.org/10.36380/jwpr.2021.16



Auliyah R, Kusnoto, and Hamid IS (2021). **Histopathology Description of Chicken Liver Infected by L2 *Toxocara Vitulorum*.** *J. World Poult. Res.*, 11 (1): 123-128. DOI: https://dx.doi.org/10.36380/jwpr.2021.16

ABSTRACT: Transmission of *Toxocara vitulorum* Infection causes a decrease in livestock productivity and results in various types of diseases in humans. Chickens are one of the parasitic hosts of toxocariasis which has the potential for transmission of toxocariasis to humans. The main location affected by *T. vitulorum* larval infection is the liver. The current study aimed to analyze the description of histopathological changes in the liver of broiler chickens infected by L2 *Toxocara vitulorum.* The present study was a true experiment using a completely randomized design. A total number of 28 broiler chickens aged 14 days were selected as the sample in this study. Samples were infected using L2 *Toxocara vitulorum* larvae and were grouped in accordance with observations of the 1, 2, 3, 7, 14, and 21 days after the larvae were given to the samples. *Toxocara vitulorum* larval infection caused changes in histopathological features of broilers chickens. This infection caused hydropic inflammation and degeneration of liver cells, cholangitis, and eventually necrosis of the cells. Exposure to infection over a long period of time can worsen liver cell and other organ damages as well as increasing the potential for the transmission of *Toxocara vitulorum* larvae.
Keywords*:* Chicken, Histopathology of liver, Infection, *Toxocara vitulorum***.**

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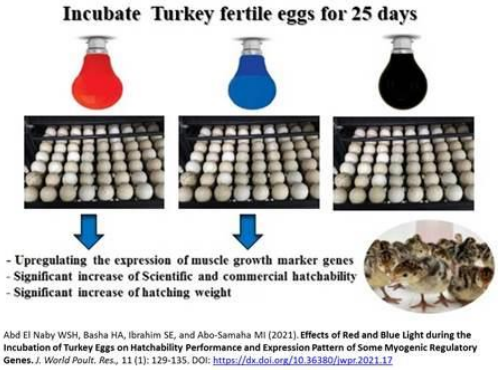
Research Paper

Effects of Red and Blue Light during the Incubation of Turkey Eggs on Hatchability Performance and Expression Pattern of Some Myogenic Regulatory Genes.



Abd El Naby WSH, Basha HA, Ibrahim SE, and Abo-Samaha MI.

J. World Poult. Res. 11(1): 129-135, 2021; pii: S2322455X2100017-11
DOI: https://dx.doi.org/10.36380/jwpr.2021.17

ABSTRACT: The present study aimed to investigate the effects of different light colors on hatching potential traits, including egg weight loss, scientific and commercial hatchability, mortality percentages, hatching wight as well as mRNA expression levels of some muscle growth marker genes (Myogenin, MyoD1, and FGF2) of pectoralis muscle in hatched and non-hatched non pipped Black Bronze turkey chicks. A total of 1500 hatching Black Bronze turkey eggs were assigned equally to three incubation treatment groups, namely dark (control group), red, and blue LED light (treated groups) for 25 days of the incubation period. Results indicated that colored lighting stimuli (red and blue) significantly affected hatching capability. This issue could also affect the expression of muscle growth marker genes in hatched and non-hatched non pipped turkey chicks. Incubation of turkey eggs under red or blue LED light showed an insignificant effect on mortality percentages. It can be concluded that the use of a red or blue light system during turkey eggs' incubation could improve hatchability via upregulating the expression of muscle growth marker genes.
Keywords: Hatchability, Incubation, Light color, Marker Gene expression, Turkey

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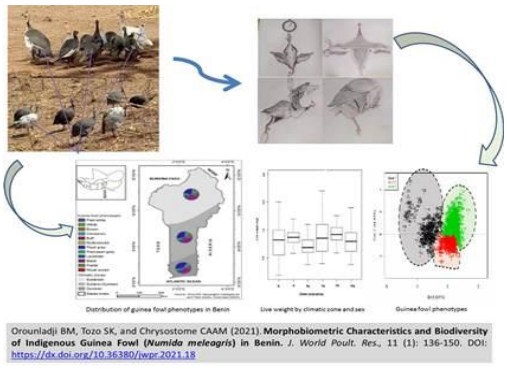
Research Paper

Morphobiometric Characteristics and Biodiversity of Indigenous Guinea Fowl (*Numida meleagris*) in Benin.



Orounladji BM, Tozo SK, and Chrysostome CAAM.

J. World Poult. Res. 11(1): 136-150, 2021; pii: S2322455X2100018-11
DOI: https://dx.doi.org/10.36380/jwpr.2021.18

ABSTRACT: The present study aimed to describe the morphobiometric characteristics of indigenous guinea fowl (Numida meleagris) populations in Benin. The current study was carried out on 1320 (529 males and 791 females) adult (at least 24 weeks old) indigenous guinea fowls from three climatic zones (Sudanian, Sudano-Guinean, and Guinean) of Benin. Each guinea fowl was subjected to a direct phenotypic description, biometric measurements, and photography. The results showed that the plumage coloration of indigenous guinea fowl in Benin was significantly diverse, but the most widespread plumage colors were pearl grey (30%), black (29.5%), and cinnamon (9.8%). The most common beak colors were grey (64.9%) and yellow-orange (24.8%). The eyes were predominantly black-white (67.1%). Grey-orange (33.7%), grey (32%), and black-orange (21%) colorations were more represented on the shanks with wattles relatively dominated by red-white (59.4%) and white-red (30.5%). The average live weight of guinea fowl was 1.34 kg in males which was 4.38% heavier than females. All the biometric measurements were significantly higher in males. The live weights of guinea fowl in the Sudanian zone (1.40 ± 0.18 kg) were higher than those of guinea fowl found in the Sudano-Guinean zone (1.27 ± 0.24 kg) and Guinean zone (1.33 ± 0.28 kg). Principal Component Analysis indicated that three distinct groups of guinea fowl can be formed based on their biometric measurements (live weight, chest circumference, body length, drumstick length, shank length, shank diameter, and wingspan). The phenotypes' diversity was relatively abundant (1-Hill: 0.69) in all climatic zones. The phenotypic biodiversity observed in the populations of indigenous guinea fowl in Benin can guide farmers to select specific phenotypes to meet consumer preferences.
Keywords: Benin, Biodiversity, Climatic zone, Indigenous guinea fowl, Phenotypic characteristic.

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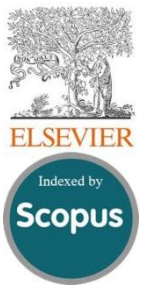

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Effects of Diet Containing Fermented Canola Meal on Performance, Blood Parameters, and Gut Health of Broiler Chickens

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ABSTRACT

The current research aimed to study the effects of the fermented canola meal (*Lactobacillus*) diet on productive performance, blood parameters, and gut health of broiler chickens under high ambient temperature conditions. A total number of 320 (Ross-308) one-day-old broiler chickens were allocated randomly into four experimental groups for 42 days. Four experimental groups with four types of diet, including the control group (CON) received basal diet, and three other experimental groups were supplemented with 20% of the canola meal (CM), 20% fermented canola meal (FCM), and 20% canola meal with probiotic (PCM). The chickens that fed FCM presented improvement in live body weight, feed conversion ratio, and higher nutrient digestibility, compared to CM and PCM groups. Serum glucose, total protein, albumin, and aspartate aminotransferase (AST) of levels of chickens fed by FCM were higher than chickens fed CM and PCM, while there was a decrease in cholesterol. Fermented canola meal resulted in some noticeable beneficial changes in the cecum microflora communities through increasing the population of *Lactobacillus* spp. and decreasing the *Escherichia coli* and improved its morphology by increasing villus height. The results indicated that the fermentation of canola meal has enhanced performance, nutrient digestibility, and gut health, which allow using greater amounts of fermented canola meal as a replacement of soybeans meal in the broiler diet.

Keywords: Broiler, Canola meal, Fermentation, Gut health, Performance, Serum parameter

INTRODUCTION

The increase in the price of feed ingredients during recent years in Egypt had a strong impact on the production cost of the poultry industry. This issue could put breeders under pressure since most of the feed ingredients are imported from abroad. Agricultural waste and oil extraction waste are a source of environmental pollution for their enormous quantities, which are useless and difficult to dispose of. This has compelled nutritionists to explore any viable method for incorporating alternative feedstuffs in poultry diets. Protein and energy sources in poultry feed are the most important and expensive ones; however, the protein sources are more expensive. Several studies have focused on the efficient use of alternative plant protein sources, or agricultural waste (unconventional feed ingredients). However, many problems would be encountered in the use of these alternatives such as antinutritional factors, low protein contents, and high fiber, which affect utilization and digestion of feed in monogastric (Alshelmani et al., 2016; Zhang et al., 2016). The chicken's digestive tract produces no enzymes to hydrolyze crude fiber, therefore, the utilization of unconventional feed ingredients in the chickens' diet would be limited and in need of processing.

Hence, many researchers have thought about solving these problems either by heat, or fermentation. As a result of the developments in microbiology, the poultry feed industry has found an effective method to improve the utilization of some unconventional feed ingredients through the fermentation process. Many studies indicated that the fermentation process leads to an increase in crude protein content, a decrease in antinutritional factors (such as tannins and glucosinolates), and crude fiber content (Mukherjee et al., 2016; Soumeh et al., 2019). One of the most important and effective methods to eliminate the antinutritional factors in unconventional feed ingredients is through fermentation, which improves its nutritional value (Croat et al., 2016; Mahata et al., 2019). Many studies presented that the fermentation process or adding microorganisms enhanced digestive enzyme activities and modulation of the immune system, improved intestinal morphology, increased gut beneficial microbes, and prevented the colonization of enteropathogens through

competitive exclusion and antagonistic activities leading to an improvement in growth performance (Chachaj et al., 2019; Mahata et al., 2019; Abdel-Moneim et al., 2020). Additionally, the fermentation process effectively reduces the glucosinolates level of Rapeseed Meal (RSM, Hu et al., 2016), hence, fermentation may improve the nutritional value of RSM when presented in broiler feeds. The current study was conducted to evaluate the performance, plasma biochemistry variables, intestinal microflora, and histomorphology of broilers fed with Fermented Canola Meal (FCM) and also to determine the possibility of using fermented canola meal (CM) at 20% as a replacement of soybean meal in broiler diet.

MATERIALS AND METHODS

Ethical approval

The current experiment was conducted at the poultry farm of the Poultry Research Unit (Siwa station) following the instructions of the Experimental Animals Care Committee, approved by the Institutional Ethics Committee, Desert Research Center, Egypt.

Fermentation of canola meal

Canola meal was purchased from the Desert Research Center Environmental Division. The Lactobacillus fermentum (CGMCC No. 0843) was brought from the Department of Microbiology, Faculty of Agriculture, Ain Shams University, Egypt. The required amount of canola meal for the experiment was weighed (80 kilograms) and mixed with distilled water (to raise the humidity) then mixed with the microbe (at a rate of 1 gram per kilogram of canola meal), placed the mixture in a polythene bag (5kg capacity), and created suitable anaerobic conditions to activate the L. Fermentum followed by 30-day incubation at room temperature (ranging from 30 to 32°C). The fermented canola was dried in the oven at 45°C for 3 days (Rodriguez-Leon et al., 2008). The freshly dried fermented canola was ground and kept at room temperature for chemical analysis and preparing the diets.

Chemical analysis of canola meal and fermented canola meal

The CM and FCM samples were analyzed for their chemical composition in the official accredited feed analysis laboratory in Egypt. Results indicated that crude protein increased from 36.31% in CM to 39.65% in FCM (Association of Official Analytical Chemists, 1990), crude fat increased from 1.61% to 2.18% (Wang et al., 2004). Meanwhile, crude fiber (12.80% to 8.56%), tannin (1.53% to 0.96%), and total aflatoxin (36 ng/g to 22 ng/g) decreased (determined according to Van Soest et al., (1991), International Organization for Standardization (1988), and Howell et al., (1981) respectively).

Experimental design and chicken housing

The presented experiment was conducted at the poultry farm of the Poultry Research Unit (Siwa station) for 42 days. The experiment started on August 10, 2019. A total number of 320, one-day-old broiler chicks (Ross-308, commercial hatchery, from a poultry company in Cairo, Egypt) were randomly allotted to cages (80 chickens in each group, four replicates of 20 chickens per each group) in a total of four treatments. The control chickens (CON) were fed with a corn-soybean diet, and three other experimental diets were supplemented with 20% of the Canola Meal (CM), 20% Fermented Canola Meal (FCM), and 20% Canola Meal with Probiotic (PCM) to replace part of soybean meal. *Lactobacillus fermentum* 1 kilo/ton was added as a source of probiotic. The starter (1–21 days) and grower (22–42 days) diets were formulated as presented in Table 1. The experimental diets were formulated to meet the nutritional requirements of the chickens at each phase of development according to the recommendations of the National Research Council (NRC, 1994). The diets and water were provided ad libitum for 42 days. The lighting for each cage was 60-watt for 24 hours in the first week and 22 hours until the end of the experiment. The chickens were vaccinated against Newcastle disease at the age of 7, 19, and 30 days as well as gumboro disease at the age of 15 days, and against influenza at the age of seven days. The chickens were raised at 33.0 ± 1°C for the first three days, and then the temperature was gradually reduced to 30.5 ± 1°C by day 10, then left with ambient temperatures to the end of the experiment.

Growth performance and nutrient digestibility

At the age of 42 days, Live Body Weight (LBW), and Feed Intake (FI) were recorded individually for each replicate, and used to calculate Feed Conversion Ratio (FCR). At the end of the experiment, four chickens from each experimental group were randomly selected for slaughtering to measure the carcass traits. The percentage of carcass yield, spleen, gizzard, heart, abdominal fat, thymus, bursa of Fabricius, and small intestine relative weights were measured. At the age of 42 days, the digestion experiment started. Four chickens from each treatment group were weighed and housed in metabolic

cages individually then starved for 12 hours. During the age of 42 to 45 days, unpolluted excreta were collected, three times a day from the bottom of each cage, weighed dried, and then froze (-20 ^{0}C) to analyze and measure the digestibility determination for Crude Protein (CP) and Dry Matter (DM) according to Association of Official Analytical Chemists, (AOAC, 2003) which are presented in Table 2.

Biochemical serum analysis

Blood samples were collected at the age of 42 days from the wing vein before slaughtering. Blood samples were collected from four chickens of each experimental group then centrifuged at 3000 rpm for 15 min to obtain the serum and then the serum was stored at -10°C until analyzed. Cholesterol, total protein, triglycerides, glucose, albumin, uric acid, and hepatic enzyme activity (Alanine aminotransferase [ALT], and Aspartate aminotransferase [AST]) were estimated using an automatic biochemical analyzer (CX9, Beckman).

Microflora and histomorphology

Cecal samples were taken for a microbial count as described by Zhu et al. (2002) to investigate gut health. Four chickens from each group (one from each replicate) were selected as above, the caecum was quickly dissected, and 3 g of their contents were collected in sterilized sampling tubes. Then, 10-fold serial dilutions of one g of sample were serially made in phosphate buffer solution. Subsequently, 100 µl were removed from 10^{-4}, 10^{-5}, and 10^{-6} dilutions, and poured onto Petri dishes containing the agar (culture media). *Escherichia coli* were cultured in eosin methylene blue agar and incubated at 37°C for 48 hours under aerobic conditions. *Lactobacilli* were cultured in De Man, Rogosa, and Sharpe's agar and incubated at 37 °C for 72 hours under anaerobic conditions. Cecal contents were counted for microbial populations using a conventional method (spread plate method) by Casagrande Proietti et al. (2009). Histological slides were prepared to form the ileum samples of the slaughtered chickens (approximately 2 cm taken from the ileum mid-part). Segments were fixed in 10% neutral buffered formalin solution and were embedded in paraffin wax. Parts of the ileum were prepared and placed on a glass slide and stained with Alcian Blue stain. Histological evidence (villus height and crypt depth) slides were examined by using the electron light microscope (ZEISS Axio Imager A2, Germany) after stained with Haematoxylin and Eosin.

Statistical analysis

Data were analyzed using the analysis of variance in SAS (2002) followed by performing the Duncan Multiple Range Test. The chosen level of significance for all comparisons was $p < 0.05$.

Table 1. Ingredient composition and nutrient content of different experimental diets

		Starter (day 1 to 21)				Grower (day 22 to 42)			
		CON	**CM**	**PCM**	**FCM**	**CON**	**CM**	**PCM**	**FCM**
Ingredient (%)	Corn	57.86	50.45	50.45	50.65	61.57	53.41	53.41	53.86
	Soybean meal (48%)	32.50	17.55	17.55	17.54	26.55	13.10	13.10	12.64
	Canola meal (CM)	00.00	20.00	20.00	00.00	00.00	20.00	20.00	00.00
	Fermentation (CM)	00.00	00.00	00.00	20.00	00.00	00.00	00.00	20.00
	Corn gluten meal	4.00	5.00	5.00	4.80	5.00	5.00	5.00	5.00
	Corn oil	1.50	2.90	2.90	2.95	3.10	4.85	4.85	4.85
	Di calcium phosphate	2.20	2.20	2.20	2.20	1.90	1.80	1.80	1.80
	Calcium Carbonate	1.20	1.15	1.15	1.15	1.15	1.05	1.05	1.05
	Premix *	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30
	Salt	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
	DL-methionine	0.14	0.12	0.12	0.10	0.13	0.09	0.09	0.10
	Lysine	0.00	0.03	0.03	0.05	0.00	0.10	0.10	0.10
	Sodium bicarbonate	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
	Total	100	100	100	100	100	100	100	100
Calculated composition	ME (kcal/kg**)	3000	3000	3000	3000	3200	3200	3200	3200
	CP	23	23	23	23	21	21	21	21
	Calcium	1.0	1.0	1.0	1.0	0.95	0.95	0.95	0.95
	Available Phosphorus	0.5	0.5	0.5	0.5	0.45	0.45	0.45	0.45

* Vitamin A 12,000 IU, Vitamin D3 3,000 IU, Vitamin E 40 mg, Vitamin K3 3 mg, Vitamin B1 2 mg, Vitamin B2 6 mg, Vitamin B6 5 mg, Vitamin B12 0.02 mg, niacin 45 mg, biotin 0.075 mg, folic acid 2 mg, pantothenic acid 12 mg, manganese 100 mg, zinc 600 mg, iron 30 mg, copper 10 mg, iodine 1 mg, selenium 0.2 mg, cobalt 0.1 mg. **Metabolizable energy for canola meal (kcal/kg) 2,000, CON: Control, CM: Canola Meal, FCM: Fermentation Canola Meal. CP: Crude Protein, Metabolizable energy for canola meal (kcal/kg) 2,000

Table 2. Growth performance and nutrient digestibility of broiler chickens fed different experimental diets for 42 days

		CON	CM	PCM	FCM	SEM	p value
Growth performance (g)	Live body weight	2066[a]	1957[c]	2034[b]	2081[a]	31.50	0.022
	Feed intake	3398	3367	3382	3401	18.66	0.170
	Feed conversion ratio	1.645[bc]	1.719[a]	1.667[b]	1.633[c]	0.091	< 0.001
Nutrient digestibility (%)	Dry matter	87.15[a]	81.38[c]	83.95[b]	88.03[a]	0.860	0.028
	Crude protein	78.33[ab]	72.5[c]	76.06[b]	80.43[a]	0.944	0.006

[a, b, c]: Means in the same row with different superscripts are significantly different at $p < 0.05$. CON: control (without canola), CM: 20% canola meal, FCM: 20% fermentation canola meal, PCM: 20% canola meal with probiotic, SEM: Standard Error of Means

RESULTS AND DISCUSSION

Productive performance, nutrient digestibility, and carcass traits are presented in Tables 2 and 3. Nutrient digestibility of DM and CP were significantly higher ($p < 0.05$) for chickens fed FCM than those fed unfermented canola meal. Previous studies indicated that the fermentation process helps with increasing crude protein and crude fat content (Wang et al., 2010). As a result, fermenting CM should reflect beneficial effects on the health and performance of chickens. Statistical analysis of the data indicated a better digestibility of DM and CP in diets containing FCM, compared to the other experimental groups. The same results were reported by Afsharmanesh et al. (2010) who stated that there was an improvement in nutrient utilization in chickens fed with diets containing yeast. The higher nutrient digestibility might be due to the reduction in pathogenic load (modulator microbial content) in the gut which led to a positive effect on the absorption of nutrients and improved nutritional value (Khidr et al., 2017).

The chickens fed FCM presented significantly higher LBW and an enhanced FCR, compared to chickens fed with CM and PCM ($p < 0.05$). Feed intake decreased in the broilers fed CM, compared to groups fed PCM and FCM. These findings were in agreement with the earlier findings of Feng et al. (2007) Mathivanan et al. (2006) who observed a significant difference in body weight due to the fermentation of the broiler diet. Significant improvements in performance parameters of broiler chickens might be caused by the enhancement of nutritional quality and lowering the content of antinutritional factors in the fermented canola meal. Similar improvements in productive performances were reported by Chiang et al. (2010) with the fermentation of unconventional feed ingredients in broilers.

There was an increase in carcass yield and relative weight of the small intestine, and a decrease in the abdominal fat of the FCM group, compared to CM and PSM groups (Table 3). The relative weight of Bursa of Fabricius was significantly higher in FCM and PCM groups ($p < 0.05$), compared to CM and CON groups. However, there were no significant differences between different experimental diets on weights of spleen, liver, thymus, gizzard, and heart. The observed increase in carcass yield in FCM and PCM groups might be due to the positive effect of the fermentation process on nutrient utilization leading to more gain in body weight in the chickens of these groups. Those improvements could be a result of the cumulative effect of *Lactobacillus* in the fermentation process, including increasing digestive enzyme activity and balancing beneficial microbial populations in the gut environment to improve digestion and nutrient utilization (Shim et al., 2010). The increase in carcass weight is a result of adding beneficial bacteria to the diet which enhanced protein availability (Nahashon et al., 1996). The significant increase in the relative weight of the bursa of Fabricius in FCM and PCM groups might be due to an increase in the number of lymphocytes in primary lymphoid organs, as a result of the beneficial bacteria effect on the functional activities of the immune system which led to an improvement in the immune system responses of the chickens (Willis et al., 2007). The highest weight of the small intestine and the lowest abdominal fat ($p < 0.05$) were observed in the chickens fed FCM. This positive effect of the beneficial microbes helps in a better distribution of fat in the carcass, which indicates that probiotics efficiently improve energy usage (Santoso et al., 1995).

Experimental treatments have affected serum biochemistry as presented in Table 4. The chickens, which fed FCM and PCM had higher levels of glucose, total protein, and albumin than those fed a control diet and CM. The levels of cholesterol and triglycerides of FCM and PCM groups were significantly lower than those of control groups ($p < 0.05$). The concentration of AST increased in chickens fed FCM, compared to CON and other groups, while their values of Alanine aminotransferase were not

affected ($p < 0.05$). In the current experiment, an increase in the level of the serum total protein and albumin was observed in broilers fed with FCM leading to an improvement in the deposition of protein in the tissues (Xu et al., 2012). The lower content of total serum cholesterol and triglycerides of broiler fed with FCM and PCM were observed, compared to other groups, which indicated that fermented CM feed might significantly improve the utilization of lipids in dietary. Studies by Hu et al., (2016) and Elbaz and ELshiekh (2020) showed a reduction in total serum cholesterol and triglycerides levels when the chickens were fed fermented diets or diets supplemented with probiotics. There was an increase in AST concentration in FCM chickens of the current study and this result was in agreement with Chachaj et al., (2019) who reported that fermented soybean meal increased the AST concentration in turkeys. The increase in the activity of the AST enzyme may be an indication of liver functional changes resulted from the increase in amino acid transforming rate during the metabolism of many tissues (Fevery, 2008). Microbial count results of cecal samples are presented in Table 5. Fermenting the diet had a significant effect on the microbial population in the gut. The total number of lactobacilli in the ceca of the chickens fed FCM and PCM were significantly higher; meanwhile, the numbers of *Escherichia Coli* were lower than those fed CM or the control diet. Similar observations were recorded by different studies (Engberg et al., 2009; Sun et al., 2013; Khidr et al., 2017). Similarly, fermented feeds may act similar to probiotics in improving gut health by balancing the microbial population (the competitive flora) and the production of organic acids by lactobacillus (Paton et al., 2006; Liang et al., 2012).

Morphological measurements of small intestines are presented in Table 5. An increase in villus height of ileum was noticeable in broilers fed with FCM and PCM ($p < 0.05$). However, chickens fed either FCM or PCM diet had lower crypt depth in the ileum than those fed CM or CON ($p < 0.05$). These results were similar to those recorded by Chiang et al., (2010) and Zhang et al., (2016) showed that feeding chickens on fermented rapeseed meal led to an increase in villus height. The increase in the weight of the intestine can also be explained by the increase in length villus height in the ileum in chickens fed with FCM. The beneficial changes in the morphology of the intestine led to an increase in the surface of absorption, and thus enhanced the absorption of nutrients, which could explain the improvement in the productive performance.

Table 3. Carcass traits and immune organic of broiler chickens fed different experimental diets

Items		CON	CM	PCM	FCM	SEM	p value
Carcass trite (g/100 g body weight)	Carcass yield	77.91^{a}	74.26^{b}	76.15^{ab}	78.22^{a}	0.751	0.020
	Liver	2.76	2.63	2.94	3.09	0.089	0.295
	Gizzard	1.82	1.70	1.72	1.74	0.052	0.791
	Heart	0.526	0.585	0.513	0.528	0.029	0.831
	Abdominal fat	1.82^{a}	1.79^{a}	1.80^{a}	1.67^{b}	0.075	0.037
	Small intestine	3.43^{b}	2.89^{c}	3.72^{ab}	4.08^{a}	0.092	0.018
Immune organic (g/100 g body weight)	Spleen	0.114	0.095	0.166	0.148	0.010	0.094
	Thymus	0.262	0.247	0.275	0.250	0.010	0.165
	Bursa of Fabricius	0.125^{b}	0.112^{b}	0.217^{a}	0.225^{a}	0.027	0.021

[a, b, c]: Means in the same row with different superscripts are significantly different at $p < 0.05$. CON: control (without canola), CM: 20% canola meal, FCM: 20% fermentation canola meal, PCM: 20% canola meal with probiotic, SEM: Standard Error of Means.

Table 4. Serum biochemical parameters of broiler chickens fed different experimental diets

Parameters	CON	CM	PCM	FCM	SEM	p value
Glucose (mmol/L)	7.16^{b}	6.78^{b}	9.65^{a}	10.08^{a}	0.041	0.032
Cholesterol (mmol/L)	2.92^{a}	3.55^{a}	2.14^{b}	1.97^{b}	0.038	0.014
Triglycerides (mmol/L)	0.51^{a}	0.37^{b}	0.46^{ab}	0.42^{ab}	0.093	0.040
Total protein (g/L)	27.80^{ab}	23.73^{b}	28.13^{ab}	30.73^{a}	1.188	0.019
Albumin (g/L)	12.26^{ab}	8.24^{c}	11.76^{b}	13.96^{a}	0.677	0.000
Uric acid (µmol/L)	178.15	169.95	196.20	201.05	4.595	0.169
ALT ($U.l^{-1}$)	45.24	47.84	40.50	41.02	6.657	0.205
AST ($U.l^{-1}$)	161.5^{a}	140.9^{b}	155.0^{ab}	169.3^{a}	5.775	0.023

[a, b, c]: Means in the same row with different superscripts are significantly different at $p < 0.05$. CON: Control (without canola), CM: 20% canola meal, FCM: 20% fermentation canola meal, PCM: 20% canola meal with probiotic. ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, SEM: Standard Error of Means.

Table 5. Morphology and microbial counts of the cecum of broiler chickens fed different experimental diets

Items		CON	CM	PCM	FCM	SEM	p value
Morphology (µm)	Villus height	545.60[b]	536.00[b]	585.57[a]	599.60[a]	42.9	0.001
	Crypt depth	125.61[a]	127.50[a]	116.94[b]	117.67[b]	25.0	0.030
Microbial counts (log10 CFU g-1 digesta)	Lactobacilli	6.52[b]	6.30[b]	7.14[ab]	8.49[a]	0.35	0.050
	Escherichia coli	2.49[ab]	3.15[a]	1.87[b]	1.75[b]	0.40	0.030

[a, b, c]: Means in the same row with different superscripts are significantly different at $p < 0.05$. CON: control (without canola), CM: 20% canola meal, FCM: 20% fermentation canola meal, PCM: 20% canola meal with probiotic, SEM: Standard Error of Means

CONCLUSION

The results obtained from the current study indicate that fermenting canola meal (*Lactobacillus* spp.) in broiler diets resulted in a beneficial effect on performance traits, which include an improvement in body weight gain, feed conversion ratio, nutrient digestibility, and gut health. Therefore, it can be concluded that using fermented canola meal in broiler diets might be effective to achieve the maximum benefits in broiler production by reducing dietary costs.

DECLARATIONS

Competing interests

The author has declared that there was no conflict of interest in the current research work.

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Clinicopathological Findings in Suspected Cases of Virus-induced Neoplastic Diseases in Commercial Layer Chickens in Nigeria

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ABSTRACT

Avian neoplastic diseases, including Marek's disease (MD), avian leukosis (AL), and reticuloendotheliosis (RE), are of economic importance in the chicken industry. However, it is difficult to differentiate MD from AL and RE by clinical signs and postmortem examination. Therefore, the present study aimed to classify the avian neoplastic diseases affecting commercial layer chickens in Nigeria using clinical history, postmortem examination, and histopathological technique. Carcasses of commercial layer chickens from 7 and 20 poultry farms in Kaduna and Plateau States were studied, respectively, from February 2017 to March 2018. The age, morbidity, and mortality rates in each of the affected farms were determined. Detailed postmortem examinations were carried out on the carcasses from the affected farms, and organs observed to have neoplastic lesions were fixed in 10% neutral buffered formalin for histopathology. The age means of the affected layers were 20.6 weeks and 20.8 weeks in Kaduna and Plateau States, respectively. The average morbidity rates of neoplasm in the affected layers were 3.9% and 9.3% in Kaduna and Plateau States, respectively, while the average mortality rates were 8.6% and 8.5% in Kaduna and Plateau States, respectively. The clinical observation of affected chickens indicated that they were anorexic and emaciated. Generally, the neoplastic lesions were characterized by white to gray, multifocal, firm nodules of varying sizes on the affected organs. In Kaduna State, the neoplasms were commonly observed on the liver (85.7%), spleen (71.4%), heart (42.9%), and kidneys (42.9%), while in Plateau State, the affected organs included liver (50%), spleen (25%), proventriculus (25%) and lungs (25%). The histopathological changes in the affected tissues were similar and characterized predominantly by the infiltration of lymphocytes, lymphoblasts, and macrophages. The patterns of distribution of the pleomorphic neoplastic cells within the liver were multifocal and perivascular in most cases. Findings from the current study indicated that cases of neoplasms in commercial layer chickens in Kaduna and Plateau States, Nigeria, could be attributed to MD.

Keywords: Avian neoplastic diseases, Layer chickens, Pathology

INTRODUCTION

Avian neoplastic diseases, including Marek's disease (MD), avian leukosis (AL), and reticuloendotheliosis (RE), are of economic importance (Payne and Venugopal, 2000). The clinical signs of these diseases are in most cases not pathognomonic and include inappetence, abnormal feathering, paleness of comb and wattles, emaciation, depression, paralysis, and death (Okoye et al., 1993; Abdel-Latif and Khalafalla, 2005). In addition to encouraging tumor growth, avian neoplastic disease viruses may induce immunosuppression and other production problems including reduced growth rate and livability, decreased fertility and hatchability, and decreased egg production in an affected flock (Palya et al., 2000; El-Sebelgy et al., 2014; Zeghdoudi et al., 2017).

Available literature indicates that there was an increase in outbreaks of virus-induced avian neoplastic diseases in Kaduna and Plateau States, and most of the outbreaks have been attributed to Marek's disease, despite vaccination of commercial layer chickens (Wakawa et al., 2012; Sani et al., 2017). Diagnosis of virus-induced avian neoplastic diseases in Kaduna and Plateau States have

mostly been by clinical observations and gross examination of carcasses for tumors (Jwander et al., 2013; Musa et al., 2013; Sani et al., 2017). Gross examination of neoplastic tissues in most cases cannot be relied upon to differentiate one virus-induced avian neoplastic disease from another (Dong et al., 2015; Meng et al., 2018). However, MD, AL, and RE have distinct histopathological changes that may be used to differentiate MD from AL and RE, hence the present study was set up for that. Therefore, the current study aimed to classify the neoplastic diseases affecting commercial layer chickens in Kaduna and Plateau States, Nigeria using clinical history, postmortem examination, and histopathological technique. Results from this study provided insights on the neoplastic diseases affecting commercial layer chickens in Kaduna and Plateau States, Nigeria, which is pivotal in instituting control measures for the diseases.

MATERIALS AND METHODS

Ethics committee approval

The research protocol was approved by the University of Abuja Ethics Committee on Animal Use (UAECAU), with the assigned number: UAECAU/2017/0020.

Study areas

The study was carried out in Kaduna and Plateau States, Nigeria. The Nigeria Galleria (2015) described Kaduna State as located at the center of Northern Nigeria, and comprised 23 Local Government Areas. Kaduna State is located between latitudes 9°03' and 11°32'N and longitudes 6°05' and 8°38'E of the Greenwich Meridian (Kaduna State, 2019). Kaduna State shares boundaries with Federal Capital Territory, Abuja and Nasarawa State to the south, Bauchi and Plateau States to the east, Niger State to the west, and Zamfara, Katsina, and Kano States to the North. Plateau State is in North Central, Nigeria, and is located between latitude 80°24'N and longitudes 80°32' and 100°38'E (Plateau State, 2019). The State shares boundary with Bauchi State to the Northeast, Kaduna State to the Northwest, Nasarawa State to the Southwest, and Taraba State to the Southeast (NIPC, 2019).

Selection of commercial layer chickens' farms for the study

A total of 7 layer farms (designated Z1 to Z7) and 20 layer farms (designated J1 to J20) in Kaduna and Plateau States were selected for the study, respectively, in which neoplastic lesions were observed during the postmortem examination of carcasses. The seven commercial layer chickens' farms studied in Kaduna State were cases reported to the Poultry Clinic of the Veterinary Teaching Hospital, Ahmadu Bello University, Zaria, during the study period. The 20 farms studied in Plateau State were cases reported to the Evangelical Church Winning All Veterinary Clinic, Bukuru, Jos South, during the study. The seven farms in Kaduna State had commercial layer chickens with flock sizes ranging from 323 (farm Z4) to 907 (farm Z3) with an average flock size of 581.1 chickens. The 20 farms in Plateau State had commercial layer chickens with flock sizes ranging from 86 (farm J19) to 4703 (farm J9) with an average flock size of 872.9 chickens. The information about the affected layer flocks, such as the age, vaccination history against MD, morbidity and mortality rates as well as the rate of egg production were documented. The study lasted 13 months, from February 2017 to March 2018.

Gross pathology

Detailed postmortem examination of carcasses of commercial layer chickens from the farms affected with avian neoplastic diseases in Kaduna and Plateau States was carried out according to the method described by Collett et al. (2020). The lesions observed on the organs (liver, spleen, proventriculus, intestine, pancreas, mesentery, kidneys, heart, lungs, sciatic nerve, and ovary) were properly described based on the changes in their size, shape, color, consistency, and distribution of the lesions (Brown, 2012), and promptly documented. Lesions that were firm, nodular to diffuse, white to grey, deep or superficial with smooth surface were considered neoplastic (Duguma et al., 2005). It was ensured that personal protective equipment, such as gloves, coveralls, and boots, were used throughout the necropsy. Photographs of the lesions were taken using a digital camera (SONY® Cybershot, 16.2 megapixels, DSC-TX20 model, Japan).

Histopathology

The tissues observed to have neoplasms at postmortem from carcasses of commercial layer chickens with suspected cases of virus-induced neoplastic diseases in the study areas were subjected to standard histopathological procedures as described by Aughey and Frye (2001) at the Histopathology Laboratory, Department of Veterinary Pathology, Faculty of Veterinary Medicine, ABU, Zaria. The slides were examined for features of neoplasia which included a high mitotic index, nuclear hyperchromacia, cellular pleomorphism, and cell types

(Payne and Venugopal, 2000) using a light microscope at different magnifications (X40, X100, X200, and X400). Photomicrographs of the changes observed in each slide were taken with a digital camera (SONY®Cybershot 16.2 megapixels, DSC-TX20 model, Japan), transferred to a computer and labeled appropriately.

Data analysis

The mean ages of the affected commercial layer chickens in Kaduna and Plateau States were determined. The egg production, morbidity, and mortality rates of the affected layer chickens in Kaduna and Plateau States at the time of sampling were summarized into percentages using descriptive analysis in the Statistical Package for Social Sciences (SPSS) software version 22.

RESULTS

Clinical history

The most common clinical manifestations of affected chickens in the farms in both states were anorexia, emaciation, ruffled feathers, somnolence, and shriveled combs and wattles. The age range of the commercial layer chickens in the seven farms from Kaduna State was from 12 weeks (farm Z6) to 31 weeks (farm Z4) with an average age of 20.6 weeks. The commercial layer chickens in four farms (Z1, Z2, Z3, and Z7) in Kaduna State were vaccinated against MD, while the vaccination history of commercial layer chickens in the other farms (Z4, Z5, and Z6) could not be ascertained. The morbidity of neoplastic disease in the commercial layer chickens in Kaduna State ranged from 0.7% (farm Z3) to 12.3% (farm Z1) with an average morbidity rate of 3.9%, while the mortality ranged from 2.6% (farm Z1) to 16.5% (farm Z6) with an average mortality rate of 8.6%. The average level of egg production by the commercial layer chickens from the seven farms in Kaduna State was 62% (Table 1).

The age range of the commercial layer chickens in the 20 farms from Plateau State was 11 weeks (farms J4 and J8) to 39 weeks (farm 11) with an average age of 20.8 weeks. Fourteen of the farms only in Plateau State were vaccinated against MD. The morbidity rates of neoplastic disease in the commercial layer chickens from the farms in Plateau State were within the range of 1.1% (farm J11) to 33% (farm J20) with average morbidity of 9.3%, while mortality ranged from 1.6% (farm J11) to 22.3% (farm J3) with an average mortality of 8.5%. The average level of egg production by the commercial layer chickens in the 20 farms in Plateau State was 65.5% (Table 2).

Gross lesions

The neoplastic lesions were characterized by white to gray, multifocal, firm nodules of varying sizes on the affected organs. Marked enlargement of the affected organs was also observed. In Kaduna State, the organs of carcasses of commercial layer chickens were observed grossly to have neoplasms, included the liver (85.7%), spleen (71.4%), heart (42.9%), kidney (42.9%), mesentery (28.6%), proventriculus (14.3%), lung (14.3%), intestine (14.3%), and pancreas (14.3%). Other observed gross lesions included emaciated carcasses (85.7%), enlarged liver (85.7%), spleen (71.4%), proventricular glands (71.4%), kidneys (57.1%), and sciatic nerve (14.3%). Petechial hemorrhages were observed on the mucosa of the proventriculus (71.4%) and caecal tonsils (14.3%). The ovarian follicles atrophied in all the carcasses of the commercial layer chickens that were laying.

In Plateau State, the organs of carcasses of commercial layer chickens were observed grossly to have neoplasms, included the liver (50%), spleen (25%), proventriculus (25%), lung (25%), kidney (15%), intestine (10%), heart (5%) and mesentery (5%). Other observed gross lesions were emaciated carcasses (95%), hepatomegaly (85%), enlarged proventricular glands (60%), splenomegaly (40%), renomegaly (15%), and enlarged sciatic nerve (10%). Petechial hemorrhages were detected on the mucosa of the proventriculus (35%), caecal tonsils (25%) and intestine (15%). The ovarian follicles atrophied in all the carcasses of the commercial layer chickens that were laying.

Histopathology

The infiltrating neoplastic cells in the affected organs were pleomorphic, comprising small, medium and large lymphocytes, macrophages, and plasma cells. The distributional patterns of the pleomorphic neoplastic cells within the liver of commercial layer chickens from Kaduna State were multifocal (85.7%) and perivascular (85.7%). Other histopathologic changes observed in the livers included degeneration and necrosis of the hepatocytes (100%), congested blood vessels (100%), thick vascular walls (57.1%), dissociated hepatic cords (57.1%), and hemorrhage within the parenchyma (14.3%).

On the other hand, the distributional patterns of the pleomorphic neoplastic cells within the liver of commercial layer chickens from Plateau State were multifocal (70%), perivascular (50%), diffuse (20%), and coalescing (5%). Other histopathologic changes observed in the livers included degeneration and necrosis of the hepatocytes (95%), congested blood vessels (35%), dissociated hepatic cords (30%), and hemorrhage within the parenchyma (5%).

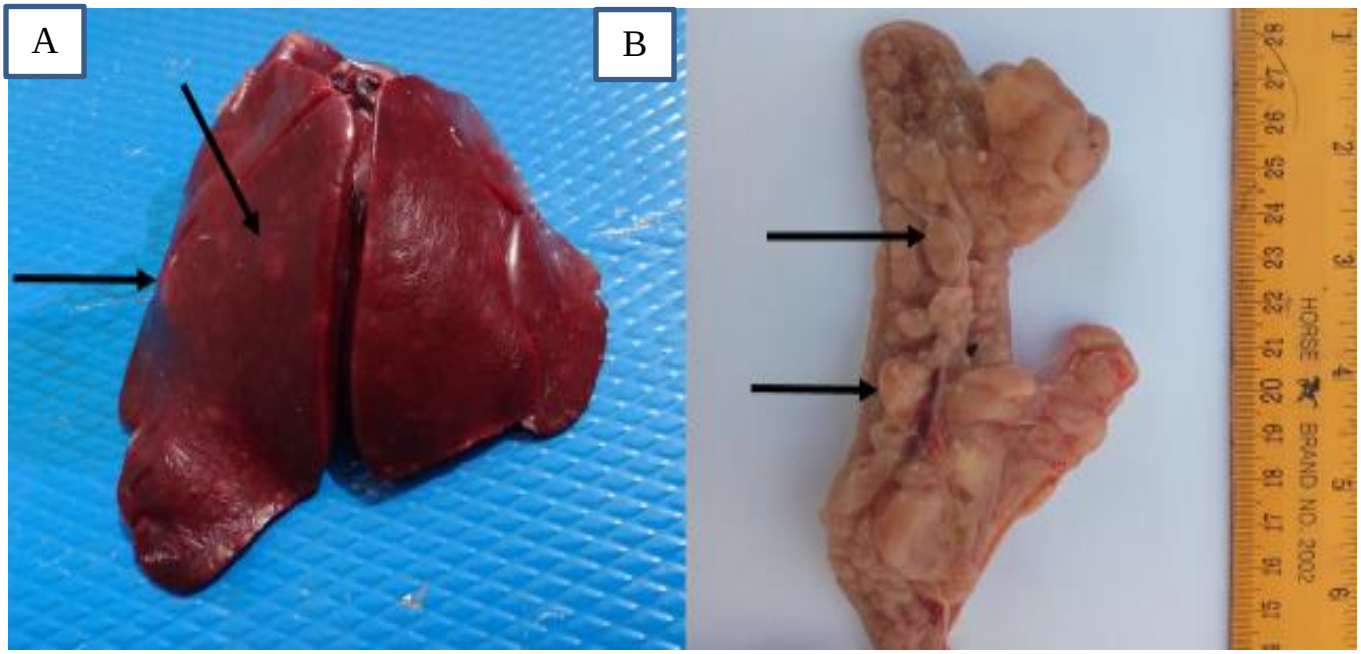

Figure 1. A: Liver of a 14-week-old commercial layer chicken (Z1). Note the hepatomegaly and white to gray, multifocal, firm neoplastic nodules of varying sizes ranging from 0.2 to 1.0 cm in diameter (arrows) affecting all the lobes of the liver. **B:** Intestine (duodenum) of a 27-week-old commercial layer chicken (Z3). Note the yellow, nodular neoplastic growths ranging from 0.2 to 2.0 cm in diameter, involving the pancreas and the mesentery (arrows).

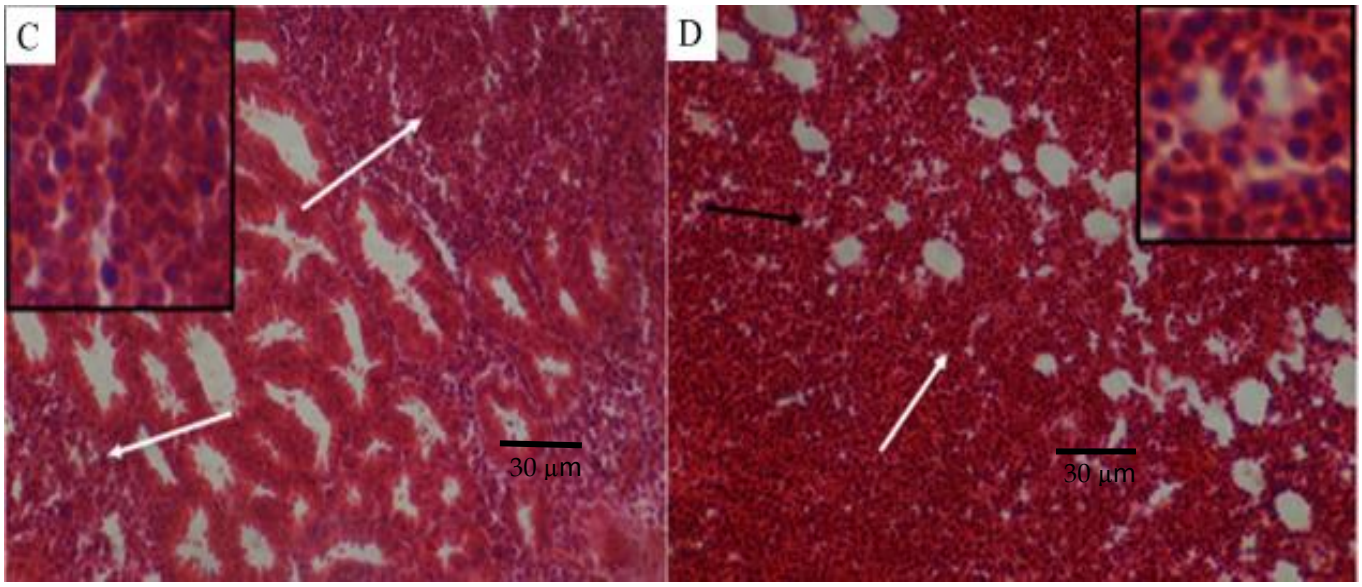

Figure 2. A: Photomicrograph of the section of the liver of a 37-week-old commercial layer chicken affected by the neoplastic disease (J6). Note the multifocal aggregates of neoplastic mononuclear cells predominantly lymphocytes (arrows and insert); H & E stain, 30 µm. **B:** Photomicrograph of the section of the heart of a 12-week-old commercial layer chicken (J15). Note the neoplastic mononuclear cellular infiltrates, predominantly lymphocytes (black arrow and insert), crowding out the fragmented cardiomyocytes (white arrow); H & E stain, 30 µm. **C:** Photomicrograph of the section of the proventriculus of a 12-week-old commercial layer chicken (J15). Note the neoplastic mononuclear cells predominantly lymphocytes (white arrows and insert), and hyperplasia of the epithelial cells of the glands; H & E stain, 30 µm. **D:** Photomicrograph of the section of lung of a 13-week-old commercial layer chicken (J19). Note the neoplastic mononuclear cells predominantly lymphocytes, occluding the air sacs (black arrow and insert). The inter air sac walls were also thickened (white arrow), H & E stain, 30 µm.

Table 1. Morbidity and mortality rates and the level of egg production of commercial layer chickens from the farms with neoplastic diseases in Kaduna State, Nigeria.

Farm	Age (weeks)	Flock size	MDVS	Morbidity (%)	Mortality (%)	EP (%)
Z1	14	640	Vaccinated	22 (3.4)	17 (2.6)	NA
Z2	16	480	Vaccinated	12 (2.5)	28 (5.8)	NA
Z3	27	907	Vaccinated	6 (0.7)	32 (3.5)	55
Z4	31	323	Unknown	17 (5.3)	43 (13.3)	63
Z5	29	542	Unknown	9 (1.7)	67 (12.4)	68
Z6	12	351	Unknown	43 (12.3)	58 (16.5)	NA
Z7	15	825	Vaccinated	51 (6.2)	104 (12.6)	NA
Average	20.6	581.1		22.9 (3.9)	49.9 (8.6)	62

MDVS: Marek's disease vaccination status, EP: Egg production, NA: Not applicable.

Table 2. Morbidity and mortality rates and the level of egg production of commercial layer chickens from the farms with neoplastic diseases in Plateau State, Nigeria.

Farm	Age (weeks)	Flock size	MDVS	Morbidity (%)	Mortality (%)	EP (%)
J1	33	290	Vaccinated	12 (4.1)	47 (16.2)	62
J2	29	180	Vaccinated	6 (3.3)	13 (7.2)	68
J3	17	242	Vaccinated	31 (12.8)	54 (22.3)	NA
J4	11	1,304	Vaccinated	218 (16.7)	174 (13.3)	NA
J5	13	3,362	Vaccinated	408 (12.1)	376 (11.2)	NA
J6	37	387	Unknown	16 (4.1)	12 (3.1)	72
J7	25	178	Unknown	5 (2.8)	11 (6.2)	42
J8	11	793	Vaccinated	23 (2.9)	52 (6.6)	NA
J9	14	4,703	Vaccinated	271 (5.8)	285 (6.1)	NA
J10	15	586	Vaccinated	93 (15.9)	77 (13.1)	NA
J11	39	1,118	Vaccinated	12 (1.1)	18 (1.6)	69
J12	27	89	Unknown	5 (5.6)	8 (9.0)	78
J13	13	672	Vaccinated	108 (16.1)	73 (10.9)	NA
J14	14	215	Unknown	34 (15.8)	23 (10.7)	NA
J15	12	738	Vaccinated	54 (7.3)	61 (8.3)	NA
J16	27	299	Vaccinated	13 (4.4)	12 (4.0)	62
J17	33	406	Unknown	9 (2.2)	8 (2.0)	71
J18	16	1,261	Vaccinated	112 (8.9)	87 (6.9)	NA
J19	13	86	Unknown	7 (8.1)	13 (15.1)	NA
J20	16	548	Vaccinated	181 (33)	79 (14.4)	NA
Average	20.8	872.9		80.9 (9.3)	74.2 (8.5)	65.5

MDVS: Marek's disease vaccination status, EP: Egg production, NA: Not applicable.

DISCUSSION

The age range of commercial layer chickens in the farms with cases of avian neoplasm in Kaduna State was 12 to 31 weeks, whereas it was 11 to 39 weeks in Plateau State. This finding was similar to the finding of Gao et al. (2012), where most of the layer chickens with neoplasms associated with AL virus subgroup J infection were between 15 and 29 weeks of age. In a retrospective study by Musa et al. (2013), 88.7% of the cases diagnosed as MD were from layer chickens with ages between 11 and 30 weeks. Furthermore, Haq et al. (2001) reported a high prevalence of MD in chickens that were between 13 and 30 weeks old.

The average morbidity rate of avian neoplasm in layer chickens from farms in Kaduna State was 3.9%, while the average morbidity was 9.3% in Plateau State. This was in line with previous findings indicating that the morbidity of avian neoplastic diseases was generally low (Okwor and Eze, 2011). Okonkwo (2015) reported the morbidity rate of MD as 4.7% and 9.5% in two-layer flocks, whereas Cheng et al. (2010) reported the morbidity rates between 5 and 15% in cases of avian neoplastic disease due to ALV subgroup J infection. However, morbidity rate as high as 67.9% in local chickens that were not vaccinated against MD was reported by Duguma et al. (2005). Similarly, Gao et al. (2010) reported a morbidity rate of 60% in chickens with ALV subgroup J associated neoplasms.

In the present study, the average mortality rate of avian neoplastic disease in layer chickens from farms in Kaduna State was 8.6%, whereas it was 8.5% in Plateau State. A wide range of mortality, ranging from low to high, due to avian neoplastic diseases was commonly observed in affected flocks. While Okonkwo (2015) reported MD related mortality of 2.3% and 2.7% in two flocks of layers, Okwor and Eze (2011) reported low mortality due to MD in several flocks of chickens, but up to 25% mortality in a few flocks, with morbidity and mortality decreasing as the chickens grew older. In unvaccinated local chickens, MD was responsible for a very high mortality of 66.2% and a case fatality rate of 97.9% (Duguma et al., 2005). Similar high mortalities have been reported in outbreaks due to AL. Gao et al. (2010) reported a mortality of more than 20% in a case of hemangioma due to AL virus subgroup J infection. Three flocks were reported to have mortalities of 12%, 18%, and 20% due to erythroblastosis (Wang et al., 2013). Zeng et al. (2015) reported a wide range of mortalities in flocks with avian neoplasms when many of the tested chickens having mixed infections with avian neoplastic disease viruses. Mortalities of 16.4% and 20% in two flocks of pullets due to RE was reported by Okoye et al. (1993).

In the current study, the liver was also observed to be the most affected organ as reported by some authors (Cheng et al., 2011; Gao et al., 2015). The liver plays an important role in digestion, metabolism, regulating the production, storage and releasing of lipids, carbohydrates, and proteins (Denbow, 2000). These strategically vital functions of the liver may not be carried out when the structural integrity of the liver is severely compromised, as observed in this study.

Pathognomonic lesions for avian neoplastic diseases were not commonly observed in this study. For example, the involvement of peripheral nerves is generally regarded as a pathognomonic lesion observed in chickens with MD. However, this finding was observed in only one (Z3) out of the seven layer chickens' farms with cases of avian neoplasms in Kaduna State, and in only two (J3 and J8) of the layer chickens' farms (20) with cases of avian neoplasms in Plateau State. This situation has made the differential diagnosis of avian neoplastic diseases by gross examination of affected chickens' carcasses almost impossible. The nature of infiltrating neoplastic cells, characterized by cellular pleomorphism comprising lymphocytes, lymphoblasts, macrophages, and neutrophils, was similar in all the organs examined. These histopathological findings were consistent with those observed in cases of MD as reported in many studies (Mitra et al., 2013; Okonkwo, 2015; Abreu et al., 2016). The distributional pattern of the neoplastic cells was multifocal (85.7%) and perivascular (85.7%) in the livers of layer chickens from the farms in Kaduna State, whereas the distribution was multifocal (70%), coalescing (5%), diffuse (20%), and perivascular (50%) in the livers from the farms in Plateau State.

Vaccination has been widely used to control MD all over the world. However, the emergence of more virulent strains of MD has made some MD vaccines ineffective (Sun et al., 2017). The majority of the layer chickens' farms with cases of avian neoplastic disease in both Kaduna and Plateau States had a history of vaccination against MD. Although the type of vaccine used by the farmers could not be ascertained, available reports suggest that the Herpesvirus of Turkeys (HVT) vaccines are widely used for the control of MD in the two States (Jwander et al., 2012; Adedeji et al., 2017). In a study by Tian et al. (2011), no difference was observed between HVT vaccinated chickens and unvaccinated chickens in terms of mortality patterns and tumor development. However, no mortality or tumor development was observed in the chickens challenged with a field strain of MDV and vaccinated with Rispens. The presence of immunosuppressive diseases such as chicken infectious anaemia and infectious bursal disease, may also be responsible for MD vaccination failure in chickens (Zhang et al., 2017). These immunosuppressive diseases are endemic in Nigeria (Mbuko et al., 2010; Adedeji et al., 2016).

The average egg production rates of commercial layer chickens from the farms with cases of avian neoplasm in Kaduna State was 62%. Similarly, the average egg production status of layer chickens from the farms with cases of avian neoplasm in Plateau State was 65.5%. Low egg production in layer chickens from the farms with cases of either MD, AL, or RE have been widely reported (Okoye et al., 1993; Abdel-Latif and Khalafalla, 2005; Okonkwo, 2015). Grossly, the ovarian follicles were regressed in all the carcasses from the layer flocks that were in lay, from the farms in Kaduna and Plateau States. This finding suggests the reason for low egg production recorded in the laying flocks with the outbreak of the avian neoplastic disease in the current study.

CONCLUSION

The clinical history and gross changes in the affected chickens were not pathognomonic, therefore, it could not

be used to differentiate MD from AL or RE. The nature of infiltrating neoplastic cells, characterized by cellular pleomorphism comprising lymphocytes, lymphoblasts, macrophages, and neutrophils, was similar in all affected organs, and suggestive of MD. However, RE may present similar histological changes. Therefore, further study is recommended to determine the different avian neoplastic disease viruses infecting chickens in Nigeria, using specific diagnostic tools, such as immunohistochemistry and polymerase chain reaction.

DECLARATIONS

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Authors' contributions

All authors contributed equally to this work. NAS, SBO, PAA, and CN conceptualized the work. NAS, CIU, SEA, AS, and MSM participated in the collection, processing, and analysis of data. NAS drafted the manuscript while CIU, SEA, AS, MSM, SBO, PAA, and CN critically revised the manuscript for important intellectual content. All authors approved the final version of the manuscript for publication.

Competing interests

The authors declare that they have no competing interests.

Consent to publish

Not applicable

Ethics and consent to participate

Not applicable

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Effect of Pre-Slaughter Antacid Supplementation of Drinking Water on Carcass Yield and Meat Quality of Broiler Chickens

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ABSTRACT

Antacid is a mixture of sodium bicarbonate, bicarbonate, and citric acid, which can neutralize stomach acidity and may stabilize the pH of post-mortem carcass and meat. Therefore, the present study aimed to investigate the carcass and meat quality of broiler chickens by supplementing the antacid in drinking water. A total of 48 male broiler chickens (Ross 308) were divided into two groups that the first group was the control group (did not receive antacid supplementation in the drinking water) and the second group was supplemented with antacid in drinking water (0.10%) for three days pre-slaughter. It was found that the antacid supplementation increased the percentage of breast meat, while carcass yield, and thigh, drumstick, and wing were not significantly affected. The pH of breast meat 45 minutes and the drip loss at 24 hours post-slaughter was significantly higher. The shear-force of breast meat was reduced ($P < 0.05$) by antacid supplementation. For the color of the breast meat, there were no significant differences in lightness (L*), redness (a*), and yellowness (b*) between the two groups, but the total difference in the color of meat was slightly increased. It can be concluded that supplementing the drinking water with an antacid for three days before slaughter improves the carcass and meat quality of broiler chickens by maintaining the pH and water holding capacity of the meat.

Key words: Antacid, Broiler chickens, Carcass yield, Meat quality.

INTRODUCTION

Meat quality is influenced by the interaction between the genotype and the environment, particularly the stresses undergone before slaughter (Ali et al., 2008). Under stress, it is known that self-oxidative peroxidation increases free radicals, oxygen-reactive species, resulting in destabilization of the cell wall (Puthpongsiriporn et al., 2001). Excessive levels of free radicals enhance lipid peroxidation and changes in meat organoleptic characteristics (Fellenberg and Speisky, 2006). Furthermore, muscle from the broiler chickens stressed pre-slaughter by transportation or high temperatures normally becomes pale, soft, moist, or exudative after a normal 18-24 hours chilling period. Due to rapid pH decline and protein denaturation, this often results in lower possessing yields, increased cooking losses, and reduced juiciness (Ali et al., 2008).

Under heat stress, hyperventilation normally decreases blood carbon dioxide, thereby inducing respiratory alkalosis (increased blood pH) in chickens (Borges et al., 2007). Glycolysis and creatine kinase activity are stimulated, resulting in more pyruvate conversion to lactate (anaerobic metabolism), and inducing acidosis in meat under heat stress (Song and King, 2019). Sodium bicarbonate is an absorbable systemic buffer that supplies sodium and bicarbonate to the body; dietary sodium bicarbonate supplementation to laying hens improves body acid-base balance and eggshell quality (Jiang et al., 2015). In broiler chickens, under heat stress, supplementation of sodium bicarbonate in the drinking water or feed could reduce mortality rate (Mushtaq et al., 2007), and improve growth performance (Mushtaq et al., 2014). Furthermore, using sodium bicarbonate (5.49 g/kg diet) resulted in better growth and carcass performance in broiler chickens compared to other dietary electrolyte balances (Mushtaq et al., 2014).

An antacid is a mixture of sodium bicarbonate, bicarbonate, and citric acid, which can neutralize stomach acidity in humans. Respiratory alkalosis increases the excretion of citric acid, while acidosis decreases the

excretion of citrate (Jiang et al., 2015). For example, ingestion of an alkali load (citrate mixture and sodium bicarbonate) results in an increase in urinary citrate excretion (Xue et al., 2020). The reaction between sodium bicarbonate and citric acid forms carbon dioxide (quickly absorbed) and sodium citrate (high buffering capacity). Since meat quality is directly related to the metabolic acidosis that converts more pyruvate to lactate (anaerobic metabolism), an increase of pH in muscle cells can improve the meat quality. However, the effects of therapeutic antacid chemicals on post-mortem carcasses and meat quality have not been reported. Therefore, the current study was conducted to evaluate the effect of supplementing drinking water with antacid on carcass yield and meat quality of broiler chickens (3 days pre-slaughter).

MATERIALS AND METHODS

Ethical approval

The experimental animals were kept, maintained, treated, and handled in accordance with accepted standards for the humane treatment of animals under license number U1-07385-2561.

Animals and management

A total of 48 male broiler chickens (Ross 308) were kept in an evaporative cooling system from day 1 to day 35 of age, and maintained, treated, and handled under accepted standards for the humane treatment of animals (according to animal welfare). Management and vaccination were provided according to commercial practices. Water and feed were offered *ad libitum*. At 32 days of age, the chickens were divided into two experimental groups of 24. The basal diet was formulated to contain 23% CP and 3,000 ME kcal/kg for age 1 to 10 days, 21.50% CP and 3,100 ME kcal/kg for age 11 to 24 days, and 19.50% CP and 3,200 ME kcal/kg for age 25 to 35 days). All nutrient requirements were formulated according to the recommendations for the strain (Table 1).

Antacid supplementation in the drinking water

During 32-35 days of age, the drinking water was supplemented with antacid in order to evaluate the carcass and meat quality. The experimental groups were assigned to the control group in which the chickens received no supplementation in the drinking water and the antacid group where Citric acid (43.33%), sodium bicarbonate (46.67%), and bicarbonate (10.00%) were added to the drinking water at 0.10%.

Table 1. Composition and nutritional content of the experimental diets

Item	Age 1 to 10 days	Age 11 to 24 days	Age 25 to 35 days
Corn	49.11	51.92	56.57
Soybean meal (48% Crude protein)	40.73	37.30	32.27
Rice bran oil	4.95	5.99	6.71
Monodicalciumphosphate (22% Phosphorus)	1.54	1.36	1.22
Limestone	1.43	1.30	1.19
Salt	0.58	0.48	0.29
Sodium bicarbonate	-	0.15	0.30
DL-Methionine	0.34	0.28	0.26
L-Lysine	0.19	0.12	0.12
L-Threonine	0.10	0.07	0.04
Vitamin and mineral premix	0.24	0.24	0.24
Choline chloride (60%)	0.08	0.08	0.08
Antioxidant and toxin binder	0.16	0.16	0.16
Anticoccidial	0.05	0.05	0.05
corncob	0.50	0.50	0.50
Total	100.00	100.00	100.00
Nutrients by calculation			
Metabolisable energy (Kcal/Kg)	3000.00	3100.00	3200.00
Crude protein (%)	23.00	21.50	19.50
Fiber (%)	3.57	3.43	3.24
Fat (%)	7.32	8.40	9.21
Methionine (%)	0.68	0.61	0.56
Methionine+cystine (%)	1.08	0.99	0.91
Lysine (%)	1.44	1.29	1.16
Threonine (%)	0.97	0.88	0.78
Valine	1.11	1.04	0.95
Calcium (%)	0.96	0.87	0.79
Total phosphorus (%)	0.72	0.67	0.62
Available phosphorus (%)	0.48	0.44	0.39
Sodium (%)	0.23	0.23	0.20

Carcass and meat quality

At 35 days of age, the feed was removed for 12 hours before processing. Twenty-four broilers from each group were killed using CO_2 asphyxiation in an atmosphere of less than 2% oxygen (air displaced by CO_2) for 1.5-2.0 minutes. The breast meat, thigh, wing, and drumstick of all chickens were collected, weighed, and calculated as a percentage of the live body weight. Meat quality measurements were carried out on pectoralis major. The pH values at 0 and 45 minutes were measured using a pH measuring instrument (model HI 99163; Hanna Instruments, Wilmington, MA, USA, (Glamoclija et al., 2015). The breast muscles were refrigerated overnight at 4°C, and then kept at room temperature before cooking. For cooking loss, the breast muscle from each broiler chicken was cooked to an internal temperature of 70°C measured using a digital thermostated water bath (HH-4, Jiangbo Instrument, Jiangsu, China), then cooled to the room temperature, and then weighed. The Warner-Bratzler shear force of the breast meat was determined using an Instron universal tensile tester (Instron model 4411, Instron Corp., Canton, MA, Jiang et al., 2007).

Meat color was measured 45 minutes post-mortem using a chromameter (CR-410, Minolta Co. Ltd, Suita-shi, Osaka, Japan) to measure the CIE LAB values (L* measures relative lightness, a* measures relative redness, and b* measures relative yellowness). Readings were taken at the surface of the sample representing the whole surface of the muscle (Jiang et al., 2007). Deltas for L* (ΔL*), a* (Δa*), and b* (Δb*) may be positive (+) or negative (-). The total difference, Delta E (ΔE*), however, is always positive (AMSA, 2012). The total difference, Delta E (ΔE^*), however, is always positive (AMSA, 2012).

ΔL^* (L^* sample – L^* standard): difference in lightness and darkness (+ : lighter, – : darker); Δa^* (a^* sample – a^* standard): difference in red and green (+ : redder, – = greener); Δb^* (b^* sample – b^* standard) : difference in yellow and blue (+ : yellower, – = bluer).

ΔE^*: $\sqrt{(L_2^* - L_1^*)^2 + (a_2^* - a_1^*)^2 + (b_2^* - b_1^*)^2}$

Statistical analysis

A t-test was used to compare measured values obtained from the two independent groups of the carcass and meat quality of the broiler chickens. Statements of statistical significance were based on $p < 0.05$.

RESULTS AND DISCUSSION

Carcass yield

The effects of supplemented drinking water with antacid on the carcass yield of broiler chickens are presented in Table 2. The weight of breast meat increased significantly with supplemental antacid in the drinking water ($p = 0.02$), while there were no significant effects of the supplementation on carcass yield, carcass percentage, or thigh, drumstick, and wing weight.

Dietary sodium bicarbonate has been reported to improve the body weight, carcass yield, breast meat yield, and abdominal fat of broiler chickens (Yasoob and Tauqir, 2017) although the addition of sodium bicarbonate to the drinking water alone during pre-slaughter did not influence the carcass yield or meat quality of broiler chickens (Petrolli et al., 2016). In terms of mixed solutions, however, Ma et al. (2015) found that electrolyte solutions (containing sugar, sodium chloride, potassium chloride, sodium bicarbonate, and citric acid) significantly increased the body weight of stressed pigs by 15.64%. Moreover, supplementing drinking water using commercial electrolyte packs at 0.10% for 3 days prior to slaughter also improved weight gain and percentage of carcass water uptake (no significant difference) of turkeys, broiler chickens, and pigs during the final 25 days (Ma et al., 2015). Accordingly, the current study showed that adding antacid (a mixture of sodium bicarbonate, bicarbonate, and citric acid) to drinking water during the three days pre-slaughter increased the breast meat weight of the broiler chickens. This indicates that antacid does not neutralize the acidity only in the stomach, but it improves the acid-base balance at the cellular level. Consequently, it maintains cellular metabolism in the breast muscle of the chickens.

Table 2. Effect of antacid supplementation in drinking water on carcass quantity of broiler chickens

Item	Control group	Antacid group	p value	SEM
Live weight (g)	2,004.58 ± 58.69	1,987.33 ± 50.11	0.44	11.04
Carcass yield (g)	1,890.04 ±71.94	1,887.42 ± 46.45	0.91	12.09
Carcass (%)	94.29 ± 2.55	94.98 ± 1.31	0.41	0.41
Breast (%)	21.69 ± 0.73	22.99 ± 1.66*	0.02	0.29
Thigh (%)	13.90 ± 1.08	13.75 ± 1.27	0.76	0.23
Drumstick (%)	10.52 ± 0.62	10.16 ± 0.39	0.10	0.11
Wing (%)	8.07 ± 0.25	7.84 ± 0.27	0.07	0.05

Values presented as mean ±SD. * Means within a row with different letters indicate a significant difference ($P < 0.05$). SEM: Standard Error of Measurement

Meat quality

The effects of adding antacid to the drinking water on the quality of breast meat of the broiler chickens are presented in table 3. There was no significant difference among the pH of the meat of the experimental groups at 0 minutes, while the pH of the antacid group was significantly higher than that of the control group at 45 minutes ($P < 0.05$). Adding antacid to the drinking water decreased the drip loss of the meat at 24 hours ($P < 0.05$) and 48 hours ($p = 0.06$). Moreover, the shear force value was significantly reduced by supplementation of the drinking water ($p < 0.01$) although the cooking loss was not affected. For the color of the breast meat, there were no significant differences between the two groups for the L* a* and b* values ($p > 0.05$) while the total difference in color was slightly increased.

Since the aerobic metabolism in muscle changes to anaerobic metabolism after the oxygen supply is stopped, the rate of pyruvate conversion to lactate is increased (Zhang et al., 2009). Rapid post-mortem glycolysis while carcass temperature is still high results in a rapid drop in pH, protein denaturation, pale meat color, reduced water holding capacity, and poor texture (Song and King, 2019). Bicarbonate is a blood buffer that maintains both extracellular and intracellular pH, and sodium bicarbonate supplementation increases blood bicarbonate concentrations, resulting in blood alkalosis (Lancha et al., 2015). Several investigators have reported that supplementing with sodium bicarbonate results in high pH in chicken meat (Woelfel and Sams, 2001) and pork (Wynveen et al., 2001). Therefore, a high pH in meat reduces the percentage of drip loss by an improvement in the water holding capacity (Fischer, 2007). The water holding capacity of meat is minimal when the pH is close to the isoelectric point of myofibrillar proteins (about 5.2-5.3 in poultry meat) while the ionic strength could be steadily increased by adjusting the PH, thus it leads to an increase in the water holding capacity of meat products (Barbut, 2002; Petracci and Cavani, 2012). Therefore, it is clear that supplementing drinking water with antacid will improve the water holding capacity of meat by maintaining the PH.

Table 3. Effect of antacid supplementation in drinking water on meat quality of broiler chickens

Item	Control group	Antacid group	p value	SEM
pH_0	7.17 ± 0.20	7.26 ± 0.21	0.34	0.04
pH_{45}	6.63 ± 0.22	6.84 ± 0.22*	0.03	0.05
Drip loss24 hr. (%)	2.63 ± 0.61	2.16 ± 0.42*	0.03	0.11
Drip loss48 hr. (%)	3.50 ± 0.79	2.92 ± 0.68	0.06	0.15
Cooking loss (%)	26.52 ± 3.26	26.60 ± 2.89	0.94	0.61
Shear force (N)	43.47 ± 5.01	29.92 ± 5.29**	<0.01	2.65
L^{*}	42.70 ± 2.17	41.64 ± 3.99	0.35	0.65
a^{*}	1.77 ± 0.74	2.15 ± 0.99	0.29	0.18
b^{*}	13.15 ± 0.76	13.16 ± 1.17	0.97	0.19
ΔL^{*}	0	-1.06	-	-
Δa^{*}	0	0.38	-	-
Δb^{*}	0	0.01	-	-
Total color difference between the control group (ΔE^{*})	0	1.12	-	-

Values presented as mean ±SD. * $P < 0.05$; ** $P < 0.01$, L*: Lightness, a*: Redness, b*: Yellowness, SEM: Standard Error of Measurement

An increase in the pH of meat at 45 minutes by supplementing the antacid in drinking water may be due to the high buffering capacity and ionic strength of bicarbonate and sodium citrate. the reason is that sodium bicarbonate reacted with citric acid to produce sodium citrate, which is dissociated from citrate anion (Stephens et al., 2008). Therefore, a reduction in H+ and an elevation in bicarbonate in plasma resulting in an increase of the extracellular buffering capacity (Tugnoli et al., 2020). The increase in blood pH through sodium citrate supplementation induces more efflux of H+ and lactate from the active muscles via monocarboxylate transporter (Requena et al., 2005). This implies that sodium citrate may provide a high buffering capacity to improve the acid-base balance in meat.

Moreover, the findings of the present study showed that the shear-force of the meat was reduced by antacid supplementation of the drinking water. There are reports

indicating that sodium bicarbonate reduced the shear force and improved the yield of pork and poultry meat (Mudalal and Petracci, 2019). However, broiler chickens given 0.50% sodium bicarbonate from five to eight weeks of age had meat of higher shear force than those given a combination of 0.50% sodium bicarbonate and 0.50% potassium chloride (Hao and Gu, 2014). It may be said that supplementing with antacid (electrolyte solution) may have more impact on the shear-force than sodium bicarbonate alone.

A rapid drop in pH may be associated with low redness, high lightness and high drip, and cooking losses in chickens' breast meat (Hao and Gu, 2014). The use of bicarbonate to minimize the problem of pale, soft, and exudative meat has been reported in pork (Wynveen et al., 2001; Mudalal and Petracci, 2019) and poultry (Woelfel and Sams, 2001; Alvarado and Sams, 2003). However, a low incidence of Pale Soft Exudative (PSE) meat was presented in the current study (all pH values were above 5.8 and L* values were below 52). This pigmentation/redness ratio was used to assess myoglobin oxidation, with high redness values indicating high myoglobin oxidation (Kim et al., 2010). Although the L*, a*, and b* colors were not significantly affected, a slight increase in the total color difference in the antacid group may be due to the maintenance of the pH and water holding capacity.

CONCLUSION

Since the supplementation can maintain the pH and water holding capacity of the meat, it is concluded that the antacid (a mixture of sodium bicarbonate, bicarbonate, and citric acid) should be added to the drinking water for three days before slaughter to improve carcass (percentage of breast meat) and meat quality (pH at 45 minutes, drip loss percentage at 24 and 48 hours and shear force) of broiler chickens.

DECLARATIONS

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Authors' contribution

Namted S and Rakangthong C developed the concept, analyzed data, and wrote the manuscript. Namted S and Srisuwan K assisted in data collection while Bunchasak C designed the graphical abstract. All authors reviewed and confirmed the manuscript before submission.

Competing interests

The authors declare that they have no conflict of interest.

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Effects of Acetaminophen and Vitamin Supplement on Feed intake, Body Weight, and Acute Pain Responses of Pullets Subjected to Beak-trimming

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ABSTRACT

The first experiment aimed to compare the effects of manual and electric beak-trimming on feed intake, body weight, and some biochemical parameters of eight-week-old pullets. A total of 20 pullets aged 3 weeks were assigned to two treatment groups; those in group A were manually debeaked while the samples in group B were electro debeaked. The findings indicated that 2 hours post-beak-trimming (PBT), the packed-cell volume of group A was significantly higher than that of group B. Plasma cortisol level of group A was significantly higher than that of group B at 2 and 6 hours PBT while total plasma protein level of group A was significantly lower than that of the group at 2 and 72 hours PBT. Furthermore, feed intake and body weight markedly decreased in the pullets debeaked with both methods until 72 hours PBT. The chickens' beak trimmed with both methods experienced intense pain of varying degrees lasting up to 72 hours. In the second experiment, 40 eight-week-old pullets were assigned to four groups; group A was the control, group B was treated with a vitamin supplement, group C was treated with acetaminophen, and group D was treated with vitamin supplement plus acetaminophen. After 24 hours, chickens were beak-trimmed using a manual cutter. The results revealed that 2 hours PBT, plasma cortisol level in groups B, C, and D were significantly lower than that of group A. Blood glucose was lowest in groups A and D at 6 and 24 hours PBT, respectively. It is concluded that the pre-treatment with vitamins and NSAIDs could reduce stress and pain in debeaked chickens.

Keywords: Anti-stress, Debeaking, Pain, Pullet

INTRODUCTION

Beak-trimming, formerly known as debeaking, is a routine husbandry procedure performed in many poultry species, such as laying hens, turkeys, ducks, and quails (Gentle et al., 1995; Gustafson et al., 2007; Fournier *et al.*, 2015). It is the act of amputation or cauterization of approximately one-quarter to one-third of the upper beak or both upper and lower beak of a bird (Gentle et al., 1995; Van Liere, 1995) to prevent aggressive behaviors, feather pecking, and cannibalization (Dennis *et al.*, 2009). There are several methods for debeaking, including mechanical beak-trimming using simple blades or scissors (Gentle et al., 1995; Gustafson et al., 2007), cold blade method (Van Liere, 1995), hot blade method (Gentle et al., 1995; Van Liere, 1995), chemical beak-trimming (Van Liere, 1995), electrical debeaking (Gentle et al., 1995), freeze-drying method, as well as robotic and laser beak-trimming (Marchant-Forde et al., 2008). The choice of the method to be used depends on the financial capability of the farmer. Therefore, smallholder farmers inevitably use manual and hot blade methods while large-scale commercial farmers and hatcheries can afford to utilize the electrical, laser, or infrared methods.

The practice of beak-trimming has raised numerous welfare concerns due to its potential to cause acute and chronic pain as well as the loss of function in poultry (Gentle, 2011). Researches have demonstrated that beak-trimming results in acute pain in young poultry, whether performed by the conventional hot-blade method or the new infra-red procedure (Marchant-Forde et al., 2008). Moreover, beak-trimming of adults can lead to the incidence of neuroma and chronic pain (Breward and

Gentle, 1985; Duncan et al., 1989; Gentle, 1991). The utilization of the conventional hot-blade debeaking method is not usually encouraged since it is an invasive method which can cause stress, pain, and intense suffering in poultry. However, most small-scale farmers still employ this method of beak-trimming with the intention of achieving better productivity (Dennis *et al.*, 2009; Dennis and Cheng, 2010). Furthermore, all manual methods of beak-trimming seem to be inefficient, time-consuming, and are often poorly performed (Dennis *et al.*, 2009). As reported, infra-red trimming can result in a higher decrease of growth and feed intake in the following weeks, compared to the hot-blade trimming method. This means that the pain induced by infra-red beak-trimming may be similar to or more severe than the pain caused by the hot-blade method (Honaker and Ruszler, 2004; Marchant-Forde et al., 2008).

Environmentalists condemn beak-trimming since it is perceived as painful mutilation of the beak which impairs feed and water intake days after trimming (Marchant-Forde et al., 2008) leading to stress and mortality among poultry. Thus, the egg production industry is under intense pressure to stop beak-trimming. However, the abolishment of this procedure in layer farms may negatively impact animal welfare, as it increases the aggressive behaviors of the subjected poultry. (Guesdon *et al.*, 2006; Dennis and Cheng, 2010). Thus, to address welfare concerns and reduce stress and pain following beak-trimming, it is a common practice for veterinarians and farmers to administer anti-stress medications to poultry a day before beak-trimming. However, the efficacy and implications of this practice have not been experimentally supported.

This study was conducted to compare the stress level experienced by eight-week-old pullets following the use of a manual cutter and an electric debeaker for beak-trimming. The study further investigated the effect of pre-beak-trimming administration of a vitamin supplement, acetaminophen, and vitamin plus acetaminophen combination on stress-related changes and acute pain responses in pullets.

METHODOLOGY

Ethical approval

The protocols used for this research were performed in conformity with the National Institutes of Health revised guidelines for laboratory animals' care and use (NIH, 1985) and approved by the Faculty of Veterinary Medicine, University of Nigeria, Nsukka (Approval no. UNFVM/11/19/021).

Animals

One hundred day-old ISA brown pullets were procured from a commercial hatchery and raised in the poultry pen of the Department of Veterinary Medicine, University of Nigeria, Nsukka for eight weeks. The chickens were routinely vaccinated against Newcastle disease, Infectious bursa disease, and fowlpox disease before the start of the study. Water and feed were provided *ad libitum* throughout the experiment. Before beak-trimming, chickens were dewormed with levamisole Hcl (100 g in 200 L water). The diet contained 15% crude protein, 8% fat, 15 crude fibre, 0.9-1.2% calcium, 0.35% phosphorus and 2600 Kcal/Kg of metabolisable energy

Experiment I: Comparison of acute pain induced by beak-trimming using manual beak cutter and electric debeaker

Pre-beak-trimming measures

A total of 20 eight-week-old pullets were assigned to two treatment groups (n=10). Each group of pullets was housed separately. Both groups of chickens were fed pre-determined weights (1000 g) of growers ration (Vital feed®, Nigeria) for three days to ascertain their feed intakes. The body weights, heart rates, and rectal temperature of chickens were measured 10 minutes before beak-trimming. Blood (2 ml) was also collected from the jugular vein of each pullet for the determination of packed cell volume (PCV), plasma cortisol level, and total plasma protein level.

Beak-trimming

One-third of the upper beak of each pullet was trimmed using a manual cutter (group A) and an electric debeaker (group B). Following beak-trimming, chickens were returned to their cages and provided with a weighed quantity (1000 g) of growers ration. Clean medication-free drinking water was also provided for the chickens.

Measured parameters post-beak-trimming

Feed intake of chickens was determined at 24, 48, 72, and 96 hours post-beak-trimming. Feed intake by the chicken was determined by weighing the remained feed rations given at 24, 48, 72, and 96 hours and subtracting it from the weight of the initial 1000 g of growers ration fed the previous day. Percentage changes in feed intake per group were subsequently determined. The weight of each chicken was re-determined 24 and 168 hours (day 7) after beak-trimming using a weighing scale.

At 2, 6, and 24 hours post-beak-trimming, blood was collected from the pullets into EDTA plastic vacutainers for the following laboratory investigations. The PCV was determined using the microhaematocrit methods as described by Natt and Herrick (1952). Plasma was obtained by the centrifugation of anti-coagulated blood to determine cortisol and total plasma protein. Plasma cortisol was measured using a Cortisol ELISA Kit (Medix biotech kit®, USA) according to the manufacturer's instructions. Total plasma protein was determined using the Randox® kit (United Kingdom) under standard conditions.

Heart rate (HR) and temperature were taken 24 hours after beak-trimming. The heart rate was determined with the use of veterinary multi-parameter monitor TM-9009 (Technocare Medisystems, India) and the temperature was determined with a clinical thermometer.

Experiment II: Effect of pre-beak-trimming administration of vitamin supplement, acetaminophen, and vitamin supplement plus acetaminophen on changes in feed intake, body weight, and acute pain response

Pre-beak-trimming protocols

Forty 8-week-old pullets of similar weights were selected and assigned to four treatment groups. Each group (n=10) was housed separately and was fed pre-determined weights (800g) of growers ration (Vital feed®, Nigeria) for three days to ascertain their feed intakes. Medications administered to the groups consisted of vitamin supplement (Vitalyte Extra® United Kingdom; group B), acetaminophen (Sigma Aldrich® Germany; group C), vitamin supplement plus acetaminophen (group D). The vitamin supplement and acetaminophen were included in drinking water of chicken in groups B and C at dose rates of 2 and 0.4 g/4 liters of water, respectively, based on manufacturer recommendations. Drinking water (4 liters) of birds in group D was medicated with 2 g of a vitamin supplement and 0.4 gram of acetaminophen. The pullets in the treatment groups were allowed access to the medicated water for 1 hour prior to beak trimming. No medication was administered to chickens in group A (control group). Regarding post-beak-trimming, medicated water was provided for chickens in the treatment groups *ad libitum* for 5 days.

Ten minutes before beak-trimming, blood (2 ml) was collected from the jugular vein of each pullet for the determination of baseline values of PCV, blood glucose, plasma cortisol level, and total plasma protein level.

Beak-trimming

One-third of the upper beak of each pullet was trimmed using a manual cutter. Following beak-trimming, chickens were returned to their cages and provided with a weighed quantity (800g) of growers ration. They were also provided with clean medication-free water.

Parameters measured post-beak-trimming

Feed intakes were determined 24, 48, 72, and 96 hours after beak-trimming. Feed intake was calculated by subtracting the weight of the remnant feed 24, 48, 72, and 96 hours after the initial 800 g of growers ration fed the previous day. Percentage change in feed intake per group was subsequently determined. At 2, 6, and 24 hours post-beak-trimming, blood was collected from the pullets into EDTA plastic vacutainers for the following laboratory investigations. Blood glucose was determined using a handheld glucometer (Accucheck advantage Π®). PCV, plasma cortisol, and total plasma protein were determined as described in Experiment I. Percentages of changes in the glucose levels of pullets were calculated.

Statistical analysis

The obtained data were reported as mean ± standard error of the mean. Mean values of the investigated parameters obtained in Experiment I were compared using the student's T-test while those of Experiment II were compared using one-way ANOVA. Post hoc comparison was performed using LSD at a probability of less than 0.05.

RESULTS

Experiment I

Feed intake

As shown in Figure 1, feed intake of pullets decreased markedly in group A by 98.7%, compared to a 62.97% decrease in the weight of consumed feed by pullets in group B when compared with the weight of feed consumed before beak-trimming. Furthermore, a decrease in feed intake of group A (19.34%) was higher than that of group B (17.07%) 48 hours after beak-trimming. As indicated, 72 hours after beak-trimming, feed intake increased in group B, however, there was a decrease in group A. The findings indicated that 96 hours after beak-trimming, the feed intake of pullets in groups A and B increased by 5.28% and 22.41%, respectively.

Body weight and vital parameters

Twenty-four hours after beak-trimming, body weights of chickens in groups A and B decreased by 4.09% and 2.26% 24, respectively. The body weights of

chickens in groups A and B increased by 11.5% and 15.98%, respectively, 7 days after beak-trimming (Figure 2). Heart rates and rectal temperatures of both groups were not significantly ($p > 0.05$) different at the investigated post-beak-trimming time points (Table 1).

Packed cell volume, plasma cortisol, total plasma protein

By 2 hours post-beak-trimming, there was a significant decrease of PCV of group A than group B ($p < 0.05$). There was no significant difference between the PCV of groups at other time points (Table 2). Plasma cortisol of group A was significantly higher than that of group B by 2 and 6 hours post-beak-trimming ($p < 0.05$). No significant difference was observed among the groups 24 hours after beak-trimming (Table 2). The total protein level of group A was significantly lower than that of group B by 2, 6, and 24 hours post-beak-trimming ($p < 0.05$, Table 2).

Experiment II

Feed intake

The results of the percentage change in feed intake are presented in Figure 3. By 24 hours post-beak-trimming, feed intake in all four groups decreased by 98.97% (group A), 97.7% (group B), 93.66% (group C), and 61.48% (group D), compared to pre-beak-trimming feed intake. The feed intake decreased 48 hours after beak-trimming in groups A, B, C, and D by 79.7%, 92.88%, 42.25%, and 45%, respectively. The results obtained 72 hours post-beak-trimming indicated that feed intake increased in groups C and D while there was a decrease in the feed intake in groups A and B were 69.6% and 10.56%. Although 72 and 96 hours after beak-trimming the feed intake of chickens increased above the baseline, feed intake in groups C and D remained low.

Plasma cortisol, total plasma protein, and blood glucose

The results obtained 2 hours after beak-trimming revealed that plasma cortisol levels of group B, C, and D were significantly lower than that of group A ($p < 0.05$). Cortisol levels of all the groups were not significantly different 6 and 24 hours post-beak-trimming ($p > 0.05$, Table 3). Total plasma protein levels of all the groups were not significantly ($p > 0.05$) different 2, 6, 24, and 72 hours post-beak-trimming (Table 3). Glucose levels of chickens in all groups decreased after beak-trimming. Glucose levels in groups A, B, C, and D decreased to 13.85%, 15.09%, 27.1%, and 20.5%, respectively, 6 hours after beak-trimming. However, 24 hours after beak-trimming, glucose levels of groups A, B, C, and D decreased to 26.83%, 25.19%, 24.88%, and 5.28%, respectively (Figure 4).

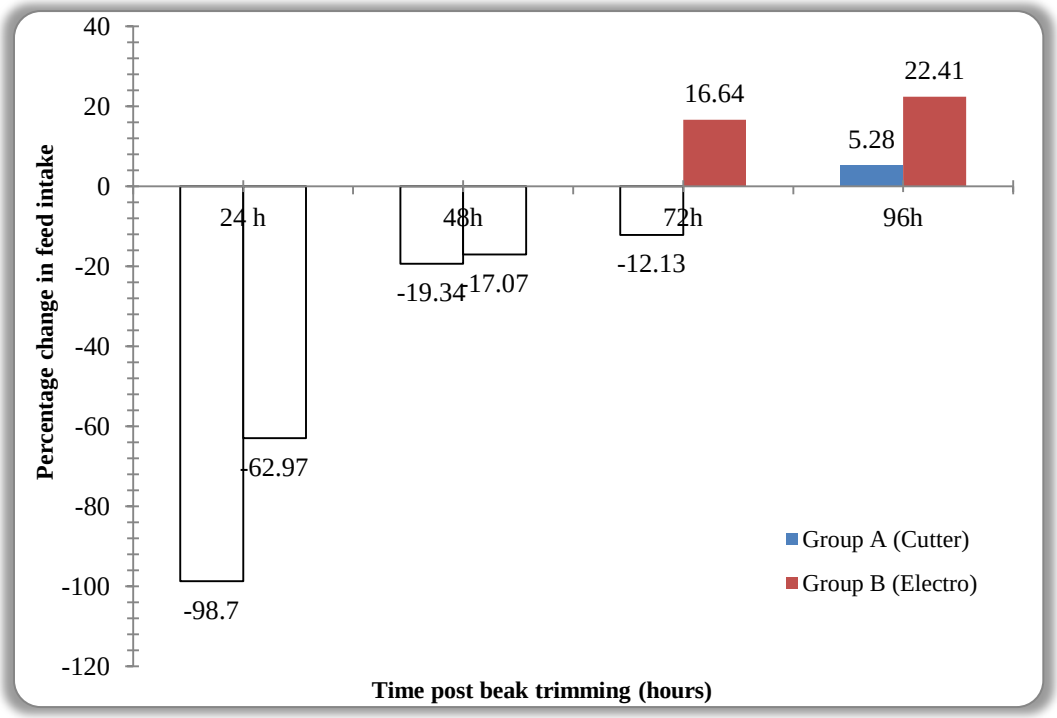

Figure 1. Percentage changes in feed intake of pullets post-beak-trimming using a manual cutter and electro debeaker.

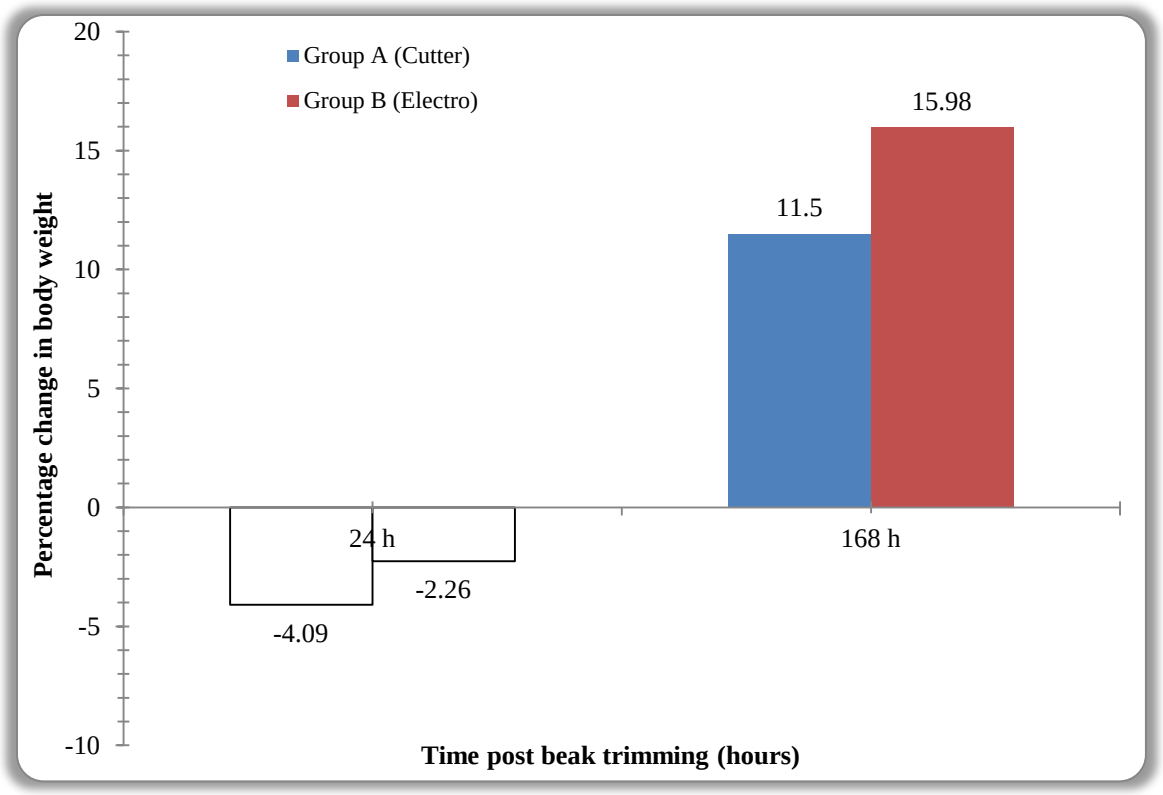

Figure 2. Percentage changes in body weight of pullets post-beak-trimming using the manual cutter and electro debeaker.

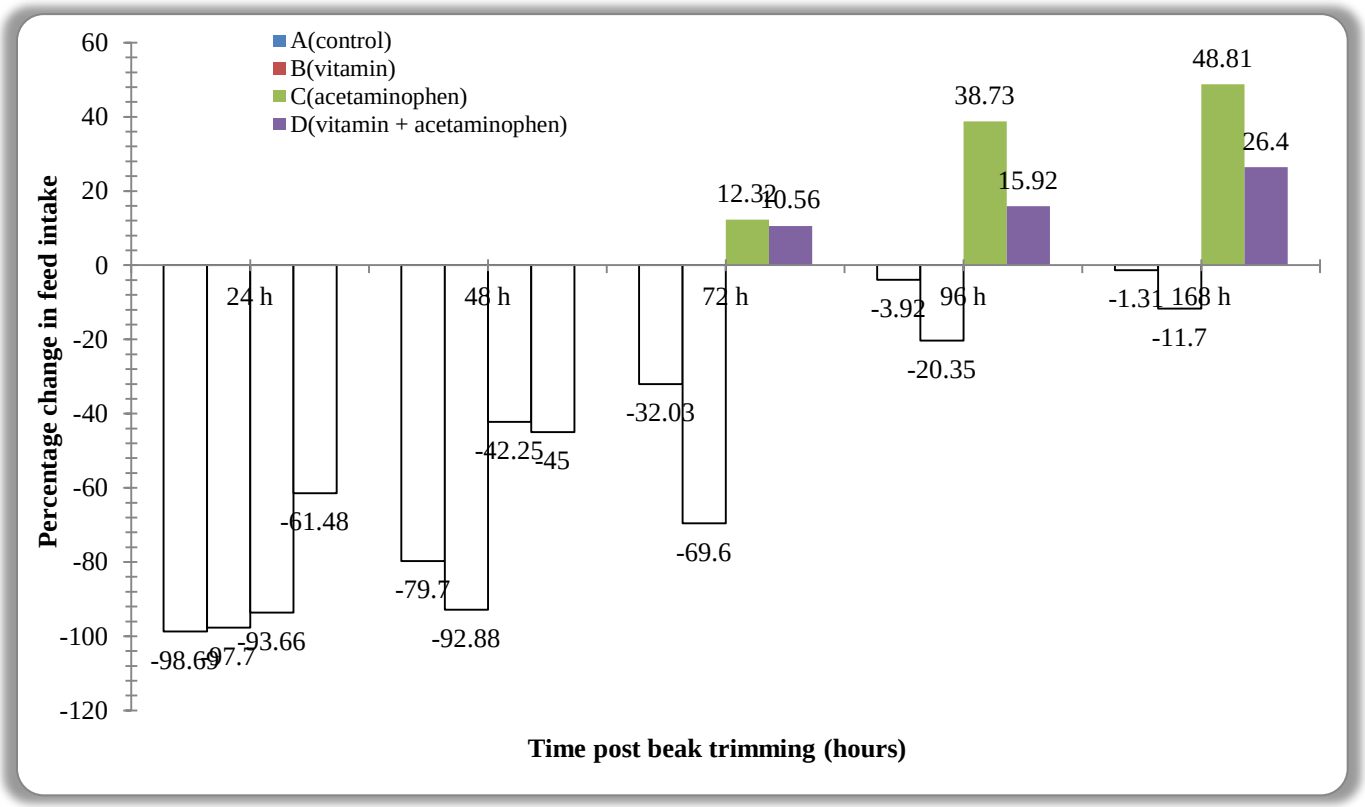

Figure 3. Percentage changes in feed intake of pullets treated with different medications.

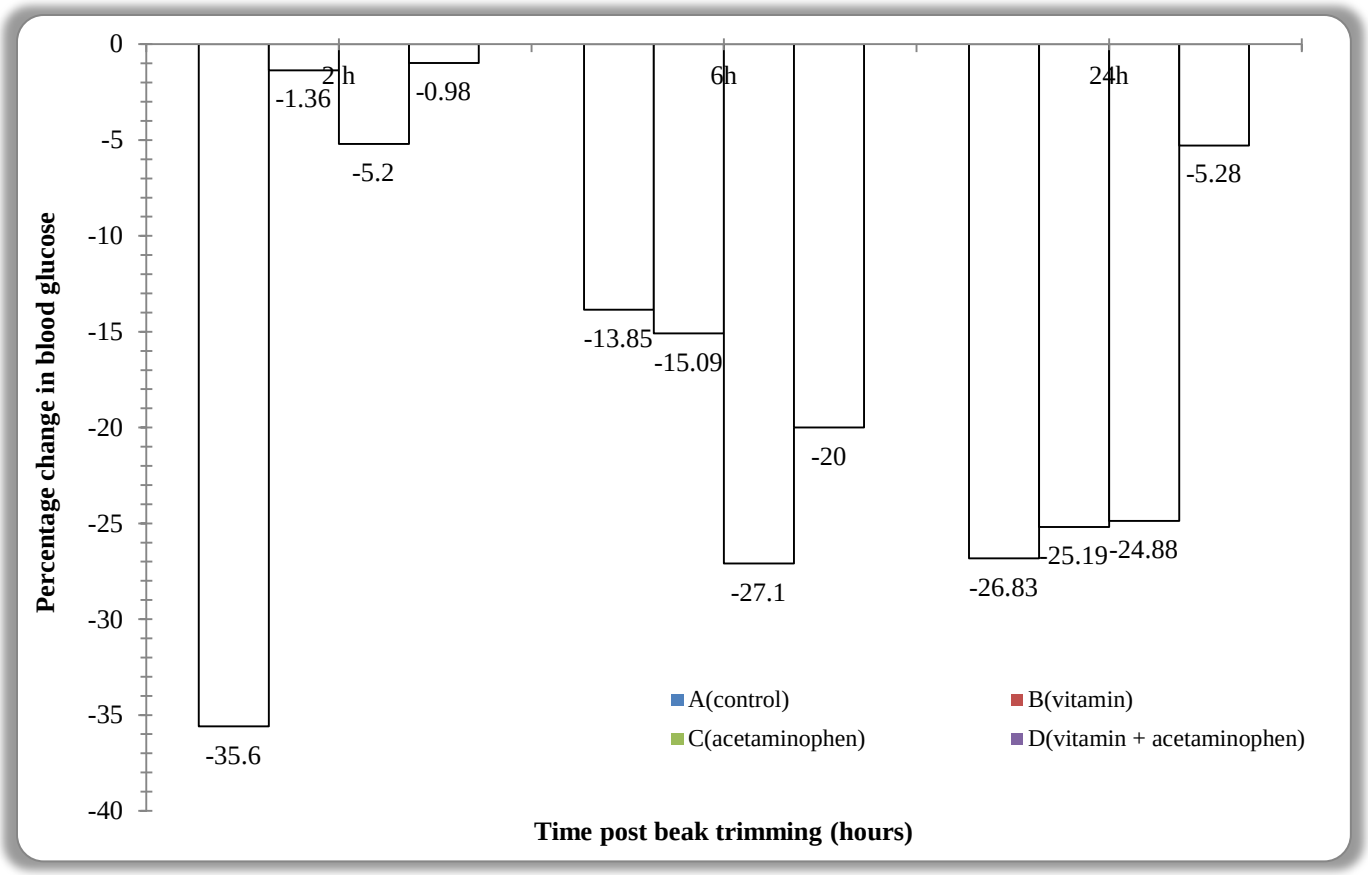

Figure 4. Percentage change in blood glucose of pullets treated with different medications.

Table 1. Heart rate and body temperature of pullets pre and post-beak-trimming using different methods.

Parameters	Groups	Pre-beak-trimming	24 hours PBT
Heart rates (beats/min)	Group A	343.20 ± 7.73[a]	325.60 ± 8.10[a]
	Group B	341.60 ± 11.28[a]	356.30 ± 3.66[a]
Temperature (°C)	Group A	41.30 ± 0.14[a]	41.06 ± 0.06[a]
	Group B	40.96 ± 6.08[a]	41.08 ± 0.08[a]

*Different superscript letters in a column indicate a significant difference between mean values of groups A and B at $p < 0.05$. PBT: Post-beak-trimming. Chickens of group A were beak-trimmed using a manual cutter and group B by electro debeaker.

Table 2. Packed cell volume, cortisol, and total plasma protein levels of pullets pre- and post-beak-trimming using different methods.

Parameters	Groups	Pre-beak-trimming	2 hours PBT	6 hours PBT	24 hours PBT
Packed cell volume (%)	Group A	27.2 ± 0.37[a]	20.6 ± 0.49[a]	23.0 ± 1.05[a]	24.0 ± 5.03[a]
	Group B	27.6 ± 0.6[a]	23.7 ± 0.24[b]	26.6 ± 0.34[a]	27.2 ± 0.37[a]
Cortisol (µg/dl)	Group A	0.48 ± 0.05[a]	0.71 ± 0.05[a]	1.04 ± 0.36[a]	0.898 ± 0.54[a]
	Group B	0.53 ± 0.07[a]	0.66 ± 0.01[b]	0.51 ± 0.28[b]	0.57 ± 0.13[a]
Total plasma protein (g/dl)	Group A	3.58 ± 0.62[a]	3.04 ± 0.30[a]	3.22 ± 0.16[a]	3.50 ± 0.13[a]
	Group B	3.32 ± 0.96[a]	3.24 ± 0.04[b]	3.00 ± 0.20[a]	4.18 ± 0.42[b]

*Different superscript letters in a column indicate a significant difference between mean values of groups A and B at $p < 0.05$. PBT: Post-beak-trimming. Chickens of group A were beak-trimmed using a manual cutter and group B by electro debeaker.

Table 3. Cortisol and total plasma protein levels of pullets treated with different medications.

Parameters	Groups	Pre-beak-trimming	2 hours PBT	6 hours PBT	24 hours PBT
Cortisol (µg/dl)	Group A	0.67 ± 0.15	1.51 ± 0.40[a]	0.83 ± 0.08[a]	0.76 ± 0.09
	Group B	0.83 ± 0.18	0.68 ± 0.13[b]	0.62 ± 0.08 [b]	0.62 ± 0.18
	Group C	0.64 ± 0.16	0.55 ± 0.97[b]	0.71 ± 0.17 [b]	0.73 ± 0.09
	Group D	0.68 ± 0.11	0.46 ± 0.03[b]	0.63 ± 0.02 [b]	0.57 ± 0.09
Total plasma protein (g/dl)	Group A	3.65 ± 0.26	2.95 ± 0.14	3.10 ± 0.06	4.45 ± 0.49
	Group B	3.4 ± 0.00	3.45 ± 0.26	3.45 ± 0.32	3.4 ± 3.34
	Group C	3.45 ± 0.23	3.03 ± 0.06	3.35 ± 0.30	3.85 ± 0.27
	Group D	3.73 ± 0.06	3.73 ± 0.42	2.95 ± 0.14	3.80 ± 0.36

*Different superscripts in a column indicate a significant difference between mean values of groups A, B, C, and D at $p < 0.05$. PBT: Post-beak-trimming. Group A (control), Group B (vitamin supplement), Group C (acetaminophen), and Group D (vitamin supplement plus acetaminophen).

DISCUSSION

Beak-trimming is acutely painful, as many sensory nociceptors are present in the beak (Van Liere, 1995). Post-beak-trimming behaviors in chickens can be reflected through reduced feed intake, reduced activity, and beak guarding (Gentle et al., 1997; Marchant-Forde et al., 2008). According to Dubbeldam et al. (1995), there was a decrease in the feeding intake time of chickens during the first post-beak-trimming weeks as a result of pain. Thus, the severe decrease in feed intake and body weight of samples in manual beak cutter and electric debeaker groups of the current study could suggest that the samples experienced intense pain at different levels lasting up to 72 hours. This result was not in accordance with previous reports on the outcome of debeaking in very young chickens indicating no clear evidence of prolonged acute pain in chickens. According to these studies, young chickens experienced less pain and no scar tissue or neuroma development after beak-trimming due to rapid re-growth of their beaks (Dubbeldam et al., 1995; Lunam et al., 1996; Gentle et al., 1997). However, in chickens as old as the samples in the current study, studies have shown that the trimmed beak rapidly heals but does not regenerate. The tips of the beaks underlying the epidermis were composed of scar tissues (Breward and Gentle, 1985). In addition, branches of the trigeminal nerve innervating the beak are damaged during beak-trimming while neuromas formed subsequently at the tip of the beak often regress in the early stage of healing (Duncan et al., 1989). However, if severe beak-trimming methods are used, neuromas may persist and exhibit ectopic activity leading to chronic pain in the beak stump (Gentle et al., 1990; Gentle, 1991; Crespo and Shivaprasad, 2003). Therefore, it can be concluded that the short duration of pain in chickens subjected to manual and electric beak-trimming methods in the current study suggests low severity of these two methods. Moreover, a higher decrease in feed intake cum weights of pullets in the group debeaked with manual cutter could suggest that more pain was felt by chickens in this group. Less pain might have been felt in the electro-debeaked pullets since the use of the electric current in debeaking causes complete loss of afferent nerve supply and sensory nerve endings with no neuroma formation (Gentle et al., 1995). Thus, chickens debeaked with the electric debeaker might not experience beak guarding and can resume feeding earlier than their counterparts debeaked with the manual cutter.

The primary glucocorticoid released in response to stress and pain is cortisol (Molony and Kent, 1997). Therefore, serum cortisol assay is recognized as one of the objective methods of assessing stress and pain in many species (Carroll et al., 2006; Keita et al., 2010). Higher plasma cortisol levels among chickens 2 and 6 hours after beak-trimming using the manual cutter suggest that the use of the cutter for chickens could lead to higher levels of stress and pain. This probably leads to higher rates of weight loss and a decrease in the total plasma protein level as also seen in this group.

PCV of pullets in both groups studied decreased following beak-trimming probably because bleeding occurred in the pullets. However, the PCV of the manual cutter group was significantly lower PCV than that of the electro debeaker group in the immediate period (2 hours) post-beak-trimming. This suggests that more beak bleeding might have occurred in this group. This finding highlights the advantage of the electro debeaker method of beak-trimming over manual beak-trimming since the beaks are immediately cauterized by the electro debeaker as they are being cut. However, despite this advantage of the electro debeaker, its use is not common in our locality due to their non-availability and cost leaving farmers with no choice but to recourse to the use of manual cutters, such as a simple blade or scissors-like devices.

Experiment II was conducted to ascertain the efficacy of drugs administered to pullets as anti-stress medications prior to beak-trimming. The outcome of the study showed that administration of acetaminophen and a combination of vitamin plus acetaminophen significantly ameliorated stress and pain in pullets. The use of acetaminophen alone gave the best outcome. This can be seen in the results obtained by 72 hours from the assessment of feed intake and post beak-trimming plasma cortisol assay after 2 and 6 hours. At the aforementioned time points, there was a marked decrease in feed intake and high plasma cortisol levels, compared to results obtained in the control group (that were debeaked using the manual cutter without any form of drug intervention) and the vitamin group. Similarly, Glatz (2000) revealed that analgesics have the potential to ameliorate the initial pain felt by chickens post-beak-trimming, According to Glatz (2000), a mixture of bupivacaine and phenylbutazone can be swabbed unto the beak immediately after beak-trimming. Therefore, the addition of mild analgesics, such as acetaminophen in water for preemptive analgesia prior to manual beak-trimming should be encouraged in layer farms in order to hasten the return to feeding and drinking in chickens.

The current study also showed that blood glucose levels of pullets in all the groups remained decreased significantly for up to 24 hours post-beak-trimming. This was not expected since an increase in blood glucose had characterized the immediate post-trauma period in other animals, such as dogs (Lemke et al., 2002; Ugwu et al., 2020), goats (Udegbunam et al., 2013), and rats (Udegbunam et al., 2012). Blood glucose rise post-trauma had been attributed to post-operative pain which leads to the increased production of epinephrine, norepinephrine, cortisol, growth hormones, and adrenocorticotropic hormone (Kuo et al., 2015). Cortisol also leads to hyperglycemia (Singh, 2003). In addition, catecholamines mainly epinephrine and glucagon activate glycogenolysis and mobilization of glucose principally from muscles with a consequent increase in the production of lactate and glucose (Prunier et al., 2005). There has been no study assessing the glucose response in chicken post-beak-trimming so far. However, it may be assumed that the avian species do not respond to trauma as other species probably due to their small body muscle mass. Regarding glucose response, it is required to include glucose powder in drinking water for pullets during the first few days post-beak-trimming to energize the chickens which exhibit zero to poor feeding due to severe pain induced by beak-trimming. However, this finding excludes the use of glucose rise as an index of severe pain in chicken.

CONCLUSION

The manual beak cutter and electric debeaker led to intense pain of varying degrees lasting up to 72 hours. This period of acute pain was characterized by a severe decrease in feed intake, weight loss, blood glucose level, PCV, and total plasma protein levels of pullets as well as the increase in serum cortisol level. Accordingly, the administration of acetaminophen and a combination of vitamin plus acetaminophen significantly ameliorated pain in chickens.

DECLARATIONS

Authors' contribution

All authors contributed equally to this work.

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Competing interests

The authors declare that they have no conflict of interest.

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Effect of Egg Storage Length on Hatchability and Survival of Koekoek Chickens

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ABSTRACT

Chicken production plays a major in the livelihood of rural people due to the provision of eggs and meat which are high sources of protein. This calls for sustainable production of chickens through strategies aimed at improving the hatchability of eggs and survival of chickens. Therefore, the present study was conducted to determine the effect of egg storage length on egg hatchability and survival of the Koekoek chickens. A total number of 270 eggs were divided into three treatment groups, and the eggs of each group were stored for 3, 7, and 11 days before incubation. Each treatment consisted of three replicates. The General Linear Model procedure was used to analyze the data. The eggs that were stored for three days before incubation had a higher hatching percentage, compared to those that were stored for 7 and 11 days before incubation. Storing eggs for few days before incubation resulted in reduced embryonic mortality rate and lower mortality of chickens during the first seven days after hatching. Based on these results, is recommended that Koekoek chicken eggs should be stored for three days before incubation to maximize hatchability and survival of chickens before the age of seven days.

Keywords: Eggs, Storage, Embryo mortality, Hatchability, Koekoek chicken

INTRODUCTION

The small-scale poultry production system is the main source of income and protein for the majority of poor people, especially in rural areas (Wong et al., 2017). The demand for Koekoek chickens is high and their performance is better than indigenous breeds (Yirgu et al., 2019). They are one of the best-suited chicken breeds for a free-range environment under the rural communities setting and it is unnecessary to confine them in a shelter (Belay et al., 2018). The average egg production performance of the Koekoek chickens was reported 176 per year with an average egg weight of 45.33 grams (Abadi et al., 2020).

However, Molapo and Kompi (2015) highlighted that the majority of farmers rearing Koekoek chickens depend on hatcheries for the supply of one-day-old chickens while a small number of farmers hatch their chickens through natural incubation. Therefore, Boleli et al. (2016) stated that the continuous supply of chicken meat and eggs in the market is influenced by increasing the hatchability of healthy chickens that would survive under different rearing field conditions. On the other hand, Salamon (2020) emphasized that the storage conditions of the eggs before incubation have a significant effect on hatchability. Among these factors, Nasri et al. (2019) emphasized that the pre-incubation egg storage time has an impact on the hatching percentage of eggs. Therefore, to have an improved hatching percentage, eggs that are unsuitable for incubation should not be used for hatching purposes but could be used as a source of protein for families. Storage of hatching eggs for more than 7 days seems to negatively affect the hatching percentage and embryonic development (Fasenko, 2007). Addo et al. (2018) also explained that the extended storage of chicken eggs beyond seven days is harmful to chicken quality and hatchability. Reirink et al. (2010) and King'ori (2011) emphasized that egg storage duration reduces hatchability and chicken quality. The higher embryonic mortality of eggs that were stored for a longer period was observed in a study that was conducted by Fasenko (2007). Lima et al. (2012) also reported higher embryo mortality in eggs that were stored for 14 days before incubation. Prolonged egg storage (14 days) resulted in chickens with unhealed

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navels, deformities, and general symptoms of weakness (Fasenko, 2009). In addition, Yassin et al. (2008) clarified that pre-incubation egg storage for a long time adversely affects the chicken embryonic development due to reduced egg quality. Moreover, Reirink et al. (2010) stated that the chickens that are deformed at hatching may die within two days after hatching.

Currently, farmers who are engaged in Koekoek chicken's hatching industry have been facing a problem of lower hatchability. The reason is that there has been no study addressing pre-incubation egg storage length since the introduction of Koekoek chickens in Lesotho. Therefore, the current study aimed to determine the effect of egg storage length on hatchability and survival of Koekoek chickens up to seven days after hatching.

MATERIALS AND METHODS

Ethical approval

The research and ethics committee in the Department of Animal Science of the National University of Lesotho approved the current study based on international welfare standards for use of animals in conducting research.

Study site

The study was conducted at the Department of Animal Science Experimental farm of the National University of Lesotho which is located 36 km from Maseru, the capital city of Lesotho.

Management of chickens

Koekoek chickens were given laying mash bought from the commercial feed manufacturer with the following chemical composition (Table 1).

Table 1. Nutrient composition of Koekoek chickens laying mash at the age of 18 to 32 weeks

Nutrients	Ingredient (g/kg)
Crude protein	130.0
Moisture	120.0
Fiber	70.0
Calcium (minimum)	27.0
Calcium (minimum)	45.0
Phosphorus	5.0
Methionine	0.5
Lysine	0.8
Metabolizable Energy (Kcal/kg)	2900

The feed and water were prepared *ad libitum* daily. They were raised in a deep litter system with a floor space of $0.25m^2$ per chicken. The house was constructed in a way that there was ventilation. The artificial light and heat were not provided for chickens. All Koekoek chickens were given a stress pack dissolved in water on arrival. Those Koekoek chickens that presented signs of illness were isolated and treated accordingly.

Experimental design

Eggs were collected from 150 Koekoek hens aged 30-32 weeks in the morning and afternoon. In each rearing pen, there were 10 hens and 1 cock. The study was conducted for a period of 42 days from February to March 2020. A total of 400 eggs were collected and cleaned using damp cloths immersed in warm water to avoid alterations in the egg embryo. The eggs were checked for any cracks, abnormal shapes, spots, and transparent markings before being put in the trays for storage. Eggs weighing between 50 and 55 g were candled using a led torch to ensure that the fertile ones were used as experimental units. The eggs were stored in a house with opened windows during the day for ventilation purposes. The collected eggs were stored for 3, 7, and 11 days before being taken to the sure hatch incubator (SH680, automatic digital model, South Africa) for 21 days. The incubator was turned on for 24 hours before eggs were placed inside. The humidity was 70% and the temperature was 37.6ºC. A total number of 270 eggs were allotted to three different treatment groups through a completely randomized design and each treatment was replicated three times with 30 eggs per replicate.

Data collection

On day 22, unhatched eggs were removed from the incubator and were broken to check the stage at which chicken embryonic development was stopped based on the guidelines provided by Hamburger and Hamilton (1951). The chicken embryonic mortality was recorded as early embryonic (1-5 days), mid embryonic (6-10 days), and late embryonic (15-21days) mortality. The hatching percentage was calculated as follows:

$$\text{Hatching (\%)} = \frac{\text{Total number of eggs hached}}{\text{Total number of fertile eggs incubated}} \text{ x } 100$$

After hatching the chicken mortality was observed for seven days.

Statistical analysis

The data were analyzed using the Statistical Package for Social Sciences (SPSS, version 20). General Linear Model Univariate was used to establish the effect of pre-incubation egg storage duration on egg hatchability and

survival of hatched Koekoek chickens. The differences between treatments were tested by Duncan's new multiple range test. In all the analyses, the confidence level was at 95% while the threshold for significance level was $p < 0.05$.

RESULTS AND DISCUSSION

Egg hatchability

As can be seen in Table 2, the hatching percentages were 87.33, 78.67, and 71 for eggs that were stored for 3, 7, and 11 days, respectively. It could be concluded that eggs that were stored for three days before incubation had a higher hatching percentage ($p < 0.05$) followed by those that were stored for 7 days. The hatchability of eggs that were stored for at least 11 days before incubation was 16.33% and 8.66%, less than those stored for three and seven days, respectively.

Table 2. Hatching percentage of Koekoek eggs stored for 3, 7, and 11 days.

Treatment	Hatching percentage	S.E
3 days	87.33[a]	4.54
7 days	78.67[b]	2.15
11 days	71.00[c]	3.81

[abc] Means within a column without common superscript differ significantly ($p < 0.05$), S.E: Standard Error.

A similar trend of results was recorded by Ayeni et al. (2020) who indicated that the elongated storage of eggs reduced hatchability and increased the amount of incubation time required for hatching. Khan et al. (2014) also illustrated that storing eggs beyond 7 days resulted in a hatching percentage of 33.89. The studies that were performed on Japanese quail presented that the extended period of egg storage before incubation resulted in lower hatchability (Lacin et al., 2008; Mani et al., 2008; Seker et al., 2005). Furthermore, Romao (2008) reported that excessive egg storage duration can adversely affect hatchability. In support of the results obtained from the current study, Senbeta (2016) indicated that the eggs that were stored for the shortest period (5 days) had the largest hatchability rate. On the other hand, Petek and Dikmen (2006) explained that the hatchability of fertile eggs was not significantly affected by the length of the egg storage period. In addition, Günhan and Kirikçi (2017) explained that storage time did not affect the hatchability of fertile eggs.

Embryonic mortality

The findings of the current study indicated that eggs stored for more than seven days before incubation had higher embryonic mortality (Table 3). The mid and late embryonic deaths were significantly lower in eggs that were stored for three days ($p < 0.05$) and mid and late embryonic death was higher for those that were stored for 11 days before incubation ($p < 0.05$). Generally, egg storage for three days before incubation could reduce embryonic mortality by 68.35% and 128.88% than storage periods of 7 and 11 days prior to incubation, respectively.

Table 3. Embryonic mortality of Koejoek eggs stored for 3, 7, and 11 days.

Treatments	EEM	SE	MEM	SE	LEM	SE
3 days	4.07[a]	2.35	3.00[a]	1.05	5.60[a]	1.20
7 days	5.00[a]	3.00	5.60[b]	1.98	10.73[b]	0.83
11 days	7.33[a]	1.56	6.67[b]	0.95	15.00[c]	1.45

[abc] Means within a column without common superscript differ significantly ($p < 0.05$), SE: Standard Error, EEM: Early embryonic mortality, MEM: Mid embryonic mortality, LEM: Late embryonic mortality.

In support of the results obtained from the present study, Grochowska et al. (2019) indicated that early embryonic mortality increased with the lengthening of egg storage time. Similarly, Fasenko (2007) emphasized that long-term egg storage induces cell death, and hence, higher embryonic mortality. Fasenko (2007) also reported the negative effect of long egg storage on embryonic development and metabolism. In order to reduce the chicken embryonic mortality in eggs stored for more than seven days before incubation, Tag EL-Din et al. (2017) recommended that eggs should be warmed for 2.5 hours after every five days. In the same vein, Khan et al. (2014) reported more embryonic deaths in eggs that were stored for at least eight days. Schmidt (2009) also reported a linear relationship between storage time and embryonic mortality in chickens. In a study conducted by Nasri et al. (2019), it was discovered that the pre-incubation egg storage of more than seven days has a negative impact on the internal egg quality and embryonic survival during the storage and incubation period. The same results were shared by Petek and Dikmen (2006) who found that egg storage for three days can result in a satisfactory hatching percentage in quails. The embryos of eggs stored for nine days revealed lower hatchability and higher mortality during incubation (Khan et al., 2014). The longer storage period significantly increased late embryonic mortality (Gómez-de-Travecedo et al., 2014). Other researchers

reported that storing eggs longer than seven days before incubation is accountable for slowing and damaging embryonic development and livability (Elibol et al., 2002; Christensen et al., 2001). On the other hand, Sujata et al. (2019) reported that pre-incubation egg storage did not affect the embryonic throughout all the stages of embryonic development. Fasenko et al. (1992) also observed that egg storage duration did not affect embryonic mortality.

Chickens' mortality

As can be seen in Table 4, indicated that the survival rate of hatched chickens from eggs stored for three days was significantly higher, compared to hatched chickens from eggs stored for 7 and 11 days ($p > 0.05$). In support of results from the current study, Addo et al. (2018) highlighted that prolonged egg storage beyond 7 days is detrimental to chicken quality. In a study performed by Fasenko (2007) chickens with unhealed naves, deformities, and signs of weakness were hatched from eggs that were stored for more than 14 days. Nasri et al. (2019) also stipulated that prolonged egg storage duration resulted in lower chicken quality in both young and old breeders because storage duration has been shown to affect egg quality and have adverse effects on embryonic development and post-hatch performance. These findings were in agreement with Petek and Dikmen (2006) who reported that egg storage for more than seven days deteriorated post-hatch performance and chicken quality. On other hand, El-Sagheer (2012) reported that the number of chickens' deaths was not significantly affected by the pre-incubation storage period.

Table 4. The mortality rate of Koekoek chickens hatched from eggs stored for 3, 7, and 11 days

Treatments	Chick mortality (%)	Standard Error
3 days	7.33[a]	1.92
7 days	11.33[b]	2.56
11 days	11.67[b]	1.54

[ab] Means within a column without common superscript differ significantly ($p < 0.05$).

CONCLUSION

The pre-incubation storage of Koekoek eggs for a maximum period of three days improved the hatching percentage, embryonic survival as well as post-hatch chicken survival. Therefore, it is recommended that farmers should not store eggs for longer than three days to improve the reproductive performance of Koekoek chickens.

DECLARATIONS

Authors' contribution

Setsumi Motsoene Molapo conceptualized and wrote the manuscript. Motselisi Mahlehla contributed to data collection and statistical analysis. Paseka Pascalis Kompi collected data and designed the graphics. Monaheng Taoana collected data and drafted the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors have not declared any conflict of interest.

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The Effect of Substitution of Fish Meal by Maggot Meal (*Hermetia Illucens* L) on the Relative Length of Digestive Tract, Histomorphology of Small Intestines and the Percentage of Carcass Parts in Native Chickens

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ABSTRACT

The development of the digestive tract organs is closely related to the increased body weight growth in chickens. The present study aimed to determine the effect of using maggot meal as an antibacterial and protein source of fish meal substitution in diets on the relative length of the digestive tract organs, small intestine histomorphology, and the percentage of the native chicken carcass. A total of 140 one-day-old chickens were randomly assigned to one of the five treatments according to a completely randomized design with four replications for each treatment. The treatments included P0 (basal diet + 15% fish meal + 0% maggot meal), P1(basal diet + 11.25% fish meal + 3.75% maggot meal), P2 (basal diet + 7.5% fish meal + 7.5% maggot meal), P3(basal diet + 3.75% fish meal + 11.25% maggot meal), and P4 (basal diet + 0% fish meal + 15% maggot meal). The results showed that the use of maggot meal in P3 had a significant effect ($P < 0.05$) on the relative length, villi height, depth of duodenal crypt, jejunum and ileum, villi surface area, the density of jejunum and ileum villi, and percentage of thigh and wing weight. Besides, the treatment tended to increase the relative length of the caecum and colon, surface area of the duodenal villi, and chest weight percentage. However, the treatment did not affect the duodenal villi density and percentage of back weight in native chickens. The use of maggot meal up to 11.25% in diets can improve the relative length of intestinal, histomorphology of small intestine's villi, and the percentage of carcass parts of native chickens.

Keywords: Carcass parts, Digestive tract, Histomorphology, Maggot meal, Native chicken

INTRODUCTION

The poultry business development has now become an industry with complete components from upstream to downstream, which supports the growth of agribusiness, especially in the livestock sector. In the past, the business was only a side business, and was defined narrowly as a business that produced meat and eggs to fulfill the food needs of families. However, this business is currently developing in a broad sense, namely as the spearhead of improving nutrition, determining the nations' intelligence and health, the strength of national food security, job creation, economic growth, and increasing per capita income. In Indonesia, especially in rural areas, native chickens, has an important role in providing meat and eggs to improve public health. The community raises free-range chickens in their yards using the umbaran system, especially for family savings, which can be sold quickly when they are in need of money.

Meanwhile, in addition to hatching eggs, some are consumed as nutritional sources. Along with the increasing demand for native chickens, meat, and eggs, many local chickens are cultivated semi-intensively and intensively. Domestic chickens are not only taken for meat, but many are also egg-oriented. The obstacle faced in the development of native chickens is the low productivity due to their slow growth. However, domestic chickens have a quite strong disease resistance. One factor influencing chickens' rapid growth is feed efficiency (Willems et al. 2013; Rahmanto 2012). This is due to the digestive process of feed, correlated with the digestive histological conditions. Small intestine is one of the digestive organs that functions in the process of absorption of nutrients. The small intestine is the main organ that plays an essential role in transferring nutrients, and a place for digestion and absorption of digestive products (Suprijatna et al., 2008). Additives function to stimulate growth which can be used in obtaining a good carcass. The additives given are usually based on

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synthetic/chemical antibiotics. However, it is believed that this diet ingredient is not proper for humans to consume. This is due to a residual effect on chicken carcasses, and if the antibiotics are given continuously, it can result in antibiotic resistance for livestock. Consumer desires vary in choosing pieces of the free-range chicken carcass (breast, back, wings, and thighs). These pieces can increase the selling value because, in addition to animal protein sources, the price of the individual pieces are also affordable. One source of natural animal protein that can be used as a feed ingredient is maggot meal, which comes from Black Soldier Fly (BSF) larvae, produced in the second phase of metamorphosis after the egg phase and before the pupal stage, which can play an important role in improving the performance of the digestive tract. According to Jayanegara (2017) maggot meal (*Hermetia illucens*) has a crude protein content of 44.9%, crude fat of 29.1%, crude fiber of 16.4%, and 8.1% ash. The nutritional content of maggots, especially crude protein, is quite high, and this shows that maggot (*Hermetia illucens*) has a considerable potential to be replaced instead of fish meal. Furthermore, the maggot is known to have antibacterial activity in the form of Antimicrobial Peptide (AMP) (Park et al., 2014). As also reported by the research results (Kim and Rhee, 2016), maggot contains high lauric acid, and functions as a natural antibacterial agent. This antibacterial activity dramatically affects the health and development of the digestive tract organs in absorbing nutrients.

With this in mind, the current study aimed to evaluate the effect of using maggot meal (*Hermetia illucens*) as a source of antibacterial substitution and fish meal protein in diets on the relative length of the digestive tract organs, small intestine's histomorphology, and the percentage of carcass parts in native chickens

MATERIAL AND METHODS

Ethical approval

This research was conducted at the Poultry Production Laboratory, Faculty of Animal Science Hasanuddin University Makassar, Indonesia in 2020. This study was approved by the Experimental Animal Ethics Committee of the Faculty of Animal Science, Hasanuddin University Makassar, Indonesia.

Experimental design, native chickens, and feeding

A total of 140 one-day-old native chickens with an average body weight of 28.74 ± 1.58 g were placed in a colony-shaped cage, and each drum contained seven chickens. The trial design used was a completely randomized design with five treatments and four replications for each one, consisting of seven chickens. The treatment diet was formulated as follows; P0: basal diet + 100% fish meal (15% in diet) + 0% maggot meal (0% in diet), P1: basal diet + 75% fish meal (11.25% in diet) + 25% maggot meal (3.75% in diets), P2: basal diet + 50% fish meal (7.5% in diets) + 50% maggot meal (7.5% in diets), P3: diets basal + 25% fish meal (3.75% in diets) + 75% maggot meal (11.25% in diets), P4: basal diet + 0% fish meal (0% in diets) + 100% maggot meal (15 % in the diet). The maggot used in this study was maggot that has been dried and grounded into a meal. Maggot that has become meal was mixed with other feed ingredients (Mawaddah and Hermana, 2018) The feed given in the current study was a starter phase in the form of commercial feed for two weeks from the Japfa Confeed Indonesia factory as an adaptation period (Harlystiarini, 2017), then continued with basal feed for the grower and finisher phases. The diets were given from one day to twelve weeks of age every morning and evening according to the requirement of each maintenance phase mentioned by Mawaddah and Hermana (2018), and drinking water was also given *ad-libitum*. The composition and chemical composition of the diet can be seen in Table 1.

Preparation and measurement of digestive tract samples and carcass parts

Intestinal sample preparation was carried out after 90 days of maintenance. Before being slaughtered, chickens fasted for 12 hours, while they were given drinking water. The sample was selected after weighing. The chicken slaughter began with cutting the jugular vein and carotid artery located between the head bone and the first neck segment. Subsequently, the chicken was hung with its legs above, the head was bowed to accelerate the blood-thinning process after the livestock was confirmed to die, then immediately was immersed in the warm water with a temperature of 50-55 ºC for 90-120 seconds to facilitate the feather removal (Soeparno, 2009). Following that, the offal was removed, the collected samples were part of the digestive tract, and proceed with the separation of the thighs, wings, chest, and back for weighing. Data on the small intestine's length were obtained by separating the digestive tract, namely the small intestine (duodenum, jejunum, and ileum), caecum, and colon, then measured the length of the duodenum, jejunum and ileum, cecum, and colon using a tape measure (Satimah et al., 2019). The small intestine, caecum, and colon organs measured were uncleaned organs. The obtained data were calculated using the following formula of the measurement of the length of the intestinal organs (Incharoen, 2013).

$$\text{Relative length of organ} : \frac{\text{length of organ}}{\text{Live weight}} \text{ x } 100\%$$

Furthermore, each piece of the carcass was weighed to determine its weight, and compared to carcass weight multiplied by 100% (Soeparno, 2009).

$$\text{Percentage of carcass parts} : \frac{\text{Weight of carcass parts}}{\text{Weight of carcass}} \text{ x } 100\%$$

Preparation and measurement of small intestine's histomorphology

The histological preparations were performed by making Hematoxylin Eosin (HE) preparations and collaborated with the Maros Veterinary Center. According to Gulo (2013), the histological testing procedure was as follows: For histological tests, small intestine samples were prepared. The samples used were parts of the small intestine consisting of the duodenum, jejunum, and ileum. The duodenum stretched into a parallel loop, starting from the base of the gizzard to the bile ducts' meeting. The jejunum was defined as the small intestine segment between the duodenum and Meckel's diverticulum. Ileum is a segment of the small intestine that ranges from Meckel's diverticulum to the beginning of the caeca branch. The small intestine samples were cut into 2 cm long pieces for each segment of the small intestine, namely duodenum, jejunum, and ileum, then fixed in 10% formalin, allowed to soak for 24-48 hours, and then histological preparations were made. To perform the hematoxylin-eosin preparations, a piece of the small intestine segment with a thickness of about 2 mm was inserted into a tissue basket, then dehydrated. Dehydration action was done by soaking these preparations in succession for 2 hours each in an alcohol solution of 70%, 80%, 90%, 95%, and after that, absorbed in absolute alcohol for 2 hours.

Table 1. Ingredients and chemical analysis of experimental diets.

Feedstuff composition (%)	Treatment				
	P0	**P1**	**P2**	**P3**	**P4**
Maize	64	64	64	64	64
Fine rice bran	12.5	12.5	12.5	12.5	12.5
Soybean meal	1	1	1	1	1
Fish meal	15	11.25	7.5	3.75	0
Maggot meal	0	3.75	7.5	11.25	15
Coconut cake meal	2.5	2.5	2.5	2.5	2.5
Vegetable oil	2	2	2	2	2
CaCO3	2	2	2	2	2
Premix	1	1	1	1	1
Total	100	100	100	100	100
Nutrien composition*					
Protein (%)	18.46	18.44	18.42	18.39	18.37
Fat (%)	3.31	3.79	4.27	4.75	5.24
Crude fiber (%)	7.11	7.43	7.76	8.09	8.42
Ca (%)	0.99	0.97	0.96	0.94	0.93
P (%)	0.91	0.81	0.71	0.6	0.5
Ash (%)	8.48	7.96	7.44	6.92	6.4
BETN (%)	61.56	59.33	59.06	58.79	58.53
ME** (kkal/kg)	3063.16	3090.54	3117.92	3145.30	3172.68

*) Calculated based on the results of the analysis and calculation of proximate analysis table data at the Laboratory of Animal Feed Chemistry and Nutrition, Faculty of Animal Science, Hasanuddin University, Indonesia. P0: Basal ration + 100% fish meal (15% in diet) + 0% Maggot meal (0% diet), P1: Basal diet + 75% fish meal (11.25% in diet) + 25% Maggot meal (3.75% in diet), P2: Basal diet + 50% fish meal (7.5% in diet) + 50% Maggot meal (7.5% in diet), P3: Basal diet + 25 % fish meal (3.75% in diets) + 75% Maggot meal (11.25% in diet), P4: Basal diet + 0% fish meal (0% in diet) + 100% Maggot meal (15% in diet). **) Metabolic Energy (EM) Value is calculated based on the Balton formula; EM: 40.81 × (0.87 (PK + (2.25 x LK) + BETN) + 2.5

Furthermore, according to Zainuddin et al. (2016), the purification process was carried out with xylol for an hour. The tissue was then put into paraffin (in the filtering process), which was in an oven at 60°C for one hour. The network was then inserted into a printer whose arrangement was made so that it was filled with liquid paraffin using the paraffin embedding console. Afterward, the preparation was left until the paraffin was hardened enough to cut. Tissue cutting was performed with microtomes at a thickness of 3-4 µm. The cut results were attached to the object-glass, then

were dried and ready to be colored. The staining stage began with deparaffinization and rehydration. The process was carried out by inserting the preparations into a solution of xylol, absolute alcohol, 95% alcohol, 90%, 80%, 70% alcohol, and water with the time required to insert the preparations into each solution for 2 minutes. After that, the preparations were stained with hematoxylin dye for 10 minutes, then rinsed with running tap water. After that, it was put into eosin for about 20 minutes, then aquadest and dehydrate were added again. In the final stage, the preparation was dripped with adhesive, then covered with a cover glass, and was ready to be viewed using a light microscope with a magnification of 10x. Furthermore, the villi's height, apical width of the villi, width of the basal villi, crypt depth, and villi density were measured. Villi surface area was calculated according to Iji et al. (2001).

Villi surface area: $\frac{(a+b)}{2}$ x c

a: the width of the basal villi; b: the apical width of the villi; c: villi height

Statistical analysis

Data were analyzed using analysis of variances (ANOVA) according to a completely randomized design. The significant effects of treatments were further determined using Duncan's Multiple Range Test at a 5% level of significance (Steel and Torrie, 1980).

RESULT AND DISCUSSION

The relative length of the digestive tract

Table 2 shows the length of the duodenum of native chickens fed with the substitution of fish meal and maggot meal. The P3 had a longer average value of 3.44% of the live weight than the treatment without using maggot meal (P0). The variance analysis showed that the use of maggot meal as a substitute for the fish meal had a significant effect on the duodenal length ($P<0.05$). A significant increase indicated that the maggot meal can respond to the small intestine's growth, and improve digestive function according to a study conducted by Suprijatna et al. (2008) that the first digestive process occurred in the duodenum. Bile from the liver and enzymes from the pancreas were sent to the duodenum plus the enzymes produced by the intestines digest food to impact the increasing growth. The duodenum's length was closely related to the villi's length and the duodenum's relative weight. The longer the intestinal villi, the optimal surface area for nutrient absorption, and nutrient absorption cause the duodenum to become heavier and more extended. This is in line with the statement of Lenhardt (2001) that the small intestine villi's height was closely related to the potential of the small intestine to absorb nutrients, so that the higher the small intestinal villi, the greater the effectiveness of nutrient absorption through the small intestinal epithelium.

Table 2. The relative length of the digestive tract with substitution of fish meal and maggot meal in native chickens aged 12 weeks.

Variables (%)	Treatment					P-Value
	P0	P1	P2	P3	P4	
Duodenum	2.34 ± 0.14^{a}	2.69 ± 0.25^{a}	2.80 ± 0.37^{a}	3.44 ± 0.23^{b}	2.58 ± 0.36^{a}	0.00
Jejunum	4.26 ± 0.35^{a}	4.71 ± 0.64^{a}	4.93 ± 0.63^{ab}	5.68 ± 0.29^{b}	4.61 ± 0.65^{a}	0.02
Ileum	4.07 ± 0.22^{a}	4.41 ± 0.42^{a}	4.71 ± 0.46^{ab}	5.39 ± 0.48^{b}	4.42 ± 0.77^{a}	0.02
Caecum	2.37 ± 0.21	3.13 ± 0.43	2.89 ± 0.22	3.21 ± 0.37	2.57 ± 0.42	0.09
Colon	0.58 ± 0.06	0.62 ± 0.06	0.68 ± 0.06	0.77 ± 0.16	0.70 ± 0.03	0.09

Different superscripts on the same line show significant differences ($P < 0.05$). P0: Basal diet + 100% fish meal (15% in diet) + 0% maggot meal (0% diet), P1: Basal diet + 75% fish meal (11.25% in diet) + 25% maggot meal (3.75% in diet), P2: Basal diet + 50% fish meal (7.5% in diet) + 50% maggot meal (7.5% in diet), P3: Basal diet + 25 % fish meal (3.75% in diets) + 75% maggot meal (11.25% in diet), P4: Basal diet + 0% fish meal (0% in diet) + 100% maggot meal (15% in diet)

The use of 11.25% maggot meal (P3) significantly increased the length of the jejunum compared to the control diets (P0) and other treatments ($P<0.05$, Table 2). The increase in the jejunum length corresponded to the increased absorption of nutrients into the intestines' blood. In a study conducted by Bardocz et al. (1995), the increase in the relative length of the jejunum and the intestine's tensile strength can be caused by high levels of complex carbohydrates, including resistant starch oligosaccharides, non-starch polysaccharides, and the percentage of lectins in the diet. The increase in the length of the digestive tract showed the expansion of nutrient absorption and the effectiveness of absorption of food juices. The longer the jejunal organ, the greater the jejunal villi's effect, so that the villi surface area for nutrient absorption was more significant (Nyoman et al., 2018).

The results of the Duncan region test showed that the use of maggot meal in P3 (3.75% fish meal + 11.25% maggot meal) significantly increased ileal length compared to P0, P1, P2, and P4 treatments (P<0.05, Table 2). The average relative length of the ileum ranged 4.07-5.39%. The intake of crude fiber influenced the increase in ileal length. Treatment P3 consumed more crude fiber, followed by P2, P4, P1, and P0. This was supported by Lundin et al. (1993) reporting that fibers can increase the volume density of epithelium and villus in the jejunum, ileum, and duodenum. High crude fiber can stimulate the small intestine's growth, which causes an increase in the small intestine's performance to digest the crude fiber (Savory and Gentle 1976). Providing diets with maggot meals can increase the length of the digestive tract organs, suspected of having an AMP, which functions to inhibit pathogenic microbes from improving intestinal balance benefiting beneficial microorganisms (Harlystiarini, 2017). In addition to AMP, it also contains lauric fatty acid in medium-chain fatty acid, affecting microbes in the small intestine (Spranghers et al., 2017).

The caecum is two bags located on the border between the small intestine and the large intestine. Variance analysis showed that the use of maggot meal does not affect the caecum length. However, P3 treatment (3.75% fish meal + 11.25% maggot meal) of 3.21% of live weight tended (P: 0.09) to increase the caecum length, compared to other treatments. This is probably due to the use of maggot meal affecting microbial activity on the caecum. This finding was in line with the obtained results of a study conducted by Krismiyanto et al. (2013) revealing that the decreasing population of *Escherichia coli* (*E. coli*) can improve the digestion of crude fiber fermented by cellulolytic bacteria caecum, thereby promoting healthy digestion.

The mean relative length of the large intestine or colon was 0.58-0.77% of the live weight. Based on the analysis of variance, it was indicated that the use of maggot meal can not increase the relative length of the large intestine in the absorption of nutrients but tended (P: 0.09) to increase the length of the colon compared to the control diet (P0, Table 2). Low colon activity resulted in a low relative length of the large intestine. This was in line with the study performed by Harlystiarini (2017) revealing that the absorption of crude fiber in the large intestine takes place in small amounts, so it does not affect the increase of colon length of native chickens. There was a close relationship between the increase in colonic length and colonic function in birds. The colon's role is to distribute food waste from the small intestine to the cloaca and where water and some minerals are absorbed. It also functions as a place for fermentation of crude fiber, especially hemicellulose, apart from the caecum in poultry (Gunawan, 2011).

Histomorphology of small intestinal villi

The measurement of the surface area of the small intestine's villi in native chickens is presented in Table 3. The variance results showed that the use of maggot meal with fish meal substitution did not affect the duodenal villi's surface area at the age of 12 weeks. However, P3 treatment tended to increase it, compared to other treatments. In contrast, the P3 treatment had a significant effect (P < 0.05) on the villi of the jejunum and ileum surface area. The largest duodenal villi surface area was found in treatment P3 (50049.48 μm^2) and the smallest in P0 (24128.85 μm^2). The largest surface area of the villi of jejunum and ileum was at P3 (48066.49 µm2 and 43787.35 μm^2), and the smallest in the control (P0) which was 20916.01 μm^2 and 17956.18 μm^2. In the current study, the surface area of villi in all parts of the small intestine (duodenum, jejunum, and ileum) tended to increase with increasing levels of use of maggot meal (P1, P2, P3, and P4). The duodenal villi surface area's size was relatively larger than the surface area of the villi in the jejunum and ileum. Villi growth is closely related to the development of microbes in the intestine. The use of maggot meal as a substitute for fish meal is thought to have an active compound in the form of an AMP which inhibits the activity of pathogenic microorganisms and is considered to have a relatively better protective effect on maintaining the morphometry of the chickens' villi of the small intestine. This was in line with Harlystiarini (2017) stating that the AMP found in maggots plays an active role in inhibiting pathogenic microbes from improving the gut's balance benefiting beneficial microorganisms.

Villi are small finger-like protrusions found in the small intestine and play an important role in the absorption of nutrients from food in the intestine (Frandson, 1981). Hamdi et al. (2013) stated that the duodenum's intestinal villi are more numerous and longer than the villi in the ileum. The results of the analysis of variance showed a significant effect (P<0.05) on villi's height in the duodenum of chickens aged 12 weeks (Table 3). The mean villi's height in the duodenum ranged from 413.69 to 613.36 µm. Table 3 shows that the duodenal villi's height at P3 was 613.36 ± 40.72 µm higher than P0, P1, P2, and P4. This indicates that using an 11.25% maggot meal can trigger the proliferation of intestinal cells during the embryonic period in native chickens to impact cell development to the next phase positively. The difference in the results presented in the table was caused by several factors, including differences in feed consumption and differences in levels of use of maggot meal. However, the villi's height in the small

intestine represented an area for the complete absorption of nutrients. The transportation of nutrients throughout the body is better and smoother. This was in line with the opinion of Miles et al. (2006) revealing that the increase in villi's height and villi's width is indicated by a wider area of the villi's surface to absorb nutrients into the bloodstream. It was also reported by Ensminger (1992) who claimed that the absorption of nutrients in the small intestine is influenced by the surface area of the small intestine (folds, villi, and microvilli). There was a close relationship between an increase in the number of epithelial cells on the small intestine villi's surface and increased villi's height (Fan et al., 1997).

Table 3. Villi surface area, villi height, villi density and depth of duodenal crypt, jejunum, and ileum treated with fish meal substituted for maggot meal in native chickens aged 12 weeks.

Variables	Treatment					p-value
	P0	P1	P2	P3	P4	
Villi surface area (µm^2)						
Duodenum	24128.85±8883.13	35822.88±9382.55	36198.56±8572.41	50049.48±21589.54	26419.29±7360.65	0.06
Jejunum	20916.01±4512.87^{a}	30521.59±9278.96^{ab}	33380.80±17213.92^{ab}	48066.49±15827.33^{b}	22666.59±3251.36^{a}	0.03
Ileum	17956.18±4516.05^{a}	25979.01±7847.10^{a}	33175.99±13434.29^{ab}	43787.35±10487.10^{b}	30131.29±8518.81^{ab}	0.02
Villi height (µm)						
Duodenum	413.69±114.28^{a}	429.03±53.56^{a}	464.03±101.61^{a}	613.36±40.72^{b}	507.49±29.14^{b}	0.01
Jejunum	253.72±63.07^{a}	400.26±38.73^{b}	432.65±96.79^{ab}	457.89±31.51^{b}	377.01±62.60^{b}	0.00
Ileum	252.67±16.26^{a}	344.39±43.75^{b}	406.41±18.98^{c}	460.59±17.83^{d}	307.23±18.41^{b}	0.00
Depth of crypt (µm)						
Duodenum	209.58±72.49^{a}	244.80±34.85^{a}	253.16±39.51^{a}	361.48±87.27^{b}	276.01±36.06^{b}	0.02
Jejunum	210.08±36.48^{a}	230.03±63.36^{a}	253.89±26.81^{ab}	307.76±52.68^{b}	280.25±21.91^{ab}	0.04
Ileum	168.58±46.93^{a}	191.54±45.11^{a}	210.25±61.62^{ab}	279.61±44.27^{b}	244.48±30.68^{ab}	0.03
Villi density (µm)						
Duodenum	5500±1290.99	6250±957.42	6750±4787.13	8000±2449.48	7500±1732.05	0.68
Jejunum	6750±957.42^{a}	7500±577.35^{ab}	8250±957.42^{bc}	9000±816.49^{c}	9500±1290.991^{c}	0.00
Ileum	7500±577.35^{a}	8250±500.00^{a}	9000±1414.21^{abc}	10250±2061.55^{c}	9500±577.35^{bc}	0.04

Different superscripts on the same line show significant differences (P < 0.05). P0: Basal diet + 100% fish meal (15% in diet) + 0% Maggot meal (0% diet), P1: Basal diet + 75% fish meal (11.25% in diet) + 25% maggot meal (3.75% in diet), P2: Basal diet + 50% fish meal (7.5% in diet) + 50% maggot meal (7.5% in diet), P3: Basal diet + 25 % fish meal (3.75% in diets) + 75% maggot meal (11.25% in diet), P4: Basal diet + 0% fish meal (0% in diet) + 100% maggot meal (15% in diet)

The function of the small intestine villi will be enhanced by the increase in the number of high-size jejunum and ileum villi, which have an important role in the process of absorption of nutrients (Yamauchi and Isshiki, 1991). The variance analysis showed that the use of maggot meal with fish meal substitution had a significant effect (P<0.01) on the height of the jejunum and ileum's villi. The P3 treatment with the use of maggot meal showed better small intestinal villi's height than the control group. The use of maggot meal (*Hermetia illucens* L) feed is known to have the potential to increase the surface area of the small intestine villi of the duodenum, jejunum, and ileum. It is suspected that the presence of AMP as an active compound in maggot meal that suppresses pathogenic microorganisms such as *E. coli* and creates ideal conditions for intestinal microecology and benefits beneficial microorganisms, such as *Lactobacillus*, thereby increasing the performance of the digestive organs. This was reported by Auza et al. (2020) stating that extracts of BSF larvae or maggot have antibacterial activity against *Salmonella typhimurium*, *E. coli Pseudomonas aeruginosa* bacteria. The active substance in maggot meal is AMP, which plays an active role in inhibiting pathogenic microbes from improving intestinal balance and benefiting beneficial microorganisms (Harlystiarini, 2017). In addition to AMP, it is suspected that lauric acid acts as a natural antimicrobial (Barros-Cordeiro et al., 2014). This condition has an impact on the improvement of mucosal immunity. The decreased number of Gram-negative (pathogenic) bacterial colonies dramatically affects the small intestinal villi's height. Likewise, if the pathogenic bacteria increases, it can inhibit the growth of small intestinal villi's height. Fuller (1992) in his research revealed that the balance of the intestinal microflora would occur in case the beneficial microbes

could suppress the harmful microbes by pushing out the pathogenic microbes. The higher the number of non-pathogenic bacterial colonies, the better the performance in suppressing pathogenic bacteria. One way to obtain a high proportion of non-pathogenic bacteria or lactic acid bacteria is to use several protein peptides, such as antimicrobial peptides and support beneficial bacteria, such as lactic acid bacteria (LAB); these bacteria can dominate the population. Animal health is influenced by the number of microbes in the microflora (Philip, 1993).

The use of maggot meal showed a significant effect on the depth of crypt in the duodenum, jejunum, and ileum of native chickens at 12 weeks of age (P<0.05, Table 3). The depth of crypt in treatment P3 and P4 was better than P2, P1, and P0. The highest value for the depth of crypt in treatment P3 was 361.48 µm, and the lowest was at P0 of 209.58 µm. This was because the level of using maggot meal to substitute fish meal impacts the growth of cells in the small intestine of the duodenum, jejunum, and ileum. There are also suspected antimicrobial peptides and lauric acid in maggot meal, which play an active role in inhibiting pathogenic bacteria such as *E. coli*. This was in agreement with the research by Harlystiarini (2017) who states that BSF larvae extract inhibitory activity against *E. coli* and *Salmonella sp*.

The AMP in the treated feed would positively affect the balance and microecological conditions of the gut that inhibit pathogenic microorganisms, such as Coliform, and benefit non-pathogenic microorganisms, including Lactobacillus (Wang et al., 2006; Tang et al., 2009). This condition triggered the increase in the value of the crypt's depth, and the villi's height can affect improving digestive ability. The higher the size of the villi and the depth of the crypt, the wider the area of absorption of nutrients by the small intestinal wall to stimulate the growth (Rahmawati, 2016). Based on the morphology and function, nutritional requirements in the early growth period depend on the digestive tract (Zhou et al., 2013). The histological growth of the small intestine can assist the chickens in digesting the feed earlier. At the beginning of hatching, the chickens still have the remaining yolk sac, which is absorbed into the intestines as nutrients before getting reliable feed. The fast growth of the digestive tract organs can adjust and adapt quickly to the previously given feed.

Percentage of carcass parts

The results of measurements of small intestinal villi density in native chickens are presented in Table 3. The results of the current study showed no difference in villous density in the duodenum, but it had a significant effect on the jejunum and ileum (P < 0.05). The greatest duodenal villi density at 12 weeks was P3 (8000 µm), and the smallest was P0 (5500 µm). The greatest villous density in the jejunum was at P4 (9500 µm), and the smallest at P0 (6750 µm). Meanwhile, the largest villi density was in the ileum, namely at P3 (10250 µm), and the smallest at P0 (7500 µm). Table 3 shows the density of villi (number of villi / 1 µm) is the smallest in the duodenum and the largest in the ileum. The effectiveness of using maggot meal (P3 and P4) on villous density was seen in the jejunum and ileum. This indicated that the possibility of AMP and high lauric acid in maggot meal inhibit pathogenic microbes and benefit beneficial microorganisms, such as LAB, so that LAB colonized the jejunum and ileum which can affect villi development. As stated by Harlystiarini (2017) and Barros-Cordeiro et al. (2014), the active substances contained in maggot meal are AMP and lauric acid, which play an active role in inhibiting pathogenic microbes, so that they can improve balance and benefit from the intestine. According to Ferrer et al. (2019), the density and size of villi in the small intestine can explain the rate of change in absorption in the villi surface area. This indicated a correlation with the absorption capacity of poultry (Ebadias, 2011). The small intestine of livestock with heavier body weight is characterized by a more extended absorption area of the small intestine and a wider absorption area than the small intestine of birds with lighter bodyweight (Yamauchi and Isshiki,1991).

Table 4. Average percentage of carcass parts weight of village chickens aged 12 weeks.

Variables	Treatment					P-Value
	P0	P1	P2	P3	P4	
Chest (%)	16.20±2.83	19.26±4.75	19.38±2.90	23.54±1.82	21.82±4.17	0.07
Back (%)	16.47±3.07	17.28±1.91	18.93±7.59	23.72±3.45	23.62±4.58	0.10
Thighs (%)	22.70±3.65[a]	25.51±2.84[a]	26.61±2.56[a]	36.49±3.63[b]	34.95±5.93[b]	0.001
Wings (%)	11.00±1.13[a]	10.97±1.18[a]	13.72±1.30[b]	15.58±0.57c	11.94±0.91[a]	0.001

Different superscripts on the same line show significant differences (P < 0.05). P0: Basal diet + 100% fish meal (15% in diet) + 0% Maggot meal (0% diet), P1: Basal diet + 75% fish meal (11.25% in diet) + 25% maggot meal (3.75% in diet), P2: Basal diet + 50% fish meal (7.5% in diet) + 50% maggot meal (7.5% in diet), P3: Basal diet + 25 % fish meal (3.75% in diets) + 75% maggot meal (11.25% in diet), P4: Basal diet + 0% fish meal (0% in diet) + 100% maggot meal (15% in diet)

The results of the present study revealed that the use of maggot meal did not affect the percentage of chest weight. However, it tended to (P = 0.07) increase the percentage of breast weight in treatment P3. Based on Table 4, it can be seen that the P3 treatment (3.75% fish meal + 11.25% maggot meal) had the highest average percentage value of breast weight, namely 23.54%, while the lowest average percentage of breasts was in the control treatment (15% fish meal + 0% maggot meal) which was 16.20%. This shows that using 11.25% maggot meal in the diet can increase the percentage of breasts in native chickens. The increase in the percentage of breasts at the level of 11.25% using maggot meal was most likely due to AMP, which can improve the digestive tract, thereby contributing to the growth of body weight and carcass. This is reinforced by Harlystiarini (2017) stating that AMP in feed can improve intestinal balance and create intestinal microecological conditions that suppress pathogenic bacteria. Besides, maggot meal is known to have a high enough fat content. The high and low percentage of the carcass is believed to be related to the high and low-fat content in the diet leading to an increase in the digestibility of feed in the intestine which is correlated with an increase in the carcass, including the percentage of native chickens' breast. In addition, maggot flour is known to have a high enough fat content. The high and low percentage of the carcass was related to the high and low-fat content in the diet, which allows an increase in the carcass, including the percentage of native chickens' breast. Whereas in P4 treatment, there was a decrease in the percentage of breasts with the use of 15% maggot meal in the diet, presumably due to the presence of crude fiber and chitin compounds which were high enough to inhibit the chickens' digestion leading to slow growth, and consequently decreases the percentage of carcass weight. As reported by Purnamawati (2015), the high content of chitin will make the diet to be amba (voluminous), thus reducing the consumption of chicken feed. This shows that maggot meal can work optimally up to a usability level of 11.25% in spurring the growth of carcass weight, including the percentage of native chickens' breasts.

The results of Duncan's multiple area test showed that the use of maggot meal substituted instead of fish meal did not significantly affect the percentage of back weight, but tended to increase the percentage of back weight. Table 4 shows that the highest percentage value of back weight was found in the P3 treatment (23.72%) and the lowest was in the control at 16.47%. This was probably because the mineral content in each diet treatment was not much different so that some components were composed of more bones, such as back and wings. The back is dominated by bones so that not too much muscle is formed. The results of the present study were not much different from those reported by Marfuah (2016) revealing that the percentage of backs of 6 weeks old broiler chickens ranged 21.36-22.31% of the carcass weight. It was also reported in the study by Merkley et al. (1980) that feed is one factor affecting the percentage of carcass pieces in poultry consisting of the breast, back, thighs, and wings.

The thigh's carcass is the part that grows earlier than the other parts (Swatland, 1984). The results of the current study showed that the use of maggot meal had a significant effect on the percentage of thigh weight (P<0.05). The average percentage of thigh weight was 22.70-36.49%. Table 4 shows that the P3 treatment has a high carcass percentage value than the control and other treatments. The increase in the percentage of thigh weight in P3 (3.85% fish meal + 11.25% maggot meal) is thought that maggot meal with a high enough protein and fat content can increase the availability and digestibility of feed nutrients in the digestive tract of chickens so that it will result in an increase in carcass including the chickens' thighs. As stated by, oil or lipids have an important role in the growth of chickens (Fadilah et al., 2007). In this case, it functioned as an energy source, helping the absorption of fat-soluble vitamins, reducing dustiness in feed, and may also help palatability of food, as well as serving as a source of heat. Meanwhile, protein plays an essential role in the growth of muscle meat, so that a ratio with a high protein content will result in a high percentage of thigh weight. According to Fahmi (2015), the protein content from maggots or BSF larvae was 44.26%.

The results of the analysis of variance showed that the use of maggot meal had a significant effect (P < 0.05) on the percentage of wing weight. The current study indicated that the mean wing weight percentage varied from 11.00 to 15.58% (Table 4). The highest percentage value of back weight was found in the P3 treatment, which was 15.68%, and the lowest was in the control at 11%. The results were lower than those reported by Suartiningsih et al. (2017), which amounted to 16.8-17.86% in native chickens. The increase in the percentage of wing weight was probably due to the high mineral content of calcium and phosphorus in maggot meal, and not much different from fish meal. The results of this study were in line with Pesik et al. (2016) claiming that the maggot meal (*Hermetia illucens*) used in this study is

thought to have calcium (Ca), which has a better biological value than calcium (Ca) in fish meal. According to Nita et al. (2015), food substances in the form of protein and energy, and minerals are used to form bones, meat, and feathers based on the size and structure of wing feathers.

CONCLUSION

The use of maggot meal on P3 (3.75% fish meal + 11.25% maggot meal) had a significant effect (P<0.05) on relative length, villi height, depth of duodenal crypt, jejunum and ileum, villi surface area, the density of jejunum and ileum villi and percentage of thigh and wing weight. Besides, the treatment tended to increase the relative length of the caecum and colon, surface area of the duodenal villi, and chest weight percentage. However, the treatment did not affect the duodenal villi density and percentage of back weight in native chickens. The use of maggot meal up to 11.25% in diets can improve the relative length of the digestive tract organs, a histomorphological feature of the small intestine villi, and the percentage of carcass parts in native chickens

DECLARATIONS

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Competing interests

The authors did not have any conflict of interest.

Author's contributions

Fuji Astuty Auza designed, conducted the experiment, collected and analyzed samples in the laboratory, obtained data, drafted the paper, read and approved the final draft of the manuscript. Sri Purwanti and Jasmal A. Syamsu designed and directed the experiment, including laboratory analysis, data analysis, writing, read and approved the final draft of the manuscript. Asmuddin Natsir was responsible for the whole experiment, including designing, directing, monitoring, data analysis, writing, read and approved the final draft of the manuscript.

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The Effects of Mixed Vitamins, Minerals, Fatty Acids and Amino Acids Supplementation into Drinking Water on Broiler Chickens' Performance and Carcass Traits

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ABSTRACT

The present study was conducted to evaluate the effects of different levels of the feed supplement containing minerals, fatty acids, vitamins, and amino acids added to drinking water on broiler chickens' performance and carcass traits. A total of 100 one-day-old Cobb 707 (mean weight 46.7 g) were randomly assigned into four treatments, including control group (C), C + 2.25 ml/L Viterna Plus (V1), C + 2.50 ml/L Viterna Plus (V2), and C + 2.75 ml/L Viterna Plus (V3). Each treatment group contained 5 replicates of 5 birds in each (25 birds per treatment). Birds were maintained for 28 days. The results suggested that feed supplement at 2.50 ml/L could successfully improve final body weight, performance index, and carcass weight ($P < 0.05$). Concurrently, the treatment also reduced the percentage of abdominal fat ($P < 0.05$). In the current study, there were no significant differences in terms of feed intake and feed conversion ratio among treatment groups ($P > 0.05$). In conclusion, the incorporation of commercial feed supplement containing mixed of minerals, vitamins, and amino acids at 2.50 ml/L into drinking water improved the overall performance of the broiler chickens.

Keywords: Broiler chicken, Carcass, Feed supplement, Tropics, Viterna plus.

INTRODUCTION

Optimal nutrient formula and high-quality raw materials are two key factors to support the fast-growing rates of broiler chickens. In the tropics, high ambient temperature and humidity are the major constraints for maximum broilers' productivity (Yousaf et al., 2019), which directly impact their health, welfare, and performance (Zhou et al., 2020). Under heat stress conditions, broilers use more energy to dissipate body temperature as a result of high metabolic rates, thus leading to energy deficiency or energy imbalance for maintenance and production function (Bin et al., 2017). A large number of studies have indicated that high environmental temperature impaired nutrient absorption and utilization, broiler growth, feed efficiency, and considerable economic losses (Zhou et al., 2019). In addition, it is also known to induce several metabolic disorders in modern broilers' genetics (Olubodun et al., 2015; Yousaf et al., 2019).

In such conditions, nutrient modification by providing instant nutrients and additives are essential to support the normal biological function and metabolic activities of broiler chickens. Among additives, vitamins and amino acids are the most widely used both for preventing heat stress and improving productivity. Amino acids supplementation is important to compensate dietary crude protein (CP) since it is known that dietary CP produces higher heat production which can compromise the broiler hyperthermic condition (Gonzalez-Esquerra and Leeson, 2006; Santos et al., 2020). In addition, studies reported that broiler chickens with heat stress experienced a reduction in the levels of plasma ascorbic acid, α tocopherol, and ascorbic acid (Saiz del Barrio et al., 2020).

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Therefore, it is also important to increase the density of dietary vitamins, such as vitamin A, E, and C above the minimum requirement to support the antioxidant status of the animals (El-Senousey et al., 2018; Gan et al., 2020).

Viterna Plus is a commercial additive containing complete minerals, vitamins, amino acids, and fatty acids (aspartic acid, glutamic acid) in a single product which is produced from natural substances (Supartini, 2008). Those essential nutrients can be directly absorbed in the small intestine, allowing them to be directly used by broiler chickens for metabolism. A previous study revealed that supplementing Viterna Plus via drinking water improved weight gain and feed conversion ratio (FCR, Sutomo et al., 2015). It can be supplemented via drinking water or feed. Accordingly, the present experiment aimed to evaluate the effects of different levels of supplementation with Viterna Plus via drinking water on the performance and carcass traits of broiler chickens.

MATERIALS AND METHODS

Ethical approval

All procedures performed in this study involving broiler chickens were approved by the Ethical Committee of Faculty of Agriculture and Animal Sciences, Universitas Islam Negeri Syarif Kasim, Riau, Indonesia.

Animals, diets, and experimental design

The present experiment was conducted at the experimental farm of the Faculty of Agriculture and Animal Science, State Islamic University of Sultan Syarif Kasim, Riau, Indonesia. A total of 100 one-day-old (DOC) Cobb 707 (mixed sex and average weight of 46.7 gram) were randomly assigned to one of the following four treatment groups: control group (C), C + 2.25 ml/L Viterna Plus (V1), C + 2.50 ml/L Viterna Plus (V2), and C + 2.75 ml/L Viterna Plus (V3). Each treatment group contained five replicates of five chickens (25 birds per treatment). The chickens had a similar initial weight of 46.7 g. In the current study, all birds received a similar diet purchased from a commercial retailer, and the diets were prepared according to two feeding phases of starter (1-20 days, in crumble form) and finisher (21-28 days, in pellet form). The chemical composition of the diet was analyzed according to AOAC (2005) for crude protein (CP), ether extract (EE), crude fiber (CF), calcium (Ca), phosphorus (P), and ash, and Rostagno (2011) for metabolizable energy (ME) calculation. The nutritional composition of each diet (commercial feed) is presented in Table 1.

Broiler chickens were maintained on an open house system equipped with typical Indonesian farmer litter. They had free access to feed and drinking water. Starter feed was provided in a crumble form, while finisher diet was given in a pellet form. In addition, water was provided in a five-litter chicken water jar. Lighting and other rearing management were in according to Cobb 700 manual guide. Viterna Plus was obtained commercially. The product contained vitamins (C and B complexes), minerals (N, P, K, Ca, Mg, Na, Cl, S, Fe, Zn, Cu, Mn, I, Co, Mb, Se, Cr, F), fatty acids (aspartic acid and glutamic acid), and amino acids (Lysine, Methionine, Leucine, Iso-leucine, Valine, Arginine, Threonine, Tryptophan, Tyrocine, Serine, Phenylalanine; PT. Natural Nusantara).

Table 1. The nutritional composition of starter and finisher diets of broiler chickens used for this experiment

Nutrient composition	Feeding phase	
	Starter (1–20 days)	Finisher (21–28 days)
Metabolizable energy (ME, kcal/kg)	2781	2910
Crude protein (CP, %)	23.5	20.5
Crude fiber (CF, %)	5.0	5.0
Ether extract (EE, %)	5.0	5.0
Ca (%)	0.9	0.9
Total Phosphorus (%)	0.6	0.6
Ash (%)	7.0	7.0
Lysine (%)	1.30	1.10

Sampling and measurements

Feed intake (FI), body weight (BW), and FCR were recorded on weekly basis. Body weight was determined by weighing all of the birds in each replicated. The FCR was calculated as cumulative FI/ final BW. Mortality (if any) was recorded daily. In addition, index performance was calculated according to European Production Efficiency Factor (EPEF) at 28 days with the formula of (average daily gain (g) × (100–% mortality) / (FCR × 10). Broilers were slaughtered after 28 days to determine the carcass yield, percentage, and other body components.

Statistical analysis

This study was arranged according to the completely randomized design of ANOVA with four experimental treatments and five replicates of each. Raw data were initially subjected to an outlier test. Data with more than ~2 of standard deviations of the mean were defined as outlier thus were excluded from the analysis. All data were subjected to ANOVA, followed by Duncan's multiple range test to compare means among the treatments. Significant was declared when $P < 0.05$. The statistical analysis was conducted using IBM SPSS statistics version 20.

RESULTS AND DISCUSSION

Broiler chickens' performance

The performance of broiler chickens as affected by levels of Viterna Plus is presented in Table 2. Throughout the experiment, there was no mortality and culling recorded, indicating that all birds were healthy and comfortable with the house condition. Dietary Viterna Plus in any levels did not affect FI and FCR ($P > 0.05$). However, it had a significant effect on body weight gain (BWG), water intake, and performance index reflected from EPEF value ($P < 0.05$). Final BW was higher for the chickens that received 2.50 ml/L of Viterna plus, compared to the control group ($P < 0.05$). However, supplementing at 2.25 and 2.75 ml/L Viterna Plus did not influence the BWG. In line with BWG, the EPEF value for V2 also indicated the highest achievement than any other treatments (Table 2). This result suggested that Viterna Plus containing essential nutrients is beneficial for broiler chickens in terms of improving their overall performance.

Generally speaking, incorporating vitamins and minerals mixed with additive into the diet or drinking water can alleviate the heat stress on broiler chickens. As the experimental chickens did not experience any heat stress indications, the effect of feed supplement in this study can not be fully expected. The major findings were the improvement in broiler performance by adding feed supplement, compared to the control group. This result can be primarily related to higher micro and macronutrient density imported from the supplement as it contains minerals, vitamins, and amino acids which are essential for broilers' growth (Saiz del Barrio et al., 2020). Considering the composition of the supplement used, there may be a synergistic effect among electrolytes such as Na, K, Cl. Previous studies reported that supplementation multiple micronutrients on broilers' diet and drinking water improved the average daily gain (ADG) and FCR (Panda et al., 2008; Imik et al., 2013; Saiz del Barrio et al., 2020). Furthermore, vitamins also play a crucial role in maintaining broilers' health and immune system. It was demonstrated that supplementation of vitamin A at 15.000 IU successfully improved BW, FCR, and carcass yield of broiler chickens exposed to heat stress (Kucuk et al., 2003) while ascorbic acid (vitamin C) was also known to promote a higher carcass percentage and quality (Kutlu, 2001), ameliorate the bacterial challenge, and improve the structure of cecal microbiota and intestinal health (Gan et al., 2020). Moreover, mixed of vitamins A, D, E, and B complex was also reported to improve the immune system under heat stress (Lin et al., 2006). The primary role of vitamins can be related to lowering the oxidative stress that is susceptible for broiler chickens in a hot climate (Akbarian et al., 2016).

Additionally, the beneficial effects of Viterna Plus could also be attributed to the role of amino acids. Amino acids are known to have specific and multiple biological functions primarily for growth, maintenance, reproduction as well as defense mechanisms in the immune regulatory pathways (Trevisi et al., 2015). Indeed, the specific effect of each amino acid could not be elucidated since the amounts of amino acids were not quantified individually in the present study. However, there are a large number of explanations in the literature that amino acids are involved almost in all metabolic pathways including maintaining the balance of free radicals, diminish cell damage, and body protein synthesis (Bin et al., 2017). In an appropriate amount, the effect would be likely as an additive similar to the product used in the current study, the percentage of each individual amino acids was low.

Viterna Plus in the current study significantly increased water intake (Table 2). A higher water intake in some cases was detrimental because it may increase the risk of wet litter, and then increase the risk of other problems, such as skin dermatitis, bacterial infection, footpad lesions, which increases the potential risk of rejection at slaughterhouses (Shepherd and Fairchild, 2010). As wet litter was not observed in the present study, the amount of water intake was assumed in the normal range.

Table 2. Performance of the broiler chickens affected by different levels of Viterna Plus

Performance parameters	Treatment groups				
	C	V1	V2	V3	p-value
Water intake (L)	4.76 ± 0.46^{b}	5.11 ± 0.44^{ab}	5.52 ± 0.16^{a}	$4.89 \pm 0,50^{b}$	0.048
Feed intake (g)	1.99 ± 0.07	2.00 ± 0.06	2.04 ± 0.05	1.99 ± 0.39	0.552
Body weight gain (kg)	1.11 ± 0.04^{b}	1.14 ± 0.04^{ab}	1.19 ± 0.01^{a}	1.11 ± 0.04b	0.011
Feed conversion	1.80 ± 0.07	1.75 ± 0.04	1.71 ± 0.04	1.80 ± 0.08	0.111
Mortality (%)	0	0	0	0	-
EPEF	220 ± 14.5^{b}	233 ± 12.6^{ab}	249 ± 6.11^{a}	222 ± 18.1^{b}	0.017

EPEF: European Production Efficiency Factor; Different superscripts on the same line show significant differences ($p < 0.05$), C: Control, V1: 2.25 ml/L Viterna Plus, V2: 2.50 ml/L Viterna Plus, V3: 2.75 ml/L Viterna Plus

Table 3. Carcass traits of broiler chickens affected by different levels of Viterna Plus

Variables	Treatment groups				
	C	V1	V2	V3	p-value
Carcass weight (g)	793 ± 33.8^{bc}	843 ± 44.6^{ab}	906 ± 71.7^{a}	752 ± 52.1^{c}	0.002
Carcass (%)	68.8 ± 4.45	71.0 ± 5.86	73.2 ± 6.19	64.9 ± 4.68	0.125
Abdominal fat (g)	28.4 ± 5.85^{ab}	27.2 ± 1.39^{ab}	24.1 ± 2.05^{a}	30.4 ± 1.33^{b}	0.049
Abdominal fat (%)	2.45 ± 0.47^{b}	2.29 ± 0.17^{ab}	1.95 ± 0.19^{a}	2.62 ± 0.17^{b}	0.011

Different superscripts on the same line show significant differences ($p < 0.05$), C: Control, V1: 2.25 ml/L Viterna Plus, V2: 2.50 ml/L Viterna Plus, V3: 2.75 ml/L Viterna Plus

Carcass traits

As indicated in Table 3, carcass weight was higher for chickens receiving 2.50 ml/L of the treatment (V2), which had 14.3% higher than that of the control group ($P<0.05$). This result was in line with the BW of the V2 (Table 2) in which concurrently produced a higher carcass portion. Interestingly, the group of V3 treatment had the lowest carcass weight among others. However, there was no difference among treatments regarding carcass percentage.

The carcass of broiler chickens ranged around 60-65% of their BW at the end of harvest time (Ahmad et al., 2020). The percentage of the carcass can be related to the type of chickens reared, length or duration of maintenance, amount and quality of feed paid for during the maintenance and management. One of the maintenance management that needs to be known is feeding management. Errors in implementation, which will have an impact on the mobilization of feed nutrients into abdominal fat. Overall results of the present study suggested that adding more essential nutrients, such as amino acids with constant basal CP and energy improved their balance as well as productive energy released from the diet. This can be elucidated by the fact that amino acids promote body protein components.

Concurrently, higher carcass weight in the V2 also resulted in significantly lower abdominal fat and fat percentage in comparison with any other treatments ($P < 0.05$; Table 3). Fat deposition is attributable to minimal heat and is enhanced by increasing body temperatures. Apparently, the productive advantage from supplement improvements under the present terms of experimentation did not relieve heat formation to the extent that muscle formation could benefit. Moreover, Rahmatnejad and Saki (2015) suggested that fat deposits in body tissues are a form of excess energy in poultry. This means that chickens consuming higher energy above their requirement are likely to deposit higher body fat. However, feed containing high energy (metabolizable energy, ME) will increase the cost or production resulting in higher feed price as well as feed cost. In addition to fat deposits from excess feed energy, Redka et al. (2019) suggested that fat deposits in the body of broilers can also come from triglycerides. Triglycerides in the tissues of broilers and poultry are generally a component that comes from the feed by 95%, and only 5% are synthesized alone in the liver.

According to Lohakare et al. (2004), the addition of vitamin C in feed at 10-20 ppm resulted in 2.70-2.94% abdominal fat of live weight. A decrease in the percentage of broilers' abdominal fat, consuming drinking water with the addition of 2.50% commercial supplements, may affect the potentiality of commercial supplements to improve the balance of microorganisms in broilers' intestines, and furthermore improve nutrient absorption. The factors to reduce the percentage of broilers' abdominal fat are considered to be the nutritional content of the feed, age, and type or strain of broilers (Henry et al., 2001).

CONCLUSION

In conclusion, the incorporation of commercial feed supplements containing mixed of minerals, vitamins, and amino acids at the dose of 2.50 ml/L into the drinking water improved the overall performance of broiler chickens reflected from higher final body weight and carcass weight. Concurrently, the treatment also reduced the percentage of abdominal fat relative to body weight which is beneficial for carcass quality. Further research evaluating economic feasibility is therefore demanding.

DECLARATIONS

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Competing interests

Authors have no competing interests to declare.

Authors' contribution

Sadarman, Abdul Hamid, Eniza Saleh, and Wieda N. H. Zain conceptualized supervised the experiments, Rizki Arisandi did the investigation and data collection, Muhammad M. Sholikin, Tri R. Prihambodo, and Rakhmad P. Harahap did the data curation and statistical analysis, Rondius Solfaine, Ahmad Sofyan, and Agung Irawan were responsible for reviewing and revising the manuscript. All authors checked and approved the statistical results and final proof of the article.

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Sero-Epidemiology of Marek's Disease Virus on Local and Exotic Chickens in Northwest Ethiopia

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ABSTRACT

Marek's disease (MD) is a lymphoproliferative and neuropathic disease of domestic fowl caused by alphaherpesviruses. The current cross-sectional study with a simple random sampling method was undertaken from January 2018 to May 2020. The current study aimed to estimate sero-epidemiology and assess potential risk factors, it is very important to determine MD sero-positivity. Moreover, to measure the association of host and environmental risk factors, the occurrences and spread of MD were identified in local and exotic chickens in Northwest Ethiopia. A total of 768 serum samples from 3 zones were collected and assayed for Marek's Disease Virus (MDV) antibodies using the indirect enzyme-linked immunosorbent assay test. A questionnaire survey was also conducted to gather information on the potential risk factors of MDV sero-positivity, as well as the status of occurrences and spread in the chicken flocks. In the present preliminary study, a high flock and chicken level of MDV was demonstrated, with an overall seroprevalence of 59.11%. The mixed-effect logistic regression analysis of the host potential risk factors showed that the odds of seropositive for MD was significantly higher in local chickens (OR: 1.70, 95% CI: 1.26-2.28) than exotic chickens, higher in chickens getting non-proper feed (OR: 0.26, 95% CI: 0.13-0.54) than getting proper feed, higher in vaccinated chickens (OR: 1.04, 95% CI: 0.76-1.43) than non-vaccinated chickens. Rearing chicken of different batches in one house was decreased the odds of occurrence of MD by 55% (95% CI: 0.38-0.80) than all-in-all-out, higher in the well-ventilated type farms decreased the odds of occurrence of MD by 60% (95% CI: 0.39-0.80) than in poor ones. Litter management when farmers used chickens as fertilizer has decreased the odds of occurrence of MD by 55% (95% CI: 0.01-0.47) and chickens were tossed into open sheds 40% (95% CI: 0.01-0.31), compared to buried chickens management. The study results indicated that the number of farms where farmers wearing no clothes and shoes (95% CI: 0.10-0.58) were significantly decreased the occurrence of MD by 24% than those where farmers were equipped with clothes and shoes. The study area was highest in West Gojjam (OR: 0.40, 95% CI: 0.27-0.58) and South Gondar (OR: 0.19, 95% CI: 0.13-0.28) compared to North Gondar zone. In conclusion, the present study revealed a high flock and chicken seroprevalence level of MDV among chicken flocks in northwest Ethiopia, suggesting that environmental dust/dander and farm management systems might be a source of this disease for chicken infection. Besides, the observed association of MD, sero-positivity with environmental dust/dander, and farm management systems may suggest the economic importance of the disease for chicken production. Therefore, it warrants control attention to reduce its economic and disease spread burden in the study areas. Further works on the economic impacts, virus isolation, and molecular characterization of the disease are suggested.

Keywords: Chicken, Marek's Disease, Northwest Ethiopia, Risk factors, Sero-epidemiology

INTRODUCTION

Marek's Disease Virus (MDV), recently classified as the Office International des Epizooties list B disease, and is a lymphoproliferative disease of chickens (Kamaldeep et al., 2007; Couteaudier and Denesvre, 2014). Marek's Disease Virus is a highly cell-associated oncogenic α-herpesvirus that causes immunosuppression (Puro et al., 2018), neurological disorders, chronic polyneuritis, lymphomas in

visceral organs and other tissues, transient paralysis (Atkins et al., 2013; McPherson and Delany, 2016; Wang et al., 2018). The virus is transmitted to inexperienced chickens via the respiratory region (Witter and Burmester, 1967). Macrophages or dendritic cells subsequently transfer the virus to the major lymphoid organs (Woźniakowski and Samorek-Salamonowicz, 2014), where it infects B and T cells (Boodhoo et al., 2019). The route of infection is by inhalation of airborne cell-free virus particles within the contaminated dust and dander, and then shed from the infected host produced in terminally differentiated feather follicle epithelium, into a naive respiratory track (Boodhoo et al., 2016). The virus replicates within the feather follicle and spreads horizontally in poultry houses with dirt and dander (Davidson, 2019). It has a complex life with four main phases: an early cytolytic phase at 2-7 days post-infection (dpi) (Gurung et al., 2017), a latent section around 7-10 dpi, a late lysis section with the presence of tumors that are triggered between the 14 and 21 dpi, and a final proliferation section at 28 dpi. Throughout the primary lysis part, MDV preliminary uses B cells as targets for its replication before targeting activated CD4 T cells to adjust a persistent latent phase (Bai et al., 2019).

In infected chickens, unhealthful field strains of MDV will cause mortality of up to 100% in susceptible flocks; In infected chickens, unhealthful field strains of MDV will cause mortality of up to 100% in susceptible flocks; however, commercial losses due to MD are effectively controlled by the use of antigenically-related live attenuated vaccines (Cadmus et al., 2019; Neerukonda et al., 2019). In Ethiopia, the village system contributes to more than 90% of the national chicken meat and egg output (Ibrahim et al., 2019).

A number of challenges and obstacles warning the success and profitability of both backyard and semi-intensive production have been identified, including infectious diseases, low effort of veterinary services, poor housing, poor biosecurity, predators, as well as the quality and cost of feed (Sambo et al., 2015). The economic encumbrance of MDV hail from both direct losses due to chicken mortality and morbidity (egg production loss) and indirect losses caused by the wide use of vaccines and control measures in the industry (Rozins et al., 2019). Furthermore, immunosuppression resulting in increased coexisting disease and decreases in weight gain are both important indirect effects associated with MDV infection (Atkins et al., 2013). The concentration of MDV in dust can vary by numerous orders of magnitude between farms and within the farms over time. The underlying cause of this variation is unknown (Kennedy et al., 2018). Periodic disease outbreaks always affect the chicken production system in Ethiopia in different ways by losing almost the entire flock in the village chicken. The other by the constant presence of infectious disease can lead not only to illness and death but also reduction of the product (Habte et al., 2017). The mortality rate due to natural MD challenge varied among four indigenous chickens in Ethiopia by ecotypes Fayoumi (94.8%), Tepi (62.2%), Horro (51.4%), and Jarso (36.3%) (Duguma et al., 2006), and the mortality rate was 46% (Lobago and Woldemeskel, 2004) in non-vaccinated poultry, and about 5% in MDV vaccinated chickens (Duguma et al., 2005). Nevertheless, estimates of overall mortality (56.5%) and morbidity (58.1%) were reported (Asfaw et al., 2021).

Amhara region also has the second (10,368,274) reserves of poultry in Ethiopia (9,983,180 native breeds; 339,046 crossbreeds and 46,049 exotic breeds (Alemu et al., 2008). The majority (94-99%) of the chicken population in Ethiopia was estimated as 49 million in 2011 (Sambo et al., 2015). More than 60% of the Ethiopian families kept, managed, and controlled chickens as a source of income by selling them in cash (Dessie and Ogle, 2001). The reported mortality rate of Lohmann white breeds and Rhode Isle land Red were 29.34% and 16.18%, respectively. The other mortality rate reported was also in exotic chickens in low altitude (52.98%), (48.88%), and mid-altitude (43.25%) (Mazengia et al., 2012). In spite of their low productivity, and poultry health management, especially prevention, control of MDs was poor and particularly no seroprevalence study has been done in the northwest part of the country to address this issue. The findings of previous studies have shown that the seroprevalence of MDV antibodies in chickens ranges from 58% to 74.9%. Within the country, the most important causes of MDV (feeding status, farm type, farm size, production type, rearing method, house sanitation, litter management, carcass management, and farm shoes and clothes within the flocks of chicken are not well documented, and also the association with the seroprevalence of MDV has not been well noted.

When the counts of chickens were high, the farmers did not benefit from the sector disease outbreak in Ethiopia (Yitbarek and Atalel, 2013). However, in the current study area, there was no clear evidence about MD challenges, epidemiological information, sero-prevalence, and status in Northwest Ethiopia.

In Ethiopia, greater efforts have been made to transform the production system into a more commercialized and intensive large-scale system but they

have faced some challenges and serious problems that cause morbidities and mortalities in chickens, especially in the Northwest, Ethiopia. There was no enough data regarding the production constraints, opportunities, and MDV outbreak control for poultry production in the current study.

The current study was the first preliminary one in northwestern Ethiopia. Data on the seroprevalence of MDV in chickens would be caring for planning and implementing precautionary measures for economic impacts. Therefore, the objectives of the study were firstly to estimate the seroprevalence and the possible risk factors of MDV infection in local and exotic chickens in Northwest Ethiopia, and secondly to assess the relationship between sero-positivity of MDV through the environmental and management systems of farms for local and exotic chickens.

MATERIAL AND METHODS

Ethical approval

For the research team members to conduct the current study, after permitted ethical approval and statement given by the University of Gondar, Ethiopia. The current study was reviewed by the Institutional Ethical Review Board of the University of Gondar for its ethical soundness, and it is found to be ethically acceptable. Thus, the Research and Community Service Vice President Office has awarded on R. No. - O/V/PRCS/05/495/2018.

Study areas

Amhara Regional National State is located in the northwestern part of Ethiopia (Fentie et al., 2013). Study zones included North Gondar, West Gojjam, and South Gondar. According to Dachew and Bifftu (2014), North Gondar zone is located between geographically coordinates 12.3º to 13.38º north latitudes and 35.5º to 38.3º east longitudes. The altitude ranges from 550 meters below the sea level (masl) which is lowland (in western parts) and 4620 meters above the sea level (masl) which is highland (in the north part). The average annual rainfall varies from 880mm to 1772 mm, which is characterized by a monomodal type of distribution. The mean annual minimum and maximum temperatures are 10ºC and 44.5ºC in the highland and lowland, respectively. The indigenous chicken flock in the study zone was estimated at 3.75 million. Most of the poultry flocks were found in the highland and mid-highland areas of the region. This was due to the ecosystem and demography variations from low land. In this area, the local chickens were major poultry flocks in each household level as an income generation with a free-range production system (Fentie et al., 2013). According to Solomon et al. (2013), West Gojjam zone is situated at 11° 09' 60.00" N latitude and 37° 14' 60.00" E longitude with an altitude ranging from 1500 to 3420 m above the sea level. West Gojjam zone is one of the zones in Amhara Region where elevation varies from 1500 to 3500 m.a.s.l. Agriculture is the chief foundation to support the community (Motbaynor et al., 2020). South Gondar zone was encompassed in the study and is located in the Amhara Region, 660 km northeast of Addis Ababa, the Capital of Ethiopia. This Zone is well-known with diverse topography ranging from flat and low grazing land to high cold mountains. The altitude is 1500 to 3,600 m.a.s.l. The average yearly rainfall varies from 700 mm to 1300 mm whereas the average daily temperature is 17°C in 2017 (Alelign et al., 2019).

Study animals

Seroprevalence investigation was conducted in a total of 768 chickens, where 384 local chickens and 384 exotic Sasso T lines (Ruby T) a dual-purpose chicken for meat and eggs, in a farm from Northwestern Ethiopia. Chickens were kept under intensive and extensive poultry farm management in the mixed production system of the area. Chickens, which two and above months of age were included in the sampling process. Farm rearing methods were all-in-all-out and different batches in one house.

Study design and sample size

A present cross-sectional study with a cluster random sampling was undertaken from January 2018 to May 2020. The sample size was calculated using a formula given by Greiner and Gardner (2000). Expected prevalence (Pexp) of 50%, desired absolute precision (d), and a confidence level of 95% was determined by significance. Therefore, 384 Chickens were considered for sampling purposes. However, to account for the design effect associated with the clustering of study units within flocks and locations, the sample size was multiplied by two and the resulting in a total sample size of 768 Chickens.

Blood sample collection and serum separation

Whole blood samples (about 2-3 ml) were collected aseptically through wing vein puncture using 5 ml plain vacationer tubes (Birhan et al., 2019). All necessary information related to each chicken included age, breed, sex, vaccine history, feeding status, farm type, farm size, production type, rearing method, house sanitation, litter management, house sanitation, ventilation type, zone, and

farm shoes and cloth were properly labeled on the blood collection tubes and the data recording sheet. The blood samples were then allowed to clot in a slant position for a few minutes and centrifuged at 4000 rpm for 5 minutes to separate the sera. Subsequently, sera were decanted into 1.5 ml Eppendorf tubes and kept at -20°C until the serological analysis for the presence of Marek's antibodies. In the attempts to screen and detect the MDV antibody at the National Veterinary Institute (NVI), all the materials were International Organization for Standardization. Regarding MD, the overall prevalence (Antibody prevalence) using indirect enzyme-linked immunosorbent assay was 59.11%.

Questionnaire survey

A pretested semi-structured questionnaire survey was conducted during the blood sample collection by interviewing owners or attendants to assess the potential risk factors of MDV in chicken flocks. Farm supervisor veterinarians and farm owners were purposively selected. A total of 46 respondents were interviewed using semi-structured questions for epidemiological data regarding total chickens, number of affected birds, number of birds reported dead due to MDV, age, breed, sex, vaccine history, feeding status and farm type, farm size, production type, rearing method, house sanitation, litter management, carcass management, and farm shoes and clothes as a source of the pathogen.

The hypothesized potential risk factors for MDV to assess included having information on the role of vaccine history, feeding status and farm type, farm size, rearing method, house sanitation, litter management, carcass management, and farm shoes and wearing as a source of a pathogen (yes/ no), flock size (50-200, 201–500 and >501), presence of a house as well for sick chickens in or around the farm (yes/no), presence of clothes and shoes for farm management (yes/no), chicken feed sources (properly feed and non-properly feed/millhouse grinder leftover), dead chickens' disposal method (buried, throw away in the field or use as fertilizer), and chicken feed contamination by dust/dander (yes/no). Moreover, the owners/attendants were interviewed about previous experiences of the occurrence of MDV transmissions at the flock/farm or individual chickens' level.

Serological analysis

Marek's disease virus Antibodies were assayed in the collected serum samples using the indirect ELISA kit test (Shenzhen Zhenrui Biotechnology Co., Ltd., China) following the previous reports (Zelnik et al., 2004) and manufacturer's recommendations. Indirect ELISA tests were detected using the naked eye and stereomicroscope. The presence of an indirect ELISA test was considered as positive at a titer of > 0.2, and showed an antibody concentration equal to or greater than 100 ul. The flocks also were considered when at least one chicken was found positive. The laboratory procedures were performed at the NVI, Serology Laboratory.

Data management and analysis

The data generated was stored in the Microsoft Excel spreadsheet (2010), and analyzed using Stata software Version 16 for windows. The data were summarized using descriptive statistics. Seroprevalence of MDV infection was calculated by dividing the total number of seropositive chickens by the total number of examined poultry. Similarly, chicken flocks' seroprevalence was calculated as the total number of positive divided by the total number of the examined chicken flocks. Mixed effect logistic regression analysis was used to identify potential risk factors associated with MDV. First, invariable logistic regression analysis with the flock as a random effect was performed and potential risk factors (explanatory variables) with p values ≤ 0.25 were screened for the multivariable mixed-effect logistic regression. Before running the multivariable mixed-effect logistic model, the explanatory variables were checked for multi-collinearity using the variance inflation factor (VIF) value of greater than 10 was considered the cut-off point for the collinearity diagnostics (Steinfeld et al., 2015). The associations were considered statistically significant when $P < 0.05$ at 95% confidence level. Odds ratios at a 95% confidence interval were used to express the strength of the risk of the diseases associated with the tested factors. Association of environmental risk factors (feeding status, farm type, farm size, rearing method, house sanitation, litter management, carcass management, and farm shoes and wearing as a source of a pathogen) with sero-positivity for MDV at flock level was analyzed similarly using mixed-effect logistic regression considering reproductive flosses as outcome variables and sero-positivity as the explanatory variable. The cutoff value of the receiver operating characteristic curve in the current study was 0.5% (Figure 1). This indicated that the expected probability from the model may have been a 50% probability of success.

The Area Under the Roc Curve (AUC) is an effective way to summarize the overall diagnostic test. It takes values from 0 (perfectly inaccurate test) to 1 (perfectly accurate test). The AUC can be computed using the general rules,

in these rules the value of AUC (0.5) suggests no discrimination, 0.7-0.8 is considered acceptable, and 0.8-0.9 is considered excellent, and more than 0.9 is considered outstanding (Mandrekar, 2010). In dichotomous outcome (positive/negative test results) was used to plot sensitivity versus 1-Specificity is called receiver operating characteristic (ROC) curve, and AUC, as an effective measure of the accuracy of meaningful interpretations (Hajian-Tilaki, 2013). The AUC was an effective way to summarize the overall diagnostic test. It takes values from 0 (perfectly inaccurate test) to 1(perfectly accurate test). Considering the obtained results of the current study, the AUC of the curve was 0.75. This suggested a 75% chance that the indirect ELISA test was correctly distinguished non-Marek's Disease from Marek's Disease chicken based on the standard of the optical density value with indirect ELISA reader. In this outcome, the result of sensitivity and specificity were 80.18% and (54.46%), respectively, and also the correctly classified was 69.66%. However, in this consequence of score value, the postulation was that indirect ELISA test was subjectively gives considered laboratory results of MD in chickens.

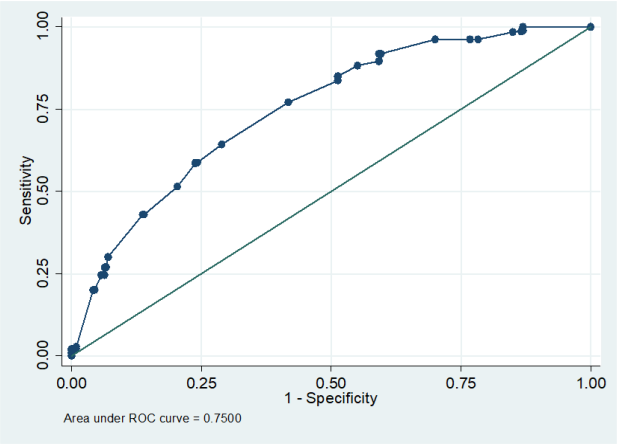

Figure 1. Receiver operator characteristic curve analysis and estimation of the study.

RESULTS

Seroprevalence and host-related risk factor analysis

A total of 768 serum samples were examined and 454 (59.11%) were positive for MDV. As can be seen in Table 1, the results of different age groups indicated a relatively higher prevalence of MDV in adult chickens 61.1% (0.61-0.57), compared to that of young age groups 52.7% (0.45-0.60). Similarly, a relatively higher prevalence of MDV was observed on the extensive farming system (60.4%), as compared to that of the intensive (55.6%) based on chicken farming systems. In the present study, different chicken farm sizes were examined, relatively a higher prevalence of MDV was observed in the medium farm's size (66.2%), as compared to small (60.7%) and large (25%) farms size. Moreover, a relatively higher prevalence of the MDV was observed in the exotic breeds (67.7%) than the local breeds (52.1%). On the other hand, vaccinated chickens higher prevalence (66.3%) than non-vaccinated ones (53.7%).

The results presented in Table 1 indicated that there were statistically significant differences observed ($p < 0.05$) among the prevalence of MDV, according to the age, breed, and vaccine history, feeding status, and rearing methods.

Questionnaire survey related to farm owners/attendants as risk factors for Marek's Disease Virus occurrences

A total of 46 chickens' farm owners/attendants participated in the questionnaire survey aimed at collecting data about potential risk factors for MDV. Out of 46 farm owners'/attendants' respondents, 69.57% did not know concerning the role of house litters and dust/dander as a pathogen source. All of them practiced an extensive management system. Among the respondents, 76.1% had no isolation house in their farms for diseased/sick chickens. The most frequently stated feeding status of chickens was properly fed (83.6 %) followed by non-properly fed (57.2 %). Respondents also stated that litter management, including buried chickens, was 67% whereas chickens that accumulate near the farmhouse were 23.9% and the other that used chickens as fertilizer were 9.1%. The respondents informed about rearing methods, different batches in one house were 71.74% and all-in-all-out methods were 28.3%. The most accurate timetable for farm disinfections was 54.35%, compared to the inaccurate timetable was 45.65%. In the study areas, 10.87% of the farm owners/attendants dress in special clothes and shoes, but 89.13% were not used special clothes and shoes.

Table 1. Invariable and multivariable mixed-effect logistic regression analysis of hosting potential risk factors for Marek's Disease Virus

Variables	Category	Number of examined	Number of positive	Prevalence (%) (95% CI)	Invariable		Multivariable	
					COR (95% CI)	*p value*	AOR (95%CI)	p value
Breed	Exotic	344	233	67.7 (0.63-0.73)	1.0			
	Local	424	221	52.1 (0.47-0.57)	0.52 (0.39-0.70)	0.000*		
Sex	Male	219	128	58.4 (0.52-0.65)	1.0			
	Female	549	326	59.4 (0.55-0.64)	1.04 (0.76-1.43)	0.812		
Farm Type	Intensive	198	110	55.6 (0.49-0.63)	1.0			
	Extensive	570	344	60.4 (0.56-0.64)	1.22 (0.87-1.86)	0.237		
Farm Size	Small (50-200)	567	344	60.7 (0.57-0.65)	1.0			
	Medium (201-500)	145	96	66.2 (0.58-0.74)	1.27 (0.87-1.86)	0.221		
	Large (>501)	56	14	25 (0.13-0.37)	0.22 (0.12-0.40)	0.000*		
Production Type	Layer	445	278	62.5 (0.58-0.67)	1.0			
	Broiler	170	102	60 (0.40-0.56)	0.90 (0.63-1.29)	0.573		
	Dual	153	74	74 (0.40-0.56)	0.56 (0.39-0.82)	0.002		
Age	Young	184	97	52.7 (0.45-0.60)	1.0			
	Adult	584	357	61.1 (0.61-0.57)	1.41 (1.01-1.97)	0.043	0.41 (0.20-0.87)	0.020
Vaccination history	Vaccinated	436	234	53.7 (40.49-0.58)	1.0			
	Non-Vaccinated	332	220	66.3 (0.61-0.71)	1.70 (1.26-2.28)	0.000*	2.15 (1.54-2.99)	0.000*
Feeding Status	Properly Feed	55	46	83.6 (0.74-0.94)	1.0			
	Non-Properly Feed	713	408	57.2 (0.54-0.61)	0.26 (0.13-0.54)	0.000*		
Rearing Method	All-in-All-out	157	110	70.1 (0.63-0.77)	1.0			
	Different Batches in One House	611	344	56.3 (0.52-0.60)	0.55 (0.38-0.80)	0.002		

COR: Crude odds ratio, AOR: Adjusted odds ratio, CI: Confidence Interval, p values ≤ 0.05 was statistically significant, p values = 0.00* were strongly significant.

Table 2. Invariable and multivariable mixed-effect logistic regression analysis of environmental risk factors for Marek's Disease Virus

Variables	Category	Number of examined	Number of positive	Prevalence (%) (95 % CI)	Invariable		Multivariable	
					COR (95% CI)	*p value*	AOR (95% CI)	p value
Ventilation Type	Good	609	378	48 (0.40-0.56)	1.0			
	Poor	159	76	62.1 (0.58-0.66)	0.56 (0.39-0.80)	0.001		
House Sanitation	Good	448	252	47.8 (0.40-0.56)	1.0			
	Poor	320	202	56.3 (0.52-0.61)	1.33 (0.99-1.79)	0.056	0.17 (0.07-0.33)	0.000*
Litter Management	Buried	25	24	63.1 (0.58-0.68)	1.0			
	Use as fertilizer	570	344	96 (0.88-1.04)	0.06 (0.01-0.47)	0.007		
	Accumulate near space	173	86	60.4 (0.56-0.64)	0.04 (0.01-0.31)	0.002		
Zone	North Gondar	256	200	78.1 (0.73-0.83)	1.0			
	South Gondar	256	104	40.6 (0.35-0.47)	0.19 (0.13-0.28)	0.000*	0.05 (0.03-0.10)	0.000*
	West Gojjam	256	150	58.6 (0.53-0.65)	0.40 (0.27-0.58)	0.000*	0.07 (0.03-0.15)	0.000*
Farm Shoes and clothes	Have	40	34	60.4 (0.57-0.65)	1.0			
	Have-No	728	420	85 (0.73-0.97)	0.24 (0.10-0.58)	0.002	0.10 (0.034- 0.27)	0.000*

COR: Crude odds ratio, AOR: Adjusted odds ratio, CI: Confidence Interval, p values ≤ 0.05 was statistically significant, p values = 0.00* were strongly significant.

DISCUSSION

Chicken production is the distinctive local economic development of urban centers through the development of related micro-enterprises wholly or partly responsible for the provision of inputs and processing, packaging, and marketing of outputs as well as the delivery of services to the sector (Ebsa et al., 2019). The current study has indicated the widespread occurrence of MDV infection in the chicken flock in northwest Ethiopia with a high seroprevalence of 59.11%. This coincides with the previous studies in Ethiopia, which similarly reported high seroprevalence of MDV in chickens (Duguma et al., 2005). Similarly, the present findings are compatible with the reports of Oni and Owoade (2009) and Wajid et al. (2013) from Nigeria, and Wajid et al. (2013) from Southern Iraq. However, the current result was higher than other previous reports from the Central African Republic (Snoeck et al., 2012), and lower than reports from Tanzania (Sailen et al., 2017).

In fact, many factors attributed to why the results of the current study differ from other findings. Considering the investigated factors, different diagnostic test kits, the diverse geographical location and climate changes of the study, and the distinctive farm management system can be listed. The variation observed among the different studies in the prevalence of MDV might be due to the difference in the breed, management system, and the epidemiological status of the animals among the different study areas.

Among the risk factors considered in the current study, age, breed, vaccination history, feeding status, rearing method, litter management, ventilation type, zone, and farm shoes and wearing were found to be statistically significant ($P < 0.05$). There was a significant difference in the prevalence among chickens of different ages ($P < 0.05$). The highest prevalence (61.1%) was found in adult chickens, followed by the lowest prevalence (52.7%) in young chickens. The high prevalence of the MDV revealed in adult chickens might be due to the physiology of the exhausted canal which is more dilated, and remains partially open because there have been years of repeated exposure, and it is a chronic disease. Susceptibility to MDV was considered to be much higher in older chickens than in younger ones. Although the age-resistance remained unclear, the immune system maturation likely participated in this event. Marek's disease virus can occur at any time, beginning at 3-4 weeks of age (Ikezawa et al., 2010).

However, there were significant differences in the occurrence of MD between exotic and local breed outcomes (Table 1). A higher prevalence (67.7%) was recorded in the exotic breed, and a lower prevalence (52.1%) was verified in chickens with the local breed. Due to low genetic-diseases-resistant and other environmental stress, exotic breeds were high exposed than local breeds (Duguma et al., 2005).

Furthermore, non-vaccinated chickens (66.3%) were more susceptible and had the chance of acquiring the MD, whereas the vaccinated chickens (53.7%) less susceptible. However, vaccines are commonly used in the commercial poultry industry because small numbers of doses cannot be purchased for use. The best protection mechanism for the backyard chicken's management systems is to disinfect the area. Predominantly the commercial flocks, it is important to have proper biosecurity to ensure that vaccinated chickens will develop immunity before they are exposed to a severe challenge of MDV (Mazengia, 2012).

Among the environmental risk factors, feeding status, rearing method, ventilation type, litter man management system, and farm shoes and wearing were statistically significant ($P < 0.05$, Table 2). Since chickens are required to be reared separately, they are free from the infected fluff and dust of older birds. Standard hygiene measures are also important, including a thorough clean-out and disinfection of the sheds and equipment among batches of chickens with a disinfectant effective against viruses. Decent nutrition and maintenance of freedom from other diseases and parasites are also vital. These practices will help to maintain the flock's health, and ensure that the birds have optimum resistance against MD infection (Rwuaan et al., 2012). The virus matures into a fully infective, enveloped form in the epithelium of the feather follicle, from which it is released into the environment (Morrow and Fehler, 2004).

The current study showed significant results among various zones ($P < 0.05$). A higher prevalence was recorded in the north Gondar zone (78.1%) followed by the West Gojjam zone (58.6%) and South Gondar zone (40.6%), respectively. According to these studies, this might be associated with the herding practices of chickens. The absence of research-based investigation approaches resulted in a lack of knowledge of the prevalent strain of viruses and information on the overall epidemiological patterns of the MDV (Witter et al., 1970).

CONCLUSION

In conclusion, the current preliminary study revealed a high seroprevalence of Marek's disease (MD) in the chicken flocks in northwest Ethiopia, suggesting that by

applying proper farm management systems with biosecurity, such as Good rearing method, litter management, and follow up seasonal vaccines, the disease can be prevented and controlled. The study also demonstrated that sero-positivity for MDV was associated with age, breed, and vaccine history, feeding status, rearing method, litter management, ventilation type, zone, and farm shoes and wearing with an important economic significance for poultry farm owners. Therefore, it warrants control to reduce its economic and disease burden. Further works on the virus isolation and economic impacts are needed in the study area and it is also given attention to further investigation on the effectiveness of vaccines and their delivery system. In order to know the circulation virus and strain type, the molecular study of the diseases is suggested.

DECLARATIONS

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Authors' contributions

Mastewal Birhan, Nega Berhane, Molalegne Bitew, Esayas Gelaye, and Takele Abayneh are substantial contributors to the conception and design, acquisition of data, or analysis and interpretation of data, drafting the article or revising it critically for important intellectual content. Belayneh Getachew, Aragaw Zemene, Kassahun Birie, and Wudu Temesgen are given some technical comments. Mastewal Birhan, Nega Berhane, Molalegne Bitew, Esayas Gelaye, and Takele Abayneh approved the final draft of the manuscript to be published and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Competing interests

All authors declare that they have no competing interests.

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Biosecurity Practices and Characteristics of Poultry Farms in Three Regions of Cameroon

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ABSTRACT

The outbreak of diseases is the main factor affecting poultry production in Cameroon. The implementation of biosecurity measures in poultry farms is essential to reduce disease outbreaks. This study aimed to assess biosecurity practices in poultry farms in three regions of Cameroon. The study was carried out using a structured questionnaire on 90 randomly selected poultry farms. Most of the farmers were men (85%) with deep litter (77.8%), battery cage (2.2%), and both deep litter and battery cage (20.0%) housing systems. Amongst the farms surveyed, 9/30 (30.0%) in the Centre; 8/30 (26.7%) in the Littoral; and 13/30 (43.3%) in the West were aware of biosecurity measures. The biosecurity score (BS) of surveyed farms ranged between 2 and 3. The findings indicated that 39 farms (12 in the Centre, 14 in the Littoral, and 13 in the West) were at moderate risk, and 51 farms (18 in the Centre, 16 in the Littoral, and 17 in the West) were at high risk. Reasons for keeping chickens and the number of chickens per farm did not significantly influence BS, while the farm category could significantly affect it. The outbreak of diseases correlated with BS, showing a tendency of increase in the outbreak of diseases with increasing BS. This study underlines the fact that biosecurity practices in Cameroon have not been well implemented by chicken farmers. This leads to disease outbreaks, and consequently, important economic losses as well as massive use of drugs that may be unsafe for human consumption. Therefore, the effective monitoring of biosecurity in chicken farming should be encouraged by extension of training to the farmers to support the efficient production of chickens by respecting biosecurity that drastically reduces the risk of disease outbreaks and provides good quality chicken products for human consumption.

Keywords: Assessment, Biosecurity practices, Biosecurity scores, Cameroon, Poultry farms

INTRODUCTION

Chicken farming is a growing sector in Cameroon that creates income generation in rural and urban areas (Guetiya et al., 2016), representing a good source of essential nutrients. Amongst food animals, chicken production is quicker and cheaper than other meat sources. In addition, chicken products gain more attention in Islamic countries due to religious rules forbidding the consumption of some animal meats, such as pork, and therefore, play an important role in public nutrition (Paryad and Mahmoudi, 2008; Melesse, 2014; Sambo et al., 2015). The necessity of securing the food supply in terms of quality and quantity, consumers' awareness, and tendency to maintain a healthy and balanced diet, have all made the poultry sector a significant industry throughout the world (Aral et al., 2013).

Some infectious diseases, such as Highly Pathogenic Avian Influenza (HPAI) are zoonotic, resulting in a range of mild to serious diseases having fatal consequences in both poultry and humans (Beeckman and Vanrompay, 2009; WHO, 2011). The unprecedented widespread outbreaks of HPAI, which has occurred in many countries in Asia, Europe, and Africa since 2003, call for rapid and active response at regional, national, and international levels. Biosecurity is a key strategy to reduce the incidence of outbreak diseases, such as HPAI, by applying technical recommendations at the farm or poultry house (DAFF, 2011; Newell et al., 2011). Biosecurity measures are necessary to prevent the negative consequences of infectious diseases during chicken farming because they reduce the introduction, persistence, or dissemination of infectious agents (Loth et al., 2011), and minimize the direct and indirect negative economic effect of infections

on stakeholders (farmers, customers, and suppliers) (Can and Altuğ, 2014). Furthermore, biosecurity measures are vital for better performance and quality of chicken production in the competitive world.

Various assessment studies have highlighted substantial weaknesses in the implementation of biosecurity measures in chicken farms (Abdurrahman et al., 2016; Maduka et al., 2016; Yitbarek et al., 2016). In Cameroon, few studies have assessed biosecurity practices. Kouam and Moussala (2018) studied the level of implementation of biosecurity measures on small-scale broiler farms in the Western Highlands of Cameroon and found that level of implementation was poor and there was a significant relationship between farm biosecurity score (BS) and farm production system.

The HPAI H5N1 virus has caused widespread mortality in the poultry sector among many African countries (Egypt, Ethiopia, and Nigeria). This situation of disease outbreaks, particularly in Cameroon has raised a concern about the level of implementation of biosecurity measures on chicken farms (MINEPIA, 2009). The present article outlines the biosecurity practices implementation on chicken production with the aim of contributing to improving chicken management practices in resource-limited conditions, specifically in Cameroon. The current study had three objectives of appraising the chicken production system, assessing the level of biosecurity practices, and examining any relationship between the biosecurity practices and the socio-technical characteristic of farms and farmers in three regions of Cameroon.

MATERIALS AND METHODS

Study areas

The study was conducted in three regions of Cameroon, including the center, the littoral, and the west regions (Figure 1). The central region covers 68926 km^2 and is composed of rolling hills on a vast plain with a mean altitude of 700–800 m, with lowered mounds. The climate has two wet seasons. The population density is low, with about 36 inhabitants/km^2 (NIS, 2006; BUCREP, 2010). The littoral region has an area of 20239-km^2 with more than 2,202,340 inhabitants. The population density is 124 inhabitants/km^2. The west region represents an area of 13872 km^2 characterized by highlands with a mean altitude of 1600 m and narrow valleys with catchments separating them. The population density is estimated at 143 inhabitants/km^2 (BUCREP, 2010).

Study design and data collection

Study areas were selected as they could represent a high potential for chicken production due to favorable conditions (Teleu Ngandeu and Ngatchou, 2006). Data were collected using structured questionnaires. The data were collected from governmental authorities and poultry farmers by data collector/field assistants. The research team worked in partnership with local veterinary agencies to recruit participants since most of them are scared of sanitary control by governmental authorities. The farms were randomly selected; 30 farms in the center, 30 farms in the littoral, and 30 farms in the western regions. The objective of the study was explained to the farmers and their verbal informed consent was obtained before administering the questionnaire. Chicken farms were evaluated for biosecurity practices and scored. Biosecurity measures were grouped according to some indicators of biosecurity events (events outside the premises, events at the farm boundary, events between farm boundary and poultry house, events inside poultry house).

Questionnaire design

A set of preliminary questionnaires were prepared and tested with 10 farmers in Yaoundé, Cameroon for biosecurity practices a few months after the avian influenza outbreak. Thus, necessary modifications were made based on the feedback and the final questionnaire was prepared based on the pilot survey. The structured questionnaire with both open-ended and closed questions was applied to 90 chicken farmers or farm employees by a team of two persons, the interview lasted for 45-60 minutes. The questionnaire had three parts: 1)socio-demographic characteristics of farmers, 2) information about poultry farming systems, 3) inquiry into biosecurity measure implementation.

Biosecurity scoring system

The scoring system used in this study was developed from the biosecurity indicators observed in the evaluation of biosecurity practices on the farm as previously reported with scores of 0-3. Criteria used for scoring biosecurity practices were adopted from USAID (2009). Thirty-three biosecurity indicators were recorded and characterized with scores of 0-3 (3.00 for the worst incorrect practice,2.00 for the occasional respect of biosecurity measures, 1.00 for average compliance with biosecurity measures of biosecurity measures, and 0.00 for the best correct practice). The BS of each farm was the average of

the scores of the biosecurity indicators. Accordingly, a low mean BS value indicates a higher level of biosecurity.

Statistical analysis

Data were analyzed by SPSS software, version 20.0. Categorical variables were expressed using frequencies and percentages while continuous variables, such as BS of farms, were expressed as means and standard deviations. The associations and relationships were assessed using the Chi-square test and Pearson's correlation, respectively, and variations in means were assessed by one-way ANOVA followed by Tukey post hoc test. The difference was considered significant for a p-value <0.05 with a confidence interval of 95%.

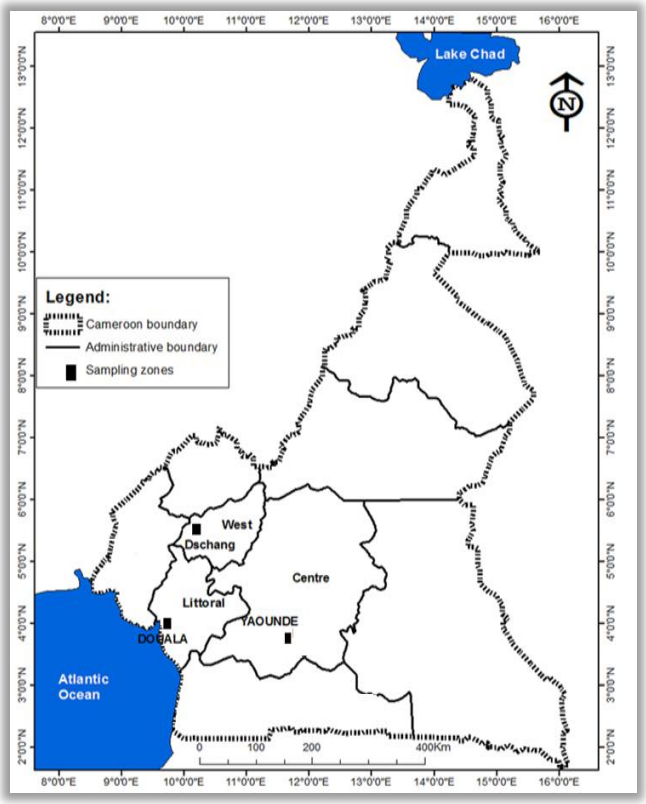


Figure 1. The map of Cameroon showing study areas marked by small black squares.

RESULTS

General characteristics of the studied farms

The participating farmers were mostly men (85%), and most of the farmers (74.4%) had no formal training in chicken production and 60% of farmers had more than three years of experience (Figure 2). Diseases were the most constraint faced by chicken farmers (100.0%), followed by financial constraints (93.3%). Some farmers (13.3%) produced chickens for only family consumption, while others (20.0%) produced for family consumption and also sold the surplus. The higher percentage of farmers (66.7%) who own the majority of chickens (97.5%) produced chickens for only commercial purposes. Farms make income from the sale of live chickens and table eggs to fecal droppings to crop farmers and carcasses of dead chickens to breeders. The flock size in the surveyed farms ranged from 50-10,000 chickens. About ten (12.2%) of farms hosted 200-500 chickens, followed by 501-1000 birds (15.6% of farms), and 1001-2000 chickens (13.3% of farms). Regarding the age of the chickens, 33.3% were < 4 weeks, 38.9% were between 4 to 8 weeks and 27.8% were ≥ 8 weeks. The three main types of chicken were broiler (55%), layer (43%), and backyard chickens (2%). Three housing systems of chicken farming were deep litter system (77.8%), combination of the deep litter with battery cages (20.0%), and battery cages (2.2%). The deep litter housing system hosted 97.9% of the total chicken population (TCP). Ninety-three percent of farms affirmed using veterinary drugs for disease control. The veterinary drugs used were obtained from veterinary pharmacies, markets, and uncertified/unknown sources**.** Reasons for production, flock size, age of birds, housing system, as well as sources of day-old chicks, feed, and veterinary drugs are presented in Table 1.

Table 1. Characterization of different poultry farms surveyed

Variables	Number of farms (%)	Number of birds (%)
Reason for keeping birds		
Commercial	60 (66.7)	107100 (97.5)
Family consumption	12 (13.3)	140 (0.1)
Both (semicommercial)	18 (20.0)	2590 (2.4)
Number of birds per farm		
<200	11 (12.2)	7930 (7.2)
200-500	16 (17.8)	6900 (6.3)
501-1000	14 (15.6)	29000 (26.4)
1001-2000	12 (13.3)	35800 (32.6)
2001-10000	37 (41.1)	30200 (27.5)
Age of birds at study time		
< 4 weeks	30 (33.3)	45170 (41.1)
4-8 weeks	35 (38.9)	33450 (30.5)
≥ 8 weeks	25 (27.8)	31210 (28.4)
Housing system		
Deep litter	70 (77.8)	107490 (97.9)
Battery cage	2 (2.2)	2000 (1.8)
Both	18 (20.0)	340 (0.3)
Source of day-old chicks and feed		
AGROCAM	5 (5.6)	7500 (6.8)
ALIVET	5 (5.6)	3000 (2.7)
BELGOCAM	6 (6.7)	10000 (9.1)
NAPCAM	5 (5.6)	3000 (2.7)
SPC	18(20.0)	36900 (33.5)
ALIVET/SPC	5 (5.6)	1400 (1.2)
SOCAVE	6 (6.7)	4500 (4.1)
NAPCAM/SPC	5 (5.6)	4000 (3.6)
SPC/AGROCAM	9 (10.0)	16000 (14.6)
Unknown	26 (28.6)	23530 (21.4)
Source of veterinary drugs		
Veterinarypharmacy	52 (57.8)	89980 (82.1)
Market	22 (24.4)	7300 (6.7)
Unknown	16 (17.8)	12280 (11.2)

Total number of farms questioned = 90 Total number of chickens in farms questioned = 109830.

Biosecurity implementation in farms surveyed

Positive responses on biosecurity indicators for each region are presented in Table 2. Biosecurity indicators with more than 80% of positive responses in all three regions were concerning appropriate carcass disposal, rodent-proof, disinfecting feeders/drinkers regularly, prophylactic chemotherapy to healthy chickens, usage of veterinary drugs, and presence of diseases in the past three months. Biosecurity indicators with less than 50% positive responses in all three regions included awareness of biosecurity practices, washing/disinfecting of vehicles, on-farm necropsy, separation of chicken according to types and age, chickens occasionally allowed to move out of the poultry house. The mean BS ranged between 2 and 2.8 (Table 3). The difference was significant among the mean BS of Centre, Littoral, and West regions ($p< 0.001$). Regarding the obtained data, 12 chicken farms in the Centre region, 14 chicken farms in the Littoral region, and 13 chicken farms in the West region were at moderate risk (BS=2), while 18 chicken farms in the Centre, 16 chicken farms in the Littoral regions, and 17 in the West region were at high risk (BS=3). The BS means varied with the reason of keeping chickens as well as with the flock size (Table 3). The BS was significantly influenced by the farmer category ($p = 0.004$). Table 4 shows that the flock size did not significantly ($p > 0.05$) affect the BS and disease outbreaks. However, variations of mean BS amongst farms were significant ($F = 4.171$, $p = 0.046$) and the highest mean BS was in farms with ≤ 1,000 chickens. Disease outbreaks did not correlate with BS, showing a tendency to increase disease outbreaks with increasing BS. The reason for keeping chickens did not significantly ($p>0.05$) affect disease outbreak within the last three months. In general, chicken farms with low flock size were more at risk than high flock size farms and had disease outbreaks as a major constraint.

Table 2. Percentage of poultry farms with positive responses on indicators of biosecurity events

Indicators of biosecurity events	Number (%) of farms with "yes" response		
	Centre	Littoral	West
Events outside the premises			
Awareness of biosecuritypractices	9 (30.0)	8 (26.7)	13 (43.3)
Certified sources of quality chicks and feeds	19 (63.3)	8(26.7)	23(76.7)
Acquisition of second-hand equipment	7 (23.3)	20 (66.7)	13 (43.3)
Purchase of veterinary drugs in veterinary pharmacy	18 (60.0)	14 (46.7)	13 (43.3)
Farm boundary events			
Visitorsallowedintopremises	11(36.7)	14 (46.7)	5 (16.7)
Washing/disinfecting of vehicles	12(40.0)	4 (13.3)	8 (26.7)
Events between farm boundary and poultry house			
Presence of good feed storage facility	27 (90.0)	15(50.0)	24 (80.0)
Appropriate carcass disposal	22 (73.3)	4 (13.3)	9 (50.0)
On-farm necropsy	14 (46.7)	0 (0.0)	11 (36.7)
Certified commercial feed sources only	19 (63.3)	8 (26.7)	15 (50.0)
On-farm carnivores (dogs and cats)	19 (63.3)	8 (26.7)	13 (43.3)
Washing hands/shower before and after handling chickens	19 (63.3)	10 (33.3)	22 (73.3)
Rodent-proof	25 (83.3)	28 (93.3)	27 (90.0)
Residence of farm workers within premises	18 (60.0)	28 (93.3)	11 (36.7)
Functional footbath at the entrance of poultry house	12 (40.0)	8 (26.7)	17 (56.7)
Events inside the poultry house			
Separation of chicken according to types and age	27 (90.0)	22 (73.3)	19 (63.3)
Proper ventilation	15 (50.0)	11 (36.7)	19 (63.3)
Availability of clean water	15 (50.0)	14 (46.7)	22 (73.3)
Frequent changing of bedding with dry ones	22 (73.3)	14 (46.7)	17 (56.7)
Chickens occasionally allowed to move out of the poultry house	15 (50.0)	1 (3.3)	5 (16.7)
Washing/disinfecting poultry house prior to restocking	30 (100.0)	30 (100.0)	30 (100.0)
Washing feeders/drinkers regularly	30 (100.0)	27(90.0)	27 (90.0)
Disinfecting feeders/drinkersregularly	30 (100.0)	26 (86.7)	8 (26.7)
Isolation of apparently sick chickens	30 (100.0)	25 (83.3)	25 (83.3)
Prophylactic chemotherapy to apparently healthy chickens	30 (100.0)	30 (100.0)	30(100.0)
Usage of veterinary drugs	30 (100.0)	5 (16.7)	27 (90.0)
Consultation of veterinarians only in case of problems	30 (100.0)	25 (83.3)	11 (36.7)
Presence of diseases in the past three months	30 (100.0)	30 (100.0)	30 (100.0)

Total number of farms surveyed = 90

Table 3. Mean biosecurity score of poultry farms classified according to different parameters

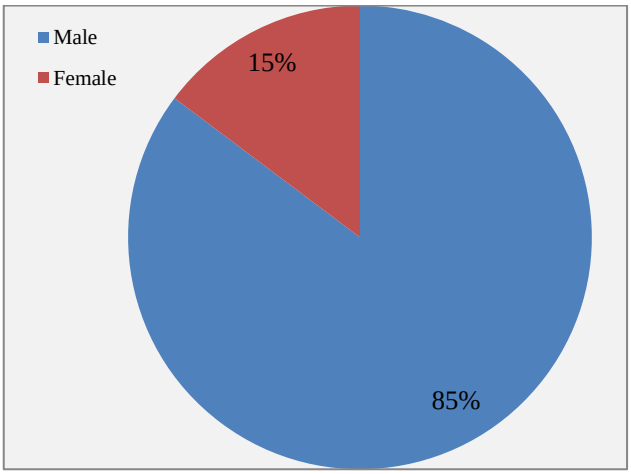
Parameter	Mean biosecurity score (Number of farms)
Reason for keeping chickens	
Commercial	2.3 ± 0.2 (60)
Semi-commercial	2.6 ± 0.1 (12)
Family use only	2.8 ± 0.1 (18)
Number of chickens per farm	
<200	2.7 ± 0.2 (11)
201-500	2.6± 0.1 (16)
501-1000	2.5 ± 0.1 (14)
1001-2000	2.4 ± 0.1 (12)
>2000	2.0 ± 0.2 (37)
Farmer category	
Trained	2.2 ± 0.2 (23)
Untrained	2.6 ± 0.1 (67)

Total number of farms surveyed = 90

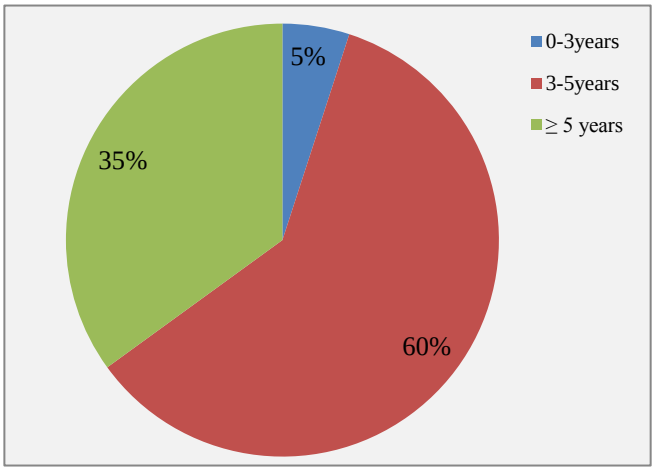
Table 4. Mean biosecurity scores in poultry farms in three regions of Cameroon

Study areas	Mean biosecurity scores	Number of farms (%)	Number of farms with the disease outbreak	Flock size
Center	2	12 (40.0)	5 (16.7)	>1000
	3	18 (60.0)	6 (20.0)	≤1000
Littoral	2	14 (46.7)	4(13.3)	>1000
	3	16 (53.3)	6 (20.0)	≤1000
West	2	13(43.3)	4 (13.3)	>1000
	3	17 (56.7)	8 (26.7)	≤1000

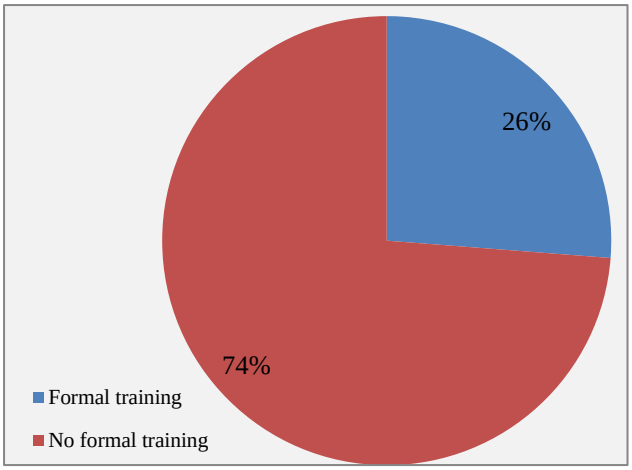
Total number of farms surveyed = 90



(a) Gender



(b) Work experience



(c) Qualifications in poultryfarming

Figure 2. Characteristics of participating poultry farmers (n = 90) in the present study

DISCUSSION

The present study assessed the level of implementation of biosecurity practices in chicken farming and their possible impacts on poultry quality in three regions of Cameroon. Poultry farmers were mostly males and most of them were not trained in poultry production or biosecurity measures. Similar to the present study, previous studies have also shown that poultry farming is male dominant. Women are usually more involved in activities, such as trade, and growing crops (Abdurrahman et al., 2016; Fongang Fouepe et al., 2017). The level of farmer training significantly influenced biosecurity scores. Farms surveyed were using three main systems of production, but the main production system was the commercial system. This production system is mainly used because of its high productivity and incomes. Semi-commercial and family production systems also contribute to the provision of chicken and eggs as well as income generation but at a low level.

Unfortunately, during the present study, disease outbreaks were still the main constraint faced by all the farmers (100.0 %) as earlier reported by Fongang Fouepe et al. (2017). This constraint could be responsible for the uncontrolled usage of veterinary antibiotics as previously mentioned by Gondam et al. (2016) and Guetiya et al. (2016). In the present study, the flock size of most farms ranged from 200 to 2000 chickens and the chicken population was more concentrated in this range. Abdurrahman et al. (2016) reported a different situation in poultry farms of Zamfara State, Nigeria, where the total chicken population was represented by flock sizes of < 200 chickens and 2001-10000 chickens. The same common flock size was reported by Maduka et al. (2016) in Jos state, Nigeria. Flock size less than 200 chickens was found in family farms, while flock size of 200-500 was found in farms with both semi-commercial and strictly commercial production systems. In fact, family farms in this study were used for only family consumption purposes, and normally, the flock size should be small. Previous studies in Nigeria reported that flock size with less than 200 poultry was found in family farms (Geidam et al., 2011). However, flock sizes higher than 500 chickens were found in both family and commercial farms (Esiobu et al., 2014). A report in Cameroon on the traditional poultry sector revealed the flock size within the range of 4,000-10,000 broilers and 2,000 to 5,000 layers per farm (Ekue et al., 2000; Fongang Fouepe et al., 2017). Broilers were more representative in TCP, followed by layers, while backyard chickens were less. The high representativity of broilers could be explained by the short time of production (six weeks) compared to the production times of the backyard (at least 4 months) and layer (at least 18 months). Muhammad et al. (2010) and Maduka et al. (2016) reported the presence of a higher number of broiler farms than layer farms but more layers represented the TCP in stocking capacity. Chickens were mainly housed on a deep litter during the survey and this could be explained by the fact that the litter is cheaper and available. This observation was reported in previous studies conducted in Nigeria (Muhammad et al., 2010; Geidam et al., 2011; Maduka et al., 2016).

Biosecurity is an important tool for the limitation of disease outbreaks and economic losses as earlier mentioned by Conan et al. (2012) but in the present study, the level of awareness on biosecurity amongst the surveyed chicken farmers was too low. This lack of awareness about biosecurity could be explained by the fact that the majority of chicken farmers were not trained in poultry farming. Poultry farms with trained farmers had a lower mean BS than farms with untrained farmers. During training on poultry production, farmers become aware of biosecurity measures and their importance to prevent the occurrence of some common diseases.

In fact, biosecurity involves a set of measures known as biosecurity measures that can be used for farm classification according to the biosecurity score system. In the present study, biosecurity was attributed to each biosecurity measure, leading to the classification of chicken farms surveyed in two groups according to the USAID (2009) biosecurity score system. These two groups include moderate and high-risk levels. This classification could be justified by the weak awareness of farmers on biosecurity as observed during the survey. Maduka et al. (2016) reported different results in Jos, Nigeria, where chicken farms were classified as good, very good, and excellent according to BS. Nigerian farmers had a good knowledge of the basic biosecurity measures needed for day to day running of poultry farms especially the ones dealing with sanitation of the farm (Ajewole and Akinwumi, 2014). Diseases were the major constraints in investigated farms as a result of a failure in biosecurity implementation. Small-scale chicken farms were at a high-risk level, probably because their flock sizes were small, they did not care about it and the economic loss could not be important. In addition, Negro-Calduch et al. (2013) reported that biosecurity measures are rarely applied in small-scale production units. In the

present study, the flock size and the training level of the farmer were major reasons for not complying with certain biosecurity measures. This observation can be justified by the simple fact that failure in biosecurity was remarkable in chicken farms of small flock sizes where farmers were not trained. Furthermore, chicken farms hosting small flock sizes were mostly classified at high risk.

It was also noted that farms located in the Centre were mainly in proximity to humans compared to those in the other two regions, but the movement of people and other animals as well as allowing vehicles inside the gate were observed in the three regions. Several studies identified the proximity of poultry sheds to humans, roads or water bodies, and the movement of objects people and other animals in and out of the sheds, allowing vehicles inside the gate as risk factors for H5N1 outbreaks (Ahmed et al., 2012; Gilbert and Pfeiffer, 2012; Osmani et al., 2014). Failure in biosecurity implementation was remarkable in isolation of apparently sick chickens and functional footbath at the entrance of poultry house in the west region. In the present study, the low percentages of positive responses on biosecurity measures, such as vehicles drove into farm premises without washing and disinfection, farmworkers living outside the farm premises in the majority of farms, separation of chickens according to the type and age, and the use of second-hand equipment, were important components of risk factors. The occurrence of disease outbreaks within the last three months of the present study was one of the consequences of this failure to comply with biosecurity measures. This consequence could be great as several chicken farmers in the three regions investigated affirmed to consult veterinarian only in case of a problem. Other risk factors associated with disease outbreaks were receiving visitors to the farms, rodent-proof, on-farm carnivores, absence of good storage facilities, and non-realization of on-farm necropsy. Receiving visitors to the farms and farm workers living outside the farm premises were risks reported in Nigeria by Fasina et al. (2011), Wakawa et al. (2012), and M'etras et al. (2013). These risks could have serious implications on the spread of contagious poultry diseases by people as well as being of public health importance regarding zoonoses such as avian influenza as reported by Abdurrahman et al. (2016). In this present study, it was observed that failure in biosecurity implementation was associated with the number of chickens in the farm, thus more important was the flock size, more attention was paid to the biosecurity although farms of all flock sizes were at risk. The mean BS was higher in the West region than that in the other regions. Similarly, Kouam and Moussala (2018) revealed a higher BS for poultry farmers from the West region. A previous study reported that farmers have greater experience in the West (Ngandeu and Ngatchou, 2006). In addition, the capital city of the West region, Bafoussam, is the headquarter of the Cameroonian Association of poultry production professionals known under the acronym of IPAVIC. Thus, West region farmers are supposed to be more endowed with the latest information and innovations in poultry production compared to farmers in the other regions.

Poultry farming is a growing sector in Cameroon and contributes to income-generating (Esiobu et al., 2014; Maduka et al., 2016; Fongang Fouepe et al., 2017), and its products constitute good sources of proteins of high quality and other nutrients for human nutrition (Altan et al., 1993; Seuss-Baum and Nau, 2011). Failure in biosecurity measures could lead to disease outbreaks, causing mortality and important economic losses (Conan et al., 2012). Moreover, disease outbreaks result in the improper use of veterinary drugs, leading to the occurrence of their residues in chicken products (Guetiya et al., 2016; Gondam et al., 2016). It could also lead to drug resistance and transmission of diseases from one farm to another, one animal species to another, and from animal to human.

CONCLUSION

Three chicken farming systems, including family, semi-commercial, and commercial, are applied by chicken farmers in Cameroon. Chicken production generates both incomes and nutrients of high quality but biosecurity measures are not well-practiced and implemented, and this can lead to disease outbreaks and widespread use of veterinary drugs for disease control, resulting in drug resistance and residuals of veterinary drugs in chicken products. It is, therefore, urgent to put in place a good management system that will contribute to increase farm productivity and provide safer chicken products to consumers to fulfill the potential of chicken farming as an engine for the development of Cameroon.

DECLARATIONS

Authors' contributions

Fabrice De Paul Tatfo Keutchatang designed the research protocol, collect data, and drafted the manuscript under the guidance of Gabriel Medoua Nama and Germain Kansci. Data were analyzed by Fabrice De Paul Tatfo

Keutchatang and. Isabelle Sandrine Bouelet Ntsama All activities were coordinated by Gabriel Medoua Nama and Germain Kansci. Finally, all authors read and approved the final edition of the manuscript.

Competing interests

The authors declare that there is no conflict of interest

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Egg Production, Fertility, Hatchability and Luteinizing Hormone Profile of Progesterone Hormone Injected to Arabic Gold Chicken (*Gallus turcicus*)

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ABSTRACT

The production and reproduction performance of chicken depends on their hormonal status, especially progesterone hormone, which has been known to correlate with egg production. The present study aimed to analyze the effect of progesterone hormone injection on Arabic Gold chickens (*Gallus turcicus*) regarding egg production and luteinizing hormone concentration in blood plasma. A total number of 60 Arabic Gold chickens aged 26-weeks were divided into three groups based on injected hormone concentration (P_0: control; P_1: 1 mg/chicken; P_2: 2 mg/chicken). The study was conducted using a completely randomized design and the obtained data were analyzed with a descriptive analysis for qualitative data and one-way analysis of variance followed with Duncan's Multiple Range Test as a post hoc test for the quantitative data. The results presented that progesterone hormone injection had a significant effect on hen day production two and six weeks after injection. The P_1 group was able to reach its peak production (82.9%) at week 29, while the P_2 group reached its peak at week 26 (78.9%). In addition, it was found that the P_2 group produced a soft-shelled egg and double egg yolk. Progesterone injection led to no significant effect on the egg weight, shape index, fertility, embryo viability, hatchability, and chick weight at hatch. The luteinizing hormone concentration was higher in P_2 (1.52 ng/ml), compared to P_0 (1.36 ng/ml) and P_1 (1.34 ng/ml) groups. It was concluded that progesterone hormone injection during the production phase of Arabic Gold chicken had a significant effect on egg production and caused varying egg production peak and luteinizing hormone concentration.

Keywords: Arabic Gold chicken, Egg quality, Hen day production, Luteinizing hormone, Progesterone

INTRODUCTION

Arabic gold chicken (*Gallus turcicus*) is a chicken breed widely reared in Indonesia for egg production. The breed is preferred by the local farmers due to its better Feed Conversion Ratio (FCR), which is around 3 to 4 (Rizal et al., 2015), compared to the native Kampung chicken for which the ratio is 6.73 on average (Pagala et al., 2019). The Arabic Gold hens begin to lay eggs by the age of five months and its peak production would be achieved at the age of 8-9 months (Indra et al., 2013). Some factors that have been known to determine egg production of laying hens include the genetic characteristics, age, nutrition intake, and rearing system (Sarica et al., 2012). Pirsaraei et al. (2008) added that hormonal status also plays a significant role in egg production.

Egg production is a complex process that involves hormonal interaction within chickens. Reproductive hormones, such as progesterone, play an important role in reproductional functions in fowls (Scanes, 2015; Han et al., 2017), including the development of reproductive organs, ovulation, albumen synthesis, eggshell's formation, and egg oviposition (Mishra et al., 2019). The progesterone hormone is one of the important steroid hormones that affects cattle and fowl (turkeys and laying egg hens) reproduction (Scanes, 2015). The progesterone hormone has been acknowledged to have a positive correlation with egg production. The progesterone hormone would promote the pre-ovulation release of luteinizing hormone (LH) that impacts egg production (Zaghari et al., 2009).

A previous study indicated that the secretion of progesterone hormone correlates with egg production on

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Guinea Fowl by providing an exogenous progesterone hormone to the feed and drinking water (Adeyinka et al., 2007). Aside from feed and drinking water, the exogenous progesterone can also be given by injection. Zaghari et al. (2009) conducted a study on the effect of progesterone injection on broiler chickens. In a study performed by Ito et al. (2011), it was found that progesterone hormone injection 20 hours before ovulation affects the release of sperm storage tubules in the ureterovaginal junction.

Although effort has been made to increase egg production (Samadi et al., 2020), there is still no report on the enhancement of egg production in Arabic Gold chicken regarding hormone application. Therefore, this study aimed to analyze the effect of progesterone hormone injection on Arabic gold chicken during the production phase towards the performance of egg production, fertility, and LH hormone profile.

MATERIALS AND METHODS

Ethical approval

This study has been approved by the Animal Ethics Committee of Universitas Brawijaya with number 001-KEP-UB-2020 and has been declared to have fulfilled the ethical feasibility of the research. All procedures and care for chickens were in accordance with institutional guidelines for the use of animals in the study.

Experimental design

The study was conducted as experimental research using a completely randomized design in the poultry installation unit of the Politeknik Pembangunan Pertanian Malang, Indonesia, from August to December 2019. A total of 60 Arabic Gold chickens were divided into three treatment groups with 10 replications each and 2 chickens for each replication. The current study had three roosters at approximately 12 months of age to produce semen for the artificial insemination process twice a week. The data of this study were collected from a week of first chicken insemination or at the age of 26 weeks old.

Research sample

The Arabic Gold chickens used in the current study were obtained from "Mitra Ternak Juara Group" farm in Probolinggo, Indonesia, and has been vaccinated with Marek's Disease, Infectious Bursal Disease (IBD), Infectious Bronchitis (IB), Infectious Laryngo Tracheitis (ILT), and Newcastle Disease (ND) from the age of 1 day to 4 months. The chickens were firstly reared from the age of 16 weeks for battery cage and feed adaptation by using commercial laying hens feed containing 14-16% protein, 5-7% crude fiber, 4-7% crude fat, 5-7% ash, and 2500-2700 Kcal/kg metabolizable energy, given *ad libitum* along with the drink. The hens were inseminated at the age of 25 weeks until 33 weeks and kept in individual coops with 14 hours of light and 10 hours of dark system daily.

Research treatments

The research treatment of this study entailed the injection of different concentrations of progesterone hormone to the Arabic gold chickens. The progesterone hormone used for the present study was P0478-5G produced by Tokyo Chemical Industry (TCI) Co, Ltd. The progesterone hormone injection doses for experimental hens included 0% or no injection (control), 1 mg/chicken (P_1), and 2 mg/chicken (P_2). The solvent used in the current study was sesame oil (0.5 ml of oil/ chicken, Correa et al., 2005). The progesterone hormone injection was performed subcutaneously. The hormone was injected five hours prior to the estimated ovulation as calculated from the previous egg-laying time. The injections were administered twice a week for four consecutive weeks (on weeks 28 to 31). Eggs were collected from two weeks before the injection time until two weeks after the injection to observe the egg production performance. The eggs from hormone-injected hens were then hatched at the temperature of 37.5°C with a humidity level of 55-60% (Mohan et al., 2015).

Hen day production

The observed variables in the present study included hen day production (HDP), egg qualities, and LH profile. The HDP was measured by comparing the number of eggs produced in a day with the number of hens; the result was then multiplied by 100% (Pirsaraei et al., 2008). The HDP values were calculated for weekly production.

Egg qualities

The observed egg quality parameters included weight, size (length and width), shape index, and eggshell quality. Egg weight was measured using an analytic scale. The shape index is the comparison of egg width and length diameters multiplied by 100% (Duman et al., 2016). The width and length of the egg were measured using vernier calipers. The exterior egg quality was observed by checking its surface, whether smooth, rough, or soft. The egg fertility percentage is achieved by dividing the number of fertile eggs by the total amount of observed eggs, the result is then multiplied by 100%. The egg fertility was measured by the candling method. The candling was performed on the seventh day of the hatching period.

Furthermore, the egg candling was also performed on the fourteenth and eighteenth days of the hatching period to determine the embryo viability. The embryo viability is the percentage of the live embryo at 14 or 18 hatching days of fertile eggs multiplied by 100% (Reijrink et al., 2010). The hatchability is the percentage of hatched eggs of the embryo of fertile eggs multiplied by 100% (Indrawati et al., 2015).

Luteinizing hormone profile

The concentration of LH in the blood plasma of subjects was measured two hours before and after the injection, the result was analyzed using Enzyme-linked Immunosorbent Assay (ELISA).

Statistical analysis

The qualitative data were analyzed descriptively, while the quantitative data were analyzed using analysis of variance, and followed by Duncan's Multiple Range Test. P-value less than 0.05 was considered statistically significant ($P<0.05$). The results were expressed as the mean ± standard deviation (SD).

RESULTS

Hen day production

The Arabic Gold chickens began to lay eggs at the age of 20 weeks and the initial egg production was 5.2%. The HDP of Arabic Gold chicken injected with different progesterone levels in this study is presented in Figure 1. Figure 1 demonstrates that egg production gradually decreases as the chickens matured indicating varying peak production time frames. The control group receiving no treatment (P_0) experienced a decrease in production and its peak was on week 27 with HDP at 75.7±22.6% when other groups did not receive any progesterone hormone injection. The P_1 group reached its peak production on week 29 with HDP at 82.9±9.64% while the P_2 group reached its peak production on week 26 with the HDP at 77.9±12.3%. The HDP data during the progesterone hormone injection treatment until week 33 is presented in Table 1.

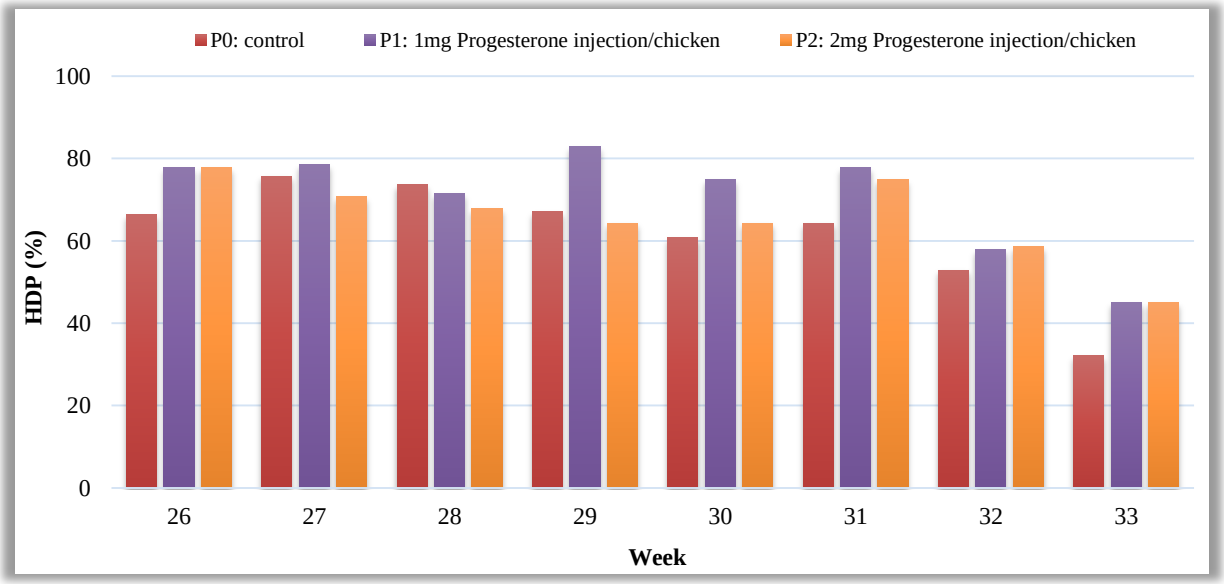

Figure 1. Hen day production of Arabic Gold chickens from week 26 until week 33 with different injected progesterone levels.

Table 1. Average hen day production of Arabic Gold chickens injected with different progesterone levels.

Treatment \ HDP (%)	26	27	28	29	30	31	32	33
P_0	66.4±25.4^a	75.7±22.6^a	73.6±14.7^a	67.1±19.4^a	60.71±23.6^a	64.3±11.7^a	52.9±11.8^a	32.1±13.6^a
P_1	77.9±18.9^a	78.5±16.5^a	71.4±4.8^a	82.9±9.64^b	75.00±15.52^a	77.8±12.8^a	57.9±12.8^a	45.0±15.1^b
P_2	77.9±12.3^a	70.7±13.2^a	67.9±8.4^a	64.3±14.67^a	64.29±16.1^a	75.0±13.6^a	58.6±11.6^a	45.0±10.7^b
SEM	3.81	2.30	1.67	5.77	4.29	4.13	1.80	4.29
p value	0.35	0.61	0.45	0.02	0.22	0.11	0.52	0.05

Different superscripts on each column indicate significant differences ($P < 0.05$). The progesterone hormone was injected at 1 mg/chicken for P_1 and 2 mg/chicken for P_2 from week-28 to 31.

Egg qualities

The egg quality parameters observed in the current study included egg weight, size (width and length), shape index (%), and the eggshell. The exterior egg quality measurement in the present study is presented in Table 2, while the eggshell quality is indicated in Table 3.

Egg fertility and hatchability are two essential parameters in egg reproduction to produce the day-old chick. The egg fertility percentage was measured by conducting the candling test on the seventh day of the incubator. Table 4 indicates the ANOVA test of fertility among treatments. The obtained results revealed no significant difference between the treatment groups and the control group (P>0.05). The embryo viability during the hatchery period was observed through the candling test. In the present study, the embryo observed on day 14 and day 18 based on grouping indicated that the death occurred in the mid-phase. The embryo viability showed the number of living ones from the fertile eggs that fit the hatchery stage. The results of this study showed that the embryo viability due to hormone injection was not different (P>0.05) among all treatments. Therefore, it could be concluded that the progesterone hormone injection did not affect the chicken embryo viability which was supported by a relatively high result of all groups. The hatchability found in this study did not significantly differ among treatment groups (P>0.05), the hatched eggs were derived from layer hens with similar age (28 to 32 weeks).

Luteneizing hormone profile

Progesterone stimulates the LH hormone release prior to ovulation. The ELISA analysis results on the LH hormone concentration in the current study are presented in Figure 2, and the results of ANOVA analysis of LH concentrations are presented in Table 5. Figure 2 presents the progesterone hormone injection five hours before the ovulation could stimulate the LH hormone release. The ANOVA analysis (Table 5) indicated that the LH concentration was not significantly different (P > 0.05) among treatment groups although P_2 had a higher LH concentration than other treatments. A high dose of progesterone treatment could increase the LH concentration in the plasma to 1.52 ng/ml three hours before the predicted ovulation, or around 28 hours before the next oviposition.

Table 2. Exterior egg quality of Arabic Gold chickens injected with different progesterone levels.

Parameter / Treatment group	P_0	P_1	P_2	SEM	*P*-value
Egg weight (g)	38.9±1.58	39.6±1.09	39.7±1.06	0.27	0.27^{ns}
Egg width (cm)	35.7±0.65	36.1±0.67	36.2±0.57	0.15	0.18^{ns}
Egg length (cm)	46.4±1.16	46.8±0.63	46.4±0.54	0.13	0.49^{ns}
Shape index (%)	77.1±1.10	77.3±3.62	78.2±10.8	0.34	0.19^{ns}

ns: no significant difference between each treatment group (P > 0.05)

Table 3. Eggshell quality of Arabic Gold chickens injected with different progesterone levels.

Parameter / Treatment group	P_0	P_1	P_2
Smooth eggshell (%)	93.2	88.1	87.7
Speckled smooth eggshell (%)	4.44	5.03	8.10
Rough eggshell (%)	2.37	6.85	4.25
Soft shell (%)	0	1.96	3.49
Double egg yolk	0	0	1.62
White colour (%)	82.9	63.9	72.8
Brown colour (%)	17.1	36.1	27.2

Table 4. Egg fertility evaluation of Arabic Gold chickens injected with different progesterone levels.

Parameter \ Treatment group	P_0 N = 123	P_1 N = 129	P_2 N = 121	SEM	*P*-value
Egg fertility (%)	88.2±8.74	83.6±3.62	88.1±10.8	1.51	0.54^{ns}
Embryo viability to day 14 (%)	97.2±5.91	96.1±12.2	97.4±4.16	0.40	0.93^{ns}
Embryo viability to day 18 (%)	94.9±8.86	91.6±14.6	94.1±6.07	1.08	0.73^{ns}
Hatchability (%)	89±13.7	84.4±14.8	83.9±10.4	1.62	0.64^{ns}
DOC weight (g)	25.9±1.05	26.8±1.43	26.8±0.95	0.32	0.13^{ns}

N: Number of hatched eggs, ns: No significant difference between each treatment group (P > 0.05).

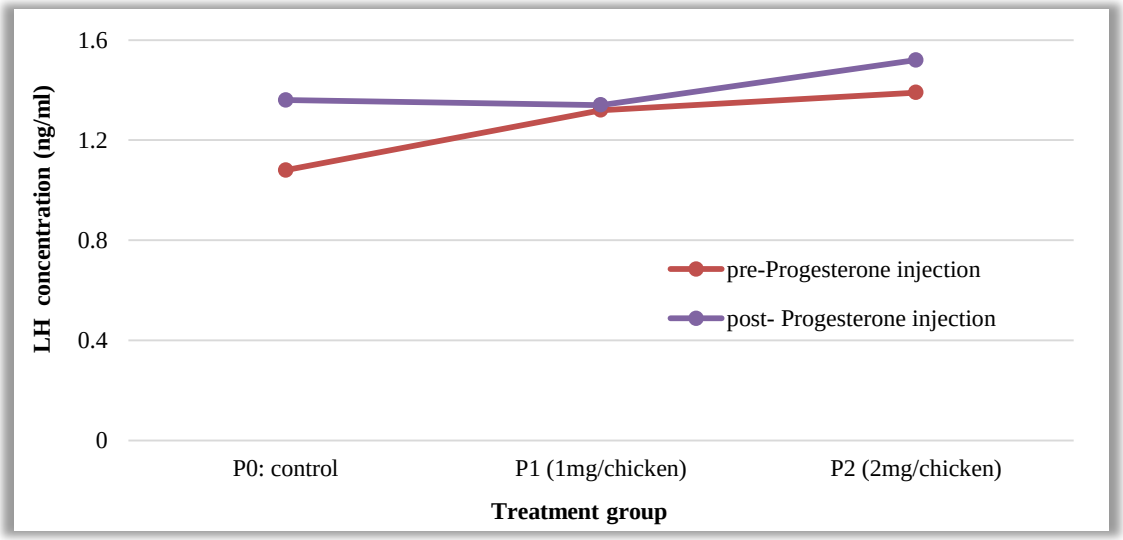

Figure 2. Luteinizing hormone concentration on each treatment group of Arabic Gold chickens from week 26 until week 33 with different injected progesterone levels.

Table 5. Luteinizing hormone concentration of Arabic Gold chickens injected with different progesterone levels.

Treatment	P_0	P_1	P_2	*P*-value
Pre-progesterone injection	1.08±0.21	1.32±0.61	1.39±0.52	0.32^{ns}
Post-progesterone injection	1.36±0.35	1.34±0.44	1.52±0.36	0.52^{ns}

ns: No significant difference between each treatment group (P > 0.05).

DISCUSSION

The performance of egg production can be analyzed from the number of eggs produced by each chicken (Javed et al., 2003). The production parameter is achieved from the number of daily egg production, which is then converted to HDP, after that, the HDP collection is calculated to determine the weekly average. The HDP provides essential information to realize the daily egg production (Farooq et al., 2002; Pirsaraei et al., 2008).

The Progesterone hormone injection was administered during the production phase (28-31 weeks), the egg production data was collected from two weeks before the treatment (week-36) until the egg production reached the percentage of 74.2%. Production data was calculated for 8 weeks started from week 26 until 33 (2

weeks before the treatment, 4 weeks during the treatment, and 2 weeks after the treatment).

Table 1 indicates that progesterone hormone injection affects egg production ($P<0.05$) in week two of the treatment process (week-29) and two weeks after the treatment (week 33). The present study applied a low-dose progesterone hormone injection (1 mg/chicken), which could improve egg production only until week 29 or similar to the second week of the treatment, followed by a decrease in egg production. The low-dose progesterone treatment is proven to achieve the highest production compared to the control group. Moreover, the high dose of progesterone injection (2 mg/chicken) represented that the egg production dropped for three weeks after the initiation of treatment, but started to increase again from the last week of the treatment (week 31) before the egg production decreased again. This finding was in line with a study by Zaghari et al. (2009) that indicated that progesterone injection could significantly affect egg production.

The chicken that gets progesterone injection produces fewer stable eggs than those who receive no treatment. A similar finding by the current study presented that after the progesterone injection was administered, egg production of turkeys (Bacon and Liu, 2004), broilers (Liu and Bacon, 2005), and Japanese quail (Liu and Bacon, 2004) decreased. On the last observation of week 33, all groups experienced a decrease in egg production, but treatment groups had a higher decrease (45%), compared to the control group (32%).

The characteristics of egg quality depend on several factors, namely the age and genus of the chicken, the nutrient of the feed, and chicken weight (Sarica et al., 2012). Besides, other factors that affect egg production include the maintenance system, the oviposition time, molting induction, general stress, heat stress, diseases, addition of an exclusive product to the feed, and the hormonal status of the chicken (Pirsaraei et al., 2008; Ahmadi and Rahimi, 2011). A stressful situation and other physiological conditions can generally affect oviposition, which eventually affects egg quality (Ahmadi and Rahimi, 2011). The treatment of progesterone hormone injection in this study for four weeks during the egg production had no significant effect on the exterior egg quality ($P>0.05$) due to the similar treatment on feed, maintenance method, and careful treatments provided to all chicken to avoid stress.

Factors that affect the egg weight are the chicken age, strain and breed, feed nutrients, chicken weight, egg-laying time, environmental temperature, and diseases (Bell and Weaver, 2002). The egg weight of Arabic Gold chicken breed in the present study was in the normal range. The range was in agreement with a study conducted by Bakar et al. (2005), which indicated that the weight of Arabic chicken breed could range from around 31 to 52 grams per egg. Table 3 presents that the three treatments of the Arabic chicken breed do not differ significantly regarding the egg weight ($P>0.05$). The result of the study was lower in value than the results from a study conducted by Yumna et al. (2011) in which the weight of eggs was 46.81 ± 2.22 grams during the age of 32 to 36 weeks. This difference could be due to the age difference of the subjects used in these two studies. The progesterone hormone affects the eggshell formation in the reproductive tract (Zhang et al., 2019) which is assumed to affect the egg weight; nevertheless, the finding showed different results. This means that the hormone injection does not have any impact on the egg weight.

Egg shape index (SI) is the width and length ratio of an egg, it is an essential criterion in determining the quality of an egg. An egg that does not have a standard shape, such as misshapen, too round, or a flat surface on one side, is not included in grade AA (almost perfect) or grade A (slightly under AA) since it does not comply with the standard oval egg shape (Duman et al., 2016). The shape index correlates with the egg shape. The variation of the egg index is between 0.65-0.82 (Yuwanta, 2010). The higher the egg shape index (0.82), the more round the egg shape. On the contrary, the lower the egg shape index, the more oval and tapering the egg. Yuwanta (2010) also explains that the egg shape index will progressively decrease as laying time increases; right after the egg is laid, the egg shape index ranges around 0.77, but at the end of laying time, the index is around 0.74. The current study used eggs derived from chickens during the mid-production time, eventually, their shape index was about 77-78%.

The variable of eggshell quality underwent a descriptive analysis. On treatment P_2, there was a double egg yolk occurrence with a percentage of 1.62%. The double egg yolk occurred due to the progesterone hormone injection that stimulated the LH hormone release before the ovulation which resulted in the occurrence of double ovulation from the mature follicle. After the double egg yolk started the hatching stage, on day seven, it is found infertile as a result of the candling test. This finding was in line with Buchanan et al. (2002) who reported two or more follicles on each position of the hierarchy that could produce two or more follicles in a day, an egg with multiple ovulation cannot be suitable to undergo the incubation.

Several studies have indicated that progesterone correlated with eggshell quality. The Progesterone hormone affects the ovary and hypothalamus leading to stimulating the surge of LH used for the ovulation process. The progesterone injection can have an impact on the quality of the eggshell during the calcification initiation (Zhang et al., 2019). The findings of the current study indicated that the progesterone hormone injection resulted in the soft-shell egg whose percentage was higher for the high-dose treatment P_2 (4.25%), compared to the P_1 treatment that reached 1.96%. The soft eggshell occurrence was similar to a study performed by Zaghari et al. (2009) concluding that progesterone injection caused an increase in eggshell qualities with a softshell during the first week of the hormone injection initiation. The double egg yolk and soft shell are considered to increase ovulation although these eggs are not suitable for hatching and not to be counted as good eggs (Zaghari et al., 2009). Liu and Bacon (2005) also observed a higher occurrence of the softshell eggs after the injection of progesterone in the broiler chicken farm.

The shell color is one of the parameters of eggshell quality (Liu et al., 2010). Eggshell color is a result of a stack of pigment during the egg formation process in the oviduct. The type of pigment depends significantly on breed type and its genetic characteristics (Liu and Cheng, 2010). The Arabic chicken breed is known to produce eggs with white to the light brown shell which resembles the eggs of free-range chicken (Yumna et al., 2011). In the present study, the color of the shell was still within the normal range. The shell color depends on the pigment produced by shell glad during the shell formation. The color of the egg can be determined when it is inside the uterus and the end of the oviduct. During the shell formation, the epithelial cells on the surface of the shell glands (uterus) start to synthesis the color pigments (Baylan et al., 2017).

Egg fertility and hatchability are two of the essential parameters in egg reproduction to produce the day-old chick (King'ori, 2011). These parameters depend on genetic and environmental factors. Both egg fertility and hatchability measurement in the current study was estimated 83-89%. King'ori (2011), explained the factors affecting fertility and hatchability included nutrient, chicken in the flock, egg factors, hatchery (natural and artificial incubation), and environmental factors.

The spermatozoa are inseminated to the cloaca, and consequently stored for several days in the sperm storage tubule (SST) located between the uterovaginal junction (UVJ) and infundibulum. The site has a receptor for progesterone and estrogen (Yoshimura et al., 2000) that is shown to determine the fertility rate. The correlation between the progesterone hormone and egg fertility was supported by the finding of Ito et al. (2011) that reported progesterone as one of the factors causing the spermatozoa release towards the fertilization site, which could be active for 20 hours after the oviposition. The highest egg fertility of Arabic gold chickens in this study was found at 2 mg progesterone hormone injection (P_2 treatment) which was at 88.1±10.8%. This finding was higher compared to the finding by Astomo et al. (2016), showing that the egg fertility of the Arabic chicken breed was around 62.5-79.2%.

Hatchery process, the death of embryo detected through the candling test and is classified into three phases, including the death of the initial embryo on day 10 (5 to 10 days), death of the embryo in the middle phase, observed on day 18, and death in the final phase of hatchery process which means day 21, and the embryo would not hatch (Larivière et al., 2009). This study observed the embryo death on days 14 and 18indicating that the death occurred in the mid-phase. The embryo viability indicated the number of living ones from the fertile eggs that fit the hatchery stage. The results showed that the progesterone hormone injection did not give significant differences ($P>0.05$) on embryo viability between all treatments Therefore, it can be concluded that the progesterone hormone injection did not affect the chicken embryo viability proven by a relatively high result of all treatment groups.

The hatchability is a critical factor of hatching in the breeding farm, it depends on many factors, such as hens in the flocks, egg fertility, and egg handling (Liu and Ngadi, 2012). There was no significant difference among treatment groups in terms of hatchability ($P>0.05$) for the hatched eggs derived from layers aged 28-22 weeks. The obtained result was higher in value, compared to a study conducted by Astomo et al. (2016) that reported the hatchability of the Arabic chicken breed for natural breeding to get the highest percentage of 76.7%. It was also stated that the affecting factors included egg quality, nutrition of hens, duration of egg storing, cage quality (temperature and humidity), and the hens quality as the egg producer. In the current study, the layer hens were selected according to specific parameters, qualified feed, good egg quality, the eggs were stored at 18ºC temperature before entering the incubation machine, therefore, the hatchability rate was relatively high.

The hatching weight of Arabic chicken breed due to progesterone hormone injection in this study did not

significantly differ among treatment groups (P>0.05). The result from hatching weight in this study reported a relatively lower one than a study performed by Astomo et al. (2016) that stated the hatching weight of Arabic chicken breed ranges around 29.4 g, and was significantly affected by initial egg weight before entering the incubation machine. A bigger egg has more nutrition than a smaller one. The embryo that is lacking nutrients would not grow properly and affect the hatched chicks. The current study indicated that the progesterone hormone injection did not have any significant effect (P>0.05) on the egg weight, it does not have a substantial impact on the hatching weight. The hatched weight of this study was lower than the one performed by Rohmad and Fitri (2016) that presented the hatched weight range of Arabic chicken breed as 31.2-32.53 g and this difference was due to the egg weight and different age of layer hens.

A high-dose of progesterone injection could increase the LH concentration in the plasma up to 1.52 ng/ml at three hours before the time that predicted ovulation, or around 28 hours prior to the next oviposition. This finding was in accordance with a study by Bronneberg et al. (2007) that the plasma LH concentration increases by 1-2 ng/ml and starts from 11 hours before the oviposition. The obtained results of a study by Lewis et al. (2005) also reported that the LH concentration in chicken was within the range of 1.1-1.2 ng/ml during different photoperiods.

The increase of LH concentration in P_2 was still within normal levels despite the multiple ovulation caused by the double egg yolk. The high LH in P_2 treatment was then expected as the cause of double-yolked egg, as both factors known to be correlated (Bédécarrats et al., 2016). Another research found that multiple ovulation occurs due to the defect in the follicle hierarchy than the distraction on the LH concentration profile (Buchanan et al., 2002).

CONCLUSION

The progesterone hormone injection in Arabic gold chicken during the production phase (28-31 weeks) had a significant effect on egg production two and six weeks after the injection. The progesterone hormone injection caused a varying production peak, and reduce the overall egg production. The progesterone hormone injection did not significantly affect the egg weight, shape index, fertility, embryo viability, hatchability, and hatched weight. Nevertheless, a high-dose of progesterone injection (2 mg/chicken) increased the occurrence of softshell and double egg yolk, as well as LH concentration in plasma blood.

DECLARATIONS

Competing interests

The authors declare that there are no competing interests.

Author's contributions

Iswati and Muhammad Halim Natsir designed the research. Iswati and Muhammad Halim Natsir performed the research and analyzed the data. Iswati wrote the manuscript. Gatot Ciptadi, Muhammad Halim Natsir, and Trinil Susilawati participated in the revision of the manuscript. All authors have read and approved the final version of the manuscript.

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Etiology of Respiratory Diseases of Poultry Farms in the North Coast of Egypt

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ABSTRACT

The current study aimed to identify the respiratory problems in poultry farms located in the north coast of Egypt from October 2018 to November 2019. A total of 89 poultry flocks (79 broilers, 5 layers, 3 ducks, and 2 turkeys) were investigated for four major viral respiratory pathogens, namely avian influenza (AI) H9N2, AI H5 subtypes, Newcastle Disease (ND), and Infectious Bronchitis (IB) viruses. All 89 flocks were subjected to real-time PCR to investigate AI H9N2 virus. The samples of 31, 43, and 15 out of 89 flocks were selected for the investigation of ND, IB, and AI H5 subtypes viruses, respectively, using real-time PCR. Sample selection was performed according to the mortalities, clinical signs, and post mortem lesions. The positive findings indicated that 22 out of 89 flocks were positive for AI H9N2 virus (2 layers + 20 broilers), 32 out of 43 flocks were positive for IB virus (2 layers + 30 broilers), 24 out of 31 flocks were positive for ND virus (1 Duck + 1 layer+ 22 broilers) and 9 out of 15 flocks were positive for AI H5N8 virus (1 turkey + 1 duck + 7 broilers). Partial sequencing for selected isolates of six ND, five IB, four H9N2, and three H5N8 viruses was applied, then nucleotide sequences were accessed on GenBank. Six ND isolates belonged to genotype VII viruses circulating in Egypt. Two IB isolates were related to the classical strain circulating in Egypt, while the other three IB isolates belonged to EGY/Variant II. Four H9N2 AI isolates were related to G1-lineage of H9 viruses circulating in the Middle East and Egypt. Three H5N8 AI isolates belonged to the highly diverse clade 2.3.4.4.b viruses circulating in Egypt. It was concluded that ND and IB viruses isolated in this study were not related to their vaccinal strains. Also, AI H5N8 circulating alone in affected flocks while AI H9N2 circulating alone and/or mixed with either IB or ND viruses. Finally, there is a need to devise a complete strategy to control the isolated respiratory viruses on the north coast of Egypt.

Keywords: Poultry, Respiratory, RRT-PCR, Sequence, Viruses

INTRODUCTION

Respiratory affection is a major problem in the commercial poultry industry leading to the annual loss of a hundred million dollars (Easterday et al.,1997). During the last few years, Egyptian commercial chicken flocks have been suffering from the co-circulation of multiple respiratory viruses leading to variable mortality rates and different clinical manifestations. Many important diseases can affect the respiratory system of poultry, including Avian Influenza (AI), Newcastle Disease (ND), and Infectious Bronchitis (IB) viruses (Shankar, 2008). The AI-H5N1 viruses affected poultry flocks in many countries including Egypt, and have become endemic (Aly et al., 2008). An additional challenge facing the poultry industry occurred in Egypt when AI-H9N2 subtype was detected during 2010-11 in chickens and commercial quails (El-Zoghby et al., 2012). In addition to high pathogenic avian influenza (HPAI) H5N1 and low pathogenic avian influenza (LPAI) H9N2, there was the incursion of HPAI H5N8 to Egypt in November 2016 via wild birds followed by spreading into commercial poultry flocks, further complicated the situation (Hassan et al., 2019). The ND virus still represents a serious problem for poultry production in many countries, although strict vaccination and other controlling regimes. In Egypt, ND virus strains of recent outbreaks in poultry farms have belonged to class II, genotype VII which might be introduced through the trading of poultry and poultry products with China and the Middle Eastern countries (Mohamed et al., 2011; Radwan et al., 2013). Furthermore, IB virus threatens the poultry industry worldwide and is considered as one of the most economically important respiratory viral diseases (Cook et al., 2012), and it was described as a natural infection in

different countries of Asia and the Middle-East in association with both HPAI and/or LPAI virus (Hassan et al., 2016). Therefore, the present study aimed to determine the current field situation of these major avian respiratory pathogens in the north coast region with the molecular identification of certain selected isolates to monitor and record their genetic properties through sequencing, phylogenetic analysis, and GenBank accessions.

MATERIALS AND METHODS

Samples collection and processing

Tracheal and/or oropharyngeal swabs and tissues of 89 different poultry flocks (10 samples per each flock) suffering from respiratory symptoms, a severe drop in egg production, and high mortalities were collected between October 2018 and December 2019. The area of investigation included the north coast area (west Alexandria, El-Hamam, El Alamein, and Matrouh governorates) in Egypt. All 89 flocks were subjected to real-time PCR for the investigation of AI H9N2 virus. The samples of 31, 43, and 15 out of 89 flocks were selected for the investigation of ND, IB, and AI H5 subtypes viruses, respectively, using real-time PCR. Sample selection was performed according to the mortalities, clinical signs, and post mortem lesions. Tissue samples were grounded in phosphate buffer saline with a pH of 7.0 to 7.4 containing gentamycin (50 µg/mL) and Mycostatin (1,000 units/mL) in a 1:5 (w/v) dilution, centrifuged, and tissue supernatant was collected (OIE, 2005; Naguib et al., 2017). The transport medium used for collected swabs composed of glycerol (50%), Phosphate buffer saline (PBS) (50%) with $2x10^6$ U/liter penicillin, 200 mg /liter streptomycin, and 250 mg/liter amphotericin B. Samples were stored at - 80°C until being tested (Gelb and Jackwood, 2008). All tissue samples were tested using RRT-PCR, then certain selected positive samples for each virus were purified through intra-allantoic inoculation of Specific Pathogen Free Embryonated Chicken Egg (SPF-ECE, De Wit, 2000), and these allantoic fluids were subjected to RT-PCR and sequencing.

Viral RNA extraction

All steps were carried out according to the manufacturer's instructions. Whole nucleic acid extraction from the samples was performed using the QIAamp® minielute virus spin kit (Qiagen, Germany, GmbH). Briefly, 200 µl of the sample suspension was incubated with 25 µl of Qiagen protease, and 200 µl of AL lysis buffer at 56°C for 15 min, then 250 µl of ethanol 100% was added to the lysate. After that, the sample was washed and centrifuged. The nucleic acid was eluted with 100 µl of elution buffer**.**

Real-time reverse transcription-polymerase chain reaction

For AI viruses, type A (matrix) gene primer was used (Spackman et al., 2002), followed by H5 (Löndt et al., 2008), H9 (Ben Shabat et al., 2010), and N8 primers (Hoffmann et al., 2016), while for ND virus and IB virus, M primer and N primer were utilized, respectively (Wise et al., 2004; Meir et al., 2010). Viral RNA extraction with QIAamp® was performed using viral RNA mini kit buffers (Qiagen, Germany) and Master Mix kit (Quantitect probe RT_PCR kit), and all steps were carried out according to the manufacturer's instructions (Table 1).

Conventional polymerase chain reaction

The H gene primer was used with the length of amplified product 311 bp and 920 bp for AI H5 virus and AI H9 virus, respectively (Slomka et al., 2007; Adel et al., 2016), while for ND virus, M & F gene primer with the length of amplified product 766 bp (Mase et al., 2002), and for IB virus, Spike SP1 gene primer with the length of amplified product 457 bp were used (Naguib et al., 2017). Viral RNA extraction with QIAamp® was performed using viral RNA mini kit buffers (Qiagen, Germany) and Master Mix kit (Quantitect probe RT_PCR kit), and all steps were performed according to the manufacturer's instructions (Table 2).

Analysis of the polymerase chain reaction products

The products of PCR were separated by electrophoresis on agarose gel 1.5% (Applichem, Germany, GmbH) in 1x Tris Borate EDTA (TBE) buffer at room temperature using gradients of 5V/cm. For gel analysis, 15 µl of the products were loaded in each gel slot. A gene ruler 100 bp DNA ladder (Fermentas, Thermofisher, Germany) was used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra), and the data were analyzed using the computer software (Automatic Image Capture Software, protein simple formerly cell biosciences, USA).

Sequencing and phylogenetic analysis

The PCR products of selected different isolated respiratory viruses (six ND, five IB, four H9, and three H5 subtypes viruses) were purified using QIAquick PCR Product extraction kit (Qiagen, Valencia). Bigdye Terminator V3.1 cycle sequencing kit (Perkin-Elmer) was used for the sequence reaction, and then it was purified using Centrisep spin column. Sequence analysis was done by Applied Biosystems3130 genetic analyzer (HITACHI, Japan), and the sequence identity was initially performed by A BLAST® analysis (Basic Local Alignment Search Tool) (Altschul et al., 1990) to be established to GenBank accessions. The phylogenetic tree was created by the MegAlign module of Laser gene DNAStar version 12.1 (Thompson et al., 1994), and Phylogenetic analyses were performed using maximum likelihood, neighbor-joining, and maximum parsimony in MEGA6 (Tamura et al., 2013).

Ethical approval

The present study was affirmed by the Ethics of Animal Health Committee, Desert Research Center, Egypt.

Table 1. Oligonucleotide primers and probes used for RRT-PCR.

Virus	Gene	Primer/ probe sequence 5'-3'	Virus	Gene	Primer/ probe sequence 5'-3'
AIV	H5	H5LH1 ACATATGACTAC CCACARTATTCA G	NDV	M	AGTGATGTGCTCGGACCTTC-3’
		H5RH1 AGACCAGCT AYC ATGATTGC			CCTGAGGAGAGGCATTTGCTA-3’
		H5PRO [FAM]TCWACA GTGGCGAGT TCCCTAGCA[TAMRA]			[FAM]TTCTCTAGCAGTGGGACAGCCTGC[TAMRA]-3’
	H9	H9F GGAAGAATTAATTATTATTGGTCGGTAC	IB	N	IBF: ATGCTCAACCTTGTCCCTAGCA
		H9R GCCACCTTTTTCAGTCTGACATT			IBR: TCAAACTGCGGATCATCACGT
		H9 Probe [FAM]AACCAGGCCAGACATTGCGAGTAAGATCC [BHQ]			IBTM: (FAMTTGGAAGTAGAGTGACGCCCAAACTTCA-TAMRA)
	N8	N8-1296F TCC ATG YTT TTG GGT TGA RAT GAT			
		N8-1423R GCT CCA TCR TGC CAY GAC CA			
		N8-1354 FAM- TCH AGY AGC TCC ATT GTR ATG TGT GGA GT-Tamra			

Table 2. Oligonucleotide primers used for conventional PCR.

Target agent	Target gene	Primer sequence (5'-3')	Length of amplified product (bp)
H9	H	H9F GGAAGAATTAATTATTATTGGTCGGTAC	920 bp
		HT7R TAA TAC GAC TCA CTA TAA GTA CAA ACA AGG GTG	
IB	Spike SP1	IBV-HVR1-2-FW GTK TAC TACTAC CAR AGT GC	457 bp
		IBV-HVR1-2-RV GAA GTG RAA ACR AGA TCA CCA TTT A	
ND	M and F	M2 TGG-AGC-CAA-ACC-CGC-ACC-TGC-GG	766 bp
		F2 GGA-GGA-TGT-TGG-CAG-CAT-T	
H5	H	H5-kha-1 CCT CCA GAR TAT GCM TAY AAA ATT GTC	311 bp
		H5-kha-3 TAC CAA CCG TCT ACC ATK CCY TG	

RESULTS

Prevalence of respiratory viruses

Concerning the results of the RRT-PCR used for detection of respiratory viruses (Figure 1), 22 out of 89 flocks (24.7%) were positive (+ve) for AI H9N2 virus, 9 out of 15 flocks (60%) were positive for AI H5 subtype virus, 24 out of 31 flocks (77.4%) were positive for ND virus, and 32 out of 43 flocks (74.4%) were positive for IB virus (Tables 3 and 4). All AI H5 subtypes were H5N8 viruses.

Single and mixed viral infection

There were 16 out of 89 poultry flocks negative for RRT-PCR. Furthermore, 59 flocks were recorded with a single viral infection, 8 flocks with AI H9N2, 22 with IB, 20 with ND, and 9 with AI H5N8 viruses. Mixed infection with AI H9N2 virus occurred in 14 poultry flocks, 10 flocks with mixed IB, and 4 flocks with mixed ND virus. On the other hand, there was no mixed infection with AI H5N8 subtype **v**irus (Table 5).

Results of conventional polymerase chain reaction

The PCR product revealed the specific amplification of 920, 766, 457, and 311 bp fragments for all selected AI H9N2, ND, IB, and AI H5N8 isolates of viruses, respectively (Figure 2).

Sequencing and phylogenetic analysis of avian influenza H9N2 isolated viruses

The isolates of four selected AI H9N2 viruses were genetically related to G1-lineage of H9 viruses circulating in the Middle East and were clustered in the same branch of the isolated Egyptian viruses during 2011-2019. The phylogenetic tree of HA gene showed that the first three analyzed isolates (A/Chicken/Egypt/North-coast/19 H9N2, A/Chicken/Egypt/Bahig/19 H9N2, and A/Chicken/Egypt/Matrouh/19 H9N2) were genetically related and significantly closer to each other (99 bootstrap value), and with the fourth isolate (A/chicken/Egypt/King-Mariot/18 H9N2) 54 bootstrap value (Figure 3). King-Mariot 2018 AI H9N2 virus isolate had an identity of 98.7% with other selected isolates which were closely related (100%) to each other. The four selected isolates were identical with other Egyptian strains of 2011-2019 by 94.8-100%, while with Hong Kong strains in 1997 were identical by 87.3-87.4%, Israel strains isolated in 2018 were identical with the four isolates from 97.1-97.2% and Korean strains in 1996 and 2007 had the identity of 82.7-83.1% (Figure 4).

Sequencing and phylogenetic analysis of avian influenza H5N8 isolated viruses

The selected three H5N8 HPAIV isolates were compared with Egyptian strains during 2014-2019, and it was found that these isolates belonged to the highly diverse clade 2.3.4.4.b viruses circulating in Egypt. On the basis of the phylogenetic tree, the analyzed isolates (A/chicken/Egypt/El-hamam/1/2019 H5N8, A/chicken/Egypt/El-hamam/10/2019 H5N1, and A/turkey/Egypt/North-coast k38/2019 H5N1) were clustered together and related highly genetically of 86 bootstrap value with each other, and 53 bootstrap value with other Egyptian viruses isolated during 2014-2019 (Figure 5). An amino acid sequence identity of the three isolates was 100% with each other and 98.9-99.3% with the Egyptian strains from 2017 to 2019 (Figure 6).

Sequencing and phylogenetic analysis of infectious bronchitis isolated viruses

The isolates of five selected IB viruses were compared with Egyptian classic, variant, QX, and their vaccinal strains. Two IB isolates (MT324521_IBV Matrouh and MT324522_IBV El-hamam) were genetically related to the classical strain circulating in Egypt (71 bootstrap value), and 33 bootstrap value with each other. The mentioned isolates had an amino acid sequence identity of 100% with each other and 71.8 - 91.2% with the Egyptian strains. The other three IB isolates (MT324523_IBV North-coast-k70, MT324524_IBV El-Alamin, and MT324525_IBV El-hawaria) belonged to EGY/Variant ll. MT324523_IBV North-coast-k70, and MT324525_IBV El-hawaria were genetically related to each other, 97 bootstrap value with an amino acid sequence identity of 100%, and had 41 bootstrap value with an amino acid sequence identity of 97.5% and 70.6-75.2% when compared with MT324524_IBV El-Alamin and the Egyptian strains respectively (Figures 7 and 8).

Sequencing and phylogenetic analysis of Newcastle disease viruses

The isolates of all selected six ND viruses were compared with Egyptian strains of 2012- 2018. The findings indicated that the isolates belonged to the genotype Vll viruses circulating in Egypt. The phylogenetic tree revealed that the analyzed isolates, MT324529_North coast-4 and MT324530_North coast-5 were closely related to each other, 66 bootstrap values. MT324531_North coast-6 virus isolate was clustered separately alone, 63 bootstrap value, while the other three virus isolates (MT324528_North coast-3, MT324526_North coast-1, and MT324527_North coast-2) were clustered together, and related genetically to each other with 57 bootstrap value (Figure 9). All selected six isolates showed an amino acid sequence identity of 98.6-100% with each other, and 97.6-100% and 97.6-98.1% when compared with the Egyptian and Israel strains respectively (Figure 10).

GenBank accessions of all selected viruses' isolates

Nucleotides sequences of all selected AI H9N2, AI H5N8, IB, and ND viruses' isolates were submitted to GenBank, and given accession numbers (Table 6).

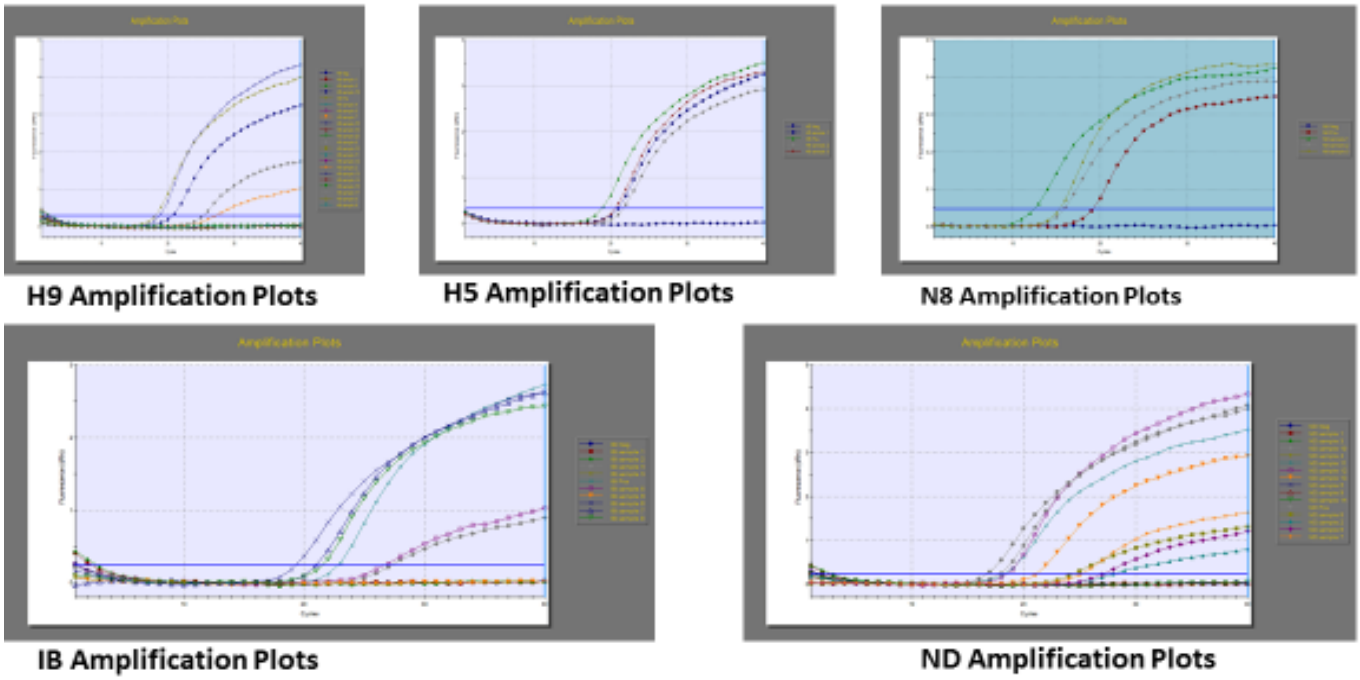

Figure 1. Results of RRT-PCR for collected samples of different poultry flocks showed amplification plots for the following primers H9, H5, N8, M, and N of H9N2, H5N8, ND, and IB viruses' isolates, respectively. ND: Newcastle disease IB: Infectious Bronchitis.

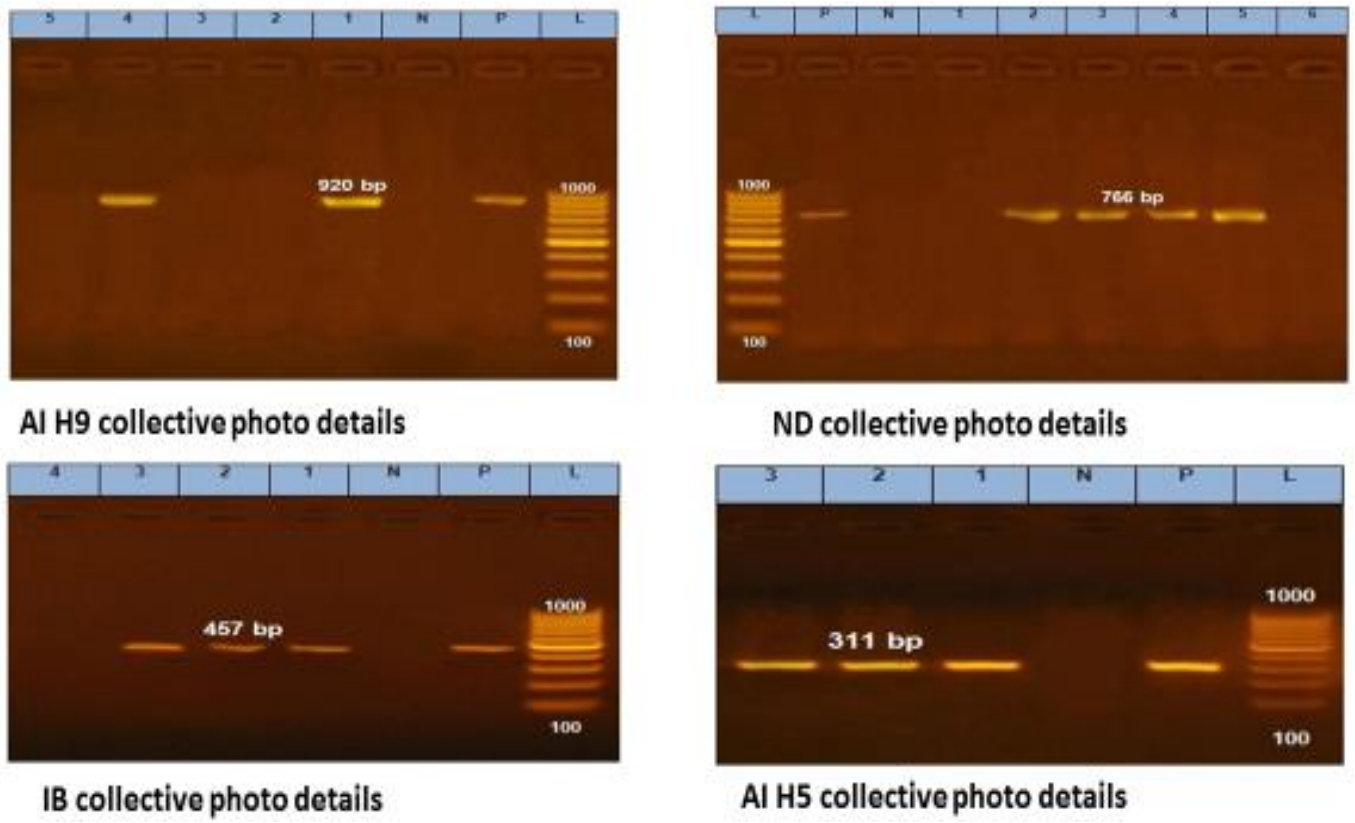

Figure 2. Results of conventional PCR showed the specific amplification of 920, 766, 457, and 311 bp fragment for all selected AI H9N2, ND, IB, and AI H5N8 viruses' isolates, respectively. ND: Newcastle disease IB: Infectious Bronchitis.

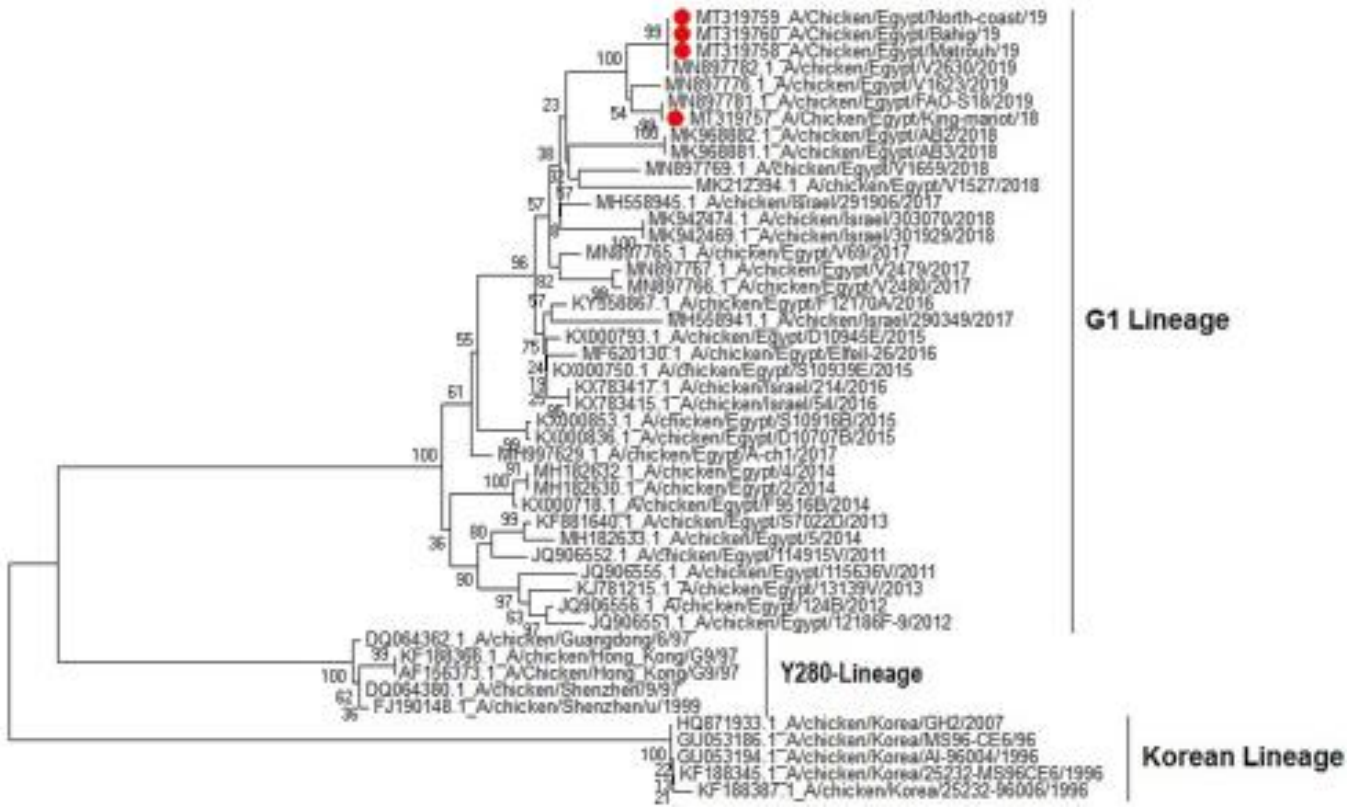

Figure 3. Phylogenetic tree showing the genetic relationships between circulating LPAI H9N2 and the four selected isolates for the HA gene (indicated by red dots).

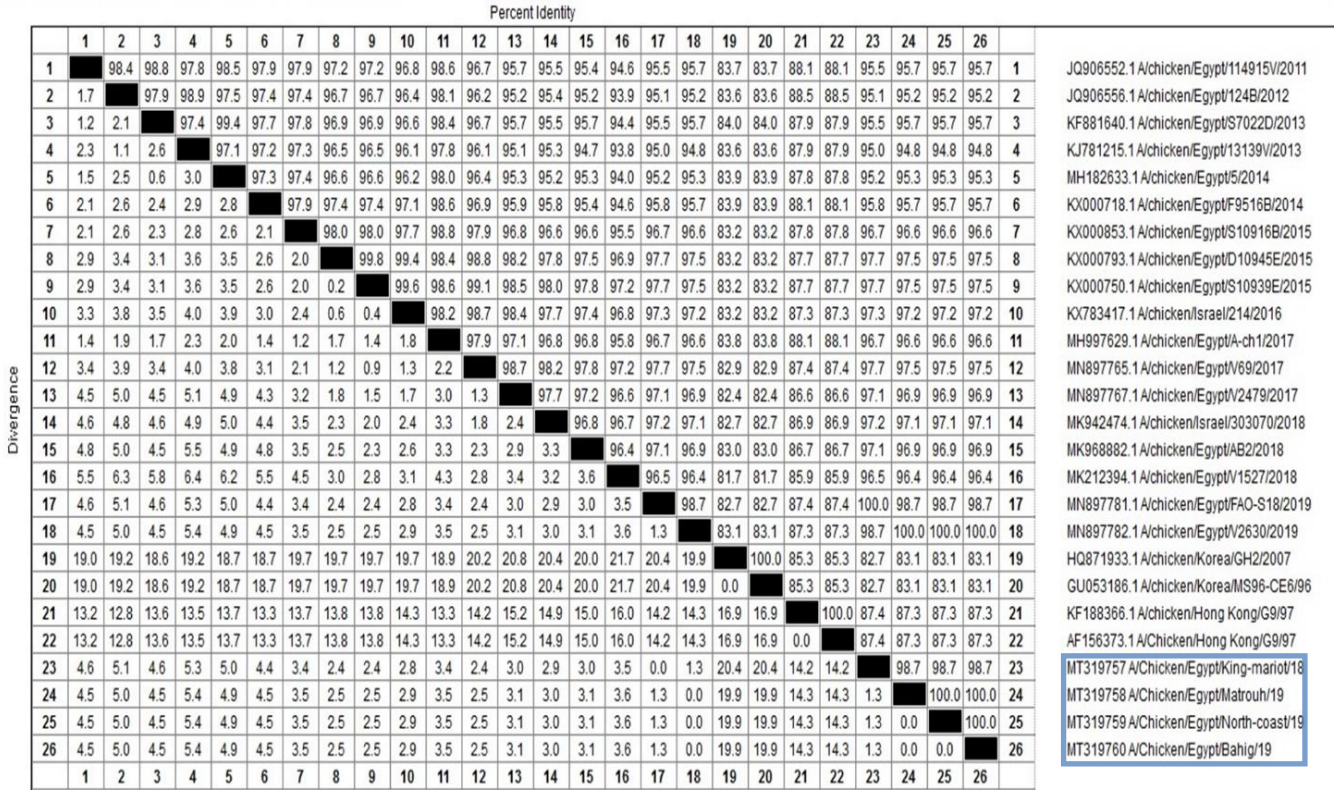

Figure 4. Genetic identity between circulating LPAI H9N2 and the selected four isolates (blue text box).

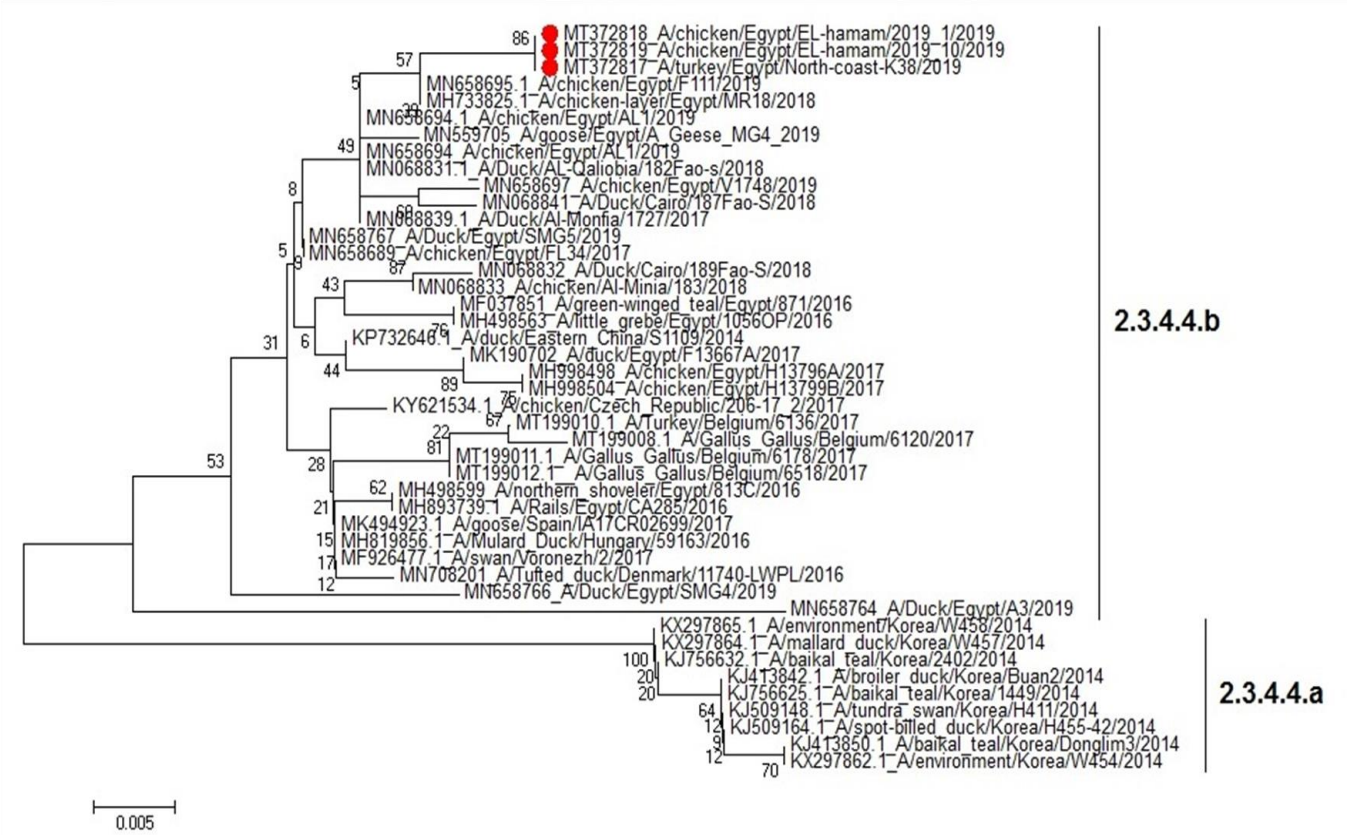

Figure 5. Phylogenetic tree showing the genetic relationships between circulating HPAI H5N8 and the selected three isolates for the HA gene (indicated by red dots).

Percent Identity

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	
1		99.6	99.3	98.6	98.9	97.9	97.5	97.9	97.9	98.6	98.9	93.3	92.9	93.3	93.6	93.6	92.9	98.2	98.2	98.2	98.2	98.2	98.6	98.6	1
2	0.4		98.9	98.2	98.6	97.5	97.2	97.5	97.5	98.2	98.6	93.3	92.9	93.3	93.6	93.6	92.9	97.9	97.9	97.9	97.9	97.9	98.2	98.2	2
3	0.7	1.1		99.3	99.6	98.6	98.2	98.6	98.6	99.3	99.6	94.0	93.6	94.0	94.3	94.3	93.6	98.2	98.2	98.2	98.9	98.9	99.3	99.3	3
4	1.4	1.8	0.7		99.6	98.6	98.2	98.6	98.6	99.3	98.9	94.0	93.6	94.0	94.3	94.3	93.6	98.9	98.9	98.9	99.6	99.6	100.0	100.0	4
5	1.1	1.4	0.4	0.4		98.9	98.6	98.9	98.9	99.6	99.3	94.3	94.0	94.3	94.7	94.7	94.0	98.6	98.6	98.6	99.3	99.3	99.6	99.6	5
6	2.2	2.5	1.4	1.4	1.1		97.5	97.9	98.6	98.6	98.2	93.3	92.9	93.3	93.6	93.6	92.9	97.5	97.5	97.5	98.2	98.2	98.6	98.6	6
7	2.5	2.9	1.8	1.8	1.4	2.5		99.6	98.2	98.9	97.9	93.6	93.3	93.6	94.0	94.0	93.3	97.2	97.2	97.2	97.9	97.9	98.2	98.2	7
8	2.2	2.5	1.4	1.4	1.1	2.2	0.4		98.6	99.3	98.2	94.0	93.6	94.0	94.3	94.3	93.6	97.5	97.5	97.5	98.2	98.2	98.6	98.6	8
9	2.2	2.5	1.4	1.4	1.1	1.4	1.8	1.4		99.3	98.9	94.0	93.6	94.0	94.3	94.3	93.6	97.5	97.5	97.5	98.2	98.2	98.6	98.6	9
10	1.4	1.8	0.7	0.7	0.4	1.4	1.1	0.7	0.7		98.9	94.7	94.3	94.7	95.0	95.0	94.3	98.2	98.2	98.2	98.9	98.9	99.3	99.3	10
11	1.1	1.4	0.4	1.1	0.7	1.8	2.2	1.8	1.1	1.1		93.6	93.3	93.6	94.0	94.0	93.3	97.9	97.9	97.9	98.6	98.6	98.9	98.9	11
12	7.2	7.2	6.4	6.4	6.0	7.1	6.8	6.4	6.4	5.6	6.8		99.6	100.0	99.6	99.6	99.6	92.9	92.9	92.9	93.6	93.6	94.0	94.0	12
13	7.6	7.6	6.8	6.8	6.4	7.5	7.2	6.8	6.8	6.0	7.2	0.4		99.6	99.3	99.3	100.0	92.6	92.6	92.6	93.3	93.3	93.6	93.6	13
14	7.2	7.2	6.4	6.4	6.0	7.1	6.8	6.4	6.4	5.6	6.8	0.0	0.4		99.6	99.6	99.6	92.9	92.9	92.9	93.6	93.6	94.0	94.0	14
15	6.8	6.8	6.0	6.0	5.6	6.8	6.4	6.0	6.0	5.2	6.4	0.4	0.7	0.4		100.0	99.3	93.3	93.3	93.3	94.0	94.0	94.3	94.3	15
16	6.8	6.8	6.0	6.0	5.6	6.8	6.4	6.0	6.0	5.2	6.4	0.4	0.7	0.4	0.0		99.3	93.3	93.3	93.3	94.0	94.0	94.3	94.3	16
17	7.6	7.6	6.8	6.8	6.4	7.5	7.2	6.8	6.8	6.0	7.2	0.4	0.0	0.4	0.7	0.7		92.6	92.6	92.6	93.3	93.3	93.6	93.6	17
18	1.8	2.2	1.8	1.1	1.4	2.5	2.9	2.5	2.5	1.8	2.2	7.6	8.0	7.6	7.2	7.2	8.0		100.0	100.0	99.3	99.3	98.9	98.9	18
19	1.8	2.2	1.8	1.1	1.4	2.5	2.9	2.5	2.5	1.8	2.2	7.6	8.0	7.6	7.2	7.2	8.0	0.0		100.0	99.3	99.3	98.9	98.9	19
20	1.8	2.2	1.8	1.1	1.4	2.5	2.9	2.5	2.5	1.8	2.2	7.6	8.0	7.6	7.2	7.2	8.0	0.0	0.0		99.3	99.3	98.9	98.9	20
21	1.8	2.2	1.1	0.4	0.7	1.8	2.2	1.8	1.8	1.1	1.4	6.8	7.2	6.8	6.4	6.4	7.2	0.7	0.7	0.7		100.0	99.6	99.6	21
22	1.8	2.2	1.1	0.4	0.7	1.8	2.2	1.8	1.8	1.1	1.4	6.8	7.2	6.8	6.4	6.4	7.2	0.7	0.7	0.7	0.0		99.6	99.6	22
23	1.4	1.8	0.7	0.0	0.4	1.4	1.8	1.4	1.4	0.7	1.1	6.4	6.8	6.4	6.0	6.0	6.8	1.1	1.1	1.1	0.4	0.4		100.0	23
24	1.4	1.8	0.7	0.0	0.4	1.4	1.8	1.4	1.4	0.7	1.1	6.4	6.8	6.4	6.0	6.0	6.8	1.1	1.1	1.1	0.4	0.4	0.0		24
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	

Divergence

1 MT199012A/Gallus Gallus/Belgium/6518/17
2 MT199010 A/Turkey/Belgium/6136/17
3 MK494923 A/goose/Spain/IA17CR02699/17
4 MN658694 A/chicken/Egypt/AL1/19
5 MN658767 A/Duck/Egypt/SMG5/19
6 MN068832 A/Duck/Cairo/189Fao-S/18
7 MH998498 A/chicken/Egypt/H13796A/17
8 MK190702 A/duck/Egypt/F13667A/17
9 MF037851A/green-winged teal/Egy/871/16
10 KP732646 A/duck/Eastern China/S1109/14
11 MH893739 A/Rails/Egypt/CA285/16
12 KJ413842 A/broiler duck/Korea/Buan2/14
13 KJ413850A/baikal teal/Korea/Donglim3/14
14 KJ756625 A/baikal teal/Korea/1449/14
15 KX297865 A/environment/Korea/W458/14
16 KX297864 A/mallard duck/Korea/W457/14
17 KX297862 A/environment/Korea/W454/14
18 MT372817A/turkey/Egy/North-coast-K38/19
19 MT372818A/chicken/Egy/EL-hamam/19-1
20 MT372819A/chicken/Egy/EL-hamam/19-10
21 MN658695 A/chicken/Egypt/F111/19
22 MH733825A/chicken-layer/Egypt/MR18/18
23 MN068839 A/Duck/Al-Monfia/1727/17
24 MN658694 A/chicken/Egypt/AL1/19

Figure 6. Genetic identity between circulating HPAI H5N8 and the selected three isolates (blue text box).

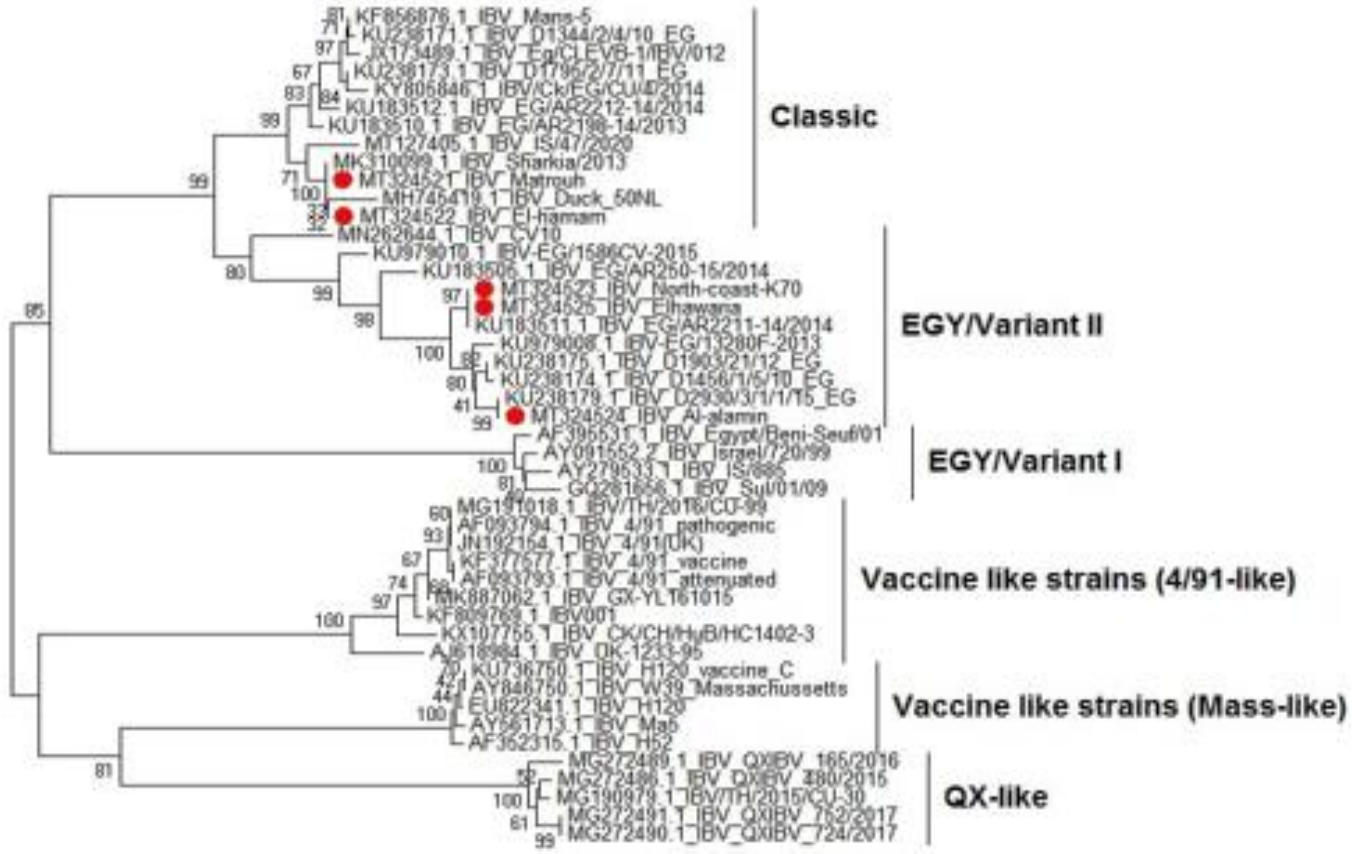

Figure 7. Phylogenetic tree showing the genetic relationships between representative IB viruses and the selected five isolates for the S1 gene (indicated by red dots) IB: Infectious Bronchitis.

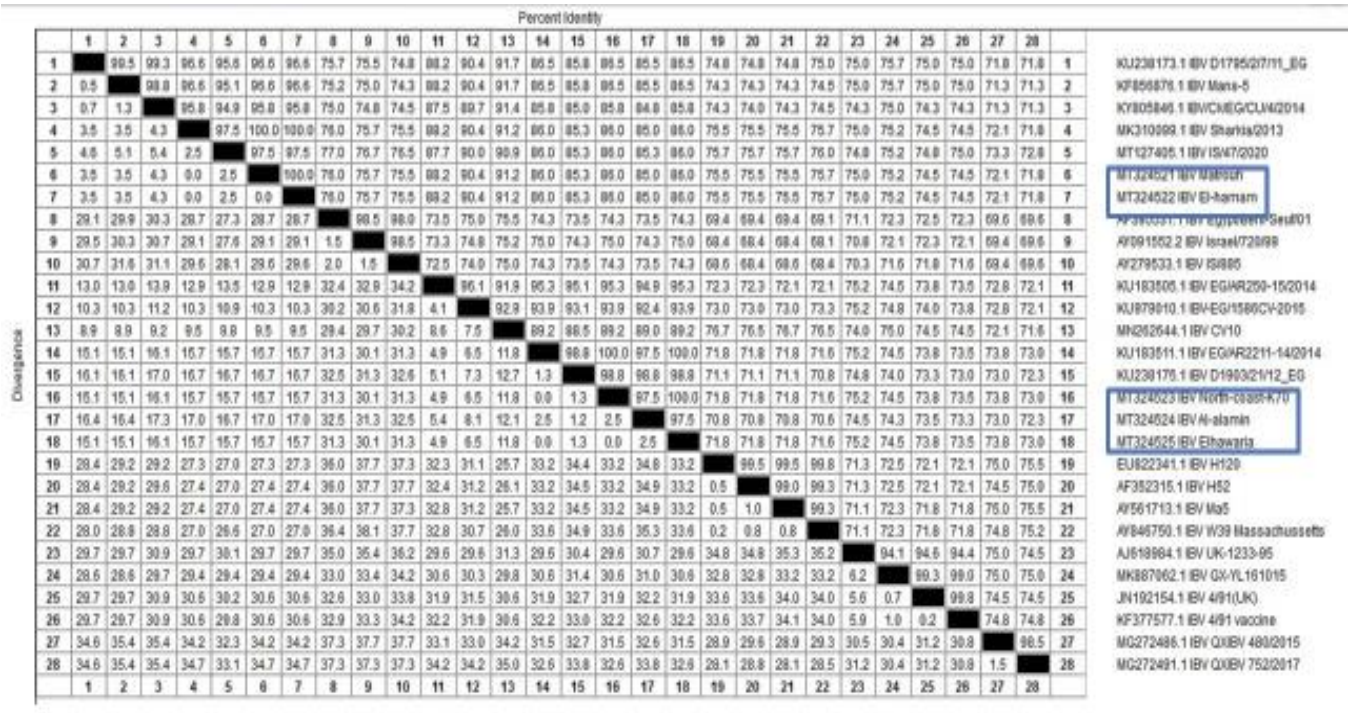

Figure 8. Genetic identity between circulating IB and the selected five isolates (blue text box) IB: Infectious Bronchitis.

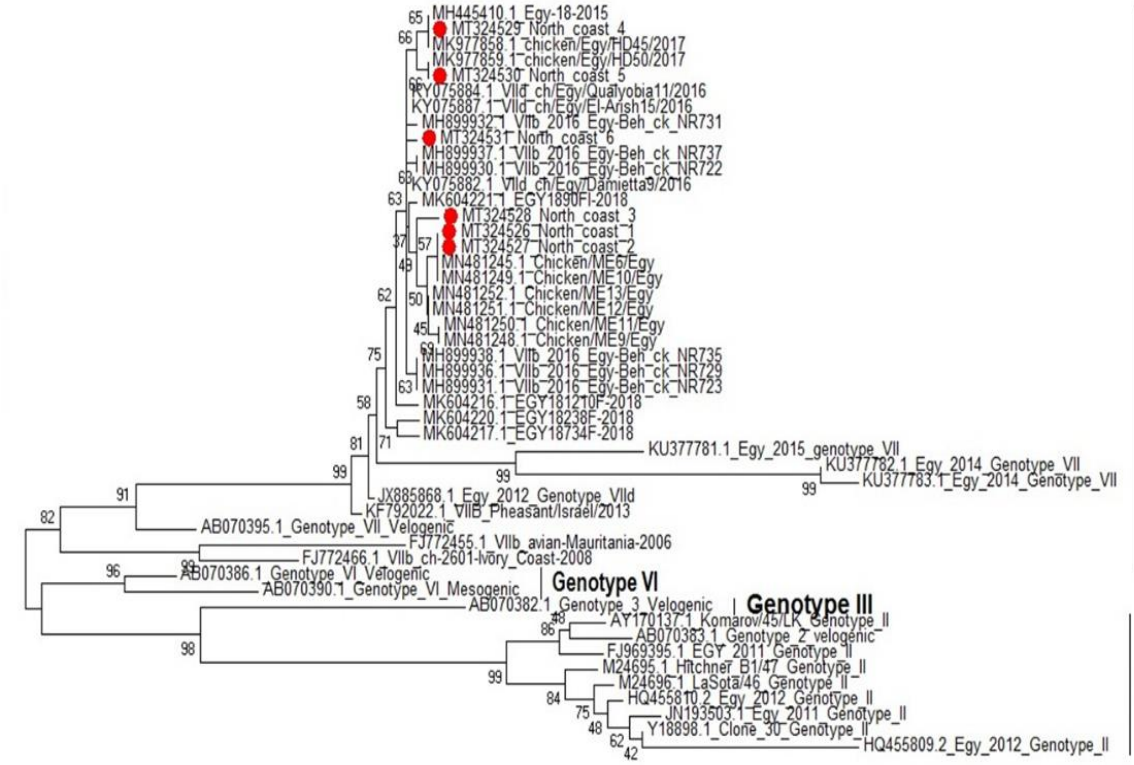

Figure 9. Phylogenetic tree showing the genetic relationships between circulating ND viruses and the selected six isolates for the F gene (indicated by red dots) ND: Newcastle disease.

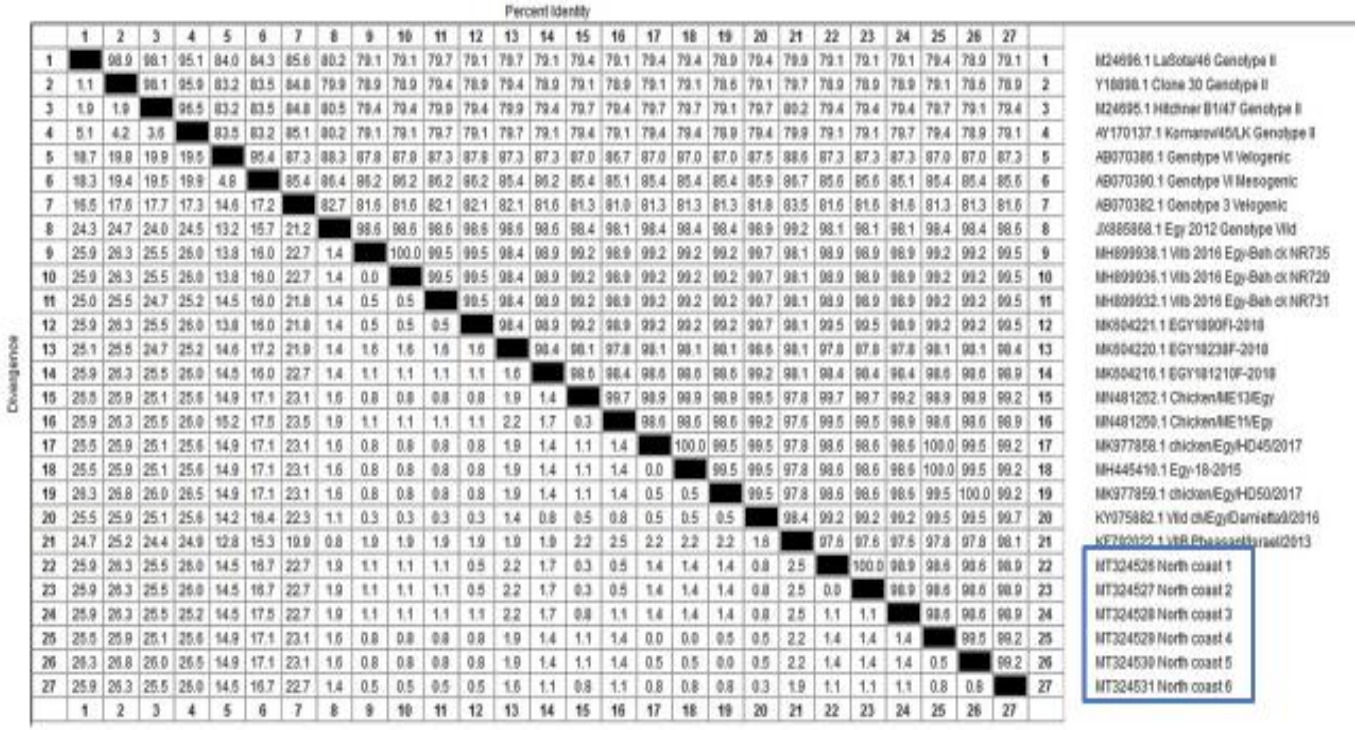

Figure 10. Genetic identity between circulating ND viruses and the selected six isolates (blue text box). ND: Newcastle disease.

Table 3. Results of RRT-PCR of surveyed poultry flocks.

virus		AI H9N2		Total
		+ve	-ve	
IB	+ve	10	22	32
	-ve	5	6	11
ND	+ve	4	20	24
	-ve	3	4	7
AI H5N8	+ve	0	9	9
	-ve	0	6	6
Total		22	67	89

ND: Newcastle disease IB: Infectious bronchitis +ve: Positive -ve: Negative AI H9N2: Avian influenza H9 subtype AI H5N8: Avian influenza H5N8 subtype.

Table 4. Prevalence of different respiratory viruses in poultry flocks in the north coast of Egypt.

Virus	AI H9N2	IB	ND	AI H5N8
Total flocks	89	43	31	15
+ve	22	32	24	9
percent	24.7%	74.4%	77.4%	60%

ND: Newcastle disease IB: Infectious bronchitis +ve: Positive -ve: Negative AI H9N2: Avian influenza H9N2 subtype AI H5N8: Avian influenza H5N8 subtype.

Table 5. Single and mixed infection with respiratory viruses in the surveyed poultry flocks.

Viruses	AI H9N2	IB	ND	AI H5N8	Total
Single infection	8	22	20	9	59
Mixed infection with AI H9N2	-	10	4	-	14

ND: Newcastle disease IB: Infectious bronchitis AI H9N2: Avian influenza H9 subtype AI H5N8: Avian influenza H5N8 subtype.

Table 6. Data and GenBank accession numbers of selected virus isolates for sequencing.

ND virus samples					
sample	**Code name**	**Governorates**	**Species**	**Collection date**	**accession numbers**
1	North cost 1	Matrouh	Broiler	10/2018	MT324526
2	North cost 2	Alexandria	Broiler	12/2018	MT324527
3	North cost 3	Alexandria	Layer	1/2019	MT324528
4	North cost 4	Alexandria	Broiler	2/2019	MT324529
5	North cost 5	Alexandria	Duck	4/2019	MT324530
6	North cost 6	Matrouh	Broiler	11/2019	MT324531
IB virus samples					
1	Matrouh	Matrouh	Broiler	11/2019	MT324521
2	El hamam	Alexandria	Broiler	9/2019	MT324522
3	North coast K70	Alexandria	Broiler	1/2019	MT324523
4	Al alamin	Matrouh	Layer	10/2018	MT324524
5	Elhawaria	Alexanderia	Layer	12/2018	MT324525
H9 virus samples					
1	King mariot	Alexandria	Broiler	11/2018	MT319757
2	Matrouh	Matrouh	Layer	9/2019	MT319758
3	North coast K48	Alexandria	Broiler	1/2019	MT319759
4	Bahig	Alexandria	Broiler	10/2019	MT319760
H5 virus samples					
1	North coast K38	Alexandria	Turkey	8/2019	MT372817
2	EL hamam	Alexandria	Broiler	1/2019	MT372818
3	EL hamam	Alexandria	Broiler	10/2019	MT372819

ND: Newcastle disease IB: Infectious bronchitis.

DISCUSSION

Avian viral respiratory pathogens investigated in the north coast poultry farms were detected in the current study using RRT-PCR. The obtained results indicated that ND viruses had the highest rate of 77.4% followed by 74.4%, 60%, and 24.7% for IB, H5N8, and H9N2 viruses, respectively. These results matched with those reported by Hassan et al. (2019) who found that 35, 27, 12, 9, and 9 samples out of 39 flocks were positive for AI H5N8, AI H9N2, IB, AI H5N1, and ND viruses, respectively, and detected an increase in the rate of positive flocks for AI H5N8 from 23% in 2017 to 66.6% during 2018. Shakal (2013) recorded that IB viruses had the highest incidence rate with 64% of the total investigated poultry farms, during January-July 2012 at 19 Egyptian governorates. Taher et al. (2017) showed that the incidence of IB, ND, AI H5, and AI H9 was 13.3%, 5.6%, 2.8%, and 1.1% respectively. Amer et al. (2018) found that H5, H9, and H5+ H9 subtypes were rated as 8.3%, 16.7%, and 6.5% respectively.

Co-infection with circulating H9N2 occurred in 14 poultry flocks, 10 flocks with IB, and 4 flocks with ND viruses. Abd El-Hamid et al. (2018) found that 12 out of 36 H9N2 affected poultry flocks co-infected with H5N1 (19.4%), ND (11.1%), and IB viruses (8.3%). Hassan et al. (2016) showed that mixed infections of IB with AI H9N2 viruses were the most common infection (41.7%). Amer et al. (2018) detected the mixed infections in chicken flocks of Egypt with AI H9N2 and ND genotype VII viruses. Davidson et al. (2014) and Hassan et al. (2016) reported that natural co-infections of AI H9N2 with ND viruses have occurred in poultry in Egypt.

Regarding the results of sequencing and phylogenetic analysis in the current study, AI H9N2 isolated viruses belonged to G1-lineage circulating in the Middle East, and were clustered with the isolated Egyptian H9N2 viruses during 2011-2019 with the similarity rate of 94.8-100%. Amer et al. (2018) found that H9 sequences belonged to the G1 lineage clustered with Egyptian H9N2 strains during 2015-2016. Abd El-Hamid et al. (2018) reported that the H9 isolates were clustered with recent Egyptian isolates of G1/97-like lineage of HA gene sequencing, and were similar to A/Quail/Egypt/113413v/2011 with about 92.3%-97.1% similarity rate. Monne et al. (2012) reported that all Egyptian AI H9N2 isolates were grouped within group B of G1-like lineage.

AI H5N8 isolated viruses were closely related to clade 2.3.4.4.b circulating in Egypt from 2014 to 2019 with an identity of 98.9 - 99.3%. Hassan et al. (2019) revealed that H5N1 and H5N8 were grouped within Clade 2.2.1.2 and Clade 2.3.4.4b, respectively**.** WHO (2019) reported that the higher the number of H5N8 infected poultry farms, the lower the number of human cases in Egypt (2016: 10; 2017: 3; 2018: 0). Also, Grund et al. (2018) reported that Clade 2.3.4.4b H5N8 viruses were known to have grossly reduced zoonotic propensity versus clade 2.2.1.2 H5N1 (Samir et al., 2015; Ghazi et al., 2016).

Two IB isolated viruses were located with classic strain circulating in Egypt with an identity of 71.8-91.2%, and the other three IB isolates belonged to EGY/Variant ll with an identity of 70.6-75.2%. Abou El-Fetouh et al. (2016) revealed that the results of partial sequencing and phylogenetic analysis of 400 bp of S1 gene for the isolates of IB viruses were separated into two distinct groups, namely variant 2 and classic. Also, Zanaty et al. (2016) isolated 20 IB viruses, four belonging to the classic group and 16 belonging to the variant group (six isolates with Egy /Var-1 and 10 isolates with Egy /Var -2).

Abd El-Moneim et al. (2012) partially analyzed the S1 gene of IB virus isolates and found that these isolates were closely related to recent Egyptian IB viruses isolated in northern and middle Egypt, and belonged to genotype variant 2. Meir et al. (2004) found that isolates of IB viruses were closely related to the Israeli nephropathogenic isolate (IS/885/00).

Finally, the selected isolated ND viruses belonged to genotype Vll circulating in Egypt from 2012 to 2018 with an identity of 97.6-100%. Amer et al. (2018) found that NDV isolates belonged to the class II genotype VII. Ramzy (2016) mentioned that genetic classification has divided NDV into two classes (I and II); class I was composed of only 1 genotype (class I, genotype I), and class II was divided into 18 genotypes (class II, genotypes I–XVIII). The predominant genotypes and virulent viruses circulating worldwide were genotypes V, VI, and VII. Genotype VII was associated with the most recent outbreaks in Africa, Asia, and the Middle East.

CONCLUSION

The current study concluded that the most predominant etiology of respiratory diseases in the north coast of Egypt were ND followed by IB, AI H5N8, and AI H9N2 viruses, furthermore ND and IB viruses isolated in this study were not related genetically to their vaccinal strains. Also, AI H5N8 circulating alone in affected flocks while AI H9N2 circulating alone and/or mixed with either IB (most

common) or ND viruses (less), finally, The north coast of Egypt is a zone of sustainable development, so there is a need to devise a complete strategy to control the isolated respiratory viruses.

DECLARATIONS

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Competing interests

The authors declare that they do not have any Competing interests.

Authors' contribution

Dr. Hanan El-Samahy planned the work, article writing, and revision, Dr. Disouky Mourad designed the protocol and helped in sample collection and laboratory analyses. All authors have read and approved the final manuscript.

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Isolation and Identification of Newcastle Disease Virus from Ducks Sold at Traditional Livestock Market Center in Indonesia

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ABSTRACT

Newcastle disease (ND) is one of the important infectious diseases in the poultry industry. The traditional poultry markets have great potential in ND transmission. The ducks infected by the ND virus rarely show clinical symptoms, thus they can potentially spread the disease to other fowls. The current study aimed to isolate and identify the ND virus from ducks in a traditional live bird market center in East Java, Indonesia. Cloacal swab samples were taken from 300 ducks. The study consisted of 100 pooled samples, each containing a cloacal swab sample obtained from 3 individual ducks. The samples were inoculated in specific antibody-negative embryonated chicken eggs for 8-10 days. Hemagglutination and hemagglutination inhibition tests were performed for confirmation and identification of ND virus. Based on the result of the current study, out of 100 pooled samples, there were three to nine ducks infected with the ND virus.

Keywords: Cloaca Swab, Duck, Livestock, Newcastle Disease

INTRODUCTION

Newcastle disease (ND) is one of the important infectious diseases in the poultry industry. Since 1926, ND has been reported as an endemic disease occurring in several countries of the world (Samal, 2011). This disease causes a significant loss for the poultry industry (Hu et al., 2010). The ND is caused by various virus strains. Based on the severity of the disease, ND can be classified into three pathotypes namely lentogenic, mesogenic and velogenic. Although vaccination of commercial chickens is routinely performed in Indonesia, ND remains a major problem in the poultry industry (Samal, 2011).

The traditional poultry markets have great potential in transmitting the ND virus (NDV). Transmission of the disease from the infected fowls to the healthy fowls is caused by various species of fowls which being marketed together. Transmission of NDV can also be caused by different duck maintenance systems ranging from the intensively to extensively maintenance system (Yuliana et al., 2015). The spread of NDV can occur because the fowls which are not sold by the merchant will be taken to other poultry markets, thus the fowls have the potential to be a source of disease in the area (Yuliana et al., 2015).

Unfortunately, there is no information regarding the importance of white Pekin duck as the carrier of NDV, or the potential risk of spreading the disease by Pekin duck to other food-producing fowls that may be looked after close to the Pekin duck habitat (Nishizawa et al., 2007). Although the type of white Pekin duck is resistant to the development of ND clinical signs when challenged with velogenic NDV, the white Pekin duck can shed the virus 20-30 days after the challenge, hence vaccination against ND is very important to reduce virus elimination in the field (Nishizawa et al., 2007).

Remembering insufficient breeders' awareness addressing the importance of biosecurity, vaccination, and separation of ducks and other waterfowl fowls, ducks can act as virus carriers or disease sources that can transmit the disease to other sensitive fowls. Thus, the present study was carried out to isolate and identify the presence of NDV in ducks sold at the traditional livestock market in Indonesia.

MATERIAL AND METHOD

Ethical approval

All experimental protocols and procedures were approved by the Institutional Animal Care of Indonesia.

Samples

This study was conducted on 300 ducks from the traditional live bird market in Sepanjang area, Sidoarjo, East Java, Indonesia from March to August 2019. The ducks were originated from the regions of Mojosari, Jombang, Lumajang, and Sidoarjo. A purposive sampling method was used to take cloacal swab samples. Sampling was conducted during five weeks and 100 pooled samples (each containing cloacal swab sample from 3 individual ducks) were collected.

Inoculation

Before sampling, transport media was prepared from 100 ml of 0.9% NaCl solution plus penicillin (1000 IU/ml of 0.9% NaCl solution) and streptomycin (1 mg/ml of 0.9% NaCl solution). Cloacal swab sample was inserted in a microtube containing the transport media. Each microtube contained three cloaca swabs for one duck. Samples were collected as many as 100 pooled samples. The collected samples were stored in an icebox and taken immediately to the laboratory. Then, the cotton swab was taken using tweezers. The samples were then centrifuged at 2500 rpm for 15 minutes. Furthermore, the virus isolation was carried out by inoculating the sample in the embryonic chicken egg. In the process of inoculation, the embryonic chicken egg used was Specific Antibody Negative (SAN) and aged 8-10 days. This was due to the optimal area of allantoic thus it easier to do inoculation and reduce the risk. After further inoculation, embryonic chicken eggs were incubated in an incubator at 37 ^{o}C for 5 days.

Tests

The tests were conducted at the Virology and Immunology Laboratory of the Veterinary Microbiology Department, Faculty of Veterinary Medicine, Univesitas Airlangga, Surabaya. The hemagglutination (HA) test was conducted by filling 0.9% NaCl solution in all wells (1-12) microplate as much as 25 µl. Next, 25µl Ag from the allantoic fluid was inserted into the first row of wells, homogenized Ag, and 0.9% NaCl solution using a micropipette, then transferred to the second well. The same procedure was repeated until the 11th well, then 25 µl was discarded from the wells 11, and well 12 was used as an erythrocyte control. Following that, 0.5% of chicken erythrocytes 50 µl was added to all wells, shaken slowly, and incubated for 30 minutes at room temperature or 4^{o}C for 60 minutes (OIE, 2012). The result was positive in the case of perfect hemagglutination (100%), where the erythrocyte layer was seen evenly (diffuse) on the base of the microplate. The HA test was negative if erythrocyte sedimentation was in the form of a dot in the middle of the microplate and would form teardrops if tilted.

Hemagglutination inhibition (HI) test was conducted similar to HA, but 0.9% NaCl solution was also added with positive antiserum of ND as much as 25 µl mixed with 0.9% NaCl solution to be homogeneous (used as a replacement, compared with HA test), in wells 1-11. In the HI test, well 12 was used as erythrocyte control. Then, the wells 1-12 were filled with 4 HAU antigens as much as 25 µl using a 25 µl micropipette. This antigen was derived from allantoic fluid of embryonic chicken egg which has been diluted using 0.9% NaCl solution, according to the titer obtained in the HA test. Microplate incubation was at room temperature for 30 minutes. Furthermore, all the wells (1-12) were filled with 50 µl of 0.5% chicken erythrocytes using a 50 µl micropipette. Incubation was performed again at room temperature for 30 minutes or 4^{o}C for 45 minutes. The data obtained were presented in descriptive form by calculating the percentage of positive samples. The total number of samples was compared with the number of samples collected.

RESULTS

Hemagglutination and hemagglutination inhibition tests

The results showed that ducks sold in the market were infected by ND virus. The results of sampling from ducks by region of origin are presented in Table 1.

In the first week of sampling, 3 out of 14 pooled samples of duck cloaca derived from Mojosari showed positive results during the HA test, however, the result was negative when the HI-ND test was conducted. Meanwhile, 5 of 6 pooled samples of ducks which derived from Lumajang showed positive result during the HA test, however, only 1 pooled sample showed positive result during HI-ND test. In the second week of sampling, 7 out of 9 pooled samples of ducks from Mojosari showed positive results during the HA test, however, the result was negative when HI-ND test was conducted. Meanwhile, for the samples derived from Lumajang, there were 10 of 11 pooled samples of ducks with positive result during the HA test, however, only 1 pooled sample was positive

during the HI-ND test. In the third week, the ducks from which cloacal swab samples were taken, were monitored and kept separately from the flock. When the HA test was conducted on the samples, all samples showed negative results. In the fourth week, a positive result was observed in 9 of 10 pooled samples of ducks derived from Mojosari using the HA test, however, the result was a negative result during the HI-ND test. Meanwhile, 7 out of 10 pooled samples of ducks from Lumajang showed positive results during the HA test, however, 6 pooled samples were positive when the HI-ND test was conducted. In the fifth week, 19 out of 20 pooled samples of ducks from Jombang showed positive results during the HA test, but there was only 1 positive pooled sample during the HI-ND test. In addition, this study also calculated the percentage of samples infected by NDV in the ducks throughout the pooled sample (Table 2).

Table 2. Number of positive and negative samples for Newcastle disease virus by the region of origin

Duck's origin	Number of pooled samples	∑ Positive ND	% Positive ND	∑ Negative ND
Mojosari	33	0	0 %	33
Lumajang	27	2	7 %	25
Sidoarjo	20	0	0 %	20
Jombang	20	1	5 %	19

Each pooled sample contained a cloacal swab sample from three ducks. ND: Newcastle disease

Table 1. Results of hemagglutination and hemagglutination inhibition tests for Newcastle disease virus isolated from ducks' cloacal swabs at the traditional live bird market center, Indonesia

Week of sampling	Regions											
	Mojosari			Lumajang			Jombang			Sidoarjo		
	Total samples	Positive HA samples	Positive HI samples	Total samples	Positive HA samples	Positive HI samples	Total samples	Positive HA samples	Positive HI samples	Total samples	Positive HA samples	Positive HI samples
I	14	3	0	6	5	1	---	---	---	---	---	---
II	9	7	0	11	10	1	---	---	---	---	---	---
III	---	---	---	---	---	---	---	---	---	20	0	0
IV	10	9	0	10	7	0	---	---	---		---	---
V	---	---	---	---	---	---	20	19	1	---	---	---

HA: Hemagglutination, HI: Hemagglutination inhibition

DISCUSSION

Based on the obtained results of the study, 3-9 out of 100 pooled samples of ducks derived from several areas were infected with the NDV. The NDV prevalence was relatively low in ducks in the traditional livestock market. In previous studies, the isolation rate of NDV in ducks infected with JSD0812 strain was relatively low but the ducks showed clinical signs of disease or death (Dai et al., 2014).

Observation results during embryonic chicken egg incubation showed that the time of embryo death varied between three and five days after inoculation. The rapid death of an embryo could be caused by several factors, including the malignant strain virus, the contamination caused by germs, or the trauma to the embryo during inoculation. Moreover, "viral toxicity" caused by a partial replication cycle can lead to the death of infected cells, but it was not responsible for the production of infective offspring (Friend and O Trainer, 1972).

The current study was conducted on a type of Pekin ducks from several regions in Indonesia. Based on the result of the study, ducks coming from Mojosari and Sidoarjo were not infected by the NDV. In addition, the relatively low percentage of NDV was observed in the white Pekin duck being resistant to the development of clinical signs of ND, compared to the velogenic NDV. This was important for NDV epidemiology since the white Pekin ducks could shed the virus 20 to 30 days after the challenge, and vaccination against ND was very important to reduce virus elimination in the field (Nishizawa et al., 2007).

In the current study, there were samples which had high titer values during the HA test, but they indicted negative results after the HI test. This could occur because the virus isolated might not an NDV but another type of virus which had the characteristic of erythrocyte

agglutination in the HA test, one of which is the avian influenza virus (Kim, Paldurai and Samal, 2017). In a study conducted in Brisbane, Australia, it was also found that HI antibody titers were much higher in ducks than chickens in the result of the experiment, and higher than the titers usually recorded in chickens (Bouzari, 2014). The immune response in the in-contact ducks indicated the transmission of the virus among ducks (Bouzari, 2014). In addition, differences in the composition of these proteins affected the speed of virus replication and determined the degree of virulence. The efficiency of protein F0 division and virulence of NDV depended on the presence of one or two arginines at the positions of 112 and 115 and phenylalanine at the position of 117 (Hines and Miller, 2012). The F1 active polypeptides mediated fusion between the virus lipid membrane and the host cell membrane (Rout, 2007).

The lentogenic virus had a *single basic amino acid* motif on the F *cleavage site* $_{112}$G/E-K/ R-Q-G/E-R^{116} and L (leucine) in the residue of 117 and could be cleaved by protease enzymes, such as trypsin, found in the digestive tract and breathing (Kim et al., 2008; Choi et al., 2010). Mesogenic and velogenic strains had multiple basic amino acid motifs (*arginine* or *lysine*) on the F cleavage site $_{112}$R/K-R-Q/K/R-R$_{116}$ and F (*phenylalanine*) in the residue of 117 (Meulemans et al., 2002) and could be cleaved intracellularly by protease enzymes, such as *furin*, that was in various host tissues, and thus it could cause fatal systemic infection (OIE, 2012).

The management conditions are a factor affecting the spread of NDV in ducks. Ducks infected by the NDV generally showed different physical symptoms from healthy ducks. However, if symptoms are detected late by duck breeders it can cause the rapid spread of the NDV. Based on the results of previous studies, it was indicated that it is difficult to avoid contact between the natural waterfowl and the fowls. Accordingly, the reservoir of natural waterfowl and sensitive fowls operations, circulating lentogenic NDV strain, could represent a potential reservoir for velogenic NDV strain leading to the epidemic to the chickens (Meng et al., 2015).

The current study faced some limitations, for instance, there was no other type of observations to complete the results of the study regarding biological characteristics of fowls, especially ducks infected by NDV. The microscopic observation of cells infected by NDV could definitely shed more light on the findings. However, the obtained results of the current study revealed that NDV-infected ducks were present in the animal market in the Sidoarjo area, but at a relatively safe level. The findings of the current study can be useful for veterinarians, practitioners, breeders, scientists, and livestock entrepreneurs in the efforts to prevent NDV infection.

DECLARATIONS

Authors' contributions

Aisyah Nur Azizah designed the study. Chairul Anwar collected the samples. Adi Prijo Rahardjo performed laboratory analysis. Aisyah Nur Azizah and Adi Prijo Rahardjo interpreted the results. All authors approved the final draft of the manuscript.

Conflict of interest

All authors have no conflicts of interest to declare.

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Control of Intestinal *E. coli* Infection in Broiler Chicks Using *Lactobacillus casei* Isolated from *Nono*

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ABSTRACT

The current study aimed to evaluate whether the probiotic *Lactobacillus casei* could be effective in controlling chicken intestinal colibacillosis. Avian pathogenic *Escherichia coli* (*E. coli*.) and *Lactobacillus casei* (*L. casei)* isolates were obtained from *nono* (a sour milk product produced by Fulani tribe of Nigeria), and were used for the chicken infection and probiotic treatment, respectively. The experimental design was conducted on three-week-old broiler chicks, which were divided into five groups, namely A (healthy control), B (infected without treatment), C (infected and treated with antibiotic), D (infected and treated with *L. casei*), and E (initially given *L. casei* before infecting with *E. coli*). Groups C and D were treated using 15 g/L norfloxacin and 1.5 ml of $1.1x10^9$cfu/ml *L. casei*, respectively. Group E was given the oral infusion of 1.5 ml of 1.1×10^9 cfu/ml *L. casei* before infection with 1.5 ml of 1.3×10^7 cfu/ml avian pathogenic *E. coli*. Weight, hematological parameters, liver function, and fecal *E. coli* counts of the chicks were monitored and used to evaluate the level of protection elicited by the probiotic organism. There was weight gain in chicken groups, except for group B. There was a significant difference in the sodium, chlorine, and bicarbonate levels amongst the groups. The hematological profile revealed a significant difference in the hemoglobin, white blood cells, lymphocyte, and neutrophil counts of the chicken groups. Assessment of liver enzymes showed no significant difference amongst the chick groups except in group B. Similar results were obtained for the urea, creatinine, and C-reactive protein levels. The microbial tests revealed a decrease in the total *E. coli* count for groups C, D, and E. The results of the current study indicated that *L. casei* could be used as a probiotic in the control of chicken colibacillosis.

Keywords: Broiler, Colibacillosis, *Escherichia coli*, *Lactobacillus*, *Nono*, Probiotics

INTRODUCTION

Antibiotic resistance has become a major threat to disease treatment in recent years and the use of antibiotics for controlling infection in food animal production has been figured as one of the reasons for increased resistant strains (WHO, 2014; CDC, 2019).

Escherichia coli (*E. coli*) is one of the top seven pathogens of public health concern (CDC, 2014). Avian pathogenic *E. coli* (APEC) is a type of *E. coli* strain responsible for colibacillosis, one of the major infections that threaten biodiversity conservation of poultry (De Preter et al., 2007; Dziva and Mark, 2008; Lutful-Kabir, 2010). The *E. coli* and some other bacterial infections in poultry have been controlled over the years with conventional antibiotics, such as monensin, chlortetracycline and oxytetracycline (Vesković Moračani et al., 2014). Nigerian farmers employ metronidazole and chloramphenicol as major antibiotics for controlling bacterial infections in poultry. This infection control method has major drawbacks, some of which include the selection of multidrug-resistant strains of *E. coli* in food animals and subsequent high antibiotic float in the human ecosystem (Ikele et al., 2020a). Therefore, the focus has been shifted to a search for antibiotic alternatives in food animal production.

Probiotics are microorganisms that confer health benefits to the host when consumed in adequate quantity (Holzapfel et al., 2001; Haben, 2019). The use of probiotics in animal production is an expanding research area as antibiotic alternatives for infection control in food

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animals (Vantsawa et al., 2017; Nugraheni et al., 2019). *Lactobacillus casei* (*L. casei*) is a Gram-positive anaerobic rod belonging to the lactic acid bacteria (LAB) group and is well-known for its wide probiotic values. It is a facultative homo-fermenter found in dairy products, such as cheese and *nono* (Ikele et al., 2020b). *Nono* is a locally fermented milk beverage consumed in Nigeria. It is a functional food that contains high numbers of LAB (Vantsawa et al., 2017). This study sought to evaluate the effectiveness of *L. casei* in the control of intestinal colibacillosis in broiler chicks.

MATERIALS AND METHODS

Ethical approval

This study was in accordance with the provisions of the National Institute of Health Guidelines for Care and Use of Laboratory Animals (PUB No. 85-23, revised 1985) and under the approval of the Nnamdi Azikiwe University Awka, Nigeria Ethical Committee, Nigeria, on the use of laboratory animals.

Isolation of bacteria

Lactobacillus and *E. coli* isolates were isolated from different *nono* samples obtained from different vendors at different locations in Awka, Anambra State, Nigeria. For bacterial isolation, 1 ml of *nono* was aspirated with a sterile pipette and subjected to 10-fold serial dilutions in sterile peptone water. The diluted samples (0.1 ml each) were cultured on de Man, Rogosa and Sharpe (MRS) agar and Eosin Methylene Blue (EMB) agar as selective media for *Lactobacillus* and *E. coli*, respectively. Cultures were incubated at 35 °C for 24 h in an anaerobe jar for *Lactobacillus* isolates and aerobically for *E. coli* isolates according to the methods proposed by Makut et al. (2014).

Identification of isolates

Lactobacillus and *E. coli* isolates were presumptively identified using routine cultural and biochemical tests and confirmed by 16s rDNA molecular typing at Macrogen Incorporate, South Korea.

Screening of *Escherichia coli* isolates for avian pathogenicity

The samples include 20 broiler chicks aged 3 weeks that were raised in a battery cage system and divided into four groups. Each group was orally infected with 10^7 cfu/ml of different presumptively identified *E. coli* isolates in phosphate-buffered saline (PBS, pH 6.8), with the aid of a sterile Pasteur pipette. The chicks were then monitored for 30 days for clinical signs, such as weakness, watery, and bloody stools (Ezema, 2013). Isolate that produced the most severe signs in a group was selected for molecular typing and used for further investigations.

Probiotic screening of *Lactobacillus* isolates

L. casei isolates were screened for acid tolerance, cell surface hydrophobicity, bile tolerance, sodium chloride tolerance, crude bacteriocin activity, and cell-free supernatant antibacterial activity against APEC (Dunne et al., 2001). The agar well diffusion assay was used to assess the antibacterial activity of cell-free supernatant (CFS) of *L.* casei isolates. A 10-ml aliquot of MRS broth was added to the *Lactobacillus* culture and incubated at 37 °C for 48 h in an anaerobic jar. After incubation, the culture was subjected to centrifugation (8000 rpm for 10 minutes), and the supernatant was obtained. A 100-µl aliquot of CFS was placed into wells on Mueller-Hinton agar plates (20 ml) seeded with *E. coli* (0.1 ml), and incubated at 35 °C for 24 h. Subsequently, the diameters of inhibition zones were measured in mm (Ronnqvist et al., 2007). The *L.* casei isolate with the highest zone of inhibition against the chosen *E. coli* isolate was selected for the animal studies.

Crude bacteriocin activity assay

Lactobacillus spp. were grown in MRS broth for 24 h at 35 °C in an anaerobic chamber. Cell-free supernatant was obtained by being centrifuged at 4000 rpm for 10 minutes. Ammonium sulfate (0.425 g in 5 ml solution) was used to directly precipitate the crude bacteriocin, after which the mixture was refrigerated overnight at 4 °C, and vortexed again at the same speed and time. The supernatant was discarded while the trapped precipitate was dissolved using phosphate buffer pH 6.0, and was assayed for antibacterial activity using the agar well diffusion method (Joshi et al., 2006).

Standardization of pure cultures of isolates

Cultures of *L.* casei and APEC were grown in 25 ml MRS broth and nutrient broth, respectively, and incubated at 30 °C for 24 h in an anaerobic jar for *Lacotbacillus* and aerobically for *E. coli*. The cultures were serially diluted to achieve concentrations of 10^9 cfu/ml for *L. casei* and10^7 cfu/ml for *E. coli* according to the method introduced by Hartmann et al. (2011).

Animals

Day-old broiler chicks (*Gallus domesticus*) were obtained from Aroma Farms, Awka, Anambra State

Nigeria, and raised in battery cages with wood sawdust as bedding materials, at the temperature of 28-33 °C. The chicks were vaccinated for Newcastle disease until they aged three weeks before they were used for the experiment. They were subjected to no antibiotic administration. They were fed with a starter diet for the first seven days and a finisher diet for the remaining weeks. Blood samples (1.5 ml) were collected from each chick starting from the third week, with a sterile syringe and needle through their wing vein.

Experimental design

Five groups of 10 three-week-old chicks were set up as follows: healthy control (Group A), infected with APEC (Group B), infected with APEC and treated with norfloxacin (Norflox-200, Interchemie, Netherlands; Group C); infected with APEC and treated with *L. casei* (Group D), given oral dose of *L. casei* 48 h before infection with APEC (Group E).

Groups B, C, and D orally received initially 1.5 ml of 1.3×10^7 cfu/ml of *E. coli* mixed with 0.5 ml PBS (pH 6) with the aid of a sterile pipette and left for two days to allow for proper pathogen colonization and disease establishment (evidenced by the discharge of watery and bloody stool). Afterwards, group D was dosed orally with 1.5 ml of 1.1×10^9 cfu/ml of *L. casei* to initiate competitive inhibition. Group C was treated with norfloxacin (15 g/L in drinking water) for two weeks. Group E was administered with an oral initial dose of 1.5 ml of 1.1×10^9 cfu/ml of *L. casei* and left for two days, then was infected with 1.5 ml of 1.3×10^7cfu/ml of *E. coli*. This group was used to ascertain the preventive ability of the *L. casei* on colibacillosis (Pascual et al., 2009; Ikele et al., 2019).

Growth performance parameters

Weights, percentage weight gain, and specific growth rates of the chicks were monitored using the method introduced by Radhakrishman et al. (2015). The body weights of the chicks were weekly measured using Mettler weighing balance of 0.01 g sensitivity. Percentage weight gain and percentage specific growth rate (SGR) were determined with the following formulas:

$$\text{Weight gain (\%)} = [(Wf - Wi) / Wi] \times 100;$$

$$\text{SGR (\%)} = \frac{\ln Wf - \ln Wi}{\text{Number of experimental days}} \times 100$$

Where, Wf is the final weight and Wi refers to the initial weight.

Hematological and biochemical profile

Hemoglobin concentration was determined using an automated hemoglobin reader (Accu-check Roche JB-09927130, Germany). Total and differential white blood cell counts were determined with the methods put forward by Cheesbrough (2006). Blood electrolytes (sodium, potassium, chlorine, and bicarbonate), urea, creatinine, and C-reactive protein were determined according to the methods adopted by Reddy et al. (2011). Serum aspartate aminotransferase, alanine aminotransferase, and acid phosphatase were monitored according to the methods employed by Reitman and Frankel, (1957), Babson and Read, (1959) and Eissa and Zidan, (2010).

Intestinal *Escherichia coli* load

Intestinal washing with 1 ml of PBS (pH 6.8) was performed weekly from 4 weeks of age until the end of experiment on sacrificed chicks (they were sacrificed by using inhalation anesthesia with chloroform, and cuting the cervical vein) in each group; then, 1 ml of lavage fluid was serially diluted in 9 ml peptone water and 0.1 ml of suspension was cultured on EMB agar using the pour plate method. Colony-forming units from lavage cultures after 24 h incubation were used to determine the intestinal loads of *E. coli* (Pascual et al., 2009).

Statistical analyses

One-way ANOVA was applied to determine the means at 95% confidence interval. Tukey test was used for the comparison of means. P-value less than 0.05 was considered statistically significant.

RESULTS

Identification and molecular typing of isolates

The *E. coli* isolate with the most pathogenic effect on broiler chicks was identified as *E. coli* O157:H7 strain Sakai.

Probiotic characteristics of *Lactobacillus casei*

The probiotic screening of *L. casei* was suggestive of an acceptable level of tolerance to acidic pH, 10% NaCl and bile, as well as good adherence to xylene. *In-vitro* antibacterial activity evaluation showed that cell-free supernatant produced 10 mm diameter zone of inhibition,

and crude bacteriocin exhibited 10.4 mm zone of inhibition diameter against APEC.

Effect of administration of *Lactobacillus casei* on body weight

Except for the infected and untreated group B, all other groups recorded weight gain. However, the highest weight gain was recorded in the control (uninfected chicks). The mean weights and specific growth rates of the chicks over the experimental period are shown in Figure 1 and Table 1, respectively.

Effect of *Lactobacillus casei* administration on hematological parameters and serum electrolytes of chicks

While the obtained results of the infected group indicated a significant decrease in hemoglobin levels, the groups treated with antibiotics and *L. casei* showed improved hemoglobin levels. There were also significant differences in the total white blood cell, neutrophils, and lymphocyte counts of infected and treated chicks. For these three parameters, the infected chicks had significantly ($p < 0.05$) elevated counts (Table 2). No significant ($p > 0.05$) differences were recorded in eosinophil, basophil, and monocyte counts. For serum electrolytes, there were significant ($p < 0.05$) increases in sodium, chlorine, and bicarbonate levels in treated groups, compared to the infected group, but no increase in potassium. Urea, creatinine, and C - reactive protein levels were moderate when compared to those of the antibiotic-treated and control groups (Table 3).

Effect of *Lactobacillus casei* administration on liver function of chicks

There was a marked increase in serum aspartate aminotransferase, alanine aminotransferase, and acid phosphatase values in the infected and untreated chicks, compared to the infected chicks treated with *L. casei* (Table 4). These differences were significant ($p < 0.05$).

Effect of *Lactobacillus casei* administration on intestinal *E. coli* counts

There was a recorded steady increase in *E. coli* counts of infected and untreated chicks (B), from the first week to the fifth week of monitoring, compared with other groups. However, there was a slight decrease in intestinal *E. coli* count in the sixth week, which was still also the highest in value when compared to other groups. The antibiotic-treated chicks (C) showed a sharp decline in *E. coli* count after the first week of infection, and the same was observed in the probiotic-treated group (D). In the prophylactic group (E), *E. coli* growth was decreased, compared to other investigated groups (Figure 2).

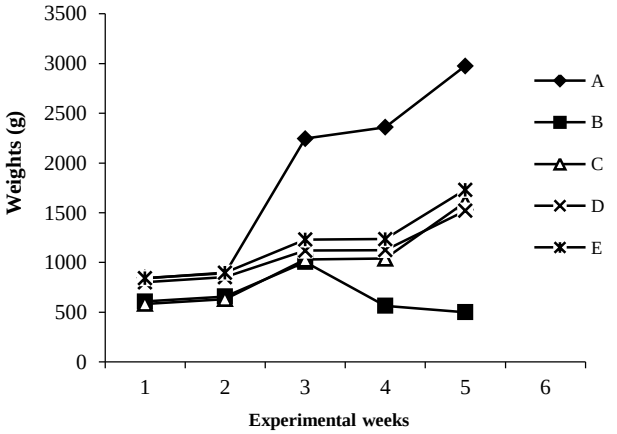

Figure 1. Comparison of mean body weights of broiler chicks infected with APEC in different treatment groups. APEC: Avian pathogenic *E. coli*. A: Healthy control, B: Infected with APEC and untreated chicks, C: Infected with APEC and treated with norfloxacin, D: Infected with APEC and treated with *L. casei*, E: Administered with *L. casei* 48 h before infection with APEC.

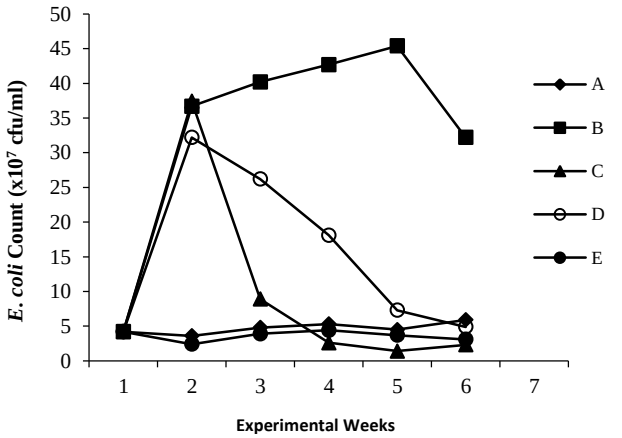

Figure 2. Intestinal *E. coli* counts of broiler chicks infected with APEC in different treatment groups. APEC: Avian pathogenic *E. coli*. A: Healthy control, B: Infected with APEC and untreated chicks, C: Infected with APEC and treated with norfloxacin, D: Infected with APEC and treated with *L. casei*, E: Administered with *L. casei* 48 h before infection with APEC.

Table 1. Growth parameters of broiler chicks infected with APEC in different treatment groups

Experimental Groups	Weight gain		Specific Growth Rate (%)
	(g)	(%)	
A	2130.96	252.4	4.5
B	-109.26	-17.9	-0.7
C	1019.54	174.1	3.6
D	717.83	89.5	2.28
E	887.80	105.4	2.57

APEC: Avian pathogenic *E. coli*. A: Healthy control, B: Infected with APEC and untreated chicks, C: Infected with APEC and treated with norfloxacin, D: Infected with APEC and treated with *L. casei*, E: Administered with *L. casei* 48 h before infection with APEC.

Table 2. Hematological profile of broiler chicks infected with APEC in different treatment groups

Parameters		Reference range	Treatment groups	Values after 21 days of treatment	Values after 28 days of treatment
Hemoglobin (g/dl)		5.05 ± 1.05^{a}	A	6.50 ± 1.15^{b}	6.05 ± 1.15^{b}
		4.86 ± 0.55^{a}	B	3.60 ± 0.55^{a}	2.45 ± 0.35^{a}
		4.66 ± 0.33^{a}	C	5.05 ± 1.15^{b}	8.35 ± 1.25^{b}
		4.82 ± 1.05^{a}	D	4.10 ± 1.15^{b}	9.50 ± 1.15^{b}
		5.25 ± 1.26^{a}	E	5.35 ± 1.20^{b}	7.35 ± 1.20^{b}
Total white blood cell count ($\times 10^{9}$cells/L)		9.05 ± 1.2^{a}	A	10.80 ± 1.45^{a}	12.79 ± 1.45^{a}
		9.22 ± 1.2^{a}	B	12.90 ± 1.15^{b}	14.10 ± 1.20^{b}
		9.12 ± 1.15^{a}	C	13.20 ± 0.85^{b}	12.60 ± 1.20^{a}
		9.22 ± 1.2^{a}	D	11.80 ± 1.20^{b}	12.70 ± 1.70^{a}
		9.22 ± 1.15^{a}	E	13.40 ± 1.20^{b}	12.88 ± 1.25^{a}
Differential white blood cell counts (%)	**Neutrophil**	26.20 ± 1.10^{a}	A	28.20 ± 1.15^{b}	30.30 ± 1.20^{b}
		26.05 ± 1.15^{a}	B	22.60 ± 1.45^{a}	26.00 ± 1.15^{b}
		32.65 ± 1.15^{b}	C	29.65 ± 1.75^{b}	28.65 ± 0.82^{b}
		32.60 ± 1.12^{b}	D	27.50 ± 1.20^{b}	31.60 ± 1.45^{b}
		26.15 ± 1.15^{a}	E	30.25 ± 1.20^{b}	23.00 ± 1.20^{a}
	Lymphocyte	60.06 ± 1.75^{b}	A	65.60 ± 1.20^{ab}	65.60 ± 6.60^{b}
		60.25 ± 1.15^{b}	B	86.60 ± 1.20^{b}	86.40 ± 1.40^{c}
		51.33 ± 1.50^{a}	C	62.65 ± 1.70^{ab}	68.00 ± 1.10^{a}
		50.15 ± 1.20^{a}	D	70.00 ± 1.10^{a}	64.00 ± 1.70^{a}
		60.33 ± 0.80^{b}	E	61.60 ± 1.70^{a}	72.30 ± 1.45^{b}
	Eosinophil	0^{a}	A	0.65 ± 0.65^{a}	0.00 ± 0.00^{a}
		1.00 ± 0.01^{a}	B	1.00 ± 1.00^{a}	1.00 ± 1.00^{a}
		0.67 ± 0.67^{a}	C	1.00 ± 1.00^{a}	0.67 ± 0.67^{a}
		0.00 ± 0.00^{a}	D	1.00 ± 1.00^{a}	1.00 ± 1.00^{a}
		0.67 ± 0.67^{a}	E	1.00 ± 1.00^{a}	1.33 ± 1.33^{a}
	Basophil	0^{a}	A	0.00 ± 0.00^{a}	0.33 ± 0.33^{a}
		0.00 ± 0.00^{a}	B	0.00 ± 0.00^{a}	0.33 ± 0.33^{a}
		0.33 ± 0.33^{a}	C	0.33 ± 0.33^{a}	0.67 ± 0.33^{a}
		0.33 ± 0.33^{a}	D	0.67 ± 0.33^{a}	0.00 ± 0.00^{a}
		0.67 ± 0.03^{a}	E	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
	Monocytes	1.00 ± 0.37^{a}	A	1.33 ± 0.05^{a}	2.00 ± 1.05^{a}
		1.00 ± 0.67^{a}	B	0.66 ± 0.66^{a}	0.55 ± 0.16^{a}
		1.00 ± 0.68^{a}	C	2.00 ± 0.48^{a}	2.48 ± 0.67^{a}
		1.33 ± 0.58^{a}	D	2.26 ± 0.58^{a}	2.67 ± 0.67^{a}
		1.00 ± 0.33^{a}	E	1.33 ± 0.58^{a}	1.37 ± 0.33^{a}

Means within the same column with different suffixes are significantly different ($p<0.05$). APEC: Avian pathogenic *E. coli*. A: Healthy control, B: Infected with APEC and untreated chicks, C: Infected with APEC and treated with norfloxacin, D: Infected with APEC and treated with *L. casei*, E: administered with *L. casei* 48 h before infection with APEC.

Table 3. Blood chemistry parameters of broiler chicks infected with APEC in different treatment groups

Parameters	Reference range	Treatment groups	Values after 21 days treatment	Values after 28 days treatment
	124.00 ± 1.15[b]	A	145.00 ± 1.15[c]	146.00 ± 3.06[c]
	138.34±1.45[c]	B	132.32 ± 1.20[a]	130.00 ± 1.15[a]
Sodium (MEq/L)	110.22±1.5[a]	C	140.32 ± 1.45[b]	135.32 ± 1.40[ab]
	136.00±1.15[c]	D	134.00 ± 1.15[a]	136.00 ± 1.15[abc]
	110.00±1.15[a]	E	135.00 ± 1.75[a]	139.30 ± 1.40[b]
	2.15 ± 1.00[a]	A	3.37 ± 1.0[a]	2.15 ± 1.80[a]
	3.00 ±1.05[a]	B	2.35 ± 1.15[a]	2.25 ± 0.80[a]
Potassium (MEq/L)	2.40±1.10[a]	C	2.00 ± 1.05[a]	2.20 ± 1.10[a]
	3.02±1.10[a]	D	2.15 ± 1.00[a]	2.21 ± 1.10[a]
	2.50±1.15[a]	E	2.85 ± 0.83[a]	2.20 ± 1.15[a]
	94.06 ± 1.15[a]	A	138.00 ± 1.15[c]	145.00 ± 1.15[d]
	105.55±1.40[b]	B	115.60 ± 1.40[a]	105.00 ± 1.15[a]
Chlorine (MEq/L)	96.20±1.75[a]	C	120.65 ± 1.75[b]	135.00 ± 1.15[c]
	116.60±0.06[b]	D	120.00 ± 1.15[b]	130.30 ± 1.45[b]
	116.28±1.08[b]	E	122.00 ± 1.15[b]	135.30 ± 1.20[c]
	14.00±1.15[a]	A	25.30 ± 1.45[a]	27.00 ± 1.15[c]
	25.20±1.15[b]	B	20.00 ± 1.5[a]	15.00 ± 1.15[a]
Bicarbonate (MEq/L)	20.33±1.10[b]	C	18.00 ± 1.15[a]	24.00 ± 1.10[b]
	22.21±1.10[b]	D	25.67 ± 34.5[b]	21.00 ± 1.10[b]
	17.01±1.15[a]	E	22.02 ± 1.15[b]	24.02 ± 1.15[b]
	4.19 ± 2.81[a]	A	6.06 ± 1.52[a]	7.02 ± 0.78[a]
	4.92±0.78[a]	B	11.96 ± 1.34[b]	17.41 ± 3.22[b]
Urea (mg/dl)	4.33±0.59[a]	C	8.87 ± 3.02[ab]	9.40 ± 2.33[a]
	4.22±1.45[a]	D	9.73 ± 1.01[ab]	9.83 ± 1.15[a]
	4.31±1.12[a]	E	9.41 ± 0.89[ab]	10.83 ± 1.54[a]
	0.13 ± 0.03[a]	A	0.83 ± 0.60[a]	0.51 ± 0.31[a]
	0.24±0.33[a]	B	2.55 ± 1.96[b]	1.19 ± 0.16[b]
Creatinine (mg/dl)	0.22±0.25[a]	C	0.52 ± 0.19[a]	0.57 ± 0.25[a]
	0.13±0.17[a]	D	0.65 ± 0.08[a]	0.39 ± 0.25[a]
	0.09±0.15[a]	E	0.81 ± 0.33[a]	0.85 ± 0.08[a]
	4.00 ± 1.15[a]	A	8.00 ± 3.46[a]	10.00 ± 3.46[a]
	4.17±1.33[a]	B	20.00 ± 3.42[b]	18.00 ± 6.00[b]
C-reactive Protein (mg/L)	4.31±0.23[a]	C	8.67 ± 2.31[a]	6.00 ± 0.00[a]
	4.45±0.15[a]	D	8.00 ± 3.06[a]	6.00 ± 0.01[a]
	4.93±0.05[a]	E	10.00 ± 1.46[a]	8.00 ± 3.42[a]

Means within the same column with different suffixes are significantly different ($p<0.05$). APEC: Avian pathogenic *E. coli*. A: Healthy control, B: Infected with APEC and untreated chicks, C: Infected with APEC and treated with norfloxacin, D: Infected with APEC and treated with *L. casei*, E: Administered with *L. casei* 48 h before infection with APEC.

Table 4. Liver function parameters of broiler chicks infected with APEC in different treatment groups

Parameters	Reference range	Treatment Groups	Values after 21 days Treatment	Values after 28 days Treatment
	24.12 ± 3.33[a]	A	33.33 ± 7.64[a]	35.00 ± 7.55[a]
	33.86 ± 10.24[c]	B	74.33 ± 7.02[c]	88.00 ± 6.64[c]
AST (IU/L)	34.32 ± 3.35[b]	C	52.67 ± 6.86[ab]	74.00 ± 6.56[b]
	32.68 ± 4.05[b]	D	62.33 ± 4.16[b]	75.00 ± 9.64[b]
	35.15 ± 6.33[c]	E	63.33 ± 7.64[b]	74.00 ± 8.68[b]
	28.22 ± 5.35[a]	A	34.00 ± 8.19[a]	36.00 ± 5.29[b]
	31.00 ± 6.16[a]	B	63.67 ± 6.66[b]	48.67 ± 6.66[c]
ALT (IU/L)	29.66 ± 3.13[a]	C	47.67 ± 4.93[ab]	27.00 ± 7.55a
	33.00 ± 8.19[b]	D	59.33 ± 3.58[ab]	33.33 ± 2.58[a]
	33.33 ± 7.26[b]	E	55.67 ± 4.73[ab]	36.67 ± 1.08[a]
	15.15 ± 3.33[a]	A	18.10 ± 5.87[a]	28.87 ± 0.03[a]
	17.33 ± 6.66[a]	B	44.94 ± 7.27[c]	47.19 ± 6.26[b]
ACP (IU/L)	15.06 ± 2.15[a]	C	23.70 ± 4.77[a]	24.56 ± 5.40[a]
	16.28 ± 4.05[a]	D	28.83 ± 7.10[b]	26.75 ± 6.95[a]
	15.25 ± 6.33[a]	E	20.64 ± 7.73[a]	30.43 ± 1.30[a]

Means within the same column with different suffixes are significantly different ($p<0.05$). AST: Aspartate transaminase; ALT: Alanine transaminase; ACP: Acid phosphatase, APEC: Avian pathogenic *E. coli*. A: Healthy control, B: Infected with APEC and untreated chicks, C: Infected with APEC and treated with norfloxacin, D: Infected with APEC and treated with *L. casei*, E: Administered with *L. casei* 48 h before infection with APEC.

DISCUSSION

Attributes expressed by *L. casei* during probiotic screening suggest that it is a good probiotic organism as mentioned in studies conducted by Liu (2003) and Haben (2019).

Observation of the experimental groups showed a steady increase in the weight of uninfected chicks (A), compared with other groups. The infected, but untreated chicks (B) decreased in weight from about three weeks post-infection until the end of the experiment. On the other hand, the infected chicks treated with antibiotics and probiotic *L. casei* (C, D, E) showed weight gain, compared to untreated chicks (Figure 1 and Table 1) although the growth rate remained below that of the healthy control. Researchers have reported that probiotic administration in poultry usually results in weight gain as a result of improved absorption in the walls of the intestine and thus suggested that they could be adopted in place of antibiotics as growth promoters (Ezema, 2013; Vantsawa et al., 2017; Gulmez et al., 2019; Nugraheni et al., 2019). The findings in the current study support the claim that probiotics possibly affect weight gain in chicks, as seen in the mean body weight gain of chicks in group D.

Chicken colibacillosis is a localized or systemic infection caused by APEC. It is one of the common infectious diseases of farmed poultry and may manifest in several forms, including enteritis, septicemia, sub-acute pericarditis, salpingitis, peritonitis, and cellulitis (Lutful-Kabir, 2010). In the current study, disease progression was monitored in the chicks by the presence of watery and bloody diarrhea, which are signs of enteritis along with the analysis of haematological and biochemical parameters, which are useful as physiological indicators and diagnostic tools in chicks (Lloyd and Gibson, 2006; Keçeci and Çöl, 2011). The results obtained in the current study showed that there was a marked reduction in hemoglobin count in the infected and untreated chicks, compared to the infected chicks treated with *L. casei* (Table 2). There was also a significant difference in total white blood cell count, neutrophil, and lymphocyte counts between groups B and D; group B showed increased white blood cell and lymphocyte counts, compared to D. However, no significant differences were recorded in eosinophil, basophil, and monocyte counts. A severe reduction in red cell count is suggestive of anemia and may be attributed to the observable bloody diarrhea in the infected chicks. On the other hand, increased white blood cell and lymphocyte count, observed in the infected chicks, are usual indicators of the disease condition. All these signs were ameliorated in the current study by the administration of *L. casei* probiotic, either as a treatment option, post-infection, or as a prophylactic measure. This is in line with previous studies that have reported the immune-boosting capacities of *L. casei*, *L. delbrueckii*, and *L. plantarum* (Kumar et al., 2010; Kechagia et al., 2013).

Oral administration of *L. casei* also produced some significant ($p < 0.05$) effects on some blood chemistry profiles of the broiler chicks. There was a marked reduction in sodium, chlorine, and bicarbonate values in the infected and untreated chicks compared to the infected chicks treated with *L. casei*. This indicates diarrhea severity and severe electrolyte loss and imbalance in infected untreated chicks but was not the case for the probiotic administered groups. There was also an observable difference in urea, creatinine, and C-reactive protein values between the two groups (B and D). There was proper creatinine clearance in the probiotic control and prophylactic groups, indicating proper kidney functions. There were also indications of poor liver function in the diseased chicks, probably as a result of systemic colibacillosis. However, the probiotic control and prophylactic groups had a normal liver function which implies that *L. casei* could not cause adverse effects in the liver function of chicks when taken orally.

A significant decrease in fecal *E. coli* counts reflects the probiotic effectiveness in controlling colibacillosis in the broilers. This finding could be further explained considering the report of Gogineni et al. (2013), which states that probiotic treatment of intestinal diseases cuts the infection cycle shorter almost like that of antibiotic treatment. As seen in Figure 2, chick groups treated with antibiotics and *L. casei* (C and D), respectively, showed a significant ($p<0.05$) reduction in fecal *E. coli* counts. In the groups administered *L. casei* prophylaxis, however, there was complete inhibition of infection, as chicks in that group (E) had similar counts as the healthy control (Figure 2). It is also noteworthy that the *L. casei* counts were sustained until the end of the experiment. This is in line with the report of Gaynor (2019) that *L. casei* can gut colonize and could provide some health benefits when used as a food ingredient.

CONCLUSION

The *L. casei* has positive probiotic qualities that can be used in the prevention and treatment of chicken colibacillosis.

DECLARATIONS

Competing interests

Authors declare no competing interests.

Authors' contributions

Onyeka Michael Ikele conceptualized and carried out the research work, manuscript writing, and data analyses. Ifeoma Maureen Ezeonu assisted in the second supervision, data curation, and manuscript proof-reading. Chibuzo Nneka Umeh designed and supervised the research work before Ifeoma Maureen Ezeonu completed the supervision. All authors approved the final draft of the manuscript.

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Formalin Potentials in the Pathogenic Attenuation of *Eimeria tenella* based on Oocyst Productions

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ABSTRACT

Coccidiosis is a disease found in poultry caused by parasitic protozoa, namely *Eimeria tenella* (*E. tenella*), which may lead to high rates of morbidity and mortality. To prevent coccidiosis, vaccination is required to inactivate and attenuate *E. tenella* protozoa. One of the compounds applied for attenuation is formaldehyde. Formaldehyde reduces the pathogenicity of an organism by creating rigidity in its structure. As a result, the organism cannot inflict disease and has a higher impact on building antibodies although it is still alive. The current research was an experimental study aimed to determine the formalin potential in attenuation of *E. tenella* pathogenesis in terms of oocyst production. The present study was conducted using the completely randomized design method. A total number of 25 broiler chickens were applied and their feces were tested to observe oocysts production and clinical symptoms. The obtained data would be analyzed by the ANOVA statistical test. The treatment groups presented clinical symptoms of *E. tenella* infection. The number of oocysts in treatment group I fluctuated from the lowest number which was zero on day five and then increased by day six, seven, and eight and it has reached the peak with the most significant amount of 4,050,460 oocysts on day nine. The treatment group II with the same condition reached its peak with the highest number of 1,363,160 oocysts on day nine. The treatment group III peaked with the most significant number of 618,960 oocysts on day nine. In addition, the treatment IV group attained the apex with the highest number of 719,480 oocysts on day nine. Meanwhile, the treatment V group reached the highest number of 284,200 oocysts on day nine. The difference in formalin concentration affected the amount of *E. tenella* oocyst production of broiler chickens. Formalin soaking with a concentration of 1.2% was the most optimal concentration to attenuate *E. tenella*.

Keywords: Broiler chicken, *Eimeria tenella*, Formalin, Oocyst

INTRODUCTION

There are some threats to inhibit the sustainability of the poultry sector, one of them is poultry disease (Gussem, 2007; Kadhim, 2014). Poultry can suffer from several diseases due to parasitic protozoa, which may cause high morbidity and mortality. One of the diseases is coccidiosis, caused by a protozoa type and a genus of *Eimeria* (Dalloul and Lillehoj, 2006; Hafex, 2011; Kadhim, 2014). *Eimeria tenella* (*E. tenella*) is one of the *Eimeria* species that causes plump-formed coccidiosis in chickens. This disease often ravages chickens aged around three weeks. The parasite lives in the cecum epithelium and experiences multiplication in the epithelium. Consequently, it causes weight loss, stunted growth, decreased appetite, pale combs and pallor, tangled fur, diarrhea, reduced meat production, bloody diarrhea, and even death (Fanatico et al., 2007). Coccidiosis is one of the most prevalent diseases, which occurs during the chicken-breeding period (Kadhim, 2014). Various attempts to control coccidiosis have been carried out, although they are not entirely successful. Maintaining clean sanitation is known to break the *Eimeria* life cycle. This method, nonetheless, cannot completely prevent coccidiosis (Ayed et al., 2012). The use of coccidiostat for a long period has led to problems. The side effect of coccidiostat includes resistance to the coccidiostat, even poisoning. Adding coccidiostat to chicken feed for the long term may cause residues in meat and eggs, which threatens consumers (Srinivasu et al., 2019). Vaccination is an effective and safe alternative to control coccidiosis (Bahrami and Bahrami, 2006; Meeusen et al., 2007). A vaccine is produced through inactivation and attenuation. Pathogenicity attenuation means reducing the ability of an organism to cause disease in infected organisms (Bahrami

and Bahrami, 2006). If chemicals are utilized for attenuation, that material must produce a minuscule change to stimulate protective immunity in the antigen. One of the compounds used for attenuation is formaldehyde. Formaldehyde reacts with amino and amide groups in proteins, and with amino bound to non-water substances with the essential ingredients of purine nucleic acids and pyrimidines (Tizard, 2004). The chemical forms cross-bonding, and thus provides structural rigidity in the organism. The structural rigidity of these organisms can reduce organism pathogenicity. Even though the organism is alive, it cannot cause any disease. Furthermore, it is highly effective to form antibodies (Tizard, 2004). Based on the explanation above, this research aimed to determine the formalin potential in attenuation of *E. tenella* pathogenesis in terms of oocyst production.

Ethical approval

All experimental protocols and procedures were approved by the Institutional Animal Care of Indonesia.

MATERIALS AND METHODS

The present research was an experimental study using the completely randomized design with an assumption that the experimental animal cages were uniform. The isolations employed were *E. tenella* oocysts collected from the cecum of domestic chickens, and developed in the Department of Veterinary Parasitology, Universitas Airlangga, Surabaya, Indonesia. Oocysts isolates were field strains known to cause cecal lesions according to Jordan et al. (2011). Sporulation was obtained by constant stirring and also aeration of oocysts for 48 hours at 28°C.

In the current study, 25 male broiler chickens aged two weeks old, were randomized based on the completely randomized design. Each pen (cage) was provided with water and an individual feeder. Room temperature was maintained at 21°C. Meanwhile, the experimental design pattern was conducted on five treatment groups with five replications in each group. The present study consisted of five treatment groups. Treatment group I (P-I) was the control group in which oocysts were not immersed in formalin, then about 10,000 oocytes were inoculated. Treatment group II (P-II) involved *E. Tenella* immersed in formalin with a concentration of 0.15% for 96 hours, and then inoculated, amounting to 10,000 oocysts per oral. In treatment group II (P-III), *E. Tenella* was immersed in formalin with a concentration of 0.3% for 96 hours, and then inoculated, amounting to 10,000 oocysts per oral. Treatment group IV (P-IV) entailed *E. tenella* immersed in formalin with a concentration of 0.6% for 96 hours, and then inoculated, amounting to 10,000 oocysts per oral. Finally, in treatment group V (P-V), *E. tenella* was immersed in formalin with a concentration of 1.2% for 96 hours, and then inoculated, amounting to 10,000 oocysts per oral.

Feces collection

The chicken feces were collected during 5-12 days after infection, and then they were put into plastic bags and 10% formalin was added. Then, they were labeled according to the distribution of the treatment groups. Collected chicken feces were stored for a short time before the examination of feces.

Examination and calculation of total oocyst production per day

The collected feces samples were examined to calculate the amount of oocyst production. The sequence process for calculating the total amount of oocyst production was conducted using the Mc-Master method. The feces samples were weighed as much as 2 grams and then put in a test tube. A total amount of 58 ccs of saturated salt solution was added to the tube containing feces, and then stirred using a stirring pipette until they were homogeneous. Next, the samples were taken using a Pasteur pipette and put into the Mc-Master counting chamber until both chambers became full. Following that, they were calculated under a light microscope with 100× magnification and were calculated in two Mc-Master counting chambers.

Research variable

There were three variables in the current research including independent variable, dependent variable, and control variable. The independent variables were formalin concentration and *E. tenella* immersion time in formalin. The dependent variables were the amount of oocyst production and clinical symptoms. Meanwhile, the control variables in this study were *E. tenella* oocysts, chicken species, chicken feed, cages, environment, and required tools for the research.

Data analysis

The data to obtain were the clinical symptoms and oocyst production. The date of clinical symptoms was descriptively presented while the oocysts production data was assessed by Analysis of Variant (ANOVA) in SPSS (version 18) for Windows. If each group were significantly different, a further test with the Least Significant Different

(LSD) would be carried out. Differences were considered significant when $p < 0.05$.

RESULTS AND DISCUSSION

Clinical symptoms

In the control group (P-I) with non-attenuated *E. tenella* infection, the chickens presented clinical symptoms, such as looking weak, loss of appetite, and bloody diarrhea five to six days after the infection. Clinical symptoms in P-II, P-III, and P-IV were relatively similar. In general, the clinical symptoms included looking rather limp chickens, loss of appetite, and watery stool (diarrhea). On the other hand, the P-IV group represented non-significant clinical symptoms ($p > 0.05$). The chickens in this group had good health, good appetite, and a proper drink. Moreover, the chickens in this group looked more agile than the chickens in the other groups.

Test results of oocyst production

The results of the *E. tenella* oocysts production on feces was commenced on day 5 to 12. The oocysts production on day five was zero for all treatment groups. Therefore, the data included in this section is the production data of oocysts per gram feces on days six to seven after the infection. The examination was performed using the Mc-Master method and was observed under a light microscope with 100× magnification. Table 1 describes the research data.

Feces samples

Table 1 indicates that the average amount of oocyst production of the broiler chickens infected by *E. tenella* in the control group was significantly different in terms of the average result per day, compared to the other groups ($p < 0.05$).

Figure 1 demonstrates that the number of oocysts in the P-I group fluctuated from the lowest number of zero on day five and then increased on days six, seven, and eight and reaching the peak with the highest number of 4,050,460 oocysts on day nine. in the same condition, P-II achieved the summit with the highest number of 1,363,160 oocysts on day nine. However, P-III hit the culmination with the highest number of 618,960 oocysts on day nine. In addition, treatment IV reached the peak with the highest number of 719,480 oocysts on day nine. Eventually, the P-V group reached the top result with the highest number of 284,200 oocysts on day nine. As a matter of fact, the oocysts productions in the P-I group were always higher during 7-12 days when compared to the other groups. It was due to factor that the treatment in this group was not immersed in formalin. The oocyst production peaked on day nine after infection in the P-I group. In treatment groups with different concentrations of formaldehyde immersion, the oocyst production on day nine was lower than the P-I, without any formaldehyde immersion. After the oocyst production reached the summit on day nine, the amount of oocyst production decreased on the following day.

Based on figure 2, it can be observed that there is a significant difference in the number of oocyst production in each treatment group on the same day ($p < 0.05$). The control group (P-I) always had the highest number of oocyst production, compared to the treatment groups that *E. tenella* was immersed in formalin with various concentrations. The number of oocysts in P-II was lower than P-I and was higher than P-III. The number of oocytes in P-III was generally higher than in P-IV. Nevertheless, P-III had a lower oocyst production than P-IV on days 9 and 10. The number of oocysts in P-V was lower than P-III and P-IV.

Table 1. Average and standard deviation of oocyst production in feces samples of the broiler chickens infected by *Eimeria tenella* during 6-12 days after infection.

Group	Day 6 ($\bar{x}\pm SD$[1])	Day 7 ($\bar{x}\pm SD$)	Day 8 ($\bar{x}\pm SD$)	Day 9 ($\bar{x}\pm SD$)	Day 10 ($\bar{x}\pm SD$)	Day 11 ($\bar{x}\pm SD$)	Day 12 ($\bar{x}\pm SD$)
Control	$9.4^{a} \pm 8.013$	$30.9^{a}\pm12.630$	$355.6^{a}\pm215.20$	$4050.5^{a}\pm3095.22$	$2488^{a} \pm1902.34$	$1341^{a}\pm627.393$	$120.34^{a}\pm78.415$
Formalin 0.15%	$3.54^{ab} \pm 4.862$	$19.9^{b}\pm4.621$	$231.04^{ab}\pm129.462$	$1363.2^{b}\pm732.344$	$601.38^{b}\pm343.373$	$238.4^{b}\pm136.706$	$42.240^{b}\pm26.988$
Formalin 0.3%	$2.35^{b}\pm3.609$	$14.6^{b}\pm 2.897$	$130.4^{b}\pm87.656$	$618.96^{b}\pm324.259$	$340.74^{b}\pm311.717$	$172.38^{b}\pm135.353$	$35.980^{b}\pm35.231$
Formalin 0.6%	$0.82^{b}\pm1.833$	$3.8^{a}\pm2.456$	$128.87^{b}\pm55.665$	$719.48^{b}\pm446.684$	$354.60^{b}\pm116.976$	$109.4^{b}\pm77.105$	$20.16^{b}\pm16.353$
Formalin 1.2%	0^{b}	$2.309^{a}\pm1.3$	$40.14^{b}\pm33.644$	$98.644^{b}\pm25.718$	$176.60^{b}\pm147.92$	$116.34^{b}\pm75.349$	$12.38^{b}\pm11.47$

Note: Different superscripts in the same columns represent a significant difference ($p < 0.05$) of the treatments per day. [1]standard deviation

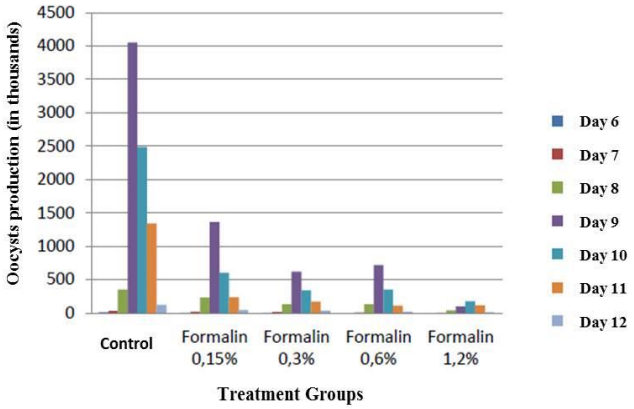

Figure 1. Formalin Potential Graphic of *Eimeria tenella* pathogenicity which attenuated with different formalin concentrations regarding oocyst production of the broiler chickens infected by *Eimeria tenella* per day.

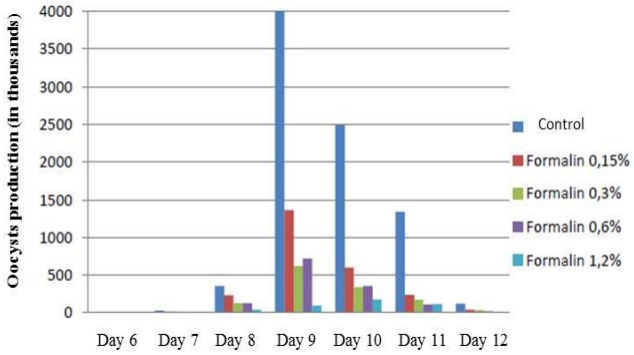

Figure 2. Formalin Potential Graphic of *Eimeria tenella* pathogenicity which attenuated with different formalin concentrations in terms of oocyst production of the broiler chickens infected by *Eimeria tenella* during 6-12 days after infection.

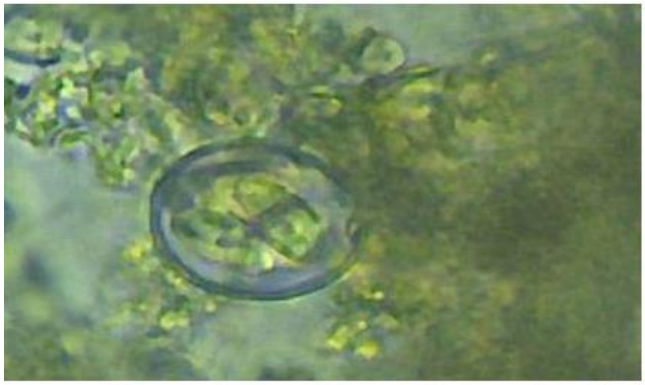

Figure 3. *Eimeria tenella* not immersed in formalin in feces sample of infected broiler chicken.

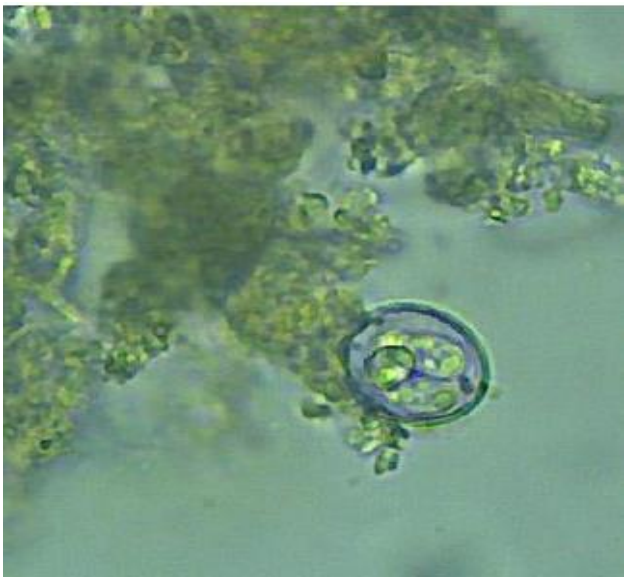

Figure 4. *Eimeria tenella* immersed in formalin with 0.6% concentration in feces sample of infected broiler chicken.

DISCUSSION

The analyzed data from oocytes production demonstrated significant differences among the chickens in the control group inoculated with *E. tenella* oocysts which were not immersed in formalin and the treatment groups that inoculated with *E. tenella* oocysts immersed in formalin. Formalin reacts with amino and amide groups in proteins, as well as with amino bound to non-water substances with the basic ingredients of purine nucleic acids and pyrimidines to form cross-bonds. Hence, it results in *E. tenella* structural rigidity, and consequently reduced pathogenicity. Although *E. tenella* is alive, it cannot inflict any disease and it still has significant influences on forming antibodies (Tizard, 2004).

There are several vital factors affecting the pathogenicity of *E. tenella* infection as follows, the number of swallowed oocysts, oocyst strains, environmental factors, the number of formed merozoites, and the amount of merozoites production in each cycle of schizogony. These cycles commence from the damage in host epithelial cells, the parasites settling in the tissue and cells of the host, the rate of re-infecting, and the level of immunity. Young animals are more sensitive to this disease than older ones. However, it generally does not cause death (Cui et al., 2016).

The most pathogenic stage in *E. tenella* life cycle is the second schizont generation which is large and contains hundreds of merozoites. The schizont develops in the lamina propria so that the mucosa will experience necrosis

when the schizont grows and releases merozoites. Generally, death occurs five and six days after infection. In acute infections, death is possible to occur within a few hours after initial clinical symptoms arise (Srinivasu et al., 2019).

The attenuation of *E. tenella* can be conducted using formaldehyde (formalin) chemicals. Formaldehyde reacts with amino and amide groups in proteins, as well as with amino bound to non-water substances with the necessary ingredients of purine nucleic acids and pyrimidines to form cross-bonds. It, thus, provides structural rigidity in the organism. The structural rigidity of the organism can decrease organism virulence. As a result, the organism is alive, however, it cannot cause any disease. It significantly influences the antibodies forming (Tizard, 2004).

The coccidiosis can be acute or fatal, depending on the dose of infective oocysts swallowed by the host. In an infection with less than 150 oocysts, there will be bleeding points on the mucosa of the cecum with slight discoloration of the cecum wall. The number of 150-500 oocysts may cause bleeding in the mucosa with little bleeding, injury, and thickening of the cecum wall. Meanwhile, 1,000-3,000 oocysts cause high mortality, bleeding, some blood clots, and abnormal cecum contents. In addition, 5,000 or more oocysts will lead to the highest rate of death and bleeding, blood clots in the cecum, which are frozen and calcified (Srinivasu et al., 2019).

The clinical symptoms in coccidiosis are characterized by mucoid and hemorrhagic diarrhea. The symptoms of diarrhea are usually followed by dry feather, dehydration, anemia, lethargy, bending of the head and neck, and drowsiness. The chicken begins to excrete bloody feces 96 hours post-infection. The bleeding reaches its summit five or six days post-infection and in a severe case, it can be followed by death. In case an animal survives, the disease can be healed and the animal gets immune after seven days. In acute events, death occurs four to five days after infection (Boulton et al., 2018; Fortuoso et al., 2019).

Formaldehyde functions to denature protein enzymes which cause the alteration of the enzyme structure. Therefore, the enzyme activity is inhibited resulting in an inability to catalyze the metabolic processes in cells, and consequently microorganisms' death. Formalin can chemically react with almost all substances in the body cells to suppress cell function and cause cell death (Sarot et al., 2017). Formalin aldehydes are easy to react with proteins (Musiał et al., 2016). Formalin can react with *E. tenella* oocysts. This is because the outer layer of the *E. tenella* oocyst wall consists of protein, meanwhile, the inner layer consists of fat which binds to protein. Formalin will bind to proteins and cause protein death in the *E. tenella* oocyst wall. Formalin can dehydrate the cell of an organism (lack of water), consequently, the organism will dry up and structural rigidity occurs (Fortuoso et al., 2019).

Formalin concentration levels that are effective in attenuating *E. tenella* can cause low oocyst production. *E. tenella* immersion in 0.3% formalin can significantly affect antibody response of the host against *Eimeria* infection and protects chickens against severe cecum histopathological changes (Kadhim, 2014).

CONCLUSION

Formalin could attenuate the *Eimeria tenella* pathogenicity in broiler chickens. The difference in formalin concentration affected the amount of *Eimeria tenella* oocyst production in broiler chickens. In addition, a concentration of 1.2% formalin was the most optimal dose of formalin for *Eimeria tenella* attenuation.

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Diallel Analysis on Breast and Thigh Muscle Traits in the Cross of Three South African Indigenous Chicken Genotypes

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ABSTRACT

The present study aimed to estimate carcass characteristics of pure and crossbred chickens produced from three parental populations. A 3 × 3 complete diallel mating system involving three indigenous breeds, namely Potchefstroom Koekoek (P), Venda (V), and Ovambo (O), was used to produce three purebred (P × P, V × V, O × O), three crossbreds (P × O, P × V, O × V) and three reciprocals (O × P, V × P, V × O). The nine genetic groups were reared from hatch to 10 weeks of age in an open house with deep litter. At 10 weeks of age, six chickens per genetic group were randomly selected for slaughter. After slaughtering the breast and thigh muscles samples for analysis of the carcass characteristics (Meat colour, meat pH, and Shear force). The results showed that the Potchefstroom Koekoek breed had higher values in all colour indicators, L* (lightness), a* (redness), and b* (yellowness), compared to the other chicken breeds. The Potchefstroom Koekoek and P × O breed had higher pH values ranging from 5.66 to 6 at two hours post-slaughter and from 5.54 to 6.38 at 24 hours post-slaughter. The pH declines in all the nine genetic groups after two to 24 hours, with the exception of the crossbred P × O, which increased from 6.06 to 6.38. In terms of shear force, the O × P had the highest shear value, ranging from 35.89N to 74.80N, compared to other genetic groups. Potchefstroom Koekoek had normal meat colour and pH, whereas the Venda breed had tougher meat than other genotypes. The results of the present study might be useful for local chicken farmers to improve carcass traits.

Keywords: Crossbred, Meat colour, Meat pH, Purebred, Shear force.

INTRODUCTION

Diallel cross is the crossbreeding among several genotypes and data analysis from such crosses (Khalil and Khadiga, 2020). Crossbreeding is one of the tools that can be used in genetic variation (Wang et al., 2019). This crossbreeding which is the mating of two individuals with different breed compositions, is one type of a larger class of mating systems known as out-breeding (Khalil and Khadiga, 2020). There are many South African indigenous chicken genotypes, including Potchefstroom Koekoek, Venda, and Ovamba chicken (Manyelo et al., 2020). The Potchefstroom Koekoek chicken genotype, developed by crossing Black Australorp cockerels with White Leghorn hens and Plymouth Rock, reaches sexual maturity after 130 days and is mainly categorized by black and white speckled colour patterns (Mutibvu et al., 2020). The Venda chicken genotype, discovered in the Venda region of South Africa, is a dual-purpose breed with multi-coloured patterns predominated by black and white colours (Manyelo et al., 2020). On the other hand, the Ovambo chicken genotype, first discovered in the Ovamboland of northern Namibia, is predominantly dark to black in colour and can reach sexual maturity after 140 days (Mutibvu et al., 2020). Tyasi et al. (2019) conducted a diallel cross of three South African indigenous chicken genotypes, namely chicken genotypes Potchefstroom Koekoek, Venda, and Ovamba, to investigate the crossbreeding effects on growth traits. Thus far, there had been limited studies of the crossbreeding effects on breast and thigh muscles in Potchefstroom Koekoek, Ovambo, and Venda chicken breeds. Hence, the objective of the study was to determine the effect of crossbreeding on meat pH, meat colour and meat tenderness on breast and thigh muscle traits. The current study will aid indigenous chicken farmers to improve carcass characteristics through crossbreeding.

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MATERIALS AND METHODS

Ethical approval

This research was carried out in accordance with the standard operation procedures of the Animal Research and Ethics Committee (AREC) at the University of Limpopo, South Africa.

Study area

The present study was conducted on the experimental farm of the University of Limpopo in the Limpopo Province of South Africa. The farm is located about 10 kilometers northwest of the university. The mean temperatures in winter (April to July) range between 10.1 and 28.4 °C and in summer (August to March) between 18 and 36 °C. The annual rainfall ranges between 446.8 and 468.4 mm.

Preparation of the house

The house was prepared as explained in Alabi et al. (2020). Briefly, the incubator and all the equipment such as drinkers, feeders, and wire separators were thoroughly cleaned and disinfected.

Chickens management

Ten hens of each breed were assigned randomly to be mated with two roosters of each breed. The eggs were collected daily by breeds and crossbreds and then hatched separately according to breeds and crossbreds. Accordingly, nine genetic groups of P × P, V × V, O × O, P × V, P × N, V × P, V × O, O × P, and O × V chickens were obtained. The hatched chickens were wing banded until eight weeks of age, followed by leg bands to keep their breed and crossbred group identity. The chickens were kept together on a litter floor in a semi-open house, which was partitioned according to their breeds and crossbreds. They were medicated in a similar way and were subjected to the same management, hygiene, and climatic conditions. During the brooding and rearing periods, all chickens were fed *ad libitum* using a standard commercial starter (21 % Crude Protein (CP) and 3,000 kcal Metabolizable Energy (ME)/kg) from hatching time until four weeks of age, followed by a grower diet (18 % CP and 2,900 kcal ME/kg) up to ten weeks of age. Water was provided *ad libitum*. Artificial heat (32 °C) using infrared lights and a continuous light program was provided. The ventilation was controlled using curtain rails.

Meat pH measurements

A portable pH meter with a fibre-optic probe (CRISON pH 25 Instruments S.A., Alella, Spain) was used to measure the pH (pH 2 and 24 hours post mortem) of the carcasses. The pHu was first calibrated using standard solutions of pH 4, pH 7, and pH 9 (CRISON Instruments, SA, and Spain). The measurements were then performed with a sharpened metal sheath to prevent probe breakage due to the raw meat contamination.

Determination of meat colour

The colour of the meat (L*: Lightness, a*: Redness, and b*: Yellowness) was determined two hours after slaughter (Commission International De l` Eclairage, 1976). A refrigerated portable vehicle was used to move the carcass from the slaughtering place to the University of Limpopo, Department of Animal Production laboratory one hour after the slaughter. A Minolta colour-guide 45/0 BYK-Gardener GmbH machine with a 20mm diameter measurement and an illuminant D65-daylight, from 100 standard observers was used for the colour measurement. The machine was calibrated each time before measurements were taken using the standard green, black and white colour samples provided for this purpose. The readings were taken by rotating the Colour Guide 900 between measurements to obtain the average value for the colour.

Determination of meat tenderness

The tenderness of the chicken was determined using the Instron- Warner-Bratzler Shear Force (WBSF). After cooking, subsamples of specified core diameter were cored parallel to the grain of the meat. Three subsamples with a core diameter of 10 mm were cored parallel to the grain of the meat. The samples were sheared perpendicular to the fiber direction using a Warner Bratzler (WB) shear device mounted on an Instron (Model 3344) Universal Testing apparatus (the crosshead speed at 400mm/minutes, one shear of each core). The mean maximum load (N) was recorded for the batch.

Data analysis

The Generalized Linear Models procedure (PROC GLM) of Statistical Analysis System (SAS, 2019), version 9.4, was used to determine the effect of the chicken genotype on the chicken quality attributes (pH, L*, a*, b* and WBSF values).

$$Yi = \mu + \alpha i + \varepsilon i$$

Where,

Yi = Response variable (pH, L, a*, b* and WBSF), μ is the overall mean, αi denoted breed effect (V × V, O × O, P × P, V × O, V × P, O × V, O × P, P × V and P × O genotypes), and εi refers to random error term. Differences considered significant ($p \leq 0.05$) were compared with Fisher's protected Least Significant Difference (LSD test).

RESULTS

The meat colour of different genotypes for the breast muscle is presented in Table 1. The results showed a significant difference ($p < 0.05$) in the colour of the breast muscle between the nine genetic groups. In terms of lightness, the Ovambo chicken genotype had a higher value, followed by O × V chicken genotype. However, there were no statistically significant differences ($p > 0.05$) between crosses (P × O, P × V, and O × V) and reciprocals (V × P, V × O, and O × P). For redness, the Potchefstroom Koekoek genotype had a higher value ($p < 0.05$) followed by the P × O chicken genotype. The results also indicated that O × V had statistically insignificant differences ($p > 0.05$) with reciprocals (V × P, V × O, and O × P) as well as V × V and P × V. In yellowness, the Potchefstroom Koekoek chicken genotype also had a higher value ($p < 0.05$) than the other chicken genotypes. The results showed that there were no statistically significant differences ($p > 0.05$) between V × V and crosses (P × O, P × V, and O × V). These results also indicated that there were no remarkable differences ($p > 0.05$) between V × V and the reciprocal values (O × P, V × P).

Table 2 shows a meat colour of different genotypes for the thigh muscle. The results showed a significant difference ($p < 0.05$) in thigh muscle colour between the nine genetic groups. In terms of lightness, Potchefstroom Koekoek genotype, and Venda chicken genotype had a higher value ($p < 0.05$), followed by Ovambo chicken genotype and the crosses (P × O, P × V, and O × V) chicken genotypes. However, no statistically significant differences ($p > 0.05$) between reciprocals (V × P, V × O, O ×P) were observed. In redness, The findings recognized that no statistically significant differences ($p > 0.05$) were observed between P × P, O × O purebreds, P × O, P × V, O × V crosses, and V × O reciprocal. In yellowness, the Potchefstroom Koekoek chicken genotype had a higher value ($p < 0.05$) than the other chicken genotypes. The results also showed that there were no statistically significant differences ($p > 0.05$) between O × O, V × V, and O × V cross and O × P reciprocal.

The meat pH values of breast muscle at 2 and 24 hours post-mortem in purebreds, crossbreds, and reciprocals are presented in Figure 1. At a pH of 2 hours post-mortem, the findings indicated that the cross of P × O had a higher pH value (5.70 ± 0.09) ($p < 0.05$) than the other genetic groups. The results indicated that no statistically significant differences ($p > 0.05$) between purebreds' P × P (5.66 ± 0.01), O × O (5.61 ± 0.02), and V × V (5.64 ± 0.03) were observed. The results also recognized that there were no remarkable differences ($p > 0.05$) between the reciprocal genetic groups O × P (5.57 ± 0.16), V × P (5.52 ± 0.02), and V × O (5.55 ± 0.01) were observed. At a pH of 24 hours post-mortem, the results indicated that the Potchefstroom Koekoek chicken genotype had a higher pH value (5.64 ± 0.03) ($p < 0.05$) than the other chicken genotypes. The results indicated that there were no statistically significant differences ($p > 0.05$) between reciprocal genetic groups O × P (5.47 ± 0.04), V × P (5.53 ± 0.02), and V × O (5.47 ± 0.04), were observed.Meat pH of thigh muscle at 2 and 24 hours' post-mortem in purebreds, crossbreds, and reciprocals is presented in Figure 2. At a pH of 2 hours post-mortem, the results showed that the cross of P × O had a higher pH value (6.06 ± 0.07) ($p < 0.05$) than the other genetic groups. The results indicated that there were no remarkable differences ($p > 0.05$) between purebreds' P × P (6.00 ± 0.04), and V × V (5.98 ± 0.08), and reciprocal V × P (5.96 ± 0.06) chicken genotype were observed. At a pH of 24 hours post-mortem, the findings recognized that the cross of P × O chicken genotype had a higher pH value (5.70 ± 0.09) ($p < 0.05$) than the other chicken genotypes. There were no statistically significant differences ($p > 0.05$) between V × V (5.98 ± 0.08), cross O × V (5.83 ± 0.07), and reciprocals V × P (5.96 ± 0.06) and V × O (5.90 ± 0.03) were observed.

Table 3 shows the results of the shear force for thigh and breast muscles. In purebreds, the results showed that P × P had a higher shear force value as compared with other purebreds but there were no statistically significant differences ($p > 0.05$) between P × P and O × O in thigh muscle. Whereas in breast muscle, there were no statistically significant differences ($p > 0.05$) observed between purebreds. In crosses, the findings indicated that there were no statistically significant differences among all crosses ($p > 0.05$). While in breast muscle, O × V had a higher shear force value ($p < 0.05$) followed by O × V, respectively. With respect to reciprocals, V × O had a higher shear force value ($p < 0.05$) but no statistically significant differences ($p > 0.05$) observed between O × P and V × P. However, in breast muscle, there were no statistically significant differences ($p > 0.05$) detected between O × P and V × O, respectively.

Table 1. Meat colour of breast muscle in purebreds, crosses, and reciprocals of three South African indigenous chicken genotypes (Potchefstroom Koekoek, Ovambo, and Venda)

Genotypes \ Meat colour indicators		L*	a*	b*
Purebreds	P × P	43.56 ± 0.02^{d}	9.39 ± 0.51^{a}	10.68 ± 0.88^{a}
	O × O	56.54 ± 1.50^{a}	1.68 ± 0.30^{e}	6.69 ± 0.61^{c}
	V × V	44.79 ± 0.89^{cd}	5.13 ± 0.81^{cd}	9.15 ± 0.77^{abc}
Crosses	P × O	48.03 ± 3.09^{bcd}	6.51 ± 0.43^{b}	8.96 ± 0.90^{abc}
	P × V	47.41 ± 0.38^{bc}	6.43 ± 0.29^{bc}	8.99 ± 0.84^{abc}
	O × V	50.93 ± 2.45^{b}	4.90 ± 0.29^{cd}	9.36 ± 1.11^{ab}
Reciprocals	O × P	48.84 ± 2.35^{bc}	5.05 ± 0.65^{cd}	7.44 ± 1.22^{bcd}
	V × P	46.93 ± 0.55^{c}	4.02 ± 0.38^{d}	7.60 ± 1.22^{bcd}
	V × O	47.64 ± 0.11^{bcd}	3.75 ± 0.47^{d}	5.13 ± 1.11^{d}

$^{a-d}$: Means in the same column with the same letters did not differ significantly ($p > 0.05$), P × P: Potchefstroom Koekoek, O × O: Ovambo, V × V: Venda, L*: Lightness, a*: Redness, b*: Yellowness.

Table 2. Meat colour of thigh muscle in purebreds, crosses, and reciprocals of three South African indigenous chicken genotypes (Potchefstroom Koekoek, Ovambo, and Venda).

Genotypes \ Meat colour indicators		L*	a*	b*
Purebreds	P × P	52.09 ± 1.12^{a}	8.95 ± 0.58^{a}	12.87 ± 1.16^{a}
	O × O	46.29 ± 0.59^{b}	8.54 ± 0.53^{a}	9.26 ± 0.31^{b}
	V × V	50.83 ± 0.36^{a}	6.47 ± 0.53^{c}	7.36 ± 1.59^{bc}
Crosses	P × O	45.92 ± 0.93^{b}	8.06 ± 1.15^{a}	6.56 ± 2.08^{c}
	P × V	43.11 ± 0.74^{b}	8.74 ± 0.62^{a}	4.53 ± 0.78^{d}
	O × V	45.67 ± 1.11^{b}	8.00 ± 1.37^{a}	7.77 ± 2.04^{bc}
Reciprocals	O × P	46.80 ± 0.71^{b}	7.43 ± 0.36^{b}	7.03 ± 0.77^{bc}
	V × P	46.60 ± 0.82^{b}	6.78 ± 0.11^{bc}	6.81 ± 1.40^{c}
	V × O	47.55 ± 0.45^{b}	8.30 ± 0.32^{a}	6.27 ± 1.62^{c}

$^{a-d}$ Means in the same column with the same letters did not significantly differ ($p > 0.05$), P × P: Potchefstroom Koekoek, O × O: Ovambo, V × V: Venda, L*: Lightness, a*: Redness, b*: Yellowness.

Table 3. Comparison of shear force in purebreds, crosses, and reciprocals of three South African indigenous chicken genotypes (Potchefstroom Koekoek, Ovambo, and Venda).

Genotypes \ Shear force		Thigh WBSF	Breast WBSF
Purebreds	P × P	44.15 ± 7.45^{a}	58.82 ± 5.92^{c}
	O × O	40.50 ± 6.78^{ab}	51.94 ± 3.87^{cd}
	V × V	32.50 ± 1.55^{bc}	59.07 ± 2.81^{c}
Crosses	P × O	31.38 ± 2.04^{bc}	55.51 ± 9.64^{c}
	P × V	29.25 ± 2.01^{c}	43.62 ± 0.64^{d}
	O × V	31.73 ± 3.05^{bc}	61.23 ± 4.36^{b}
Reciprocals	O × P	35.89 ± 1.36^{b}	74.80 ± 7.15^{a}
	V × P	42.33 ± 3.56^{ab}	65.41 ± 6.35^{b}
	V × O	46.09 ± 6.36^{a}	72.34 ± 8.26^{a}

$^{a-d}$ Means with the same column with the same letters did not significantly differ ($p>0.05$), P × P: Potchefstroom Koekoek, O × O: Ovambo, V × V: Venda. WBSF: Warner Braztler Shear Force

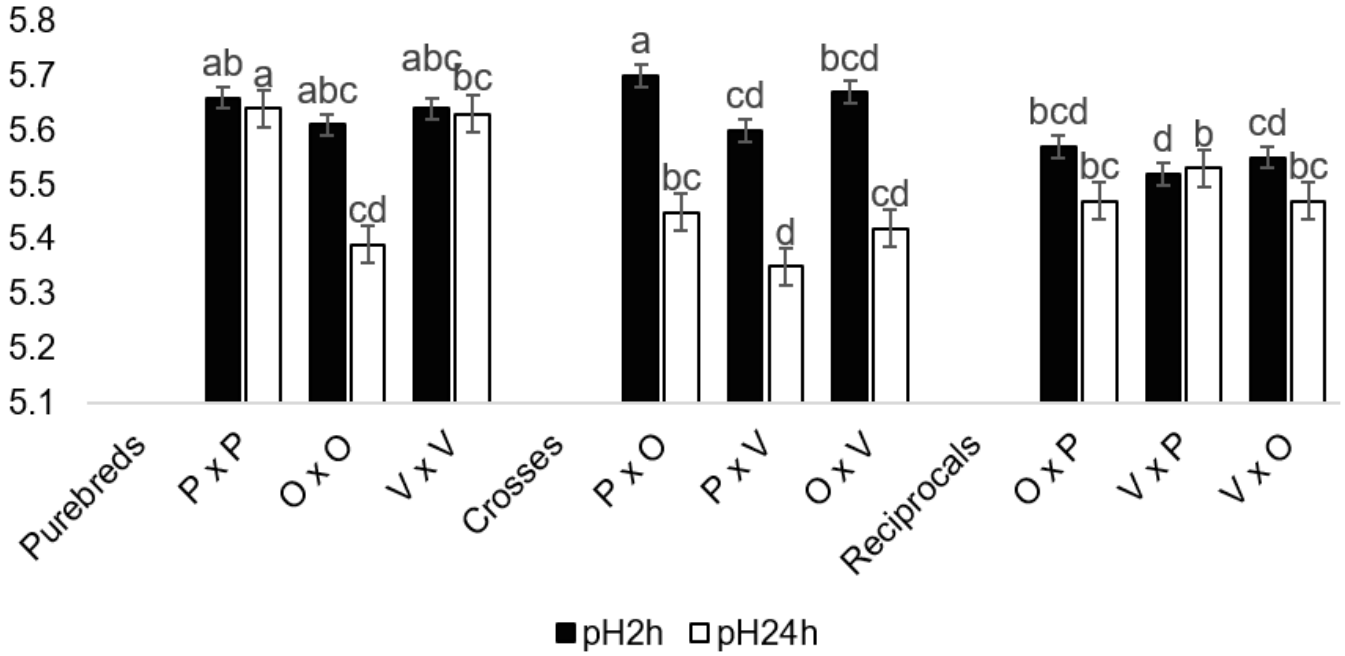

Figure 1. Meat pH of breast muscle 2 and 24 hours in purebreds, crosses, and reciprocals of three South African indigenous chicken genotypes. [a-d]: Means that the same column with the same letters did not significantly differ ($p > 0.05$), P × P: Potchefstroom Koekoek, O × O: Ovambo, V × V: Venda, pH_{2h} = pH 2 hours post-mortem, pH_{24h} = pH 24 hours post-mortem.

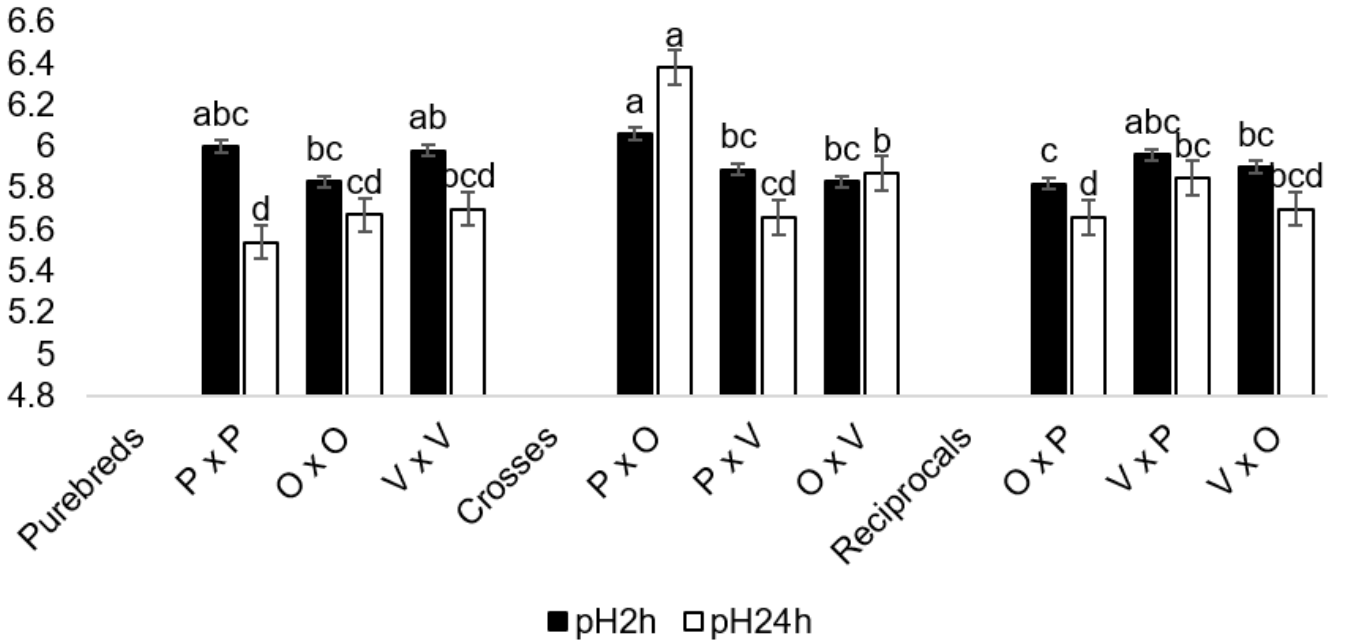

Figure 2. Meat pH of thigh muscle at 2 and 24 hours in purebreds, crosses, and reciprocals of three South African indigenous chicken genotypes. [a-d]: Means with the same column with the same letters did not significantly differ ($p>0.05$), P × P: Potchefstroom Koekoek, O × O: Ovambo, V × V: Venda, pH_{2h} = pH 2 hours' post-mortem, pH_{24h} = pH 24 hours' post-mortem.

DISCUSSION

Crossbreeding may possibly be used to achieve genetic improvement with or without genetic selection in the parental lines (Wang et al., 2019). The influence of crossbreeding on meat color, pH, and tenderness in breast and thigh muscles of nine genetic groups namely; purebreds (P × P, O × O, V × V), crossbreds (P × O, P × V, O × V) and reciprocals (O × P, V × P, V × O) were explored in this study. Meat colour is one of the factors which the consumer uses as a measure of acceptance. For example, a pink interior is considered desirable in a processed whereas a pink or reddish colour is highly undesirable in fully cooked poultry meat (Singh et al., 2021). In the present study, meat color findings suggested that the breast and thigh of the Ovambo chicken genotype were paler (high L*), more yellow (high b*) and less red (low a*) than other genetic groups. Potchefstroom Koekoek chicken genotype had higher values in all color indicators, L* (lightness), a* (redness), and b* (yellowness) as compared to the other chicken breeds. It was however observed that the Venda breed had a high L* (lightness) which was normal between 43 and 53. The pH of chicken meat for the current study was a significant source of variability amongst the nine genetic groups at different hours (2 hours, 24 hours) after slaughter. The pH declined in all the nine genetic groups at 2 to 24 hours with the exception of crossbred, P × O which increased from 6.06 to 6.38. The tenderness of the meat was also examined with a Warner-Bratzler shear force. The Warner-Bratzler shear force is an objective measure of tenderness used in the research laboratory to evaluate relative differences in tenderness or toughness of meat products (Wang et al., 2019). The results of the present study indicated that shear force values of breast muscle were higher than those of the thigh muscle. Similar results were also reported by Wang et al. (2019). Singh et al. (2021) compared the carcass characteristics of a slow-growing crossbred broiler to a fast-growing broiler and found that the pH at 24-hour post-mortem was 5.88 for a fast-growing broiler and 5.74 for a slow-growing broiler. The breast color of the fast-growing broiler had a lightness (L*) of 51.93, a redness (a*) of 208, and a yellowness (b*) of 4.97, while the slow-growing broiler had an (L*) of 45.39, an (a*) of 2.64, and a (b*) of 3.84, respectively. Mueller et al. (2020) studied the meat quality of two dual-purpose and one-layer hybrid chickens for 67 or 84 days compared to a slow-growing broiler. The results showed that the pH in the breast muscle ranged from 5.00 to 6.00 at 24 hours post mortem, while the breast colour ranged from 47.0 to 59.0 for L*, 1.11 to 3.33 for a*, and -0.22 to 1.06 for b*. Devatkal et al. (2019) recognized that the breast shear force of the slow-growing broiler was 11.09 and 12.47 for the fast-growing broiler. Mueller et al. (2018) indicated that the breast meat pH at 24-hour post-mortem for Ross PM3 chicken was 6.25, Sasso S1 chicken 5.92, Lohmann Dual chicken 5.82, Belgian Malines chicken 5.91, Schweizerhuhu chicken 5.73, and Lohmann Brown Plus chicken 5.90, while the breast colour ranged from L* 49.0 to 54.6, a* 1.26 to 3.58, and b* 0.09 to 0.72, respectively. These studies are inconsistent with the current study and the variations might be due to different genotypes, age, nutrition, and environment. Tyasi et al. (2019) discovered that the Potchefstroom Koekoek chicken genotype had a positive influence on the general ability to combine and the crossbred between the Potchefstroom Koekoek and the Venda chicken genotype had a positive influence on the specific ability to combine, and thus suggested that the Potchefstroom Koekoek chicken genotype could possibly be used for crossbreeding with other indigenous chicken genotypes to improve growth traits.

CONCLUSION

Crossbreeding had a remarkable effect on meat colour, pH, and tenderness in a diallel cross of the Potchefstroom Koekoek, Ovambo, and Venda chicken genotypes. The Potchefstroom Koekoek genotype had normal meat colour and pH whereas the Venda chicken genotype had tougher meat than other chicken genotypes. It is recommended that crossbreeding could be introduced to improve genetic effects on carcass quality traits.

DECLARATION

Acknowledgments

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Author's contributions

Thobela L Tyasi conducted the experiment, analyzed the data and wrote the manuscript. Jones W Ng'ambi and David Norris designed and oversaw the running of the experiment.

Competing interests

The authors declare that they have no conflict of interest.

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Histopathology Description of Chicken Liver Infected by L2 *Toxocara Vitulorum*

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ABSTRACT

Transmission of *Toxocara vitulorum* Infection causes a decrease in livestock productivity and results in various types of diseases in humans. Chickens are one of the parasitic hosts of toxocariasis which has the potential for transmission of toxocariasis to humans. The main location affected by *T. vitulorum* larval infection is the liver. The current study aimed to analyze the description of histopathological changes in the liver of broiler chickens infected by L2 *Toxocara vitulorum.* The present study was a true experiment using a completely randomized design. A total number of 28 broiler chickens aged 14 days were selected as the sample in this study. Samples were infected using L2 *Toxocara vitulorum* larvae and were grouped in accordance with observations of the 1, 2, 3, 7, 14, and 21 days after the larvae were given to the samples. *Toxocara vitulorum* larval infection caused changes in histopathological features of broilers chickens. This infection caused hydropic inflammation and degeneration of liver cells, cholangitis, and eventually necrosis of the cells. Exposure to infection over a long period of time can worsen liver cell and other organ damages as well as increasing the potential for the transmission of *Toxocara vitulorum* larvae.

Keywords*:* Chicken, Histopathology of liver, Infection, *Toxocara vitulorum*

INTRODUCTION

Toxocariasis is one of the worm-originating diseases that can attack ruminants, especially calves of cows and buffaloes and the mothers (Hübner et al., 2001). *Toxocara vitulorum* which attacks cows at all ages can be transmitted through food boxes or placenta that can infect the fetus in the womb (Levine, 1995). *Toxocara vitulorum* is commonly found in tropical and subtropical climates (Starke et al., 1996). This infection leads to a reduction in livestock productivity, which will be a financial burden for farmers if not controlled. In addition, *T. vitulorum* infection causes anorexia, stomach pain, diarrhea, constipation, dehydration, bad breath, and also a decrease in the body weight of cattle (Raza et al., 2013).

Humans or animals that consume raw or undercooked liver of paratenic hosts of *Toxocara* spp. are the potential to being contaminated with toxocariasis (Yoshikawa et al., 2008). Some paratenic hosts of toxocariasis are mice, rats, pigs, birds, chickens, humans, and other mammals (Azizi et al., 2007; Yoshikawa et al., 2008; Raza et al., 2013). Larvae can move to various tissues and survive for a long period of time (Azizi et al., 2007; Strube et al., 2013). The movement of larvae into the tissues (lung, liver, and kidney) or milk is thought to be a medium of transmission to humans (Kusnoto et al., 2005). The consumption amount of raw or undercooked meat increases the prevalence of toxocariasis cases (Taira et al., 2011) leading to human zoonosis diseases, such as visceral larva migrans (VLM) and ocular larva migrans (OLM).

T. vitulorum larvae can cause liver and lung lesions, inflammation of lymph nodes, as well as eosinophilia during the life cycle of the parasite (Abbott et al., 2006; Khan et al., 2007). *Toxocara* spp. larvae migrate to the liver through the porta hepatica systems and cause hepatomegaly which is a common phenomenon (Soulsby and Monnig, 1982). In humans, The human infection of *Toxocara* spp. leads to hepatocellular necrosis and inflammatory reactions (Hübner et al., 2001). On the other hand, histopathological examination of visceral organs using helminthiasis has not been performed much, especially to see the histopathological picture of the liver as the site of second-stage *T. vitulorum* larvae migration in

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chickens as paratenic host, where parasites can live but cannot develop into adulthood (Cardillo et al., 2008). Therefore, this study was conducted to describe the histopathological changes in the liver of broiler chickens after being infected by L2 *Toxocara vitulorum.*

MATERIALS AND METHODS

The present study was a true experiment using a completely randomized design performed at the Helminthology Laboratory of the Parasitology Department of Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia, in 2020. The liver histopathology examination was carried out in the Pathology Laboratory. Three variables have been investigated in the current study. The histopathological image of the liver (degeneration and necrosis of hepatocytes) was considered as the dependent variable, the effective dose of L_2 *T. vitulorum* and liver sampling time were taken into account as independent variables. Finally, controlled variables included strain, sex, age, feed, and also environmental conditions of chickens.

Experimental design

Broiler infection involves eggs containing L2 *T. vitulorum.* Phosphate Buffer Saline (PBS) (Sigma Co.) was used as the media of L2 *T. vitulorum*, while the protozoa growth and other microorganisms were avoided using formalin 0.5-1%. In addition, Trypsin 1% was used for releasing L2 *T. vitulorum* from the formed liver tissue.

Isolation and preparation of *Toxocara vitulorum* eggs

Infective eggs of *T. vitulorum* worm inoculant material comes from the intestine of 10 cows contaminated by *toxocariasis,* during visiting the slaughter-house. Worms were washed in 0.85 % saline to remove debris, and they were fixed into 70 % ethanol. The worms were then cleaned with aquadest and transferred to a container containing PBS solution as a development medium. After that, the worms were incubated at 37^{o}C for up to three days in order to lay *T. vitulorum* eggs. The *T. vitulorum* egg retrieval was performed through the worm's reproductive tract by the surgical process. Subsequently, a gradient preparation technique was used to separate the dirty debris from the eggs.

Toxocara vitulorum eggs fertilization

Egg fertilization was carried out in PBS medium with the addition of five drops of formaldehyde 10%. This addition served to prevent the interference of other microbes with the growth of *T. vitulorum* eggs until the first (L1) and second larvae were obtained (L2). The development of worm eggs was observed using a dissecting microscope (Olympus upright microscope) and documented in the form of photographs on a regular basis. The process took 21-28 days at room temperature until the egg developed into L2 (Kusnoto et al., 2011).

Calculation of *Toxocara vitulorum* eggs

The egg calculation was carried out using a modified calculation of the worm eggs per gram excretion introduced in the Lucient Brumpt method (Kusnoto et al., 2007). An amount of 1 ml of *T. vitulorum* egg suspension from the culture media was then taken and diluted 10 times until 10 ml of suspension achieved. Then, 1 ml of suspension was taken by means of a Pasteur pipette to calculate the number of drops for every 1 ml of suspension. One drop of the suspension was put on a glass object and then examined through a light microscope with 100× magnification. Eggs that appeared through the microscopic magnification were counted using the formula which is written below (Kusnoto et al., 2007).

Number of eggs: Number of drops per ml (N) × number of worm eggs per drop (n) × number of dilutions

Treatment of experimental animals

A total number of 28 broiler chickens with body weights of 100-200 gr were selected as experimental animals of the current study. The chickens were raised on the farm and the floor. The adaptation period was one week. There was no available information about the vaccination program since the study was performed during rearing. Broilers were required to be 14 days old for deviation to seven treatment groups. The chickens were quarantined (A week) before being randomly divided into seven treatments with four replications in each. The chickens were feeding *ad-libitum* every afternoon and morning with strict hygiene. Then, each broiler chicken was infected by using 3000 eggs containing *T. vitulorum* second stage larvae (L2) when it was added to the food. The broiler chicken groups were divided into six groups (Azizi et al., 2007; Taira et al., 2011). The broiler chickens in the control group (K) were not infected with *T. vitulorum*. The P1 consisted of broilers infected by L2 *T. vitulorum* with a dose of 3000 eggs per chicken, and euthanized a day after infection. The broilers in P2 group were infected by L2 *T. vitulorum* with a dose of 3000 eggs per chicken, and euthanized two days after infection. The P3 group entailed broilers infected with L_2 *T. vitulorum* with a dose of 3000 eggs per chicken, and euthanized three days after infection. The P4 had broilers infected by L2 *T. vitulorum* with a dose of 3000 eggs per chicken, and euthanized seven days after infection. Moreover, P5 was

composed of broilers infected by L2 *T. vitulorum* with a dose of 3000 eggs per chicken, and euthanized 14 days after infection. Finally, the P6 group encompassed broilers infected by L2 *T. vitulorum* with a dose of 3000 eggs per chicken, and euthanized 21 days after infection.

Liver extraction

Liver extraction for histopathological preparation was carried out 1, 2, 3, 7, 14, and 21 days after L2 *T. vitulorum* infection. Extraction of the chicken liver was done after euthanasia and surgery. Broiler's liver organs were stored in aquadest and formalin 10%. Chicken's livers were cleaned with physiological NaCl then put in a plastic pot containing aquadest and formalin 10%, and subsequently stored for 24 hours before making the histopathological preparations.

Examination of preparations

The materials used for liver histopathological preparation were multilevel ethanol (70%, 80%, 90%, and absolute), formalin 10% added to the solution, ether, physiological saline (NaCl 0.9%), paraffin, entellan (transparent adhesive), Harris's Haematoxylin-Eosin double coloring, emersion oil, and xylol. Examination of preparations was performed using a light microscope with 400× magnification of five different fields of view (LP) for each sample. The observed changes included degeneration, the swelling of cell size due to vacuoles in the cytoplasm, Infiltration of inflammatory cells around the central vein, whether porta hepatis or sinusoid. Subsequent examination of preparations was assessed according to the Knodell score method (Knodell et al., 2019).

Statistical analysis

The research data including the histopathological score of liver cells of chickens were analyzed using Kruskal Wallis test, then continued with the *Z*-test. Differences were considered significant when $p < 0.05$.

RESULTS AND DISCUSSION

The present study obtained the results from the observation of isolated *T. vitulorum* worm eggs from adult worms that were fertilized and incubated for about one month. This process also obtained a second-stage larvae (L2) (Figure1). The results of the treatment on broilers microscopically demonstrated a histopathological change in the chicken liver after being infected with L2 *T. vitulorum.* Non-parametric Kruskal Wallis test indicated a significant difference ($p < 0.05$) for each treatment in broilers (Table 1). Provision of infective larval infections (L_2) *T. vitulorum* affects the histopathology of broiler chickens' liver. This study found a significant difference between the control group (K) and treatment groups which were euthanized 1- 21 days post-L2 infection ($p < 0.05$). The obtained scores were then followed by a multiple comparison test (Z test) to determine the order of the change rate in the liver histopathological pictures among the seven treatment groups.

Histopathological pictures of the liver tissue in the treatment groups presented damage due to hydropic degeneration (cloudy swelling), necrosis, inflammation, and cholangitis. The Z test indicated significant differences in the treatment groups P1, P3, P4, P5, and P6 with the control group (K). However, there was a change in histopathological features in P2 which were not significantly different from the control group. Group P6 represented the worst results, compared to other treatment groups (Figure 2).

In Figure 3, part A, hepatocytes were normal (blue sign) and did not appear to have inflammation and degeneration, and ductal images were still normal (green signs). In figure B, the cholangitis in the P6 group was characterized by inflammatory cells (yellow marking) and epithelial proliferation (red marks) of the bile duct. In figure C, the black mark referred to the presence of hydropic degeneration, and cytoplasm appeared turbid (cloudy swelling) and the green mark referred to the necrosis of the nucleus which appeared to be picnotic. The yellow mark in figure D indicated inflammation around the portal area.

Table 1. Statistical results on the extent of liver damage to the broiler chickens infected by *T. vitulorum*

Treatment	Liver Damage Value (Mean Rank ± SE[1])
K	$2.50^{d} \pm 0.289$
P1	$15.50^{b} \pm 1.472$
P2	$9.63^{c} \pm 0.816$
P3	$12.50^{bc} \pm 1.443$
P4	$14.75^{b} \pm 1.323$
P5	$21.25^{ab} \pm 1.190$
P6	$25.38^{a} \pm 0.645$

a-d: different superscripts in the same column show significant differences ($p < 0.05$) [1]SE: Standard Error. K: control group, broilers were not infected with *T. vitulorum*. P1: Broilers were infected by L2 *T. vitulorum* at a dose of 3000 eggs per chicken, and euthanized a day after infection. P2: Broilers were infected by L2 *T. vitulorum* at a dose of 3000 eggs per chicken, and euthanized two days after infection. P3: Broilers were infected with L_2 *T. vitulorum* at a dose of 3000 eggs per chicken, and euthanized three days after infection. P4: Broilers were infected by L2 *T. vitulorum* at a dose of 3000 eggs per chicken, and euthanized seven days after infection. P5: Broilers were infected by L2 *T. vitulorum* at a dose of 3000 eggs per chicken, and euthanized 14 days after infection. P6: Broilers were infected by L2 *T. vitulorum* at a dose of 3000 eggs per chicken, and euthanized 21 days after infection.

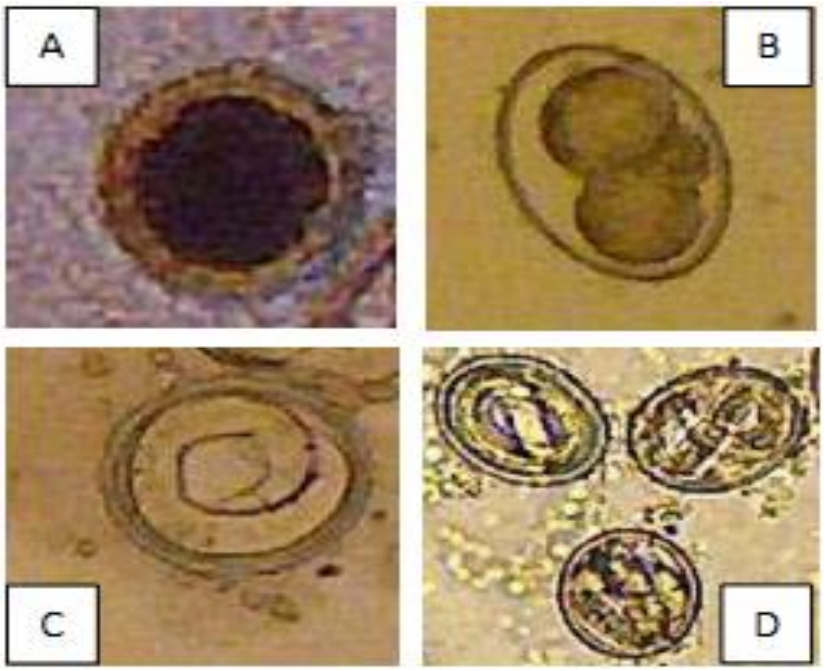

Figure 1. The results of identification of the *Toxocara vitulorum* worm eggs and their development up to L2 stage with 100× Magnification, A: worm eggs (1 cell), B: morula, C: L1, D: L2

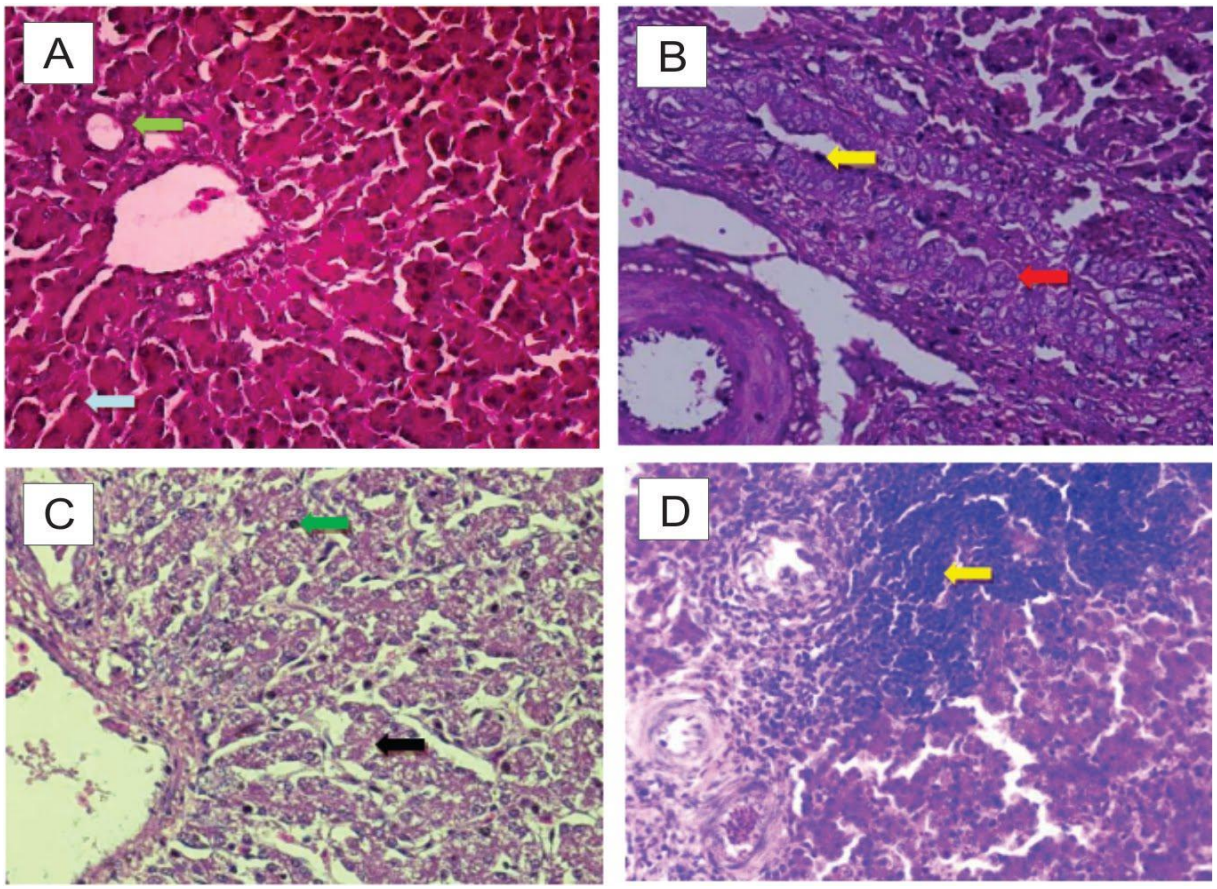

Figure 3. Histopathological picture of changes in different groups of infected broiler chickens' liver with L2 *Toxocara vitulorum*. Haematoxylin-Eosin coloring, 400× Zoom. A: control group; B: Chicken liver cholangitis P6, C: liver degeneration and necrosis P6, D: chicken liver inflammation P6

DISCUSSION

The oral administration of L2 *T. vitulorum* caused a significant change in the liver histopathology picture of broiler chickens ($p < 0.05$). This was due to the migration of larvae into the tissue. However, the larvae were not always found on liver histopathological examination (Fenoy et al., 2001). The orally administrated 3,000 eggs presented a white spot on the hepatic surface of the chickens indicating the presence of necrotic foci, eosinophil infiltration, and some lymphocytes around the necrotic area (Azizi et al., 2007; Taira et al., 2011).

Infective eggs of *T. vitulorum* hatched within 2 hours followed by penetration into the intestinal wall to reach the liver through the porta hepatica system. The life cycle of *Toxocara* spp. involves a phase of migration in tissue at every stage, starting from the egg, larvae, and adult stages. Every stage of *Toxocara* spp. growth has different antigenic devices and immunogenicity in triggering the formation of antibodies. Infective larval migration can cause histopathological changes in the cells of organs (Santos et al., 2017).

The liver experienced severe damage in the first post-infection day with the occurrence of degeneration, necrosis, severe inflammation, and cholangitis. On the second day, the liver was damaged but there were no significant differences in histopathological features which were found between treatment groups and the control group. L2 *Toxocara* spp. was most commonly found in the liver on the first day after infection and L2 migrated to another site on the second day (Taira et al., 2011). On the second post-infection day, the L2 *Toxocara* spp. was mostly found on the pulmonary of the chickens. Injuries of the liver cells were reversible and the cell would return to its original stable state within a certain time limit (Kumar et al., 2013). Histopathological picture of chicken liver in P3, P4, P5, and P6 groups indicated liver damage, especially around the central port and venous regions. *Toxocara* spp. migrated to other tissues through the circulatory system. The route of migration through the bloodstream can subsequently cause hemorrhage and multifocal necrosis in the liver. Inflammatory cell findings and epithelial proliferation in the bile duct were also observed in all treatment groups. Infective larvae *T. vitulorum* can migrate through the portal vein and then enter the bile duct through enterohepatic circulation (Azizi et al., 2007).

Histopathological pattern of liver cells infected with L2 *T. vitulorum* experienced degeneration, swelling and was accompanied by necrosis, inflammation, and cholangitis. *Toxocara* spp. larvae secrete metabolic material that caused injury to liver cells. The products or secretions of infectious organisms are toxic to the metabolism or integrity of the cell membrane (Underwood, 1996). Degenerated liver cells experiencing cloudy swelling, microscopically present the granular cytoplasm and appeared to be foggy (Thomson, 1984). This change reveals that when water accumulates in the cytoplasm, cytoplasmic organelles also absorb water which causes swelling of the mitochondria and enlargement of the rough endoplasmic reticulum accompanied by the loss of ribosomes (Cotran et al., 1994).

Liver cell necrosis is characterized by three changes in the cell nucleus, including picnosis which means the cell nucleus appears round, dark, and smaller than the normal cell nucleus, karyorrhexis is splitting the cell nucleus into several parts, and karyolysis means when the cellular nucleus chromatin disappears and leaves holes in the cell (Thomson, 1984). L2 *T. vitulorum* infection in experimental animals caused cell necrosis and disabled the cells to stimulate changes so that eventually cell death occurred. This death is a result of releasing several enzymes, such as ATP-ase, phospholipase, protease, and endonuclease. Great or lethal lesions lead to irreversible cell damages because the cell cannot defend itself against injury.

Toxocara spp. larvae secrete metabolic material that increases the production of eosinophils as an immune reaction. Cellular activity and pressure of infection can stimulate microbicidal secretions, effectors, and inflammatory mediators. This pressure responds to cells to protect and fight unwanted conditions by minimizing damage and maintaining the integrity of the host tissue. Endoplasmic reticulum and mitochondrial tissue are key cellular organelles which give signals to cellular pressure (Abbas and Lichtman, 2003). Cholangitis is inflammation of the bile wall due to lumen infection. This situation can originate from any lesion that blocks the bile duct.

Therefore, L2 *L. vitulorum* can migrate to various organs and cause damage, hence some prevention can be done by health workers such as conducting training and counseling on the importance of cleanliness and environmental management. In addition, it is also necessary to provide support and regular assistance to farmers. This aims to minimize the spread of infection by reporting the cases to health workers.

CONCLUSION

Infective larvae of (L2) *Toxocara vitulorum* administered orally could provide a change in the histopathological picture of broiler chicken's liver. Liver cell damages included cell degeneration, inflammatory cell infiltration, necrosis, and cholangitis. The P6 treatment group presented the most damage, compared to the other treatment groups, since the liver cells and other organs in chickens were exposed to toxic metabolic material released from the *Toxocara vitulorum* larvae during a longer period of time.

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Effects of Red and Blue Light during the Incubation of Turkey Eggs on Hatchability Performance and Expression Pattern of Some Myogenic Regulatory Genes

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ABSTRACT

The present study aimed to investigate the effects of different light colors on hatching potential traits, including egg weight loss, scientific and commercial hatchability, mortality percentages, hatching wight as well as mRNA expression levels of some muscle growth marker genes (*Myogenin*, *MyoD1,* and *FGF2*) of pectoralis muscle in hatched and non-hatched non pipped Black Bronze turkey chicks. A total of 1500 hatching Black Bronze turkey eggs were assigned equally to three incubation treatment groups, namely dark (control group), red, and blue LED light (treated groups) for 25 days of the incubation period. Results indicated that colored lighting stimuli (red and blue) significantly affected hatching capability. This issue could also affect the expression of muscle growth marker genes in hatched and non-hatched non pipped turkey chicks. Incubation of turkey eggs under red or blue LED light showed an insignificant effect on mortality percentages. It can be concluded that the use of a red or blue light system during turkey eggs' incubation could improve hatchability via upregulating the expression of muscle growth marker genes.

Keywords: Hatchability, Incubation, Light color, Marker Gene expression, Turkey

INTRODUCTION

The success of artificial incubation of avian eggs relies mainly on the environmental conditions inside the incubator. Temperature (Noiva et al., 2014), humidity (Bruzual et al., 2000), ventilation (Okur et al., 2018), and egg turning (Elibol and Brake, 2006) are the four main factors controlling the hatchability percentage. Many studies suggested that monitoring the light during the incubation period has a crucial role in the achievement of successful incubation of avian eggs (Fairchild and Christensen, 2000; Archer, 2015; Huth and Archer, 2015). Introducing light during incubation improved hatchability (Shafey, 2004; Archer and Mench, 2014) and embryonic growth rate (Cooper et al., 2011), as well as reducing early and late embryonic mortality (Shafey and Al-Mohsen, 2002), and post-hatch stress (Archer and Mench, 2014). Furthermore, the light during incubation stimulated opsin expression in photoreceptors (Rozenboim et al., 2013), increased growth and differentiation of myoblast, and myofiber synchronization (Halevy et al., 2006). Moreover, avian scientists have revealed that the impact of light on the embryonic development of different birds' species depends on the color (Tong et al., 2015). Generally, the light plays a crucial role in various scientific aspects. Light is electromagnetic radiation, classified into invisible and visible radiation. Invisible radiation has wavelengths too large or too small for the biological limitations of our discernment. However, the visible light is separated into different wavelengths ranged from violet color (the shortest wavelength) to red color (the longest wavelength). Yang et al. (2016) categorized the light spectrum according to the wavelength into long (red at 610-760 nm, yellow at 580-590 nm light) and short (green at 510-530 nm, and blue at 450-500 nm light).

During embryogenesis, the development of the skeletal muscle depends on myogenic regulatory factors (MRFs) which are a family of basic helix–loop–helix transcription factors important for the proliferation and differentiation of satellite cells (Schultz and McCormick,

1994). Satellite cells are the primary donors to muscle growth and regeneration (Relaix, 2006; Biressi and Rando, 2010). *MRFs* include myogenic factor 5 (*Myf5*), myogenic differentiation 1 (*MyoD1*), *Myogenin*, and *MRF4* (Buckingham and Rigby, 2014; Zammit, 2017). When muscle stem cells (satellite cells) are activated, these *MRF* genes are expressed in a consecutive pattern. During the proliferation of muscle stem cells, the *MyoD* and *Myf5* genes are expressed, and after that, *Myogenin* is expressed as the cells begin to differentiate (Cornelison and Wold, 1997). Fibroblast growth factor 2 (*FGF2*) is a potent regulator of muscle cell proliferation and differentiation (Velleman, 1999). It also plays a serious role in the maintenance of satellite cells' self-renewal by inhibiting their differentiation (Pawlikowski et al., 2017). Furthermore, *FGF2* has shown a possible influence on tissue regeneration and repair (Yun et al., 2010). Until now, few studies have investigated the relationship between hatching potentiality and molecular alterations of muscle growth related genes of turkey embryos incubated under different wavelengths of light. The present study aimed to investigate the effects of red and blue light stimuli during the incubation of Black Bronze turkey eggs on hatchability performance and expression profile of some muscle growth marker genes as a trail to understand the strategy of light color effect on hatching improvement.

MATERIALS AND METHODS

Ethical Approval

All experimental procedures and management conditions used in this study were approved by the local ethics committee of animal use, Faculty of Veterinary Medicine, Alexandria University, Egypt.

Fertile eggs and incubation condition

A total of 1500 hatching Black Bronze turkey eggs were collected from the Research Centre, Mahalet Mousa Station, Kafr El Sheikh Province, and were randomly allotted to three groups. The first group (control) was incubated at a complete dark incubator avoiding penetration of external light by covering incubator windows and door with black sheets, the second and the third ones were incubated under red LED light and blue LED light for the first 25 days of the incubation period. Light intensity was 12W/m^2 at the top surface of the eggs with a wavelength of 610-760 nm for the red light and 450-500 nm for the blue light. Incubator and hatcher were fumigated using formaldehyde gas by mixing of 40 ml formalin 40% and 20 gm potassium permanganate (KMNO4) per three cubic meters. Diluted TH4 solution (2ml/L) was sprayed on the eggs as a disinfectant before the incubation. All groups were incubated at normal incubation conditions (37.5°C, 65 % relative humidity, and turning automatically every three hours by angle ± 45). After 25 days of incubation, the eggs were transferred to the hatcher (37°C, 75% relative humidity, and no turning). During incubation, 100 eggs from each group were individually weighted every week to estimate the weight loss. Candling of the incubated eggs was performed on the seventh day of incubation to check whether the eggs are fertile or not and inspect early mortality percentage. At 14 and 25 days of incubation, eggs were candled again to determine mid, and late embryonic mortality percentages. The hatchability percentage was determined as the number of viable chicks hatched divided by the total eggs set (commercial hatchability) or fertile eggs set (scientific hatchability). The non-hatched eggs were left for an extra one day later hatching in the hatcher to give further chance to hatch. The number of hatched and non-hatched non-pipped eggs were recorded. Newly hatched chicks on the first day were weighted.

Sample collection

The pectoralis muscle samples were collected from hatched and non-hatched non-piped chicks (four samples from each group) then homogenized, immediately snap-frozen in liquid nitrogen, and stored in -80ºC.

Total RNA isolation and cDNA synthesis

Total RNA was isolated from homogenized muscle tissue using the Biozol reagent (Bioflux, Japan) according to the manufacturer's recommendations. The SensiFAST™ cDNA Synthesis Kit (Bioline, United Kingdom) was used for cDNA synthesis according to the manufacturer's instructions. Briefly, 4 µl of total RNA was mixed with 4 µl 5X buffer, 1 µl reverse transcriptase and 11 µl RNase free H_2O. The reaction was carried out as follows: 25°C for 10 min, 42°C for 15 min (reverse transcription), and 4°C holds. The cDNA was tested by amplification of the *ß-actin* gene, and then stored at -20°C.

Quantitative real-time PCR

The quantitative real-time PCR was carried out to investigate the expression levels of *Myogenin*, *MyoD1*, and *FGF2* genes as described previously (Abd El Naby and Basha, 2018). The primer sequences designed by using (https://primer3.ut.ee/) are listed in Table 1. The *β*-

actin (housekeeping gene) was used as normalizer. The relative mRNA expression was calculated using the comparative Ct method ($2^{(-\Delta\Delta ct)}$), and the results were reported as the fold change±SD (Rao et al., 2013).

Statistical analysis

The Chi-square test was used to determine the relationship between the light color (dark, red, and blue) during the incubation period and the number of hatched or non-hatched non pipped chicks of Black Bronze turkey ($p \leq 0.05$). Other data were analyzed by one-way ANOVA using SAS (Statistical Analysis System, version 6, 4th Edition, SAS Institute, USA). Data are expressed as mean ± SE and $p \leq 0.05$ were considered significant. Analyses of significant main effects were performed using multiple range comparisons with Duncan multiple range test. However, gene expression data were statistically analyzed by GraphPad Prism software version 6 (GraphPad Prism Software, La Jolla, California, USA) using one-way ANOVA.

Table 1. Primers used for quantitative real-time PCR

Genes	Primer Sequence (5`-3`)	Amplicon size (base pair)
Myogenin (NM_001303170.1)	F: CTCTCTGAGCTGGAAACGGG R: GGTCCACAGTGTTGGAGGAT	86
MyoD1 (NM_001303171.1)	F: CATGGGAAGAGTTCCGTTGT R: GGAAATCCTCTCCACAATGC	63
FGF2 (XM_003205699.3)	F: CTGGCACTGAAATGTGCAAC R: CTTCCGTGACCGGTAAGTGT	84
ß-actin (NM_001303173.1)	F: ATGGCTCCGGTATGTGCAA R: TGTCTTTCTGGCCCATACCAA	120

MyoD1: Myogenic factor D1, *FGF2*: Fibroblast growth factor, and *ß-actin*: Beta actin; F: forward, R: reverse.

RESULTS

The relationship between light color and the number of hatched or non-hatched non-pipped chicks

The statistical analysis showed that 13.85 is the estimated Chi-Square value ($p \leq 0.05$), indicating that the hatching ability of fertile Black Bronze turkey eggs depends on light color during the incubation period.

Effect of different lighting color during the incubation period on the hatching performance

The results showed that weekly weight loss of Black Bronze turkey eggs incubated at dark, red, and blue light had no significant difference at all incubation periods (Table 2). However, the incubated eggs in darkness had the highest significant total weight loss, compared to incubated eggs exposed to red and blue light.

Table 3 showed that the application of colored light significantly increased the scientific and commercial hatchability percentages of turkey eggs. Incubation under red light exhibited the highest scientific and commercial hatchability percentages, while the lowest hatchability was recorded for the dark condition. Additionally, early, mid, and late mortality percentages did not show significant differences between colored incubation or dark management. Furthermore, the hatching weight of turkey chicks showed that eggs exposed to the red light in the incubator had the highest significant hatching weight (50.87 gm) than the other groups.

Effect of different lighting color during the incubation period on the expression profile of *Myogenin*, *MyoD1*, and *FGF2* genes of turkey chicks

The effects of different light colors during the incubation period on mRNA expression levels of muscle growth marker genes in hatched and non-hatched non-pipped chicks are shown in figures 1 and 2. Red and blue light colors induced changes in the expression patterns of *Myogenin, MyoD1,* and *FGF2* genes, compared to control (figures 1 and 2). Hatched chicks exposed to the red light during incubation showed increased *Myogenin* and *MyoD1* expression levels with 14.65 ± 2.27 and 19.01 ± 2.70 folds than in case of blue light exposure (8.5 ± 1.6 and 3.64 ± 1.35 fold) relative to control (figures 1 a and b). The *Myogenin* level was significant ($p \leq 0.05$) upregulated (59.98 ± 0.04 fold) in non-hatched non-pipped chicks were exposed to blue light during the incubation period relative to control (Figure 2a). Its relative expression level also increased in non-hatched non-pipped chicks exposed to red light, but it was less than blue color. Moreover, the *MyoD1* mRNA expression profile showed significant ($p \leq 0.05$) upregulation (38.85 ± 0.96 folds) in non-hatched

chicks exposed to red light during the incubation period relative to control (Figure 2b) and increased (5.86 ± 1.33 folds) in case of blue light. Meanwhile, the *FGF2* gene revealed different expression patterns in both hatched and non-hatched non-pipped chicks. However, their relative expression levels showed a significant ($p \leq 0.05$) increase with 7.00 ± 0.45-fold in hatched chicks exposed to blue light (Figure 1c), and 5.24 ± 1.1 fold in the non-hatch non-pipped chicks exposed to red light during incubation relative to control (Figure 2c).

Table 2. Effect of the different lighting colors during incubation on egg weight loss of Black Bronze turkey eggs

Incubation period (Day)	Egg weight loss in different light conditions (g)		
	Dark	Red	Blue
1-7	1.117 ± 0.256	1.422 ± 0.079	1.300 ± 0.199
7-14	4.298 ± 0.334	2.653 ± 0.226	1.714 ± 0.256
14-21	4.941 ± 0.333	2.869 ± 0.295	2.984 ± 0.388
21-25	5.399 ± 0.297	4.395 ± 0.411	4.983 ± 0.444
Total	15.872 ± 0.541[a]	11.554 ± 0.585[b]	10.179 ± 0.442[b]

Means within the same row with different superscripts are significantly different ($p \leq 0.05$).

Table 3. Effect of the different lighting colors on hatchability, mortality percentages and hatching weight of Black Bronze turkey eggs

Variable	Light group	Dark	Red	Blue
Hatchability (%)	Scientific	80.58 ± 0.932[c]	90.43 ± 0.785[a]	87.73 ± 0.543[b]
	Commercial	69.24 ± 0.88[b]	76.67 ± 0.97[a]	73.45 ± 1.23[a]
Mortality (%)	Early	1.50 ± 0.005	2.43 ± 0.010	1.00 ± 0.033
	Mid	3.00 ± 0.002	4.55 ± 0.001	2.30 ± 0.003
	Late	18.36 ± 0.024	20.12 ± 0.096	22.88 ± 0.176
Hatching weight (gm)	Day old	46.05 ± 0.44[b]	50.87 ± 0.17[a]	45.43 ± 0.56[b]

Means within the same row with different superscripts are significantly different ($p \leq 0.05$).

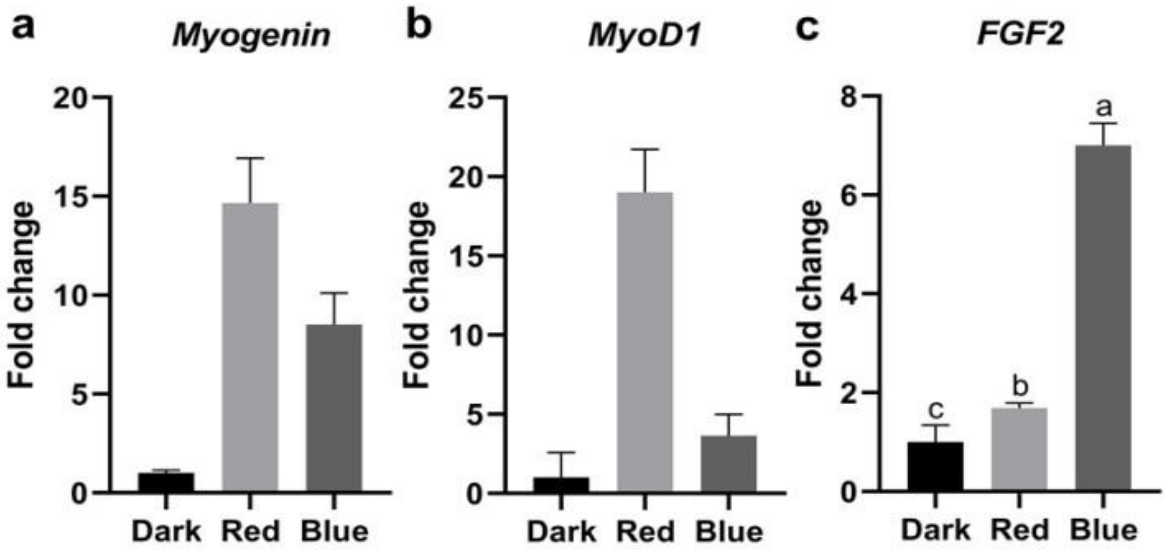

Figure 1. The relative expression levels of *Myogenin, MyoD1,* and *FGF2* genes in hatched turkey chicks with different light color treatments during egg incubation. Different letters in columns mean significant differences ($p \leq 0.05$).

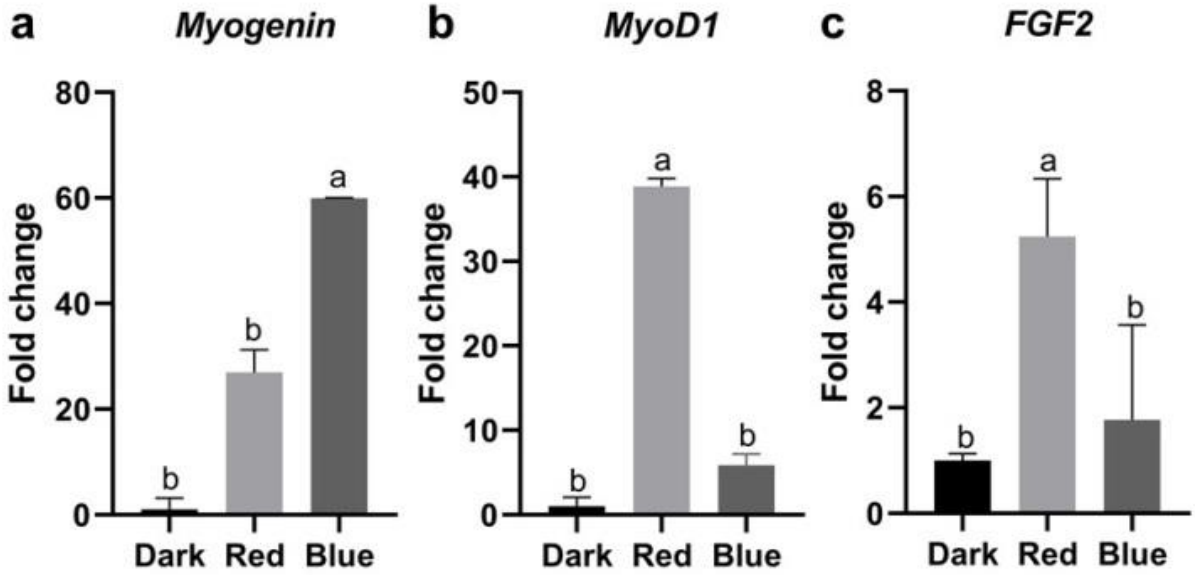

Figure 2. The relative expression levels of *Myogenin*, *MyoD1*, and *FGF2* genes in non-hatched non-pipped turkey chicks with different light color treatments during egg incubation. Different letters in columns mean significant differences ($p \leq 0.05$).

DISCUSSION

Avian embryogenesis is strongly influenced by the light wavelength and color (Shafey and Al-Mohsen, 2002; Rozenboim et al., 2013). The present study investigated the impact of colored light of different wavelengths on the hatchability performance of Black Bronze turkey. The results showed that using red or blue light in the first 25 days of incubation of Black Bronze turkey eggs improved scientific and commercial hatchability percentages. Incubation of Black Bronze turkey eggs in red light resulted in the highest improvement of hatchability percentages as well as hatching weight. However, weekly egg weight loss, mortality percentages (Early, mid, and late) were not significantly affected by colored light during incubation. These results are compatible with a study conducted by Archer (2015) indicating that red light is the success key of hatchability for broiler or layer eggs. Moreover, Archer (2017) ensured that the incubation of broiler eggs under red or white light could improve hatchery efficiency in comparison with the green light. El-Komy et al. (2017) reported increased hatchability and decreased mortality percentages for quail and Cobb 500 broiler breeder's eggs exposed to red light. Contrary to findings of the current study, providing 30 lx green LED light during the incubation of Arbor Acres fertile broiler eggs had no detrimental effect on the development of eyes, heart, and liver of embryos (Zhang et al., 2016). Santos (2019) also found no impact of red or blue light on the hatching behavior of Lohmann White and Brown eggs during incubation.

In this study, both light color stimuli (red and blue) upregulated *Myogenin*, *MyoD1*, and *FGF2* in the embryonic stage and improved hatching capability compared to the group incubated in the dark condition. The *Myogenin* gene was upregulated in non-hatched and non-pipped chick's muscle at the red light, however, this increase was less when compared to the exposure to blue color light, which revealed a significant increase compared to non-hatched non-pipped chicks in a dark condition. The *MyoD1* mRNA levels were significantly upregulated in the group exposed to red light during incubation in non-hatched and non-pipped chicks and also increased in hatched chicks. Otherwise, the *Myogenin* expression level in hatched chicks exposed to red light during incubation was more than the case of using blue light. As several studies reported, *MyoD1* is responsible for the activation and proliferation of skeletal satellite cells, however, *myogenin* plays a pivotal role in myoblast differentiation (Yablonka-Reuveni and Paterson, 2001; Cao et al., 2006). Furthermore, Zhang et al. (2014) suggested that the use of green light during incubation until hatching enhanced proliferation and differentiation of skeletal muscle satellite cells in the late embryonic stage and newly hatched chicks. This enhancement was a result of an increase in *MyoD1* expression level on day 17 of the embryo until day 3 of hatched chicks as well as an increase in *Myogenin* expression level on the first day to the fifth day after

hatch. Additionally, Halevy et al. (2006) showed that *in ovo* green light illumination has a stimulatory effect on skeletal muscle development in chicks during late embryogenesis and post-hatch period through enhanced proliferation and differentiation of adult myoblasts. regarding broiler chicken, a recent study reported that monochromatic green light stimulation during incubation increased the mRNA expressions of *Pax7* 18.77%, *MyoD*, 10.85%, *Myf5*, 13.48%, and *Myogenin*, 17.79%, which performed the satellite cell myogenic program (Bai et al., 2019).

Fibroblast growth factor 2 is a vital regulator of muscle cell proliferation and differentiation (Pawlikowski et al., 2017). the findings of the current study indicated that red light significantly increased *FGF2* mRNA expression level during incubation in non-hatched non-pipped chicks while in hatched chicks, blue light showed a significant increase in *FGF2* mRNA expression level. Based on the results of this study, it could be suggested that the red or blue light spectrum during incubation of turkey eggs is better than darkness. Furthermore, myoblast growth may be affected by light color through its influence on myogenic regulatory genes and consequently on hatching performance.

CONCLUSION

The findings suggest that using different LED light colors (red and blue) during the incubation period may affect myogenesis through its effect on the expression profiles of muscle growth marker genes. However, there should be future studies addressing the effect of light color on the intracellular events, such as the expression of *Myogenin MyoD1* and *FGF2*.

DECLARATIONS

Authors' contributions

Walaa Slouma Hamouda Abd El Naby and Heba Abdo Basha designed the plan of methodology, performed the experimental work, and wrote the main draft of the manuscript. Samya Erian Ibrahim, Magda Ismail Abo-Samaha, collected the samples and analyzed the data. All authors critically interpreted the data, revised the manuscript, and approved the final version of the manuscript.

Competing interests

The authors declare that they have no competing interests.

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Morphobiometric Characteristics and Biodiversity of Indigenous Guinea Fowl (*Numida meleagris*) in Benin

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ABSTRACT

The present study aimed to describe the morphobiometric characteristics of indigenous guinea fowl (*Numida meleagris*) populations in Benin. The current study was carried out on 1320 (529 males and 791 females) adult (at least 24 weeks old) indigenous guinea fowls from three climatic zones (Sudanian, Sudano-Guinean, and Guinean) of Benin. Each guinea fowl was subjected to a direct phenotypic description, biometric measurements, and photography. The results showed that the plumage coloration of indigenous guinea fowl in Benin was significantly diverse, but the most widespread plumage colors were pearl grey (30%), black (29.5%), and cinnamon (9.8%). The most common beak colors were grey (64.9%) and yellow-orange (24.8%). The eyes were predominantly black-white (67.1%). Grey-orange (33.7%), grey (32%), and black-orange (21%) colorations were more represented on the shanks with wattles relatively dominated by red-white (59.4%) and white-red (30.5%). The average live weight of guinea fowl was 1.34 kg in males which was 4.38% heavier than females. All the biometric measurements were significantly higher in males. The live weights of guinea fowl in the Sudanian zone (1.40 ± 0.18 kg) were higher than those of guinea fowl found in the Sudano-Guinean zone (1.27 ± 0.24 kg) and Guinean zone (1.33 ± 0.28 kg). Principal Component Analysis indicated that three distinct groups of guinea fowl can be formed based on their biometric measurements (live weight, chest circumference, body length, drumstick length, shank length, shank diameter, and wingspan). The phenotypes' diversity was relatively abundant (1-Hill: 0.69) in all climatic zones. The phenotypic biodiversity observed in the populations of indigenous guinea fowl in Benin can guide farmers to select specific phenotypes to meet consumer preferences.

Keywords: Benin, Biodiversity, Climatic zone, Indigenous guinea fowl, Phenotypic characteristic.

INTRODUCTION

Indigenous animal genetic resources represent an original and unique heritage due to the fact that they have developed particularly useful traits, in terms of production performance and adaptation qualities (Naves et al., 2011). In Benin, similar to other sub-Saharan countries, poultry production has remained dependent on family poultry farming. This form of poultry farming brings together 99% of the domestic poultry population (Chrysostome et al., 2013). This sub-sector contributes significantly to the food security, fight against poverty, and well-being of the people while requiring low levels of investment (Boko et al., 2012; Avornyo et al., 2016; Kone et al., 2018; Traoré et al., 2018; Kouassi et al., 2019). The optimal management of guinea fowl production as a rural poultry enterprise gives farmers huge advantages as well as revenue generation through sales of live guinea fowl and eggs (Agbolosu et al., 2015).

One of the problems that farmers face in animal husbandry and particularly in guinea fowl breeding is the lack of knowledge of the characteristics of indigenous genetic resources. This problem constitutes a constraint for the adequate use of their potential, which could lead to the promotion of these indigenous genetic resources in the context of ecological constraints. The characterization and conservation of various genetic attributes of indigenous species are crucial in order to maintain the genetic biodiversity of these animals, improve the food security of the rural population, and enhance economic empowerment in developing countries (Oguntunji, 2013; Haoua et al., 2015; Ould Ahmed and N'Daw, 2015). The strong

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distribution of guinea fowl is an indication of their adaptability to local environmental conditions, such as drought and high temperatures caused by climate variability (Panyako et al., 2016). Strategies for the management and enhancement of indigenous poultry resources are necessary both for rural economic development and for safeguarding biodiversity (Mahammi et al., 2014; Elkana and Gwaza, 2019). However, from these local populations of guinea fowl, some communities have conserved due to their local knowledge, types of birds with good production performance, such as varieties of guinea fowl (Chrysostome, 1995; Houndonougbo et al., 2017). However, the determination of the morphobiometric diversity of guinea fowl has not been done systematically in all the different climatic zones of Benin. The present study aimed to investigate the genetic diversity of the indigenous guinea fowl population available throughout the Beninese national territory. This knowledge would contribute to combating poverty through appropriate management and improvement of present poultry genetic resources.

MATERIALS AND METHODS

Ethical approval

All procedures were conducted in accordance with the guide for the care and use of agricultural animals in research, and approved by the department of animal production of the Faculty of Agricultural Sciences, University of Abomey-Calavi (Benin Republic).

Study areas

The study covered the Beninese national territory divided into three climatic zones which were Sudanian zone (9°45'-12°25'N), Sudano-Guinean zone (7°30'-9°45'N), and the Guinean zone (6°25'-7°30'N, Figure 1). From 1987 to 2016, the average rainfall in the Sudanian zone was less than 1000 mm in a year, and the average relative humidity during those years was 59%, and the average temperature also during those years was 28.4°C. The Sudano-Guinean zone has unimodal rainfall, from May to October with 113 days of rain, and an average annual rainfall of 900-1100 mm. The annual temperature varies between 21.2°C and 33.5°C, and the relative humidity ranges 31-98%. The Guinean zone is characterized by a bimodal rainfall with an annual average rainfall of 1200 mm; the average temperature varies between 25°C and 31°C, and the relative humidity ranges 69-97%.

Sampling

The study was carried out on a total of 1320 adult guinea fowls on which direct observations and body measurements were taken. This sampling included 529 males and 791 females with the same number of 440 guinea fowls per climatic zone. In each climatic zone, the study area was considered by taking into account the localities where guinea fowl production was more widespread. Then, in each locality considered, the main villages involved in this activity were identified. This identification of the study localities was carried out through discussions with administrative authorities, territorial agricultural development agents, guinea fowl farmers, traders, and consumers.

The study area was large and the inclusion criteria for the sample collection from livestock and animal households entailed villages with a minimum distance of 10 km, a large number of guinea fowl farmers in the villages, ownership of at least 10 guinea fowls by each farmer. Accordingly, 16 villages on average per climatic zone were used for data collection.

Data collection and calculated indices

Qualitative and quantitative data were collected. The qualitative variables (plumage colors, colors of shanks, wattles, eyes, and beaks) were used for the morphological characterization of the guinea fowl. As for the biometric characterization, it was performed through quantitative variables which included linear body measurements (chest circumference, body length, drumstick length, shank length, shank diameter, wingspan, and live weight) of each guinea fowl. The body weights were taken using an electronic balance of 1 g precision, while the shank diameters were measured using a Vernier caliper of 0.01 mm precision, and other body measurements were performed using a tape measure. All the data were collected by the same investigator using protocols consistent with the guidelines of the Food and Agriculture Organization of the United Nations (FAO, 2013) for phenotypic characterization of animal genetic resources. The schematic representation of biometric data collection

following the recommendations of FAO (2013) is presented in Figure 2. The phenotype diversity of guinea fowl was determined by defining several indexes of biological diversity.

Shannon diversity index (1949)

$$H : -\sum_{i=1}^{S} p_i \log_2 p_i$$

Where, *H* refers to the Shannon diversity index, *i* is a phenotype of the study area, *S* denotes the phenotype richness. p_i is the proportion of a phenotype (*i*) in relation to the total number of phenotype (*S*) in the study area which was calculated by the following formula:

$p_i : \frac{n_i}{N}$; *where*, n_i is the number of individuals for phenotype, and *i* and *N* indicate the total number of individuals of all phenotype combined.

Simpson index (1949)

It measures the probability that two individuals selected randomly from the sampled population belonging to the same phenotype.

$$D : \sum \left[n_i (n_i - 1) / N(N-1) \right],$$

Where, n_i is the number of individuals for phenotype *i*, and *N* refers to the total number of individuals of all phenotypes combined.

Hill index (1973)

Hill Index is a proportional abundance measure that combines Shannon and Simpson index. It provides a more precise view of the observed diversity. It was calculated by the following equation:

$$Hill : (1/D) / e^H$$

Where, *1/D* is the inverse of Simpson index, and e^H signifies the exponential of Shannon index.

Statistical analysis

The data collected was encoded in a database designed on Excel 2013. Statistical analysis of the data was performed using R software (R Core Team, 2019). Descriptive statistics were used to determine the color frequencies of the plumage, eyes, and beak, as well as the colors of shanks and wattles. These frequencies were specified by sex and by climatic zone. The biometric measurements were subjected to a non-parametric Kruskal Wallis test due to the non-normality of the distribution. The multiple comparisons procedure of the means was performed with Student Newman Keuls test when results were significant at a 5% level of probability. The variability of live weights of guinea fowl by climatic zones and sex was represented using boxplots. The Principal Component Analysis (PCA) was used to split guinea fowl with the same biometric characteristics through the FactoMineR package. In order to define more the different biometric groups of indigenous guinea fowl populations from the PCA examination, an Ascending Hierarchical Classification (AHC) was carried out with all the data made up of seven (07) variables. Hill's diversity index was used to measure the proportional abundance of guinea fowl phenotype by putting together the Shannon index (more sensitive to the numbers of rare phenotypes) and Simpson index (more sensitive to the numbers of abundant phenotypes). Accordingly, Hill index thus seems to be the most synthetic. The closer the index is to the value 1, the lower the diversity is. However, in order to facilitate interpretation, 1-Hill index was used, where the maximum diversity is represented by the value 1, and the minimum by the value 0.

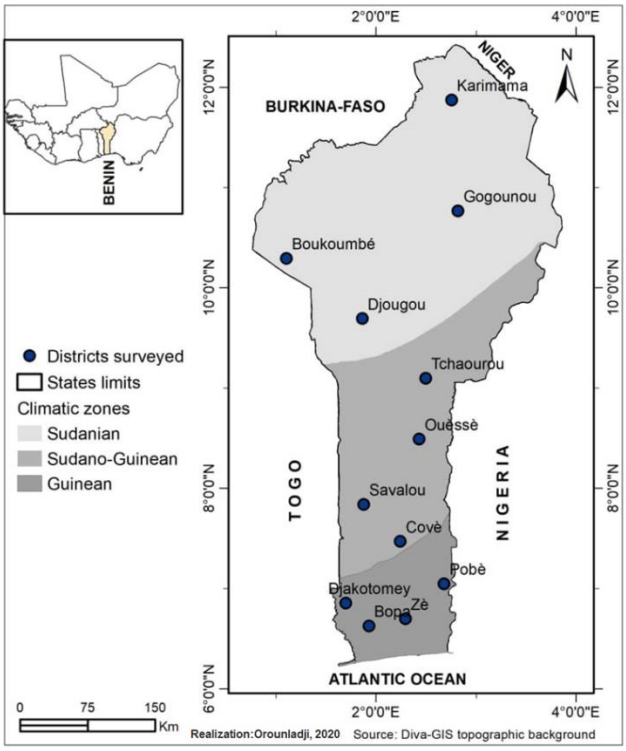

Figure 1. Materialization of the study areas.

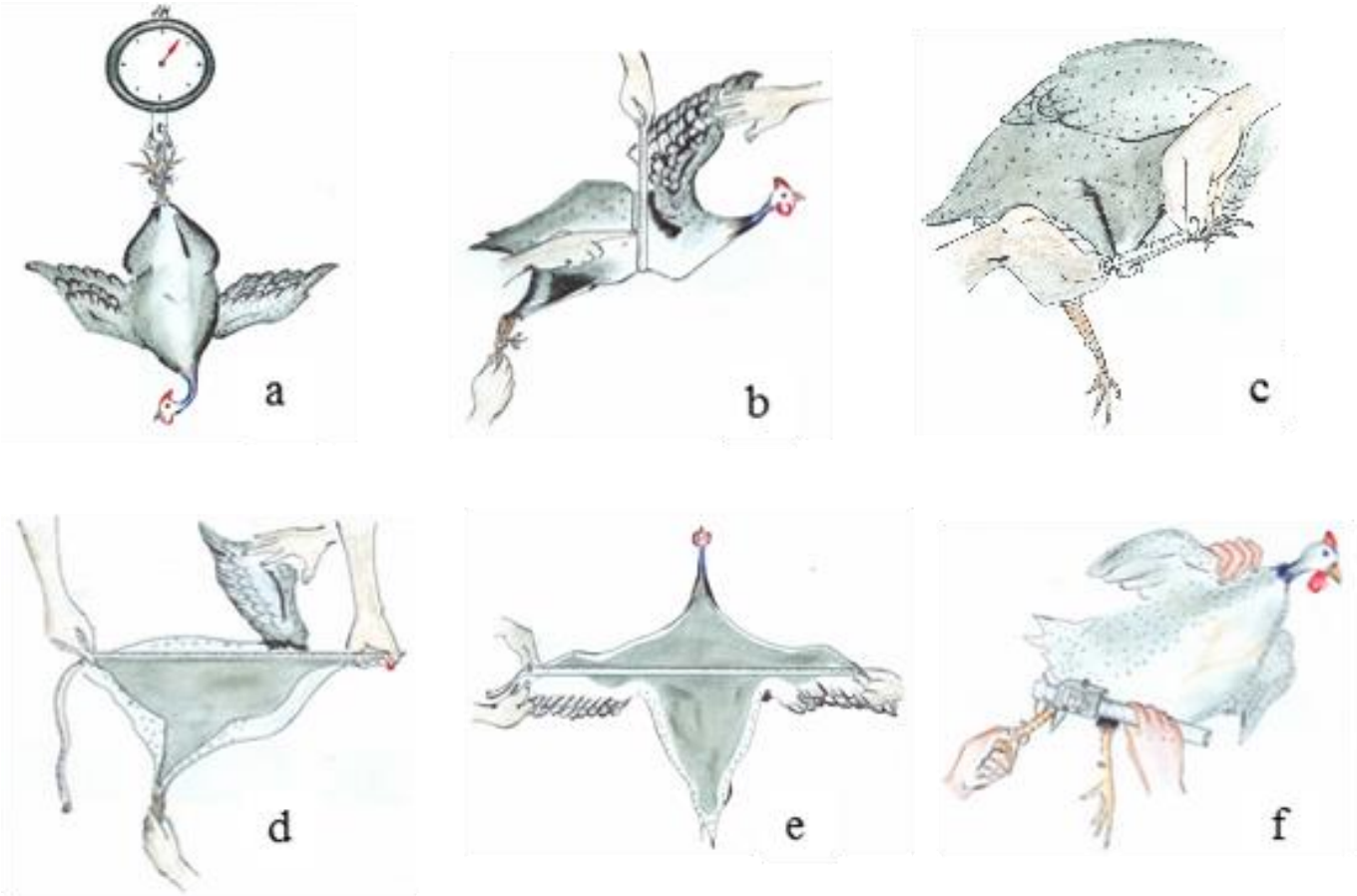

Figure 2. Body measurements; a: Live weight; b: Chest circumference; c: Shank length; d: Body length; e: Wingspan; f: Shank diameter

RESULTS

Qualitative variables describing indigenous guinea fowl populations

Descriptive appearance of guinea fowl plumage colors

Based on the colors chart of Guinea Fowl International Association (GFIA), a diversity of plumage colors was observed in the three climatic zones of Benin. A total of 12 phenotypes were identified, including pied white, white, brown, cinnamon, buff, multicolored, pearl grey, pied pearl grey, lavender, black, pastel, and royal purple (Figure 3). From the analysis of the three climatic zones, regardless of the sex of the guinea fowl, the results showed that pearl gray (30%), black (29.5%), and cinnamon (9.8%) plumages were the most represented plumage colors (Table 1). The buff, multicolored, and pastel plumages were not observed in the Guinean zone, whereas they were found in the Sudanian and Sudano-Guinean zones with pied white (3.6%), white (2.6%), brown (2.7), pearl grey (6.8%), lavender (7.7%), and royal purple (3.4%) plumages which were under-represented.

Descriptive appearance of other parts of guinea fowl

Regarding the beak, the most common color was grey (64.9%). It was accompanied successively by yellow-orange (24.8%), white (6.6%), black (3.4%), and orange (0.2%, Figure 4). As for the coloration of eyes, the major color was black-white (67.1%). Other colors, such as black-orange (12.3%), black-red (12%), and brown (8.5%) were also observed through the eyes of guinea fowl populations in all climatic zones. The wattles colorations were mainly represented by red-white (59.4%) and white-red (30.5%). White (6.4%), red (3.1%), and pink (0.7%) were other wattle colorations that were infrequent in this indigenous population of guinea fowl throughout Benin republic. The colors of shanks were more marked by grey-orange (33.7%), grey (32%), and black-orange (21%). The poorly represented colors were orange (3.9%), orange-grey (3.4%), orange-black (2.2%), grey-black (2%), black (1.1%) and pink-grey (0.8%). The black shanks were only observed in guinea fowl from the Sudanian and Sudano-Guinean zones, regardless of sex.

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Quantitative variables describing indigenous guinea fowl populations

Morphobiometric measurements by climatic zones

The analysis of the variance of the biometric measurements is presented in Table 2, showing that the live weight, the drumstick length, the shank length, and the shank diameter of guinea fowl varied significantly from one climatic zone to another ($p < 0.05$). The live weights of guinea fowl in Sudanian zone (1.40 ± 0.18 kg) were higher than those of guinea fowl found in the Sudano-Guinean zone (1.27 ± 0.24 kg) and Guinean zone (1.33 ± 0.28 kg). The drumstick length was higher ($p < 0.05$) in Guinean zone (11.77 ± 1.12 cm) than those of Sudanian and Sudano-Guinean zones which were10.48 ± 0.94 cm and 10.19 ± 0.58 cm, respectively. Guinea fowl from Sudanian zone showed higher values for shank length (6.23 ± 0.54 cm) and shank diameter (1.09 ± 0.08 cm). The other biometric measurements (chest circumference, body length, and wingspan) showed similar values ($p > 0.05$) in all climatic zones.

Morphobiometric measurements by sex

All traits measured had significantly higher values ($p < 0.05$) in males, compared to females (Table 3). Therefore, gender induced significant values of live weight, chest circumference, body length, drumstick length, shank length, shank diameter, and wingspan. Males had an average live weight of 1.37 kg, compared to 1.31 kg in females. In male guinea fowl, the values for chest circumference (32.60 ± 1.35 cm) and body length (41.71 ± 1.29 cm) were respectively higher than those observed in females (31.07 ± 2. 25 cm and 40.1 ± 2.84 cm).

Morphobiometric measurements by phenotypes

All morphobiometric parameters were significantly ($p < 0.05$) influenced by phenotypes (Table 4). According to live weight, buff phenotypes (1.51 ± 0.32 kg) were heavier than all other phenotypes. The chest circumference (33.09 ± 0.09 cm) and body length (42.31 ± 0.74 cm) of buff phenotypes were also greater than those of pied pearl grey phenotypes (30.36 ± 2.29 cm and 39.38 ± 3.29 cm) and pastel phenotypes (29.33 ± 2.75 cm and 37.81 ± 3.12 cm). Pearl grey (11.04 ± 1.15 cm), multicolored (11.04 ± 1.16 cm), and lavender (11.17 ± 1.06 cm) phenotypes had a greater ($p < 0.05$) drumstick length than pied white (10.18 ± 0.71 cm) and pastel (9.45 ± 0.78 cm) phenotypes. The shank lengths of pastel (5.79 ± 0.70 cm) phenotypes were smaller ($p < 0.05$) than those of buff (6.31 ± 0.66 cm), multicolored (6.33 ± 0.65 cm), pearl grey (6.22 ± 0.59 cm), and lavender (6.28 ± 0.42 cm) phenotypes. For the shank diameter, brown (1.14 ± 0.07 cm) and multicolored (1.13 ± 0.05 cm) phenotypes showed higher values, compared to pastel phenotypes (1.04 ± 0.08 cm). Pastel (39.12 ± 7.59 cm) phenotypes had the smallest values of wingspan compared to all other phenotypes described in the current study.

Variability of live weight by climatic zone and sex

Great variability in weights was observed within the guinea fowl populations of the Guinean zone (Figure 5). The live weight was respectively within the ranges of 0.90-2.2 kg and 0.60-2 kg in the males and females in this zone. The live weight of 50% of females in this area ranged between 1.1 and 1.5 kg, while it ranged from 1.2 to 1.65 kg in males. The Sudanian zone showed less variability. The live weights varied from 1.09 to 1.75 kg and from 1.09 to 1.76 kg, respectively, in females and males of the Sudanian zone. In 50% of males and females in the Sudanian zone, the live weight varied from 1.40 to 1.60 kg and from 1.35 to 1.45 kg, respectively. As for the males and females of the Sudano-Guinean zone, their live weights varied respectively from 0.90 to 1.8 kg and from 0.88 to 1.67 kg.

Characterization of guinea fowl populations

The cumulative contribution to the total inertia of the three axes which were retained for the interpretation of the results of the Principal Component Analysis (PCA) was 70.40% (Table 5). The dendrogram (Figure 6) and the representation of the PCA (Figure 7) were used to differentiate three biometric groups in all climatic zones of Benin. The analysis of the variance of the groups on the graphs of the AHC and the PCA allowed identifying the characteristics of each group (Table 6).

Group one contained 16.89% of the guinea fowl sampled. They had significantly lowest values ($p < 0.05$) of chest circumference (28.31 ± 1.26 cm), body length (36.13 ± 2.27 cm), drumstick length (9.44 ± 0.78 cm), shank length (5.35 ± 0.54 cm), and shank diameter (1.01 ± 0.11 cm, Table 6). The second group had a proportion of 47.5%. This group was made up of relatively half of the guinea fowl sampled. It was correlated with six biometric parameters whose values were significantly higher ($p < 0.05$) than those of group 1. These parameters were such as the chest circumference (32.19 ± 1.60 cm), body length (41.57 ± 1.22 cm), drumstick length (10.83 ± 0.86 cm), shank length (6.26 ± 0.48 cm), shank diameter (1.12 ± 0.05 cm), and wingspan (41.99 ± 1.04 cm). The third group contained 35.61% of the whole guinea fowl measured which significantly ($p < 0.05$) had the highest values of body weight (1.55 ± 0.20 kg) and drumstick length (11.44 ± 1.05 cm).

Diversity of indigenous guinea fowl populations

Of the total population considered, 12 phenotypes were identified. These 12 phenotypes were found both in the Sudanian zone and in the Sudano-Guinean zone, unlike the Guinean zone which had only 9 phenotypes (Table 7). Shannon index was similar in Sudanian (2.88) and Sudano-Guinean (2.85) zones, and they were higher than that of Guinean zone (2.5). The values of Simpson's index (0.20) and Hill's index (0.69) were relatively high for the whole population. The geographical distribution of each phenotype is presented in Figure 8.

Figure 3. Plumage colors; pearl grey[a], black[b], cinnamon[c], lavender[d], pied pearl grey[e], pied white[f], royal purple[g], brown[h], white[i], pastel[j], buff[k] and multicolored[l].

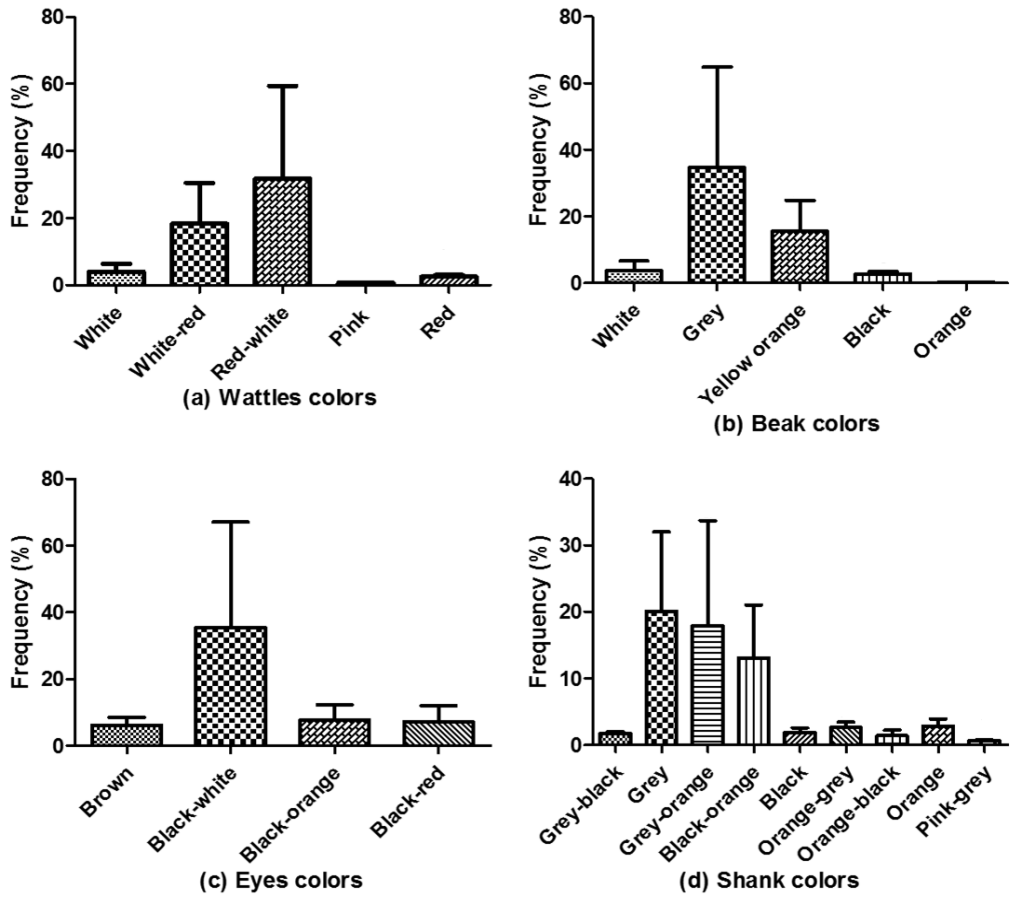

Figure 4. Morphological colors; (a) wattles, (b) beak, (c) eyes and (d) shank.

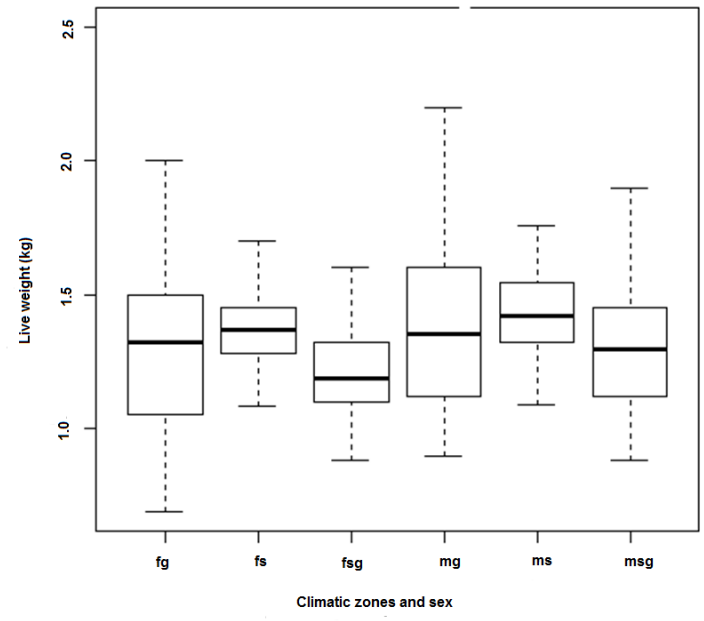

Figure 5. Variability of live weight by climatic zone and sex. fg : female of Guinean zone; fs : female of Sudanian zone; fsg : female of Sudano-Guinean zone; mg : male of Guinean zone; ms : male of Sudanian zone; msg : male of Sudano-Guinean zone.

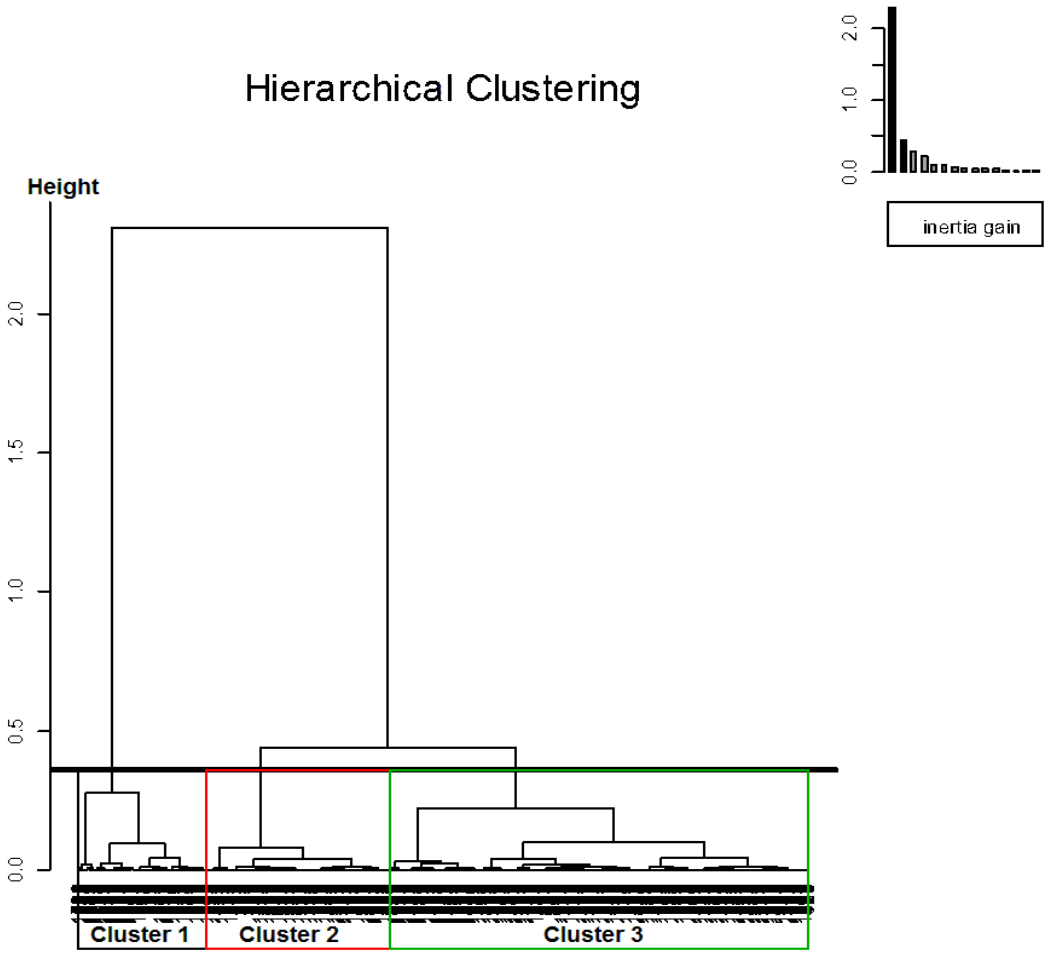

Figure 6. Dendrogram of the ascending hierarchical classification of biometric measurements

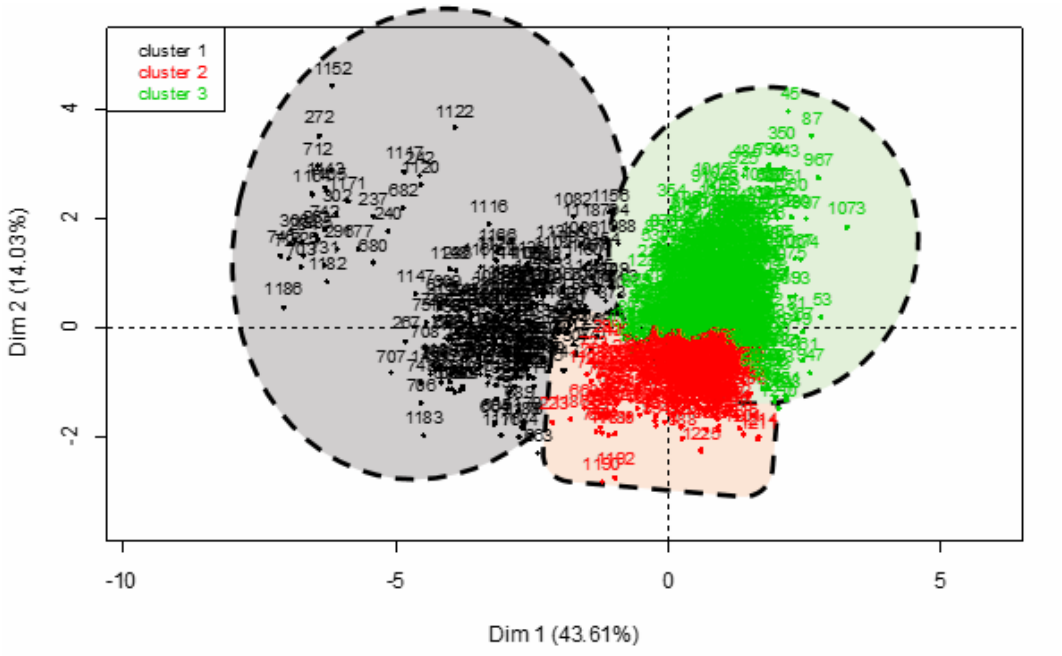

Figure 7. Graphic representation of the principal component analysis of biometric measurements

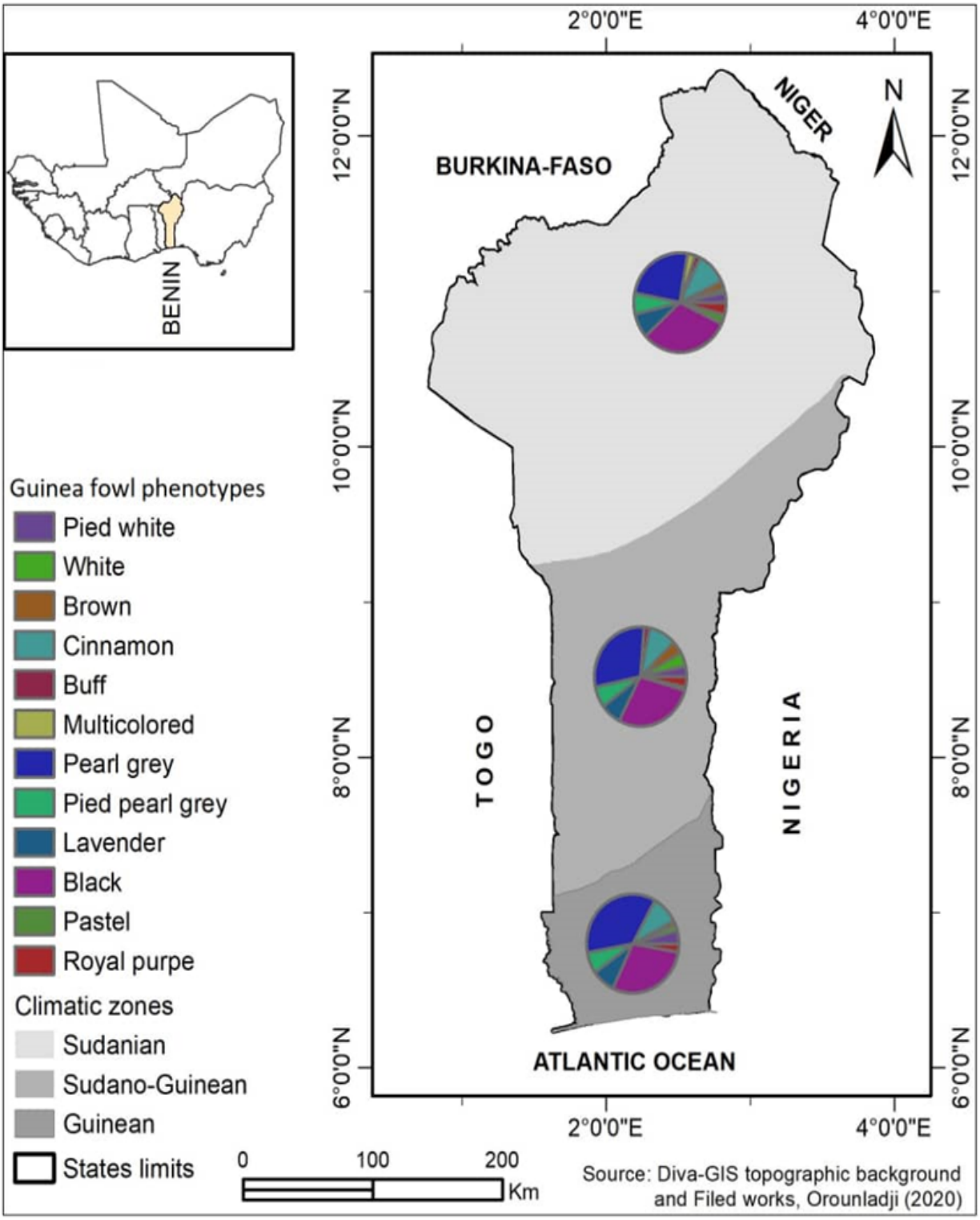

Figure 8. Geographical distribution of guinea fowl phenotypes in Benin

Table 1. Descriptive appearance of the plumage colors of guinea fowl in Benin.

Plumage colors (%)	Sudanian zone		Sudano-guinean zone		Guinean zone		Whole population		
	Male	Female	Male	Female	Male	Female	Male	Female	Overall
	151	289	208	232	170	270	529	791	1320
Pied white	1.3	4.2	5.3	1.7	5.3	3.7	4.2	3.3	3.6
White	2.0	1.0	6.2	3.0	1.2	2.2	3.4	2.0	2.6
Brown	2.6	2.4	4.3	4.3	0.6	1.9	2.6	2.8	2.7
Cinnamon	8.6	11.8	7.7	10.8	8.8	10.0	8.3	10.9	9.8
Buff	3.3	1.0	2.9	1.3	0.0	0.0	2.1	0.8	1.3
Mulicolored	4.6	1.7	1.4	0.0	0.0	0.0	1.9	0.6	1.1
Pearl grey	31.8	21.1	31.7	27.2	45.9	29.6	36.3	25.8	30.0
Pied pearl grey	4.6	8.0	5.8	7.8	4.1	8.5	4.9	8.1	6.8
Lavender	7.9	8.0	4.8	9.1	6.5	9.3	6.2	8.7	7.7
Black	25.8	33.9	24.0	31.5	27.6	30.4	25.7	32.0	29.5
Pastel	0.0	4.5	1.4	0.9	0.0	0.0	0.6	1.9	1.4
Royal purple	7.3	2.4	4.3	2.6	0.0	4.4	3.8	3.2	3.4

Table 2. Morphobiometric measurements of guinea fowl according to the climatic zones of Benin

Variables	Climatic zones		
	Sudanian zone	Sudano-guinean zone	Guinean zone
Size (n = 1320 guinea fowls)	440	440	440
Live weight (Kg)	1.40 ± 0.18^{a}	1.27 ± 0.24^{c}	1.33 ± 0.28^{b}
Chest circumference (cm)	31.71 ± 1.96	31.77 ± 2.08	31.57 ± 2.18
Body length (cm)	40.82 ± 2.49	40.75 ± 2.34	40.60 ± 2.60
Drumstick length (cm)	10.48 ± 0.94^{b}	10.19 ± 0.58^{c}	11.77 ± 1.12^{a}
Shank length (cm)	6.23 ± 0.54^{a}	6.03 ± 0.70^{b}	6.19 ± 0.54^{a}
Shank diameter (cm)	1.09 ± 0.08^{a}	1.10 ± 0.08^{a}	1.08 ± 0.08^{b}
Wingspan (cm)	41.57 ± 2.37	41.35 ± 2.37	41.58 ± 2.41

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

Table 3. Morphobiometric measurements in guinea fowl populations of Benin by sex of the individuals

Variables	Sex	
	Male	Female
Size (n = 1320 guinea fowls)	529	791
Live weight (Kg)	1.37 ± 0.25^{a}	1.31 ± 0.24^{b}
Chest circumference (cm)	32.60 ± 1.35^{a}	31.07 ± 2.25^{b}
Body length (cm)	41.71 ± 1.29^{a}	40.10 ± 2.84^{b}
Drumstick length (cm)	11.15 ± 1.10^{a}	10.59 ± 1.12^{b}
Shank length (cm)	6.30 ± 0.49^{a}	6.10 ± 0.66^{b}
Shank diameter (cm)	1.11 ± 0.10^{a}	1.08 ± 0.10^{b}
Wingspan (cm)	41.84 ± 1.07^{a}	41.28 ± 2.94^{b}

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

Table 4. Morphobiometric measurements of guinea fowl by phenotypes in Benin

Phenotypes	Size (n = 1320 guinea fowls)	Live weight (Kg)	Chest circumference (cm)	Body length (cm)	Drumstick length (cm)	Shank length (cm)	Shank diameter (cm)	Wingspan (cm)
Pied white	48	1.25 ± 0.27^{b}	31.99 ± 1.96^{ab}	40.72 ± 2.83^{abc}	10.18 ± 0.71^{b}	6.07 ± 0.54^{ab}	1.11 ± 0.09^{ab}	41.48 ± 2.70^{a}
White	34	1.29 ± 0.21^{b}	31.68 ± 1.74^{b}	40.84 ± 3.08^{abc}	10.48 ± 0.91^{ab}	6.11 ± 0.78^{ab}	1.05 ± 0.11^{bc}	40.52 ± 3.07^{a}
Brown	36	1.28 ± 0.21^{b}	31.46 ± 2.02^{b}	41.31 ± 1.92^{ab}	10.57 ± 0.78^{ab}	5.92 ± 0.80^{ab}	1.14 ± 0.07^{a}	41.12 ± 1.81^{a}
Cinnamon	130	1.30 ± 0.23^{b}	31.28 ± 2.28^{b}	39.99 ± 2.75^{bc}	10.54 ± 1.08^{ab}	5.99 ± 0.67^{ab}	1.08 ± 0.08^{bc}	41.24 ± 2.23^{a}
Buff	17	1.51 ± 0.32^{a}	33.09 ± 0.09^{a}	42.31 ± 0.74^{a}	10.58 ± 0.18^{ab}	6.31 ± 0.66^{a}	1.05 ± 0.15^{bc}	41.81 ± 0.67^{a}
Multicolored	15	1.29 ± 0.12^{b}	32.06 ± 1.47^{ab}	41.02 ± 2.06^{ab}	11.04 ± 1.16^{a}	6.33 ± 0.65^{a}	1.13 ± 0.05^{a}	41.45 ± 1.10^{a}
Pearl grey	396	1.33 ± 0.25^{b}	31.86 ± 1.99^{ab}	41.09 ± 2.07^{ab}	11.04 ± 1.15^{a}	6.22 ± 0.59^{a}	1.10 ± 0.06^{abc}	41.62 ± 2.54^{a}
Pied pearl grey	90	1.29 ± 0.21^{b}	30.36 ± 2.29^{c}	39.38 ± 3.29^{c}	10.41 ± 1.21^{ab}	5.92 ± 0.58^{ab}	1.07 ± 0.06^{bc}	41.43 ± 2.40^{a}
Lavender	102	1.30 ± 0.19^{b}	32.44 ± 1.20^{ab}	41.68 ± 1.33^{ab}	11.17 ± 1.06^{a}	6.28 ± 0.42^{a}	1.10 ± 0.05^{abc}	41.72 ± 1.14^{a}
Black	389	1.38 ± 0.26^{b}	31.72 ± 2.08^{b}	40.60 ± 2.49^{bc}	10.87 ± 1.18^{ab}	6.19 ± 0.58^{ab}	1.09 ± 0.10^{abc}	41.61 ± 2.10^{a}
Pastel	18	1.22 ± 0.12^{b}	29.33 ± 2.75^{d}	37.81 ± 3.12^{d}	9.45 ± 0.78^{c}	5.79 ± 0.70^{b}	1.04 ± 0.08^{c}	39.12 ± 7.59^{b}
Royal purple	45	1.34 ± 0.20^{b}	32.09 ± 1.95^{ab}	41.08 ± 2.21^{ab}	10.66 ± 0.87^{ab}	6.20 ± 0.61^{ab}	1.09 ± 0.06^{abc}	41.86 ± 0.79^{a}

[a, b,c,d] Means with unlike superscripts in the same column differ significantly ($p < 0.05$).

Table 5. Cumulative contribution to the total inertia of the factorial axis

Factorial axis	Inertia (%)	Cumulative inertia (%)
1	43.61	43.61
2	14.03	57.64
3	12.76	70.40

Table 6. Variables describing the Principal Component Analysis groups of guinea fowl in Benin based on their morphobiometric traits

Variables	Group 1	Group 2	Group 3
Size (n : 1320 guinea fowls)	223	627	470
Live weight (Kg)	1.24 ± 0.20^{b}	1.20 ± 0.16^{c}	1.55 ± 0.20^{a}
Chest circumference (cm)	28.31 ± 1.26^{b}	32.19 ± 1.60^{a}	32.62 ± 1.15^{a}
Body length (cm)	36.13 ± 2.27^{b}	41.57 ± 1.22^{a}	41.78 ± 0.87^{a}
Drumstick length (cm)	9.44 ± 0.78^{c}	10.83 ± 0.86^{b}	11.44 ± 1.05^{a}
Shank length (cm)	5.35 ± 0.54^{b}	6.26 ± 0.48^{a}	6.39 ± 0.46^{a}
Shank diameter (cm)	1.01 ± 0.11^{c}	1.12 ± 0.05^{a}	1.09 ± 0.07^{b}
Wingspan (cm)	38.99 ± 4.67^{b}	41.99 ± 1.04^{a}	42.04 ± 0.80^{a}

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

Table 7. Diversity index of indigenous guinea fowl populations of Benin by climatic zone

Diversity index	Sudanian zone	Sudano-guinean zone	Guinean zone	Whole population
Number of guinea fowl	440	440	440	1320
Phenotypic richness	12	12	9	12
Shannon	2.88	2.85	2.5	2.78
Simpson	0.18	0.19	0.24	0.20
1-Hill	0.70	0.69	0.65	0.69

DISCUSSION

The strong distribution of plumage colors within the genetic resources of indigenous guinea fowl in Benin probably indicates the existence of genetic variability. A total of 12 plumage colors were identified with the dominance of pearl grey and black colors in the present study. Agbolosu et al. (2015) identified nine plumage colors in Ghana, and eight were observed in Cameroon by Meutchieye et al. (2017), while seven were observed in Ghana (Brown et al., 2017) as well as in Benin (Houndonougbo et al., 2017). This higher number (12) of phenotypes observed, compared to the results of Chrysostome (1995) and Houndonougbo et al. (2017), would be due to the large-scale of the study area considered. The presence of major effect genes and the interactions among several of them were plausibly at the basis of this color diversity. The multiple crossbreeding that have not been controlled for several decades, among the birds with different plumage colors, give rise to other combinations, probably those existing in low proportion (Akouango et al., 2004). This multi-coloration of guinea fowl plumage observed in the current study was similar to the results of some authors (Chrysostome, 1995; Ogah, 2013; Fajemilehin, 2014; Agbolosu et al., 2015; Panyako et al., 2016; Houndonougbo et al., 2017). The dominance of pearl grey colors shows the color by which guinea fowl is recognized from its origin. However, the colors that were found in low proportions, notably buff, multicolored and pastel plumages, in the total population in the present study may indicate a relatively high level of gene dilution across all-round crosses. The absence of buff, multicolored, and pastel plumages in the Guinean zone may be due to the interaction between genotype and

environment (Santoni et al., 2000; Bahy et al., 2003). The higher pearl grey color recorded in the current study was in agreement with the observation reported earlier in Ghana (Agbolosu et al., 2015; Brown et al., 2017), Nigeria (Fajemilehin, 2014), Benin (Chrysostome, 1995; Houndonougbo et al., 2017), and in Burkina-Faso (Bouda, 2017). Apart from the varieties of guinea fowl listed by Houndonougbo et al. (2017), other plumage colors were identified during this study, as documented by the Guinea Fowl International Association color chart (GFIA, 2009).

The multiplicity of ornamental details, such as the coloration of the beak, wattle, and shank, also indicates the diversity of the genetic resources of indigenous guinea fowl in the three climatic zones of Benin. Guinea fowl with grey beaks and black-white eyes predominate among the phenotypes. The diversity of eye color could be attributed to genes of an animal influencing blood supply and melanin levels, environmental effect in terms of availability of carotenoids, as well as the interaction among blood supply, melanin, and carotenoids (Ngeno et al., 2014). The red-white color was the relatively predominant coloration of the wattles. As color plays an important role in the absorption and reflection of solar radiation, the wattles' color also plays a role in thermoregulation (Agbolosu et al., 2015). As for the shank, their colorations were more marked by grey-orange, grey, and black-orange.

As for body measurements, the average live weights of guinea fowl obtained were comparable to those of Bouda (2017) in Burkina Faso. On the other hand, they were higher than the live weights reported in Ghana (Agbolosu et al., 2015; Brown et al., 2017) and lower than those obtained in Nigeria (Ogah, 2013). The body lengths of guinea fowl observed in the current study were similar to those reported in an intensive production system of indigenous guinea fowl in Botswana (Tjetjoo et al., 2013) and Ghana (Brown et al., 2017).

The significantly higher values obtained in males agreed with the findings of Bouda (2017). These values were also different from the measurements obtained by Brown et al. (2017). These differences in the results relating to the biometric variables reflecting sexual dimorphism can be explained by the breeding conditions of the birds (guinea fowl) studied. Moreover, intrinsic factors, such as the genotype, age of the animal, and its physiological state (in the female) could influence the results. According to Keambou et al. (2007), the dimorphism in favor of males suggests that a breeding program for meat production would be more advantageous with males than with females, primarily for traits of economic importance, such as weight, drumstick development, and shank diameter. However, care should be taken to maintain the reproductive capacity of these animals.

Buff phenotypes were heavier than all other phenotypes (pied white, white, pearl grey, pied pearl grey, multicolored, brown, cinnamon, black, pastel, royal purple, and lavender), and buff phenotypes would be the most useful phenotype for improving growth performance. These results could be explained by the small number of observations on buff phenotypes, and most of them were mature. These observations differ from the findings of Duodu et al. (2018) who reported a higher live weight in the pearl grey phenotype, compared to the lavender, white, and black guinea fowl.

Live weight depends on the biotope and the condition of the animals. It is, therefore, an important attribute of farm animals, and thus, it forms the basis not only for assessing growth and feed efficiency but also for making economic decisions (Fajemilehin, 2014). It has been reported by Nwosu et al. (1985) that live weight is the best parameter for making management, health, production, and marketing decisions. The differences in body weight revealed in the present study indicated the inherent genetic makeup of each phenotype of guinea fowl depending on the climate variability of the areas. Variation in body weight could be useful in determining overall adaptive genetic diversity (Toro and Caballero, 2005).

Three groups were categorized on the basis of quantitative variables. While the first and the second groups respectively had low values and averages of the biometric variables, the third group constituted the category of guinea fowl to prioritize in a process of genetic improvement of the species through its high values. Thus, to promote indigenous guinea fowl in Benin, studies on the production performance of the third group can be used to produce labeled indigenous guinea fowl by seeking to strengthen their diet with unconventional food resources such as maggots, termites, etc.

The diversity of phenotypes within the three climatic zones was relatively maximal in the analysis of the value of Hill index which constitutes a compromise between the Shannon and Simpson index. This indicates the strong diversity of the guinea fowl in Benin. These results are similar to the values obtained by Kerboub et al. (2017). However, diversity was more abundant in Sudanian and Sudano-Guinean zones than in the Guinean zone. This difference is probably linked to the climatic conditions which vary between these three climatic zones, but with higher rainfall in the Guinean zone, and which are not too

favorable to the proliferation of guinea fowl within this zone. Some factors, such as physiological state, age of the guinea fowl, climate and genetic variabilities, measurements techniques, management practices, and the interaction among all these factors could explain the discrepancy in the morphobiometric parameters observed by the authors in diverse areas of tropical Africa.

CONCLUSION

The current study showed great variability in the genetic resources of indigenous guinea fowl in Benin both in its phaneroptic and in their biometric characteristics. Within the population of these guinea fowls, pearl grey plumage color was more widespread, regardless of the climatic zone. Guinea fowl with grey beaks and black-white eyes predominated among the phenotypes. The red-white color was the relatively predominant coloration of the wattles. As for the shanks, their colorations were more marked by grey-orange, grey, and black-orange. Sexual dimorphism for weight growth, as for the other biometric variables considered, was in favor of males. These quantitative variables were used to distinguish three groups of guinea fowl. The great biological diversity observed during the present study can constitute a basis for the establishment of hardy and more efficient phenotypes through genetic improvement programs integrating selection and controlled breeding. At the same time, improved breeding conditions would significantly increase the productivity of the indigenous guinea fowl as a source of quality protein, particularly in rural areas.

DECLARATIONS

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Competing interests

The authors reported no conflict of interest regarding the publication of this article.

Authors' contributions

This work was carried out by the collaboration of all authors. OBM conceptualized the work, collected and analyzed data, and wrote the manuscript. TSK and CCAAM conceptualized and supervised the work and, corrected the manuscript. All authors read and approved the final version of the manuscript.

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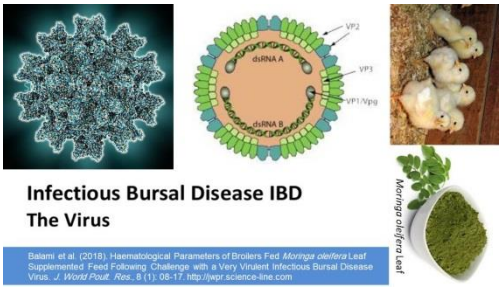

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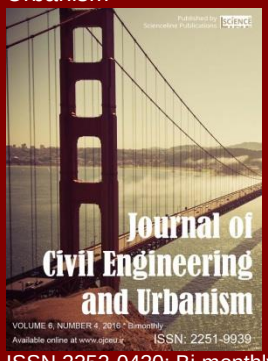

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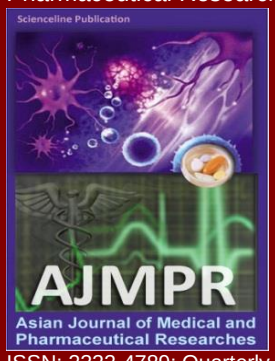

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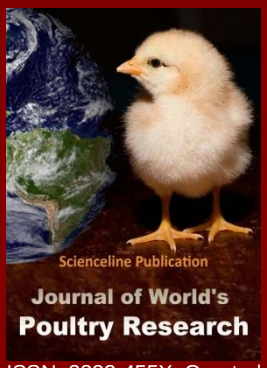

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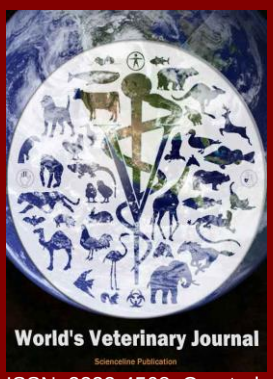

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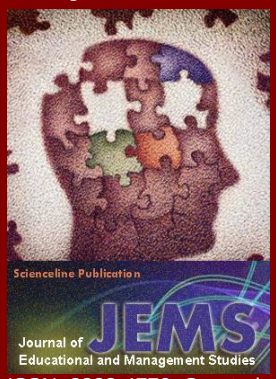

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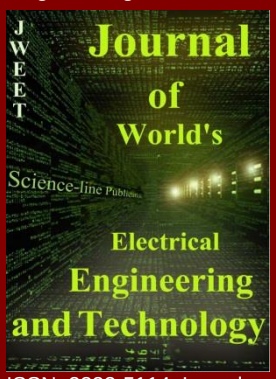

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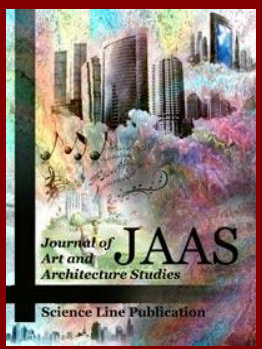

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