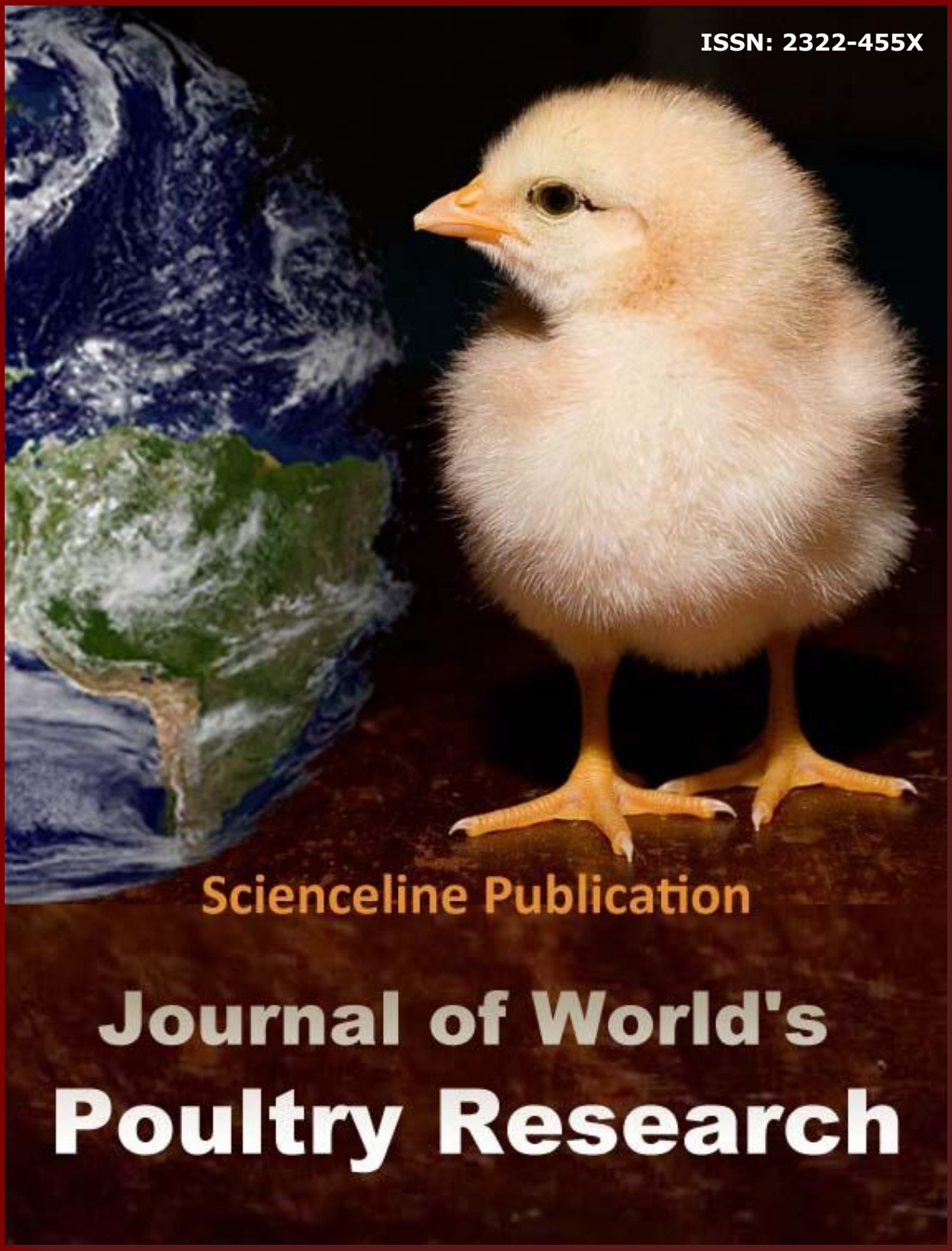
In the Name of God



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Editorial Team

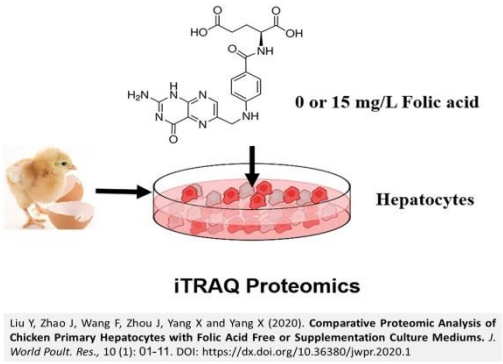
Advisory Board

Volume 10 (1); March 25, 2020

Research Paper

Comparative Proteomic Analysis of Chicken Primary Hepatocytes with Folic Acid Free or Supplementation Culture Mediums.

Liu Y, Zhao J, Wang F, Zhou J, Yang X and Yang X.
J. World Poult. Res. 10(1): 01-11, 2020; pii: S2322455X2000001-10
DOI: https://dx.doi.org/10.36380/jwpr.2020.1

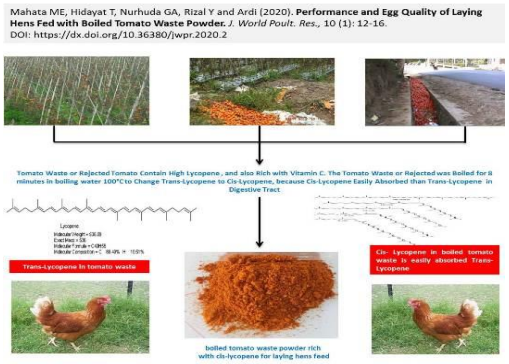


ABSTRACT: Folic acid had been reported to develop much metabolic regulation function in animals and human beings due to its roles in one carbon metabolism. The current study was conducted to explore folic acid regulation function in primary chicken hepatocytes via supplement and deprivation culture models based on proteomic analysis. Results have shown that folic acid supplement significantly increased intracellular folic acid, 5-Me-THF and SAM contents when compared with folic acid free group ($P < 0.05$). Whereas, there was no difference about genome 5mC levels and DNMTs mRNA expression between these two groups. Proteomic analysis found 85 differential expressed proteins with 35 down and 50 up regulation. COG and KEGG pathway analysis revealed that amino acid metabolism, carbohydrate metabolism and antioxidant function were affected by folic acid. Posttranslational modification, protein turnover, chaperones and transcription were gathered by COG analysis in relative high proportion. PRMT7 and ARID4B which were associated with histone methylation were up-regulated in the folic acid supplement group, suggesting that folic acid was likely to take part in metabolism regulation of hepatocytes via histone methylation manner in the study. In conclusion, proteomic analysis found 85 differential expressed proteins in hepatocytes with folic acid free and supplementation medium. Folic acid might be involved in amino acid and carbohydrate metabolism and oxidation resistance by its epigenetic modifications functions. Our study also provided fundamental differential protein profiles mediated by folic acid, which can facilitate the understanding of folic acid regulation function in hepatic metabolism.
Key words: Folic acid, Histone methylation, Primary chicken hepatocytes, Proteomics
[Full text-PDF]

Research Paper

Performance and Egg Quality of Laying Hens Fed with Boiled Tomato Waste Powder.

Mahata ME, Hidayat T, Nurhuda GA, Rizal Y and Ardi.
J. World Poult. Res. 10(1): 12-16, 2020; pii: S2322455X2000002-10
DOI: https://dx.doi.org/10.36380/jwpr.2020.2

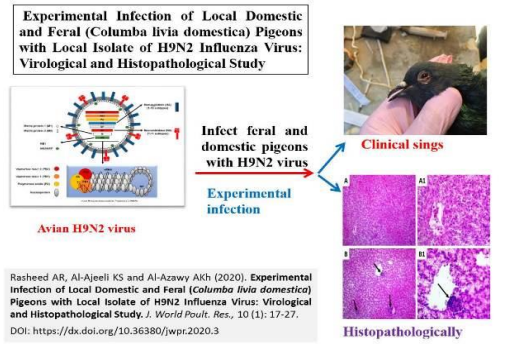


ABSTRACT: This study was performed to evaluate the effect of inclusion of boiled tomato waste powder in laying hens diet on growth performances and egg quality. Tomato waste used in this experiment was surplus and unsold mature tomato from the last harvest on the field. The tomato waste was boiled in boiling water (100 ºC) for 8 minutes to convert their lycopene structure from *trans* to *cis*, which is easily absorbed by the poultry digestive tract. In a completely randomized design, 200 Isa Brown laying hens were divided into five groups with different inclusion levels of boiled tomato waste powder (0, 3, 6, 9 and 12%) in an iso-nitrogenous (16%) and iso-caloric (2600 kcal/kg) diet. Daily feed intake, hen day egg production, egg weight, egg mass, feed conversion ratio, eggshell thickness, and eggshell strength were measured. The obtained results showed that boiled tomato waste powder in the diet did not significantly affect daily feed intake, feed conversion ratio, egg production and egg quality. In conclusion, the tomato boiled powder could be used up to 12% in laying hens diet with no effect on performance and egg quality of laying hens.
Key words: Diet, Egg quality, Layer performance, Lycopene, Tomato
[Full text-PDF]

Research Paper

Experimental Infection of Local Domestic and Feral (*Columba livia domestica*) Pigeons with Local Isolate of H9N2 Influenza Virus: Virological and Histopathological Study.

Rasheed AR, Al-Ajeeli KS and Al-Azawy AKh.
J. World Poult. Res. 10(1): 17-27, 2020; pii: S2322455X2000003-10
DOI: https://dx.doi.org/10.36380/jwpr.2020.3



ABSTRACT: A local isolate of low pathogenic Avian Influenza Virus (AIV) H9N2 subtype was used in experimental infection of 50 domestic and 50 feral pigeons (*Columba livia domestica*) to determine the susceptibility of

these birds to H9N2 infections and to study its histopathological effects on vaccinated and unvaccinated pigeons with H9N2 commercial vaccine. The birds were divided into five groups. Groups A and C contained 20 feral pigeons, B and D contained 20 domesticated pigeons. Group E contained 10 feral and 10 domesticated pigeons that were used as unvaccinated controls. Groups A and B were vaccinated with H9N2 and Newcastle disease virus commercial vaccines. Group C and D were vaccinated with Newcastle disease virus vaccine only. All groups except E were challenged with a local isolate of H9N2 serotype. Antibodies titers against AIV were estimated pre and post-vaccination using ELISA. The results indicated low antibody titers against AIV in all groups in pre-vaccination that ranged between 152.83 ± 42.01 and 337.00 ± 150.76 with no significant differences between groups. Post-vaccination antibody evaluation indicated high titers of anti-AIV antibodies in groups A and B (740.13 ± 214.38 and 673.00 ± 242.40, respectively) in comparison to pre-vaccination levels. Clinical signs appeared on 5th day post-vaccination that included mild respiratory signs, digestive disorders, and conjunctivitis in some birds of all groups. Histopathological changes in affected tissues appeared as moderate to severe multifocal necrosis diffused in the parenchymal cells of lung tissues. Infiltration with mononuclear inflammatory cells was detected in some lung tissue areas. Necrotic foci and mononuclear cell infiltration were also observed in trachea and liver of infected pigeons but mild changes were observed in intestine. The challenge virus was re-isolated in embryonated hen's eggs of nine days old by inoculation in allantoic cavity using samples collected from tissues and cloaca of infected pigeons showing clear clinical signs. The re-isolated virus was detected by the haemagglutination test using chicken RBCs and identified by haemagglutination inhibition test using a locally prepared hyperimmune serum to H9N2 in rabbits. In conclusion, pigeons are susceptible to AIV (H9N2) that might facilitate the transmission of the virus to other bird species.
Key words: Avian Influenza viruses, H9N2, Pigeons

[Full text-PDF]

Research Paper

The Effects of Extraction Methods of Mangifera indica and Azadirachta indica Bark on in vitro Antimicrobial Efficacy and Performance of Broiler Chickens.

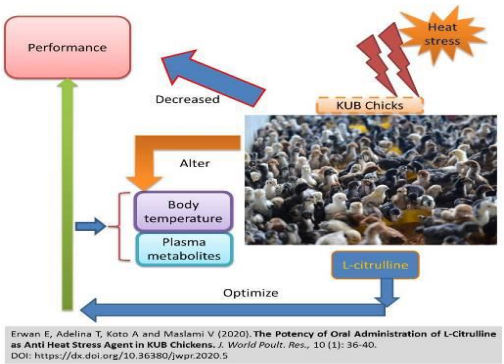
Ayoola AA, Ekunseitan D.A, Muhammad SB, Oguntoye MA and Adejola YA.
J. World Poult. Res. 10(1): 28-35, 2020; pii: S2322455X2000004-10
DOI: https://dx.doi.org/10.36380/jwpr.2020.4

ABSTRACT: This study investigated the *in vitro* efficacy of extracts of *Mangifera indica* and *Azadirachta indica* bark obtained by different extraction methods. Also, in an eight-week trial, the effect of these extracts on the performance of broiler chickens were evaluated. The barks were collected, air-dried and pulverized. The samples were extracted using maceration, infusion, and decoction methods. The extracts were screened for their activity against *Escherichia coli* and *Streptococcus aureus*. Two hundred and eighty-eight birds were divided into two groups (144 each) administered with *A. indica* or *M. indica*. Each group was subdivided into four subgroups, including control subgroup (no herb) and subgroups administered with bark extracted by one of the three extraction methods. The birds on herbal treatments were not given antibiotics. Results showed that the growth of *E. coli* was more inhibited by the various extracts irrespective of the methods of extraction. Weights were significantly influenced by the interaction between herb types and extraction methods at the starter phase. Infused herbs induced mortality at the finisher phase. In conclusion, *S. aureus* was more susceptible to the extracts compared to *E. coli*. However, decocted *A. indica* and *M. indica* bark, as well as macerated M. indica, showed antimicrobial potency against *E. coli*. It can be recommended that neem or mango bark extracted by maceration or decoction can be administered orally to broiler chickens especially at the starter phase, for improved performance and reduced mortality.
Key words: Antimicrobial assay, *Azadirachta indica*, Extraction, *Mangifera indica*, performance

[Full text-PDF]

Research Paper

The Potency of Oral Administration of L-Citrulline as Anti Heat Stress Agent in KUB Chickens.



Erwan E, Adelina T, Koto A and Maslami V.
J. World Poult. Res. 10(1): 36-40, 2020; pii: S2322455X2000005-10
DOI: https://dx.doi.org/10.36380/jwpr.2020.5

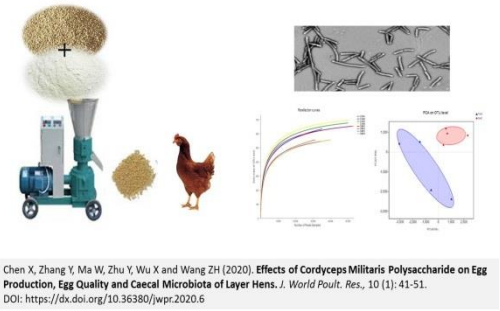
ABSTRACT: L-citrulline (L-Cit) is considered one of the potential amino acids that are able to reduce body temperature in layer chicks. However, there are no reports on functions of L-Cit in KUB chicks. Therefore, the present study aimed to evaluate the effects of oral administration of L-Cit on feed intake, body temperature and plasma biochemical parameters of KUB chicks over two hours post-administration. Neonatal KUB chicks were orally administered with different doses of L-Cit (0, 3.75, 7.5 and 15 mmol/kg body weight). At 30, 60 and 120 min, feed intake was determined, also rectal temperature of chicks was measured by using a digital thermometer. At 120 min after administration, the blood samples were immediately collected through the jugular vein, then biochemical parameters of plasma (total cholesterol, triacylglycerol and total glucose) were analyzed. The obtained results indicated that feed intake, body temperature, and plasma metabolites were not significantly influenced by different doses of L-Cit. In conclusion, L-Cit could not able to improve feed intake, decrease body temperature and change plasma metabolites in KUB chicks.

Keywords: Body temperature, Feed intake, KUB chick, L- citrulline, Plasma metabolites

[Full text-PDF]

Research Paper

Effects of Cordyceps Militaris Polysaccharide on Egg Production, Egg Quality and Caecal Microbiota of Layer Hens.



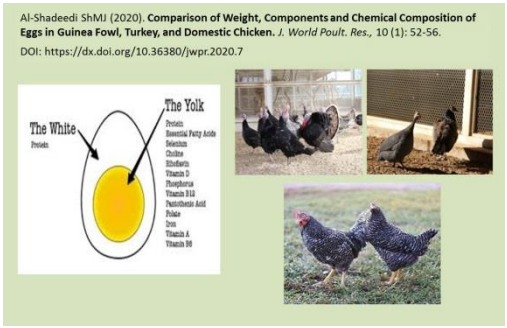
Chen X, Zhang Y, Ma W, Zhu Y, Wu X and Wang ZH.
J. World Poult. Res. 10(1): 41-51, 2020; pii: S2322455X2000006-10
DOI: https://dx.doi.org/10.36380/jwpr.2020.6

ABSTRACT: The present study was conducted to determine the effects of the diet supplementation of laying hens with Cordyceps Militaris Polysaccharide (CMP) on egg production and quality, and also caecal microbiota. A total of 360 Hy-Line Brown laying hens with 72-week-old were divided into three groups with four replicates of 30 birds each. The laying hens were fed with basal diet (control group), basal diet +100 mg CMP/kg (group 1) and basal diet + 200 mg CMP/kg (group 2). The experiment lasted 45 days. Eggs were collected daily and caecal samples were collected at the end of the experiment. Results showed that dietary supplementation with CMP did not affect albumen height, shape index, Haugh units, eggshell breaking strength and eggshell thickness. The laying rate significantly increased and the feed-egg ratio decreased in groups 1 and 2, the average daily egg weight significantly increased in group 2 compared with those in the control group. However, insignificant differences were found in broken egg rate and mortality among the three groups. The differences in caecal microbiota between group one and the control group were significant. The relative abundance of Firmicutes, Bacteroidetes and Proteobacteria at the phylum level, Christensenellaceae and Veillonellaceae at the family level, and the no rank_ Ruminococcaceae, Phascolarctobacterium and no rank Christensenellaceae at the genus level changed significantly in group one compared with those in the control group. In conclusion, dietary supplementation with 100 and 200 mg CMP/kg could improve product performance and affect the caecal microbial community structure of laying hens during the late laying period.
Key words: Cordyceps militaris polysaccharide, Egg production, Egg quality, Gut microbiome, Hens

[Full text-PDF]

Research Paper

Comparison of Weight, Components and Chemical Composition of Eggs in Guinea Fowl, Turkey, and Domestic Chicken.



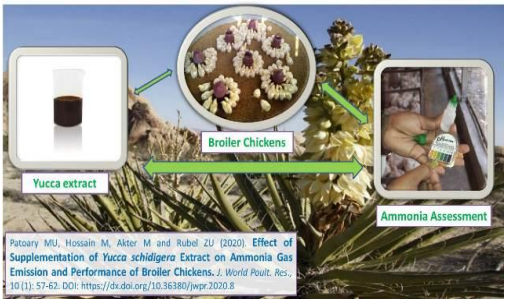
Al-Shadeedi ShMJ.
J. World Poult. Res. 10(1): 52-56, 2020; pii: S2322455X2000007-10
DOI: https://dx.doi.org/10.36380/jwpr.2020.7

ABSTRACT: The present study was carried out to compare the physicochemical characteristics of eggs of guinea fowl, turkeys and domestic chickens outdoor reared in traditional farms in Baghdad, Iraq. A total of 166 fresh eggs; 32 eggs from guinea fowls (*Numida meleagris*), 44 eggs from turkeys (*Meleagris gallopavo*) and 90 eggs from domestic chickens; were collected. Egg weight, percentage of egg components, chemical composition (protein, lipids, and ash), and lipid profile were determined. Results revealed the significant differences in egg weight among studied birds. The average egg weights for guinea fowl, turkey, and indigenous chicken were 83.15 ± 0.72, 92.41 ± 0.78 and 61.28 ± 0.62 g, respectively. No significant differences were found in egg components and the chemical composition of the edible portions of the eggs among studied birds. However, the lipid profile of egg yolk indicated that egg cholesterol and LDL levels were significantly higher in guinea fowl and turkey compared with those in indigenous chickens, whereas native chicken has high values of HDL compared to guinea fowl and turkey. There were no significant differences in the triglyceride level in egg yolks among the studied fowls. In conclusion, although egg weight was significantly different among studied birds, eggs of guinea fowl, turkeys, and domestic chickens were similar in nutritional components.
Key words: Chemical composition, Domestic chicken, Egg Components, Guinea fowl, Turkey.

[Full text-PDF]

Research Paper

Effect of Supplementation of *Yucca schidigera* Extract on Ammonia Gas Emission and Performance of Broiler Chickens.



Patoary MU, Hossain M, Akter M and Rubel ZU.
J. World Poult. Res. 10(1): 57-62, 2020; pii: S2322455X2000008-10
DOI: https://dx.doi.org/10.36380/jwpr.2020.8

ABSTRACT: The current study was conducted to investigate the efficacy of Yucca extract (YE) on ammonia gas emission from litter, evaluate the production performance, carcass characteristics and economic utility in

broiler rearing. A trial of 240 day-old commercial broiler chicks was carried out on littered floor for a period of 28 days. The birds were allocated randomly to 3 treatments and a control group with three replications (20 birds/ replication). Feeding management and rearing condition were same for all the groups, as per standard. YE was mixed with drinking water as treatment as follows: 1ml YE per 16 liters of drinking water (T_1), 1ml YE per 20 liters of drinking water (T_2), 1ml YE per 24 liters of drinking water (T_3) and no YE in drinking water i.e control group (T_0). Ammonia level of Yucca treated groups were significantly (Pth week of rearing period than control group. A significant difference (P2 group was better than other groups. This study concluded that application of YE (1ml/ 20 liters of drinking water) has an important role to reduce ammonia gas emission from broiler litter and increase the birds performance.
Keywords: ammonia control, broiler chicken, litter management, *Yucca schidigera*

[Full text-PDF]

Research Paper

Serological and Molecular Detection of Chicken Anemia Virus in Broiler and Layer Chickens in Iraq.

Al-Ajeeli KS, Al-Azawy AKh and Ghazuan H (2020). **Serological and Molecular Detection of Chicken Anemia Virus in Broiler and Layer Chickens in Iraq.** *J. World Poult. Res.*, 10 (1): 63-70. DOI: https://dx.doi.org/10.36380/jwpr.2020.9

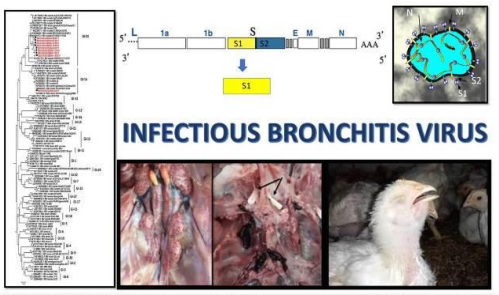
Al-Ajeeli KS, Al-Azawy AKh and Ghazuan H.
J. World Poult. Res. 10(1): 63-70, 2020; pii: S2322455X2000009-10
DOI: https://dx.doi.org/10.36380/jwpr.2020.9

ABSTRACT: Chicken Anemia Virus (CAV) infects many bird species worldwide and causes immunosuppression. This condition can facilitate the infection of affected birds with other pathogens including bacteria, viruses, and fungi. No data were available on detection or isolation of CAV from birds in Iraq, therefore this study was designed to detect CAV antibodies in broilers and layers in some poultry farms. Accordingly, 200 samples were collected from broiler and layer farms (100 samples each) from different districts of Diyala province and subjected to the ELISA test. Also, 50 tissue samples from embryonated eggs from different hatcheries, four commercial viral vaccines, and 30 ELISA positive samples were subjected to PCR assay to detect the CAV DNA. The results showed that all of broiler and layer farms sampled were serologically positive for CAV antibodies. The overall seropositivity for CAV antibodies for both chicken breeds was 51.5%. In broilers, 43 out of 100 serum samples were positive for CAV antibodies, whereas 60 out of 100 serum samples from layers were CAV antibody-positive. According to age groups, significant differences were observed among one-week-old broilers (30.2%) compared to other age groups. In layers, the age group of 30 weeks showed a seropositivity rate of 33.3%. Conventional PCR test indicated that all tissue samples collected from suspected birds and embryonated eggs were negative for CAV DNA, but only 2 out of 30 serum samples were PCR positive. It is concluded that CAV is endemic in poultry farms of Iraq and may facilitate the vaccination failure against other viruses.
Key words: Broilers, Chicken anemia virus, ELISA, Layers, PCR

[Full text-PDF]

Research Paper

Molecular Characterization and Phylogenetic Analysis of Full-length S1 Gene of GI-16 and GI-23 Infectious Bronchitis Virus in Qena, Egypt.



Sabra M, Abdellatif W, Ahmed AI and Osman N (2020). **Molecular Characterization and Phylogenetic Analysis of Full-length S1 Gene of GI-16 and GI-23 Infectious Bronchitis Virus in Qena, Egypt.** *J. World Poult. Res.*, 10 (1): 71-80. DOI: https://dx.doi.org/10.36380/jwpr.2020.10

Sabra M, Abdellatif W, Ahmed AI and Osman N.
J. World Poult. Res. 10(1): 71-80, 2020; pii: S2322455X2000010-10
DOI: https://dx.doi.org/10.36380/jwpr.2020.10

ABSTRACT: Infectious Bronchitis Virus (IBV) is a highly evolving virus that affects respiratory, urinary and reproductive systems. This virus is recognized as an important pathogen due to the continuous genesis of new variants that threaten the poultry industry worldwide. The aim of this study was to characterize emerging IBV variants originated from field outbreaks in Qena province, Egypt, and to study their genetic relationships with global strains. From September 2017 to January 2019, 52 field samples were collected from broiler flocks suspected of being infected with IBV. The collected samples were inoculated into embryonated chicken eggs via allantoic route for virus isolation. The IBV presence was confirmed using real-time reverse transcriptase PCR (rRT-PCR) assay targeting nucleocapsid (N) gene and finally, nine samples were selected from 29 positive samples with rRT-PCR for further genetic characterization through full-length spike (S1) gene sequencing. Phylogenetic analyses indicated that one isolate (IBV/CK/EG/QENA-4/2017) clustered within genotype I lineage 16 (GI-16). On the other hand, the remaining eight isolates (2017-2018) belonged to genotype I lineage 23 (GI-23) and clustered separately in monophyletic clade. The isolates in this study were found to share only 74.6-82.1% amino acid identity with the commonly used vaccine strains in Egypt. In conclusion, findings of this study provide informative data on circulating IBVs in the study area and highlight the importance of adopting a convenient vaccination strategy that can be more efficient for controlling the emergence of new IBV variants.
Key words: Full-length spike gene, GI-16, GI-23, Infectious bronchitis virus, Phylogenetic, RT-PCR.

[Full text-PDF]

Research Paper

Influence of Adding Fermented Whey Cheese into Drinking Water of Laying Hens.

Hilmi M, Prastujati UA, Khusnah A, Khirzin MH and Yannuarista D.
J. World Poult. Res. 10(1): 81-86, 2020; pii: S2322455X2000011-10
DOI: https://dx.doi.org/10.36380/jwpr.2020.11

ABSTRACT: Cheese production waste contains carbon source, one of which is lactose as an energy source in the development of fermentation, especially in the manufacture of probiotics for poultry. The research method used 3% fermented cheese whey in 17-week-old laying hen drinking water to analyze the productivity of laying hens and microbiology. The purpose of the research is to determine the effective concentration of fermented whey cheese to improve productivity, physical and chemical composition of eggs, and evaluate fat metabolism in laying hens. The present study used 120 laying hens aged 17 weeks. The experimental method was designed using the T-test method of control (P0), fermented whey cheese (P1). The observed variables were productivity (feed consumption, water consumption, daily egg production, feed conversion ration), and microbiology of small intestine and excreta (lactate acid bacteria, salmonella, *Escherichia coli*.). The effect of adding fermented whey cheese into drinking water decreased feed consumption and FCR in contrast to the control group (P0) but increased egg production, egg weight, and egg mass weight. The effect of adding fermented whey cheese into drinking water decreased the number of, *Escherichia coli* bacteria and increases lactic acid bacteria in the digestive tract of broiler chickens, especially in the ileum, caeca, so also with those in the stool. The number of Salmonella Bacteria was significantly decreased and very significantly increased lactic acid bacteria in the feces during the fermentation of whey cheese. The decrease in ammonia in the stool was very significant compared to the control group. In conclusion, adding fermented whey cheese into drinking water can reduce feed consumption, feed conversion ratio (FCR), *Escherichia coli*, salmonella, ammonia (NH_3), and increase egg production, egg weight, egg mass weight, final body weight, and lactate acid bacteria. The use of fermented whey cheese can be used as a nutraceutical feed additive to inhibit pathogenic bacteria in the intestine and increase lactic acid bacteria.
Key words: Drinking water, Fermented, Laying hens, Whey cheese

[Full text-PDF]

Research Paper

A Novel Mutation in the Promoter Region of Avian Uncoupling Protein3 Associated with Feed Efficiency and Body Composition Traits in Broiler Chicken.

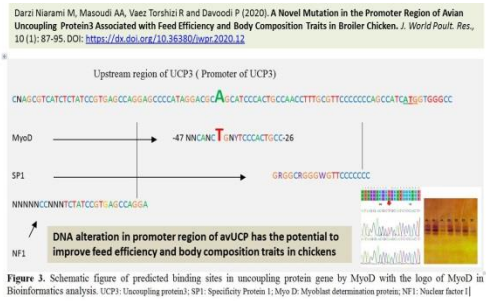


Figure 3. Schematic figure of predicted binding sites in uncoupling protein gene by MyoD with the logo of MyoD in Bioinformatics analysis. UCP3: Uncoupling protein3; SP1: Specificity Protein 1; Myo D: Myoblast determination protein; NF1: Nuclear factor 1|

Darzi Niarami M, Masoudi AA, Vaez Torshizi R and Davoodi P.
J. World Poult. Res. 10(1): 87-95, 2020; pii: S2322455X2000012-10
DOI: https://dx.doi.org/10.36380/jwpr.2020.12

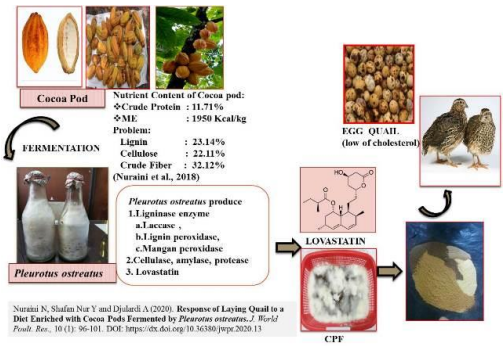
ABSTRACT: The Avian Uncoupling Protein (avUCP) belongs to the mitochondrial anion transporter family. It has a pivotal homeostatic mechanism that associated with energy regulation and lipid metabolism. The avUCP considered as a candidate gene for chicken growth-related traits according to its predominant expression is in skeletal muscle. To address genetic distance pattern of UCP3 between mammalian and avian species, sequence similarity analysis using the protein alignment of UCP3 identified the high amino acid identity between the species and complementarily detected two protein conserved regions which are known as the ADP/ATP transporter translocase and the Mitochondria-carrier. Likewise, for mutation detection, samples were genotyped, afterward PCR-SSCP method implemented. In addition, association analysis was performed for investigating single nucleotide polymorphism within the UCP3 gene relating to the given economic traits. A detected polymorphic site, on the promoter region of UCP3 (-40 T/A substitution), has displayed significant influences on the Feed Conversion Rate (FCR), Residual Feed Intake (RFI), Average Daily Gain (ADG), and Carcass Weight (CW%). In the case that, birds with genotype AA had better FCR, ADG, RFI as compared to the genotype BB and birds with genotype AA revealed a higher CW% as compared to the genotype BB. According to the obtained results from the in-silico survey, Myoblast determination protein (MyoD) was predicted as a best-matched transcription factor with a consensus sequence harboring the -40 T/A -novel SNP- in the promoter region of UCP3, where might be responsible for phenotypic variation between two genotypes. In conclusion, the result suggests important roles for UCP3 polymorphism in feed efficiency and growth traits which is better to be used in broiler chicken breeding programs.
Key words: Association analysis, Avian uncoupling protein3, Body composition traits, Broiler chicken, Feed efficiency, Novel mutation

[Full text-PDF]

Research Paper

Response of Laying Quail to a Diet Enriched with Cocoa Pods Fermented by *Pleurotus ostreatus*.

Nuraini N, Shafan Nur Y and Djulardi A
J. World Poult. Res. 10(1): 96-101, 2020; pii: S2322455X2000013-10
DOI: https://dx.doi.org/10.36380/jwpr.2020.13



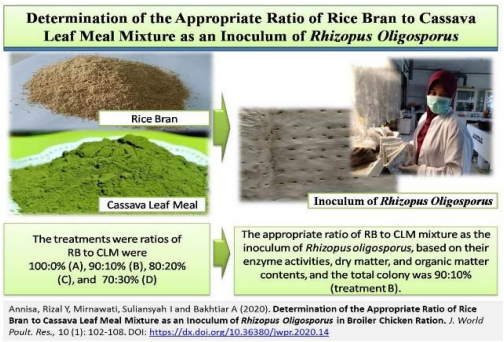
ABSTRACT: Improving the quality of cocoa pods through fermentation with *Pleurotus ostreatus* reduced its crude fiber content, especially for lignin and cellulose. Fermentation of cocoa pods product increased their utilization in poultry diet. An experiment was conducted to determine the effect of feeding cocoa pods waste fermented by *Pleurotus ostreatus* on the laying performance and egg quality of quail. This experiment employed completely randomized design with five dietary treatments: 0%, 5%, 10%, 15% and 20% cocoa pods fermented by *Pleurotus ostreatus* in the diets and four replications. 200 laying quail (7 weeks of age) were randomly allocated into 5 treatments (10 birds per treatment) and 4 replications. Diets included iso nitrogen 20% and iso metabolism energy 2800 kcal/kg. The results of the experiment indicated that feed intake, hen-day egg production, egg weight, egg mass production, and feed conversion were not affected but egg cholesterol was affected by increasing cocoa pods fermented products in the diet. In this experiment, a diet which utilized 15% cocoa pods fermented using *Pleurotus ostreatus* maintained the laying performance of quail and reduced egg yolk cholesterol (20.30%).
Key words: Cocoa pods, Egg quality, laying Quail, Performance, *Pleurotus ostreatus*

[Full text-PDF]

Research Paper

Determination of the Appropriate Ratio of Rice Bran to Cassava Leaf Meal Mixture as an Inoculum of *Rhizopus Oligosporus* in Broiler Chicken Ration.

Annisa, Rizal Y, Mirnawati, Suliansyah I and Bakhtiar A.
J. World Poult. Res. 10(1): 102-108, 2020; pii: S2322455X2000014-10
DOI: https://dx.doi.org/10.36380/jwpr.2020.14



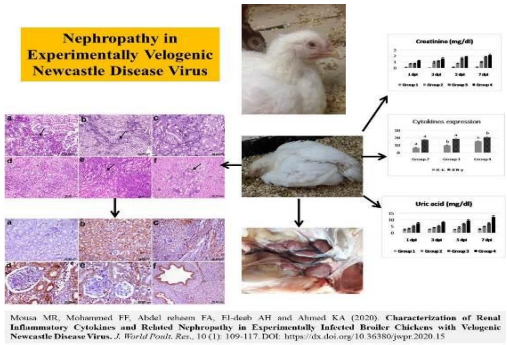
ABSTRACT: Rice bran (RB) was used as a medium for the fermentation process because it contained complete nutrients necessary for the growth of microbes. The ability to induce a fermentation substrate by microbes dependent on the availability of an inducer in the medium. The enzyme activity would increase when a suitable inducer was in the medium. In order to increase protease and cellulase activities of *Rhizopus oligosporus* in processing Cassava Leaf Meal (CLM), it was necessary to add CLM in medium producing an inoculum *Rhizopus oligosporus*. This study was conducted in a completely randomized design with 4 treatments and 5 replications. The treatments were ratios of RB to CLM as follows: 100:0% (A), 90:10% (B), 80:20% (C), and 70:30% (D). The observed variables were protease activity, cellulase activity, dry matter content, organic matter content and the total colony of *Rhizopus oligosporus* numerically. The different ratios of RB to CLM significantly affected protease and cellulase activities as well as the content of dry matter and organic matter. The addition of 10% CLM to the RB medium increased protease and cellulase activities and reduced dry matter and organic matter contents. However, when more than 10% CLM was added, it reduced the protease and cellulase activities and increased dry matter and organic matter content. The highest total colony of *Rhizopus oligosporus* was numerically in treatment B (90:10% of RB to CLM ratio). The appropriate ratio of RB to CLM mixture as the inoculum of *Rhizopus oligosporus,* based on their enzyme activities, dry matter, and organic matter contents, and the total colony was 90:10% (treatment B).
Key words: Cassava leaf meal, Enzyme activity, Inoculum, *Rhizopus oligosporus,* Rice bran.

[Full text-PDF]

Research Paper

Characterization of Renal Inflammatory Cytokines and Related Nephropathy in Experimentally Infected Broiler Chickens with Velogenic Newcastle Disease Virus.

Mousa MR, Mohammed FF, Abdel reheem FA, El-deeb AH and Ahmed KA
J. World Poult. Res. 10(1): 109-117, 2020; pii: S2322455X2000015-10
DOI: https://dx.doi.org/10.36380/jwpr.2020.15



ABSTRACT: Velogenic Newcastle disease virus (VNDV) shows systemic dissemination and it affects the histological structure and function of the kidneys. Present study is an novel attempt to correlate the changes in renal biochemical functions and gene expression of different inflammatory cytokines with related renal pathology induced by VNDV in experimentally infected broilers in different ages. One hundred broiler chickens were divided into 4 groups: group 1 served as control (non-infected group), and groups 2, 3 and 4 were inoculated with 10^6 EID_{50} of NDV on the 10^{th}, 20^{th} and 30^{th} days of age, respectively. Serum samples were collected to evaluate uric acid and

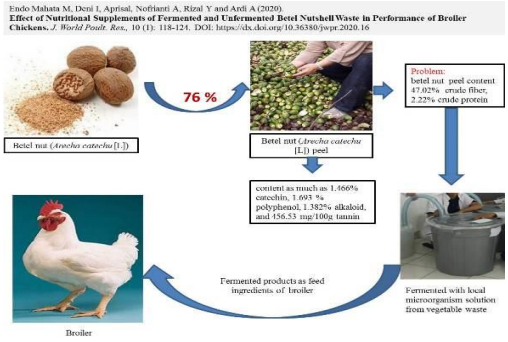
creatinine levels. Kidney specimens were collected for virus detection using real time RT-PCR, evaluation of gene expression of IL-6 and IFN-γ as well as histopathological, histochemical and immunohictochemical analysis. The highest concentration of uric acid and creatinine profile were found in Group 4 (12.06 ± 1.25 and 2.16 ± 0.12, respectively) on 7 dpi. IL-6 and IFN-γ were significantly elevated at the 7th dpi in Group 4 compared to other infected groups. The renal histopathological lesions included tubulointerstitial nephritis and glomerulopathy with expression of virus antigen in different areas of renal tissue. histopathology was the hallmark of VNDV infection in broiler chickens; their severity was related to the increased expression of inflammatory cytokines genes (IL-6 and IFN-γ) and virus antigen residence in renal tissue.

Key words: IL-6, IFN-γ, Immunohistochemistry, Nephropathy, Velogenic NDV

[Full text-PDF]

Research Paper

Effect of Nutritional Supplements of Fermented and Unfermented Betel Nutshell Waste in Performance of Broiler Chickens.



Endo Mahata M, Deni I, Aprisal, Nofrianti A, Rizal Y and Ardi A.
J. World Poult. Res. 10(1): 118-124, 2020; pii: S2322455X2000016-10
DOI: https://dx.doi.org/10.36380/jwpr.2020.16

ABSTRACT: An experiment was conducted to see the broiler's performance fed with fermented and unfermented betel nutshell. Harvesting betel nut seed leaved peel as a waste that contains some nutrients and active ingredients. This waste was useful for poultry feed, to maintain poultry performance and to reduce cholesterol. The aim of this research was to fermented betel nutshell waste with indigenous microorganism from a vegetable waste mixture that produced cellulase to reduce their crude fiber before feeding to the broiler. The nutrient profile and metabolizable energy of fermented betel nutshell in dry matter basis showed 15.96 % water, 84.04 % dry matter, 23.69 % crude fiber, 10.39 % crude protein, 0.31 % crude fat, 2.60% calcium, 1.26 % phosphorus, and 1360,44 kcal/kg. In this experiment betel nutshell waste from a local farmer in Indonesia, and 80 birds from the broiler strain CP 707 from Charoen Pockphand were used. The experiment was conducted in a completely randomized design with different levels of fermented betel nutshell waste (0, 2, 4, and 6%) and 6 % of unfermented of betel nutshell waste in the broiler ration. Each treatment was repeated four times. The measured values included daily feed intake, daily weight gain, feed conversion, body weight, carcass content, abdominal fat pads, lipoprotein profile (total cholesterol, low-density lipoprotein (LDL), and high-density lipoprotein (HDL) in serum of broiler chikens. The results indicated that fermented betel nutshell waste significantly increased daily feed intake, daily body weight gain, carcass content, and decreased abdominal fat pad, total cholesterol, HDL, and LDL of broiler chickens, however did not affect the feed conversion. The fermentation of betel nutshell waste with indigenous microorganism from vegetable waste mixture could be used up to 6 % in the broiler ration and had a positive effect on performance, blood serum lipoprotein, and carcass quality of broiler chickens.
Key words: Betel nutshell waste, Broiler, Crude fiber, Fermented betel nutshell waste, Performance

[Full text-PDF]

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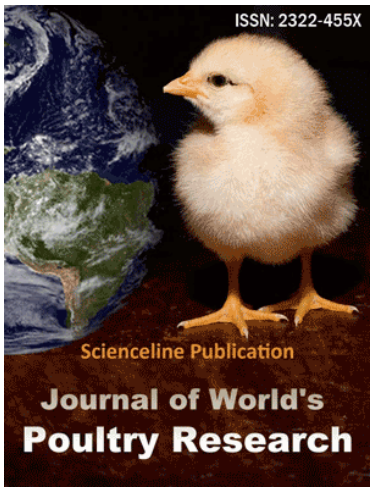

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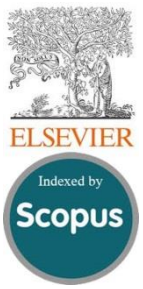

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Comparative Proteomic Analysis of Chicken Primary Hepatocytes with Folic Acid Free or Supplementation Culture Mediums

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ABSTRACT

Folic acid had been reported to develop much metabolic regulation function in animals and human beings due to its roles in one carbon metabolism. The current study was conducted to explore folic acid regulation function in primary chicken hepatocytes via supplement and deprivation culture models based on proteomic analysis. Results have shown that folic acid supplement significantly increased intracellular folic acid, 5-Me-THF and SAM contents when compared with folic acid free group ($P<0.05$). Whereas, there was no difference about genome 5mC levels and DNMTs mRNA expression between these two groups. Proteomic analysis found 85 differential expressed proteins with 35 down and 50 up regulation. COG and KEGG pathway analysis revealed that amino acid metabolism, carbohydrate metabolism and antioxidant function were affected by folic acid. Posttranslational modification, protein turnover, chaperones and transcription were gathered by COG analysis in relative high proportion. PRMT7 and ARID4B which were associated with histone methylation were up-regulated in the folic acid supplement group, suggesting that folic acid was likely to take part in metabolism regulation of hepatocytes via histone methylation manner in the study. In conclusion, proteomic analysis found 85 differential expressed proteins in hepatocytes with folic acid free and supplementation medium. Folic acid might be involved in amino acid and carbohydrate metabolism and oxidation resistance by its epigenetic modifications functions. Our study also provided fundamental differential protein profiles mediated by folic acid, which can facilitate the understanding of folic acid regulation function in hepatic metabolism.

Key words: Folic acid, Histone methylation, Primary chicken hepatocytes, Proteomics

Abbreviations: MTHFR: methylenetetrahydrofolate reductase; FA: folic acid; DNMT: DNA methyltransferase; GO: Gene ontology; COG: cluster of orthologous groups of proteins; DEP: differential expressed protein; ROS: Reactive oxygen species; KEGG: Kyoto Encyclopedia of Genes and Genomes.

INTRODUCTION

Folic acid, as an essential B vitamin, had been reported to develop many metabolic regulation functions in animals and human beings. For instance, folic acid addition could reduce hypoxia-induced inflammatory response by Reactive oxygen species and JAK2/STAT3 pathway in human pro-myelomonocytic cells (Ma et al., 2018), and also could anises acetate-induced hepatotoxicity by down-regulating NF-κB, IL-1β production and lipid peroxidation caused by cell injury (Allah and Badary, 2017). What's more, maternal use of folic acid can prevent many neural tube defects (Molloy et al., 2017). The previous study also revealed that folic acid decreased homocysteine level and improved antioxidative capacity in atherosclerotic rats (Cui et al., 2017). In addition, folate was reported to have prevention function in breast cancer risk (Chen et al., 2014). On the other hand, many study reported that folic acid developed function by changing DNA methylation because of its roles in one-carbon transfer reactions; Yu et al. (2014) has found that folic acid could reduce lipid accumulation of chicken adipocytes by increasing DNA methylation of C/EBPα promoter, thereby reducing FAS and PPARγ expression. It was reported that the mouse sperm epigenome would be altered under the condition of low paternal dietary folate (histone H3 methylation or DNA methylation), which was also associated with many negative pregnancy outcomes (Lambrot et al., 2013). Therefore, it's confirmed to some extent that folic acid could have anti-inflammation and anti-oxidation effect, and also play positive roles in some diseases.

The liver is a metabolic organ owning synthesis, transportation, detoxication functions and also a major place of folic acid metabolism. Folic acid is transported inside the cell via different processes involving membrane embedded folate receptors or reduced folate carrier (Nazki, et al., 2014), then 5,10-methylenetetrahydrofolate could be distributed towards methionine pathways, which involves in remethylation of homocysteine for genomic and non-genomic methylation, catalyzed by methylenetetra-hydrofolate reductase (MTHFR) through a non-reversible process (Lucock, 2000). In poultry industry, many metabolic diseases occur under the conditions of intensive breeding environment and higher improvement of growth performance by genetic breeding. It's aimed to come up with an assumption that whether folic acid could take part in hepatic metabolism regulation through DNA methylation capacity to solve the potential problems in chickens.

Hepatocytes culture *in vitro* is a suitable model to study metabolism, pharmacology and toxicology (Hou et al., 2001, Xu et al., 2012, Chen et al., 2017). And given the importance of liver organ itself in body metabolism and the metabolism site of folic acid, primary chicken hepatocytes will be used to explore our hypothesis mentioned above preliminarily in virtue of proteomics analysis technique. In addition, folic acid supplemented and folic acid deficient culture media are used to establish two cells culture models.

MATERIALS AND METHODS

Culture of chicken primary hepatocytes

Hepatocytes were isolated from male one-day-old layer chicks by collagenase digestion and filtration according to our previous description (Liu et al., 2018). We confirm that all animals' procedures used in the current study were approved by the ethical standards of the Animal Care and Use Committee of the College of Animal Science and Technology of the Northwest A&F University (Shaanxi, China). After 12 h attachment incubation, hepatocytes were washed with PBS and replaced with growth medium; when the confluence reached to about 80%, folic acid-free (0 mg/L) or folic acid supplemented medium (15 mg/L) was used to replace the normal medium (1 mg/L folic acid) for another 12 h treatment. RPMI 1640 culture medium with folic acid-free was purchased from Gibco (Life Technologies, Carlsbad, CA) and folic acid from Sigma (St. Louis, MO). There are three replicates in each group for proteomics analysis, and six replicates for other detections. The folic acid was dissolved in the10% ammonium hydroxide with minimal volume, then diluted to the concentration of 500 mg/L using deionized water (Yu et al., 2014), finally filtered by 0.22-μm filters. The stock solution was diluted further in culture medium to reach the final concentrations required.

5mC level

Genomic DNA from hepatocytes was isolated using the TIANamp Genomic DNA Kit (Tiangen, Beijing, China) according to standard procedures. Then 100 ng of each DNA sample was used to measure global DNA methylation level using 5-mC DNA Elisa Kit (Zymo Research, Irvine, California, USA). The amount and percentage of methylated DNA (5mC) in the total DNA was calculated based on a standard curve.

Determination of folic acid, 5-Me-THF and SAM contents

Upon treatments, cells were rinsed with ice-cold PBS and trypsinized. Hepatocytes were centrifuged, washed and suspended in PBS. After ultrasonic decomposition, cells were centrifuged at 1500 g for 15 min at 4^{o}C to remove cellular debris. The supernatant was collected to examine levels of folic acid, 5-Me-THF and SAM by Enzyme-linked Immunosorbent Assay Kits (Cloud-Clone Corp, USA). All the results were expressed as ng/10^6 cells.

RNA isolation and gene quantification

After the removal of treatment medium, cells were washed twice with ice-cold PBS. Total RNA was extracted based on the TRIZOL reagent instruction (Invitrogen, Carlsbad, CA). Its concentration and purity were determined by the absorbance at 260 nm and A260/A280 value using a NanoDrop 2000c spectrophotometer (Thermo Scientific, Wilmington, USA). 500 ng of total RNA were used to complete cDNA synthesis by Primer Script RT Reagent Kit (TaKaRa, Dalian, China). Then the SYBR Premix Ex Taq kit (TaKaRa, Dalian, China) was used to carry out the assay for gene expression. Primers sequences were shown in table 1. Detailed procedures were operated as our previous description (Liu, et al., 2016). The $2^{-\Delta\Delta\ Ct}$ method was used for gene relative expression (Livak and Schmittgen, 2001).

Protein extraction

After treatment, cells were completely homogenized with a STD buffer (4% SDS, 1 mM DTT, 150 mM Tris-HCl pH 8.0, protease and phosphatase inhibitors), then the mixture was heated at 100 ℃ for 10 min. After centrifugation at 12000 g for 10 min when cooled to room

temperature, the supernatants were collected and protein concentration was determined using the Bicinchoninic acid (BCA) assay kit (Bio-Rad) based on its protocols.

Protein digestion and iTRAQ labelling

A total of 200 μg protein were digested following the reported methods (Du et al., 2015), and the peptide content was quantified by UV light spectral density at 280 nm. Then 80 μg peptide for each sample were used for iTRAQ labelling (Applied Biosystems). The three samples in 0 mg/L group were labelled with reagents 113, 114 and 115. The samples in 15 mg/L group were labelled with 118, 119 and 121. After labelling, all samples were pooled and dried. The mixed labeled peptides were carried out fractionating by strong cationic-exchange (SCX) chromatography separation. About 36 fractions were collected and combined, then desalted on C18 Cartridges. Each fraction was detected for liquid chromatography-tandem mass spectrometry (LC-MS/MS). Detailed procedures are on the basis of previous report (Dong, et al., 2017, Cao, et al., 2018).

Protein identification and quantification

The protein identification and iTRAQ quantification were operated using a Mascot 2.2 (Matrix Science, London, UK) and Proteome Discoverer 1.4 (Thermo Electron, San Joes, CA) as described (Wang, et al., 2013). The corresponding parameters were set as same as the description by Du et al. (2015). Database search was performed against the Gallus (Uniprot) database. For statistical analysis, student's *t* test was used to identify significant changes between two group samples. Proteins with a statistically significant iTRAQ ratio of > 1.2 or < 0.83 ($P<0.05$) were considered differentially abundant proteins.

Functional analysis

Gene ontology (GO), cluster of orthologous groups of proteins (COG), KEGG pathways and proteins interaction of identified differential proteins were analyzed respectively according to previously reported method (Wu, et al., 2006, Wu, et al., 2016). A schematic workflow illustrating the steps about iTRAQ process applied in this study is shown in figure 1.

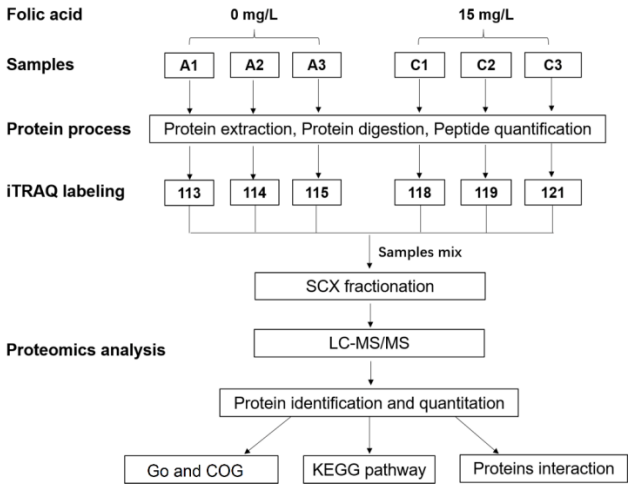

Figure 1. Experimental design and schematic diagram of proteomics analysis in the study.

Table 1. Primers of genes for RT-PCR analysis

Gene	Accession number	Primer sequences, 5′ to 3′	Product size, base pair	Reference
β-actin	L08165	Forwards: ATTGTCCACCGCAAATGCTTC Reverse: AAATAAAGCCATGCCAATCTCGTC	113	Liu et al. (2016)
DNMT1	NM206952	Forwards: ACAGCCTTCGCCGATTACA Reverse: CTCTCCACCTGCTCCACCAC	81	Liu et al. (2016)
DNMT3A	NM001024832	Forwards: CAACAACCACGACCAGGAGT Reverse: ACCATGCCCACAGTGATAGAGT	84	Liu et al. (2016)
DNMT3B	NM001024828	Forwards: CCCGTTATGATCGACGCTAT Reverse: GGGCTACTCGCAGGCAAA	92	Liu et al. (2016)

Statistical analysis

Experimental data on DNMTs expression, genomic 5mC level, folic acid, 5-methyl-THF and SAM contents in chicken hepatocytes were analyzed using *t*-test in SPSS 21.0 software (SPSS Inc., Chicago, IL, USA). The identification of differential expression proteins (DEPs) between two groups depended on the ratio of protein contents in folic acid free group to folic acid supplement group. The ratio $\geqslant 1.20$ or $\leqslant 0.83$ was regarded as differentially expressed proteins. In addition, a value of $P < 0.05$ was considered to be statistically significant.

Ethics Committee Approval

All the birds and experimental protocol in this study were approved by the Institution Animal Care and Use Committee of the Northwest A&F University (protocol number NWAFAC1008).

RESULTS

5mC level and some metabolites content

As shown in table 2, intracellular folic acid, 5-Me-THF and SAM contents were significantly higher in folic acid group when compared with folic acid free group (P<0.05). Whereas, there was no difference about genome 5mC levels between these two groups.

Table 2. Levels of genome 5mC and some metabolites in hepatocytes of layer chicks

Parameters	FA-free	FA-sup	SEM	P value
5mC (%)	1.00	0.81	0.106	0.096
FA (ng/10^6 cell)	24.00	29.94*	0.430	<0.001
5-Me-THF (ng/10^6) cell)	0.26	0.37*	0.024	0.002
SAM (ng/10^6 cell)	1.69	1.96*	0.091	0.021

Note: The symbol * showed difference significantly in statistics between folic acid free and supplement groups (P < 0.05). SEM= Standard error; FA= folic acid; 5-Me-THF=5-methyl tetrahydrofolic acid; SAM= S adenosylmethionine.

mRNA expression of DNMTs

As exhibited in figure 2, 15 mg/L folic acid supplement didn't affect genes expression about DNA methyltransferases in comparison with those in folic acid free group.

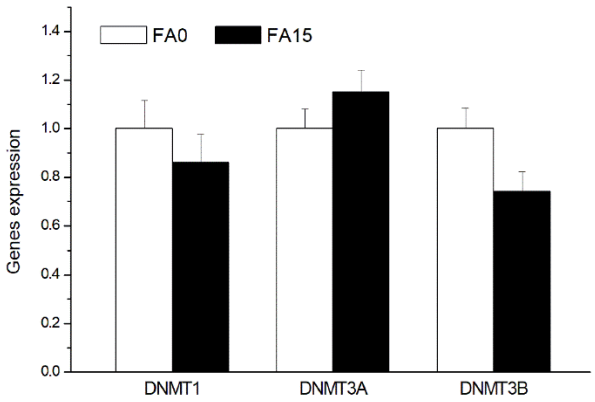

Figure 2. Gene expression of DNA Methyltransferases (DNMT1, DNMT3A, DNMT3B) in layer chicken hepatocytes between groups with folic acid free and supplement medium. Data were presented as means ± SEM (n=6).

Protein profiling

Using the Mascot software, a total of 28725 unique peptides and 4660 proteins were identified. Among these proteins, 547 were between 0 to 20 kDa, 2393 between 20 to 60 kDa, 965 between 60 to 100 kDa and 755 over 100 kDa (Figure 3A). 1405 proteins had one unique peptide, 670 had two, 667 had more than 11, and the left had 3–10 (Figure 3B). Because iTRAQ quantification indicated the amount of real fold change between groups to some extent, proteins with a fold-change > 1.2 or < 0.83 (P<0.05) were regarded as differential expressed proteins (DEPs). Based on this standard, 85 DEPs (35 down-regulation and 50 up-regulation) were detected shown in table 3.

Table 3. Differential expression proteins between folic acid free and supplement groups

Accession	Gene name	Protein name	[1]Ratio Sup/free	P value
Down-regulation				
F1N804	PLXNA1	Plexin A1	0.529	0.012
F1NL76	GAK	Cyclin G associated kinase	0.641	<0.001
Q8AWB6	SLC35B1	Solute carrier family 35 member B1	0.675	<0.001
R4GF71	TMSB4X	AM-8-amino-7-oxononanoate aminotransferase	0.707	0.002
E1B2Y2	SLC7A3	Cationic amino acid transporter-3	0.709	0.006
B5AIG4	PNPLA2	Adipose triglyceride lipase	0.711	0.007
A0A1L1S044	LOC420368	Predicted GTPase	0.711	0.038
F1P4D1	SLC30A7	Zinc transporter 7	0.746	0.031
A0A165FX80	CATH1	Cathelicidin-1	0.754	0.046
P12276	FASN	Fatty acid synthase	0.759	0.010
F1P3G3	CHN1	Chimerin 1	0.760	0.003
A0A2K6TZL8	TSNAXIP1	Translin associated factor X interacting protein 1	0.768	0.009
F1NDN6	KRT12	Keratin 12	0.768	0.001
H9L107	KRT4	Myosin heavy chain	0.771	0.003
Q5ZJ43	EXOC8	Exocyst complex component 8	0.774	0.018
F1NGI6	SGSH	N-sulfoglucosamine sulfohydrolase	0.779	0.011
E1C483	ACBD6	Acyl-CoA-binding protein	0.779	0.016

E1BS86	AIG1	Androgen induced 1	0.780	0.043
F1NQ90	C11H19ORF12	Mu-like prophage protein	0.781	0.005
F1NNN3	TCERG1L	Transcription elongation regulator 1 like	0.783	0.022
F1NKU2	MELK	Non-specific serine/threonine protein kinase	0.783	0.017
P08286	P08286	Histone H1.10	0.787	0.011
E1BSR9	RBX1	Ring-box 1	0.787	0.049
F1NRK3	RPP38	Ribonuclease P/MRP subunit p38	0.792	0.013
F1NZ92	DNAH3	Dynein, heavy chain	0.798	0.017
F1P4C2	RIPK1	Receptor interacting serine/threonine kinase 1	0.805	0.018
F1P2M3	MTIF3	Translation initiation factor 3	0.810	0.001
E1C4V2	Gga.15193	Zn-finger	0.810	0.003
E1BSI3	ENSGALG00000006435	Ubiquitin-protein ligase	0.814	0.036
F1NW64	TPX2	Microtubule nucleation factor	0.817	0.012
E1C8Q1	CEP164	Centrosomal protein 164	0.821	0.005
E1BV18	CAPSL	Calcyphosine like	0.824	<0.001
A0A0A0MQ61	ENSGALG00000016325	Glutathione S-transferase	0.825	0.017
F1NWX7	SEC61B	Transcription factor about chromatin remodeling	0.826	0.023
R4GLJ6	VHL	Phosphotransferase	0.829	0.002
Up-regulation				
F1NF85	PEMT	Phosphatidylethanolamine N-methyltransferase	1.200	0.019
E1C3M0	USP45	Ubiquitin specific peptidase 45	1.213	0.006
F1NPJ3	CCDC127	Translation initiation factor 2	1.215	0.036
E1BVP5	ASPA	Aspartoacylase	1.216	0.036
F1P2G6	PIGT	Phosphatidylinositol glycan anchor biosynthesis	1.220	0.036
R4GGH1	ENSGALG00000028833	NAD-dependent aldehyde dehydrogenases	1.223	0.029
E1C0T3	PDZRN3	PDZ domain containing ring finger 3	1.225	0.013
F1NST0	DHX58	ERCC4-like helicases	1.228	0.027
E6N1V0	LAO	Amine oxidase	1.229	0.008
P07031	ACYP2	Acylphosphatase-2	1.235	0.006
Q5ZLB2	ARL6IP1	Phosphoribosylaminoimidazole carboxylase	1.240	0.018
E1C8Q2	ETNPPL	4-aminobutyrate aminotransferase	1.243	0.001
F1ND79	ZNF644	Zn-finger	1.254	0.013
Q5F366	IDUA	Iduronidase	1.259	0.020
F1NS64	MFSD4B	Major facilitator superfamily domain containing 4B	1.270	0.034
F1NK39	ARID4B	histone trimethylation	1.271	0.007
F1P3K7	PCBD2	Pterin-4-alpha-carbinolamine dehydratase 2	1.275	0.027
F1N8L2	TECR	Very-long-chain enoyl-CoA reductase	1.285	0.034
F1NJK5	RRP7A	Ribosomal RNA processing 7 homolog A	1.295	0.006
Q5ZJC0	RCJMB04	Uncharacterized protein	1.303	0.022
E1BUS8	CRYZL1	Quinone oxidoreductase-like protein 1	1.317	0.011
Q9W7G2	SALL3	Spalt protein	1.355	0.017
F1P054	CYP1A5	Cytochrome P450	1.363	0.001
E1C7X0	VRK2	Serine/threonine protein kinase	1.367	0.018
F1NB56	PDE4D	Phosphodiesterase	1.368	0.019
B8XA33	ADAM23	Disintegrin and metalloprotease 23	1.374	0.016
F1P3X6	YTHDF2	Membrane proteins	1.377	0.001
R4GG24	AKR1B1L	Aldo/keto reductases	1.380	0.041
E1BZD6	RIPK4	Ankyrin repeat	1.382	0.013
F1NWY7	MRPL42	Mitochondrial ribosomal protein L42	1.391	0.010
Q5ZL23	APBB1IP	Protein-binding family interacting protein	1.395	0.014
Q5ZJ36	PLK1	Serine/threonine-protein kinase PLK	1.413	0.008
F1P586	SFSWAP	Splicing factor SWAP	1.428	0.002
O73884	PHOSPHO1	Phosphoethanolamine/phosphocholine phosphatase	1.429	0.042
Q5ZLE1	PRPF4B	Permeases	1.438	0.045
G4XJS0	TLR1LB	Toll-like receptor 1 type 2	1.447	0.004
Q5ZHK9	LLPH	Protein LLP homolog	1.456	0.015
F1NW34	KDM8	Lysine demethylase 8	1.459	0.024
E1C4M9	SLC43A2	Solute carrier family 43 member 2	1.473	0.027
F1P5K8	APTX	Aprataxin	1.575	0.009
F1NI14	TXLNG	Taxilin gamma	1.594	0.011
E1C6E5	TSPAN3	Tetraspanin	1.630	0.036
E1C6D5	KDM4A	PHD zinc finger-containing protein	1.910	0.028
P28568	SLC2A3	Solute carrier family 2	2.119	0.020
Q5ZIB9	PRMT7	Protein arginine N-methyltransferase 7	2.169	0.020
R4GJY5	FAM108A1	Protein ABHD17B	2.179	0.049
Q5F4A8	AK6	Adenylate kinase isoenzyme 6	2.246	0.041
G8HUH5	BACT	Beta-actin (Fragment)	2.331	0.006
Q5ZK96	BTBD9	BTB domain containing 9	2.518	0.045
Q5F3Q0	NUP205	Asp-tRNAAsn/Glu-tRNAGln amidotransferase	3.167	0.014

[1] Ratio sup/free = Protein expression in folic acid supplement group / that in folic acid free group.

Classification of DEPs

Of the 85 DEPs, 79 DEPs could be assigned to 21 categories using the COG database. As shown in figure 4, the largest group was general function prediction only (26.6%) followed by amino acid transport and metabolism (10.1%), replication, recombination and repair (10.1%). carbohydrate transport and metabolism (7.6%), transcription (7.6%), posttranslational modification, protein turnover, chaperones (6.3%), and signal transduction mechanisms (6.3%). Further, GO classification analysis of DEPs was performed. The number for significant enriched biological process, cell component and molecular function is 162, 29 and 75 respectively (data not shown). In terms of GO term distributions in the second level as presented in figure 5, for biological processes, more than 60% of the notable proteins were respectively related to regulation of cellular process, single–organism process, and metabolic process; for cell component, about 68%, 57% and 35% were correlated with cell, organelle and membrane respectively; for molecular function, about 59% and 50% were respectively associated with binding and catalytic activity.

To characterize the functional consequences of DEPs associated with folic acid intervention in chicken primary hepatocytes, KEGG pathway mapping based on DEPs were also carried out and demonstrated in figure 6. Results indicated that folic acid could significantly affect metabolism of xenobiotics by cytochrome P450, drug metabolism- cytochrome P450, retinol metabolism, steroid hormone biosynthesis, pyruvate metabolism, tryptophan metabolism and glutathione metabolism. It was worth mentioning that some proteins such as ENSGALG00000016325, CYP1A5 and ACYP2 were involved in these pathways. ENSGALG00000016325 could code glutathione S-transferase which was down-regulated in 15 mg/L folic acid group, while CYP1A5 and ACYP2 were up-regulated when compared with the no folic acid group which coded cytochrome P450 and acylphosphatase proteins respectively.

Proteins interaction analysis

The protein-protein interaction networks were performed by the web-tool STRING 10.5 (https://string-db.org/cgi/input.pl). The DEPs interactions were shown in figure 7, in which the stronger associations are represented by thicker lines. The results showed that some functional modules were clustered in the network and formed tight connections with DEPs in chicken primary hepatocytes between folic acid free and supplement groups. Disconnected nodes in the network were hided. The functional modules were mainly involved in cell cycle (SKP1, RBX1, SKP2, CDC27, CDC20, MAD2L1, CUL1, BUB1B, PLK1, BUB1 and CCNB2), ubiquitin mediated proteolysis (CUL4A, CUL2, TCEB1, RBX1, SKP1, FBXW7, SKP2, CDC27, VHL, CUL1 and CDC20), protein export (SEC63, SEC61A1, SEC61B, SEC61G and SEC61A2), protein processing in endoplasmic reticulum (SEC63, SEC61A1, SEC61B, SEC61G, SEC61A2, SKP1, RBX1 and CUL1), phagosome (SEC61A1, SEC61B, SEC61G, SEC61A2, and ACTB), lysosome (IDUA, GALNS, CLTC and CLTCL1), ribosome biogenesis in eukaryotes (LOC425215, RRP7A and RPP38), TGF beta signaling pathway (SKP1, RBX1 and CUL1) and fatty acid biosynthesis (FASN and ENSGALG00000005439).

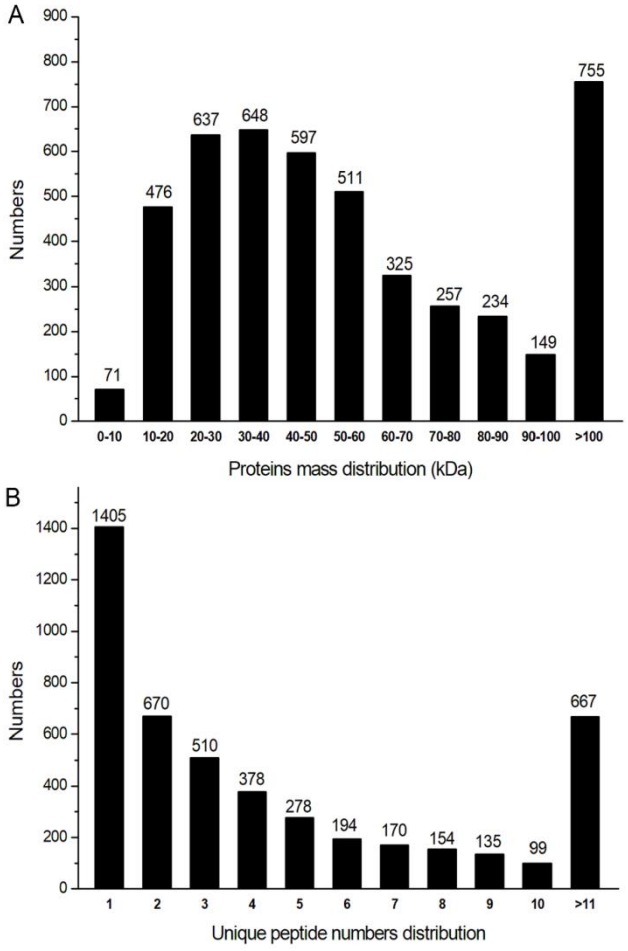

Figure 3. Basic information of iTRAQ identification. **A:** Different molecular weights distribution of proteins identified among samples. **B:** The number of unique peptides that identified proteins in the current study.

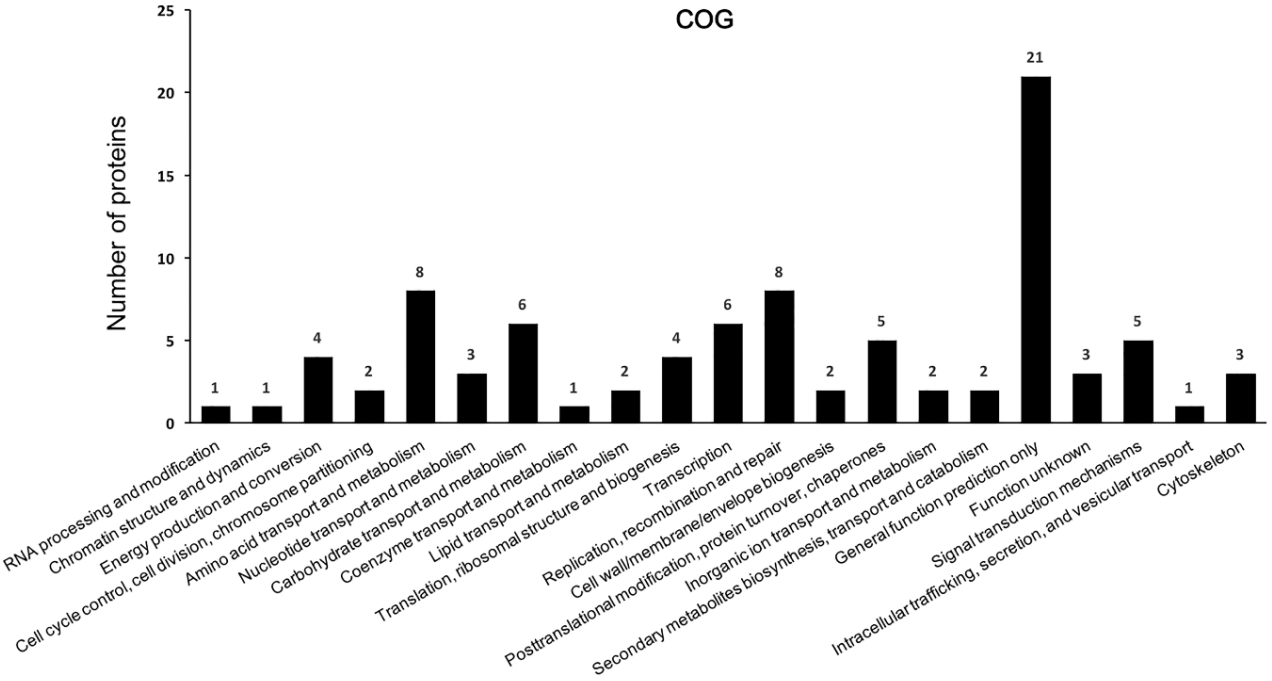

Figure 4. Clusters of Orthologous Groups (COG) of proteins classification of DEPs between folic acid free and supplement groups. The Y-axis is the numbers of DEPs annotated to the category.

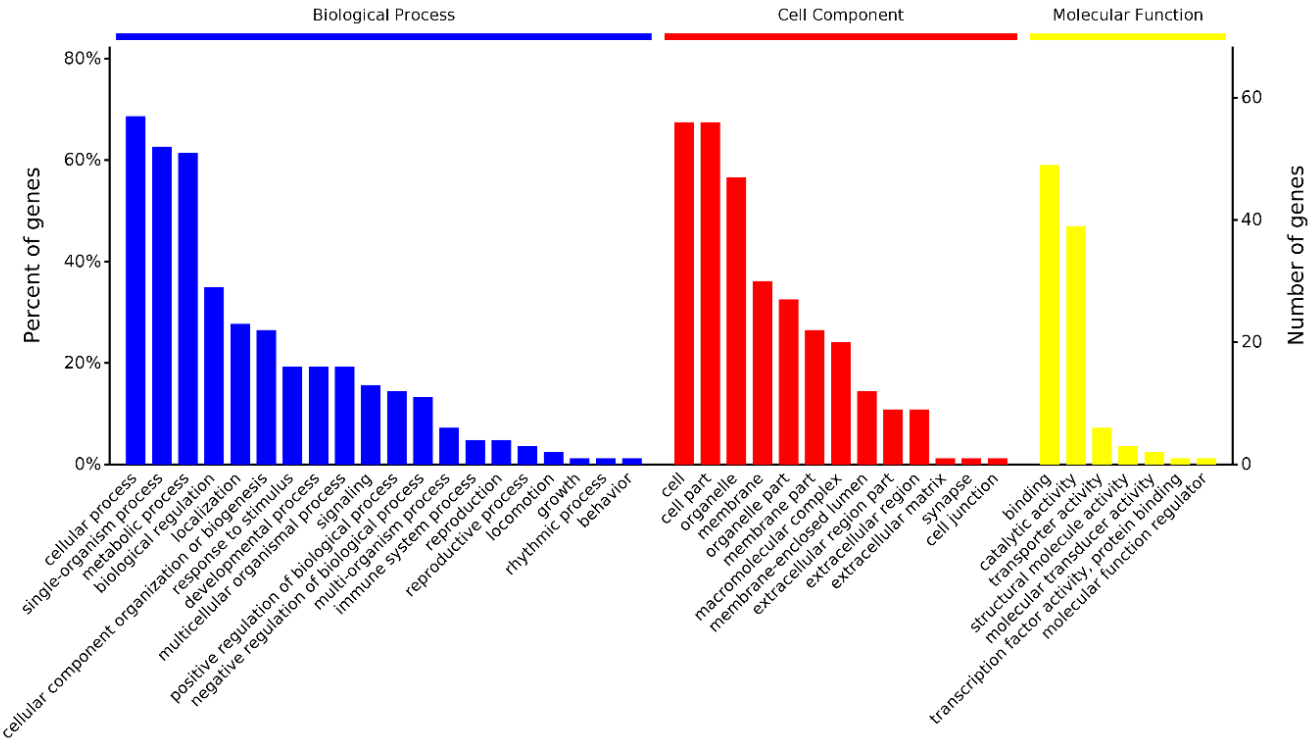

Figure 5. Functional classification of differential proteins by Gene Ontology analysis including biological process, cellular component, and molecular function. All data are presented based on GO second-level terms. The Y-axis is on behalf of the numbers of DEPs annotated to the corresponding category.

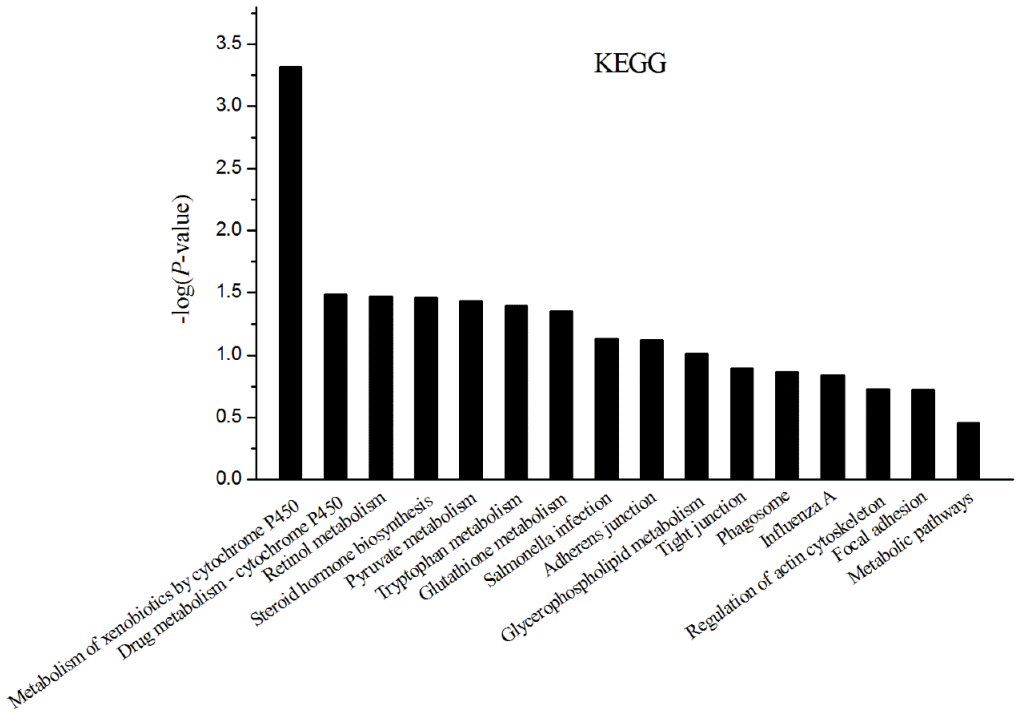

Figure 6. Distribution of enriched KEGG pathway according to DEPs between folic acid free and supplement groups.

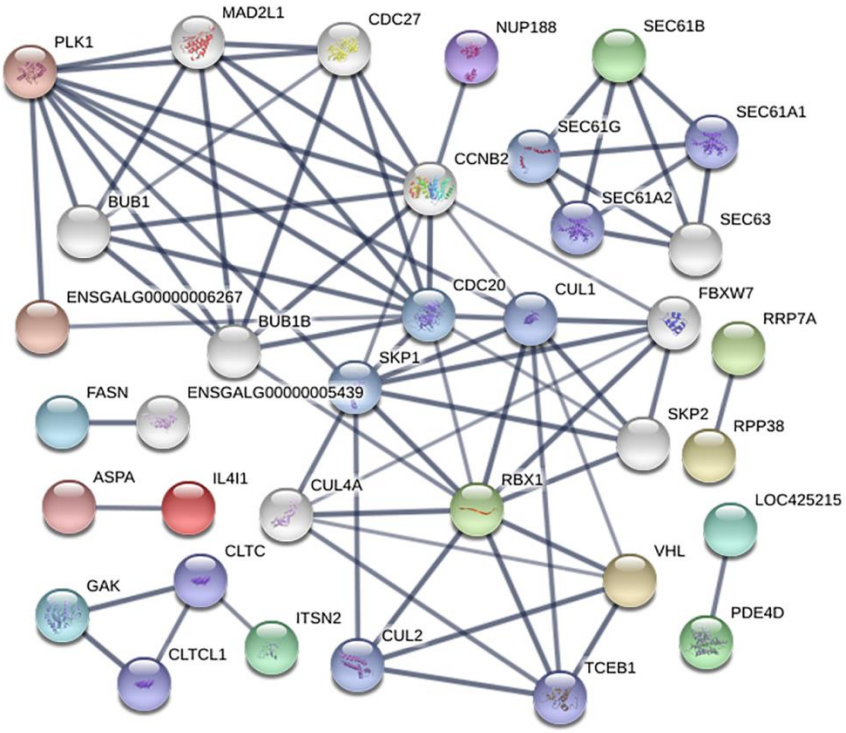

Figure 7. Interaction network analysis of DEPs using STRING software (http://string-db.org). In this network, nodes are proteins; lines represent functional associations between proteins. The resulting networks were constructed with confidence scores higher than 0.7. The gray lines between bodes represent functional associations between proteins and the thickness of the lines represents the level of confidence in association reported.

DISCUSSION

In the current study, chicken primary hepatocytes are used as the model to explore folic acid metabolism regulation function through deprivation and supplementation ways. As we all known, folic acid was commonly recognized due to its significance for the development of neurological systems in newborns. Many study have stated that there existed negative correlations between dietary or plasma folic acid and the occurrence rate of some diseases (Sie et al., 2011, Chen et al., 2014, Molloy et al., 2017). But the causal mechanisms that define the role of folic acid in these complex diseases are not established. It's generally accepted that folic acid-mediated 1-carbon metabolism could affect genes expression by DNA methylation and chromatin structure, thereby disturbing metabolic pathways about pathologies (Stover, 2009). Previous study pointed out that folic acid could slow down the aggressiveness of glioma by increasing methylation levels of DNA repeats element and genes related to apoptosis and proliferation (Hervouet et al., 2009). It was reported that low folate intake could result in genomic DNA hypomethylation and improve the risk of colorectal neoplasia, and daily supplementation with 400 mg/day folic acid for 10 weeks resulted in a marginal increase in leucocyte DNA methylation and rectal mucosa DNA methylation in patients with colorectal adenoma (Pufulete et al., 2005).

Considering the role of folic acid in DNA methylation and the fact that DNA methylation is critical to normal genome regulation and development (Crider et al., 2012), we examined genomic 5-methylcytosine (5mC) contents in hepatocytes with folic acid free and supplementation medium. Surprisingly, folic acid didn't increase DNA methylation level in the folic acid addition group. DNA methylation is catalyzed by DNA methyltransferases (DNMTs). DNMT1 is a maintenance methyltransferase and responsible for restoring the methylated status of newly synthesized daughter strands; DNMT3a and DNMT3b are de novo methyltransferases (Li et al., 2016). Consistently, these DNMTs expression were also not affected by folic acid supplementation in the current study. However, intracellular folic acid, 5-Me-THF and SAM concentrations were higher in culture medium with folic acid supplemented when compared with folic acid free group. These results may be illogicality taken together, but the relationship between folic acid and DNA methylation is complex. DNA methylation also involved in the participation of other substances such as choline, betaine and other B vitamins (Niculescu and Zeisel, 2002). On the other hand, SAM could inhibit MTHFR activity, which provides 5-Me-THF by catalyzing a unidirectional reaction (Smith et al., 2013). But other review also suggested that there was no correlation between global DNA methylation and folate status (Crider et al., 2012).

In addition, there was no difference about cell viability, albumin and lactic dehydrogenase concentration in culture medium between folic acid free and addition groups (data not shown), which suggested that the dosage of folic acid used in the study was reasonable and non-toxic for cells growth. Hence, proteomic analysis was further employed to assess folic acid metabolism regulation function in primary chicken hepatocytes. We found folic acid changed some metabolic pathways enriched by 85 DEPs including cytochrome P450 metabolism, retinol metabolism, steroid hormone biosynthesis, pyruvate metabolism, tryptophan metabolism and glutathione metabolism. Cytochrome P450 was reported to be involved in oxidation-reduction reactions (Meunier, et al., 2004), and up-regulated in the current study indicating that folic acid improved antioxidant ability. ENSGALG00000016325 which coded glutathione S-transferase (GSTs) was also contained in the pathway of cytochrome P450 metabolism, and was down-regulated in folic acid addition group. GSTs are the ubiquitous enzymes that play a key role in cellular detoxification (Jain et al., 2010), and its lower protein abundance suggested that folic acid seemed to be protective for hepatocytes. Folic acid, as an antioxidant (Gliszczynskaswiglo, 2007), has good therapeutic effects on hypoxia-induced inflammatory response by decreasing ROS activity (Ma et al., 2018).

Besides, retinol metabolism, steroid hormone biosynthesis, pyruvate metabolism, and tryptophan metabolism were also enriched. These could be contained amino acid and carbohydrate metabolism as COG analysis that amino acid or carbohydrate transport and metabolism were clustered in relative high proportion. However, how does folic acid affect these metabolism change? It is interesting to note that arginine N-methyltransferase 7 (PRMT7) and ARID4B were up-regulated proteins by folic acid addition based on proteomics though no evidence was found about DNA methylation. PRMT7 has been implicated in roles of transcriptional regulation, DNA damage repair, RNA splicing, cell differentiation, metastasis and epigenetic regulation by transferring methyl groups to arginine residues on protein substrates (Feng et al., 2013). Biological process analysis of GO has

suggested that ARID4B was associated with histone H3K9 and H4K20 trimethylation which were all related to nucleosome and chromatin structure (Xu et al., 2008, Hahn et al., 2011). These results indicated that folic acid might take part in metabolism regulation by histone methylation which contributed to transcription and post-transcriptional modification. And posttranslational modification, protein turnover, chaperones and transcription were gathered by COG analysis based on DEPs. Li et al. (2016a) has reported that folic acid increased H3K9 methylation of IL-6 promoter. Therefore, we speculated that folic acid might regulate hepatocellular metabolism via the histone methylation manner rather than DNA methylation in the present study.

CONCLUSION

In conclusion, the present proteomic analysis found 85 differential expressed proteins in primary chicken hepatocytes with folic acid free and supplementation medium. The pathways of those altered proteins are related to amino acid and carbohydrate metabolism, and oxidation resistance. Folic acid regulated these metabolisms more likely by histone methylation rather than DNA methylation. These results indicated that proteomics with bioinformatics analysis is a good starting point for understanding regulation function of some substances. A deep and broad understanding of the DEPs identified is ongoing to make clear their specific role. Our findings might provide comprehensive protein expression information that can facilitate the understanding of folic acid regulation function in hepatic metabolism.

DECLARATIONS

Competing interests

The authors declare that they have no competing interests.

Author`s contributions

XJY and YLL designed the research; JFZ, FYW, JHZ and YLL performed the research and analysed the data; YLL wrote the manuscript; XY and XJY have taken part in the revision of the manuscript. All authors read and approved the final version of the manuscript.

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REFERENCES

Allah ESHA and Badary DM (2017). Folic acid protects against lead acetate-induced hepatotoxicity by decreasing nf-κb, il-1β production and lipid peroxidation mediataed cell injury. Pathophysiology, 24 (1): 39-44. DOI: https://doi.org/10.1016/j.pathophys.2017.02.002

Cao Z, Meng B, Fan R, Liu M, Gao M, Xing Z and Luan X (2018). Comparative proteomic analysis of ovaries from huoyan geese between pre-laying and laying periods using an itraq-based approach. Poultry Science, 1: 1-13. DOI: https://doi.org/10.3382/ps/pey029

Chen P, Li C, Li X, Li J, Chu R and Wang H (2014). Higher dietary folate intake reduces the breast cancer risk: A systematic review and meta-analysis. British Journal of Cancer, 110 (9): 2327-2338. DOI: https://doi.org/10.1038/bjc.2014.155

Chen X, Che C, Korolchuk VI, Gan F, Pan C and Huang K (2017). Selenomethionine alleviates afb1-induced damage in primary chicken hepatocytes by inhibiting cyp450 1a5 expression via upregulated selw expression. Journal of Agricultural and Food Chemistry, 65 (12): 2495-2502. DOI: https://doi.org/10.1021/acs.jafc.6b05308

Crider KS, Yang TP, Berry RJ and Bailey LB (2012). Folate and DNA methylation: A review of molecular mechanisms and the evidence for folate's role. Advances in Nutrition: An International Review Journal, 3 (1): 21-38. DOI: https://doi.org/10.3945/an.111.000992

Cui S, Li W, Lv X, Wang P, Huang G and Gao Y (2017). Folic acid attenuates homocysteine and enhances antioxidative capacity in atherosclerotic rats. Applied Physiology Nutrition & Metabolism, 42: 1015-1022. DOI: https://doi.org/10.1139/apnm-2017-0158

Dong WT, Xiao LF, Hu JJ, Zhao XX, Liu JX and Zhang Y (2017). ITRAQ proteomic analysis of the interactions between bombyx mori nuclear polyhedrosis virus and silkworm. Journal of Proteomics, 166: 138-145. DOI: https://doi.org/10.1016/j.jprot.2017.07.013

Du M, Liu X, Liu X, Yin X, Han S, Song Q and An S (2015). Glycerol-3-phosphate O- acyltransferase is required for PBAN-induced sex pheromone biosynthesis in Bombyxmori. Scientific Reports, 5: 8110-8119. DOI: https://doi.org/10.1038/srep08110

Feng Y, Maity R, Whitelegge JP, Hadjikyriacou A, Li Z, Zurita-Lopez C, Al-Hadi Q, Clark A, Bedford M, Masson J and Clarke S (2013). Mammalian protein arginine methyltransferase 7 (prmt7) specifically targets rxr sites in lysine- and arginine-rich regions. Journal of Biological Chemistry, 288 (52): 37010-37025. DOI: 10.1074/jbc.M113.525345

Gliszczynskaswiglo A (2007). Folates as antioxidants. Food Chemistry, 101 (4): 1480-1483. DOI: https://doi.org/10.1016/j.foodchem.2006.04.022

Hahn MA, Wu X, Li AX, Hahn T and Pfeifer GP (2011). Relationship between gene body DNA methylation and intragenic h3k9me3 and h3k36me3 chromatin marks. Plos One, 6 (4): e18844. DOI: https://doi.org/10.1371/journal.pone.0018844

Hervouet E, Debien E, Campion L, Charbord J, Menanteau J, Vallette F and Cartrone P (2009). Folate supplementation limits the aggressiveness of glioma via the remethylation of DNA repeats element and genes governing apoptosis and proliferation. Clinical Cancer Research, 15 (10): 3519-3529. DOI: 10.1158/1078-0432.

Hou DX, Kunitake T, Kusuda J and Fujii M (2001). Primary culture of chicken hepatocytes as an in vitro model for determining the influence of dioxin. Bioscience, Biotechnology, and Biochemistry, 65 (1): 218-221. DOI: https://doi.org/10.1271/bbb.65.218

Jain M, Ghanashyam C and Bhattacharjee A (2010). Comprehensive expression analysis suggests overlapping and specific roles of rice glutathione s-transferase genes during development and stress responses. BMC Genomics, 11 (1): 73-90. DOI: https://doi.org/10.1186/1471-2164-11-73

Lambrot R, Xu C, Saintphar S, Chountalos G, Cohen T, Paquet M, Suderman M, Hallett M and Kimmins S (2013). Low paternal dietary folate alters the mouse sperm epigenome and is associated with negative pregnancy outcomes. Nature Communications, 4 (4): 2889-2903. DOI: https://doi.org/10.1038/ncomms3889

Li S, Zhi L, Liu Y, Shen J, Liu L, Yao J and Yang X (2016a). Effect of in ovo feeding of folic acid on the folate metabolism, immune function and epigenetic modification of immune effector molecules of broiler. British Journal of Nutrition, 115 (03): 411-421. DOI: https://doi.org/10.1017/S0007114515004511

Li S, Zhu Y, Zhi L, Han X, Shen J, Liu Y, Yao J and Yang X (2016b). DNA methylation variation trends during the embryonic development of chicken. Plos One, 11 (7): e0159230. DOI: https://doi.org/10.1371/journal.pone.0159230

Liu Y, Guo W, Pu Z, Li X, Lei X, Yao J and Yang X (2016). Developmental changes of insulin-like growth factors in the liver and muscle of chick embryos. Poultry Science, 95 (6): 1396-1402. DOI: https://doi.org/10.3382/ps/pew043

Liu Y, Wu S, Sun W, Chen S, Yang X, and Yang X (2018). Variation in proteomics and metabolomics of chicken hepatocytes exposed to medium with or without folic acid. Journal of Cellular Biochemistry, 119: 6113-6124. DOI: https://doi.org/10.1002/jcb.26810

Livak KJ and Schmittgen TD (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2 (-Delta Delta C(T)) method. Methods, 25 (4): 402-408. DOI: https://doi.org/10.1006/meth.2001.1262

Lucock M (2000). Folic acid: Nutritional biochemistry, molecular biology, and role in disease processes. Molecular Genetics & Metabolism, 71 (1-2): 121-138. DOI: https://doi.org/10.1006/mgme.2000.3027

Ma J, Zhen X, Huang X and Jiang X (2018). Folic acid supplementation repressed hypoxia-induced inflammatory response via ros and jak2/stat3 pathway in human pro-myelomonocytic cells. Nutrition Research, 53: 40-50. DOI: https://doi.org/10.1016/j.nutres.2018.03.007

Meunier B, de Visser SP and Shaik S (2004). Mechanism of oxidation reactions catalyzed by cytochrome p450 enzymes. Chemical Reviews, 104 (9): 3947-3980. DOI: https://doi.org/10.1021/cr020443g

Molloy AM, Pangilinan F and Brody LC (2017). Genetic risk factors for folate responsive neural tube defects. Annual Review of Nutrition, 37 (1): 269-291. DOI: https://doi.org/10.1146/annurev-nutr-071714-034235

Nazki FH, Sameer AS and Ganaie BA (2014). Folate: Metabolism, genes, polymorphisms and the associated diseases. Gene, 533 (1): 11-20. DOI: https://doi.org/10.1016/j.gene.2013.09.063

Niculescu MD and Zeisel SH (2002). Diet, methyl donors and DNA methylation: Interactions between dietary folate, methionine and choline. Journal of Nutrition, 132 (8 Suppl): 2333s-2335s. DOI: https://doi.org/10.1093/jn/132.8.2333S

Pufulete M, Al-Ghnaniem R, Khushal A, Appleby P, Harris N, Gout S, Emery PW and Sanders TAB (2005). Effect of folic acid supplementation on genomic DNA methylation in patients with colorectal adenoma. Gut, 54 (5): 648-653. DOI: 10.1136/gut.2004.054718

Sie KK, Medline A, Van Weel J, Sohn K-J, Choi S-W, Croxford R, and Kim Y (2011). Effect of maternal and postweaning folic acid supplementation on colorectal cancer risk in the offspring. Gut, 60 (12): 1687-1694. DOI: http://dx.doi.org/10.1136/gut.2011.238782

Smith DE, Hornstra JM, Kok RM, Blom HJ and Smulders YM (2013). Folic acid supplementation does not reduce intracellular homocysteine, and may disturb intracellular one-carbon metabolism. Clinical Chemistry and Laboratory Medicine, 51 (8): 1643-1650. DOI: https://doi.org/10.1515/cclm-2012-0694

Stover PJ (2009). One-carbon metabolism-genome interactions in folate-associated pathologies. Journal of Nutrition, 139 (12): 2402-2405. DOI: https://doi.org/10.3945/jn.109.113670

Wang L, Liang W, Xing J, Tan F, Chen Y, Huang L, Chen C and Chen W (2013). Dynamics of chloroplast proteome in salt-stressed mangrove kandelia candel (l.) druce. Journal of Proteome Research, 12 (11): 5124-5136. DOI: https://doi.org/10.1021/pr4006469

Wu J, Mao X, Cai T, Luo J and Wei L (2006). Kobas server: A web-based platform for automated annotation and pathway identification. Nucleic Acids Research, 34 (Web Server issue): 720-724. DOI: https://doi.org/10.1093/nar/gkl167

Wu Y, Tang J, Zhou C, Zhao L, Chen J, Zeng L, Rao C, Shi H, Liao L and Liang Z (2016). Quantitative proteomics analysis of the liver reveals immune regulation and lipid metabolism dysregulation in a mouse model of depression. Behavioural Brain Research, 311: 330-339. DOI: https://doi.org/10.1016/j.bbr.2016.05.057

Xu L, Simon MD, Chodaparambil JV, Hansen JC, Shokat KM, and Luger K (2008). The effect of h3k79 dimethylation and h4k20 trimethylation on nucleosome and chromatin structure. Nature Structural & Molecular Biology, 15 (10): 1122-1124. DOI: https://doi.org/10.1038/nsmb.1489

Xu X, Diao J, Wang X, Dang Z, Zhang P, Li Y and Zhou Z (2012). Enantioselective metabolism and cytotoxicity of the chiral herbicide ethofumesate in rat and chicken hepatocytes. Pesticide Biochemistry and Physiology, 103 (1): 62-67. DOI: https://doi.org/10.1016/j.pestbp.2012.03.005

Yu X, Liu R, Zhao G, Zheng M, Chen J and Wen J (2014). Folate supplementation modifies ccaat/enhancer-binding protein alpha methylation to mediate differentiation of preadipocytes in chickens. Poultry Science, 93 (10): 2596-2603. DOI: https://doi.org/10.3382/ps.2014-04027

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Performance and Egg Quality of Laying Hens Fed with Boiled Tomato Waste Powder

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ABSTRACT

This study was performed to evaluate the effect of inclusion of boiled tomato waste powder in laying hens diet on growth performances and egg quality. Tomato waste used in this experiment was surplus and unsold mature tomato from the last harvest on the field. The tomato waste was boiled in boiling water (100 ºC) for 8 minutes to convert their lycopene structure from *trans* to *cis*, which is easily absorbed by the poultry digestive tract. In a completely randomized design, 200 Isa Brown laying hens were divided into five groups with different inclusion levels of boiled tomato waste powder (0, 3, 6, 9 and 12%) in an iso-nitrogenous (16%) and iso-caloric (2600 kcal/kg) diet. Daily feed intake, hen day egg production, egg weight, egg mass, feed conversion ratio, eggshell thickness, and eggshell strength were measured. The obtained results showed that boiled tomato waste powder in the diet did not significantly affect daily feed intake, feed conversion ratio, egg production and egg quality. In conclusion, the tomato boiled powder could be used up to 12% in laying hens diet with no effect on performance and egg quality of laying hens.

Key words: Diet, Egg quality, Layer performance, Lycopene, Tomato

INTRODUCTION

Tomato production in Indonesia continues to increase, in 2016 as much as 883,234 tons and in 2017 reached 962,849 tons (Badan Pusat Statistik Indonesia, 2017). Raw tomato contains lycopene as much as 9.25 mg/100g (Perveen et al., 2015). The lycopene compounds can act as antioxidants (Muller et al., 2011) and also reduce cholesterol in blood serum, egg yolk, and broiler meat (Mahata et al., 2016a; and Mahata et al., 2016b). The lycopene inhibits cholesterol synthesis through inhibition of HMG-CoA reductase activity, modulation of low-density lipoprotein receptor activity and inhibition of acyl-CoA:cholesterol acyltransferase (Palozza et al., 2012). In fresh tomato, lycopene is in *trans* form, which is difficult to absorb in the digestive tract of poultry, while *cis*-lycopene can be more easily absorbed (Knockaert et al., 2012; Cooperstone et al., 2015). The heating process of tomatoes by steaming, boiling and roasting increases the availability of lycopene in *cis* form (Sahlin et al., 2016; and Handayani et al., 2018). The boiled tomato waste can be used up to 7% in broiler rations to lower cholesterol in meat and blood serum with no effect on the performance (Mahata et al., 2016a). There are little report on the use of boiled tomato waste in rations of laying hens, therefore, this research was conducted to evaluate the effects of boiled tomato waste on performance and egg quality of laying hens.

MATERIALS AND METHODS

Ethical approval

The present study was approved by the Animal Ethics Committee of the Universitas Andalas, Padang, Indonesia (No: 456/KEP/FK/2019).

Poultry

The experiment was performed by using 200 laying hens strain of ISA Brown at the age of 32 weeks with 80% hen day egg production condition.

Boiled tomato waste powder preparation

Fresh tomato waste was boiled in boiled water at 100 °C for 8 minutes. Then, it was sun-dried and powdered by a grinder.

Experimental design

The experiment was conducted in a completely randomized design with five treatment group, including different inclusion levels (0, 3, 6, 9 and 12%) of boiled tomato waste powder in laying hens diets. Each treatment was replicated four times. Each replicate consisted of five birds. Diets were formulated iso-protein (16%) and iso-energy (2990 kcal/kg diet) and composition of diets are presented in table 1. The experiment lasted for 60 days.

Table 1. Diet composition and chemical analysis of experimental diets

Ingredients (%)	Experimental diets supplemented with different levels of boiled tomato waste powder				
	0%	3%	6%	9%	12%
Concentrate for laying hens	32.50	32.25	32.00	31.75	31.50
Yellow corn	46.00	44.75	43.50	42.25	41.00
Rice bran	14.25	12.50	10.50	8.75	6.75
Palm oil	4.00	4.25	4.75	5.00	5.50
Oyster shell flour	3.00	3.00	3.00	3.00	3.00
Boiled tomato waste powder	0.00	3.00	6.00	9.00	12.00
Premix	0.25	0.25	0.25	0.25	0.25
Total	100.00	100.00	100.00	100.00	100.00
Calculated analysis					
Crude protein (%)	16.05	16.12	16.16	16.23	16.28
Fat (%)	8.28	8.46	8.87	9.05	9.46
Crude fiber (%)	3.89	4.42	4.92	5.46	5.96
Calcium (%)	3.58	3.57	3.56	3.54	3.53
Phosphor available (%)	0.40	0.40	0.40	0.41	0.41
Methionine (%)	0.13	0.13	0.12	0.11	0.11
Lysin (%)	0.16	0.16	0.15	0.14	0.13
Lycopene (ppm)	0.00	1.89	3.77	5.66	7.55
Metabolizable energy (kcal/kg)	2629.53	2617.46	2622.81	2610.73	2616.09

Measured variables

Daily feed intake

Daily feed intake was calculated by the following equation:

$$\text{Daily feed intake} = \frac{\text{The amount of diet given for 1 week} - \text{The amount of diet left over 1 week}}{7 \text{ day}}$$

Hen day egg production

Hen day egg production was measured by method described by Cesari et al. (2014) according to following equation:

$$\text{Hen day egg production} = \frac{\text{The number of eggs produced on the day of measurement}}{\text{The number of laying hens that live on the day measurement}} 100\%$$

Egg weight

Egg weight was estimated by the following formula:

$$\text{Egg weight} = \frac{\text{Egg weight produced by laying hens in each unit}}{\text{The number of eggs produced each unit}}$$

Egg mass

The egg mass was calculated by the method described by Cesari et al. (2014) according to following formula:

$$\text{Egg mass} = \text{Hen day egg production (\%)} \times \text{Egg weight (g)}$$

Feed conversion ratio

Feed conversion ratio was calculated by the method described by Kulshreshtha et al. (2014).

$$\text{Feed conversion ratio} = \frac{\text{Ration consumed (kg)}}{\text{Egg production} \times \text{Egg weight (kg)}}$$

Haugh unit

Haugh unit is an indicator of albumen quality. Eggs were weighed using a digital scale and then broken. The egg shards were placed on a flat glass, then the height of the albumen was measured using a caliper. Haugh unit was calculated according to (Haugh, 1937).

Eggshell thickness

The eggshell thickness was measured by using the method described by Aydin et al. (2008). The eggshell thickness was measured in three parts, namely the equator, the air bag, and the tip of the egg section. Measurement results from the three sections were averaged to get the eggshell thickness. The instrument used was a screw micrometer.

Eggshell strength

Eggshell strength was measured using the Egg Force Reader (SHIMPO FGV-10XY).

Statistical analysis

Data were analyzed by analysis of variance (ANOVA) using a general linear model procedure using SPSS software version 16.0. The difference among treatment means was determined by using Duncan's multiple range test ($p<0.05$).

RESULTS AND DISCUSSION

The inclusion of boiled tomato waste powder in laying hens diet did not significantly affect feed conversion ratio, egg mass, egg weight, hen day egg production, and daily feed intake (Table 2). The boiled tomato waste powder also did not significantly affect Haugh unit, eggshell thickness, and eggshell strength of laying hens (Table 3). The inclusion of boiled tomato waste powder in laying hens diet up to 12% did not affect the daily feed intake due to similar palatability of treatment diets. Similarly, An et al. (2019) demonstrated that inclusion of synthetic lycopene or tomato paste in laying hens diet did not affect daily feed intake. Furthermore, Lee et al. (2016) reported that the inclusion of lycopene on broiler's ration did not affect feed consumption. The average daily feed intake of laying hens in this study ranged from 116.47 to 121.30 g/bird. This finding was approximately similar to results obtained by Panaite et al. (2019) who found daily feed intake of laying hens fed a diet supplemented with flaxseed and dried tomato ranged from 120 to 217 g/bird. All inclusion levels of boiled tomato waste powder used in this experiment did not affect hen day egg production, egg weight, and egg mass. Some previous studies reported the inclusion of 190 g/kg dried tomato pomace, 6% tomato waste meal, 25 g/mg lycopene, and 16% dried tomato pomace had no effects on hen day egg production, egg weight, egg mass, and feed conversion ratio (Salajegheh et al., 2012; Habanabashaka et al., 2014; Jalalinasab et al., 2014).

The inclusion of boiled tomato waste powder in laying hens diet did not affect Haugh unit, eggshell thickness, and eggshell strength of laying hens (Table 3). It has been reported that the inclusion of lycopene in laying hens diet did not have an effect on the Haugh unit (Honda et al., 2019; An et al., 2019). The average Haugh unit of laying hens in this study ranged from 69 to 81. Honda et al. (2019) reported that the Haugh unit of eggs from laying hens fed with lycopene-supplemented diet ranged from 5.80 to 91.90. In this experiment, the inclusion of boiled tomato waste powder up to 12% in diet also did not affect the eggshell thickness and eggshell strength. This finding can be attributed to the similar content of calcium and phosphorus in each treatment diet. According to Ahmed et al. (2013), calcium is one of the nutrients that influence the eggshell quality and production of laying hens. Eggshell strength of laying hens fed 2.62% calcium in the diet was more weakness than that of laying hens fed diet containing 3.70 to 4.4% calcium (Jiang et al., 2013). Some studies reported that the addition of tomato powder, flaxseed and dried tomato flour to laying hens diet did not affect the thickness and strength of eggshell (Akdemir et al., 2012; and Panaite et al., 2019). The average of eggshell thickness in this study ranged from 0.409 to 0.430 mm, and the eggshell strength ranged from 3.43 to 4.62 kg/cm^2. These results were similar to that reported by An et al. (2019) who found eggshell thickness and eggshell strength of eggs from laying hens fed with lycopene or tomato paste were 0.35-0.36 mm and 4.39-4.74 kg/cm^2, respectively.

Table 2. Average of daily feed intake, hen day egg production, egg weight, and egg mass of laying hens fed with different inclusion levels of boiled tomato waste powder

Inclusion levels of boiled tomato waste powder in diet	Daily feed intake (g/bird/day)	Hen day egg production (%)	Egg weight (g)	Egg mass (g)	Feed conversion ratio
0%	116.47	83.34	61.98	51.62	2.29
3%	121.59	89.83	62.18	55.89	2.18
6%	119.41	88.33	62.09	54.89	2.19
9%	118.37	86.92	62.9	54.77	2.18
12%	121.30	89.17	62.65	55.89	2.18

Table 3. Average of Haugh unit, eggshell thickness, and eggshell strength of laying hens fed with different inclusion levels of boiled tomato waste powder

Inclusion levels of boiled tomato waste powder in diet	Haugh unit	Eggshell thickness (mm)	Eggshell strength (kg/cm^2)
0%	81.21	0.425	3.89
3%	77.47	0.409	4.29
6%	69.22	0.429	4.62
9%	79.44	0.430	4.63
12%	70.19	0.421	3.43

DECLARATIONS

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Author's contribution

Maria Endo Mahata created the idea, designed the study and drafted the manuscript. Taufik Hidayat and Gina Amalia Nurhuda collected data and performed statistical analysis. Yose Rizal and Ardi created the idea and designed the study.

Competing interests

The authors declared that they have no competing interests.

REFERENCES

Abudabos AM, Al-Owaimer AN, Hussein EOS and Ali MH (2019). Effect of natural vitamin C on performance and certain haemato-biochemical values in broiler chickens exposed to heat stress. Pakistan Journal of Zoology, 50(3): 951-955. DOI: http://dx.doi.org/10.17582/journal.pjz/2018.50.3.951.955.

Ahmed NM, Atti KAA, Elamin KM, Dafalla KY, Malik HEE and Dousa BM (2013). Effect of dietary calcium sources on laying hens performance and egg quality. Journal of Animal Production Advances, 3(7): 226-231. DOI: https://doi.org/10.3390/ani9030110

Akdemir FC, Orhan N, Sahin, Sahin DK and Hayirli A (2012). Tomato powder in laying hen diets: effects on concentrations of yolk carotenoids and lipid peroxidation. British Poultry Science, 53(5): 675-680. DOI:https://doi.org/10.1080/00071668.2012.729142.

An BK, Choo WD, Kang CW, Lee J and Lee KW (2019). Effects of dietary lycopene or tomato paste on laying performance and serum lipids in laying hens and on malondialdehyde content in egg yolk upon storage. Japan Poultry Science Association, 56: 52-57. DOI: https://doi.org/10.2141/jpsa.0170118.

Aydin R, Karaman M, Cicek T and Yardibi H (2008). Black cumin (*Nigella sativa* L.) supplementation into the diet of the laying hen positively influences egg yield parameters, shell quality, and decrease egg cholesterol. Poultry Science, 87: 2590-2595. DOI: https://doi.org/10.3382/ps.2008-00097.

Badan Pusat Statistik Indonesia (2017). Statistik tanaman buah-buahan dan sayuran semusim Indonesia 2017. No. Publikasi: 05120.1808. Katalog: 5205009. Available at: https://www.bps.go.id/publication/2018/10/05/bbd90b867a6ee372e7f51c43/statistik-tanaman-sayuran-dan-buah-buahan-semusim-indonesia-2017.html.

Cesari V, Mangiagalli MG, Giardini A, Galimberti P, Carteri S, Gallazzi D and Toschi I (2014). Egg quality and productive performance of laying hens fed different levels of skimmed milk powder added to a diet containing *Lactobacillus acidophilus*. Poultry Science, 93(5): 1197-1201. DOI: https://doi.org/10.3382/ps.2013-03518.

Cooperstone JL, Ralston RA, Riedl KM, Haufe TC, Schweiggert RM, King SA, Timmers CD, Francis DM, Lesinski GB, Clinton SK and Schwartz SEJ (2015). Enhanced bioavailability of lycopene when consumed as cis-isomers from tangerine compared to red tomato juice, a randomized, cross-over clinical trial. Molecular Nutrition and Food Research, 59(4): 658-669. DOI: https://doi.org/10.1002/mnfr.201400658

Habanabashaka M, Sengabo M and Oladunjoye IO (2014). Effect of tomato waste meal on lay performance, egg quality, lipid profile and carotene content of eggs in laying hens. Iranian Journal of Applied Animal Science, 4(3): 555-559. DOI: https://doi.org/10.1.1.845.4280.

Handayani UF, Wizna, Suliansyah I, Rizal Y and Mahata ME (2018). Effects of heating method on lycopene, dry matter and nutrient content of tomato (*lycopersicon esculentum*) waste as laying hen feed. International Journal of Poultry Science, 17(2): 63-70. DOI: https://doi.org/10.3923/ijps.2017.63.70

Haugh RR (1937). The Haugh unit for measuring egg quality. United States Egg and Poultry Magazine, 43: 522-555.

Honda M, Ishikawa H and Hayashi Y (2019). Alterations in lycopene concentration and *Z*-isomer content in egg yolk of hens fed all-*E*-isomer-rich and *Z*-isomer-rich lycopene. Animal Science Journal, 90(9): 1261-1269. DOI: https://doi.org/10.1111/asj.13276

Ilahy R, Siddiqui MW, Tlili I, Montefusco A, Piro G, Hdider C and Lenucci MS (2018). When color really matters: horticultural performance and functional quality of high-lycopene tomatoes. Critical Reviews in Plant Sciences, 37(1): 1–39. DOI: https://doi.org/10.1080/07352689.2018.1465631.

Jalalinasab A, Nobakht A and Razzaghzadeh S (2014). The effect of different levels of tomato pomace and processing methods on production performance and blood metabolites of native laying hens. Iranian Journal of Applied Animal Science, (2): 379-385. Available at: http://ijas.iaurasht.ac.ir/article_513701.html.

Jiang S, Cui L, Shi C, Ke X, Luo J and Hou J (2013). Effects of dietary energy and calcium levels on performance, engshell quality and bone metabolism in hens. The Veterinaty Journal, 198: 252-258. DOI: https://doi.org/10.1016/j.tvjl.2013.07.017.

Knockaert G, Pulissery SK, Colle I, Van Buggenhout S, Hendrickx M and Loey AV (2012). Lycopene degradation, isomerization and *in vitro* bioaccessibility in high pressure homogenized tomato puree containing oil: Effect of additional thermal and high pressure processing. Food Chemistry, 135: 1290–1297. DOI:https://doi.org/10.1016/j.foodchem.2012.05.065,

Kulshreshtha G, Stratton G, Rathgeber B, Thomas N and Evan F (2014). Feed supplementation with red seaweeds, *Chondrus crispus* and *Sarcodiotheca gaudichaudii*, affects performance, egg quality, and gut microbiota of layer hens. Poultry Science, 93: 2991–3001. DOI: https://dx.doi.org/ 10.3382/ps.2014-04200.

Lee KW, Choo WD, Kang CW and An BK (2016). Effect of lycopene on the copper-induced oxidation of low-density lipoprotein in broiler chickens. Springer Plus, 5: 389. DOI: https://doi.org/10.1186/s40064-016-2035-6

Mahata ME, Manik J, Taufik M, Rizal Y and Ardi I (2016a). Effect of different combinations of unboiled and boiled tomato waste in diet on performance, internal organ development and serum lipid profile of broiler chicken. International Journal of Poultry Science, 15(7): 283-286.

Mahata ME, Rizal Y, Ardi, Hermansyah D and Nurhuda GA (2016b). Effects of boiled tomato waste utilization in the diet on serum lipid profile and egg quality of laying-hens. International Journal of Poultry Science, 15 (12): 493-496. DOI: https://doi.org/10.3923/ijps.2016.493.496

Marti R, Leiva-Brondo M, Lahoz I, Campillo C, Cebolla-Cornejo J and Rosello S (2018). Polyphenol and L-ascorbic acid content in tomato as influenced by high lycopene genotypes and organic farming at different environments. Food Chemistry, 239: 148–156. DOI: https://doi.org/10.1016/j.foodchem.2017.06.102

Mezbani A, Kavan B P, Kiani A and Masouri B (2019). Effect of dietary lycopene supplementation on growth performance, blood parameters and antioxidant enzymes status in broiler

chickens. Livestock Research for Rural Development, 31: article *#12*. Available at: http://www.lrrd.org/lrrd31/1/bahma31012.html.

Muller L, Goupy P, Frohlich K, Dangles O, Caris, Veyrat C and Bohm V (2011). Comparative study on antioxidant activity of lycopene (Z) isomers in different assays. Journal of Agricultural and Food Chemistry, 59: 4504–4511. DOI: https://doi.org/10.1021/jf010888q.

Palozzza P, Catalona A, Simone RE, Mele MC and Cittadini (2012). Effect of lycopene and tomato products on cholesterol metabolism. Annals of nutrition and metabolism, 61: 126-134.

Panaite TD, Nour V, Vlaicu PA, Ropota M, Corbu AR and Saracila M (2019). Flaxseed and dried tomato waste used together in laying hens diet, Archives of Animal Nutrition, 73 (3): 222-238**.** DOI: https://doi.org/10.1080/1745039X.2019.1586500.

Perveen R, Suleria HAR, Anjum FM, Butt MS, Pasha I and Ahmad S (2015). Tomato *(Solanum lycopersicum)* carotenoids and lycopenes chemistry; metabolism, absorption, nutrition, and allied health claims-a comprehensive review, Critical Reviews in Food Science and Nutrition, 55(7): 919-929. DOI: https://doi.org/10.1080/10408398.2012.657809.

Phillips KM, Council-Troche M, McGinty RC, Rasor AS and Tarrago Trani MT (2016). Stability of vitamin C in fruit and vegetable homogenates stored at different temperatures. Journal of Food Composition and Analysis, 45: 147–162. DOI: https://doi.org/10.1016/j.jfca.2009.08.018.

Sahlin EGP, Savage and Lister CE (2004). Investigation of the antioxidant properties of tomatoes after processing. Journal of Food Composition and Analysis, 17 (2004): 635–647.

Salajegheh MH, Ghazi S, Mahdavi R and Mozafari O (2012). Effects of different levels of dried tomato pomace on performance, egg quality and serum metabolites of laying hens. African Journal of Biotechnology, 11(87): 15373-15379. DOI: https://doi.org/10.5897/AJB12.1031.

Sharma SK, and Le Maguer M (1996). Lycopene in tomatoes and tomato pulp fractions. Italian Journal of Food Science, 8 (2): 107-113.

Torki M, Zangeneh S and Habibian M (2014). Performance, egg quality traits, and serum metabolite concentration of laying hens affected by dietary suplemental chromium picolinate and vitamin C under a heat-stress. Biological Trace Element Research, 157: 120-129. DOI: https://doi.org/10.1007/s12011-013-9872-8.

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Experimental Infection of Local Domestic and Feral (*Columba livia domestica*) Pigeons with Local Isolate of H9N2 Influenza Virus: Virological and Histopathological Study

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ABSTRACT

A local isolate of low pathogenic Avian Influenza Virus (AIV) H9N2 subtype was used in experimental infection of 50 domestic and 50 feral pigeons (*Columba livia domestica*) to determine the susceptibility of these birds to H9N2 infections and to study its histopathological effects on vaccinated and unvaccinated pigeons with H9N2 commercial vaccine. The birds were divided into five groups. Groups A and C contained 20 feral pigeons, B and D contained 20 domesticated pigeons. Group E contained 10 feral and 10 domesticated pigeons that were used as unvaccinated controls. Groups A and B were vaccinated with H9N2 and Newcastle disease virus commercial vaccines. Group C and D were vaccinated with Newcastle disease virus vaccine only. All groups except E were challenged with a local isolate of H9N2 serotype. Antibodies titers against AIV were estimated pre and post-vaccination using ELISA. The results indicated low antibody titers against AIV in all groups in pre-vaccination that ranged between 152.83 ± 42.01 and 337.00 ± 150.76 with no significant differences between groups. Post-vaccination antibody evaluation indicated high titers of anti-AIV antibodies in groups A and B (740.13 ± 214.38 and 673.00 ± 242.40, respectively) in comparison to pre-vaccination levels. Clinical signs appeared on 5th day post-vaccination that included mild respiratory signs, digestive disorders, and conjunctivitis in some birds of all groups. Histopathological changes in affected tissues appeared as moderate to severe multifocal necrosis diffused in the parenchymal cells of lung tissues. Infiltration with mononuclear inflammatory cells was detected in some lung tissue areas. Necrotic foci and mononuclear cell infiltration were also observed in trachea and liver of infected pigeons but mild changes were observed in intestine. The challenge virus was re-isolated in embryonated hen's eggs of nine days old by inoculation in allantoic cavity using samples collected from tissues and cloaca of infected pigeons showing clear clinical signs. The re-isolated virus was detected by the haemagglutination test using chicken RBCs and identified by haemagglutination inhibition test using a locally prepared hyperimmune serum to H9N2 in rabbits. In conclusion, pigeons are susceptible to AIV (H9N2) that might facilitate the transmission of the virus to other bird species.

Key words: Avian Influenza viruses, H9N2, Pigeons

INTRODUCTION

Avian Influenza Viruses (AIVs) spread widely in birds worldwide; they are contagious but variable in their virulence. Accordingly, they were subdivided into Low Pathogenic AIV (LPAIV) and High Pathogenic AIV (HPAIV) (Imai et al., 2013). The domesticated birds and mammalian are susceptible to AIV and aquatic birds are natural virus reservoirs. AIV infections might cause severe disease in poultry with a mortality rate of more than 90%, mostly associated with HPAIV strains (MacLachlan and Dubovi, 2011).

Influenza A viruses based on genetic and antigenic differences in hemagglutinin (HA) and neuraminidase (NA) are divided into 17 HA (H1 to H17) and 11 NA (N1 to N11) subtypes (Shehata et al., 2015; Mostafa et al., 2018). All these subtypes are grouped in genus *Influenzavirus A* which is classified within the family *Orthopoxviridae*. Genus *Influenzavirus A* included viruses with linear single-stranded, negative-sense and segmented (eight segments) RNA genome (MacLachlan and Dubovi 2011). Antigenic shift and antigenic drift are the most common processes that continuously change influenza viruses and lead to the emergence of new influenza virus variants or strains (Lee et al., 2016; Kandeil et al., 2017; Arai et al., 2019).

Influenza virus subtype H9N2 is a LPAI virus and the most widespread avian influenza subtype in poultry

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worldwide (Abdelwhab and Abdel-Moneim, 2015; Nagy et al., 2017). In Iraq, H9N2 is endemic since 2004 (Kraidi et al., 2016; Kraidi et al., 2017; Mohamed et al., 2018). This virus has also been reported to cause high mortality rates in broilers (70%) as well as in breeders and layers up to 10% (Khamas, 2008).

Feral and domestic pigeons are found worldwide and can easily cross borders like the wild aquatic birds, and sometimes live close to these aquatic birds. Many reports have mentioned that wild aquatic and domestic birds are reservoirs for influenza A viruses (Abdelwhab and Abdel-Moneim, 2015; Nagy et al., 2017; Kausar et al., 2018). The possibility of transmission of such viruses to pigeons is acceptable as many studies have reported the natural infection of pigeons with the H9N2 influenza virus (Gomaa et al., 2015; Xu et al., 2015; Kandeil et al., 2017; Kausar et al., 2018; Tolba et al., 2018). No data on the isolation of H9N2 from pigeons or experimental infection of pigeons with H9N2 is available in Iraq. Accordingly, the present study aimed to determine the susceptibility of wild and domestic pigeons to the experimental infection with a local isolate of H9N2 LPAIV and to study the efficacy of H9N2 commercial vaccine in pigeons.

MATERIALS AND METHODS

Ethical approval

Scientific Ethical Committee in the University of Diyala/ Iraq, approved the research and give the ethical number (Vet 14 Medicine November 2018 A and K).

Study design

This cross-sectional study was conducted in Diyala province, Iraq, over the period from September 2018 to June 2019. In this study, 100 local domestic and feral pigeons *(Columba livia domestica*) were used and divided into five groups (A, B, C, D and E) as presented in table 1. All groups were isolated from each other in separated and completely closed animal houses to avoid any contact between them by any means of sharing feed, water, utensil, workers and environment.

Table 1. Pigeon groups used in the present study

Group	Type of birds of birds	Number	Treatment
A	Wild pigeons	20	Vaccinated with H9N2 and NDV*
B	Domestic pigeons	20	Vaccinated with H9N2 and NDV*
C	Wild pigeons	20	Vaccinated with NDV**
D	Domestic pigeons	20	Vaccinated with NDV**
E	Wild and Domestic pigeons	20 (10 for each type)	Control unvaccinated group

NDV: Newcastle disease virus, *H9N2-NDV commercial vaccine (Nobilis ®, MSD Company, Netherlands), **NDV (MSD, LaSota, Netherlands)

Detection of pre-vaccination antibodies against avian influenza virus

Avian influenza virus antibodies were measured in all groups by using ELISA kit (Zoetis proFLOK™, avian influenza virus antibody test kit, item MI 49007, USA). Accordingly, blood samples were collected from wing veins of pigeons, then the sera were separated, labeled and subjected to the ELISA test according to the manufacturer's instruction.

Vaccination of pigeons

Groups A and B were vaccinated with inactivated Newcastle Disease Virus (NDV) and influenza H9N2 inactivated vaccine (Nobilis®, MSD Company, Netherlands) by subcutaneous route (The birds are of different ages). Group C and D were vaccinated with the NDV vaccine (MSD, LaSota, Netherlands) by drinking water. This was done by fasting of pigeons from water for 24 hours and then they were supplemented with distilled water containing the LaSota NDV vaccine.

Detection of post-vaccination antibodies against avian influenza virus

Blood samples were collected after 7 days post-vaccination from vaccinated groups with influenza H9N2 vaccine to determine anti-AIV antibodies. For this purpose, ELISA was performed using the AIV ELISA kit (Zoetis proFLOK™, avian influenza virus antibodies kit-item MI 49007, USA) according to the instruction manual of the manufacturer.

Challenge virus

Influenza virus (H9N2) registered in National Center for Biotechnology Information NCBI with accession number (MH368755.1) was kindly provided by Mohammed Abdulkadhim Hussein and Prof. Dr. Emad J.

Khammas from College of Veterinary Medicine, University of Baghdad.

The stock virus (0.1 ml) was inoculated into the allantoic cavity of nine-day-old embryonated hen's eggs. The inoculation site was sealed with wax and inoculated eggs were incubated at 37 °C and observed daily for the death of the embryo. Eggs with dead embryos were removed from the incubator, chilled in a refrigerator at 4°C for a few hours and opened to collect the allantoic fluid. The collected fluid was tested for the presence of H9N2 by slide haemagglutination test using 4% avian red blood cells in sterile normal saline (Webster et al., 2002). H9N2 positive fluid from embryonated eggs was pooled together, labeled and kept frozen at -30 °C until be used.

Titration of stock virus

Propagated H9N2 was titrated in two ways, using HA and 50% Embryo Infectious Dose (EID_{50}). Haemagglutination test was performed by 2-folds serial dilution of the stock virus in sterile Phosphate Buffered Saline (PBS) using 96 well plastic plates and according to the method described by Killian (2008). The viral titers were also determined by EID_{50} using 10-folds serial dilutions of the H9N2 stock virus in sterile PBS. Five, nine-day-old embryonated hen's eggs were used for the inoculation of each viral dilution according to the method described by Reed and Muench (1938).

Preparation of hyperimmune serum against influenza virus A (H9N2)

Hyperimmune serum against subtype H9N2 was prepared in rabbits according to the method described by Horwitz and Scharff (1969). For this purpose, three rabbits were raised for hyperimmune serum preparation and two rabbits were used as control. Blood samples were collected from all rabbits before vaccination, then the serum was separated from each sample in sterile test tubes and frozen at - 30°C until use. Each rabbit was intramuscularly inoculated with 1 ml of influenza H9N2 vaccine (Nobilis®, MSD Company, Netherlands) virus. The control rabbits were inoculated with 1 ml of sterile normal saline. This inoculation was repeated weekly for successive three weeks. Blood samples were collected from inoculated rabbits one week after the last inoculation. After coagulation, the serum was separated by cold centrifugation at 3000 rpm for 30 minutes at 4 °C. Clear serum was pooled together and titrated by Haemagglutination Inhibition (HI) test according to the method described by Williams (1980) and kept at -30°C until use.

Challenge of pigeons

Each pigeon was challenged via a dropping of 0.5 ml ($10^{9.5}$ EID_{50}/ 0.1 ml) of the virus into the nose, trachea, and eyes. All the infected pigeons were observed daily for 15 days.

Detection of post-challenge antibodies

Antibodies against subtype H9N2 were determined on 5th day Post-Infection (PI) using the HI test and ELISA. Accordingly, blood samples were collected from infected birds, then sera were separated and subjected to both HI test (using the plate method and locally prepared H9N2 hyperimmune serum) and ELISA (using the same abovementioned ELISA kit).

Collection and processing of samples from infected pigeons

Cloacal swab samples and tissue samples (trachea, lung, liver, and intestine) were collected from experimentally infected pigeons at days 5, 6, 7, 8, 9 and 10 PI. Each swab sample was placed in a sterile tube containing PBS and centrifuged at 3000 rpm for 20 minutes in a cool centrifuge. Then the supernatant was collected and 0.5 ml of antibiotic- antimycotic and anti-mycoplasma were added to the sample to eliminate other infectious pathogens. After 30 minutes of incubation at room temperature, the sample was kept frozen at -30 °C until use. Tissue samples were divided into two groups. The first group was tested for virus detection by re-isolation in embryonated hen's eggs and identification by the HI test. The second group of samples was subjected to histopathological study according to methods described by Durrani et al. (2008) and Bancroft and Gamble (2008). Sections were examined by light microscopy (Olympic-Japan) and photomicrographs were taken with a digital camera (Omax, USA) for each section.

Re-isolation of H9N2

The influenza virus (H9N2) used for pigeons inoculation was re-isolated by the processing of collected tissue and the cloacal swab samples. A 10% suspension of minced tissue samples collected from challenged pigeons was made in sterile PBS, then 0.1 ml of the suspension was inoculated into the allantoic cavity of three embryonated eggs (9-11 days old), incubated at 37 °C and processed 3-4 days PI. The processed and collected allantoic fluid of these eggs was pooled and tested for the presence of the virus using slide haemagglutination test and then identified by HI test using the prepared H9N2 hyperimmune serum.

Statistical analysis

The data were analyzed using SPSS version 22. A p-value less than 0.05 was considered significant.

RESULTS

Pre-vaccination antibodies

Pre-vaccination antibodies titers against AIV are presented in table 2. Some birds, both feral and domestic pigeons, showed high titers exceeding 600 or 700 units, whereas, in many of birds no antibodies to AIV were found.

Post-vaccination antibodies

Antibodies titers against influenza virus (H9N2) after the vaccination of groups A and B are presented in table 3.

Titers of propagated stock influenza virus subtype H9N2

The titer of propagated stock virus subtype H9N2 measured by the haemagglutination test was 1024 HAU/0.1 ml. in addition, the propagated virus titer determined by 50% embryo infectious dose was $10^{10.5}$ EID_{50} / 0.1 ml. The virus was re-identified by the use of hyperimmune serum in the HI test and showed an anti-HA antibodies titer of 2048 HIU/0.1 ml of stock serum.

Post-challenge antibody immune response

Antibody titers against AIV in four challenged groups are shown in tables 4 and 5. There was no significant difference in AIV antibody titer between group A and group B, as well as between group C and group D. While significant differences ($P<0.05$) in antibody titers were observed in each of A and B groups when compared to each of C and D groups. The high titers were found in H9N2 vaccinated and challenged groups.

Table 2. Titers of avian influenza virus antibodies detected by ELISA in the pre-vaccination period

Group of pigeon*	Anti AIV antibodies (Mean ± SE)	GMT	%CV
A (wild)	274.56 ± 88.05[a]	31	23.38
B (domestic)	278.40 ± 120.45[a]	14	91.50
C (wild)	225.60 ± 114.25[a]	9	88..84
D (domestic)	152.83 ± 42.01[a]	13	89.25
E (mixed control)	337.00 ± 150.76[a]	7	134.76

***** The pigeons were of different ages. GMT: geometric mean; CV: coefficient of variation; AIV: avian influenza virus; SE: standard error. [a] No significant differences ($p > 0.05$).

Table 3. Titers of avian influenza virus antibodies detected by ELISA on 7th day post-vaccination

Groups*	Number of samples	Mean ± SE	GMT	CV (%)
A (wild pigeon)	15	740.13± 214.38[a]	65	86.55
B (domestic pigeon)	15	673.00± 242.40[a]	40	99.02

[*] The pigeons were of different ages. GMT: geometric mean; CV: coefficient of variation; SE: Standard error. [a] No significant difference ($p >0.05$).

Table 4. Detection of avian influenza virus antibodies by haemagglutination inhibition test in challenged groups of pigeons with avian influenza virus subtype H9N2 at 5 days post-challenge

	Groups			
	A	B	C	D
Mean HI titre*	256	128	64	64

*Titers were calculated in HI units (HIU)/100µl of serum sample. HI: haemagglutination inhibition

Table 5. Detection of avian influenza virus antibodies by ELISA in challenged groups of pigeons with avian influenza virus subtype H9N2 at 5 days post-challenge

Groups	N	Anti AIV antibodies (Mean± SE)	GMT	%CV
A	15	1931.00± 453.24 [a]	623	63.36
B	18	1845.61± 343.94 [a]	756	56.62
C	18	888.11± 163.57 [b]	219	51.12
D	15	955.66± 218.86 [b]	173	63.69

Different superscript letters indicate significant differences ($p<0.05$) between groups. SE: Standard error

Clinical signs

Clinical signs appeared in pigeons after 5 days PI and no mortality rates were recorded. The clinical signs disappeared at 8 and 10 days PI. Sporadic cases with mild depression were found in groups A and B, also there were two cases with bilateral conjunctivitis in each group (Figures 1A and B). Some birds showed signs of mild respiratory disorders such as nasal discharge and sneezing. Moderate clinical signs of respiratory disorders were observed in many pigeons of group C and D. One pigeon in group D showed subcutaneous hemorrhage in non-feathered skin of legs (Figure 1C). The clinical signs in groups A, B, and D disappeared on day 8 PI, whereas the clinical signs in group C disappeared on day 9 PI.

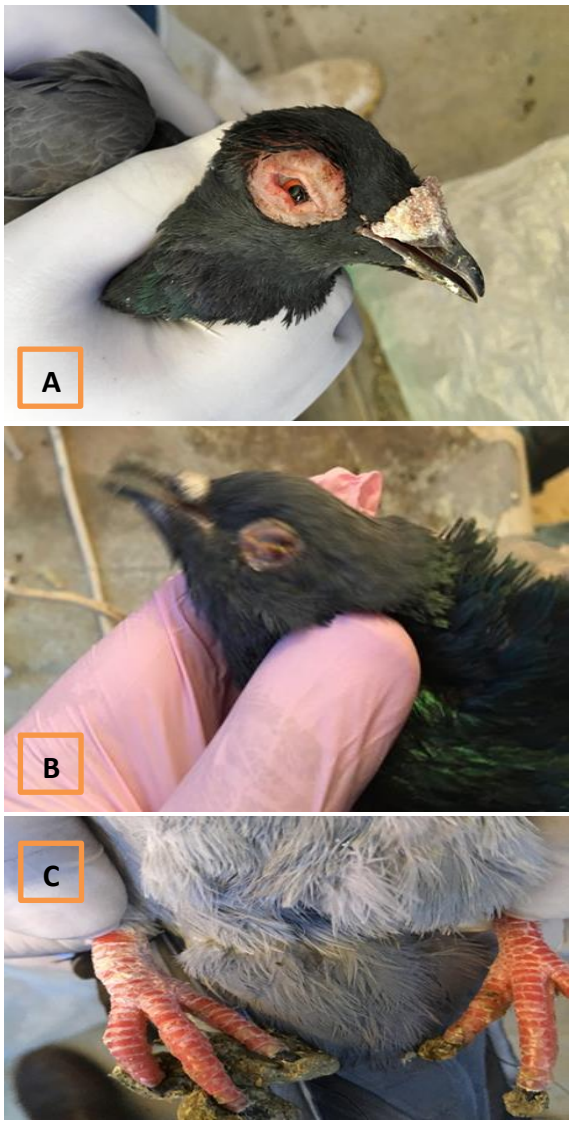

Figure 1. Conjunctivitis in feral (A) and domestic pigeons (B). Subcutaneous hemorrhagic of legs in domestic pigeons challenged with influenza virus subtype H9N2 (C).

Histopathological findings

Pathological changes in trachea and lung

The results of the histopathological examination on pigeons inoculated orally or intranasally with AIV indicated obvious pathological lesions accompanied by moderate to severe inflammation. These results revealed that there were clear pathological changes in lung some histological changes observed had parenchyma, ranging from moderate to severe multifocal necrosis diffused in the lung parenchyma. Also, infiltration with mononuclear inflammatory cells was detected in some lung areas associated with mild to severe inflammation (Figure 2). Additionally, some lung tissue exhibited clear edema and hemorrhage associated with severe congestion, degeneration and necrosis in the lung parenchyma (Figure 2). Furthermore, severe inflammatory cell infiltration was observed in the edges of lung parenchyma with clear degeneration and necrosis. Histological examination also showed alveolar damage with secretions containing blood, cell debris, and inflammatory cells.

There was obvious multifocal damage and desquamation of the pseudostratified columnar epithelium of the trachea of infected birds with AIV. Also, degeneration of mucosal gland tissue extended to the sub-mucosa was observed. Furthermore, clear desquamation of epithelial cells into the luminal space was observed and associated with hemorrhage. The histological changes in parenchymal tissue of lung showed obvious damage to vascular endothelial cells and micro-thrombosis.

In addition to the abovementioned findings, damage and severe inflammation of lung parenchyma and bronchi accompanied by edema and hemorrhage were observed in group B (Figure 3). There was an obvious degeneration and desquamation of the pseudostratified columnar epithelium of trachea as well as clear degeneration of mucous glands. Also, some areas of the tracheal ring showed severe damage and hypertrophy in chondrocytes.

Pathological changes of liver and intestine

The pathological changes in liver (Figure 4) and intestines (Figure 5) revealed a mild to moderate inflammation and local necrosis close to the hepatic veins, whereas in intestine, these changes were observed in the submucosa.

Re-isolation and identification of the challenge virus

The influenza virus (H9N2) was re-isolated from all tissue samples and cloaca swabs collected from pigeons with clinical signs in different groups. The re-isolated virus was detected by HA and identified by HI.

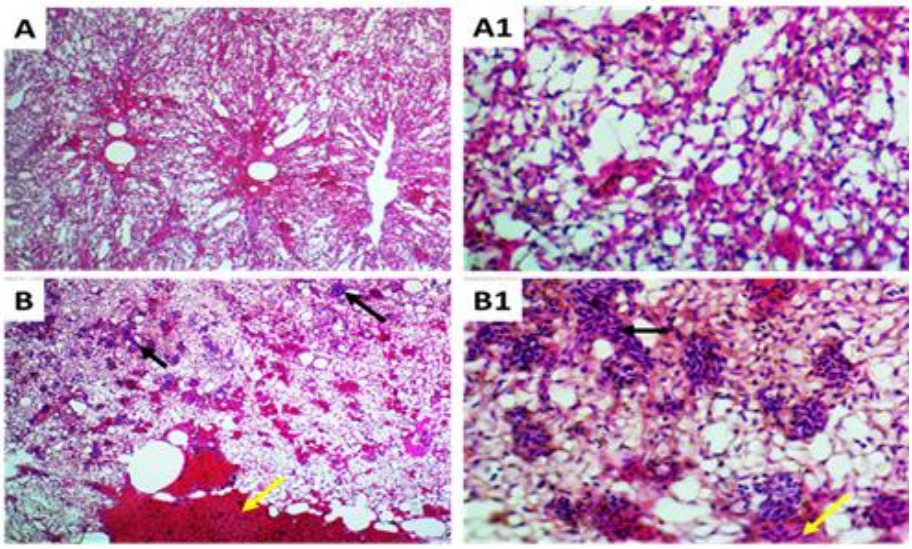

Figure 2. Photomicrographs of lung tissues of pigeons infected with influenza virus (H9N2). **A and A1:** The normal lung tissue. **B** and **B1:** The local infiltration of inflammatory cells represented by black arrows, whereas yellow arrows indicated the congestion and hemorrhage in alveoli (A and B at 10X; A1 and B1 at 40X) (H&E).

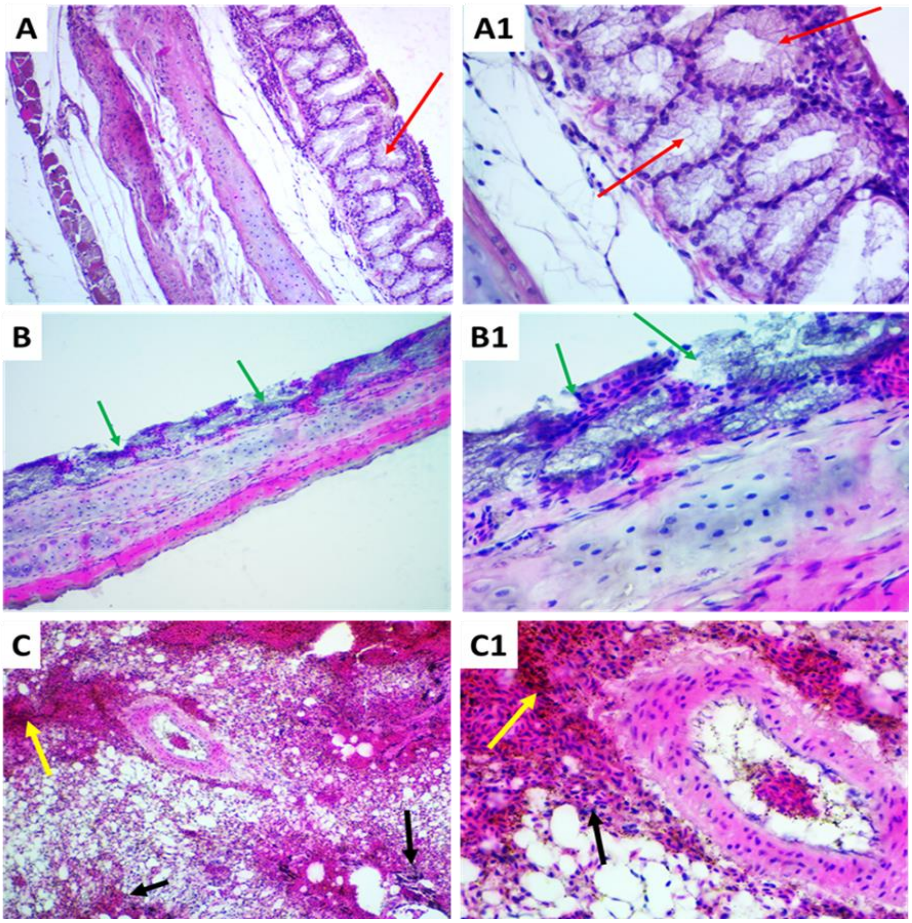

Figure 3. Photomicrographs of trachea and lung of pigeons infected with avian influenza virus (H9N2). A and A1: Normal trachea, simple alveolar mucous glands (red arrows). B and B1: Clear shrinkage and degeneration in the epithelium and mucous gland in the trachea (green arrows). C and C1: The pathological changes exhibited in bronchi and lung parenchyma accompanied by severe infiltration of inflammatory cells (black arrows) and severe hemorrhage and edema (yellow arrows). (A, B, C at 10X; A1, B1, C1 at 40X) (H&E).

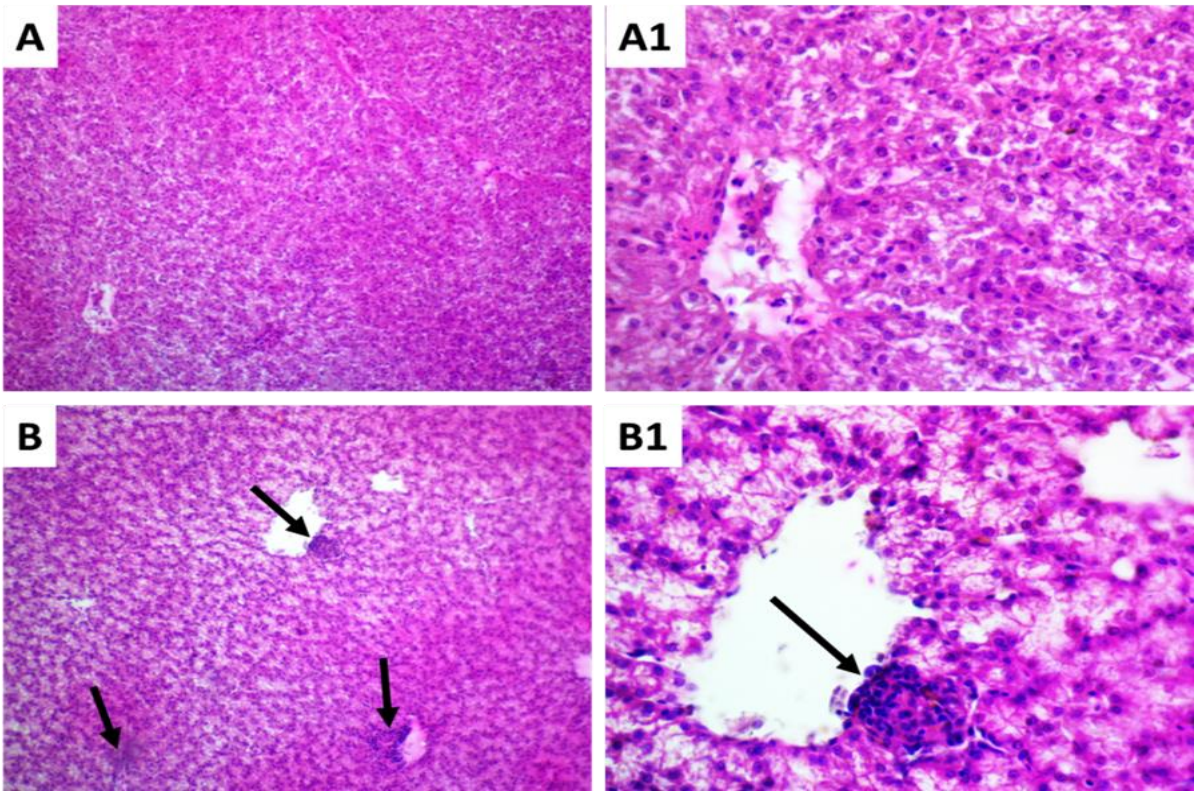

Figure 4. Photomicrographs of liver of pigeons infected with avian influenza virus (H9N2)**. A and A1:** Normal liver. **B and B1:** Focal necrosis and infiltration of the inflammatory cells (black arrows) (A and B at 10X; A1 and B1 at 40X) (H&E).

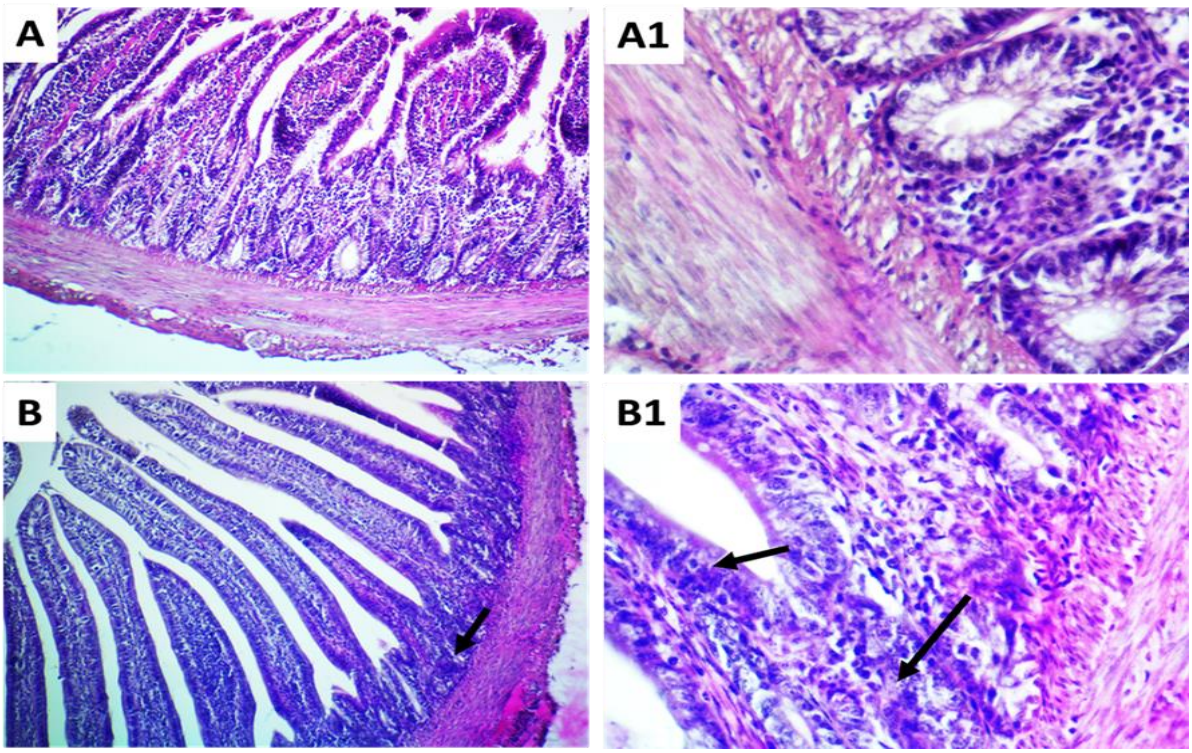

Figure 5. Photomicrographs of the intestine of pigeons infected with the avian influenza virus (H9N2). A and A1: Normal intestine. B and B1: Infiltration of the inflammatory cells (black arrows) (A and B at 10X, A1 and B1 at 40X) (H&E).

DISCUSSION

Influenza virus subtype H9N2 affects a broad spectrum of species including birds and mammals (Nagy et al., 2017). In poultry, it was reported to cause mild respiratory and digestive infection, but it may cause asymptomatic infections in other birds (Kandeil et al., 2017). Recent studies showed that co-circulation of other avian viruses like H5N1 and H9N2 in poultry farming and live bird markets had increased the risk of human exposure, resulting in complications of the epidemiological situation and raising a concern for potential emergence of new influenza A virus pandemic due to antigenic shift and drift resulted from gene exchange of the fragmented genome in case of mixed infection of the host with two subtypes of influenza virus type A (Kim, 2018). In Iraq, H9N2 is circulating among poultry farms (Khamas, 2008; Abdul-Sada, 2015; Kraidi et al., 2017; Hussein, 2019) and was serologically detected in pigeons (AL-Attar et al., 2008). The titer of hyperimmune serum in this test appeared as 2^{16} HIU / 0.1 ml of stock serum. The above-mentioned findings are mentioned by other researchers (Xu et al., 2018).

Clinical signs

The current study did not show any mortality rate among experimentally infected pigeons but only mild and moderate clinical signs. Abolnik (2014) mentioned that a very small number of pigeons infected with either HPAIV or LPAIV died in 22 different studies worldwide since 1944. Viruses such as H9N2 were proved to cause considerable morbidity but low or no mortality (Abolink, 2014; Hussein, 2019). Some other studies mentioned that ducks, gulls, starlings, and pigeons were less susceptible to AIV and displayed few or no clinical signs (MacLachlan and Dubovi, 2011). Generally, clinical signs appeared less in groups A and B compared to C and D. This may be attributed to vaccination of groups A and B with the H9N2 vaccine. It is well known that vaccination can reduce the severity of infection but cannot prevent it (Ebrahimi et al., 2011; Bahari et al., 2015). A few cases of conjunctivitis were recorded in both domesticated and wild pigeons. This result agreed with the findings obtained by Kaleta and Honicke (2004) who reported one case of conjunctivitis out of 11 experimentally infected pigeons with the influenza virus. The clinical signs in infected pigeons of the present study were similar to those appeared in experimentally infected broiler chickens with H9N2 isolated from an outbreak in Iran. Field and experimentally infected birds showed similar clinical signs including sneezing, coughing and depression (Nili and Asasi, 2003). Similar findings were reported by another study in China (Sun and Liu, 2015). Experimental infection in broiler chickens using H9N2 and LaSota NDV vaccine revealed the same abovementioned clinical signs (Ellakany et al., 2018). Signs related to enteric infection with H9N2 such as greenish diarrhea and respiratory signs were also reported by (Tolba et al., 2017) in pigeons of Egyptian commercial farms and live birds market during winter of 2015-2016. The same authors mentioned the infection of humans with both H5N1 and H9N2. In another study in Pakistan, sneezing, nasal discharge, and other respiratory signs were reported in different species of birds including sparrows, chickens, jungle fowl, and quails infected directly or by contact with the virus. These clinical signs were observed 2 to 5 days PI (Iqbal et al., 2013).

Antibody immune response

All pigeons in the present study were subjected to antibody screening against AIV before starting vaccination and challenge with the virus. The pre-vaccination anti-AIV antibody ranged from 152.83 ± 42.01 to 337.00 ± 150.76; indicating the exposure of such birds to AIV somewhere or somehow. The study conducted by Turner et al. (2017) on the live bird markets in Bangladesh showed that H9N2 viruses were detected at high frequencies (76–100%) in chickens, pigeons, and quail, while 9% of samples collected from ducks were positive for H9N2.

The presence of negative serum samples to AIV in pigeons might indicate the inexposure of such birds to AIV. The possibility of the presence of unexposed pigeons to AIV was reported by many studies. Mohammadi et al. (2010) found that 34% of serum samples collected from pigeons were positive for H9N2 antibodies. Tolba et al. (2018) detected antibodies against H9N2 in 6.5% of serum samples collected from pigeons. Serum samples collected from pigeons in Northern Saudi Arabia were negative for antibodies against H3, H5, and H9 serotypes of AIV (Alkhalaf, 2010). In a study conducted in Mosul province of Iraq, ELISA and HI tests were used for the detection of AIV antibodies in pigeons and starlings (AL-Attar et al., 2008). The results showed that 81.8% of pigeons were positive to AIV H9N2 antibodies when their sera were tested by ELISA, whereas, 50% of the same serum samples were positive when tested by the HI test. In addition, the serum samples collected from starlings were negative for AIV H9N2 antibodies and the birds did not show any clinical signs of influenza. The same authors concluded that pigeons can be infected with AIV subtype

H9N2 and they may play an important role in AIV spreading as natural carriers.

Both groups vaccinated with inactivated H9N2 (A and B) showed a significant increase ($P<0.05$) in AIV antibodies compared to antibody levels in the same groups before vaccination. Post-challenge AIV antibody levels showed a significant increase compared to post-vaccination AIV antibody levels. Increased antibody levels in vaccinated groups (A and B) with inactivated H9N2 significantly differed from the antibody level of challenged but un-vaccinated groups (C and D). The increase in antibody levels of C and D groups can be attributed to the effects of challenge virus and might be also attributed to pre-exposure of birds to circulating H9N2 before challenge (Quinn et al., 2011).

The present study demonstrated that pigeon vaccination with H9N2 inactivated vaccine did not prevent the infection with the local isolate of H9N2. All challenged groups showed clinical signs of influenza virus infection, and the virus was detected by conventional RT-PCR and by real-time RT-PCR (unpublished data). Furthermore, the virus was re-isolated from samples collected from challenged pigeons and identified by the HI test. Generally, the challenge virus was detected for longer periods of time in samples collected from unvaccinated groups compared to vaccinated groups and this might be attributed to immune responses and high antibody titers induced in vaccinated groups after challenge with live H9N2 virus that lead to the earlier clearance of the virus from infected birds (Quinn et al., 2011). Similar findings were reported in Iran when the inactivated H9N2 vaccine did not completely prevent the experimental infection with a field isolate of AIV subtype H9N2 in quails (Ebrahimi et al., 2011).

Several commercial AIV vaccines based on strains isolated during the late 20th century were widely used in domestic poultry (Sun et al., 2012). However, some studies showed that H9N2 viruses were isolated from vaccinated chicken flocks, hence some vaccines did not provide complete protection against viral infection (Bahari et al., 2015; Shen et al., 2015).

Partial protection against circulating H9N2 induced by local H9N2 inactivated vaccine was also reported in Korea (Lee et al., 2011). Accordingly, the selection of suitable local isolate and strain for vaccine production against H9N2 LPAI is recommended (Sun et al., 2012). Mutations and inter and intra-reassortment are considered as factors affecting the ability of a vaccine to protect against AIV H9N2. These factors might be associated with the emergence of new virus strains with new biological features (Ashraf et al., 2017).

Histopathology

The pathogenicity of AIVs in pigeons and the vulnerability of pigeons for various subtypes of avian influenza have investigated in some studies (Yamamoto et al 2011; Liu et al., 2015). Furthermore, the ability of pigeons in being carrier and reservoir for these viruses has been studied (Liu et al., 2007). However, there is little literature on the pathophysiological effects of AIV H9N2 on pigeons. In the current study, H9N2 subtype was found to cause severe tracheitis and pneumonia as well as mild pathological changes in liver and intestine of infected pigeons. There are similarities between these results and the histopathological observations reported in chickens infected with AIV H9N2 (Hassan et al., 2017; Arafat et al., 2018).

The presence of such pathological changes as well as direct pathogenicity in the trachea and lung indicate that H9N2 has tissue tropism for these organs (Halblolvarid et al., 2004). It can also be assumed that the presence of multifocal necrosis in the liver indicates a potential systemic viral infection with the H9N2 (Bano et al., 2003). In this study, the most important pathological changes were widespread hemorrhages with the massive edema and congestion in the lung parenchyma. Also, there was prominent degeneration in the alveolar epithelium. The histopathologic findings obtained in this study are in line with the observations of other studies on Japanese quail (Ebrahimi et al., 2010; Mehrabadi et al., 2018) and in Muscovy duck (Wang et al., 2019) infected with AIV H9N2.

The severe pathological changes observed in the trachea and lungs could be attributed to the presence of SAa2, 6 Gal receptors in the epithelial surfaces of the pharynx, trachea and bronchial tree of pigeons, which is the same receptors found for human influenza viruses (Liu et al., 2009). Although some studies reported that pigeons are less or not susceptible to AIV such as the H5N1serotypes (Liu et al., 2007), the present study demonstrated the pathophysiological aspects of H9N2 in pigeons.

CONCLUSION

The influenza virus (H9N2) can cause mild infection in wild and domestic pigeons that might facilitate the transmission of H9N2 to other birds. The mixed infection of pigeons with different subtypes of the influenza virus

may increase the risk of generation of new virulent subtypes. Further research should be conducted to monitor the virus and to investigate the interactions between the H9N2 and other serotypes in pigeons. Furthermore, commercial vaccines, regardless of the level of the antibody obtained, did not prevent the infection with serotype H9N2 but reduce its virulence.

DECLARATIONS

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Authors' contributions

Ahmed Raad Rasheed designed the study and collected the samples from the infected Pigeons. Karim Sadun Al-Ajeeli and Amer Khazaal Al-Azawy were involved in editing the manuscript and analyzing the data. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

REFRENCES

Abdelwhab EM and Abdel-Moneim AS (2015). Epidemiology, ecology and gene pool of influenza A virus in Egypt: will Egypt be the epi-centre of the next influenza pandemic. Virulence, 6: 6-18. DOI: https://doi.org/10.4161/21505594.2014.992662 .

Abdul-Sada KM (2015). Surveillance of influenza A/ H5, H7, H9 viral subtypes in domestic and wild birds at many geographical regions of Iraq. International Journal of Advanced Research, 3: 170–176. Available at: http://www.journalijar.com

Abolink C (2014). A current review of avian influenza in pigeons and doves (Columbidae). Veterinary Microbiology, 170: 181-196. DOI: https://doi.org/10.1016/j.vetmic.2014.02.042 .

AL-Attar MY, Danial FA and Al-Baroodi SY (2008). Detection of antibodies against avian influenza virus in wild pigeons and starlings. Journal of Animal and Veterinary Advances, 7(4): 448-449. Available at: http://medwelljournals.com/abstract/?doi=javaa.2008.448.449 .

Alkhalaf AN (2010). Field investigation on the prevalence of avian influenza virus infection in some localities in Saudi Arabia. Pakistan Veterinary Journal, 30: 139–142.

Arafat N, Eladi AH, Marghani BH, Saif MA and El-Shafei RA (2018). Enhanced infection of avian influenza virus H9N2 with infectious laryngotracheitis vaccination in chickens. Veterinary microbiology, 219, 8-16. DOI: https://doi.org/10.1016/j.vetmic.2018.04.009 .

Arai Y, Ibrahim MS, Elgendy EM, and Daidoji T (2019). Genetic compatibility of reassortment between avian H5N1 and H9N2 influenza viruses with higher pathogenicity in mammals. Journal of Virology, 93(4):1-17.DOI: https://doi.org/10.1128/jvi.01969-18.

Ashraf MU, Mahmood MS, Rafique A, Abbas RZ, Iqbal Z, Younas M and Ishaq MU (2017). Factors responsible for the continuous persistence and evolution of low pathogenic avian influenza virus (H9N2). World's Poultry Science Journal, 73(4): 791-802. DOI: https://doi.org/10.1017/s004393391700071x .

Bahari P, Pourbakhsh SA, Shoushtari H, and Bahmaninejad MA (2015). Molecular characterization of H9N2 avian influenza viruses isolated from vaccinated broiler chickens in northeast Iran. Tropical Animal Health and Production. 47:1195–1201.DOI: https://doi.org/10.1007/s11250-015-0848-x .

Bancroft JD and Gamble M (2008). Theory and practice of histological techniques, Elsevier health sciences. 6th Edition, Churchill Livingstone, Elsevier, China.

Bano S, Naeem K and Malik SA (2003). – Evaluation of pathogenic potential of avian influenza virus serotype H9N2 in chickens. Avian Diseases, 47: 817-822.DOI: https://doi.org/10.1637/0005-2086-47.s3.817 .

Durrani UF, Khan M and Ahmad SS (2008). Comparative efficacy (sedative and anaesthetic) of detomidine, ketamine and detomidine-ketamine cocktail in pigeons (Columba livia). Pakistan Veterinary Journal, 28 (3): 115-118.

Ebrahimi SM, Nili H and Sohrabi N (2010). Histopathological evaluation of A/Chicken/iran/339/02 (H9N2), an Iranian field isolate of influenza virus, on Japanese quail (*Coturnix coturnix japonica*). World Applied Science Journal, 9: 226-229.

Ebrahimi SM, Ziapour S, Tebianian M, Dabaghian M and Mohammadi M (2011). Study of infection with an Iranian field-isolated H9N2 avian influenza virus in vaccinated and unvaccinated Japanese quail. Avian Diseases, 55: 195–200. DOI: https://doi.org/10.1637/9714-953811-digest.1 .

Ellakany HF, Gado AR, Elbestawy AR, El-Hamid HAS, Hafez HM, El-Hack MEA, and Saadeldin, IM (2018). Interaction between avian influenza subtype H9N2 and Newcastle disease virus vaccine strain (LaSota) in chickens. BMC veterinary research, 14(1): 358-368. DOI: https://doi.org/10.1186/s12917-018-1689-4 .

Gomaa MR, Kayed AS, Elabd MA, Zeid DA, Zaki SA, El Rifay AS, Sherif LS, McKenzie PP, Webster RG, Webby RJ, er al. (2015). Avian influenza A (H5N1) and A (H9N2) seroprevalence and risk factors for infection among Egyptians: a prospective, controlled seroepidemiological study. Journal of Infectious Diseases, 211:1399_1407. DOI: https://doi.org/10.1093/infdis/jiu529 .

Halblolvarid M, Sohraby HI, Pourakhsh S and Gholami M (2004). Histopathological study of intratracheally inoculated A/Chicken/Iran/259/1998 (H9N2) influenza virus in Chicken. Archives of Razi Institute, 58: 51-62.

Hassan KE, Ali A, Shany SA and El-Kady MF (2017). Experimental co-infection of infectious bronchitis and low pathogenic avian influenza H9N2 viruses in commercial broiler chickens. Research in Veterinary Science, 115: 356-362. DOI: https://doi.org/10.1016/j.rvsc.2017.06.024 .

Horwitz MS, and Scharff MD (1969). The production of Antiserum against Viral Antigens. In: Fundamental Techniques in Virology, Academic press, p. 253

Hussein MA (2019). Studies on isolation, identification, pathotyping with sequencing and genotyping of most occurrence strains of Newcastle Virus and Avian Influenza virus in Karbala province. Ph.D. Thesis submitted to the College of Veterinary Medicine University of Baghdad.

Imai M, Herfst S, Sorrell EM, Schrauwen EJ, Linster M, De Graaf M, Fouchier RA and Kawaoka Y (2013). Transmission of influenza A/H5N1 viruses in mammals. Virus Research, 178: 15–20. DOI: https://doi.org/10.1016/j.virusres.2013.07.017 .

Iqbal M, Yaqub T, Mukhtar N, Shabbir MZ, and McCauley JW (2013). Infectivity and transmissibility of H9N2 avian influenza virus in

chickens and wild terrestrial birds. Veterinary Research, 44(1): 100-110. DOI: https://doi.org/10.1186/1297-9716-44-100 .

Kaleta EF, Honicke A (2004). Review of the literature on avian influenza A viruses in pigeons and experimental studies on the susceptibility of domestic pigeons to influenza A viruses of the haemagglutinin subtype H7. Deutsche tierärztliche Wochenschrift, 111: 453–484.

Kandeil A, El-Shesheny R, Maatouq A, Moatasim Y, Cai Z, McKenzie P, Webby R, Kayali G and Ali MA (2017). Novel reassortant H9N2 viruses in pigeons and evidence for antigenic diversity of H9N2 viruses isolated from quails in Egypt. Journal General Virology, 98: 548–562. DOI: https://doi.org/10.1099/jgv.0.000657 .

Kausar A, Anwar S, Siddique N, Ahmed S, and Dasti JI (2018). Prevalence of Avian influenza H9N2 Virus among Wild and Domesticated Bird Species across Pakistan. Pakistan Journal of Zoology, 50(4): 1347-1354. DOI: https://doi.org/10.17582/journal.pjz/2018.50.4.1347.1354 .

Khamas E (2008). Avian influenza (H9N2) outbreak In Iraq. Iraqi Journal of Veterinary Science, 32: 223–230.

Killian ML (2008). Hemagglutination assay for avian influenza virus. In Avian influenza virus, Humana Press, pp.47-52. DOI: https://doi.org/10.1007/978-1-59745-279-3_7 .

Kim SH (2018). Challenge for One health: co-circulation of zoonotic H5N1 and H9N2 avian influenza viruses in Egypt. Viruses, 10 (3): 121-128. DOI: https://doi.org/10.3390/v10030121 .

Kraidi QA, Langeroudi AG, Madadgar O and Karimi V (2016). Prevalence of AIV subtype H9 among poultry with respiratory signs in Iraq. Bulgarian Journal of Veterinary Medicine, 20 (4): 367-376. DOI: https://doi.org/10.15547/bjvm.1022.

Kraidi QA, Madadgar O, Ghalyanchi Langeroudi A and Karimi V (2017). Genetic analysis of H9N2 avian influenza viruses circulated in broiler flocks: a case study in Iraq in 2014–2015. Virus Genes, 53: 205–214. DOI: https://doi.org/10.1007/s11262-016-1407-x .

Lee DH, Bahl J, Torchetti MK, Killian ML, Ip HS, DeLiberto TJ, and Swayne DE (2016). Highly pathogenic avian influenza viruses and generation of novel reassortants, United States, 2014–2015. Emerging Infectious Diseases, 22:1283–1285. DOI: https://doi.org/10.3201/eid2207.160048 .

Lee YN, Lee DH, Park JK, Lim TH, Young HN, Yuk SS, Lee YI, Mo IP (2011).Isolation and characterization of a novel H9N2 influenza virus in Korean native chicken farm. Avian Disease, 55:724-7. DOI: https://doi.org/10.1637/9925-977411-digest.1 .

Liu Y, Han NC, Wang X, Lin J, Ma M, Shu Y, Zhou J, Yang H, Liang Q and Guo C (2009). Influenza A virus receptors in the respiratory and intestinal tracts of pigeons. Avian Pathology, 38, 263-266. DOI: https://doi.org/10.1080/03079450903055363 .

Liu Y, Yang Z, Wang X, Chen J, Yao J, Song Y and Pan J (2015). Pigeons are resistant to experimental infection with H7N9 avian influenza virus. Avian Pathology, 44(5): 342-346. DOI: https://doi.org/10.1080/03079457.2015.1055235.

Liu Y, Zhou J, Yang H, Yao W, Bu W, Yang B, Song W, Meng Y, lin J and Han C (2007). Susceptibility and transmissibility of pigeons to Asian lineage highly pathogenic avian influenza virus subtype H5N1. Avian Pathology, 36: 461-465. DOI: https://doi.org/10.1080/03079450701639335.

MacLachlan NJ and Dubovi EJ (2011). Orthomyxoviridae. In Fenner's Veterinary Virology. 4th Edition. Academic Press, Elsevier Publication. pp. 353-370.

Mehrabadi MHF, Bahonar A, Mirzaei K, Molouki A, Ghalyan Chilangeroudi A, Ghafouri SA, Tehrani F and Lim SHE (2018). Prevalence of avian influenza (H9N2) in commercial quail, partridge, and turkey farms in Iran, 2014–2015. Tropical Animal Health and Production, 50: 677-682. DOI: https://doi.org/10.1007/s11250-017-1438-x.

Mohamed N, Mohamed S, Kandeil A, AL-Zubaidy IAH, Kayali G and Ali M.A (2018). Genetic and antigenic characterization of avian influenza H9N2 viruses during 2016 in Iraq. Open Veterinary Journal, 9(2): 164–171. DOI: https://doi.org/10.4314/ovj.v9i2.12.

Mohammadi, A., Masoudian, M., Nemati, Y., and Seifi, S. (2010). Serological and RT-PCR assays for detection of avian influenza of domestic pigeons in Kavar area (Fars province, Iran). Bulgarian Journal of Veterinary Medicine, 13(2): 117-121.

Mostafa A, Abdelwhab EM, Mettenleiter TC, and Pleschka S (2018). Zoonotic potential of influenza A viruses: a comprehensive overview. Viruses, 10(9): 497. DOI.https://doi.org/10.3390/v10090497

Nagy A, Mettenleiter TC, and Abdelwahab EM (2017). A brief summary of the epidemiology and genetic relatedness of avian influenza H9N2 virus in birds and mammals in the Middle East and North Africa. Epidemiology and Infection, 145:3320–3333. DOI: https://doi.org/10.1017/s0950268817002576.

Nili H, and Asasi K (2003). Avian influenza (H9N2) outbreak in Iran. Avian Diseases, 47: 828–831. DOI: https://10.1637/0005-2086-47.s3.828.

Quinn PJ, Markey BK, Leonard FC, Hartigan P, Fanning S and Fitzpatrick EI (2011). Veterinary microbiology and microbial disease. The Veterinary Journal, 165 (3): 333. DOI: https://doi.org/10.1016/s1090-0233.

Reed LJ and Muench H (1938). A simple method of estimating fifty percent endpoints 12. American Journal of Epidemiology, 27(3): 493-497. DOI: https://doi.org/10.1093/oxfordjournals.aje.a11840 .

Shehata AA, Parvin R, Sultan H, and Halami MY (2015). Isolation and full genome characterization of avian influenza subtype H9N2 from poultry respiratory disease outbreak in Egypt. Virus Genes, 50 (3): 389-400. DOI: https://doi.org/10.1007/s11262-015-1188-7 .

Shen HQ, Yan ZQ, Zeng FG, Liao CT, and Zhou QF (2015). Isolation and phylogenetic analysis of hemagglutinin gene of H9N2 influenza viruses from chickens in South China from 2012 to 2013. Journal of Veterinary Science 16:317–324.DOI: https://doi.org/10.4142/jvs.2015.16.3.317 .

Sun Y and Liu J (2015). H9N2 influenza virus in China: a cause of concern. Protein and cell, 6(1): 18-25. DOI: https://doi.org/10.1007/s13238-014-0111-7 .

Sun Y, Pu J, Fan L, Sun H, Wang J, Zhang YI, Liu L and Liu J (2012). Evaluation of the protective efficacy of a commercial vaccine against different antigenic groups of H9N2 influenza viruses in chickens. Veterinary Microbiology, 156:193–199. DOI: https://doi.org/10.1016/j.vetmic.2011.10.003 .

Tolba HMN, Rasha MM, Abou Elez IE and Heba AA (2018). Molecular identification of avian influenza virus subtypes H5N1 and H9N2 in birds from farms and live bird markets and in respiratory patients. Peer Journal, (6): 5473. DOI: https://doi.org/10.7287/peerj.5473v0.1/reviews/4.

Turner JC, Feeroz MM., Hasan MK, Akhtar S, Walker D, Seiler P, and Krauss S (2017). Insight into live bird markets of Bangladesh: an overview of the dynamics of transmission of H5N1 and H9N2 avian influenza viruses. Emerging microbes and infections, 6(1): 1-8. DOI: https://doi.org/10.1038/emi.2016.142 .

Wang C, Wang Z, Ren X, Wang L, Li C, Sun Y, Wang M, Tong Q, Sun H and Pu J (2019). Infection of chicken H9N2 influenza viruses in different species of domestic ducks. Veterinary microbiology, 233: 1-4. DOI: https://doi.org/10.1016/j.vetmic.2019.04.018 .

Webster RG, Cox N and Stöhr K (2002) WHO Manual on Animal Influenza Diagnosis and Surveillance. Available at: http://www.who.int/csr/resources/publications/influenza/whocdscsrncs20025rev.pdf .

Williams JE (1980). Micro test methodology. In: Isolation and Identification of Avian pathogens 2nd Edition .S. B. Hitchner. C. H. Domermuth. H. G. Purchase and J. E. Williams (Editors). American Association of Avian pathologiss College Station., pp. 136 – 140.

Xu C, Ye H, Qiu W, Lin H, Chen Y, Zhang H and Liao M (2018). Phylogenetic classification of hemagglutinin gene of H9N2 avian

influenza viruses isolated in China during 2012–2016 and evaluation of selected candidate vaccine strains. Poultry Science, 97(9): 3023-3030. DOI: https://doi.org/10.3382/ps/pey154 .

Xu W, Shao Q, Zang Y, Guo Q, Zhang Y and Li Z (2015). Pigeon RIG-I function in innate immunity against H9N2 AIV and IBDV. Viruses, 7: 4131-4151. DOI: https://doi.org/10.3390/v7072813 .

Yamamoto Y, Nakamura K, Yamada M and Mase M (2011). Limited susceptibility of pigeons experimentally inoculated with H5N1 highly pathogenic avian influenza viruses. Journal of Veterinary Medical Science, 74 (2): 205-208. DOI: https://doi.org/10.1292/jvms.11-0312.

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The Effects of Extraction Methods of *Mangifera indica* and *Azadirachta indica* Bark on *in vitro* Antimicrobial Efficacy and Performance of Broiler Chickens

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ABSTRACT

This study investigated the *in vitro* efficacy of extracts of *Mangifera indica* and *Azadirachta indica* bark obtained by different extraction methods. Also, in an eight-week trial, the effect of these extracts on the performance of broiler chickens were evaluated. The barks were collected, air-dried and pulverized. The samples were extracted using maceration, infusion, and decoction methods. The extracts were screened for their activity against *Escherichia coli* and *Streptococcus aureus*. Two hundred and eighty-eight birds were divided into two groups (144 each) administered with *A. indica* or *M. indica*. Each group was subdivided into four subgroups, including control subgroup (no herb) and subgroups administered with bark extracted by one of the three extraction methods. The birds on herbal treatments were not given antibiotics. Results showed that the growth of *E. coli* was more inhibited by the various extracts irrespective of the methods of extraction. Weights were significantly influenced by the interaction between herb types and extraction methods at the starter phase. Infused herbs induced mortality at the finisher phase. In conclusion, *S. aureus* was more susceptible to the extracts compared to *E. coli*. However, decocted *A. indica* and *M. indica* bark, as well as macerated M. indica, showed antimicrobial potency against *E. coli*. It can be recommended that neem or mango bark extracted by maceration or decoction can be administered orally to broiler chickens especially at the starter phase, for improved performance and reduced mortality.

Key words: Antimicrobial assay, *Azadirachta indica*, Extraction, *Mangifera indica*, performance

INTRODUCTION

Poultry farmers are interested in raising their birds to gain market weight as early as possible within the shortest period. This has led to the use of antibiotics that can modify the intestinal microbiota and eliminate harmful bacteria, which in turn improves the growth of the birds. However, there are global concerns over the use of antibiotics for growth promotion or therapy purposes because despite rigorous withdrawal measures to prevent antibiotic residues in food some drugs enter the human food chain and lead to increased antibiotic resistance (Molbak, 2005). For these reasons, European countries have banned the use of antibiotics as growth promoters in animal feed (Castanon, 2007). Therefore, research attention has been shifted to using natural alternatives such as medicinal plants as natural feed additives in poultry diet to enhance production performance as well as to counter bacteria growth (Abaza et al., 2008).

Recent studies have tested the use of herbal extracts as alternatives to antibiotic growth promoters (Biswas et al., 2002; Landy et al., 2011; Sarker et al., 2014). The mechanism of action of bioactive components of these extracts is based on the alteration of the intestinal microbiota, increased enzyme secretion, histo-morphological maintenance of the gastrointestinal tract, and enhancement of immune system (Brugalli, 2003). Various research studies have demonstrated antimicrobial, antifungal, anthelmintic and antioxidant effects of plant extracts (Kamel, 2000). Allinson et al. (2013) reported that herbal extracts improve the performance and Feed Conversion Ratio (FCR) in poultry as well as decrease the bacterial and oocyst counts. Neem (*Azadirachta indica*) is one of the most prominent herbal medicines with different biologically active compounds such as azadirachtin, nimbin, salanin, meliacin, and triterpenoids (National Research Council, 1992; Ansari et al., 2012).

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Mangifera indica (mango) is another plant whose leaves, fruits and barks are known for their medicinal potential and are being explored. Khan et al. (1993) detected compounds such as terpenoidal saponins, polygalacturonase, fructose-1,6- diphosphatase, triterpenoid, 2- hyroxymangiferonic acid tetracyclic triterpenoid and pentacyclic triterpenoid in *Mangifera indica* extract. The bark infusion has been used as a gargle to treat mouth infections in children (Doughari and Manzara, 2008).

Methods of preparation of crude extracts and their purity greatly influence the inhibitory activity of some herbs against infectious organisms. Also, the extraction method, extraction solvent and the plant part used determines the quality of the extract. Hence, this study aimed to evaluate the performance of broiler chickens administered with neem or mango bark extract prepared by decoction, infusion and maceration techniques.

MATERIALS AND METHODS

Experimental Site

The research was carried out at the Poultry Unit of the Directorate of University Farms (DUFARMS), Federal University of Agriculture, Abeokuta, Ogun State, Nigeria.

Ethical approval

The present study was approved by the ethics and research committee of the College of Animal Science and Livestock Production, Federal University of Agriculture, Abeokuta, Nigeria.

Preparation of plant extracts

The *A. indica* and *M. indica* barks were air-dried and pulverized. Three methods of extraction used; maceration, infusion, and decoction. Maceration was performed by soaking of 100 g of dried barks of each sample in 1 L of cool water in a covered plastic for 72 hours at room temperature, and then the mixture was strained. The infusion process involved soaking of 100 g of either *A. indica* or *M. indica* dried barks in 1 L of hot water for 12 hours, then was filtered to obtain the extract. Decoction method performed by boiling 100 g dried barks in 1 L of water for 1 hour. After cooling, the extract was obtained by decantation.

Assessment of antimicrobial activity of plant extracts

The agar well diffusion method was used for the antimicrobial susceptibility test. Mueller Hilton agar was prepared according to the manufacturer's specifications. The media were autoclaved and dispensed into sterile Petri-dishes and allowed to gel. Standardized inocula of *Escherichia coli* and *Staphylococcus aureus* were streaked on the agar plate. Six wells of 6 mm each were made in each plate with a central well for positive control using a sterile cork borer. The wells were filled with 0.1 ml of different extracts of two herbs prepared by different methods (infusion, maceration, and decoction). In addition, 0.1 ml of ciprofloxacin were used in separate plates to serve as positive control while sterile distilled water was used as a negative control on separate plates. The plates were allowed to stand for 15 minutes to allow free diffusion of the extracts. After 24 hours of incubation at 37 $^{\circ}$C, a transparent plastic meter rule was used to measure the diameters of zone of inhibition, according to Dahiru et al. (2013).

Growth response trial

A total of 288 day-old broiler chicks was divided into two groups (144 birds each group) administered with *A. indica* or *M. indica*. Each group was subdivided into four subgroups (36 birds each) including control (no herb administered) and subgroups administered with herbal extracts obtained from different extraction methods: maceration, infusion, and decoction. Hence, the birds were arranged in a 2 × 4 experimental layout. Brooding was done for two weeks. Commercial broiler starter was given for the first four weeks, while commercial broiler finisher was given from four weeks to eight weeks (Table 1). The groups were given necessary medications (antibiotics, coccidiostats, and vitamins) and vaccinations (Gumboro vaccine on 7th and 15th day, and Lasota at 4 weeks of age). Birds treated with medicinal herbs were not given antibiotics. Herbs were supplied in drinking water (the extracts were added at a dosage of 150 ml to 1 L water) for three consecutive days per week for six weeks.

Table 1. Nutrient composition of feed

Parameter	Starter diet	Finisher diet
Crude protein (%)	21.00	18.00
Fat (%)	6.00	6.00
Crude fiber (5%)	5.00	5.00
Calcium (%)	1.00	1.00
Available phosphorus (%)	0.45	0.40
Lysine (%)	1.00	0.85
Methionine (%)	0.50	0.35
Salt (%)	0.30	0.30
Metabolizable energy (Kcal/kg)	2900	2800

Data collection

Feed intake

The amount of feed given to the birds and the leftover were measured weekly to determine the feed intake according to the following equation:

Feed intake = Feed given − Feed leftover

Body weight and weight gain

The birds were weighed on a replicate basis at the commencement of the experiment and subsequently every week.

Body weight (g) = Total weight of birds (g) / Total number of birds
Total weight gain (g) = Final weight (g) − Initial weight (g)
Daily weight gain = (Final weight – Initial weight) / Number of days

Feed conversion ratio

The FCR was calculated as total feed intake divided by weight gain.

FCR = Total feed intake(g) / Total weight gain(g)

Mortality rate

The mortality rate was calculated as the total number of dead birds divided by the total number of birds and expressed in percentage.

Statistical analysis

Data obtained on antibacterial assay were subjected to one-way analysis of variance while those on performance were subjected to one way of analysis of variance in a 2×4 factorial experimental layout using SPSS software (version 23.0) Significant means were separated using Duncan's multiple range test at 5% level of significance ($p<0.05$).

RESULTS

Antimicrobial assay of differently extracted *Mangifera indica* and *Azadirachta indica* bark on selected bacteria

Figure 1 shows the bar chart representation of the antimicrobial assay of differently extracted *M. indica* and *A. indica* bark on the Gram-negative organism *(Escherichia coli)* and gram-positive (*Staphylococcus aureus*) bacteria. Significant differences ($p<0.05$) were observed among the distance of inhibition zone of *E. coli* and *S. aureus* with respect to the type of herbs and extraction methods. The highest zone of inhibition of the two bacteria (33.00 mm and 29.00 mm for *E. coli* and *S. aureus*, respectively) was recorded for positive control samples (ciprofloxacin) ($p<0.05$). However, it was observed that the extracts of *A. indica* obtained by infusion and maceration methods and *M. indica* obtained by infusion did not inhibit the growth of *E. coli* while others had similar values. growth of *S. aureus* was inhibited by the extracts from the various methods of extraction except for extract of *M. indica* obtained by decoction.

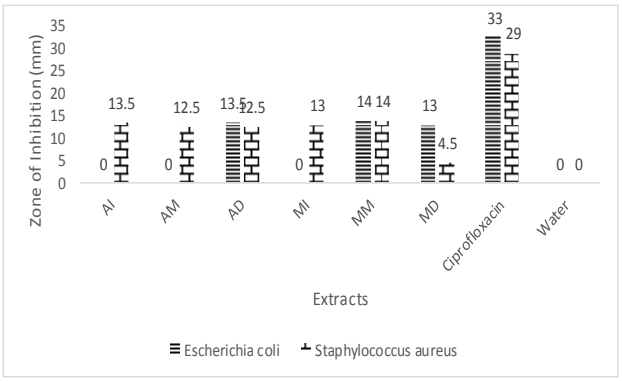

Figure 1. Antimicrobial assay of differently extracted *Mangifera indica* and *Azadirachta indica* bark on *Escherichia coli* and *Staphylococcus aureus.* AI: *Azadirachta indica* extracted by infusion; AM: *Azadirachta indica* extracted by maceration method; AD: *Azadirachta indica* extracted by decoction; MI: *Mangifera indica* extracted by infusion; MM: *Mangifera indica* extracted by maceration method; MD: *Mangifera indica* extracted by decoction.

Effect of herb types and extraction methods on the performance of broiler chickens at the starter phase

The performance traits of birds were not significantly ($p>0.05$) affected by herbs and different extraction methods at the starter phase (Table 2). The interaction effect of herbs and extraction methods on the performance of broiler chickens at the starter phase is presented in table 3. Final weight and total weight gain were highest ($p<0.05$) in the control group of birds administered with *M. indica* extract while the lowest were recorded for birds administered with *A. indica* prepared by the infusion method.

Effect of herbs and extraction methods on the performance of broiler chickens at the finisher phase

The main effect of herbs and extraction methods on the performance of broiler chickens at the finisher phase is presented in table 4. Herb type had no influence ($p>0.05$) on all parameters measured. However, the highest ($p<0.05$) mortality (1.67%) was observed in birds administered herb extract prepared by infusion. The effects of interaction between herbs and extraction methods on the performance of broiler chickens at the finisher phase are shown in table 5. All performance traits evaluated were similar ($p>0.05$) except the mortality. Mortality was highest for birds administered with *A. indica* extract prepared by infusion and the lowest was recorded for control birds and birds administered with *M. indica* and *A. indica* extracts prepared by maceration.

Table 2. Main effect of different herbs and different extraction methods on the performance of broiler chickens at starter phase

	Effect of Herbs		Effect of Extraction Methods			
Parameters	**MI**	**AI**	**Control**	**Maceration**	**Infusion**	**Decoction**
Initial weight (g/bird)	41.01±0.71	41.14±2.89	42.06±3.86	40.89±0.65	40.71±0.83	40.64±1.37
Final weight (g/bird)	760.32±41.12	738.02±50.67	764.52±61.13	770.48±47.96	722.22±40.02	739.44±22.62
Total Weight gain (g)	719.31±41.12	698.88±51.90	722.46±63.41	729.59±48.28	681.51±40.29	698.81±22.80
Weight gain/day (g/ bird /day)	25.69±1.47	24.89±1.85	25.80±2.26	26.06±1.72	24.34±1.44	24.96±0.81
Total feed (g/bird)	1580.55±220.69	1603.37±89.59	1605.78±55.33	1532.72±262.85	1538.37±70.88	1690.97±169.13
Total feed/day (g/ bird /day)	56.45±7.88	57.26±3.20	57.35±1.98	54.74±9.39	54.94±2.53	60.39±6.04
Total water intake (ml/ bird)	3588.46±264.41	3418.44±146.14	3604.62±238.75	3555.65±203.04	3417.06±167.53	3436.47±281.60
Water intake/day (ml/ bird /day)	128.16±9.44	122.09±5.22	128.74±8.53	126.99±7.25	122.04±5.98	122.73±10.06
Mortality (%)	0.50±0.80	0.25±0.62	0.17±0.41	0.50±0.84	0.17±0.41	0.67±1.03
FCR	2.20±0.33	2.31±0.22	2.24±0.26	2.10±0.38	2.26±0.16	2.42±0.27

Data are expressed as mean ± standard deviation. MI: *Mangifera indica* AI: *Azadirachta indica* FCR: Feed conversion ratio

Table 3. Interaction effects of between herbs and extraction methods on the performance of broiler chickens at starter phase

	Mangifera indica				***Azadirachta indica***			
Parameters	**Control**	**Maceration**	**Infusion**	**Decoction**	**Control**	**Maceration**	**Infusion**	**Decoction**
Initial weight (g/bird)	40.64±0.84	41.31±0.63	40.92±1.02	41.17±0.44	43.47±5.12	40.47±0.38	40.5±0.73	40.11±1.91
Final weight (g/bird)	$801.26±56.45^{a}$	$753.33±10.14^{ab}$	$738.89±41.94^{ab}$	$747.78±27.15^{ab}$	$727.78±45.90^{ab}$	$787.63±69.03^{ab}$	$705.56±37.58^{b}$	$731.11±18.28^{ab}$
Total weight gain (g)	$760.62±55.75^{a}$	$712.03±9.51^{ab}$	$697.97±42.24^{ab}$	$706.61±27.40^{ab}$	$684.31±50.75^{ab}$	$747.15±69.37^{ab}$	$665.06±38.23^{b}$	$691.00±19.13^{ab}$
Daily weight gain (g/bird)	$27.17±1.99^{a}$	$25.43±0.34^{ab}$	$24.93±1.51^{ab}$	$25.24±0.98^{ab}$	$24.44±1.82^{ab}$	$26.68±2.48^{ab}$	$23.75±1.37^{b}$	$24.68±0.68^{ab}$
Total feed intake (g/bird)	1571.29±25.26	1438.01±379.28	1561.47±90.52	1751.44±200.56	1640.28±58.70	1627.42±44.34	1515.28±52.61	1630.50±142.52
Daily feed intake (g/bird)	56.12±0.90	51.36±13.55	55.77±3.23	62.55±7.16	58.58±2.10	58.12±1.58	54.12±1.88	58.23±5.10
Total water intake (ml/bird)	3730.11±230.67	3710.28±148.90	3449.48±192.17	3463.97±409.44	3479.14±205.08	3401.01±95.71	3384.64±173.44	3408.97±168.33
Water intake/day (ml/bird/day)	133.22±8.24	132.51±5.32	123.20±6.86	123.71±14.62	124.26±7.32	121.46±3.42	120.88±6.19	121.75±6.01
Mortality (%)	0.33±0.58	0.67±1.15	0.33±0.58	0.67±1.15	0±0	0.33±0.58	0±0	0.67±1.15
FCR	2.07±0.12	2.02±0.51	2.24±0.20	2.48±0.32	2.41±0.25	2.19±0.27	2.28±0.15	2.36±0.28

Data are expressed as mean ± standard deviation. Different superscript letters in the same row indicate significant differences ($p<0.05$). FCR: Feed conversion ratio

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Table 4. Main effect of herbs and extraction methods on the performance of broiler chickens at finisher phase

	Effect of Herbs		Effect of Extraction Methods			
Parameters	**MI**	**AI**	**Control**	**Maceration**	**Infusion**	**Decoction**
Initial weight (g/bird)	760.32±41.12	738.02±50.67	764.52±61.13	770.48±47.96	722.22±40.02	739.44±22.62
Final weight (g/bird)	1847.70±77.95	1825.62±90.63	1846.09±121.54	1853.33±48.32	1836.15±39.17	1811.07±110.25
Total weight gain (g)	1087.38±52.34	1087.60±87.17	1081.57±83.27	1082.85±47.62	1113.92±38.06	1071.63±104.33
Daily weight gain (g/bird)	38.84±1.87	38.84±3.11	38.63±2.98	38.67±1.70	38.78±1.36	38.27±3.73
Total feed intake (g/bird)	3349.67±221.60	3319.51±172.89	3245.46±116.22	3334.21±217.66	3354.95±121.10	3403.74±288.70
Daily feed intake (g/bird)	119.63±7.91	118.55±6.17	115.91±4.15	119.08±7.77	119.82±4.36	121.56±10.31
Total water intake (ml/bird)	9398.34±744.80	9279.79±463.35	9424.79±517.85	9466.10±775.48	9368.98±495.06	9096.40±703.59
Water intake/day (ml/bird/day)	335.66±26.60	331.42±16.55	336.60±18.49	338.08±27.70	334.61±17.68	324.87±25.13
Mortality (%)	0.92±0.67	1.08±0.79	0.67±0.52^{b}	0.67±0.52^{b}	1.67±0.52^{a}	1.00±0.89^{ab}
FCR	3.08±0.22	3.07±0.24	3.01±0.17	3.08±0.07	3.01±0.12	3.20±0.40

Data are expressed as mean ± standard deviation. Different superscript letters in the same row indicate significant differences ($p<0.05$). MI: *Mangifera indica.* AI: *Azadirachta indica.* FCR: Feed conversion ratio

Table 5. Effects of interaction between herbs and extraction methods on the performance of broiler chickens at finisher phase

	Mangifera indica				***Azadirachta indica***			
Parameters	**Control**	**Maceration**	**Infusion**	**Decoction**	**Control**	**Maceration**	**Infusion**	**Decoction**
Initial weight (g/bird)	801.26±56.45^{a}	753.33±10.14^{ab}	738.89±41.94^{ab}	747.78±27.15^{ab}	727.78±45.90^{ab}	787.63±69.03^{ab}	705.56±37.58^{b}	731.11±18.28^{ab}
Final weight (g/bird)	1922.73±105.92	1855.15±60.58	1828.96±6.89	1783.97±57.77	1769.44±89.95	1851.52±46.43	1843.33±60.28	1838.18±157.63
Total weight gain (g)	1121.47±52.92	1101.82±59.19	1090.07±37.38	1036.19±37.01	1041.67±98.79	1063.89±32.96	1137.78±22.75	1107.07±148.56
Daily weight gain (g/bird)	40.05±1.89	39.35±2.11	38.93±1.33	37.01±1.32	37.20±3.53	37.10±1.18	40.63±0.81	39.54±5.31
Total feed intake (g/bird)	3277.53±96.02	3402.51±283.09	3332.37±148.16	3386.27±379.88	3213.38±146.49	3265.91±155.90	3377.53±117.16	3421.21±251.30
Daily feed intake (g/bird)	117.05±3.43	121.52±10.11	119.01±5.29	120.94±13.57	114.76±5.23	116.64±5.57	120.63±4.18	122.19±8.97
Total water intake (ml/bird)	9853.93±328.54	9792.97±1067.47	9130.47±408.60	8816.00±689.49	8995.66±100.11	9139.24±208.38	9607.49±524.51	9376.79±725.49
Water intake/day (ml/bird/day)	351.93±11.73	349.75±38.12	326.09±14.59	314.86±24.62	321.27±3.58	326.40±7.44	343.12±18.73	334.89±25.91
Mortality (%)	0.67±0.58^{b}	0.67±0.58^{b}	1.33±0.58^{ab}	1.00±1.00^{ab}	0.67±0.58^{b}	0.67±0.58^{b}	2.00±0^{a}	1.00±1.00^{ab}
FCR	2.92±0.10	3.08±0.09	3.06±0.09	3.27±0.37	3.10±0.20	3.07±0.05	2.97±0.14	3.13±0.49

Data are expressed as mean ± standard deviation. Different superscript letters in the same row indicate significant differences ($p<0.05$). FCR: Feed conversion ratio

DISCUSSIONS

Failure of bark extracts of infused *A. indica*, infused *M. indica* and macerated *A. indica* to inhibit *E. coli* growth (no inhibition zone diameter observed) indicates the resistance of the organism to those extracts. The appearance of the zone of inhibition by herbs prepared by decoction may indicate that this method can lead to a better release of phytochemicals and active ingredients effective in inhibiting *E. coli* activity. Since infusion and maceration techniques involve soaking in hot and cold water, respectively, thus the antimicrobial efficacy could be less potent due to reduced quality and quantity of phyto-components released, resulting in the bacteria resistant. Azwanida (2015) stated that decoction is the most effective method for extracting hard plant materials and heat-stable compounds.

It has been documented that *E. coli* can rapidly change their genetic makeup as gram-negative bacteria, this enables them to develop resistance to antibiotics (Uwimbabazi et al. 2015). This can be attributed to the lower potency of the extracts on *E. coli* compared to *S. aureus*. Resistant bacteria change their cell walls lightly, so the antibiotics cannot attach, or they produce enzymes to disable the antibiotics. Hence, the result of this study is inconsistent with findings of Gajendrasinh et al. (2012) who reported that aqueous and ethanol extracts of *A. indica* leaves were most effective against *E. coli.* The variation observed in the result could be attributed to the differences in solvent types and plant parts used.

M. indica showed a slightly stronger potency on both bacteria when extracted by the maceration method compared to infused and macerated *A. indica* and infused *M. indica* This finding indicated the role of different methods of extraction in influencing the potency of the extract.

The similar performance in birds administered with *A. indica* and *M. indica* at the starter and finisher phase is an indication that both herbs induced similar growth response in the birds. Meanwhile, in a similar study by Sarker et al. (2014), it was reported that body weight and weekly weight gain in broilers were improved with oral supplementation of 1% aqueous neem leaf extract compared to control group.

The fairly poor performance recorded for birds administered with infused *A. indica* bark indicates that growth performance decreased at the starter phase. This finding may indicate that the birds cannot well tolerate infused extract or that the potency of the herb prepared through this method is lower compared to other methods. Infusion is generally used for softer parts including leaves and flowers, thus this method may not effectively release the beneficial bioactive components in plant parts such as bark that was used in this study. Also, the temperature might not be adequate to destroy or reduce the quantity of antinutritional components of the extract which could impair feed utilization and hence suppress the growth. Tannin is a known antinutritional factor present in both herbs used and can be degraded at high temperatures. However, the temperature at infusion might not be sufficient to degrade it. Tannins in diet decrease palatability, reduce feed intake, suppress growth rate, impair net metabolizable energy and protein digestibility resulting in poor feed efficiency in animals. Tannins can also inhibit cellular protein synthesis by forming irreversible complexes with proline-rich proteins (Adejuwon et al., 2011). Contrarily, similar feed intake and FCR in the birds administered with infused bark extract with other groups in this study could be due to the fact that the herbs were administered orally and not incorporated into the diet.

The insignificant effect of the interaction of herbs and extraction methods on all performance parameters with the exception of mortality at the finisher phase corroborates the statement reported by Ayoola et al. (2015) that neem leaf had no significant effect on broiler performance at the finisher phase. Some authors found no effect of these additives on growth, feed consumption or FCR in broilers (Cross et al., 2007; Ocak, et al., 2008).

The similar effect of water intake throughout the study is in agreement with Durrani et al. (2007), who reported the non-significant effect of medicinal herbs on water intake of birds.

The highest mortality recorded in birds administered with the extract obtained by the infusion method at the finisher phase could be attributed to the accumulation of tannin which eventually became toxic and induce mortality among the birds. Also, temperature in the infusion method may not be enough to destroy toxic components present, unlike decoction which involves higher temperature and longer heating time. Thermal treatment of plant materials reduces the tannin content. (Rakic, 2004). Levels of tannin above 5% are often lethal, and it was reported that neem bark contains about 14% tannin (NRC, 1992). It is thought that the mortality rate was higher due to inability of the infusion method to properly reduce the tannin content. It can be also related to the length of time because too long infusion can cause high tannin content and tannins at high levels can result in mortality. According to Calislar (2017), poultry develops

bone problems and necrotic organs (crop, gizzard, and duodenum) resulting from liver and kidney poisoning due to excess tannin consumption. Smulikowska et al. (2001) also reported that inclusion of feed ingredients containing tannins resulted in undesirable physiological and biochemical effects including growth inhibition and negative nitrogen balances.

CONCLUSIONS

This study concludes that gram-positive (*Staphylococcus aureus*) bacteria in comparison to gram-negative *(Escherichia coli)* bacteria are more susceptible to antimicrobial effect of extracts of *Mangifera indica* and *Azadirachta indica*, regardless of the extraction methods. *Mangifera indica* and *Azadirachta indica* extracts had similar effects on the growth performance of broiler chickens at starter and finisher phases. Administration of infused neem bark decreased weight gain at the starter phase and increased mortality at the finisher phase. Hence, it can be recommended that neem or mango bark extracted by maceration or decoction can be administered orally to broiler chickens especially at the starter phase to improve performance and reduce mortality.

DECLARATIONS

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Competing interest

The authors have declared that no competing interest exists.

Authors' contribution

Ayoola A. A. conceptualized and designed the experiment, collected data and wrote the manuscript draft. Ekunseitan D. A. analyzed the data and interpreted the results. Muhammad S. B. and Oguntoye M. A. read and revised the first and second drafts and Adejola Y. A. assisted in manuscript writing.

REFERENCES

Abaza IM, Sheheta MA, Shoeb MS and Hassan II (2008). Evaluation of some natural feed additives in growing chicks' diet. International Journal of Poultry Science, 7:872-879. DOI: https://doi.org/10.3923/ijps.2008.872.879

Adejuwon AO, Agbaje EO and Idika N (2011). Antifungal and antibacterial activities of aqueous and methanolic root extracts of *Carica papaya* Linn. (*Caricaceae*). International Research Journal of Microbiology, 2(8): 270-277. Available at: https://www.interesjournals.org/archive/irjm-volume-2-issue-8-year-2011.html

Allinson IB, Ekunseitan DA, Ayoola AA, Ogunade IM and Njoku CP (2013). Effect of herbal supplement on growth response and faecal egg counts of cockerels. Online Journal of Animal Feed Resource, 3: 68-73. DOI: https://doi.org/10.3923/pjbs.2013.1022.1027

Ansari SH, Islam F and Sameem M (2012). Influence of nanotechnology on herbal drugs: A review. Journal Advance Pharmaceutical Technology Research, 3: 142-146. DOI: https://doi.org/10.4103/2231-4040.101006

Ayoola AA, Egbeyale LT, Ekunseitan DA, Adegoke AV and Adeyeri OP (2015). The effect of neem (*Azadirachta indica*) leaf meal on growth performance and carcass characteristics of broiler chickens. Nigerian Journal of Animal Production, 42(2): 141-150. Available at: https://www.ajol.info/index.php/njap/issue/view/13764

Biswas K, Chattopadhyay I, Banerjee RK and Bandyopadhyay U (2002). Biological activities and medicinal properties of Neem (*Azadirachta indica*). Current Science, 82, 1336-1345. Available at: http://medicalmate.gr/img/cms/Vivliothiki%20Gia%20Epaggelmaties%20Ygeias/wound%20management/Allegato%20NE%2002.pdf

Brugalli I (2003). Alternative feeding: The use of herbal medicines and nutraceuticals as moduladoresda immunity and animal performance. Proceedings of Symposium on Management and Nutricao Poultry and Swine. Campinas, Sao Paulo. Brazil. Campinas: CBNA. pp. 167-182.

Calislar S (2017). Tannins and their effect on poultry nutrition. Proceeding of International Conference on Agriculture, Forest, Food Sciences and Technologies (ICAFOF 2017 Cappadocia / Turkey). Available at: https://www.researchgate.net/publication/317168301_Tannins_and_Their_Effects_on_Poultry_Nutrition

Castanon JIR (2007). History of the use of antibiotic as growth promoters in European poultry feeds. Poultry Science, 86: 2466-2471. DOI: https://doi.org/10.3382/ps.2007-00249

Cross DE, McDevitt RM, Hillman K. and Acamovic T (2007). The effect of herbs and their associated essential oils on performance, dietary digestibility and gut microflora in chickens from 7 to 28 days of age. British Poultry Science, 48: 496-506. DOI: https://doi.org/10.1080/00071660701463221

Dahiru D, Malgwi AR and Sambo SH (2013). Growth inhibitory effect of *Sennasiamea* leaves extracts on selected microorganism. American Journal of Medicine and Medical Sciences, 3(5): 334- 337.

Doughari JH and Manzara S (2008). In vitro antibacterial activity of crude leaf extracts of *Mangifera indica Linn*. African Journal of Microbiology Research, 2: 067-072. Available at: https://academicjournals.org/journal/AJMR/archive/2008

Durrani FR, Sultan A, Ahmed S, Chand N, Khattak, FM, and Durrani Z (2007). Efficacy of Aniseed Extract as immune Stimulant and Growth Promoter in Broiler Chicks. Pakistan Journal of Biological Sciences, 10: 3718-3721. DOI: https://doi.org/10.3923/pjbs.2007.3718.3721

Gajendrasinh PR, Bhavika MK, Rohit, S, Hetal, A and Prajapati PK (2012). *In vitro* antibacterial study of two commonly used medicinal plants in Ayurveda: Neem (*Azadirachta indica* L.) and Tulsi (*Ocimum sanctum* L.). International Journal of Pharmaceutical and Biological Archives, 3(3): 582-586. Available at: http://www.ijpba.info/ijpba/index.php/ijpba/article/view/677

Kamel C (2000). A novel look at a classic approach of plant extracts. Feed Mix- The International Journal on Feed, Nutrition and Technology, 9(6): 19-24. Available at: https://www.worldcat.org

Landy N, Ghalamkari GH and Toghyani M (2011). Performance, carcass characteristics, and immunity in broiler chickens fed dietary Neem (*Azadirachta indica*) as alternative for an antibiotic growth promoter. Livestock Science, 142: 305-309. DOI: https://doi.org/10.1016/j.livsci.2011.08.017

Molbak K (2005). Human health consequences of antimicrobial drug-resistant *Salmonella* and other foodborne pathogens. Food Safety, 41: 1613-1620. DOI:http//doi.org/10.1086/497599

National Research Council (NRC) (1992). Neem: A tree for solving global problems. National Academy Press, Washington, DC. United State of America., pp: 141-142. Accessed in November 2017. DOI: https://doi.org/10.17226/1924

Ocak N, Erner G, Burak AK, Sungu M, Altop A and Ozmen A (2008). Performance of broilers fed diets supplemented with dried peppermint (*Mentha piperita* L.) or thyme (*Thymus vulgaris* L) leaves as growth promoter source. Czech Journal of Animal Science, 53(4): 169-175. DOI: https://doi.org/10.17221/373-cjas

Rakić S, Maletić R, Perunović M and Svrzić G (2004). Influence of thermal treatment on tannin content and antioxidation effect of oak acorn Quercus cerris extract. Journal of Agricultural Sciences, 49 (1):97- 107. DOI: https://doi.org/10.2298/jas0401097r

Sarker SK, Mostofa M, Akter F, Rahman MM and Sultana MR (2014). Effects of aqueous extract of neem (*Azadirachta indica*) leaves as growth promoter and anti-colibacillosis in broilers. Bangladesh Journal of Animal Science, 43(2): 138-141. DOI: https://doi.org/10.3329/bjas.v43i2.20715

Savage GP (2003). Chemical and Physical properties of saponins. Encyclopedia of Food Sciences and Nutrition. (Second edition). Academic Press, London, pp: 5095- 5096. DOI: https://doi.org/10.1016/b0-12-227055-x/01050-6

Smulikowska S, Pastuszewska B, Swiech E, Ochtabinska A, Mieczkowska A, Nguyen VC and Buraczewska L (2001). Tannin content affects negatively nutritive value of pea for monogastrics. Journal of Animal and Feed Sciences, 10:511–523. Available at: https://pdfs.semanticscholar.org/0bbd/e036b365d03981b02dcc1013155590fe4858.pdf

Uwimbabazi F, Uwimana J and Rutanga J (2015). Assessment of antibacterial activity of Neem plant (*Azadirachta indica*) on *Staphylococcus aureus* and *Escherichia coli*. Journal of Medicinal Plants Studies, 3(4): 85-91 Available at: http://www.plantsjournal.com/archives/?year=2015&vol=3&issue=4&part=B

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The Potency of Oral Administration of L-Citrulline as Anti Heat Stress Agent in KUB Chicks

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ABSTRACT

L-citrulline (L-Cit) is considered one of the potential amino acids that are able to reduce body temperature in layer chicks. However, there are no reports on functions of L-Cit in KUB chicks. Therefore, the present study aimed to evaluate the effects of oral administration of L-Cit on feed intake, body temperature and plasma biochemical parameters of KUB chicks over two hours post-administration. Neonatal KUB chicks were orally administered with different doses of L-Cit (0, 3.75, 7.5 and 15 mmol/kg body weight). At 30, 60 and 120 min, feed intake was determined, also rectal temperature of chicks was measured by using a digital thermometer. At 120 min after administration, the blood samples were immediately collected through the jugular vein, then biochemical parameters of plasma (total cholesterol, triacylglycerol and total glucose) were analyzed. The obtained results indicated that feed intake, body temperature, and plasma metabolites were not significantly influenced by different doses of L-Cit. In conclusion, L-Cit could not able to improve feed intake, decrease body temperature and change plasma metabolites in KUB chicks.

Keywords: Body temperature, Feed intake, KUB chick, L- citrulline, Plasma metabolites

INTRODUCTION

In general, native chickens play an important role in supporting income generation, especially in many underdeveloped countries (Padhi, 2016). KUB chickens are offspring of local chickens in several areas of West Java that are obtained after six generations of a systematically-controlled breeding system (Purbarani et al., 2019). The KUB chickens have several advantages compared to their previous generations as well as local chickens. For instance, KUB chickens have high rates of egg production (160-180 eggs/year), high hatchability, and low feed conversion ratio (Sartika, 2016). Besides that, KUB chicken is considered a meat type breed (Hidayah et al., 2019) and the high demand for chicken meat might potentially be fulfilled by increasing the productivity of KUB chicken.

In order to obtain good performance as an expression of the genetically improved breed, some efforts such as optimal management conditions (housing and feeding), is needed to increase biological and economic productivity. Many factors may influence poultry production in tropical and subtropical areas. The climatic environment is one of the important factors affect production efficiency in these regions (Renaudeau et al., 2012). Poultry experiences heat distress when the combination of relative humidity and ambient temperature rise above the comfort zone (Teeter and Belay, 1996). There are concerns about reduced poultry production due to high ambient temperatures not only in tropical regions but also in some countries that occupy the temperate where heat stress is an occasional problem during the summer months.

Several nutritional strategies have been reported to overcome the adverse effects of heat stress in poultry (Teeter and Belay, 1996; Yahav, 2000; Erwan et al., 2013a, 2014, 2017; Chowdhury et al., 2015). Heat stress reduces laying percentage, egg size, eggshell durability, and body weight gain, as well as increases mortality in layers (Sterling et al., 2003; Lin et al., 2004; Franco-Jimenez and Beck, 2007). Also, it appears to have a detrimental impact on poultry meat quality, however, it has been reported that breast meat characteristics remain unaffected by heat stress (Goo et al., 2019).

Nutrients, especially amino acids, are widely used as anti-stress agents with regard to psychological and physiological stress (Yamane et al., 2009; Hamasu et al., 2010; Erwan et al., 2012). Amino acid supplements,

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particularly essential amino acids, has been used in attempts to overcome problems of heat stress in birds (Mendes et al., 1997; Daghir et al., 2003).

It has been reported that oral administration of L-citrulline (L-Cit) decreased the body temperature in layer chicks (Chowdhury et al., 2015). However, there are no reports on the function of L-Cit in KUB chicks. Therefore, the purpose of this study was to examine the effect of orally administered L- Cit on rectal temperature and some plasma biochemical parameters in KUB chicks.

MATERIALS AND METHODS

This research was conducted at the Poultry Division Field Laboratory, Faculty of Agriculture and Animal Science, State Islamic University of Sultan Syarif Kasim Riau, Indonesia in 2018.

Ethical approval

This experiment was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Animal, at the Faculty of Agriculture and Animal Science, State Islamic University of Sultan Syarif Kasim Riau, Pekanbaru, Indonesia

Animals

Thirty-two, one-day-old, KUB chicks were purchased from a local hatchery and housed in a wire-meshed cage (50 × 35 × 31 cm) at a constant temperature of 30 ± 1°C and with continuous light. Feed (Charoen Phokpand, Indonesia) and water were provided *ad libitum*. Feed composition is presented in table 1. One day before the experiment, chicks (4 days old) were weighed individually and assigned to treatment and control groups based on their body weight to produce uniform groups. The number of animals used in each group was kept to the minimum (n = 8) that would still ensure adequate statistical power.

Table 1. The percentage of nutrient content in ration

Nutrient	Value
Crude Protein (%)	23.50
Crude Fiber (%)	1.88
Crude Fat (%)	5.87
Ca (%)	0.29
P (%)	0.15
ME (Kcal/kg)	3,050

Ca: Calcium, P: Phosphor, ME: Metabolizable Energy; *Commercial feed: 311-VIVO, PT, Charoen Pokphand, Indonesia; **Mineral Premix: Supplemented for kg of the diets: Vit. A, 12000 IU; D3, 2000 IU; E, 20 mg; K3, 3 mg; B2, 7 mg; B3, 12 mg; B5, 3 mg; B12, 0.03 mg; biotin, 0.1 mg; choline chloride, 300 mg; Mn, 130 mg; Fe, 70 mg; Zn, 60 mg; Cu,12 mg; I,1 mg; Se, 0.2 mg, and adequate antioxidant.

Experimental design

After a habituation period, chicks were randomly selected and divided into four groups each consisting of eight chicks. The chicks were reared individually in experimental cages and had *ad libitum* access to diet up to the time of the experiment. On the day of the experiment, each chick (5 days old) received L-Cit orally (treatment groups) or distilled water (control group) by the plastic needle on small syringe. Based on our recent report on L- or D-Asp and L-Cit in layer chicks (Erwan et al., 2013b, 2014; Chowdhury et al., 2015), treatment groups received 3.75, 7.5 or 15.0 mmol/ kg body weight as the low, medium and high dose, respectively. The chicks were fed *ad libitum* diets for 2 h immediately after the treatment. At the end of the experiments (2 h after L-Cit administration), birds were decapitated under chloroform anesthesia. Blood samples were collected in heparinized tubes and centrifuged for 15 min at 5,000 g, and the plasma was collected and stored at −20 °C until analysis took place.

Analysis of plasma biochemical parameters

The biochemical parameters of plasma including total glucose, total cholesterol, and triacylglycerol were determined with Microlab 300 (Vital Scientific, Netherland) as per the manufacturer's instructions. All the samples were assayed together and in a random sequence for each metabolite.

Measurement of feed intake and rectal temperature

At 30, 60 and 120 min after L-Cit administration, feed intake was calculated by measuring the reduction in the amount of feed consumed from a pre-weighed feeder. Similarly, rectal temperature of chicks was determined at 30, 60 and 120 min after L-Cit administration using a digital thermometer with an accuracy of ±0.1°C (Thermalert TH-5, Physitemp Instruments Inc., USA). It took about 5 sec to measure rectal temperature by inserting the probe.

Statistical analysis

Data on feed intake and rectal temperature were analyzed by repeated-measures two-way analysis of variance (ANOVA). Plasma metabolites were statistically analyzed by one-way ANOVA and regression equations. When significant differences were found, the comparison of means was performed using Tukey's test as a post hoc test. Differences were considered significant at $p<0.05$. Values are presented as means ± SEM. Statistical analysis

was carried out using the commercially available package StatView (Version 5, SAS Institute, Cary, USA). Data were checked by the Thompson rejection test to eliminate outliers ($p<0.05$), and the remaining data were used for the analysis among groups.

RESULTS AND DISCUSSION

The aim of the current study was to reveal whether the L-Cit amino acid influenced feed intake and have thermoregulatory functions in KUB chicks. Feed intake was not significantly affected by oral administration of several doses of L-Cit (data not shown). This result was consistent with the previous report by Erwan et al. (2016) who revealed that the inclusion of watermelon rind in ration during two weeks did not influence feed intake in broiler chicks. However, the aforementioned authors demonstrated that feed consumption increased when chicks fed for three weeks. The present findings also contrasted with previous findings in layers where L-Cit given for up to 9% caused a significant increase in feed intake in male layer chicks (Nguyen et al., 2019). These discrepancies in the feed intake in response to L-Cit may be due to variations in the experimental period, differences in doses, or species differences. Further experimentation would be needed to clarify the factors involved in this disparity.

As shown in diagram 1, rectal temperatures of KUB chicks did not change significantly by the oral administration of L-Cit ($p >0.05$). This result was not consistent with the previous report that indicated the oral administration of L-Cit decreased the body temperature in layer and broiler chicks (Erwan and Febriyanti, 2015; Chowdhury et al., 2017). The body temperature of KUB chicks ranged from 39.5 to 40.5 °C, which was still in the normal range. The different effects observed in this study can be attributed to the difference in chicken type. It is well known that indigenous chickens are more adaptable to stress than commercial chickens. Duangjinda (2017) revealed that indigenous chickens from a tropical environment had lower heterophil-to-lymphocyte ratio and mortality rate than those of commercial broilers under heat stress conditions. Similarly, Aengwanich (2007) revealed that Thai indigenous chickens and Thai crossbreed chickens can tolerate higher temperatures than broiler chickens.

Table 2 shows the effect of oral administration of different doses of L-Cit on certain biochemical parameters of plasma, indicating no significant changes were observed ($p >0.05$). The effect of oral administration of L-Cit on total cholesterol, triacylglycerol, and glucose in the plasma of indigenous chicks is unclear. Watermelon rind contains a natural source of citrulline (Rimando and Perkins-Veazie, 2005). A previous study found that oral administration of red watermelon rind juice dose-dependently increased glucose and triacylglycerol in broiler chickens (Erwan et al., 2016). In contrast, Chowdhury et al. (2017) revealed that oral administration of L-Cit decreased glucose in layer male chicks. It is thought the differences in results may be due to different strains of chicken used.

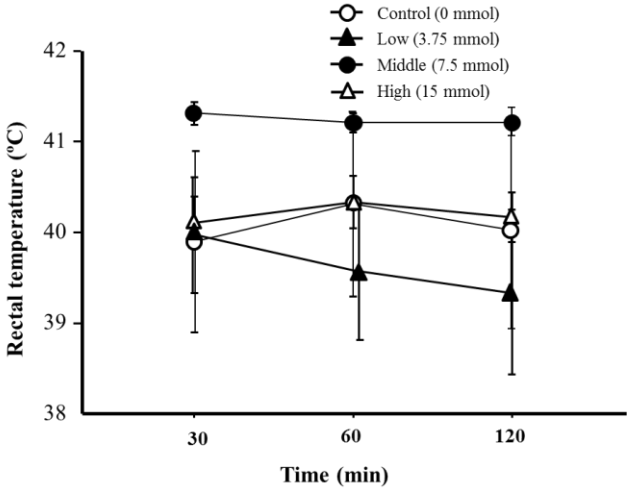

Diagram 1. The effect of different doses of L-citrulline on body temperature of KUB chicks over two hours after oral administration.

Table 2. Effects of oral administration of different doses of L-citrulline on plasma metabolites in KUB chicks

Parameters	L-citrulline (mmol)			
	0	3.75	7.5	15
Glucose (mg/d*l*)	237±20	229±14	261±8	226±18
Total cholesterol (mg/d*l*)	161±12	187±27	177±25	179±21
Triacylglycerol (mg/d*l*)	80±20	49±9	91±25	51±8

Values are expressed as mean ± SEM.

Heat stress affects plasma metabolites in poultry (Xie et al., 2015). An increase in plasma cholesterol indicates that chick has undergone heat stress (Olanrewaju et al., 2010). According to Altan et al. (2000), cholesterol in the body can come from *de novo* biosynthesis. *De novo* cholesterol biosynthesis is largely influenced by the broiler stress level (Setyadi et al., 2013). Blood cholesterol levels in this study ranged from 161 to 187 mg/dl, whereas Basmacioglu and Ergul (2005) reported that serum cholesterol contents of laying hens renged from 121to 142 mg/dl)

The results obtained in the present study indicated that triacylglycerol ranged from 49-91 mg/dl. When chickens are in a state of heat stress, plasma triacylglycerol levels drop (Sun et al., 2015). Environmental stress increases adrenocorticotropic hormone which stimulates the adrenal cortex to increase glucocorticoid secretion (von Borell and Ladewig, 1989). Blood glucose has a relationship with heat ambient temperature in poultry (Olanrewaju et al., 2010). Chowdhury et al. (2017) suggested that lower plasma glucose after oral administration of L-Cit may decrease the substrate for heat production and result in lower body temperature in chicks. Glucose in the blood serves as a buffer molecule for osmotic pressure thus normal osmotic pressure can be maintained even in a state of dehydration and heat stress (Guay et al., 2007) to maintain blood pressure and cardiovascular rhythms (Tan et al., 2010). In this study glucose concentration in chickens' blood ranged from 226-261 mg/dl. This range is included in the normal glucose levels, which indicates the chickens did not experience heat stress. Normal range glucose concentration in broiler blood is 230 - 370 mg/dl (Sulistyoningsih, 2004).

CONCLUSION

L-citrulline could not able to improve feed intake and affect on body temperature in KUB chickens. No significant changes in plasma glucose, total cholesterol and triacylglycerol were detected in the current study,indicating the oral administration of L-citrulline may not affect the metabolism of these components in KUB chicks.

DECLARATIONS

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Authors' contribution

Edi Erwan conducted the research, prepared data, performed the statistical analysis and wrote draft article. Vebera Maslami, Triani Adelina and Alaiddin Koto wrote the article. All authors checked and approved the final version of the manuscript.

Competing interests

The authors declare that they have no competing interests.

REFERENCES

Aengwanich W (2007). Comparative ability to tolerate heat between Thai indigenous chickens, Thai indigenous chickens crossbred and broilers by using heterophil/lymphocyte ratio. Pakistan Journal of Biological Sciences, 10: 1840-1844. DOI: https://doi.org/10.3923/pjbs.2007.1840.1844.

Altan O, Cabuk M and Bayraktar H (2000). Effect of heat stress on some blood parameters in broiler. Turkish Journal of Veterinary and Animal Sciences, 24: 145-148. Available at: https://www.researchgate.net/publication/279937711_Effects_of_Heat_Stress_on_Some_Blood_Parameters_in_Broilers.

Basmacioglu H and Ergul M (2005). Research on the factor affecting cholesterol content and some other characteristics of eggs in laying hens. Turkish Journal of Veterinary and Animal Sciences, 29:157-164. Available at: https://dergipark.org.tr/en/download/article-file/132841.

Chowdhury VS, Han G, Bahry MA, Tran PV, Do PH, Yang H and Furuse M (2017). L-Citrulline acts as potential hypothermic agent to afford thermotolerance in chicks. Journal Thermal Biology, 69:163-170. DOI: https://doi.org/10.1016/j.jtherbio.2017.07.007.

Chowdhury VS, Shigemura A, Erwan E, Ito K, Bahry MA, Tran V and Furuse M (2015). Oral administration of L-citrulline, but not Larginine or L-ornithine, acts as a hypothermic agent in chicks. The Journal of Poultry Science, 52: 331-335. DOI: https://doi.org/10.2141/jpsa.0150014.

Daghir NJ, Farran MT, Barbour GW and Beck MM (2003). Nutritive value of high-oil corn grown under semi-arid conditions and its impact on broiler performance and carcass composition. Poultry Science, 82:267–271. DOI: https://doi.org/10.1093/ps/82.2.267.

Duangjinda M, Tunim SI Duangdaen C and Boonkum WI (2017). Hsp70 genotypes and heat tolerance of commercial and native chickens reared in hot and humid condition. Brazilian Journal of Poultry Science, 19:7-17. DOI: http://dx.doi.org/10.1590/1806-9061-2016-0245.

Erwan E and Febriyanti R (2015). Studi potensi asam amino L-citrulline sebagai agen anti heat stress melalui oral administration pada ayam broiler. Laporan Penelitian. LPPM Uin Suska Riau.

Erwan E, Chowdhury VS, Ito K and Furuse M (2013a). Lauroyl-L-aspartate decreased food intake and body temperature in neonatal chicks. Pharmacology Biochemistry and Behavior, 13:7-11. DOI: https://doi.org/10.1016/j.pbb.2013.10.010.

Erwan E, Chowdhury VS, Nagasawa M, Goda R, Otsuka T, Yasuo S and Furuse M (2014). Oral administration of D-aspartate, but not L-aspartate, depresses rectal temperature and alters plasma metabolites in chicks. Life Sciences, 109: 65–71. DOI: https://doi.org/10.1016/j.lfs.2014.05.015.

Erwan E, Fitra D, Irawati E and Febriyanti R (2016). **S**tudi potensi kulit buah semangka sebagai sebagai agen *anti heat stress* pada ayam pedaging/broiler. Laporan penelitian, LP2M Uin Suska Riau

Erwan E, Tomonaga S, Ohmori T, Mutaguchi Y, Ohshima T, Nagasawa M and Furuse M (2013b). Oral administration of D-aspartate, but not of L-aspartate, reduces food intake in chicks. The Journal of Poultry Science, 50: 164–171. DOI: https://doi.org/10.2141/jpsa.0120116.

Erwan E, Tomonaga S, Yoshida J, NagasawaM, Ogino Y, Denbow DM and Furuse M (2012) Central administration of L- and D-aspartate attenuates stress behaviors by social isolation and CRF in neonatal chicks. Amino Acids, 43:1969–1976. DOI: https://doi.org/10.1007/s00726-012-1272-4.

Erwan E, Zulfikar, Saleh E, Kuntoro B, Chowdhury VS and Furuse M (2017). Orally administered D-aspartate depresses rectal temperature and alters plasma triacylglycerol and glucose

concentrations in broiler chick. The Journal of Poultry Science, 54:205-211. DOI: https://doi.org/10.2141/jpsa.0160010.

Franco-Jimenez DJ and Beck MM (2007). Physiological changes to transient exposure to heat stress observed in laying hens. Poultry Science, 86:538–44. DOI: https://doi.org/10.1093/ps/86.3.538.

Goo D, Kim JH, Park GH, Delos Reyes JB and Kil DY (2019). Effect of heat stress and stocking density on growth performance, breast meat quality, and intestinal barrier function in broiler chickens. Animals, 9:1-10. DOI: https://doi.org/10.3390/ani9030107.

Hamasu K, Shigemi K, Tsuneyoshi Y, Yamane H, Sato H, Denbow DM and Furuse M (2010). Intracerebroventricular injection of L-proline and D-proline induces sedative and hypnotic effects by different mechanisms under an acute stressful condition in chicks. Amino Acids, 38:57–64. DOI: https://doi.org/10.1007/s00726-008-0204-9.

Hidayah R, Ambarsari I and Subiharta S (2019). Study of physical, nutritional and sensory properties KUB chicken meat in Central Java**.** Jurnal Peternakan Indonesia, 21 (2): 93-101. DOI: https://doi.org/10.25077/jpi.21.2.93-101.2019.

Lin H, Mertens K, Kemps B, Govaerts T, De Ketelaere B, De Baerdemaeker J, Decuypere E and Buyse J (2004). New approach of testing the effect of heat stress on egg quality: mechanical and material properties of eggshell and membrane. British Poultry Science, 45:476–82. DOI: https://doi.org/10.1080/00071660400001173.

Mendes AA, Watkins SE, England JA, Saleh EA, Waldroup AL and Waldroup PW (1997). Influence of dietary lysine levels and arginine: lysine ratios on performance of broilers exposed to heat or cold stress during the period of three to six weeks of age. Poultry Science, 76: 472–481. DOI: https://doi.org/10.1093/ps/76.3.472.

Nguyen LTN, Han G, Yang H, Ikeda H, Eltahan HM, Chowdhury VS and Furuse M (2019). Dried watermelon rind mash diet increases plasma L-citrulline level in chicks. The Journal of Poultry Science, 56: 65-70. DOI: https://doi.org/10.2141/jpsa.0180018.

Olanrewaju HA, Purswell JL, Collier SD and Branton SL (2010). Effect of ambient temperature and light intensity on physiological reactions of heavy broiler chickens. Poultry Science, 89: 2668-2677. DOI: https://doi.org/10.3382/ps.2010-00806.

Padhi MK (2016). Importance of indigenous breeds of chicken for rural economy and their improvements for higher production performance, Scientifica, 2016, 1-9. DOI: http://dx.doi.org/10.1155/2016/2604685.

Purbarani SA, Wahyuni HI and Suthama N (2019). Dahlia inulin and *Lactobacillus sp.* in step down protein diet on villi development and growth of KUB chickens. Tropical Animal Science Journal, 42:19-24. DOI: https://doi.org/10.5398/tasj.2019.42.1.19

Renaudeau D, Collin A, Yahav S, de Basilio V, Gourdine JL and Collier RJ (2012). Adaptation to hot climate and strategies to alleviate heat stress in livestock production. Animal, 6:707-728. DOI: https://doi.org/10.1017/S1751731111002448.

Rimando AM and Perkins-Veazie PM (2005). Determination of citrulline in watermelon rind. Journal of Chromatography A, 1078:196-200. DOI: https://doi.org/10.1016/j.chroma.2005.05.009.

Sartika T (2016). Panen Ayam Kampung 70 hari. Penebar Swadaya, Jakarta.

Setyadi F, Ismadi VDYB dan Mangisah I (2013). Kadar kolesterol, HDL dan LDL darah akibat kombinasi lama pencahayaan dan pemberian porsi pakan berbeda pada ayam broiler. Animal Agriculture Journal, 2: 68–76. Available at: https://ejournal3.undip.ac.id/index.php/aaj/article/view/2045/2043.

Sterling KG, Bell DD, Pesti GM and Aggrey SE (2003). Relationships among strain, performance, and environmental, temperature in commercial laying hens. Journal of Applied Poultry Research, 2:85–91. DOI: https://doi.org/10.1093/japr/12.1.85.

Sulistyoningsih M (2004). Respon Fisiologis dan Tingkah Laku Ayam Brolier Starter Akibat Cekaman Tempratur dan Awal Pemberian Pakan yang Berbeda. Tesis. Magister Ilmu Ternak Program Pasca Sarjana Universitas Diponegoro. Semarang.

Sun X, Zhang H, Sheikhahmadi A, Wang Y, Jiao H, Lin H and Song Z (2015). Effects of heat stress on the gene expression of nutrient transporters in the jejunum of broiler chickens (*Gallus gallus domesticus*). International Journal Biometeorology, 59:127-135. DOI: http://dx.doi.org/10.1007/s00484-015-0996-8.

Tan GY, Yang L, Fu YQ, Feng JH and Zhang MH (2010). Effects of different acute high ambient temperatures on function of hepatic mitochondrial respiration, antioxidative enzymes and oxidative injury in broiler chickens. Poultry Science, 89: 115–122. DOI: https://doi.org/10.3382/ps.2009-00318.

Teeter RG and Belay T (1996). Broiler management during acute heat stress. Animal Feed Science and Technology, 58:127–142. DOI: https://doi.org/10.1016/0377-8401(95)00879-9.

Von Borell E and Ladewig J (1989). Altered adrenocortical response to acute stressors or ACTH (1–24) in intensively housed pigs. Domestics Animal. Endocrinology, 6:299–309. DOI: https://doi.org/10.1016/0739-7240(89)90024-6.

Xie J, Tang L, Lu L, Zhang L, Xi L, Liu HC, Odle J and Luo X (2014). Differential expression of heat shock transcription factors and heat shock proteins after acute and chronic heat stress in laying chickens (*Gallus gallus*). PLoS One, 9: 1-9. DOI: https://doi.org/10.1371/journal.pone.0102204.

Yahav S (2000). Domestic fowl-strategies to confront environmental conditions. Avian and Poultry Biology Reviews, 11:81–95.

Yamane H, Asechi M, Tsuneyoshi Y, Kurauchi I, Denbow DM and Furuse M (2009). Intracerebroventricular injection of L-aspartic acid and L-asparagine induces sedative effects under an acute stressful condition in neonatal chicks. Animal Science Journal, 80:286–290. DOI: https://doi.org/10.1111/j.1740-0929.2009.00625.x.

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Effects of Cordyceps Militaris Polysaccharide on Egg Production, Egg Quality and Caecal Microbiota of Layer Hens

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ABSTRACT

The present study was conducted to determine the effects of the diet supplementation of laying hens with Cordyceps Militaris Polysaccharide (CMP) on egg production and quality, and also caecal microbiota. A total of 360 Hy-Line Brown laying hens with 72-week-old were divided into three groups with four replicates of 30 birds each. The laying hens were fed with basal diet (control group), basal diet +100 mg CMP/kg (group 1) and basal diet + 200 mg CMP/kg (group 2). The experiment lasted 45 days. Eggs were collected daily and caecal samples were collected at the end of the experiment. Results showed that dietary supplementation with CMP did not affect albumen height, shape index, Haugh units, eggshell breaking strength and eggshell thickness. The laying rate significantly increased and the feed-egg ratio decreased in groups 1 and 2, the average daily egg weight significantly increased in group 2 compared with those in the control group. However, insignificant differences were found in broken egg rate and mortality among the three groups. The differences in caecal microbiota between group one and the control group were significant. The relative abundance of Firmicutes, Bacteroidetes and Proteobacteria at the phylum level, Christensenellaceae and Veillonellaceae at the family level, and the no rank_ Ruminococcaceae, Phascolarctobacterium and no rank Christensenellaceae at the genus level changed significantly in group one compared with those in the control group. In conclusion, dietary supplementation with 100 and 200 mg CMP/kg could improve product performance and affect the caecal microbial community structure of laying hens during the late laying period.

Key words: Cordyceps militaris polysaccharide, Egg production, Egg quality, Gut microbiome, Hens

INTRODUCTION

Antibiotics, a feed additive to improve animal production performance and disease resistance, are widely used in animal production. However, abuse of antibiotics during feeding had led to drug resistance of pathogens, which led to an imbalance in the intestinal flora of livestock and poultry (Chang et al., 2015). Antibiotics residues in livestock products and caused environmental pollution (Martinez, 2009). The researchers were therefore trying to find better antibiotic alternatives for laying hens.

Cordyceps Militaris Polysaccharide (CMP), one of the most important active substances from C. militaris, was composed of mannose, cordycepin, adenosine, arabinose, and fucose. CMPs extracted from C. militaris and C. sinensis had the same effect, and C. sinensis was rare, so Cordyceps polysaccharide was often extracted from C. militaris (Das et al., 2010). Studies had shown that Cordyceps polysaccharides had some positive effects on improving immunity, reducing blood lipids and protecting the liver and the kidney (Cheung, et al., 2009; Lee, et al., 2010a; Shin, et al., 2010). Cordyceps polysaccharides, as a feed additive had received wide attention in Asia due to their diverse functions in the livestock industry. For example, Cordyceps polysaccharides could promote the early growth of chickens, increase the feed conversion ratio and enhance the daily growth gain of broilers (Han et al., 2015). C. militaris waster medium increased the egg production rate and decreased the feed- egg ratio (F/G) (Wang et al., 2015). Hot-water extract from the mycelia of C. sinensis maintained the better microbial flora from the intestines of chickens and improved the growth performance and health index of chickens (Koh et al., 2003).

Microbial communities in the intestine played a

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crucial role in the health and function of hosts. Animal intestinal flora was a complex and diverse system and could easily be influenced by many factors, such as environment, age, nutrition, feed additive and hygiene level. Nutritional differences were the main cause of the overall variations, suggesting that food components could influence the composition and diversity of the intestinal flora (Zhang et al., 2010). Chicken egg production and quality generally decreased with age, reducing the agricultural profits of poultry farmers. It was therefore important to keep the egg production at a high level. There had been few reports on the effects of CMP as a feed additive on poultry nutrition, particularly on the intestinal flora in the late laying period. Hence, this study was conducted to determine the effects of CMP supplementation on egg production and quality, and also caecal microbiota during the late laying period of chickens and to provide valuable information on the use of CMP as feed additives.

MATERIALS AND METHODS

Ethical approval

This research did not involve the introduction of any intervention on hens. The data collcetion was obtained with humanly handled, which according of animal care and welfare standard of The People's Republic of China. Animal experimentation procedures were approved by the Institutional Animal Care and Use Committee of Henan University of Science and Technology, Luoyang, China.

Preparation of Cordyceps militaris polysaccharide

Freeze-dried C. militaris polysaccharide was purchased from Yangshao Biology and Chemical Engineering Company, Mianchi City, Henan Province, China.

Laying hens, diets and experimental design

A total of 360 Hy-Line Brown laying hens at 72-week of age were assigned to three groups with four replicates of 30 hens in each group. The hens were housed in 120 cages sized 64 cm ×35 cm ×35 cm (three hens each) During the experiment, the hens were free to obtain feed and water and were exposed to a light dark cycle of 16 hours: 8 hours. The average room temperature was 20 ± 3°C. Before the experiment started, there was no differences in the egg production rate between the three groups. The laying hens were fed basal diet (control group), and basal diet + 100 mg /kg CMP (group 1) and basal diet + 200 mg/kg CMP (group 2). The entire process included a 7-day pre-experiment and a 45-day formal experiment. The diets consisted of corn-based basal rations formulated according to the Management Guide of National Research Council (NRC, 1994) to meet the nutritional requirements of laying hens. Composition of basal feed and nutrients content in proximate analysis were showed in Table 1.

Table 1. Composition and nutrients content of basal feed

Feedstuffs ingredients	**Percentage (%)**
Corn grain	64.50
Soybean meal	13.00
Rapeseed meal	4.00
Limestone	8.00
Premix[1]	5.00
Wheat bran	5.00
Soybean oil	0.50
Total	100
Analyzed nutrient composition	**Content**
Dry matter (%)	88.89
Ash (%)	13.87
Crude Fiber (%)	4.17
Crude Protein (%)	19.90
Crude Fat (%)	4.59
Gross Energy (MJ/ kg)	16.58
Metabolizable Energy[2] (MJ/ kg)	11.61

[1] = Provide per kg of diet: vitamin A, 4000 IU; vitamin D3, 1200 IU; vitamin E, 6 mg; vitamin B1, 1.4 mg; vitamin B2, 3 mg; vitamin B6, 1.0 mg; vitamin B12, 0.01 mg; pantothenic acid, 7.5 mg; choline chloride, 500 mg; biotin, 0.15 mg; Ca,7500 mg; P, 3000 mg; Mn, 72 mg; Zn, 56 mg; Fe, 60 mg; Cu, 25 mg; I, 0.50 mg; Se, 0.10 mg. [2] = Metabolizable energy was calculated from data provided by Feed Database in China 2009. MJ/ kg = Mega Joule per kilogram

Egg production

During the experimental period, daily records of egg production, egg cracking, egg weight and mortality of hens and records of feed consumption were maintained. The Lay rate (including cracked eggs), average daily egg weight, cracked egg rate, and feed to egg ratio were analyzed.

Egg quality

Six saleable eggs (no shell defects or cracks) were randomly selected from each replication on day 45 and used to determine egg quality, egg albumen height and Haugh units were determined with a digital egg tester (TSS, England, UK), eggshell breaking strength was identified with a strength instruction (In-spec 2200, Instron Corporation, Canton, Massachusetts, USA), egg shape index was assessed using the following formula including vertical diameter/transect diameter, egg diameter

was assessed with a vernier caliper and eggshell thickness was assessed with a Spiral range finder.

Caecal microbiome

Four hens were randomly selected from the control group and group 2 (one for each replication) and sacrificed on the 45th day. The caecum samples were collected aseptically from each bird, immediately stored at −80 °C and processed promptly.

DNA extraction and PCR amplification

According to the manufacturer's protocol, an E.Z.N.A.® soil DNA kit (Omega Bio-tek, Norcross, GA, USA) was used to extract microbial DNA from cecum samples. Final DNA concentration and purification were determined using a Nano Drop 2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington, USA), and DNA quality was checked through 1% agarose gel electrophoresis. The hypervariable regions (variable 3-variable 4) of the bacterial 16S rRNA gene were amplified with the primers 341F (5′-CCTAYGGGRBGCASCAG-3′) and 806R (5′-GGACTACNNGGGTATCTAAT-3′) by using a thermocycler PCR system (GeneAmp 9700, ABI, USA). The PCR was conducted by denaturising at 95 Centigrade (°C) for 5 minutes (min.), followed by 27 cycles at 95 °C for 30 second (s), 55 °C for 30 s, 72 °C for 45 s, and a final extension at 72 °C for 10 min. PCR was performed in triplicate with a 20 μL mixture containing 4 μL of 5× FastPfu Buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL of each primer (5 μM), 0.4 μL of FastPfu polymerase and 10 ng of template DNA. PCR products were electrophoresed using a 2% agarose gel, purified using AxyPrep DNA gel extraction kit (Axygen Biosciences, Union City, CA, USA), and quantified using QuantiFluor™ -ST (Promega, USA) in accordance with the manufacturer's protocol.

Illumina MiSeq sequencing

Purified amplificates were separated pooled in equimolar and paired-end sequenced (2×300) on an Illumina MiSeq platform (Illumina, San Diego, USA) according to the standard protocols of Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China). The raw data was stored into the NCB1Sequence Read Archive database (accession nos. SRP154946 and PRJNA555247).

Sequencing data processing

Trimmomatic were used to demultiplex and do quality-filter for the raw fastq files, then merged them by FLASH according to the following criteria. First, reads were truncated at any site receiving an average quality score of < 20 over a 50 bp sliding window; then, primers were exactly matched, allowing two nucleotide mismatching, and the reads containing ambiguous bases were removed; eventually, sequences with overlaps longer than 10 bp were merged on the basis of their overlap sequence.

Using UPARSE version 7.1 the operational taxonomic units (OTUs) were clustered with a 97% similarity cut-off, the UCHIME were used to identify and remove chimeric sequences. RDP Classifier algorithm were used to analyse the taxonomy of each 16S rRNA gene sequence, which against the Silva (SSU123) 16S rRNA database with a confidence threshold of 70%.

Data analysis

Data were analyzed by one-way ANOVA using SPSS 22.0 (SPSS Inc., Chicago, IL, USA). The significance of the mean differences between the groups was identified by a Tukey test. Data significance was determined at $p < 0.05$. Alpha diversity and dilution curve analyses consisted of community diversity (simpson and shannon), richness (sobs, chao and ace) were performed using mothur（a 16S rRNA clustering tool）based on a single summary command. Beta diversity analysis was carried out to investigate the diversity between the samples. Principal component analysis (PCA) was conducted with mothur to describe the distances between the samples, and a permutative multivariate analysis of variance (PERMANOVA) was performed to analyze the significance of the OTU levels. Wilcoxon rank sum test was used to determine the significant differences in the relative abundance of bacteria at phylum and genus level between the CMP and the control group.

RESULTS

Egg production

Table 2 shows the laying performance of hens. The egg production increased significantly and the feed-egg ratio decreased ($p < 0.01$) in groups 1 and 2 compared to those in the control group. The average daily egg weight increased significantly in group 2 ($p < 0.05$). However, insignificant differences in the cracked egg rate and mortality were found among the three groups ($p > 0.05$). The egg production in group 2 increased significantly compared to group 1 ($p < 0.01$).

Egg quality

Table 3 shows the egg quality of hens. Insignificant differences were observed in the egg albumen height, the egg shape index, the Haugh units, eggshell breaking strength and eggshell thickness between three groups ($p > 0.05$).

Caecal microbiota

A total of 380,227 DNA sequence read with an average length of 415 bp were generated from all of samples. The average number of sequence readings per sample was 47,528, with a minimum number of 35,454 and a maximum number of 58,207. The sequences were further clustered into 749 OTUs by using a 97% similarity cut off. The sparse curve generated by the OTU shows that all samples achieved a high sampling coverage (~ 99%) (Figure 1). Table 4 shows the alpha diversity indices of caecal microbiota. The average values of ace, chao and sobs in the CMP group were significantly lower than those in the control group.

The PCA on the OTU level showed the changes in the community structure (Figure 2). The microbial community of the CMP group was clearly separated from that of the control group. The results revealed a significant difference in the bacterial structure in the caecum between the CMP and control groups (PERMANOVA, $p = 0.026$).

Figure 3 and table 5 demonstrate the phylum distributions of the microbial composition. In the CMP group and the control group, the main flora were Firmicutes, Bacteroidetes and Proteobacteria. Firmicutes significantly decreased in the CMP group compared with that in the control group ($p < 0.05$). In contrast, the proportions of Bacteroidetes in the CMP group increased compared to those in the control group ($p < 0.05$). The two phyla with a low relative abundance (Synergistetes and Elusimicrobia) decreased significantly in the CMP group compared to those in the control group ($p < 0.05$).

The family distribution results of the microbial composition are shown in figure 4 and table 6. The main families of the CMP and control groups were Ruminococcaceae, Bacteroidales and Lachnospiraceae. Among them, Ruminococcaceae was the most abundant. The two families with a low relative abundance (Veillonellaceae and Christensenellaceae) decreased significantly in the CMP group compared to those in the control group ($p < 0.05$).

The microbial composition was also comparable at the genus level (Figure 5 and table 7). The main genera of the CMP and control groups were norank Bacteroidales, norank Ruminococcaceae, Bacterorides Bacteroidaceae and Oscillospira. Among these genera, the relative abundance of norank Ruminococcaceae decreased significantly in the CMP group compared to those in the control group ($p < 0.05$). The two genera with a low relative abundance (Phascolarctobacterium and norank_ Christensenellaceae) decreased significantly in the CMP group compared to those in the control group ($p < 0.05$).

Table 2. Effects of Cordyceps militaris polysaccharide on laying performance in 72-week-old laying hen at Louyang,China.

Items [1] \ Treatment Groups	Control	Group 1	Group 2	SEM[2]	P value
Egg production (%)	79.95^{c}	83.26^{b}	85.55^{a}	0.508	<0.01
Cracked egg rate (%)	0.26	0.39	0.22	0.084	0.45
Average daily egg weight (g)	64.14^{b}	64.97^{ab}	65.62^{a}	0.221	0.02
Feed to egg ratio	2.17^{a}	1.96^{b}	1.98^{b}	0.023	<0.01
Mortality (%)	4.17	3.33	4.17	0.804	0.71

[1] Control, Group 1, Group 2 with 0, 100, 200 mg CMP/kg, respectively; [2] SEM= pooled standard error of mean. [a,b] = Means not sharing the same superscripts in a row differ significantly ($p < 0.05$).

Table 3. Effects of Cordyceps Polysaccharide on egg quality in 72-week-old laying hen at Louyang, China.

Items [1] \ Treatment Groups	Control	Group 1	Group 2	SEM[2]	P value
Egg shape index	1.25	1.35	1.30	0.024	0.21
Eggshell thickness (mm)	0.35	0.34	0.31	0.012	0.28
Eggshell breaking strength (kg/cm^2)[3]	2.48	3.37	3.61	0.361	0.46
Albumen height (mm)	5.41	5.43	5.34	0.282	0.49
Haugh unit	70.24	70.43	71.56	2.016	0.86

[1]Control, Group 1, Group 2 with 0, 100, 200mgCMP/kg, respectively; [2]SEM= pooled standard error of mean. [3]kg/cm^2= Kilogram per square centimetre

Table 4. Alpha Indices data of caecum samples in 72-week-old laying hen at Louyang,China.

Estimators	CON-Mean[1]	CON-Sd[2]	CMP-Mean	CMP-Sd	P Value
Ace	710.73	15.594	629.72	42.39	0.012
Chao	712.82	12.958	638.82	45.648	0.021
Coverage	0.99834	0.0007961	0.9979	0.00093558	0.502
Shannon	5.1584	0.080538	4.9022	0.25779	0.107
Simpson	0.011827	0.0018573	0.017143	0.0077245	0.230
Sobs	664.75	27.244	577.5	49.89	0.022

[1]Mean= the mean number; [2]Sd = the standard deviation; CON = control group; CMP = Cordyceps Militaris Polysaccharide

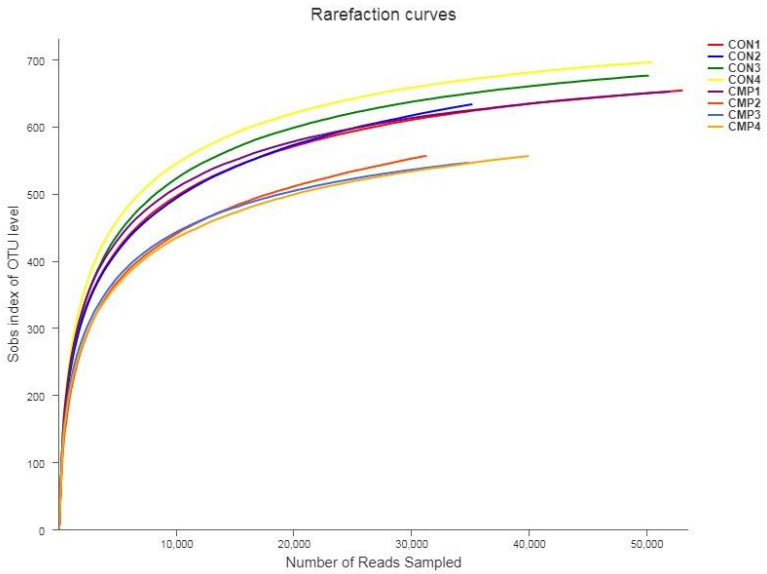

Figure 1. Rarefaction curves of samples (n=8) clustered at 97% sequences identity. OTU= Operational Taxonomic Unit; CON= control group; CMP= Cordyceps Militaris Polysaccharide.

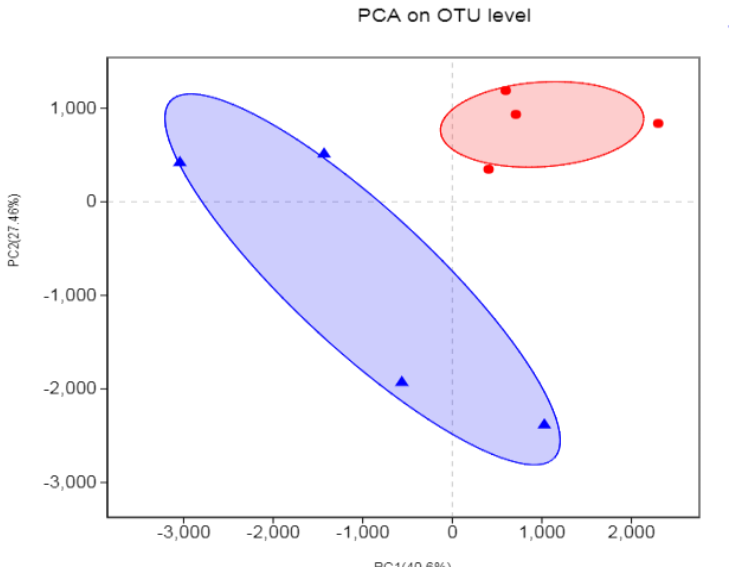

Figure 2. Principal Component Analysis (PCA) of the community membership using Bray-Curtis distance. CON = control group; CMP= Cordyceps Militaris Polysaccharide. The abscissa and ordinate represent the two selected principal components, and the percentage represents the contribution of the principal component to the difference in sample composition. Points of

different colors and shapes represent samples of different groups, and the closer the two sample points are, the more similar the composition of the two samples species is.

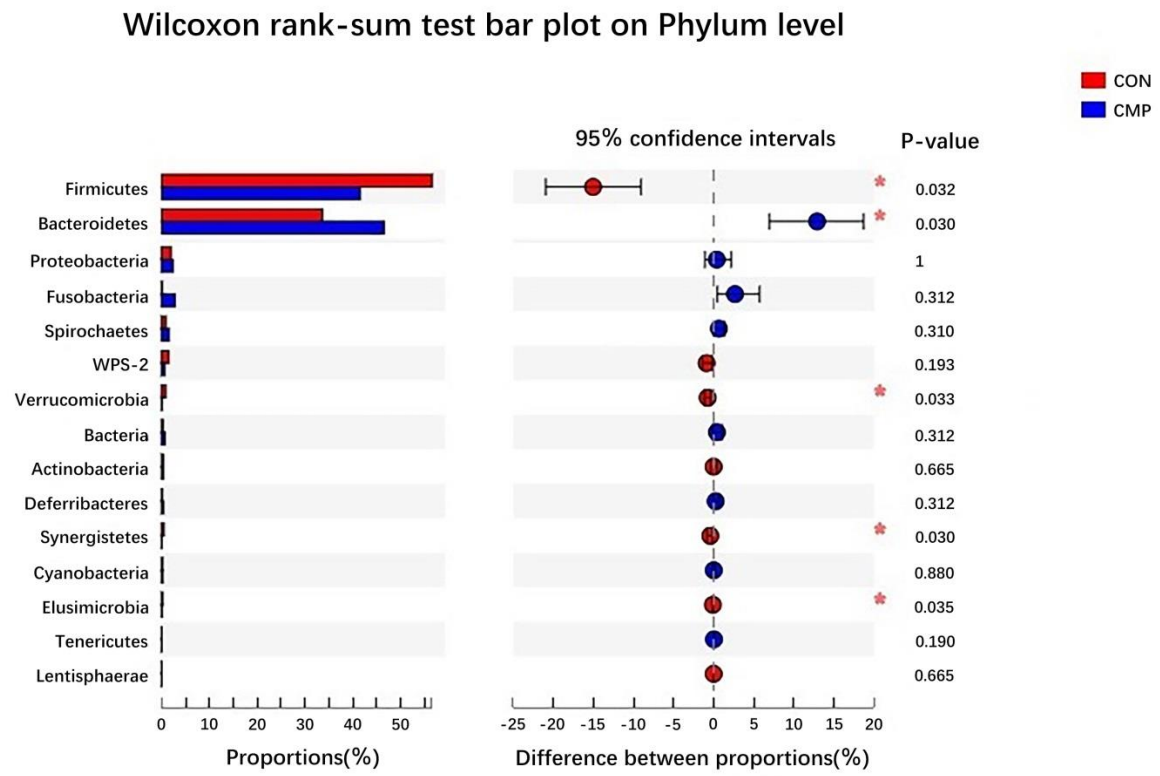

Figure 3. Welch's t-test bar plot on Phylum level. CON = control group; CMP = Cordyceps Militaris Polysaccharide. The ordinate (left) represents the phyla name and the ordinate (right) represents the *P* -Value.

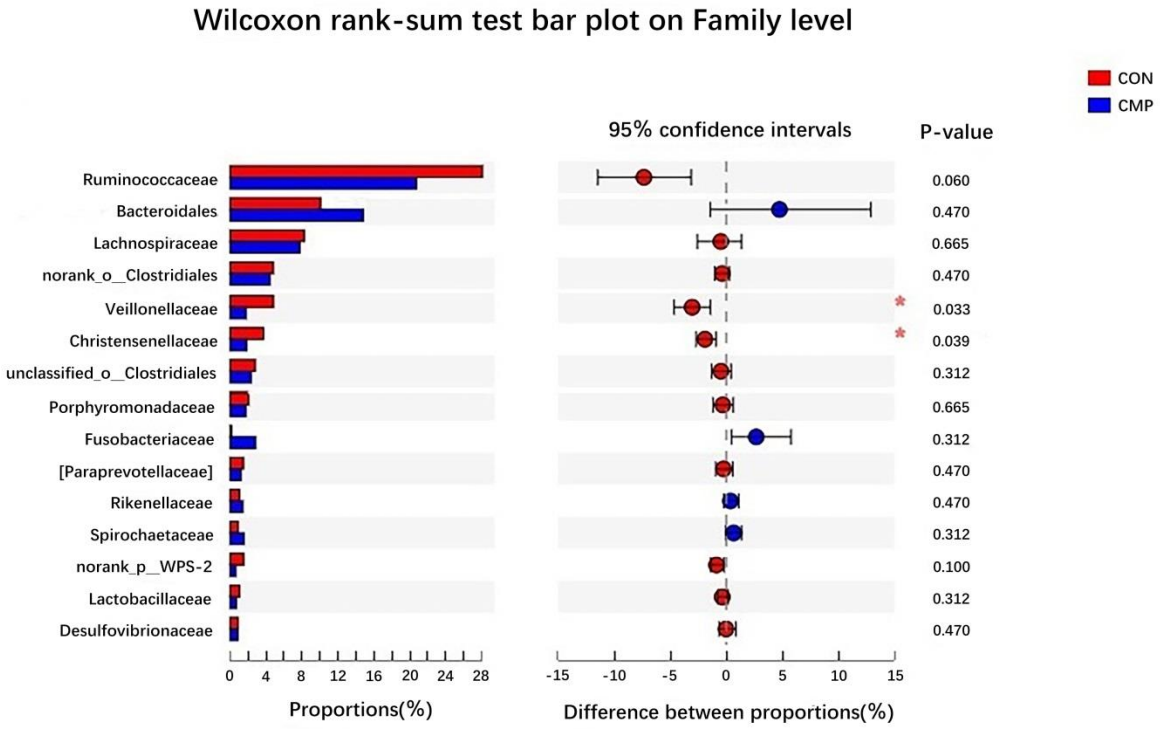

Figure 4. Welch's t-test bar plot on Family level. CON= control group; CMP = Cordyceps Militaris Polysaccharide. The ordinate (left) represents the family name and the ordinate (right) represents the *p*-Value.

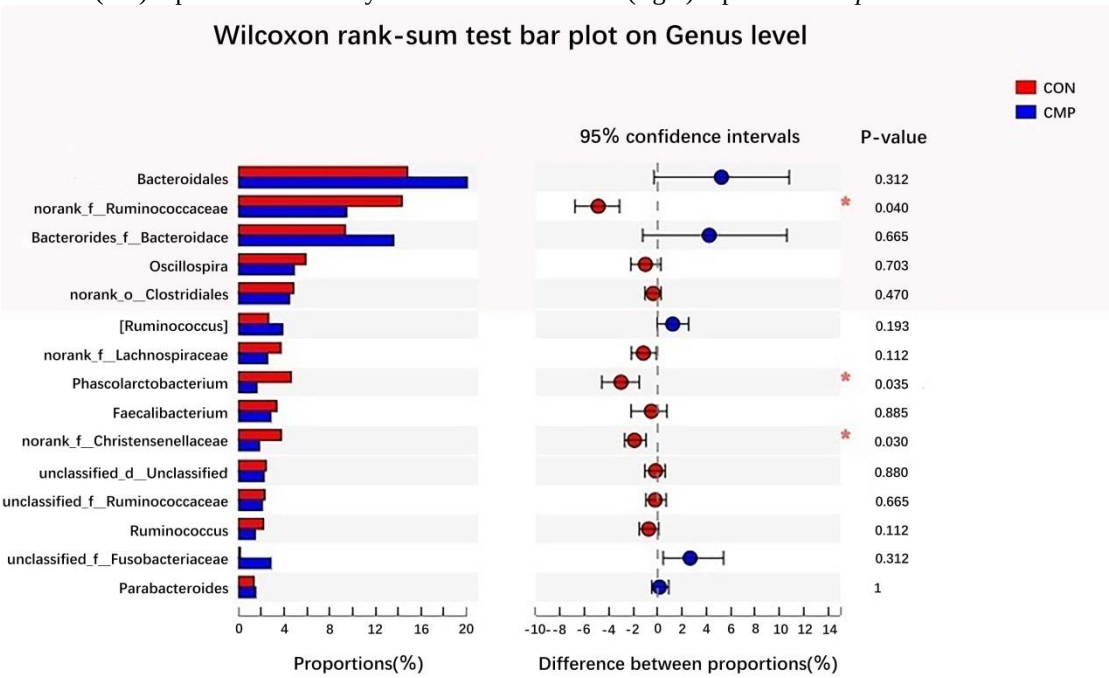

Figure 5. Welch's t-test bar plot on Genus level. CON= control group; CMP = Cordyceps Militaris Polysaccharide. The ordinate (left) represents the genus name and the ordinate (right) represents the p-Value.

Table 5. Wilcoxon rank sum test data on Phylum level (cut off at 1%).

Species name	CMP-Mean[1] (%)	CMP-Sd[2] (%)	CON-Mean (%)	CON-Sd (%)	P value
Firmicutes	41.61	6.022	56.65	4.489	0.032
Bacteroidetes	46.57	6.96	33.66	1.768	0.030
Proteobacteria	2.339	1.855	1.952	0.7248	1.000
Fusobacteria	2.827	3.139	0.1601	0.06761	0.312
Spirochaetes	1.559	0.7397	0.9031	0.3315	0.312

[1]Mean = the percentage of the mean number of Phylum; [2]Sd = the percentage of standard deviation of Phylum number; CON = control group; CMP = Cordyceps Militaris Polysaccharide.

Table 6. Wilcoxon rank sum test data on Family level (cutoff at 1%).

Species name	CMP-Mean[1] (%)	CMP-Sd[2] (%)	CON-Mean (%)	CON-Sd (%)	P value
Ruminococcaceae	20.74	4.057	28.08	3.225	0.060
Bacteroidales	14.81	8.024	10.07	0.7542	0.470
Lachnospiraceae	7.763	1.558	8.253	1.746	0.665
norank_o__Clostridiales	4.45	0.5395	4.818	0.5201	0.470
Veillonellaceae	1.784	0.9881	4.831	1.653	0.030
Christensenellaceae	1.832	0.9219	3.735	0.4204	0.039
unclassified_o__Clostridiales	2.329	0.9786	2.805	0.3763	0.312
Porphyromonadaceae	1.769	1.058	2.084	0.2	0.665
Fusobacteriaceae	2.827	3.139	0.1601	0.0676	0.312
Paraprevotellaceae	1.233	0.8325	1.478	0.4542	0.470
Rikenellaceae	1.433	0.7874	1.072	0.1486	0.470
Spirochaetaceae	1.559	0.7397	0.9031	0.3315	0.312
norank_p__WPS-2	0.6253	0.6725	1.501	0.2236	0.193

Lactobacillaceae	0.7092	0.5013	1.071	0.2273	0.312

[1]Mean = the percentage of the mean number of Family; [2]Sd = the percentage of standard deviation of Family number; CON = control group; CMP = Cordyceps Militaris Polysaccharide.

Table7. Wilcoxon rank sum test data on Genus level (cutoff at 1%).

Species name	CMP-Mean[1] (%)	CMP-Sd[2] (%)	CON-Mean (%)	CON-Sd (%)	P value
norank_o__Bacteroidales	20.05	6.837	14.82	0.3941	0.312
norank_f__Ruminococcaceae	9.476	1.981	14.35	0.838	0.040
Bacterorides_f__Bacteroidaceae	13.59	7.388	9.351	0.7964	0.665
Oscillospira	4.879	1.219	5.884	0.8032	0.470
norank_o__Clostridiales	4.45	0.5395	4.818	0.5201	0.470
Ruminococcus	3.85	1.248	2.612	0.8816	0.193
norank_f__Lachnospiraceae	2.534	0.9579	3.701	0.8048	0.112
Phascolarctobacterium	1.611	0.8582	4.618	1.682	0.035
Faecalibacterium	2.824	1.488	3.339	0.7728	0.885
norank_f__Christensenellaceae	1.832	0.9219	3.735	0.4204	0.030
unclassified_d__Unclassified	2.233	0.2937	2.405	0.9561	0.885
unclassified_f__Ruminococcaceae	2.087	0.7055	2.294	0.6626	0.665
Ruminococcus	1.447	0.7955	2.184	0.6644	0.112
unclassified_f__Fusobacteriaceae	2.827	3.139	0.1601	0.0676	0.312
Parabacteroides	1.501	0.8043	1.349	0.1146	1.000
norank_f__Rikenellaceae	1.432	0.7874	1.071	0.1485	0.470
Treponema	1.559	0.7397	0.9031	0.3315	0.312
norank_f__Paraprevotellaceae	1.122	0.7804	1.197	0.3533	0.470
unclassified_f__Lachnospiraceae	0.8786	0.1701	1.352	0.2803	0.061
norank_p__WPS-2	0.6253	0.6725	1.501	0.2236	0.194
unclassified_f__Bacteroidaceae _o__Bacteroidales	1.219	0.7175	0.7158	0.132	0.312
Lactobacillus	0.7086	0.5018	1.07	0.2276	0.312

[1]Mean = the percentage of the mean number of Genus; [2]Sd = the percentage of standard deviation of Genus number; CON = control group; CMP = Cordyceps Militaris Polysaccharide.

DISCUSSION

Effects of CMP on egg production and egg quality

It has been clinically confirmed that *C. militaries* had several functions, such as antitumor, anti-inflammatory and antioxidant effects (Chen, et al., 2010; Lee, et al., 2010b; Li, et al., 2015). *Cordyceps* polysaccharide isolated from *C. militaries* could suppress a cyclophosphamide-induced immune response to improve lymphocyte and macrophage activities, increased superoxide dismutase, catalase and glutathione peroxidase levels and reduced malondialdehyde content in the blood in mice (Wang, et al., 2012). However, information on the impact of CMP supplementation on laying hens is limited. Researchers emphasized that feeding laying hens with *C. militaries* waster medium decreased significantly feed conversion ratio (FCR) and increased egg production. They also showed that ascorbic acid and *C. militaries* waster mediuman have approximately the same reducing power (Wang et al., 2015) Cifticiet al. indicated that ascorbic acid can partially inhibit adverse oxidative protein denaturation and improve nutrient digestibility and FCR (Ciftici,et al.,2005). As such, the feed-egg ratio of the hens receiving the added CMP in the present study was likely to decrease and egg production to increased.

Eggshell thickness, eggshell strength, Haugh unit and egg shape index were important indicators for egg quality measurement, and these parameters were related to the shelling rate and freshness of eggs. In the present study, the differences in the egg quality between the three groups were insignificant. Similarly, scholars observed that supplementing a diet with *C. militaries* waster medium did not improve eggshell thickness and egg shell strength (Wang et al., 2015).

Effects of CMP on ceacal microbiota

Chicken intestinal microflora had a positive effect on food intake and the immune system (Shang et al., 2018b). Studies had shown that a well-functioning caecum could cover 10% of the body's energy needs (Hegde, et al., 1982; Józefiak, et al., 2004).

Our study determined the alpha diversity of microbial communities by measuring Sobs, Shannon's, Simpson's, Ace and Chao indices. Shannon's and Simpson's indices represent the degree of differences in the frequencies of different taxa (diversity), while Sobs, Chao and Ace indices reflect the number of different taxa present in the

sample (richness). The present study showed the significant differences in the caecal microbial density between the CMP and control groups. Consistent with previous findings (Bederska-ojewska et al., 2017), our results revealed significantly lower averages for microbial diversity and richness in the CMP group compared to those in the control group. Scientists found that the addition of cordycepin had an antimicrobial function similar to tetracycline and chloramphenicol (Ahn et al., 2000). These results indicated that CMP supplementation appeared to reduce the bacterial species in the caecal intestine of chicken. Further studies were needed to investigate the specific mode of action of CMP.

At the phylum level, differences in microbial structures could mostly be attributed to changes in the microbiome in Firmicutes, Bacteroidetes, Synergistetes and Elusimicrobia in the CMP group. In accordance with a previous research (Pan and Yu, 2014), the present study also found that Firmicutes, Bacteroidetes and Proteobacteria accounted for more than 90% in the chicken caecal microflora. The relative abundance of Bacteroidetes in the CMP group was higher than in the control group, while the Firmicutes content decreased significantly. Bacteroides could digest complex carbohydrates and maintain an intestinal micro-ecological balance (Józefiak et al., 2004; Sears 2005). Bacteroides could promote the development of the immune system (Spence et al., 2006). Bacteroidetes was related to the development of interleukin-17-producing T-helper cells (Mazmanian et al., 2005). Some studies have shown that with the increase of body mass index (BMI), the content of a increases gradually, while the content of B decreases (Koliada et al., 2017). Some studies had found differences between infants, adults and the elderly in relation to the Firmicutes to Bacteroidetes ratio. Among them, adults had the highest value (Mariat et al., 2009). Therefore, our results might had a positive effect on the health and fat accumulation of hens during the late laying period. In the present study, we also observed significant differences in the relative abundance of Synergist and Elusimicrobia between the CMP and control groups, but the functions of these phyla were unclear and should be investigated further.

At the family level, the relative abundance of Veillonellaceae and Christensenellaceae decreased significantly in the CMP group, but was low in our experimental hens. Christensenellaceae belongs to the order of the Clostridiales within the Clostridia class of Firmicutes (Morotomi et al., 2012) and had emerged as the intestinal flora component, which the abundance was most influenced by host genetics (Goodrich et al., 2014). Christensenellaceae might be a symbol of the ecosystem of long-lived people (Biagi et al., 2016), Goodrich et al reported the relationship between Christensenellaceae and the weight of humans and mice, that reduced the degree of receptor weight gain (Goodrich et al., 2014). Similarly, in the addition of a Christensella strain in a mouse model, reduced the degree of receptor weight gain (Fischbach and Segre, 2016). The colonisation of *Campylobacter jejuni*, a foodborne zoonotic pathogen, could increase the relative abundance of Christensenellaceae in a chicken intestine (Alexandre et al., 2015). However, the role of this new family in chicken intestine health was unknown. Veillonellaceae had an excellent ability to ferment lactic acid; it was known as a short-chain fatty acid acetate and propionate producer (Lecomte et al., 2015). Other studies had shown that the number of families defined as the chicken's core microbiome might increase or decrease, but families such as Veilonellaceae had been reported common members of the chicken (Qu et al., 2008; Callaway et al., 2009; Filip, et al., 2011). Veillonelaceae had a similar effect on Campylobacteraceae, so Veillonelaceae could reduce the colonisation of Campylobacteraceae in the chicken intestines (Videnska et al., 2014). However, further studies were needed to investigate the function of Veillonellaceae in the chicken intestine.

At the genus level, the main genera of the CMP and the control groups were norank Bacteroidales, norank Ruminococcaceae, Bacterorides_ Bacteroidaceae and Oscillospira. Scientists reported that the caecum of chickens consisted mainly of Costridia, followed by Lactobacillus and Ruminococcus (Gong et al., 2010). This change might be due to the addition of CMP in the diet. Other factors could also cause these changes. These factors include different types of chicken, age and environmental factors. For example, the abundance of *Clostridium* increased and the proportion of lactobacill1decreased as the chicken aged (Shang et al., 2018a). Cage type could affect the composition of caecal microflora in laying hens (Filip et al., 2011). Genotype and gender might also affect changes in the intestinal microflora of chicken (Zhao et al., 2013). Norank_ Ruminococcaceae and norank_Christensenellaceae were unknown bacterial residents, and their relative abundance decreased significantly in the CMP group. Scienticts reported that the caecum was rich in unknown and unclassified bacterial residents (Stanley et al., 2013). The relative abundance of Phascolarctobacterium also decreased significantly in the CMP group compared to

those in the control group. The Phascolarctobacterium within the *Sporomusa* branch of the *Clostridium/Bacillus* subphylum of Gram-positive bacteria and is a neighbor of *Acidaminococcus fermentans* (Del Dot et al., 1993). Phascolarctobacterium complements the lack of carbohydrate metabolism by increasing lipid metabolism with methylmalonyl-CoA mutase, and methylmalonyl-CoA carboxyltransferase was the most abundant among proteins involved in lipid metabolism (Polansky et al., 2015). However, evaluating the possible effect of the changes in these genera on the chicken's caecal microbial community was difficult because of the lack of reports about Phascolarctobacterium in the health of chicken intestine.

CONCLUSION

Dietary supplementation with 100 and 200 mg CMP/kg can improve the laying rat and decrease feed to egg ratio significantly compared to those none-supplemented hens, respectively. Dietary supplementation with CMP could alter significantly the caecal microbiota composition with the changes in the phylum, family and genus levels. These findings provided insights into egg production of chickens and intestinal microbiota modulations in response to different CMP dosages and emphasise the requirement for further research to determine the effects of CMP on the chicken intestine microbiota and predict the response of the microbiota to these agents.

DECLARATIONS

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Consent to publish

Not applicable

Competing interests

The authors declare that they have no competing interests.

Author's contribution

Xiaochen Chen carried out the molecular genetic studies, participated in the sequence alignment and drafted the manuscript. Yaowen Zhang, and Wenfeng Ma carried out the immunoassays. Xi Wu participated in the sequence alignment. Yanzi Zhu participated in the design of the study and performed the statistical analysis. Zhanbin Wang conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

REFERENCES

Ahn YJ, Park SJ, Lee SG, Shin SC and Choi DH (2000). Cordycepin: Selective growth inhibitor derived from liquid culture of cordyceps m ilitaris against clostridium spp. Journal of Agricultural & Food Chemistry, 48 (7): 2744-2748. DOI: https://doi.org/10.1021/jf990862n

Alexandre T, Philippe F, étienne Y, Julie A, Ludovic L, Ann L and Markus MH (2015). Chicken caecal microbiome modifications induced by campylobacter jejuni colonization and by a non-antibiotic feed additive. plos one, 10 (7): e0131978. DOI: https://doi:10.1371/journal.pone.013197

Bederska-ojewska D, witkiewicz S and Muszyńska B (2017). The use of basidiomycota mushrooms in poultry nutrition—a review. Animal Feed Science stechnology, 230: 59-69. DOI: https://doi.org/10.1016/j.anifeedsci.2017.06.001

Biagi E, Franceschi C, Rampelli S, Severgnini M, Ostan R, Turroni S, Consolandi C, Quercia S, Scurti M and Monti D (2016). Gut microbiota and extreme longevity. Current Biology, 26 (11): 1480-1485. DOI: https://doi.org/10.1016/j.cub.2016.04.016

Callaway TR, Dowd SE, Wolcott RD, Sun Y, McReynolds JL, Edrington TS , Byrd JA, Anderson RC, Krueger N and Nisbet DJ (2009). Evaluation of the bacterial diversity in cecal contents of laying hens fed various molting diets by using bacterial tag-encoded flx amplicon pyrosequencing. Poultry science, 88 (2): 298-302. DOI: https://doi.org/10.3382/ps.2008-00222

Chang Q, Wang W, Regev-Yochay G, Lipsitch M and Hanage WP (2015). Antibiotics in agriculture and the risk to human health: How worried should we be? Evolutionary Applications, 8 (3): 240-247. DOI: https://doi.org/10.1111/eva.12185

Chen XL, Wu GH, Gu QX and Huang ZL (2010). Optimization of extraction process of polysaccharides from fruiting body of cordyceps militaris L. Link by orthogonal test. Medicinal Plant, (7). Available at: https://www.cabdirect.org/?target=%2fcabdirect%2fabstract%2f20113129764

Cheung JKH, Li J, Cheung AWH, Zhu Y, Zheng KYZ, Bi CW, Duan R, Choi RC, Lau DT and Dong TT (2009). Cordysinocan, a polysaccharide isolated from cultured cordyceps, activates immune responses in cultured T-iymphocytes and macrophages: Signaling cascade and induction of cytokines. Journal of Ethnopharmacology, 124 (1): 61-68. DOI: https://doi.org/ 10.1016/j.jep.2009.04.010

Ciftici M, Ertas ON and Guler T (2005). Effects of vitamin E and vitamin C dietary supplementation on egg production and egg quality of laying hens exposed to chronic heat stress. Revue De Medecine Veterinaire, 156: 107-111. Available at: https://www.revmedvet.com/2005/RMV156_107_111.pdf

Das SK, Masuda M, Sakurai A and Sakakibara M (2010). Medicinal uses of the mushroom cordyceps militaris: Current state and prospects. Fitoterapia, 81 (8): 961-968. DOI: https://doi.org/10.1016/j.fitote.2010.07.010

Del Dot T, Osawa R and Stackebrandt E (1993). Phascolarctobacterium faecium gen. Nov, spec. Nov., a novel taxon of the sporomusa group of bacteria. Systematic & Applied Microbiology, 16 (3): 380-384. DOI: https://doi.org/10.1016/S0723-2020(11)80269-9

Filip VI, Jantina DV, Lotte B, Lars M, Steen N and Karl P (2011). The influence of the cage system and colonisation of salmonella enteritidis on the microbial gut flora of laying hens studied by t-rflp and 454 pyrosequencing. Bmc Microbiology, 11 (1): 187-187. DOI: https://doi.org/10.1186/1471-2180-11-187

Fischbach MA and Segre JA (2016). Signaling in host-associated microbial communities. Cell, 164 (6): 1288-1300. DOI: https://doi.org/10.1016/j.cell.2016.02.037

Gong J, Si W, Forster RJ, Huang R, Yu H, Yin Y, Yang C and Han Y (2010). 16s rRNA gene-based analysis of mucosa-associated bacterial community and phylogeny in the chicken gastrointestinal tracts: From crops to ceca. Fems Microbiology Ecology, 59 (1): 147-157. DOI: https://doi.org/10.1111/j.1574-6941.2006.00193.x

Goodrich JK, Waters JL, Poole AC, Sutter JL, Koren O, Blekhman R, Beaumont M,Van T.W, Knight R and Bell JT (2014). Human genetics shape the gut microbiome. Cell, 159 (4): 789-799. DOI: https://doi.org/10.1016/j.cell.2014.09.053

Han JC, Qu HX, Wang JG, Yan YF, Zhang JL, Yang L, Zhang M and Cheng YH (2015). Effects of fermentation products of cordyceps militaris on growth performance and bone mineralization of broiler chicks. Journal of Applied Animal Research, 43 (2): 236-241. DOI: https://doi.org/10.1080/09712119.2014.928630.

Hegde SN, Rolls BA and Coates ME (1982). The effects of the gut microflora and dietary fibre on energy utilization by the chick. British Journal of Nutrition, 48 (1): 73-80. DOI: https://doi.org/10.1079/bjn19820089

Józefiak D, Rutkowski A and Martin SA (2004). Carbohydrate fermentation in the avian ceca: A review. Animal Feed Science & Technology, 113 (1): 1-15. DOI: https://doi.org/10.1079/bjn19820089

Koh JH, Suh HJ and Ahn TS (2003). Hot-water extract from mycelia ofcordyceps sinensisas a substitute for antibiotic growth promoters. Biotechnology Letters, 25 (7): 585-590. DOI: https://doi.org/10.1023/a:1022893000418

Koliada A, Syzenko G, Moseiko V and Budovska L (2017). Association between body mass index and firmicutes/bacteroidetes ratio in an adult ukrainian population. Bmc Microbiology, 17 (1): 120. DOI: https://doi.org/10.1186/s12866-017-1027-1

Lecomte V, Kaakoush NO, Maloney CA, Raipuria M, Huinao KD, Mitchell HM and Morris MJ (2015). Changes in gut microbiota in rats fed a high fat diet correlate with obesity-associated metabolic parameters. PLoS ONE, 10 (5): e0126931. DOI: https://doi.org/10.1371/journal.pone.0126931

Lee JS, Kwon JS, Won DP, Lee JH, Lee KE, Lee SY and Hong EK (2010a). Study of macrophage activation and structural characteristics of purified polysaccharide from the fruiting body of cordyceps militaris. Journal Microbiol Biotechnol, 20 (7): 1053-1060. DOI: https://doi.org/10.4014/jmb.0910.10022

Lee JS, Kwon JS, Yun JS, Pahk JW, Shin WC, Young LS and Hong EK (2010b). Structural characterization of immunostimulating polysaccharide from cultured mycelia of cordyceps militaris. Carbohydrate Polymers, 80 (4): 1011-1017. DOI: https://doi.org/10.1016/j. carbpol.2010.01.017

Li Y, Jing Z, Shao PL, Hui F and Quan Z (2015). Quality evaluation of cordyceps through simultaneous determination of eleven nucleosides and bases by rp-hplc. Journal of Separation Science, 29 (7): 953-958. DOI: https://doi.org/10.1002/jssc.200600007

Mariat D, Firmesse O, Levenez F, Guimares V, Sokol H, Doré J, Corthier G and Furet JP (2009). Thefirmicutes/bacteroidetes ratio of the human microbiota changes with age. Bmc Microbiology, 9 (1): 123. DOI: https://doi.org/10.1186/1471-2180-9-123

Martinez JL (2009). Environmental pollution by antibiotics and by antibiotic resistance determinants. Environmental Pollution, 157 (11): 2893-2902. DOI: https://doi.org/10.1016/j.envpol.2009.05.051

Mazmanian SK, Liu CH, Tzianabos AO and Kasper DL (2005). An immunomodulatory molecule of symbiotic bacteria directs maturation of the host immune system. Cell, 122 (1): 107-118. DOI: https://doi.org/10.1016/j.cell.2005.05.007

Morotomi M, Nagai F and Watanabe Y (2012). Description of christensenella minuta gen. Nov., sp. Nov., isolated from human faeces, which forms a distinct branch in the order clostridiales, and proposal of christensenellaceae fam. Nov. International Journal of Systematic & Evolutionary Microbiology, 62 (1): 144-149. DOI: https://doi.org/10.1016/j.envpol.2009.05.051

Pan D and Yu Z (2014). Intestinal microbiome of poultry and its interaction with host and diet. Gut Microbes, 5 (1): 108-119. DOI: https://doi.org/ 10.4161/gmic.26945

Polansky O, Sekelova Z, Faldynova M, Sebkova A, Sisak F and Rychlik I (2015). Important metabolic pathways and biological processes expressed by chicken cecal microbiota. Applied & Environmental Microbiology, 82 (5): 1569. Available at:. DOI: https://doi.org/10.1128/AEM.03473-15

Qu A, Brulc JM, Wilson MK, Law BF, Theoret JR, Joens LA, Konkel ME, Angly F, Dinsdale EA and Edwards RA (2008). Comparative metagenomics reveals host specific metavirulomes and horizontal gene transfer elements in the chicken cecum microbiome, PLoS ONE. 3 (8): e2945. DOI: https://doi.org/10.1128/AEM.03473-15

Sears CL (2005). A dynamic partnership: Celebrating our gut flora. Anaerobe, 11 (5): 247-251. DOI: https://doi.org/10.1016/j.anaerobe.2005.05.001

Shang Y, Kumar S, Oakley B and Kim WK (2018a). Chicken gut microbiota: Importance and detection technology. Frontiers in Veterinary Science, 5 (254). Available at: https://www.frontiersin.org/article/10.3389/fvets.2018.00254.

Shang Y, Kumar S, Oakley BandKim WK (2018b). Chicken gut microbiota: Importance and detection technology, 5 (254). Link Retrieved from journal Available at: https://www.frontiersin.org/article/10.3389/fvets.2018.00254.

Shin SM, Kwon JH, Lee SW, Kong HS, Lee SJ, Lee CK, Cho KH, Ha NJ and Kim KJ (2010). Immunostimulatory effects of cordyceps militaris on macrophages through the enhanced production of cytokines via the activation of NF-kappaB. Immune Netw, 10 (2): 55-63. DOI: https://doi.org/10.4110/in.2010.10.2.55

Spence C, Wells WG and Smith CJ (2006). Characterization of the primary starch utilization operon in the obligate anaerobe bacteroides fragilis: Regulation by carbon source and oxygen. Journal of Bacteriology, 188 (13): 4663. DOI: https://doi.org/10.1128/JB.00125-06

Stanley D, Geier MS, Denman SE, Haring VR, Crowley TM, Hughesm RJ and Moore RJ (2013). Identification of chicken intestinal microbiota correlated with the efficiency of energy extraction from feed. Veterinary Microbiology, 164 (1-2): 85-92. DOI: https://doi.org/10.1016/j.vetmic.2013.01.030

Videnska, Petra, Rahman, Md M, Faldynova, Marcela, Babak, Vladimir, Elsheimer M, Marta et al. (2014). Characterization of egg laying hen and broiler fecal microbiota in poultry farms in croatia, czech republic, hungary and slovenia. PLoS ONE, 9 (10): e110076. DOI: https://doi.org/10.1371/journal.pone.0110076

Wang CL, Chiang CJ, Chao YP, Yu B and Lee TT (2015). Effect of cordyceps militaris waster medium on production performance, egg traits and egg yolk cholesterol of laying hens. The Journal of Poultry Science. DOI: https://doi.org/10.2141/jpsa.0140191

Wang J, Liu YM, Cao W, Yao KW, Liu ZQ and Guo JY (2012). Anti-inflammation and antioxidant effect of cordymin, a peptide purified from the medicinal mushroom cordyceps sinensis, in middle cerebral artery occlusion-induced focal cerebral ischemia in rats. Metabolic Brain Disease, 27 (2): 159-165. DOI: https://doi.org/10.1007/s11011-012-9282-1

Zhang C, Zhang M, Wang S, Han R, Cao Y, Hua W, Mao Y, Zhang X, Pang X and Wei C (2010). Interactions between gut microbiota, host genetics and diet relevant to development of metabolic syndromes in mice. Multidisciplinary Journal of Microbial Ecology, 4 (2): 232-241. DOI: https://doi.org/10.1038/ismej.2009.112

Zhao L, Gang W, Siegel P, He C, Wang H, Zhao W, Zhai Z, Tian F, Zhao J and Hao Z (2013). Quantitative genetic background of the host influences gut microbiomes in chickens. Scientific Reports, 3 (5): 1163. DOI: https://doi.org/10.1038/srep01163

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Comparison of Weight, Components and Chemical Composition of Eggs in Guinea Fowl, Turkey, and Domestic Chicken

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ABSTRACT

The present study was carried out to compare the physicochemical characteristics of eggs of guinea fowl, turkeys and domestic chickens outdoor reared in traditional farms in Baghdad, Iraq. A total of 166 fresh eggs; 32 eggs from guinea fowls (*Numida meleagris*), 44 eggs from turkeys (*Meleagris gallopavo*) and 90 eggs from domestic chickens; were collected. Egg weight, percentage of egg components, chemical composition (protein, lipids, and ash), and lipid profile were determined. Results revealed the significant differences in egg weight among studied birds. The average egg weights for guinea fowl, turkey, and indigenous chicken were 83.15 ± 0.72, 92.41 ± 0.78 and 61.28 ± 0.62 g, respectively. No significant differences were found in egg components and the chemical composition of the edible portions of the eggs among studied birds. However, the lipid profile of egg yolk indicated that egg cholesterol and LDL levels were significantly higher in guinea fowl and turkey compared with those in indigenous chickens, whereas native chicken has high values of HDL compared to guinea fowl and turkey. There were no significant differences in the triglyceride level in egg yolks among the studied fowls. In conclusion, although egg weight was significantly different among studied birds, eggs of guinea fowl, turkeys, and domestic chickens were similar in nutritional components.

Key words: Chemical composition, Domestic chicken, Egg Components, Guinea fowl, Turkey.

INTRODUCTION

Poultry such as chickens, quails, turkeys, pheasant, ostriches, ducks, and geese are domesticated birds as a source of animal protein with high nutritional value for human diets (Al-Obaidi et al., 2019). Production and consumption of table eggs have risen in the last years, global demand for table eggs will reach more than 50 million tons per year (Farrell, 2014; FAO, 2015; Farrell, 2015; Al-Shadeedi et al., 2019). Global egg production is increasing depending on global demand and is expected to increase in developing countries because it is easy to produce in a short time (USDA, 2016).

The egg has an optimal composition of essential amino acids and the favorable composition of essential fatty acids, a high percentage of polyunsaturated fatty acids and a favorable ratio of omega 6 to omega 3 fatty acids which has benefits for human health (Lass and Belluzzi, 2019). World demand and utilization of fowl eggs (raw egg consumption and for processed egg) are increased due to high nutritional value and multifunctional properties of eggs (Stadelman and Cotterill, 1995; Pingel, 2009; Al-Obaidi and Al-Shadeedi, 2016; Al-Shadeedi et al., 2019).

Since 2003, a new trend in table egg consumption was appeared through introducing a new poultry species for egg and meat production including ostrich, pheasants, turkey, and guinea fowl (Al-Obaidi and Al-Shadeedi, 2015; Al-Obaidi and Al-Shadeedi, 2017). Although chicken eggs are currently most commonly consumed by humans, the eggs from other birds are also used for daily consumption. For example, Japanese quail eggs are gaining popularity in Europe and America, and ostrich eggs in South Africa (Horanczuk et al., 2008).

Turkey (*Meleagris gallopavo*), belonging to the family Phasianidae, has good production performance and is reared in many countries of the world (Igenbayev et al., 2019). Guinea fowl (*Numida meleagris*) belongs to the Numididae family within the Galliformes order (Christopher, 2005). The peak of egg production of guinea fowl outdoor raised occurs in the rainy season (Konlan et al., 2011).

Poultry egg widely diverges in shape, volume, weight and amount of yolk and albumen material due to genetic factors (species, breed, and strain) and environmental factors (nutrition, disease and season) (Romanoff and Romanoff, 1949; Stadelman and Cotterill,

1995; Konlan et al., 2011). Al-Obaidi and Al-Shadeedi (2015) found significant differences in the components of eggs from ostrich, emu, and domestic chicken. Although, ostrich and emu eggs are so similar to chicken eggs in the chemical composition. The aim of this study was to determine the weight, chemical composition and lipid profile of eggs from guinea fowl, turkey, and indigenous chicken outdoor reared in Baghdad.

MATERIALS AND METHODS

Ethical approval

This study was in accordance with guidelines for the care and handling of animals described by the Iraq Association of Genetic and Environmental Resources Conservation (AGERC-Iraq) Committee, Ministry of Higher Education and Scientific Research, Iraq.

Birds

Guineafowl (*Numida meleagris*), turkey (*Meleagris gallopavo*) and indigenous chicken strain (Barred plumage), were used in this study. These fowls were outdoor reared in traditional farms in Baghdad city, Iraq from January 20 to December 7, 2019.

Fowl nutrition

All birds were fed in the morning with 50g/bird/day concentrated ration (Table 1). Also, birds had access to the outdoors and fed with grass.

Table 1. Proximate analysis and percentage composition of the diet used for feeding birds.

Ingredient	**(%)**
Corn	60.54
Soybean meal	25.95
Soybean oil	3.00
Calcium carbonate	8.33
Calcium phosphate	1.28
Salt	0.30
Mineral premix[1]	0.25
Vitamin premix[2]	0.35
Total	100.0
Calculated proximate analysis	
Metabolic energy (kcal)	2857
Crude protein (%)	17.00
Lysine (%)	0.77
Methionine (%)	0.30
Methionine + cysteine (%)	0.59
Calcium (%)	3.50
Available phosphorus (%)	0.35

[1]Vitamin premix contains vitamin A: 12,000 IU; vitamin D3: 2500 IU; vitamin E: 30 IU; vitamin K3: 2 mg; thiamine: 2.25 mg; riboflavin: 7.5 mg; pyridoxine: 3.5 mg; cobalamin: 0.02 mg; niacin: 45 mg; D-pantothenic acid: 12.5 mg; biotin: 0.125 mg; folic acid: 1.5 mg. [2]Mineral premix contains zinc: 50ppm; copper: 12 ppm; iodine: 0.3 ppm; cobalt: 0.2 ppm; iron: 100 ppm; selenium: 0.1 ppm.

Egg collection

A total of 166 eggs of some native fowls, 32 eggs of guinea fowl, 44 eggs of turkey and 90 eggs of indigenous chicken freshly laid were collected.

Egg components

The percentage of egg components (yolk, albumen, and eggshell) was determined according to Stadelman and Cotterill (1995) as described by Al-Obaidi (2010). All eggs were weighted using a very sensitive digital balance (Sartorius, USA). Eggs were broken, the yolk and the albumen were separated and each weighted. Then, the percentage of each component was determined using the following equation:

$$\text{Egg component (\%)} = \frac{\text{Egg component weight (g)}}{\text{Total egg weight (g)}} \times 100$$

Chemical analyses

Protein and lipid composition were determined in albumen and yolk according to AOAC (1980). All measurements were performed in triplicate. Ash content was determined by thermal decomposition of the samples in a muffle furnace oven at 600 °C for 6 hours. Lipid content determination was conducted on yolk samples using the solvents of chloroform and methanol (1:1). The mixture of yolk sample and solvents were mixed for 20 min using a magnetic stirrer, then the weight of extracted lipid in each sample was recorded and its percentage was calculated. The semi-micro Kjeldahl method was used for the determination of nitrogen and the obtained values multiplied by 6.25 to calculate protein percentage.

Cholesterol was determined calorimetrically using ethanol extraction and ferric chloride-sulfuric acid detergent according to the method described by Al-Obaidi (1999). Low-Density Lipoprotein (LDL) and High-Density Lipoprotein (HDL) were determined using EnzyChrom HDL and LDL Assay Kit (BioAssay Systems, USA). Triglycerides were eluted from yolk lipid using benzene and determined by silica gel column according to the methods described by AOAC (1980).

Statistical analysis

Data were analyzed using the general linear model procedure in SAS software (SAS Institute, USA). Means were compared by Duncan's multiple range test at 5% probability level (Steel and Torrie, 1980).

RESULTS

The results indicated significant differences ($p<0.05$) in egg weight among studied fowls. Guinea fowl had an average egg weight of 83.15 g compared to 92.41 and 61.28 g for turkey and indigenous chicken, respectively. There were no significant differences in the percentage of egg components among studied fowls, as shown in table 2. Also, the chemical composition of edible portions of eggs was not significantly different among studied birds (Table 3).

The lipid profile of egg yolk of studied birds is presented in table 4. There were significant differences ($p<0.05$) in cholesterol and LDL levels, which were high in guinea fowl (26 and 48 mg/g, respectively) and turkey (23 and 46 mg/g, respectively) compared to native chicken (17 and 32 mg/g, respectively); whereas native chicken had high values of HDL (85 mg/g) compared to guinea fowl and turkey (73 and 70 mg/g, respectively). No significant differences were found in triglyceride levels among the studied birds.

Table 2. Egg weight and percentage of egg components of guinea fowl, turkey, and domestic chicken reared outdoor in Baghdad, Iraq

Bird species	Egg weight (g)	Eggshell (%)	Yolk (%)	Albumen (%)
Guinea fowl	83.15 ±0.72^{b}	10.75 ±0.30	31.49 ±0.52	57.76 ±0.72
Turkey	92.41 ±0.78^{a}	10.61 ±0.28	31.43 ±0.46	57.96 ±0.65
Domestic chicken	61.28 ±0.62^{c}	10.35 ±0.24	31.57 ±0.39	58.08 ±0.70

Data are express as mean ±SE. Different superscript letters in a column indicate significant difference ($p<0.05$).

Table 3. Chemical composition of eggs of guinea fowl, turkey and domestic chicken reared outdoor in Baghdad, Iraq

	Fowl species	Protein (%)	Lipids (%)	Ash (%)
Yolk	Guinea fowl	17.44 ±0.38	32.53 ±1.12	1.16 ±0.10
	Turkey	17.52 ±0.41	32.50 ±1.16	1.15 ±0.10
	Domestic chicken	17.48 ±0.39	32.38 ±1.13	1.16 ±0.11
	Significance	NS	NS	NS
Albumen	Guinea fowl	11.66 ±0.21	-	1.10 ±0.10
	Turkey	11.75±0.24	-	1.11 ±0.10
	Domestic chicken	11.69 ±0.20	-	1.11 ±0.10
	Significance	NS	NS	NS

Data are express as mean ±SE. NS: non-significant ($p>0.05$)

Table 4. Lipid profile of egg yolk of guinea fowl, turkey, and domestic chicken reared outdoor in Baghdad, Iraq

Fowl species	Cholesterol (mg/g)	HDL (mg/g)	LDL (mg/g)	Triglycerides (mg/g)
Guinea fowl	26 ±1.33^{b}	73 ±2.63^{a}	48 ±1.72^{b}	66 ±1.58
Turkey	23 ±1.38^{a}	70 ±2.68^{a}	46 ±1.77^{a}	64 ±1.58
Domestic chicken	17 ±1.35^{b}	85 ±2.72^{b}	32 ±1.72^{b}	69 ±1.58

Data are express as mean ± SE. different superscript letters in a column indicate significant difference ($p<0.05$).

DISCUSSION

Outdoor rearing of fowls offer many benefits to the sustainable farm, in addition to supplying eggs and/or meat, it enhances soil fertility and control weeds and insects (Bare and Ziegler-Ulsh, 2012; Al-Obaidi and Al-Shadeedi, 2018).

In Iraq, many birds flocks such as guinea fowl, turkey, and domestic chickens are outdoor reared, free-range or semi free-range, and may grow in low levels of nutrition and poor environmental condition during summer and winter. These variable conditions may affect components and chemical composition of eggs (Al-Obaidi, 2017; Al-Shadeedi et al., 2019). Native studies revealed that significant species differences in blood serum enzyme activity during the year, which will affect egg size and components (Al-Obaidi and Al-Shadeedi, 2014). Egg weight is an expression of size, that is mainly influenced by fowl body size, evolutionary status, climate condition, and the amount of available food. Also, egg size differs among different species and between individuals within the same species (Stadelman and Cotterill, 1995; Downing and Taylor, 2010). The present study is in agreement with

Al-Obaidi and Al-Shadeedi (2014), Romero and Remage-Healey (2000) and Kordonowy et al. (2017) that reported the egg weight and chemical composition differ between fowl species.

Fowl egg is one of the most complex and highly differentiated reproductive cells, which varies in size and shape depending on the type of species, breed and strain of birds (Romanoff and Romanoff, 1949; Stadelman and Cotterill, 1995; Al-Obaidi and Al-Shadeedi, 2014, 2016). Similarly, the current study indicated that egg of guinea fowl, turkey and chicken differed in size and weight.

Fowl egg consists of approximately 10% shell, 58% white and 32% yolk (Stadelman and Cotterill, 1995; Matt et al., 2009; Al-Obaidi et al., 2019). The results obtained in the present study were similar to the mentioned values.

Egg white is a viscous colorless liquid consists of water (88%), protein (10-12%) and some minerals. The amount of lipid in the egg white is trace (0.02%) compared with the amount present in the yolk. The yolk comprises 48% water, 15-17% protein, 32-34% fat, and some minerals and vitamins. Yolk lipid content is composed of triacylglycerols (63.3%), phospholipids (29.7%) and total cholesterol (5.2%) (Stadelman and Cotterill, 1995; Marshall, 1960). The findings obtained in the current study are in agreement with the aforementioned reports. Cholesterol is the principal sterol synthesized by all kinds of animals and is important for cellular metabolism, cell membrane integrity, cellular proliferation, and steroid hormones synthesis (Brown, 2007). The high content of cholesterol in an egg is necessary to support the growth of the embryo. The high content of lipid fractions including cholesterol and LDL in guinea fowl and turkey eggs may be related to genetic variations between these birds compared to domestic chickens (Downing and Taylor, 2010; Farell, 2015; Al-Obaidi and Al-Shadeedi, 2016; Al-Shadeedi et al., 2019).

CONCLUSION

In conclusion, although egg weight in guinea fowl, turkeys, and native chickens was significantly different, nutritional components of eggs were similar among different birds**.**

DECLARATIONS

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Competing interests

The author declared that she has no competing interests.

REFERENCES

Al-Obaidi FA (1999). Evaluation of quality and chemical composition of Japanese quail egg. Ph.D. Thesis, College of Agriculture, University of Baghdad.

Al-Obaidi FA (2010). Egg morphology characteristics and components of collared dove (*Streptopelia decaocto*) in Baghdad. 11th Scientific Symposium of Arab Scientific Heritage Revival Research Center, University of Baghdad, October 27th, 2010: 219-222.

Al-Obaidi FA (2017). Effect of summer and winter seasons on outdoor rearing of blood enzymes of some native chicken strains. Journal of Genetic and Environmental Resources Conservation, 5(1):55-58. Available at: http://www.gercj.com/index.php/gercj/home

Al-Obaidi FA and Al-Shadeedi ShMJ (2014). Seasonal variation in egg phenotype and chemical composition of collard dove (*Streptopelia decaocto*) in Baghdad. Journal of Genetic and Environmental Resources Conservation, 2(1): 69-73. Available at: http://www.gercj.com/index.php/gercj/home

Al-Obaidi FA and Al-Shadeedi ShMJ (2015). Comparison study of egg morphology, component and chemical composition of ostrich, emu and native chickens. Journal of Genetic and Environmental Resources Conservation, 3(2): 132-137. Available at: http://www.gercj.com/index.php/gercj/home

Al-Obaidi FA and Al-Shadeedi ShMJ (2016). Comparison study of egg morphology, component and chemical composition of Mallard duck and domestic Peking duck. Journal of Bio Innovation, 5(4): 555-562. Available at: https://www.jbino.com/index.htm

Al-Obaidi FA and Al-Shadeedi ShMJ (2017). Comparison some native fowls (chicken, mallard ducks quail and turkey) in components and chemical composition of the eggs in Iraq. Al-Anbar Journal of Veterinary Sciences, 10(1): 65-69. Available at: https://www.iasj.net/iasj?func=issues&jId=146&uiLanguage=en

Al-Obaidi FA and Al-Shadeedi ShMJ (2018). Effect of summer and winter seasons on egg abnormalities of outdoor rearing some native chicken strains. Journal of Biodiversity and Environmental Sciences (JBES) 12: 220-225. Available at: https://innspub.net/journal/journal-of-biodiversity-and-environmental-sciences-jbes/

Al-Obaidi FA, Al-Shadeedi ShMJ and Al-Dalawi RH (2019). Table Egg and Designer Egg. 1st Edition, LAP LAMBERT Academic Publishing, ISBN-10: 3659890421; EAN: 9783659890420, Available at: https://www.lap-publishing.com/

Al-Shadeedi ShMJ, Al-Obaidi FA and Al-Dalawi RH (2019). Breeding and Management of Ducks and Geese. 1st Edition, (in Arabic). Noor Publishing, ISBN-10: 6200068372; EAN: 9786200068378

Association of Official Analytical Chemists (AOAC) (1980). Official Methods of Analysis. 13th Edition, Washington, DC, USA.

Bare M and Ziegler-Ulsh C (2012). Establishing a small-scale, sustainable, pastured poultry operation. Rodale Institute. Available at: https://rodaleinstitute.org/establishing-a-small-scale-sustainable-pastured-poultry-operation/

Brown AJ (2007). Cholesterol, statins and cancer. Clinical Experimental Pharmacology and Physiology, 34: 135–141. DOI: https://doi.org/10.1111/j.1440-1681.2007.04565.x

Downing JA and Taylor W (2010). The effect of strain and season on the performance of commercial ducks under Australian conditions. Proceedings of the Australian Poultry Science Symposium, 10: 182-185. Available at: https://az659834.vo.msecnd.net/eventsairaueprod/production-usyd-public/1330f0d28dc54ef39afea020d58a4da9

Farrell DJ (2014). Small-scale duck production: the way ahead. Journal of Animal Husbandry Science and Technology, 30(8): 73-80. Available at: http://scholarsinsight.org/journals/index.php?title=journal-of-animal-husbandry-science-and-technology

Farrell D (2015). Recent advances in the production, management and nutrition of intensively-farmed domestic ducks. The Poultry Site - Poultry News, Health, Welfare, Diseases, Markets and Economics, England. Available at: https://thepoultrysite.com/

FAO (2015). Statistical pocket book, world food agriculture. Food and Agriculture Organization of the United Nations, Rome.

Horanczuk JO, Tomasik C and Cooper RG (2008). Ostrich farming in Poland – its history and current situation after accession to the European Union. Avian Poultry and Biological Review, 1: 65-71. DOI: https://doi.org/10.3184/175815508X360470

Igenbayev A, Nurgazezova A, Okuskhanova E, Rebezov Y, Kassymov S, Nurymkhan G, Tazeddinova D, Mironova I and Rebezov M (2019). Fatty acid composition of turkey meat. Journal of Worlds Poultry Research, 9(2): 78-81. DOI: https://dx.doi.org/10.36380/jwpr.2019.9

Konlan SP, Avornyo FK, Karbo N and Sulleyman A (2011). Increasing guinea fowl eggs availability and hatchability in the dry season. Journal of Worlds Poultry Research, 1(1): 1-3. Available at: http://jwpr.science-line.com/index.php?option=com_content&view=article&id=4&Itemid=9

Kordonowy L, Lombardo KD, Green HL, Dawson MD, Bolton EA, LaCourse S and MacManes MD (2017). Physiological and biochemical changes associated with acute experimental dehydration in the desert adapted mouse, *Peromyscus Eremicus*. Physiological Reproduction, 5(6): 22. DOI: https://doi.org/10.14814/phy2.13218

Lass A and Belluzzi A (2019). Omega-3 polyunsaturated fatty acids and IVF treatment. Reproductive Biomedicine Online (RBMO), 38(1): 95-99. DOI: https://doi.org/10.1016/j.rbmo.2018.10.008

Marshall AJ (1960). Biology and Comparative Physiology of Birds, Volume 2. Academic Press, New York and London.

Matt D, Veromann E and Luik A (2009). Effect of housing systems on biochemical composition of chicken eggs. AgronomResearchch, 7(2): 662-667. Available at: https://agronomy.emu.ee/

Pingel H (2009). Waterfowl production for food security. IV World Waterfowl Conference, 11-13 Novembr, 2009, Thrissur, India. Available at: http://www.waterfowl2009.vetcos.com/proceedings%20-%20IV%20WWC%20-%20Kerala,%20India.pdf

Romanoff AL and Romanoff A (1949). The Avian Egg. John Wiley and Sons Co., New York, USA.

Romero LM and Remage-Healey L (2000). Daily and seasonal variation in response to stress in captive starlings (*Sturnus vulgaris*): corticosterone. General and Comparative Endocrinology, 119(1): 52-59. DOI: https://doi.org/10.1006/gcen.2000.7491.

SAS Institute (2001). SAS/STAT User's Guide for Personal Computer. Release 6.12 SAS Institute, INC., Cary, N.C., USA.

Stadelman WJ and Cotterill OJ (1995). Egg Science and Technology. 4th Edition. Food Products Press . An Imprint of the Haworth Press. INC. New York. London, UK

Steel RG and Torrie JH (1980). Principle and Procedures of Statistics. 2nd Edition. McGrow-Hill Book Co., Inc., New York, USA.

USDA (2016). International Egg and Poultry: FAO's Food Outlook Report. Available at: http://www.thepoultrysite.com/reports/?id=4277

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Effect of Supplementation of *Yucca schidigera* Extract on Ammonia Gas Emission and Performance of Broiler Chickens

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ABSTRACT

The current study was conducted to investigate the efficacy of Yucca extract (YE) on ammonia gas emission from litter, evaluate the production performance, carcass characteristics and economic utility in broiler rearing. A trial of 240 day-old commercial broiler chicks was carried out on littered floor for a period of 28 days. The birds were allocated randomly to 3 treatments and a control group with three replications (20 birds/ replication). Feeding management and rearing condition were same for all the groups, as per standard. YE was mixed with drinking water as treatment as follows: 1ml YE per 16 liters of drinking water (T_1), 1ml YE per 20 liters of drinking water (T_2), 1ml YE per 24 liters of drinking water (T_3) and no YE in drinking water i.e control group (T_0). Ammonia level of Yucca treated groups were significantly ($P<0.05$) lower at the 4th week of rearing period than control group. A significant difference ($P<0.05$) was noted on body weight, feed consumption, body weight gain and feed conversion ratio value of the birds treated with YE. Carcass percentage and edible portion were significantly ($P<0.05$) greater in all treatment groups than control group. Profit per bird and Benefit cost ratio were also higher ($P<0.05$) in treatment groups than control group. Among the treatments, the performance of T_2 group was better than other groups. This study concluded that application of YE (1ml/ 20 liters of drinking water) has an important role to reduce ammonia gas emission from broiler litter and increase the birds performance.

Keywords: ammonia control, broiler chicken, litter management, *Yucca schidigera*

INTRODUCTION

Ammonia in broiler farm is a major concern of this modern poultry industry especially for tropical areas. In the case of poultry, about 50% of nitrogen (N) content of freshly excreted manure is in the form of uric acid and it can be very quickly transformed into ammonia (NH_3) by hydrolysis, mineralization, and volatilization (Oenema et al., 2001). Factors that contribute to the formation of NH_3 include temperature, moisture, pH, and nitrogen content of the litter or manure. High temperatures increase bacterial activity and also ammonia production, with a 1 to 2°C rise having a huge effect on ammonia levels (Visek, 1968). Excess NH_3 in broiler house is frequently claimed for growth retardation, poor feed utilization, increase susceptibility to diseases and increase subsequent mortality. Therefore exploring safe and effective ways that control NH_3 level in broiler house and increase immunity has become a research priority (Su et al., 2016).

Yucca schidigera (Agavaceae), commonly named yucca is documented as a source of sustenance and herbal drug by native Indians due to its health-promoting activity (Patel, 2012; Su et al., 2016). The main applications of Yucca powder and juice are in animal nutrition to reduce fecal odors and ammonia, hydrogen sulfide and some other harmful volatile compounds in domestic animal excreta (Cheeke, 2000; Kelly and Kohler, 2003; Gaber, 2006). The former fractions of Yucca Extract (YE) are steroidal saponins and polyphenols (Piacente et al., 2005). The steroidal saponin is the main chemical component of YE, which physically binds ammonia, reducing the level of free ammonia and plays significant role on the improvement of economic traits on broiler chickens (Nazeer et al., 2002). YE has positive effects on the growth rates, feed efficiency, and health in livestock (Colina and Chang, 2001; Duffy et al., 2001; Flaoyen et al., 2002; Kaya et al., 2003; Su et al., 2016; Rezaei et al., 2017).

In present study, the effects of 3 different concentrations of YE on ammonia gas emission, production performance and also carcass characteristics of broiler chickens were investigated with attention to economic utility.

MATERIALS AND METHODS

Ethical approval

The present study was was conducted according ethical rules approved by Sher-e-Bangla Agricultural University, Dhaka, Bangladesh.

Experimental chicks, treatments and management

A total of 240 day-old broiler chicks of "Cobb-500" strain with 43.2±0.3g average body weight were used in the current study. They were kept in electric brooders equally by maintaining standard brooding protocol. Among them, 180 chicks were distributed randomly in three treatments of Yucca extract providing with drinking water and remaining 60 chicks were distributed as control. Each treatment was divided into three replicates and in each replicate there were 20 birds. The Yucca treated groups were: 1ml YE per 16 liters of drinking water (T_1), 1ml YE per 20 liters of drinking water (T_2), 1ml YE per 24 liters of drinking water (T_3) and the group without YE supplementation was control (T_0).Throughout the period, the chicks were raised in an open-sided broiler house with rice husk-littered floor. *Ad libitum* feeds and water were provided for rapid growth of broiler chicks up to the end of the four weeks. Nutrient content and feed composition was indicated in table 1. The chicks were vaccinated with commercial Newcastle disease vaccine (NDV) and Infectious bronchitis (IB) vaccine through eye drops at 4 days and 21 days. The Gumboro vaccines were given through drinking water at day- 9 and day-17 of the experiment respectively.

Collection of ammonia test kit and experimental chemical (Yucca extract)

To assess ammonia, commercially available Micro Essential pHydrion™ ammonia meter tester paper was collected. The paper was packaged as a 15 foot roll in a pocket sized plastic dispenser with a polypropylene case and it comes complete with a specially calibrated color chart for matching the ammonia level of the broiler farm. The experimental chemical, *Yucca schidigera* extract (No-Gas™ ACI Animal Health) was purchased and it contains saponin steroids and glycocomponents.

Data collection

Body weight and feed intake were determined weekly. The average of the daily recorded ammonia emission was calculated. Feed conversion ratio (FCR) was calculated as the total feed Intake (FI) divided by weight gain in each replication. Carcass yield of bird was obtained from live weight subtracting blood, feathers, head, shank and inedible viscera. Mortality rate was maintained on a daily basis. Then total survivability of each treatment was calculated as percentage basis.

Economic analysis

The economic feasibility of *Y. schidigera* supplement for broiler production was estimated on the basis of total expenditure and the return from the sale of live birds (Sahoo et al., 2015). The production cost was calculated by considering the expenses involved in chicks, feed, vaccine and medication, litter materials, disinfectant, electricity, labor and YE. Chicks, disinfectant, vaccine, medicine, litter materials, electricity and labor costs were considered as common costs for both the treated groups and control group. All expenses and income were calculated on the basis of market price (USD) at the time of experimental period. Return was calculated by selling the live birds per kg weight and profit was computed by subtracting the expenditure. Benefit Cost Ratio (BCR) was calculated by total income divided by total cost of production. Profit per bird (PPB) calculated by total income pee bird subtracting total expenditure per bird.

Table 1. Ingredients and nutrient composition (as-dry matter) of basal diet

Ingredients	Composition (%, unless otherwise noted)
Maize	45.5
Soybean meal (CP 46%)	17.0
Wheat flour	10.0
Bread flour	5.00
Rice bran	4.45
Crude palm oil	3.50
Corn gluten meal (CP 62%)	3.60
Distiller dried grains (CP 27%)	3.00
Meat bone meal (CP 49%)	2.80
Chicken feather meal (CP 79%)	2.00
Bone meal (CP 22%)	1.50
Lysine	0.55
Methionine	0.37
L-threonine	0.08
Salt	0.15
Premix[1]	0.50
Analysed composition	
Metabolizable energy (kcal/kg)	3,300
Dry matter	89.6
Crude protein	21.9
Crude fat	6.40
Crude fiber	5.62
Ash	6.39

[1]Mineral-vitamin premix per kg of diet: Ca 2.250 g, P 0.625 g, Fe 3.570 mg, Cu 0.640 mg, Mn 5.285 mg, Zn 0.003 mg, Co 0.001 mg, Se 0.013 mg, I 0.016 mg, vit A 375 IU, vit D 150 IU and vit E 0.080 mg.

Statistical Analysis

Total data were compiled, tabulated and analyzed in accordance with the objectives of the study. The collected data was subjected to statistical analysis by applying one way ANOVA using Statistical Package for Social Sciences (SPSS- 16.0). Differences between means were tested using Duncan's multiple range test and significance was set at $P<0.05$.

RESULTS AND DISCUSSION

Ammonia assessment

The rate of NH_3 emissions from broiler litter against the 28-days of rearing period at different concentration levels of yucca extract had shown in table 2. ANOVA analysis revealed that the average NH_3 levels in treated groups and untreated group were insignificant ($P>0.05$) in 1st three weeks but it varied statistically ($P<0.05$) at 4th week of rearing period. The T_1 reduced the rate of NH_3 emission to 1/2 of the untreated level, whereas the T_2 reduced this rate to 2/3rd and T_3 reduced 1/5th of the untreated level. Cabuk et al. (2004) also concluded that the supplementation of *Yucca schidigera* to the diet reduced NH_3 concentration in broiler house. Chepete et al. (2012) reported that when YE was applied to laying-hens, in diets significantly reduced NH_3 emission by 44% and 28% for the first two days of manure storage. However, Corzo et al. (2007) reported that the supplementation of 100 ppm of YSE and *Quillaja saponaria* was added in a corn-soybean control diet of broiler chicken, and NH_3 emission was not altered compared with control.

Table 2. Effects of Yucca extract on ammonia gas emissions of broiler litter

Treatment	First week	Second week	Third week	Fourth week
T_0	8.37±0.98	10.23±1.50	14.97±3.79	25.87^{a} ±0.73
T_1	6.87±0.27	8.33±0.91	9.67±0.84	11.87^{c} ±0.37
T_2	7.10±0.30	8.43±0.58	10.37±1.02	15.13^{c} ±1.57
T_3	7.33±0.21	9.37±1.49	11.53±0.54	20.17^{b} ±1.53

Here, T_0 = (Control), T_1= (1ml YE per 16L of drinking water), T_2 = (1ml YE per 20L of drinking water), T_3 = (1ml YE per 24L of drinking water); Different superscripts in a column means significant different ($P<0.05$).

Growth performances

Final Live weight

The relative final live weight (g) of broiler chickens in the different groups T_0, T_1, T_2 and T_3 were presented in Table 3 and highest value was found in T_2 (1632.03 g). Average final live of Yucca treated groups were about 100 g more than the control group (1515.60 g) ($P<0.05$). Results also demonstrated that the body weights varied among the treatment groups ($P<0.05$). Previous researchers stated that the positive effects of dietary supplementation with Yucca products on the growth rates and body weights in different species (Cabuk et al., 2004; Sonia et al., 2005; Sahoo et al., 2015; Su et al., 2016 ; Ahmed, 2018).

Feed intake

Total feed intake (FI) of different treatment groups and control group have been cataloged in table 3. Result in total FI demonstrated that treatment groups showed significant ($P<0.05$) difference than control group. T_2 group consumed more feed (2308.63 g) and T_0 (control) group consumed comparatively lower (2296.27 g), whereas T_1 and T_3 consumed 2306.80 g and 2306.40 g respectively. The result also presented that FI of the 1st two weeks (starter phase) significantly ($P<0.05$) greater in Yucca treated groups than control and a reversed trend was recorded at finishing stage (Table 5) that was contradictory to Cabuk et al. (2004) who reported that from 21 to 42 and 42nd day of age, the FC was not significantly different. As Yucca contains saponins that acts biological role as a membrane-permeabilising, immunostimulant and hypocholesterolaemic properties and it has found to have significant affect growth and FI in animals (Das et al., 2012).

Feed conversion ratio

There was significant difference ($P<0.05$) for FCR among the Yucca treated groups and control group (Table 3). The better FCR value was found in T_2 group (1.45), where control group performed poor (1.56). In 1st two weeks there were insignificant ($P>0.05$) difference among the treated groups with control also, but at the last two weeks (3rd and 4th) result revealed significant ($P<0.05$) difference. Lundeen, (2000), Sonia et al., (2005) and Sahoo et al., (2015) reported that the efficiency of feed utilization was significantly better in Yucca group which led to significantly ($P<0.05$) better FCR than control. The

broilers exposed to the low concentration of ammonia showed the highest feed efficiency (Shlomo, 2004; Miles et al., 2004) and the study agree with this result as YE affecting ammonia emission.

Survivability

There is not any significant difference among the groups in term of survivability (Table 3) and the reason might be proper biosecurity management. Some researchers reported that lower survivability percentage was recorded in the control group as compared to the treatment group (Aslan et al., 2005; Sahoo et al., 2015).

Carcass characteristics

Carcass characteristics of the birds had shown in table 4 and the result demonstrated that eviscerated weight percentage, breast meat and thigh yield were significantly higher in Yucca treated group ($P<0.05$) than the control group similar to Sahoo et al. (2015). Thus we also observed, overall yield of edible meat was significantly ($P<0.05$) higher in Yucca groups than the control group.

Economics

The result of economic analysis revealed that Yucca treated groups had significantly ($P<0.05$) better profit than control group (Table 5). Total expenditure per bird was slightly high in treated groups than control but was statistically insignificant ($P>0.05$). So it can be suggested that application of YE with drinking water may be cost-effective management practice to improve shed environment and in turns performance of broiler chicks.

Table 3. Effects of Yucca extract on production performances of broiler chickens.

Treatment	Final live weight (g/bird)	Average BWG (g/bird)	Total FI (g/bird)	Final FCR	Survivability (%)
T_0	$1515.60^{c}\pm2.47$	$1472.40^{c}\pm2.46$	$2296.27^{b}\pm3.73$	$1.56^{a}\pm0.01$	98.33±1.66
T_1	$1621.87^{ab}\pm9.76$	$1575.33^{ab}\pm7.48$	$2306.80^{a}\pm1.38$	$1.46^{b}\pm0.07$	100.00±0.00
T_2	$1632.03^{a}\pm3.40$	$1588.83^{a}\pm3.40$	$2308.63^{a}\pm1.02$	$1.45^{c}\pm0.03$	100.00±0.00
T_3	$1607.87^{b}\pm5.16$	$1564.67^{b}\pm5.16$	$2306.40^{a}\pm1.15$	$1.47^{b}\pm0.03$	100.00±0.00

Here, T_0 = (Control), T_1= (1ml YE per 16L of drinking water), T_2 = (1ml YE per 20L of drinking water), T_3 = (1ml YE per 24L of drinking water), BWG: body weight gain, FI: feed cintake, FCR: feed conversion ratio. Different superscripts in a column means significant different ($P<0.05$).

Table 4. Effects of Yucca extract on carcass characteristics of broiler chickens.

Treatment	*Eviscerated weight (%)	*Giblet (%)	**Breast meat (%)	**Drumstick (%)	*Edible (%)
T_0	$59.83^{c}\pm1.08$	$7.40^{a}\pm0.57$	$33.75^{c}\pm0.45$	16.80±0.35	$67.14^{c}\pm0.32$
T_1	$65.82^{b}\pm0.40$	$6.50^{ab}\pm0.26$	$35.09^{bc}\pm0.34$	17.00±0.25	$72.07^{b}\pm0.34$
T_2	$68.39^{a}\pm0.56$	$5.51^{b}\pm0.28$	$37.14^{a}\pm0.58$	17.17±0.38	$73.64^{a}\pm0.33$
T_3	$67.05^{ab}\pm0.70$	$6.11^{ab}\pm0.46$	$35.67^{b}\pm0.33$	17.13±0.31	$72.86^{ab}\pm0.69$

Here, T_0 = (Control), T_1= (1ml Yucca extract per 16L of drinking water), T_2 = (1ml Yucca extract per 20L of drinking water), T_3 = (1ml Yucca extract per 24L of drinking water), *percentage of body weight, **percentage of eviscerated weight. Different superscripts in a column means significant different ($P<0.05$).

Table 5. Effects of Yucca extract on economic aspects of broiler chicken farming.

Parameter	Treatment			
	T_0	T_1	T_2	T_3
Feed cost (USD) per bird	1.16±0.29	1.17±0.28	1.18±0.21	1.17±0.42
Cost of Yucca extract (USD) per bird	0	0.021	0.017	0.014
Common expenditure (USD) per bird	0.81	0.82	0.81	0.82
Total Expenditure (USD) per bird	1.98±0.30	2.01±0.43	2.01±0.22	1.99±0.27
Receipt per bird when sold (1.55 USD/ Kg Live weight)	$2.35^{c}\pm0.32$	$2.50^{b}\pm1.2$	$2.53^{a}\pm0.44$	$2.51^{b}\pm0.67$
Profit per bird (USD)	$0.39^{c}\pm0.60$	$0.49^{ab}\pm0.97$	$0.52^{a}\pm0.66$	$0.48^{b}\pm1.07$

Benefit cost ratio	1.18^{b}±0.03	1.24^{a}±0.06	1.25^{a}±0.03	1.24^{a}±0.01

Here, T_0 = (Control), T_1= (1ml YE per 16L of drinking water), T_2 = (1ml YE per 20L of drinking water), T_3 = (1ml YE per 24L of drinking water). Different superscripts in a column means significant different (P<0.05).

CONCLUSION

Analyzing the above research findings, this study suggested that the 1ml of *Yucca schidigera* plant extract with 20L of drinking water may be used to minimize the ammonia gas emission, for better production performance, to improve carcass quality and more economic benefit in broiler rearing. *Yucca schidigera* plant naturally not found in many areas of the world but the extract might be commercially available to other countries. The study therefore recommends for hematological parameters on birds immunity. Hence, it could be safely used in broiler rearing for higher economical return without any adversity.

DECLARATIONS

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Author's contribution

Mahfuj Ullah Patoary and Mufazzal Hossain conducted the research, prepared data and performed statistical analysis. Mofassara Akter and Zahir Uddin Rubel wrote the article. All authors checked and confirmed the final form of article.

Competing interests

The authors declared that they have no competing interests.

Consent to publish

All authors informed their consent prior to inclusion in the study.

REFERENCES

Ahmed A (2018). Effect of adding local Yucca aloifolia powder in diet in productive performance of quail. Conference: 8th International Conference for Sustainable Agriculture Development. Egypt. Available at: https://www.researchgate.net/publication/325871084

Aslan R, Dundar Y, Eryavuz A, Bulbul A, Kucukkurt I, Fidan AF and Akinci Z (2005). Effects of various quantities of *Yucca schidigera* powder (Deodorase) added to diets on the performance, some hematological and biochemical blood parameters, and total antioxidant capacity of laying hens. Revue De Medecine Veterinaire, 156: 350-355. Available at: https://www.revmedvet.com/2005/RMV156_350_355.pdf

Cabuk M, Alcicek A, Bozkurt M and Akkan S (2004). Effect of *Yucca schidigera* and Natural Zeolite on Broiler Performance. International Journal of Poultry Science, 3(10): 651-654. DOI: http://dx.doi.org/10.3923/ijps.2004.651.654

Cheeke PR (2000) Actual and Potential Applications of *Yucca Schidigera* and *Quillaja Saponaria* Saponins in Human and Animal Nutrition. In: Oleszek W., Marston A. (eds) Saponins in food, feedstuffs and medicinal plants. proceedings of the phythochemical society of Europe, 45. DOI: https://doi.org/10.1007/978-94-015-9339-7_25

Chepete HJ, Xin H, Mendes LB, Li H and Bailey TB (2012). Ammonia emission and performance of laying hens as affected by different dosages of *Yucca schidigera* in the diet. Journal of Applied Poultry Research, 21(3): 522-530. DOI: https://doi.org/10.3382/japr.2011-00420

Colina JJ and Chang EJ (2001). Dietary manipulation to reduce aerial ammonia concentrations in nursery pig facilities. Journal of Animal Science, 79: 3096-3103. DOI: https://doi.org/10.2527/2001.79123096x

Corzo A, Kidd MT, Miles DM, Dozier WA and Cheeke PR (2007). Yucca schidigera and Quillaja saponaria supplementation in broiler diets [abstract]. International Poultry Scientific Forum, p. 64. Available at: https://www.ars.usda.gov/research/publications/publication/?seqNo115=201720

Das TK, Banerjee D, Chakraborty D, Pakhira MC, Shrivastava B and Kuhad RC (2012). Saponin: Role in Animal system. Veterinary World, 5(4): 248-254. DOI: https://doi.org/10.5455/vetworld.2012.248-254

Duffy CF, Killeen GF, Connolly CD and Power RF (2001). Effects of dietary supplementation with Yucca schidigera Roezl ex Ortgies and its saponin and non-saponin fractions on rat metabolism. Journal of Agricultural and Food Chemistry, 49: 3408-3413. DOI: https://doi.org/10.1021/jf010149+

Flaoyen A, Wilkins AL and Sandvik M (2002). Ruminal metabolism in sheep of saponins from Yucca schidigera. Veterinary Research Communications. 26(2):159-69. DOI: https://doi.org/10.1023/A:1014051803104

Gaber MM (2006). The effects of plant-protein based diets supplemented with Yucca on growth, digestibility, and chemical composition of Nile Tilapia (Oreochromis niloticus, L) fingerlings. Journal of the World Aquaculture Society, 37: 74-81. DOI: https://doi.org/10.1111/j.1749-7345.2006.00008.x

Kaya S, Erdogan Z and Erdogan S (2003). Effect of different dietary levels of *Yucca schidigera* powder on the performance, blood parameters and egg yolk cholesterol of laying quails. Journal of Veterinary Medicine Series A, 50(1): 14-17. DOI: https://doi.org/10.1046/j.1439-0442.2003.00487.x

Kelly AM and Kohler CC (2003). Effects of *Yucca schidigera* extract on growth, nitrogen retention, ammonia excretion, and toxicity in channel catfish Ictalurus punctatus and hybrid tilapia O. mossambicus × O. niloticus. Journal of the World Aquaculture Society, 34: 156-161. DOI: https://doi.org/10.1111/j.1749-7345.2003.tb00052.x

Miles DM, Butcher NH, Branton SL and Lott BD (2004). atmospheric ammonia is detrimental to the performance of modern commercial broilers. Poultry Science, 83(10): 1650–1654. DOI: https://doi.org/10.1093/ps/83.10.1650

Nazeer MS, Pasha TN, Shahid A and Ali Z (2002). Effect of *Yucca* saponin on urease activity and development of ascites in broiler

chickens. International Journal of Poultry Science, 1(6): 174-178. DOI: http://dx.doi.org/10.3923/ijps.2002.174.178

Oenema O, Bannink A, Sommer SG and Velthof GL (2001), Gaseous nitrogen emissions from livestock farming systems. Nitrogen in the environment: sources, problems, and management. Elsevier, Amsterdam, pp. 255-289. Link: https://research.wur.nl/en/publications/gaseous-nitrogen-emissions-from-livestock-farming-systems-2

Panetta DM, Powers WJ, Xin H, Kerr BJ and Stalder KJ (2006). Nitrogen excretion and ammonia emissions from pigs fed modified diets. Journal of Environmental Quality, 35(4): 1297-1308. DOI: https://doi.org/10.2134/jeq2005.0411

Patel S (2012). Yucca: A medicinally significant genus with manifold therapeutic attributes. Natural Products and Bioprospecting, 2(6): 231-234. DOI: https://doi.org/10.1007/s13659-012-0090-4

Piacente S, Pizza1 C and Oleszek W (2005). Saponins and phenolics of *Yucca schidigera* Roezl: Chemistry and bioactivity. *Phytochemistry* Reviews, 4(2-3): 177-190. DOI: https://doi.org/10.1007/s11101-005-1234-5

Rezaei R, Lei J and Wu G (2017). Dietary supplementation with Yucca schidigera extract alleviates heat stress–induced growth restriction in chickens. Journal of Animal Science, 95(4): 370-371. DOI: https://doi.org/10.2527/asasann.2017.866

Sahoo SP, Kaur D, Sethi APS, Sharma A and Chandra M (2015). Evaluation of *Yucca schidigera* extract as feed additive on performance of broiler chicks in winter season. Veterinary World, 8(4): 556-560. DOI: http://doi.org/10.14202/vetworld.2015.556-560

Santoso B, Mwenya B, Sar C and Takahashi J (2006). Ruminal fermentation and nitrogen metabolism in sheep fed a silage-based diet supplemented with *Yucca schidigera* or Y. schidigera and nisin. Animal Feed Science and Technology, 129(3): 187-195. DOI: https://doi.org/10.1016/j.anifeedsci.2006.01.001

Shlomo Y (2004). Ammonia affects performance and thermoregulation of male broiler chickens. Animal Research, 53: 289–293. DOI: https://doi.org/10.1051/animres:2004015

Su JL, Shi BL, Zhang PF, Sun DS, Li TY and Yan SM (2016). Effects of Yucca extract on feed efficiency, immune and antioxidative functions in broilers. Brazilian Archives of Biology and Technology, 59: e16150035. DOI: https://doi.org/10.1590/1678-4324-2016150035

Visek WJ (1968). Some aspects of ammonia toxicity inanimal cells. Journal of Dairy Science, 51: 286. DOI: https://doi.org/10.3168/jds.S0022-0302(68)86976-0

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Serological and Molecular Detection of Chicken Anemia Virus in Broiler and Layer Chickens in Iraq

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ABSTRACT

Chicken Anemia Virus (CAV) infects many bird species worldwide and causes immunosuppression. This condition can facilitate the infection of affected birds with other pathogens including bacteria, viruses, and fungi. No data were available on detection or isolation of CAV from birds in Iraq, therefore this study was designed to detect CAV antibodies in broilers and layers in some poultry farms. Accordingly, 200 samples were collected from broiler and layer farms (100 samples each) from different districts of Diyala province and subjected to the ELISA test. Also, 50 tissue samples from embryonated eggs from different hatcheries, four commercial viral vaccines, and 30 ELISA positive samples were subjected to PCR assay to detect the CAV DNA. The results showed that all of broiler and layer farms sampled were serologically positive for CAV antibodies. The overall seropositivity for CAV antibodies for both chicken breeds was 51.5%. In broilers, 43 out of 100 serum samples were positive for CAV antibodies, whereas 60 out of 100 serum samples from layers were CAV antibody-positive. According to age groups, significant differences were observed among one-week-old broilers (30.2%) compared to other age groups. In layers, the age group of 30 weeks showed a seropositivity rate of 33.3%. Conventional PCR test indicated that all tissue samples collected from suspected birds and embryonated eggs were negative for CAV DNA, but only 2 out of 30 serum samples were PCR positive. It is concluded that CAV is endemic in poultry farms of Iraq and may facilitate the vaccination failure against other viruses.

Key words: Broilers, Chicken anemia virus, ELISA, Layers, PCR

INTRODUCTION

Chicken Anemia Virus (CAV) infection is an important disease from economic and health points of view in the poultry industry worldwide (Bhatt et al., 2011; Padhy et al., 2015; Adedeji et al., 2016). The CAV is a single-stranded circular negative sense DNA virus. It is the smallest among DNA viruses and classified within the genus *Gyrovirus* in the family *Anelloviridae* (Li et al., 2017a; Rosario et al., 2017). The virus DNA encodes viral proteins of VP1, VP2, and VP3 (Ducatez et al., 2008). This virus causes chicken anemia or blue wing disease in many species of birds (Mariya, 2018).

The CAV was first isolated from affected chicks in Japan in 1979, but Toro et al. (2006) by analyzing stock serums, reported that the virus has been circulated in the USA since 1959. Although the infection is contagious, most affected birds with CAV are aged 7 to 28 days, which shows atrophy of lymphoid tissues and severe anemia (Dhama et al., 2008). Birds infected with CAV are susceptible to infection with other pathogens as the virus causes immunosuppression and results in vaccination program failure (De Herdt et al., 2001; Hoerr, 2010; Rimondi et al., 2014). Mortality, secondary infections, and poor weight gain due to CAV infection lead to economic losses (Dhama et al., 2008).

The causative agent of the disease can be transmitted by two modes (Miller et al., 2003; Brentano et al., 2005), vertical (Cardona et al., 2000a) or horizontal transmission through oral-fecal route and infected feather's shaft (Davidson et al., 2008). Some studies have detected antibodies against CAV in specific-pathogen-free chickens, supporting the hypothesis of latent or persistent CAV infections. It is suggested that the virus persists in the reproductive system and it may be somehow reactivated (Cardona et al., 2000b; Miller and Schat 2004; Miller et al., 2008).

In case of active infections, it is recommended to depend on clinical findings for CAV diagnosis.

Furthermore, this can be confirmed by many serological tests such as immunofluorescent antibody test, Enzyme-Linked Immunosorbent Assay (ELISA) and virus neutralization using reference serum (Todd et al., 2001), hemagglutination inhibition test, agar gel precipitation test (Kataria et al., 2013) and indirect immunofluorescent test (Oluwayelu et al., 2007) or by virus isolation using cell culture (Van Santen et al., 2001). Many molecular techniques such as Polymerase Chain Reaction (PCR), whole-genome sequencing, and restriction fragment length polymorphism are used for epidemiological studies or differentiation between virus isolates of CAV (Schat, 2009; Manoharan et al., 2012; Rehman et al., 2018).

In Iraq, no data were available on screening of chicken commercial farms for presence of CAV infection; there is only one report on CAV seropositivity in Japanese quails and local fowls (Al-Ajeeli et al., 2018). Accordingly, the present study aimed to investigate the presence of CAV antibodies in broiler and layer chickens and to detect CAV DNA in positive serum samples, embryonated eggs from different hatcheries, and some commercial viral vaccines.

MATERIALS AND METHODS

This study was conducted in virology and molecular biology laboratories of the College of Veterinary Medicine, University of Diyala, Iraq over the period from October 2017 to July 2018.

Ethical approval

Scientific ethical committee in the University of Diyala/ College of Veterinary Medicine, Iraq, approved the research and give the ethical number (Vet 24 Medicine November 2017 K, A and H).

Serum samples

A total of 200 blood samples were collected from five commercial broiler farms aged 1-5 weeks old (100 samples) and four layer farms aged 8-30 weeks old (100 samples) in different areas of Diyala province, Iraq. Blood sampling was performed by wing vein puncture using sterile syringes and vacuum blood collection tubes gel clot activator (UNIMEDIC, Iraq). Then sera were separated and placed in Eppendorf sterile tube, labeled and centrifuged at 1500 rpm for 5 minutes (Cold Eppendorf centrifuge. THERMO FISHER, USA). The supernatant was collected from each sample and transferred to another sterile Eppendorf tube and stored at -20 °C until used.

Processing of samples for ELISA

The sera (1:10 dilution) were tested using a commercial ELISA kit (IDEXX Lab, Germany). Optical density values were read at 650 nm using the ELX 800™ microplate reader (BIO-TEK Instruments, USA) and data were expressed as S/N ratio (sample to negative ratio). Samples with S/N > 0.60 were considered negative for CAV antibodies, whereas samples with S/N ≤ 0.60 were considered positive for CAV antibodies.

PCR samples

PCR samples included 50 embryonated hen eggs (10 days old) collected from five different hatcheries in Diyala province, 30 CAV-ELISA positive serum samples randomly selected, and four available commercial viral vaccines against Newcastle disease, infectious bronchitis, and infectious bursal disease (Gumboro) (Table 1). These samples were subjected to DNA extraction before testing by PCR.

DNA extraction

DNA extraction was carried out using a DNA extraction kit (Genekam Biotechnology AG, Germany) according to the instructions of the manufacturer.

Table 1. Commercial vaccine samples used for detection of chicken anemia virus by PCR assay.

Vaccines	Dose	Country
Nobilis® Gumboro D78 (live)	1000	Netherlands
Nobilis® MA5 + CLONE 30 (live) / against Massachusetts type of IB and ND	1000	Netherlands
Nobilis® ND clone 30 (live)	1000	Netherlands
Nobilis® IB 4-91 (live attenuated)	1000	Netherlands

IB: Infectious bronchitis, ND: Newcastle disease

PCR procedure

To detect the presence of CAV DNA in the above-mentioned samples, PCR kit (Genekam Biotechnology AG: Ref.K132, Germany) was used according to the manufacturer's instructions. This PCR commercial kit has been designed to produce a CAV DNA fragment of 675

base pairs (bp) from the gene responsible for VP1 production (VP1 gene).

The PCR thermal cycle included a heating step at 94°C for 300 seconds, and the amplification cycle that included denaturing step at 94°C for 60 seconds, annealing step at 50°C for 60 seconds, extension step at 72°C for 120 seconds. This cycle was repeated 35 times and followed by a long extension step at 72°C for 600 seconds. The product was cooled to 4 °C and kept at -20 ºC until electrophoresed in 2% agarose gel in 1x TAE buffer along with molecular weight marker, stained with ethidium bromide (0.05µg/ml) and photographed.

Statistical analysis

The results were statistically analyzed using SPSS version 21. P-values less than 0.05 were considered significant.

RESULTS

Seropositivity rate of chicken anemia virus in broiler chickens

The overall seropositivity rate for CAV in broilers was 43%. According to age groups, there were significant differences in seropositivity between groups of two weeks old (9.3%) and five weeks old (16.3%) with groups of one (30.2%), three (20.9%) and four weeks old (23.3%). The significant differences were observed in the number of seropositive samples to the number of seronegative samples of age groups of one, two and four weeks (Table 2).

Seropositivity rate of chicken anemia virus in layer chickens

The overall seropositivity rate for CAV in layers was 60%. The age group of 30 weeks old showed a seropositivity rate of 33.3%, followed by a seropositivity rate of 31.7%, 26.7% and 8.3% for the age groups of 28, 8 and 10 weeks, respectively. Significant differences appeared between positive and negative serum samples of each age group. Also, significant differences appeared between the age group of 10 weeks and other age groups in both positive and negative serum samples (Table 3).

Seropositivity rate for chicken anemia virus according to chicken breeds

The results showed differences in seropositivity among both groups of chicken breeds (layers and broilers). In broilers, 43 (43%) samples were positive for CAV antibodies. In layers, 60 (60%) serum samples were positive for CAV antibodies. Significant differences were observed between the seropositivity of broilers' serum samples and that of layers' serum samples (Table 4).

Table 2. Seropositivity rate for antibodies against chicken anemia virus in broiler chickens of different ages

Age of birds	No of CAV-ab positive samples	No of CAV-ab negative samples	Total
One week	13[a]	7[b]	20
Two weeks	4[b]	16[a]	20
Three weeks	9[a]	11[a]	20
Four weeks	10[a]	10[a]	20
Five weeks	7[b]	13[a]	20
Total	43	57	100

Different superscript letters in a column indicate significant differences (p<0.05). CAV-ab: chicken anemia virus antibody, No: Number

Table 3. Seropositivity rate for antibodies against chicken anemia virus in layers chickens of different ages

Age of birds	No of CAV-ab positive samples	No of CAV-ab negative samples	Total
8 weeks	16[a]	9[b]	25
10 weeks	5[b]	20[a]	25
28 weeks	19[a]	6[b]	25
30 weeks	20[a]	5[b]	25
Total	60[a]	40[b]	100

Different superscript letters in a column indicate significant differences (p<0.05). CAV-ab: chicken anemia virus antibody, No: Number

Table 4. Seropositivity rate for antibodies against chicken anemia virus in layer and broiler chickens

Age of birds	No of CAV-ab positive samples	No of CAV-ab negative samples	Total
Broilers	43[a]	57	100
Layers	60[b]	40	100

Total	103	97	200

Different superscript letters in a column indicate significant differences (p<0.05). CAV-ab: chicken anemia virus antibody, No: Number

Level of chicken anemia virus antibodies

The level of antibodies in broiler serum samples appeared high (H) in 13% of samples, medium (M) in 11% and low (L) in 19% of samples, whereas 57 (57%) samples were negative for CAV antibodies. In layers, 29% were H, 19% samples were M, and 12% were L, whereas, 40% of samples were negative. There were significant differences among H level and L level of layers. The number of negative samples for CAV antibodies according to the S/N ratio was 97 samples (57 from broilers and 40 from layers). Significant differences (p<0.05) appeared between the number of negative serum samples collected from broilers (57%) and negative serum samples collected from layers (40%). Significant differences were also observed in number of CAV-positive broiler serum samples of H (13), M (11), and L (19) S/N ratio with those of H (29), M (19), and L (12) S/N ratio in layers, respectively (Table 5).

Detection of chicken anemia virus DNA by PCR assay

All 50 tissue samples collected from embryonated eggs and four commercial virus vaccines were negative for CAV DNA using PCR test. Out of 30 tested sera, only two serum samples were positive for CAV DNA using PCR that resulted in a PCR fragment of 850 base pairs (Figure 1).

Table 5. Antibody levels to chicken anemia virus in different chicken breeds.

Range of S/N ratio	Broilers	Layers	Total
High (0.001 to 0.199)	13[a]	29[b]	42
Medium (0.200 to 0.399)	11[a]	19[b]	30
Low (0.400 to 0.599)	19[b]	12[a]	31
Negative (0.600 and above)	57[a]	40[b]	97
Total	100	100	200

Different superscript letters in a column indicate significant differences (p<0.05). S/N ratio: Sample to Negative ratio

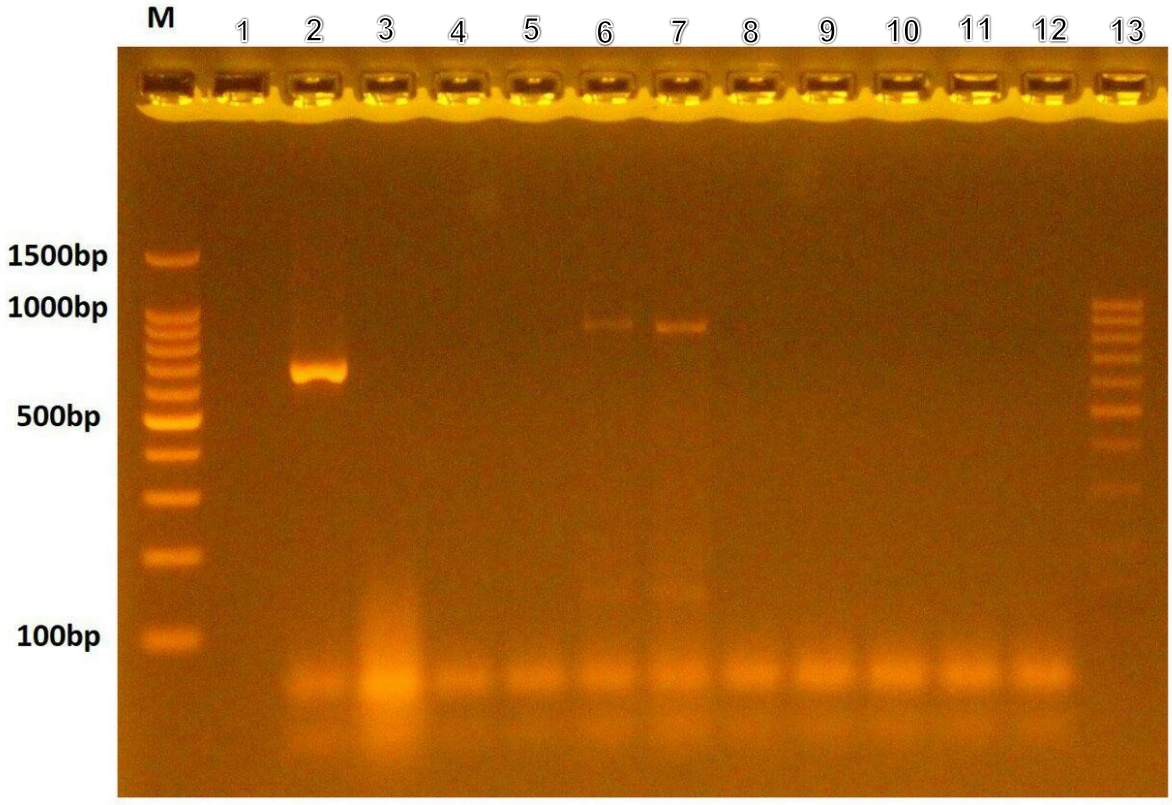

Figure 1. Results of CAV-ELISA positive serum samples subjected to PCR. Lane M (molecular weight markers), Lane 1 (negative control), Lane 2 (positive control, 675 bp), Lanes 3, 4, 5, 9, 10, 11 and 12 (negative serum samples), Lanes 6 and 7 (positive serum samples, 850 bp), Lane 13 (molecular weight markers).

DISCUSSION

Seropositivity rate of chicken anemia virus among broilers

The present study showed an overall seropositivity rate of 43% for CAV antibodies in broilers from different farms of Diyala province. Similar findings were reported from the Central African Republic and Cameroon by Snoeck et al. (2012) who found that 147 out of 400 (36.75%) chicken serum samples were positive for CAV antibodies. Furthermore, they found that the seropositivity of birds to CAV antibodies was different according to age groups and ranged from 25% to 50%, and most positive cases were reported in age groups of 4 to10 weeks old. In the present study, the majority of positive cases were in ages ranged from three to five weeks. Similar findings were reported in Malaysia (Oluwayelu et al., 2008), India (Bhatt et al., 2011) and China (Zhou et al., 1996). A seropositivity rate ranged from 66-100% was reported in Hungary by Drén et al. (1996).

The low seropositivity rate of CAV antibodies obtained in the present study is in agreement with the speculation of De Herdt et al. (2001) who mentioned that a particular chicken flock might not show homogenous seropositivity rate because the number of positive cases for CAV antibodies increases with age. Similar findings in association of seropositivity rate with increasing age of birds were reported by Owoade et al. (2004) who mentioned 40% seropositivity rate for CAV antibodies among broilers in Nigeria aged 2-6 weeks. Seroprevalence more than that of the present study was reported in many other countries; 49% in Argentina (Craig et al., 2009), 80% in Malaysia (Hailemariam et al., 2008) and 87% in China (Ducatez et al., 2008).

In Iraq, there was no vaccination program against CAV for broilers, but it seems that CAV is widely distributed among broilers. The seropositivity rate of CAV in unvaccinated broilers was also documented by Bidin et al (2010) who reported a seropositivity rate of 94.7% for CAV antibodies when 54 out of 57 samples collected from breeders were positive for CAV antibodies using ELISA assay. The same authors found that 24 out of 90 (26.6%) serum samples collected from broilers were positive for CAV antibodies. The seropositivity rate in unvaccinated birds aged 4 weeks and above was attributed to horizontal transmission of CAV that may not lead to clinical cases (Bidin et al., 2010). Furthermore, it was suggested that immunization of broilers with infectious bursal disease and Marek's disease viruses indirectly protect them from immune suppression observed in cases of CAV active infections (Bidin et al., 2010).

Seropositivity rate of chicken anemia virus among layers

In the present study, an overall seropositivity rate of 60% for CAV in layers was found. Similar findings to the seropositive rate of CAV in layers were reported by Abdelfattah (2009) who mentioned a seropositivity rate of 67.3% for CAV in layers in Sudan. Hadmili et al. (2008) reported a seroprevalence of 70.9% among layer groups in Turkey. The same authors found that 15 out of 16 layer groups were positive for CAV antibodies. In the present study, all layer farms tested were positive for CAV antibodies. Ballal et al. (2005) also reported that all tested layer groups in Sudan were positive for CAV antibodies. In the present study, the high seropositivity rate for CAV was detected in age groups of 28-30 weeks. This finding is consistent with previous findings that indicated an increase in seropositivity rate at older ages (Owoade et al., 2004; Sharma et al., 2014).

Detection of the chicken anemia virus DNA

PCR was used as a diagnostic tool for the detection of CAV in samples collected from birds suspected to be infected by the virus (Abo-Elkhair et al., 2014; Simeonov et al., 2014; Eskandarzade et al, 2015; Al-Kateb et al., 2017). Hailemariam et al. (2008) found that 40-70% of tested chicken embryos were positive for CAV when tested by PCR. However, the results obtained in the present study indicated that all embryonated tissue samples were negative for CAV. It seemed that it was dependable on the source of egg supply. The five hatcheries sampled in the present study were received their fertile eggs from one source supplier.

In the present study, the detection of CAV DNA in four commercial vaccines revealed that all used poultry commercial viral vaccines were negative for CAV. This finding is inconsistent with findings of Li et al. (2017b) who reported two batches of live viral vaccines were positive for CAV DNA in comparison to 12 negative batches of the vaccines in China, and also with findings of Varela et al. (2014) who found that 6 out of 32 tested live

viral vaccines were positive for CAV DNA and 1 out of 3 inactivated viral vaccines were also positive for the virus using PCR. The negativity of PCR detection in commercial vaccines in this study may be attributed to the small size of sampling (only four vaccines) in addition to these samples were from one company.

In the present study, CAV DNA was detected in 2 out of 30 tested serum samples. This result is in agreement with the findings of Tham and Stanislawek (1992), who detected the CAV DNA in two serum samples out of 37 PCR tested sera. This could be attributed to circulating CAV antibodies that might clear the virus from the blood circulation of infected birds and accordingly could not be detected by PCR. The PCR fragment that produced from two positive serum samples of present study was of 850 bp but the DNA fragment that should be detected by the PCR kit was assumed as 675 bp, and this difference in molecular weight of detected DNA fragment of present study and the assumed fragment's molecular weight of CAV DNA may be attributed to genetic variation among the locally detected CAV.

Many studies reported genetic variations among CAV isolates (Van Santen et al., 2007; Mohamed, 2010; Eltahir, et al., 2011; Hussein et al., 2016) that supports the finding of present study that the detected DNA PCR fragment of 850 bp rather than 675 bp of PCR kit might be attributed to genetic variation that allows the production of different DNA fragment size.

Oluwayelu et al. (2008) reported genetic differences among Nigerian CAV isolates that grouped them into four clusters. Hailemariam et al. (2008) described genetic variation in Malaysian CAV isolates due to genetic substitutions in DNA nucleotide bases resulted in changing of amino acid positions of VP1 in amino acid 75, 97,139 and 144 that led to group the isolates into two groups. Zhang et al. (2013) recorded strong evidence of genetic recombination in coding and non-coding regions of whole CAV genome in different Chinese isolates.

CONCLUSIONS

In conclusion, the detection of antibodies against chicken anemia virus in broilers and layers demonstrated that this virus widely distributes in poultry farms of Diyala province, Iraq. This condition requires restricted and effective control measures to avoid the possibility of complicated infections and vaccination failure as the virus causes immune suppression in infected birds.

DECLARATIONS

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Author's contribution

Karim Sadun Al-Ajeeli and Amer Khazaal A-Azawy proposed the hypothesis, designed the study and conducted the serological and molecular works. Haneen Ghazuan collected samples from poultry farms. All authors contributed to manuscript preparation and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

REFERENCES

Abdelfattah IEA (2009). Serological survey of CAV in Khartoum State, Sudan. M.Sc. thesis, Department of Preventive Medicine and Veterinary Public Health, Faculty of Veterinary Medicine, University of Khartoum. pp.27-42.

Abo-Elkhair M, Abd El-Razak AG and Metwally AEY (2014). Molecular characterization of CAV circulating in chicken group in Egypt. Advances in Virology, 2014: 797151. DOI: http://dx.doi.org /10.1155 .2014 /797151

Adedeji AJ, Sati N, Pewan S, Ogbu K, Adole J, Lazarus D, Ijiwo S, Okpanachi A, Nwagbo I and Joannis T (2016). Concurrent infections of chicken infectious anemia and infectious bursal disease in 5 weeks old pullets in Jos, Plateau State, Nigeria. Veterinary Sciences: Research and Reviews, 2(3): 60-65. DOI: http://dx.doi.org/10.17582/journal.vsrr/2016.2.3.60.65 .

Al-Ajeeli KS, Al-Azawy AK and Al-Rubayee H (2018). Sero-prevalence of chicken anemia virus in local fowls and Japanese quails. Indian Journal of Natural Sciences, 9 (51):15691-15699.

Al-Kateb A, Mansour L, Skeeb A, Al-Khallab E, Dardour A and Gerish EKH (2017). Molecular detection of chicken infectious anemia virus from backyard chickens in Libya. AlQalam Journal of Medical and Biological Research, 1(1): 70-75. Available at: https://www.researchgate.net/publication/331635917 .

Ballal A, Elussein AM and Igbal ASA (2005). Serological survey of chicken infectious anemia in commercial group in Khartoum state, Sudan. Journal of Animal and Veterinary Advances, 4(7):666-667.

Bhatt P, Shukla S K, Mahendran M, Dhama K, Chawak MM and Kataria JM (2011). Prevalence

Biđin M, Savić V, Biđin Z, Balenović M and Majnarić D (2010). The prevalence of antibodies against CAV in unvaccinated broilers and broiler breeders in Croatia. Veterinarski Archiv, 80(6): 753-760. Available at: https://hrcak.srce.hr/62391 .

Brentano L, Lazzarin S, Bassi SS, Klein TAP and Schat KA (2005). Detection of CAV in the gonads and in the progeny of broiler breeder hens with high neutralizing antibody titers. Veterinary Microbiology, 105(1): 65-73. DOI: https://doi.org/10.1016/j.vetmic.2004.09.019.

Cardona C, Lucio B, O'Connell P, Jagne J and Schat KA (2000b). Humoral immune responses to chicken infectious anemia virus in three strains of chickens in a closed flock. Avian Diseases, 44(3): 661-667. DOI: https://doi.org/10.2307/1593108 .

Cardona CJ, Oswald WB and Schat KA (2000a). Distribution of chicken anaemia virus in the reproductive tissues of specific-pathogen-free chickens. Journal of General Virology, 81(8): 2067-2075. DOI: https://doi.org/10.1099/0022-1317-81-8-2067 .

Craig MI, Rimondi A, Delamer M, Sansalone P, König G, Vagnozzi A and Pereda A (2009). Molecular characterization of chicken infectious anemia virus circulating in Argentina during 2007. Avian Diseases, 53(3): 331-335. DOI: https://doi.org/10.1637/8932.1 .

Davidson I, Artzi N, Shkoda I, Lublin A, Loeb E and Schat KA (2008). The contribution of feathers in the spread of CAV. Virus Research, 132(1-2): 152-159. DOI: https://doi.org/10.1016/j.virusres.2007.11.012 .

De Herdt P, Bosch V, Ducatelle R, Uyttebroek E and Schrier C (2001). Epidemiology and significance of chicken anemia virus infections in broilers and broiler parents under nonvaccinated European circumstances. Avian Diseases, 45(3):706-708. Available at: https://www.jstor.org/stable/1592916?origin=JSTOR-pdf&seq=1

Dhama K, Mahendran M, Somvanshi R and Chawak MM (2008). Chicken infectious anemia virus: an immunosuppressive pathogen of poultry-a review. Indian Journal of Veterinary Pathology, 32(2): 158-167. DOI: https://doi.org/10.1017/S0043933914000828 .

Drén C, Farkas T and Németh I (1996). Serological survey on the prevalence of chicken anaemia virus infection in Hungarian chicken group. Veterinary Microbiology, 50(1-2): 7-16. DOI: https://doi.org/10.1016/0378-1135(96)00002-8 .

Ducatez MF, Chen H, Guan Y and Muller CP (2008). Molecular epidemiology of CAV in southeastern Chinese live birds markets. Avian Diseases, 52(1): 68-73. DOI: https://doi.org/10.1637/8049-070407-reg .

Eltahir YM, Qian K, Jin W, Wang P and Qin A (2011). Molecular epidemiology of CAV in commercial farms in China. Virology Journal, 8:145-152. DOI: https://doi.org/10.1186/1743-422x-8-145 .

Eskandarzade N, Heydarnezhad A, Valizadeh Y,Shokouhi M and Kholenjani AS (2015). Molecular detection of CAV from native Larry-breed chickens in Charmahal-va-Bakhtiyari province, Iran. International Journal of Medical Laboratory, 2(2):105-111. DOI: https://doi.org/10.1016/j.meatsci.2014.02.007 .

Hadmili HH, ERGANİŞ O, Güler L and Ucan US (2008). Investigation of chicken infectious anemia virus infection by PCR and ELISA in chicken group. Turkish Journal of Veterinary and Animal Sciences, 32(2): 79-84.

Hailemariam Z, Omar AR, Hair-Bejo M and Giap TC (2008). Detection and characterization of CAV from commercial broiler breeder chickens. Virology Journal, (5): 128-138. DOI: https://doi.org/10.1186/1743-422x-5-128 .

Hangood LT, Kelly TF, Wright JC and Hoerr FJ (2000). Evaluation of chicken infectious anemia virus and associated risk factors with disease and production losses in broilers. Avian Diseases, (44): 803-808. DOI: https://doi.org/10.2307/1593052 .

Hoerr FJ (2010). Clinical aspects of immune suppression in poultry. Avian Diseases, 54(1): 2-15. DOI: https://doi.org/10.1007/978-1-4615-7237-4 .

https://doi.org/10.1111/j.1865-1682.2011.01215.x .

Hussein E, Arafa AE, Anwar N and Khafaga A (2016). Molecular and pathological analysis of CAV isolated from field infection in three Egyptian Provinces. Advances in Animal and Veterinary Sciences, 4(5): 218-229. DOI: https://doi.org/10.14737/journal.aavs/2016/4.5.218.229 .

Kataria JM, Dham K, Nagarajan S, Chakraborty S, Kaushal A and Deb R (2013). Fowl adenoviruses causing hydropericardium syndrome in poultry. Advances in Animal and Veterinary Sciences, 1(48):5-13.

Li Y, Fang L, Cui S, Fu J, Li X and Zhasng H (2017a). Genomic characterization of recent chicken anemia virus isolates in China. Frontiers in Microbiology, 8: 401-410. DOI: https://doi.org/10.3389/fmicb.2017.00401 .

Li Y, Hu, Y, Cui S, Fu J, Wang Y, Cui Z, Fang L, Chang S and Zhao P (2017b). Molecular characterization of chicken infectious anemia virus from contaminated live-virus vaccines. Poultry Science, 96(5): 1045–1051. DOI: https://doi.org/10.3382/ps/pew406 .

Manoharan AS, Ramadass P and Kumanan K (2012). Assessment of polymerase chain reaction sensitivity for the detection of chicken anaemia virus using different primers for three genes. Veterinarski Arhiv, 82(1): 89-102. Available at: https://hrcak.srce.hr/77406 .

Mariya H (2018). Blue wing disease. Approaches in Poultry Dairy and Veterinary Sciences, 5(2):407-408. DOI: https://doi.org/10.1080/03079458408418503 .

Miller MM and Schat KA (2004). Chicken infectious anemia virus: An example of the ultimate host-parasite relationship. Avian Diseases, 48(4): 734-745. DOI: https://doi.org/10.1637/7271-090304r .

Miller MM, Ealey KA, Oswald WB and Schat KA (2003). Detection of CAV DNA in embryonal tissues and eggshell membranes. Avian Diseases, 47(3): 662-671. DOI: https://doi.org/10.1637/7007 .

Miller MM, Jarosinski KW and Schat KA (2008). Negative modulation of the chicken infectious anemia virus promoter by COUP-TF1 and an E box-like element at the transcription start site binding δEF1. Journal of General Virology, 89(12): 2998-3003. DOI: https://doi.org/10.1099/vir.0.2008/003103-0 .

Mohamed MA (2010). Chicken infectious anemia status in commercial broiler chickens group in assiut-upper Egypt: occurrence, molecular analysis using PCR-RFLP and apoptosis effect on affected tissues. International Journal of Poultry Science, 9(6): 591-598. DOI: https://doi.org/10.3923/ijps.2010.591.598 .

of chicken infectious anemia virus (CIAV) in commercial poultry group of Northern India: a serological survey. Transboundary and Emerging Diseases, 58(5): 458-460.DOI:

Oluwayelu DO, Olaleye OD, Todd D, Emikpe BO and Ohore OG (2007). Evaluation of diagnostic performance and adoption of indirect immunofluorescence for serodiagnosis of chicken infectious anaemia in Nigerian poultry. Tropical Veterinarian, 25 (2): 41-47. DOI: https://doi.org/10.1016/0732-8893(88)90035-1 .

Oluwayelu DO, Todd D and Olaleye OD (2008). Sequence and phylogenetic analysis of chicken anaemia virus obtained from backyard and commercial chickens in Nigeria. Onderstepoort Journal of Veterinary Research, 75(4): 353-357. DOI: https://doi.org/10.4102/ojvr.v75i4.111 .

Owoade AA, Oluwayelu DO, Fagbohun OA, Ammerlaan W, Mulders MN and Muller CP (2004). Serologic evidence of chicken infectious anemia in commercial chicken group in southwest Nigeria. Avian Diseases, 48(1): 202-205. DOI: https://doi.org/10.1637/7075 .

Padhy A, Sahu A, Sahoo S and Ganguly S (2015). Chicken Infectious Anemia: A Review on its Potential Threat to Poultry Industry Worldwide. World Journal of Biology and Medical Sciences, 2(4): 17-19. DOI: https://doi.org/10.1002/9781119421481.ch8 .

Rehman SU, Saleemi M, Khan M, Khan A, Shahzad A, Khatoon A, Fayyaz A, Aslam B, Sajid M, Akhtar M and Abbas R (2018). Molecular epidemiology and pathology of chicken infectious anemia in day old broiler chicks in Faisalabad, Pakistan. International Journal of Agriculture and Biology, 20: 57–61.

Rimondi A, Pinto S, Olivera V, Dibárbora M, Pérez-Filgueira M, Isabel M and Pereda A (2014). Comparative histopathological and immunological study of two field strains of chicken anemia virus. Veterinary Research, 45:102. DOI: https://doi.org/10.1186/s13567-014-0102-y .

Rosario K, Breitbart M, Harrach B, Segales J, Delwart E, Biagini P and Varsani A (2017). Revisiting the taxonomy of the family Circoviridae: establishment of the genus Cyclovirus and removal of the genus Gyrovirus. Archives of Virology, 162(5): 1447 – 1463. DOI: https://doi.org/10.1007/s00705-017-3247-y .

Schat KA (2009). Chicken anemia virus. Current Topics in Microbiology and Immunology, 331:151-183. Available at: http://www.springer.com/west/home/generic/search/results .

Sharma RN, Tiwari K, Chikweto A, Thomas D, Stratton G and Bhaiyat MI (2014). Serological evidence of chicken infectious anemia in layer and broiler chickens in Grenada, West Indies. Veterinary World, 7:59-61. DOI: https://doi.org/10.14202/vetworld.2014.59-61 .

Simeonov KB, Petrova RT, Gyurov BI, Peshev RD and Mitov BK (2014). Isolation and PCR identification of CAV infection in Bulgaria. Journal of Veterinary Medicine, 17(4):276-284.

Snoeck CJ, Giscard FK, Bonya PM, Emmanuel N, Alain LF, Mbah PO and Muller CP (2012). Epidemiology of CAV in Central African Republic and Cameroon. Virology Journal, 9:189-199. DOI: https://doi.org/10.21236/ada198234 .

Tham KM and Stanislawek WL (1992). Polymerase chain reaction amplification for direct detection of CAV DNA in tissues and sera. Avian Diseases, 36(4): 1000-1006. DOI: https://doi.org/10.2307/1591561 .

Todd D, McNulty MS, Adair BM and Allan GM (2001). Animal circoviruses. Advances in Virus Research, 57:1-72. DOI: https://doi.org/10.1016/s0065-3527(01)57000-1 .

Toro H, Ewald S and Hoerr FJ (2006). Serological evidence of chicken infectious anemia virus in the United States at least since 1959. Avian Diseases, 50(1): 124-126. DOI: https://doi.org/10.1637/7442-092205r.1 .

Van Santen VL, Li L, Hoerr FJ and Lauerman LH (2001). Genetic characterization of CAV from commercial broiler chickens in Alabama. Avian Diseases, 45(2): 373-388. DOI: https://doi.org/10.2307/1592977 .

Van Santen VL, Toro H and Hoerr FJ (2007). Biological characteristics of CAV regenerated from clinical specimen by PCR. Avian Diseases, 51(1): 66-77. DOI: https://doi.org/10.1637/0005-2086(2007)051[0066:bcocav]2.0.co;2 .

Varela APM, dos Santos HF, Cibulski SP, Scheffer CM, Schmidt C, Lima FES, Silva AD, Esteves PA, Franco AC and Roehe PM (2014). CAV and avian gyrovirus 2 as contaminants in poultry vaccines. Biologicals, 42(6): 346-350. DOI: https://doi.org/10.1016/j.biologicals.2014.08.002 .

Zhang X, Liu Y, Wu B, Sun B, Chen F, Ji J, Ma J and Xie Q (2013). Phylogenic and molecular characterization of CAV in southern China from 2011- 2012. Scientific Reports, (3): 3519-3525. DOI: https://doi.org/10.1038/srep03519.

Zhou W, Yang B, Shen B, Han S and Zhou J (1996). A serologic survey of antibody against chicken infectious anemia virus by indirect immunofluorescent assay in domestic poultry in China. Avian Diseases, 40(2): 358-360. DOI: https://doi.org/10.2307/1592232.

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Molecular Characterization and Phylogenetic Analysis of Full-length S1 Gene of GI-16 and GI-23 Infectious Bronchitis Virus in Qena, Egypt

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ABSTRACT

Infectious Bronchitis Virus (IBV) is a highly evolving virus that affects respiratory, urinary and reproductive systems. This virus is recognized as an important pathogen due to the continuous genesis of new variants that threaten the poultry industry worldwide. The aim of this study was to characterize emerging IBV variants originated from field outbreaks in Qena province, Egypt, and to study their genetic relationships with global strains. From September 2017 to January 2019, 52 field samples were collected from broiler flocks suspected of being infected with IBV. The collected samples were inoculated into embryonated chicken eggs via allantoic route for virus isolation. The IBV presence was confirmed using real-time reverse transcriptase PCR (rRT-PCR) assay targeting nucleocapsid (N) gene and finally, nine samples were selected from 29 positive samples with rRT-PCR for further genetic characterization through full-length spike (S1) gene sequencing. Phylogenetic analyses indicated that one isolate (IBV/CK/EG/QENA-4/2017) clustered within genotype I lineage 16 (GI-16). On the other hand, the remaining eight isolates (2017-2018) belonged to genotype I lineage 23 (GI-23) and clustered separately in monophyletic clade. The isolates in this study were found to share only 74.6-82.1% amino acid identity with the commonly used vaccine strains in Egypt. In conclusion, findings of this study provide informative data on circulating IBVs in the study area and highlight the importance of adopting a convenient vaccination strategy that can be more efficient for controlling the emergence of new IBV variants.

Key words: Full-length spike gene, GI-16, GI-23, Infectious bronchitis virus, Phylogenetic, RT-PCR.

INTRODUCTION

Avian infectious bronchitis (IB) is a highly contagious acute viral disease of chickens that is of great economic importance in the poultry industry. This disease is caused by Infectious Bronchitis Virus (IBV) and the upper respiratory tract is considered the main site for virus replication (Raj and Jones, 1997; Jackwood and de Wit, 2013). However, some IBV strains have also a great affinity to replicate in the reproductive tract and the kidneys, as well as some other strains have been reported to replicate in other tissue such as proventriculus (Yu et al., 2001).

IBV is a member of genus *Gammacoronavirus* within family *Coronaviridae*. The virus has a positive sense, enveloped, single-stranded and non-segmented RNA genome (Boursnell et al., 1987; Cavanagh, 2007), consisting of regions that code for four structural proteins including the nucleocapsid protein (N), the membrane glycoprotein (M), the envelope protein (E), and the spike glycoprotein (S). It also includes regions 1a and 1ab expressing the replicase gene, in addition to that it comprises several accessory regions (Spaan et al., 1988; Masters, 2006; Jackwood and de Wit, 2013). The N gene is highly conserved even among IBV isolates of different serotypes, therefore, it is often chosen as the target gene for virus detection by real-time reverse transcriptase PCR (rRT-PCR) assay (Meir et al., 2010; Bande et al., 2016).

After translation, the S glycoprotein is cleaved into two subunits, S1 and S2 (Perlman and Netland, 2009). The S1 subunit encloses not only in the infectivity of the virus but also contains virus neutralization and serotype-specific epitopes which are located in three different Hyper Variable Regions (HVRs). These epitopes are responsible for the induction of neutralizing antibodies and immune responses (Cavanagh et al., 1988; Moore et al., 1997). The nucleotide sequence variation in the S1 gene may lead to lower cross-protection against serotypes and can modify the protection ability of a vaccine or immunity (Cavanagh and Gelb, 2008). Therefore, the nucleotide sequence of S1

gene is used to classify IBV strains and to identify new IBV variants that may challenge vaccination protocols (De Wit et al., 2011; Valastro et al., 2016). Recently, Valastro et al. (2016) proposed a unified IBV classification system based on S1 phylogeny that divided IBV strains into six main genotypes from GI to GVI comprising 32 viral lineages.

IBV was first documented in Egypt by Ahmed (1954) and since then, several IBV strains with continuous diversity and recombination have been reported (Abdel-Moneim et al., 2006; Zanaty et al., 2016). The majority of IBV strains circulating in Egypt especially in the last years clustered into two distinct phylogenetic groups, the GI-1 lineage which contains classical strains and the GI-23 lineage that contains field IBV variants which was further sub-divided into Egy/var I and Egy/var II which are related to IS/1494 and IS/885 (Abdel-Moneim et al., 2002; Abdel-Moneim et al., 2012; Zanaty et al., 2016). In spite of this fact, other IBV lineages such as GI-12 (Abdel-Moneim et al., 2006; Valastro et al., 2016), GI-13 (Rohaim et al., 2019) and GI-16 (Q1 like strains) (Kiss et al., 2016; Abdel-Sabour et al., 2017) have been reported in Egypt.

Although chicken flocks are routinely vaccinated with live attenuated vaccines, outbreaks of IB in vaccinated flocks have been occurred, since there is little or no cross-protection among various serotypes of IBV (Reddy et al., 2015). The present study provided a monitoring data regarding the molecular characteristics, evolutionary relationship and genetic diversity of IBV strains isolated from chickens in Qena province as one of the southern provinces of Egypt in view of the fact that the majority of scientific research in Egypt focused on studying the IBV in the north and middle of Egypt.

MATERIAL AND METHODS

Ethical approval

This research did not involve the introduction of any intervention in/on birds, but the direct collection of tissue samples from freshly dead birds was conducted in full compliance with the recommendations of Faculty of Veterinary Medicine, South Valley University, Qena, Egypt for the care and use of laboratory animals.

Sampling and flocks' history

Fifty-two tissue samples (lung, trachea, and kidneys) were collected during the period from 2017 to 2019 from nine broiler chicken flocks in Qena province, South Egypt. These flocks were suspected of being infected with IBV and exhibited IB respiratory manifestations such as nasal discharge, sneezing, coughing, bronchial rales, gasping, tracheitis, airsacculitis, lung congestion, caseous materials in the trachea and/or nephropathogenic lesions such as pale enlarged kidneys with prominent tubular pattern. Most of these flocks had been previously vaccinated against IBV with one or more of vaccines of H120, H120 + D274, 1/96 and M41. The collected samples were labeled, stored on ice, transported to the Poultry Disease Department laboratory, Faculty of Veterinary Medicine, South Valley University, Egypt where kept frozen at -80 °C for further processing.

Egg inoculation and virus isolation

Collected tissue samples from each IBV-suspected flocks were homogenized in PBS (10% w/v) containing 5,000 IU /ml penicillin G, 5 µg/ml amphotericin B, and 5 mg/ml streptomycin (Sigma Chemical Co., USA). The homogenates were then centrifuged at 3000 rpm for 10 min after incubation at 4°C overnight and then 200 ul of the supernatant from each sample was inoculated into three 9-11-day-old embryonated chicken eggs. The allantoic fluid was harvested at 5-7 days post-inoculation and used for subsequent passages. The embryos were evaluated for gross lesions at each passage and this was performed as described by Guy (2008).

RNA extraction and real-time reverse transcriptase PCR

The genomic viral RNA of 52 samples were extracted from the harvested infected allantoic fluid using QIAmp® Viral RNA Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. Presence of IBV in all samples was checked by rRT-PCR targeting the N gene of IBV using QuantiTect® probe RT-PCR kits (Qiagen, Hilden, Germany), and the reactions were carried out on Agilent Mx3005P thermocycler machine (Life Technologies, USA) using two specific primers, (AIBV-fr): 5'- ATG CTC AAC CTT GTC CCT AGC A -3' and (AIBV-as) 5'- TCA AAC TGC GGA TCA TCA CGT -3' and probe (AIBV-TM) 5'- [FAM] TTG GAA GTA GAG TGA CGC CCA AAC TTC A [TAMRA] -3' to amplify a 130 bp fragment of N gene as previously described by Meir et al. (2010) in the following conditions: 50 °C for 30 min, one cycle at 95 °C for 15 min, followed by 40 cycles at 95 °C for 15 sec and 60 °C for 45 sec.

Reverse transcriptase PCR and full S1 sequencing

RT-PCR was performed to amplify the full S1 gene using Qiagen one step RT-PCR kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol, using two specific primer pair sets (Table 1) in two overlapping PCR fragments. The RT- PCR using the first primer pair was performed under the following conditions: 50°C for 30 min, then 95°C for 15 min, 35 cycles (94°C for 1 min, 54°C for 45 s, 72°C for 1 min), 72°C for 10 min, while the same conditions were used with the second primer pair except the number of cycles were 40 cycles and the extension time changed to be 72°C for 1.30 min. The PCR product was analyzed by electrophoresis on 1.2% agarose gel stained with SYBR Green and visualized using an ultraviolet transilluminator. After gel electrophoresis confirmation, the PCR products were sent for sequencing by a commercial service provider (Macrogen, Inc., South

Korea) where the PCR products were purified and sequenced in both forward and reverse directions using the same primer pair sets.

Table 1. Primers used in this study to amplify the full-length spike glycoprotein (S1) gene

Name	Sequence (5'-3')	Band size (base pair)	Reference
SF 1-1	GCCAGTTGTTAATTTGAAAAC	980	(Pohuang et al., 2011; Yousefi et al., 2019)
SR 1-1	TAATAACCACTCTGAGCTGT		
SF 1-2	ACTGGCAATTTTTCAGATGG	1065	
SR 1-2	AACTGTTAGGTATGAGCACA		

Sequence analysis and phylogenetic analysis

All chromatograms were analyzed, assembled using DNA Baser Assembler v5.15.0 software. The sequences obtained in the current study together with the other representative IBV sequences obtained from NCBI GenBank (n=106) were aligned based on the nucleotide sequences with MUSCLE criterion (Edgar, 2004), implemented in MEGA6 software (Tamura et al., 2013). Prior to phylogenetic analysis, Gblocks software (Talavera and Castresana, 2007) was used to remove all potentially poorly aligned regions. For this analysis, a less stringent procedure, allowing for gap positions within final blocks, was applied. The phylogenetic analysis based on the full S1 gene was carried out with MEGA6 software using Maximum likelihood method with the general time reversible model as implemented in MEGA6 and a discrete gamma distribution with 1000 bootstrap replicates (Felsenstein, 1985) was used in the data analysis to assess the robustness of the branches. Evolutionary distances between the studied sequences and the reference and vaccine strains were inferred using the full S1 dataset, with pairwise comparisons of nucleotide sequences performed using BioEdit software v7.0.5.3 (Hall, 1999). Multiple alignment of deduced amino acid sequences with the amino acid sequences of the most currently used vaccine strains in Egypt was carried out using BioEdit software v7.0.5.3 (Hall, 1999). The nucleotide sequences obtained in this study were submitted to the NCBI GenBank to assign accession numbers (Table 2).

RESULTS AND DISCUSSION

Virus isolation and identification

Similar to other RNA viruses, the IBV virus is constantly evolving and mutating (Jackwood, 2012). Studies of genetic diversity and the relationships among other viruses circulating globally are very important for tracking the circulation of viruses and for better understanding of how isolates evolve to give rise to new variants of IBV. In this study, inoculation of collected tissue samples in embryonated chicken eggs revealed the ability of many isolated samples to induce typical IBV lesions such as stunting, curling, dwarfing, abnormal feathering and subcutaneous congestion (De Wit, 2000; Guy, 2008) in some inoculated embryos. Testing of harvested allantoic fluids for IBV by using rRT-PCR assay revealed that 29 out of 52 samples were positive for IBV and 9 out of 29 positive samples were selected to be amplified with one-step RT- PCR assay for full S1 sequencing and genetic characterization (Table 2).

Distance and phylogenetic analysis of full-length S1 gene

Recently, genetic characterization based on the full S1 gene of IBV has become the primary method for classifying IBV strains because of its functional significance and heterogenicity (Valastro et al., 2016). The phylogenetic analysis based on the full S1 sequences representing different IBV strains (n=106) demonstrated that all nine isolates in this current study were variants and none of them were classic or of vaccine origin. One of those nine isolates named (IBV/CK/EG/QENA-4/2017) clustered with other isolates from Italy, China, South Korea, Taiwan, Vietnam, and Peru in GI-16 (Valastro et al., 2016) (Figure 1). Interestingly, this isolate, which was isolated from a unvaccinated broiler flock, was very closely related to other strains isolated in China (GU938413.1, HM363027.1, AF286302.1 and AF286303.1), Italy (KP780179.1) and Vietnam (KY992863.1) (Yu et al., 2001; Zou et al., 2010; Ji et al., 2011; Franzo et al., 2015; Le et al., 2019) and showed high levels of nucleotide (99.7-99.9%) and deduced amino acid (99.2-99.8%) identities. These very high identities based on the nucleotide and amino acid sequences with other viruses indicate a common origin among these viruses.

The GI-16 lineage previously identified as Q1-like IBV or CK/CH/LDL/97I-type IBV was first identified in China in 1995 from layer flock with proventriculitis (Yu et al., 2001). The strains of this lineage were isolated regularly from both vaccinated and non-vaccinated chicken flocks (Yu et al., 2001; Luo et al., 2012). Also, in 2011, the GI-16 lineage was isolated from three different Middle Eastern countries (Jordan, Saudi Arabia, and Iraq) from chicken flocks suffered from respiratory manifestations, kidney affections and decrease in egg production (Ababneh et al., 2012), and in Egypt, the strains of this lineage were isolated from broiler flocks suffered from respiratory symptoms associated with renal lesions and increased mortality (Kiss et al., 2016; Abdel-Sabour et al., 2017).

The remaining eight isolates were clustered together in a separate monophyletic branch within GI-23 (Valastro et al., 2016) (Figure 1), suggesting that IBV circulating in this area is undergoing evolution. These eight variant isolates were found to be highly related among themselves with 95.4-99.9% and 94.1-99.8% nucleotide and amino acid sequence identities, respectively (Table 3). The percentage of the nucleotide and amino acid sequence identities (87.49-93.9% and 81.2-92.2%, respectively) among these eight isolates and the rest of the GI-23 viruses used in the construction of the phylogenetic tree showed higher nucleotide and amino acid diversity within the lineage.

GI-23 lineage of IBV was first recognized in Israel in 1998 from chickens suffering from respiratory and kidney lesions (Meir et al., 2004; Valastro et al., 2016) then spread rapidly to Egypt and other Middle East countries. For nearly 20 years, the strains of GI-23 have been geographically limited to Middle East countries, but have recently spread to some European countries (Valastro et al., 2016; Lisowska et al., 2017; Fischer et al., 2019). In Egypt, GI-23 lineage has become the most prevalent lineage in the field as the majority of circulating IBV variant strains reported in chickens belong to this lineage as stated in many studies (Awad et al., 2014; Valastro et al., 2016; Zanaty et al., 2016; Abdel-Sabour et al., 2017; Abozeid et al., 2017; Naguib et al., 2017), Which is consistent with the results obtained in this study.

Compared to the commonly used vaccine strains in Egypt, the nine isolates in this study showed different levels of nucleotide (77.4-82.3%) and amino acid (74.2-81.8%) identities to the H120, Ma5, Mass41, 4/91, CR88, D274 and Israel variant 1(1/96) strains. The vaccine strain D274 shared the highest nucleotide amino acid identities among the vaccine strains with isolates obtained in this study. In addition to the pairwise identity, the phylogenetic analysis revealed that these nine variant isolates had a far distant relation to these vaccine strains. The high sequence differences between our isolates and the commonly used vaccine strains in Egypt may explain the reason for the failure of the vaccines to protect against challenge with these field strains.

On the other hand, there are other factors should be taken in consideration such as immunocompromised chicken flocks with other pathogens (Cheng et al., 2018), lack of bio-security (Jackwood and Lee, 2017) as well as the improper application of vaccines (Magouz et al., 2018). However, the frequent evolution of novel IBV variants is still the main cause of vaccination failure (Reddy et al., 2015; Khataby et al., 2016).

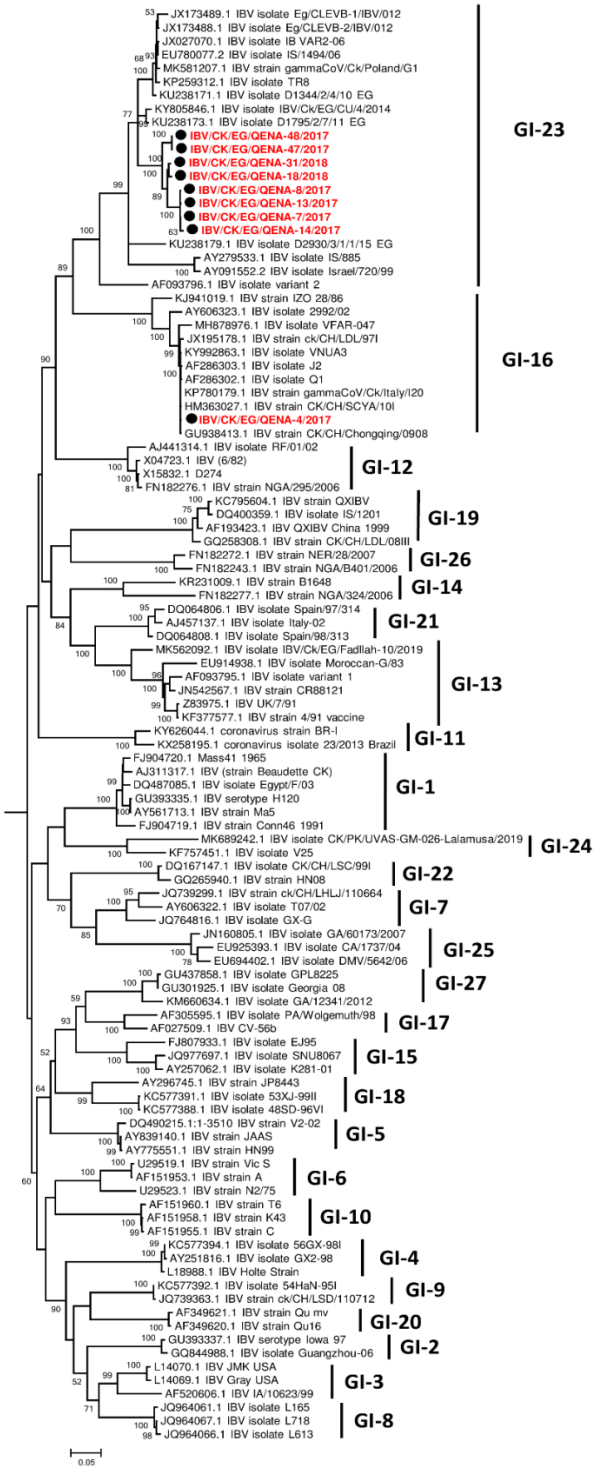

Figure 1. Phylogenetic analysis based on a full-length nucleotide sequence of the S1 gene, showing the relationship among the isolates in this study and other infectious bronchitis virus strains retrieved from Genbank. The tree was constructed using the maximum-likelihood method with (GTR+G+I) model and 1000 bootstrap replicates using MEGA 6 software. There were a total of 1570 positions in the final dataset. The isolates sequenced in this study are highlighted in bold font with red color.

Table 2. Flocks data sampled for infectious bronchitis virus isolates used for full-length spike glycoprotein (S1) gene characterization

No.	Isolate identification	Flock age (day)	Vaccines used in flock against infectious bronchitis	Collection date	Flock location	Signs and postmortem lesions	Accession No.
1	IBV/CK/EG/QENA-4/2017	29	Non vaccinated	November 2017	Qena- Dishna	Sever respiratory manifestations	MN890126
2	IBV/CK/EG/QENA-7/2017	29	M41	September 2017	Qena- Abu Tesht	Kidney damage with high morbidity and mortality rates	MN890127
3	IBV/CK/EG/QENA-8/2017	29	M41	September 2017	Qena- Abu Tesht	Kidney damage with high morbidity and mortality rates	MN890128
4	IBV/CK/EG/QENA-13/2017	29	M41	September 2017	Qena- Abu Tesht	Kidney damage with high morbidity and mortality rates	MN890129
5	IBV/CK/EG/QENA-14/2017	29	M41	September 2017	Qena- Abu Tesht	Kidney damage with high morbidity and mortality rates	MN890130
6	IBV/CK/EG/QENA-18/2018	17	H120	March 2018	Qena- Dishna	Sever respiratory manifestations	MN890131
7	IBV/CK/EG/QENA-31/2018	32	H120 & 1/96	December 2018	Qena- Qus	Sever respiratory manifestations	MN890132
8	IBV/CK/EG/QENA-47/2017	32	H120 + D274	December 2017	Qena- Abu Tesht	Kidney damage with high morbidity and mortality rates	MN890133
9	IBV/CK/EG/QENA-48/2017	32	H120 + D274	December 2017	Qena- Abu Tesht	Kidney damage with high morbidity and mortality rates	MN890134

Table 3. Nucleotide and amino acid identities of full-length spike glycoprotein (S1) gene sequence of the nine infectious bronchitis virus isolates in this study with other Egyptian strains, reference strains and vaccine strains.

		Amino acid identity (%)																				
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	
1	GU393335.1_H120		99.8	96.4	78.6	75.4	75.0	75.2	75.9	77.5	76.3	75.8	75.8	75.8	75.9	75.6	75.0	75.4	75.8	75.4	75.6	**1**
2	AY561713.1_Ma5	99.8		96.2	78.4	75.2	74.8	75.0	75.9	77.5	76.3	75.6	75.6	75.8	75.9	75.6	75.0	75.4	75.8	75.4	75.6	**2**
3	FJ904720.1_Mass41_1965	97.6	97.7		77.8	74.8	74.2	74.6	74.8	76.7	75.6	75.4	75.4	75.0	74.8	74.8	74.2	74.6	75.0	74.6	74.8	**3**
4	X15832.1_D274	80.9	80.9	80.6		80.1	79.0	78.6	79.3	82.9	83.5	81.4	81.6	81.8	81.6	81.4	81.0	81.2	81.4	81.4	81.6	**4**
5	JN542567.1_CR88121	78.1	78.0	78.4	79.6		93.1	95.4	76.7	79.0	80.3	77.8	77.8	80.3	79.9	79.7	79.3	79.0	79.2	79.0	79.2	**5**
6	KF377577.1_4/91	78.6	78.6	78.9	79.1	96.6		92.2	77.5	77.6	79.2	77.3	77.3	79.5	79.5	79.3	78.4	78.0	78.6	78.0	78.2	**6**
7	AF093795.1/IS variant1 (1/96)	78.0	78.0	78.3	79.4	97.4	95.5		76.9	78.8	79.3	77.6	77.6	79.7	79.7	79.5	78.6	78.6	78.8	78.6	78.8	**7**
8	AY279533.1_IS/885_S1	78.6	78.6	78.6	78.9	77.9	78.6	77.7		87.9	87.9	79.7	79.7	87.1	87.1	86.7	86.2	86.0	86.7	86.3	86.5	**8**
9	KY805846.1 EG/CU/4/2014	80.3	80.1	80.3	82.8	78.8	78.5	79.0	88.4		94.5	81.6	81.8	91.6	91.3	91.1	90.7	92.4	92.0	92.4	92.6	**9**
10	EU780077.2_IS/1494/06	80.1	79.9	79.9	83.1	79.2	79.2	79.2	88.1	95.4		82.0	82.2	91.4	91.1	90.9	90.5	91.8	91.8	91.8	92.0	**10**
11	AF286302.1_Q1	77.5	77.4	78.1	80.7	78.6	78.8	78.9	80.1	82.1	81.6		99.6	82.4	82.4	82.0	81.8	80.9	81.2	82.4	82.6	**11**
12	IBV/CK/EG/QENA-4/2017	77.5	77.4	78.1	80.6	78.6	78.8	78.9	80.2	82.2	81.6	99.8		82.4	82.4	82.0	81.8	81.0	81.4	82.6	82.7	**12**
13	IBV/CK/EG/QENA-7/2017	79.6	79.4	79.6	82.3	79.1	79.4	79.0	87.4	92.3	92.6	81.5	81.5		99.6	99.4	98.8	96.5	96.9	95.2	95.4	**13**
14	IBV/CK/EG/QENA-8/2017	79.7	79.5	79.6	82.1	79.0	79.4	79.0	87.4	92.2	92.5	81.4	81.4	99.8		99.4	98.4	96.7	97.1	94.8	95.0	**14**
15	IBV/CK/EG/QENA-13/2017	79.5	79.3	79.5	82.1	78.9	79.3	78.9	87.3	92.1	92.5	81.3	81.3	99.8	99.8		98.2	96.4	96.7	94.7	94.8	**15**
16	IBV/CK/EG/QENA-14/2017	79.2	79.0	79.2	82.0	78.8	79.1	78.7	87.0	91.9	92.2	81.1	81.1	99.5	99.4	99.3		95.4	95.8	94.1	94.3	**16**
17	IBV/CK/EG/QENA-18/2018	79.6	79.4	79.6	82.0	79.0	78.9	78.9	87.4	93.6	93.4	81.4	81.4	97.1	97.2	97.1	96.7		99.2	96.2	96.4	**17**
18	IBV/CK/EG/QENA-31/2018	79.7	79.5	79.7	81.9	78.8	78.9	78.7	87.6	93.6	93.5	81.3	81.3	97.2	97.3	97.2	96.8	99.3		96.2	96.4	**18**
19	IBV/CK/EG/QENA-47/2017	79.4	79.3	79.4	82.2	78.9	78.9	78.8	87.2	93.7	93.0	82.0	82.1	95.8	95.7	95.6	95.4	97.4	97.2		99.8	**19**
20	IBV/CK/EG/QENA-48/2017	79.4	79.4	79.5	82.3	79.0	78.9	78.9	87.2	93.7	93.0	82.1	82.1	95.9	95.7	95.7	95.4	97.4	97.3	99.9		**20**
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	
Nucleotide identity (%)																						

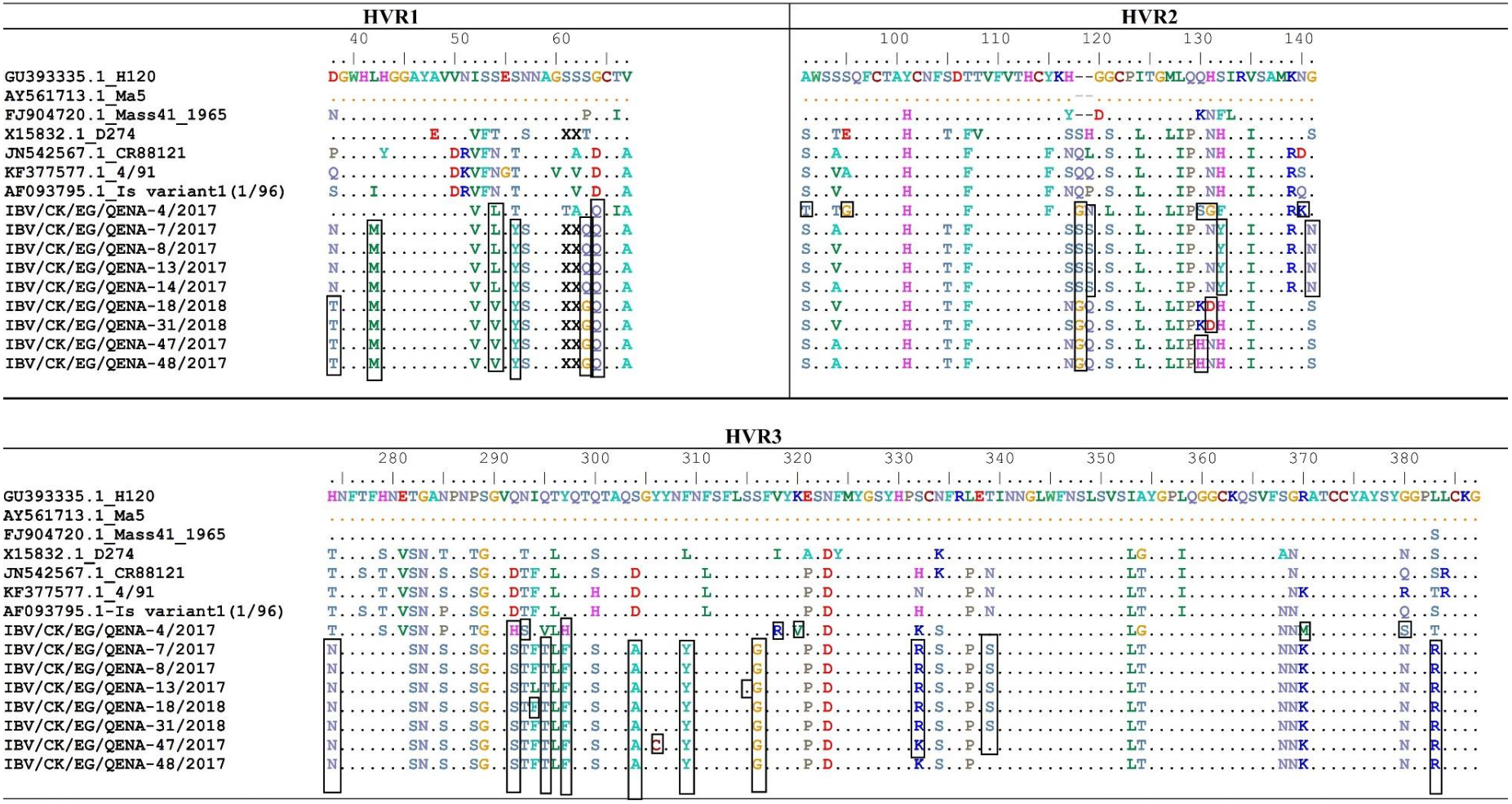

Figure 2. Multiple alignment of deduced amino acid sequences of hypervariable regions (HVR) from the infectious bronchitis virus (IBV) isolates in this study and some IBV vaccine strains. A dot indicates an identical amino acid with H120 strain. A dash indicates an amino acid deletion, "X" means any gap within the codon not translated to a valid amino acid.

Alignment analysis of deduced amino acid

The S1 protein contains three HVRs associated with serotype specificity and virus-neutralizing epitopes and located within the amino acid residues 38–67, 91–141, and 274–387 (Moore et al., 1997; Wang and Huang, 2000). In this study, full-length S1 gene sequences of obtained isolates were translated and aligned with the amino acid sequences of the most currently used vaccine strains in Egypt. Based on the alignment in three HVRs of S1, the nine isolates showed unique amino acid differences in comparison to the commonly used vaccine strains (Figure 2). It is well known that even small changes in the amino acid sequence of the spike protein can contribute to the generation of new antigenic types that can alter the protective ability of a vaccine (Adzhar et al., 1997; Casais et al., 2003).

CONCLUSION

The present study provided a robust depiction of genetic characteristics of IBVs isolated from chickens in Qena province, Egypt as well as the evidence for the emergence of IBV variants in vaccinated and unvaccinated broiler flocks. This study demonstrated the circulation of two IBV variants lineages (GI-16 and GI-23). The genetic variability among studied isolates and commonly used vaccine strains can explain the poor vaccination performance and disease outbreak in this region. The continuous disease monitoring and surveillance are required not only to elucidate sequence characteristics of prevailing strains but also to revise appropriate vaccine strategies. These data will be essential as a step for selecting appropriate vaccine strains as well as planning for future vaccine strategies.

DECLARATIONS

Authors' contributions

All authors contributed equally to this work.

Competing interests

The authors declare that there is no competing of interests.

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REFERENCES

Ababneh M, Dalab AE, Alsaad S and Al-Zghoul M (2012). Presence of infectious bronchitis virus strain CK/CH/LDL/97I in the Middle East. ISRN Veterinary Science, 2012: 201721. DOI: https://doi.org/10.5402/2012/201721

Abdel-Moneim AS, El-Kady MF, Ladman BS and Gelb J (2006). S1 gene sequence analysis of a nephropathogenic strain of avian infectious bronchitis virus in Egypt. Virology journal, 3: 78. DOI: https://doi.org/10.1186/1743-422X-3-78

Abdel-Moneim AS, Madbouly H, Gelb J and Ladman B (2002). Isolation and identification of Egypt/Beni-Seuf/01 a novel genotype of infectious bronchitis virus. Veterinary Medical Journal-Giza, 50(4): 1065-1078. Available at: https://www.researchgate.net/publication/257856074_

Abdel-Sabour MA, Al-Ebshahy EM, Khaliel SA, Abdel-Wanis NA and Yanai T (2017). Isolation and molecular characterization of novel infectious bronchitis virus variants from vaccinated broiler flocks in Egypt. Avian Diseases, 61(3): 307-310. DOI: https://doi.org/10.1637/11566-121516-RegR

Abozeid HH, Paldurai A, Khattar SK, Afifi MA, El-Kady MF, El-Deeb AH and Samal SK (2017). Complete genome sequences of two avian infectious bronchitis viruses isolated in Egypt: Evidence for genetic drift and genetic recombination in the circulating viruses. Infection, Genetics and Evolution, 53: 7-14. DOI: https://doi.org/10.1016/j.meegid.2017.05.006

Adzhar A, Gough R, Haydon D, Shaw K, Britton P and Cavanagh D (1997). Molecular analysis of the 793/B serotype of infectious bronchitis virus in Great Britain. Avian Pathology, 26(3): 625-640. DOI: https://doi.org/10.1080/03079459708419239

Ahmed H (1954). Incidence and treatment of some infectious viral respiratory diseases of poultry in Egypt. DVM Thesis. Cairo University, Cairo, Egypt.

Awad. AM, Sediek ME and El-Yamany ME (2014). Isolation and molecular characterization of novel IBV isolates from broiler chicken farms in Egypt. Alexandria Journal of Veterinary Sciences, 42(1): 74-82. DOI: https://doi.org/10.5455/ajvs.160278

Bande F, Arshad SS, Omar AR, Bejo MH, Abubakar MS and Abba Y (2016). Pathogenesis and diagnostic approaches of avian infectious bronchitis. Advances in Virology, 2016. DOI: https://doi.org/10.1155/2016/4621659

Boursnell MEG, Brown TDK, Foulds IJ, Green PF, Tomley FM and Binns MM (1987). Completion of the sequence of the genome of the coronavirus avian infectious bronchitis virus. Journal of General Virology, 68(1): 57-77. DOI: https://doi.org/10.1099/0022-1317-68-1-57

Casais R, Dove B, Cavanagh D and Britton P (2003). Recombinant avian infectious bronchitis virus expressing a heterologous spike gene demonstrates that the spike protein is a determinant of cell tropism. Journal of Virology, 77(16): 9084-9089. DOI: https://doi.org/10.1128/JVI.77.16.9084-9089.2003

Cavanagh D (2007). Coronavirus avian infectious bronchitis virus. Veterinary Research, 38(2): 281-297. DOI: https://doi.org/10.1051/vetres:2006055

Cavanagh D, Davis PJ and Mockett APA (1988). Amino acids within hypervariable region 1 of avian coronavirus IBV (Massachusetts serotype) spike glycoprotein are associated with neutralization

epitopes. Virus Research, 11(2): 141-150. DOI: https://doi.org/10.1016/0168-1702(88)90039-1

Cavanagh D and Gelb J (2008). Infectious Bronchitis. In YM Saif (Editor.), Diseases of poultry. 12th Edition. Ames, Iowa Blackwell, Iowa State University Press. pp. 117-135.

Cheng J, Huo C, Zhao J, Liu T, Li X, Yan S, Wang Z, Hu Y and Zhang G (2018). Pathogenicity differences between QX-like and Mass-type infectious bronchitis viruses. Veterinary Microbiology, 213: 129-135. DOI: https://doi.org/10.1016/j.vetmic.2017.11.027

De Wit J, Cook JK, Van der Heijden and MJF H (2011). Infectious bronchitis virus variants: a review of the history, current situation and control measures. Avian Pathology, 40(3): 223-235. DOI: https://doi.org/10.1080/03079457.2011.566260

De Wit JJ (2000). Detection of infectious bronchitis virus. Avian Pathology, 29(2): 71-93. DOI: https://doi.org/10.1080/03079450094108

Edgar RC (2004). MUSCLE: Multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Research, 32(5): 1792-1797. DOI: https://doi.org/10.1093/nar/gkh340

Felsenstein J (1985). Confidence limits on phylogenies: an approach using the bootstrap. Evolution, 39(4): 783-791. DOI: https://doi.org/10.2307/2408678

Fischer S, Klosterhalfen D, Wilms-Schulze Kump F and Casteel M (2019). Research Note: First evidence of infectious bronchitis virus Middle-East GI-23 lineage (Var2-like) in Germany. Poultry Science, 99(2):797-800 DOI: https://doi.org/10.1016/j.psj.2019.10.031

Franzo G, Listorti V, Naylor CJ, Lupini C, Laconi A, Felice V, Drigo M, Catelli E and Cecchinato M (2015). Molecular investigation of a full-length genome of a Q1-like IBV strain isolated in Italy in 2013. Virus Research, 210: 77-80. DOI: https://doi.org/10.1016/j.virusres.2015.07.008

Guy JS. (2008). Isolation and propagation of coronaviruses in embryonated eggs. In D Cavanagh (Ed.), SARS- and Other Coronaviruses: Laboratory Protocols. Totowa, NJ, Humana Press. pp. 109-117.

Hall TA (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series, 41: 95-98.

Jackwood MW (2012). Review of infectious bronchitis virus around the world. Avian Diseases, 56(4): 634-641. DOI: https://doi.org/10.1637/10227-043012-Review.1

Jackwood MW and de Wit S. (2013). Infectious bronchitis. In DE Swayne (Editor.), Diseases of poultry. 13th Edition. Hoboken, New Jersey, Wiley Blackwell. pp. 139-159.

Jackwood MW and Lee D-H (2017). Different evolutionary trajectories of vaccine-controlled and non-controlled avian infectious bronchitis viruses in commercial poultry. PloS one, 12(5): e0176709. DOI: https://doi.org/10.1371/journal.pone.0176709

Ji J, Xie J, Chen F, Shu D, Zuo K, Xue C, Qin J, Li H, Bi Y, Ma J and Xie Q (2011). Phylogenetic distribution and predominant genotype of the avian infectious bronchitis virus in China during 2008-2009. Virology Journal, 8(1): 184. DOI: https://doi.org/10.1186/1743-422X-8-184

Khataby K, Fellahi S, Loutfi C and Mustapha EM (2016). Avian infectious bronchitis virus in Africa: a review. Veterinary Quarterly, 36(2): 71-75. DOI: https://doi.org/10.1080/01652176.2015.1126869

Kiss I, Mató T, Homonnay Z, Tatár-Kis T and Palya V (2016). Successive occurrence of recombinant infectious bronchitis virus strains in restricted area of Middle East. Virus Evolution, 2(2): vew021. DOI: https://doi.org/10.1093/ve/vew021

Le TB, Lee H-J, Le VP and Choi K-S (2019). Multiple genotypes of avian infectious bronchitis virus circulating in Vietnam. Korean Journal of Poultry Science, 46(2): 127-136. DOI: https://doi.org/10.5536/KJPS.2019.46.2.127

Lisowska A, Sajewicz-Krukowska J, Fusaro A, Pikula A and Domanska-Blicharz K (2017). First characterization of a Middle-East GI-23 lineage (Var2-like) of infectious bronchitis virus in Europe. Virus Research, 242: 43-48. DOI: https://doi.org/10.1016/j.virusres.2017.09.010

Luo H, Qin J, Chen F, Xie Q, Bi Y, Cao Y and Xue C (2012). Phylogenetic analysis of the S1 glycoprotein gene of infectious bronchitis viruses isolated in China during 2009-2010. Virus Genes, 44(1): 19-23. DOI: https://doi.org/10.1007/s11262-011-0657-x

Magouz A, Abdo W, Abdelsabour A, Elbestawy A and Desouky A (2018). Molecular and histopathological investigation of avian infectious bronchitis virus in the Delta of Egypt between 2016 and 2017. Pakistan Veterinary Journal, 38(3). DOI: http://www.pvj.com.pk/pdf-files/38_3/243-248.pdf

Masters PS (2006). The molecular biology of coronaviruses. Advances in Virus Research, 66: 193-292. DOI: https://doi.org/10.1016/S0065-3527(06)66005-3

Meir R, Maharat O, Farnushi Y and Simanov L (2010). Development of a real-time TaqMan® RT-PCR assay for the detection of infectious bronchitis virus in chickens, and comparison of RT-PCR and virus isolation. Journal of Virological Methods, 163(2): 190-194. DOI: https://doi.org/10.1016/j.jviromet.2009.09.014

Meir R, Rosenblut E, Perl S, Kass N, Ayali G, Hemsani E and Perk S (2004). Identification of a novel nephropathogenic infectious bronchitis virus in Israel. Avian Diseases, 48(3): 635-641. DOI: https://doi.org/10.1637/7107

Moore K, Jackwood M and Hilt D (1997). Identification of amino acids involved in a serotype and neutralization specific epitope within the S1 subunit of avian infectious bronchitis virus. Archives of Virology, 142(11): 2249-2256. DOI: https://doi.org/10.1007/s007050050239

Naguib MM, El-Kady MF, Lüschow D, Hassan KE, Arafa A-S, El-Zanaty A, Hassan MK, Hafez HM, Grund C and Harder TC (2017). New real time and conventional RT-PCRs for updated molecular diagnosis of infectious bronchitis virus infection (IBV) in chickens in Egypt associated with frequent co-infections with avian influenza and Newcastle disease viruses. Journal of Virological Methods, 245: 19-27. DOI: https://doi.org/10.1016/j.jviromet.2017.02.018

Perlman S and Netland J (2009). Coronaviruses post-SARS: update on replication and pathogenesis. Nature Reviews Microbiology, 7(6): 439-450. DOI: https://doi.org/10.1038/nrmicro2147

Pohuang T, Chansiripornchai N, Tawatsin A and Sasipreeyajan J (2011). Sequence analysis of S1 genes of infectious bronchitis virus isolated in Thailand during 2008–2009: identification of natural recombination in the field isolates. Virus Genes, 43(2): 254-260. DOI: https://doi.org/10.1007/s11262-011-0635-3

Raj GD and Jones RC (1997). Infectious bronchitis virus: immunopathogenesis of infection in the chicken. Avian Pathology, 26(4): 677-706. DOI: https://doi.org/10.1080/03079459708419246

Reddy VR, Theuns S, Roukaerts ID, Zeller M, Matthijnssens J and Nauwynck HJ (2015). Genetic characterization of the Belgian nephropathogenic infectious bronchitis virus (NIBV) reference strain B1648. Viruses, 7(8): 4488-4506. DOI: https://doi.org/10.3390/v7082827

Rohaim MA, El Naggar RF, Hamoud MM, Bazid A-HI, Gamal AM, Laban SE, Abdel-Sabour MA, Nasr SA, Zaki MM and Shabbir MZ (2019). Emergence and genetic analysis of variant pathogenic 4/91 (serotype 793/B) infectious bronchitis virus in Egypt during 2019. Virus Genes, 55(5): 720-725. https://doi.org/10.1007/s11262-019-01693-9

Spaan W, Cavanagh D and Horzinek MC (1988). Coronaviruses: structure and genome expression. Journal of General Virology, 69(12): 2939-2952. DOI: https://doi.org/10.1099/0022-1317-69-12-2939

Talavera G and Castresana J (2007). Improvement of phylogenies after removing divergent and ambiguously aligned blocks from protein sequence alignments. Systematic Biology, 56(4): 564-577. DOI: https://doi.org/10.1080/10635150701472164

Tamura K, Stecher G, Peterson D, Filipski A and Kumar S (2013). MEGA6: Molecular evolutionary genetics analysis version 6.0. Molecular Biology and Evolution, 30(12): 2725-2729. DOI: https://doi.org/10.1093/molbev/mst197

Valastro V, Holmes EC, Britton P, Fusaro A, Jackwood MW, Cattoli G and Monne I (2016). S1 gene-based phylogeny of infectious bronchitis virus: An attempt to harmonize virus classification. Infection, Genetics and Evolution, 39: 349-364. DOI: https://doi.org/10.1016/j.meegid.2016.02.015

Wang CH and Huang YC (2000). Relationship between serotypes and genotypes based on the hypervariable region of the S1 gene of infectious bronchitis virus. Archives of Virology, 145(2): 291-300. DOI: https://doi.org/10.1007/s007050050024

Yousefi Y, Bassami MR, Kalidari GA and Seno MMG (2019). Sequence characterization of full-length S1 gene of infectious bronchitis viruses isolated from poultry farms in Khorasan Razavi, Iran. Turkish Journal of Veterinary and Animal Sciences, 43(2): 235-243. DOI: https://dergipark.org.tr/tr/download/article-file/734722

Yu L, Jiang Y, Low S, Wang Z, Nam SJ, Liu W and Kwang J (2001). Characterization of three infectious bronchitis virus isolates from China associated with proventriculus in vaccinated chickens. Avian Diseases, 45(2): 416-424. DOI: https://doi.org/10.2307/1592981

Zanaty A, Naguib MM, El-Husseiny MH, Mady W, Hagag N and Arafa A-S (2016). The sequence of the full spike S1 glycoprotein of infectious bronchitis virus circulating in Egypt reveals evidence of intra-genotypic recombination. Archives of Virology, 161(12): 3583-3587. DOI: https://doi.org/10.1007/s00705-016-3042-1

Zou NL, Zhao FF, Wang YP, Liu P, Cao SJ, Wen XT and Huang Y (2010). Genetic analysis revealed LX4 genotype strains of avian infectious bronchitis virus became predominant in recent years in Sichuan area, China. Virus Genes, 41(2): 202-209. DOI: https://doi.org/10.1007/s11262-010-0500-9

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Influence of Adding Fermented Whey Cheese into Drinking Water of Laying Hens

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ABSTRACT

Cheese production waste contains carbon source, one of which is lactose as an energy source in the development of fermentation, especially in the manufacture of probiotics for poultry. The research method used 3% fermented cheese whey in 17-week-old laying hen drinking water to analyze the productivity of laying hens and microbiology. The purpose of the research is to determine the effective concentration of fermented whey cheese to improve productivity, physical and chemical composition of eggs, and evaluate fat metabolism in laying hens. The present study used 120 laying hens aged 17 weeks. The experimental method was designed using the T-test method of control (P0), fermented whey cheese (P1). The observed variables were productivity (feed consumption, water consumption, daily egg production, feed conversion ration), and microbiology of small intestine and excreta (lactate acid bacteria, salmonella, *Escherichia coli*.). The effect of adding fermented whey cheese into drinking water decreased feed consumption and FCR in contrast to the control group (P0) but increased egg production, egg weight, and egg mass weight. The effect of adding fermented whey cheese into drinking water decreased the number of *Escherichia coli* bacteria and increases lactic acid bacteria in the digestive tract of broiler chickens, especially in the ileum, caeca, so also with those in the stool. The number of Salmonella Bacteria was significantly decreased and very significantly increased lactic acid bacteria in the feces during the fermentation of whey cheese. The decrease in ammonia in the stool was very significant compared to the control group. In conclusion, adding fermented whey cheese into drinking water can reduce feed consumption, feed conversion ratio (FCR), *Escherichia coli*, salmonella, ammonia (NH_3), and increase egg production, egg weight, egg mass weight, final body weight, and lactate acid bacteria. The use of fermented whey cheese can be used as a nutraceutical feed additive to inhibit pathogenic bacteria in the intestine and increase lactic acid bacteria.

Key words: Drinking water, Fermented, Laying hens, Whey cheese

INTRODUCTION

Whey is a liquid cheese waste or a milk serum which is produced during the cheese production and separated from a large number of curd which mainly are not utilized by the Community, and therefore resulting in environmental pollution if directly disposed without processing (Prasetyo and Kustiawan, 2012). According to Nursiwi et al. (2015) the waste of cheese production contains carbon sources such as lactose as a source of energy in the development of biotechnology fermentation, mainly producing the probiotics for livestock. Lactose content (4-7%) and proteins (0.6-1%), which could be used as a medium for bacterial growth in the fermentation process was very beneficial for livestock health and increase the productivity of organic livestock (Prasetyo and Kustiawan, 2012). Also it could be used as an increased biomass medium to develop lactic acid bacteria and yeasts and produce several bioactive components through Biofermentation (Ariyanti and Hadiyanto, 2013; Nursiwi et al., 2015; Watson et al., 2017). According to Watson et al. (2017) fermentation products had a role in assisting the absorption process of vitamin D and K, stimulating the growth of beneficial bacteria in the small intestine and assists the process of absorption of various microsubstances such as minerals such as calcium and iron ions. This chemical and nutritional content could be applied as a nutraceutical feed additive to livestock (Mellor, 2000; Charalampopoulos and Rastall, 2009; Kabir, 2009; Sugiharto, 2016; Watson et al., 2017). Nutraceutical feed Additive was a form of feed (or part of a food) that provided medical or health benefits, including prevention and treatment of diseases, as well as enhancing the performance and production of livestock (Bhattacharyya and Roy, 2015). In other words, nutraceuticals were organic chemicals or natural feedstuffs

that could improve health by providing physiological benefits by providing essential nutrients. Enzymes, prebiotics, probiotics, phytogenic additives, organic acids, and others were included in the nutraceutical. The role of probiotics for poultry had a positive impact on improving performance, productivity, and livestock health. According to Kabir (2009) and Kabir et al., (2005) who had expressed the administration of nutraceutical feed additive in the form of probiotics in broiler cattle could improve the performance, quality of carcasses, the number of lactic acid bacteria in the intestine, immune response and lowering stress levels. The addition of nutraceutical feed additive in the form of probiotics, prebiotics, symbiotic, and organic acids could also increase the production of eggs, egg mass and quality (Youssef et al. 2013).

MATERIALS AND METHODS

Ethical approval

The approval of this research was sorted by Politeknik Negeri Banyuwangi's ethics committee in Indonesiaand guidelines for care, and human handling of animals was strictly followed throughout the study (FASS, 2010).

Animal and treatment groups

This research used 120 laying hens (Lohman strain, Japfa comfeed, Indonesia), aged 17-weeks old and used two kinds of treatments including P0 (without fermented whey cheese, P1 (fermented whey cheese) with 10 replications. Each replication used six chickens (1 hen/cage, the dimensions for each cage were 35 x 36 x 42 cm). Drinking water was provided *ad libitum* and feeding was given twice a day, in the morning (07.00 am) and afternoon (05.00 pm). The research period was nine weeks. The research feed using the nutrients requirement recommendation of Leeson and Summers (2005). The diet contained 20 % crude protein and 2900 kcal/kg. The composition and nutrients content of the control diet are showed in table 1

Fermentation of whey cheese

Fermentation of whey cheese included whey cheese which was heated to 80^{o}C for 10 minutes, and then mixed with 10% of molasses. The temperature dropped rapidly to 35-40^{o}C with an immersion beaker glass containing a mixture of water and molasses in cold water. The next step was adding an inoculation starter to 5% kefir grain. After completing the inoculation process, the anaerobic fermentation of kefir was carried out for 24 hours at temperatures between 35 and 40°C. At the end of the incubation period, the quality of the fermented whey cheese was analyzed (Table 2).

Table 1. Composition and nutrients contents of basal diet fed in the experimental diet of laying hens

Ingredients	(%)
Maize	52.00
Soybean meal	24.40
Fish meal	8.60
Rice bran	1.10
Corn Gluten Meal (CGM)	2.50
Palm oil	2.65
Di-Calcium Phosphate (DCP)	1.00
Calcium carbonate ($CaCO_3$)	6.70
NaCl	0.39
Premix[1]	0.50
DL-Methionine	0.16
Total	100
Nutrient content[2]	
Metabolism Energy (kcal/kg)	2901.35
Crude protein (%)	20.03
Crude fat (%)	5.04
Crude fiber (%)	2.31
Methionine (%)	0.59
Lysine (%)	1.37
Methionine + Cysine (%)	0.96
Ca (%)	3.11
P available(%)	0.69
Na (%)	0.24

Note: [1] Premix composition Each 10 kg contains vitamin A: 12.000.000 IU, vitamin D3: 2.000.000 IU, vitamin E: 8.000 IU, vitamin K: 2.000 mg, vitamin B1: 2.000 mg, vitamin B2: 500 mg, vitamin B6, vitamin B12: 12.000 g, vitamin C: 25.000 mg, Calcium-D-anthothenate: 6.000 mg, Niacin: 40.000 mg, Choline chloride: 10.000 mg, Lysine: 30.000 mg, Manganese: 120.000 mg, Iron: 20.000 mg, Iodine: 200 mg, Zinc: 100.000 mg, Cobalt: 200 mg, Copper: 4.000 mg, Zinc Bacitracin: 21.000 mg. [2] Formulated based on nutrient requirement recommendations by Leeson and Summers (2005).

Table 2. Chemical quality characteristics of fermented whey cheese

Indices	**Contents**
Alcohol (%)	3.18
Acetic acid (%)	0.21
Lactic acid (%)	0.29
pH	3.70
Titratable Acidity	0.579
Antioxidant	38.91
Lipid (%)	0.20
Protein (%)	0.27
Lactic acid bacteria (log CFU / milliliter)	10.27
Total Plate Count (log CFU / milliliter)	2.60
Yeast (log CFU / milliliter)	8.11

Experiment design and measurements

Feed and water were given *ad libitum*. Drinking water in P1 treatment was added by 3% whey cheese fermented into the water, while P0 gave only water without fermented whey cheese. Feed intake was measured every week, while egg production and weight were recorded daily during treatment. The parameters observed in this study were the performance of laying yamsincluding feed consumption, egg production, egg mass weight, egg weight, feed conversion, and microbiology.

Data analyses

All data were expressed as mean ± standard deviation and analyzed using T-tests. The analysis was considered significant at $p < 0.05$ using the Statistical Package for Social Sciences (SPSS version 21) for windows.

RESULTS AND DISCUSSION

Production of laying hens

Analysis of the productivity of laying hens aged 17-26 weeks can be seen in table 3. The effect of adding fermented whey cheese into drinking water ($p < 0.001$) decreased feed consumption and feed conversion ratio (FCR) compared to the control treatment (P0) but increased egg production, egg weight, and egg mass weight. The decrease in feed consumption and feed conversion ratio (FCR) was around 0.9% and 11.8% ($p < 0.001$) compared to the control treatment while the increase in final body weight, egg production, egg weight, and egg mass weight were 4.13%, 6.41 %, 12.59%, and 19.27%.

Table 3 Effect of fermented whey cheese added to the drinking water of the productivity of laying hens

Variable	Treatment		SEM	P- value
	P0	P1		
Feed intake (gram /hen/ day)	105.90[a]	104.91[b]	0.15	<0.001
Initial Body Weight (gram / hen)	1455.00	1465.5	3.19	0.102
Body weight gain (gram /hen)	1776.80[b]	1850.20[a]	10,98	<0.001
Egg production (%)	75.21[b]	80.03[a]	0.69	<0.001
Egg weight (gram)	59.48[b]	66.97[a]	0.88	<0.001
Egg mass weight (gram /hen/day)	44.93[b]	53.59[a]	1.04	<0.001
FCR	1.78[a]	1.57[b]	0.03	<0.001

[a,b,c,d] = Means within a column with different superscripts differ significantly ($p < 0.05$). P0 = Control group (without fermented whey cheese), P1= treatment group with fermenting whey cheese, SEM= Standard Error Mean, FCR = Feed conversion ratio.

Feed intake

Analysis of the productivity of laying hens aged 18-26 weeks can be seen in table 3. Effect of adding fermented whey cheese into drinking water decreased ($p < 0.01$) feed consumption. Decreased feed consumption was due to the content of lactic acid bacteria and yeast that were in the fermentation of whey cheese which can split nutrients in the small intestine, so that absorption of nutrients in the body was food leaded to reduction in feed consumption (Mahfuz et al., 2017). Further, Taklimi et al. (2012) stated that giving probiotics such as lactic acid bacteria and yeast would reduce feed consumption. In contrast to the research of Toghyani et al. (2015), using molasses and fermented milk kefir grains did not affect the decrease in feed consumption.

Body weight gain

The administration of fermented whey cheese had a very significant effect on increasing the body weight of 26-week-old laying hens (P1) compared to conventional drinking water treatment (P0). This increase in body weight was due to the content of lactic acid bacteria and yeasts which present in whey cheese fermented with kefir seeds that could inhibit pathogenic bacteria in the digestive tract, so the degradation of nutrients in the feed was easily appropriately absorbed. Kefir is a natural product that contains a complex mixture of lactic acid bacteria and yeasts, which acts as a probiotic (Fuller, 1989; Toghyani et al., 2015). Probiotics can improve intestinal health by inhibiting the growth of pathogens, resulting in better digestion and absorption of nutrients. According to Yaman et al., (2006), adding kefir into drinking water significantly increased the population of Lactobacilli *spp.* also, total aerobic bacteria populations and decreased the populations of Enterobacteriaceae so the absorption of nutrients would be better.

Egg production

Increased egg production at the age of 18-26 weeks was thought to be related with the presence of lactic acid bacteria and yeast in the small intestine and large intestine, by suppressing pathogenic bacteria and stimulating the growth of usefull bacteria which would increase the absorption capacity and digestibility of proteins, as well as increasing the egg production (Sathya and Muragian, 2015; Lokapirnasari et al., 2017). Protein is necessary for poultry body growth, healing damaged tissue, and also for the production and egg-forming elements. Using lactic acid bacteria and yeasts could produce an acidic atmosphere in the digestive tract, thereby suppressing the

growth of pathogenic bacteria. A suitable condition of the digestive tract increases the metabolic process and absorption of necessary nutrients for the body , and also help increasing egg production, eggshell weight, eggshell thickness, and reducing cholesterol levels in egg yolks (Chaucheyras-Durand and Durand 2009; Delia et al., 2012; Lokapirnasari et al., 2017).

Egg weight and egg mass weight

The increase in egg weight and egg mass weight was highly significant ($p < 0.01$) by the administration of fermented whey cheese (P1) compared to the control treatment (P0) (Table 3). Increased egg weight and egg mass weight was related to the content of lactic acid bacteria in the digestive tract which inhibit pathogenic bacteria that use protein in the small intestine. According to Huda et al. (2019), the utilization of lactic acid bacteria, which was applied to animal feed, could inhibit the growth of pathogenic microbes in the small intestine so that protein absorption improved and egg weight automatically increased. Furthermore, Pradikta et al. (2018) explained that the Absorption Rate of food substances in the digestive tract would work better with the help of lactic acid bacteria; and food substances contained in the feed, such as protein and amino acids, would be better absorbed in the chicken's body. This protein and amino acids would be used later by chickens to produce eggs, so if the absorption is not optimal, then production will also be not good. According to Istinganah et al. (2013), the essential factor to influence egg weight was protein and amino acid content, because about 50% of the dry matter contained in eggs is protein, therefore amino acids supplement was essential in the process of egg formation.

Feed conversion ratio

The addition of fermented whey cheese significantly decreased the FCR of laying hens aged 18 to 26 weeks (P1) compared to conventional drinking water treatment (P0) (Table 3). The decrease in FCR was caused by a decrease in feed consumption. According to Huda et al. (2019) who stated that the effective factor of FCR was the adequate nutritional content of the feed. The high FCR in the control treatment without whey fermentation was due to a high feed consumption, so that the absorption of nutrients for egg production was due to the enhanced activity of pathogenic, the digestibility, and the metabolic energy that caused the rate of movement of food in the digestive tract to be faster (Jadhav et al., 2015). The faster movement of feed affected the efficiency of ingesting food used to make eggs (Kabir, 2009; Jadhav et al., 2015; Mousavi et al., 2018). Furthermore, Toghyani et al. (2015) stated that adding milk kefir and kefir molasses could reduce the FCR due to the increase of microbiota in fine milk, whichwas beneficial for absorption of feed nutrients and decreasing the consumption .

Gastrointestinal microflora and ammonia in laying hens

Microbiological analysis of 26-week-old laying hens can be seen in table 4. The effect of adding fermented whey cheese into drinking water decreased ($p < 0.01$) the number of *Escherichia coli* and increased lactic acid bacteria in the digestive tract of broiler chickens, especially in the ileum, caeca, and also in the stool. The number of Salmonella bacteria was significantly ($p < 0.05$) decreased and very significantly ($p < 0.01$) increased lactic acid bacteria in the feces during the fermentation of whey cheese. The decrease of ammonia in the stool was very significant ($p < 0.01$) compared to the control. The decrease in the population of *Escherichia coli* in the intestine and salmonella in the stool was related to the increase in lactic acid bacteria that produced various organic acid components such as lactic acid and acetic acid and decreased the value of intestinal pH (Engberg et al., 2009).

Table 4. Effect of whey cheese fermentation into drinking water on gastrointestinal microflora, excreta, and ammonia laying hens at 26 weeks.

Variable	Treatment		SEM	P-value
	P0	P1		
Escherichia coli				
Ileum (log CFU / gram)	5.67[a]	5.18[b]	0.08	<0.01
Caeca (log CFU / gram)	7.17[a]	6.40[b]	0.10	<0.001
Lactic acid bacteria				
Ileum (log CFU / gram)	7.12[b]	8.19[a]	0.16	<0.001
Caeca (log CFU / gram)	7.17[b]	8.39[a]	0.15	<0.001
Excreta				
Lactic acid bacteria (log CFU / gram)	5.57[b]	7.29[a]	0.23	<0.001
Escherichia coli (log CFU / gram)	4.60	4.51	0.12	0.664
Salmonella (log CFU / gram)	4.33[a]	3.79[b]	0.13	0.028
NH_3 (ppm)	3.73[a]	2.59[b]	0.17	<0.001

[a,b,c,d] = Means within a column with different superscripts differ significantly ($p < 0.05$). P0 = Control (without fermentied whey cheese), P1= Fermenting whey cheese, SEM = Standard Error Mean, log = logarithm, CFU = Colony Forming Unit, ppm = part per million

According to Canibe and Jensen (2003) and Kabir (2009) feed that contains a lot of lactic acid bacteria would produce high concentrations of lactic acid and acetic acid

and lower the pH to made a hostile environment for growth of gram-negative bacteria that are sensitive to acids such as Campylobacter, Salmonella, and *Escherichia coli*. An increase in lactic acid bacteria in the feces would also affect the production of organic acids that could break the chain of reaction of urea formation. According to Mi et al. (2019) lactic acid bacteria could produce more effective acid to reduce ammonia in chicken feces, so the amount of H^+ ions would be high. H^+ ions in chicken feces could convert ammonia to ammonium ($NH4^+$) so the formation of ammonia emissions by gram-negative bacteria could be prevented (Kabir, 2009; Pezzuolo et al., 2019).. Lactic acid bacteria produce acidand are proteolytic, so they can reduce ammonia by breaking down the protein in uric acid. Lactic acid bacteria utilized uric acid as a nutrient and broke it down to the monomers. This resulted in decreasing ammonia production because of the availability of uric acid to be converted to ammonia is reduced. Lactic acid bacteria also produced bacteriocin (antibiotics), which suppressed the growth of pathogenic gram-negative bacteria which produced ammonia (Dhama et al., 2011; Park et al., 2016; Chen et al., 2017). This suppression of bacterial growth resulted in decrease of producing the urease enzyme from gram-negative bacteria which used to convert uric acid to ammonia (Mi et al., 2019; Pezzuolo et al., 2019). Mi et al. (2019) indicated that the pH of the stool is very important for the release of ammonia in the stool. A decrease in pH will change ammonia (NH_3) to ammonium (NH_4^+), which is more water-soluble, and making it less volatile than NH_3.

CONCLUSION

Adding 3% fermented whey cheese to drinking water can reduce feed consumption, FCR, Escherichia coli, salmonella, ammonia (NH_3), and increase egg production, egg weight, egg weight, final body weight, and lactic acid bacteria. The fermented whey cheese could be used as a nutraceutical feed additive in increasing the productivity of laying hens.

DECLARATIONS

Consent to publish

All authors gave their informed consent before their inclusion in the study.

Competing interests

The authors declare that there is no competing interest in this research.

Authors' contribution

The authors contributed to arrange the experimental research, determine the method of analysis, preparation of materials method, study, and data analyzed. The authors confirmed the final revised article for publishing in this journal.

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REFERENCES

Ariyanti D and Hadiyanto H (2013). Pembuatan Bioetanol Dari Limbah Keju (Whey) Melalui Proses Fermentasi Fed-batch Dengan Kluyveromyces Marxianus. Journal Teknologi Kimia dan Industri, 2(2): 155-162. Available at: https://ejournal3.undip.ac.id/index.php/jtki/article/view/2839.

Bhattacharyya A and Roy D (2015). Nutraceuticals in livestock and poultry: New India Publishing Agency.

Canibe N and Jensen BB (2003). Fermented and nonfermented liquid feed to growing pigs: effect on aspects of gastrointestinal ecology and growth performance. Journal of Animal Science, 81(8): 2019-2031. DOI: https://doi.org/10.2527/2003.8182019x.

Charalampopoulos D and Rastall RA (2009). Prebiotics and probiotics science and technology: Springer.

Chaucheyras-Durand F and Durand H (2009). Probiotics in animal nutrition and health. Beneficial microbes, 1(1):3-9. DOI: https://doi.org/10.3920/BM2008.1002.

Chen CY, Chen SW and Wang HT (2017). Effect of supplementation of yeast with bacteriocin and Lactobacillus culture on growth performance, cecal fermentation, microbiota composition, and blood characteristics in broiler chickens. Asian-Australasian journal of animal sciences, 30(2): 211-220. DOI: https://doi.org/10.5713/ajas.16.0203.

Delia E, Tafaj M and Männerin K (2012). Efficiency of probiotics in farm animals. Probiotic in animals: Di dalam: E C Rigobelo, editor Croatia, BoD–Books on Demand. Available at: https://www.intechopen.com/books/probiotic-in-animals/efficiency-of-probiotics-in-farm-animals: 247-272.

Dhama K, Verma V, Sawant PM, Tiwari R, Vaid R and Chauhan RS (2011). Applications of Probiotics in Poultry: Enhancing Immunity and Beneficial Effects on Production Performances and Health - A Review. Journal of IImmunology and Immunopathology, 13:1-19. Available at: http://www.indianjournals.com/ijor.aspx?target=ijor:jii&volume=13&issue=1&article=001.

Engberg RM, Hammersh J, Johansen N, Abousekken MS, Steenfeldt S and Jensen BB (2009). Fermented feed for laying hens: effects on egg production, egg quality, plumage condition and composition

and activity of the intestinal microflora. British poultry science, 50(2): 228-239. DOI: https://doi.org/10.1080/00071660902736722.

FASS (2010). Guide for the Care and Use of Agricultural Animals in Research and Teaching: FASS Champaign, IL. DOI or Available at:

Fuller R (1989). Probiotics in man and animals. The Journal of applied bacteriology, 66(5): 365-378. DOI: https://doi.org/10.1111/j.1365-2672.1989.tb05105.x.

Huda K, Lokapirnasari W, Soeharsono S, Hidanah S, Harijani N and Kurnijasanti R (2019). Pengaruh Pemberian Probiotik Lactobacillus acidophilus dan Bifidobacterium terhadap Produksi Ayam Petelur yang Diinfeksi Escherichia coli. Jurnal Sain Peternakan Indonesia, 14(2): 154-160. DOI: https://doi.org/10.31186/jspi.id.14.2.154-160.

Istinganah L, Mugiyono S and Iriyanti N (2013). Penggunaan Berbagai Jenis Probiotik Dalam Ransum Terhadap Produksi Dan Bobot Telur Ayam Arab. Jurnal Ilmiah Peternakan, 1(1):338-346. Available at: https://www.e-jurnal.com/2016/10/penggunaan-berbagai-jenis-probiotik.html.

Jadhav K, Sharma K, Katoch S, Sharma V and Mane B (2015). Probiotics in broiler poultry feeds: A review. Journal of Animal Nutrition and Physiology, 1: 04-16. Available at: https://pdfs.semanticscholar.org/7e66/c9a51d93f0c7ed838f4d38d6279e446f4710.pdf.

Jin L, Ho Y, Abdullah N and Jalaludin S (2000). Digestive and bacterial enzyme activities in broilers fed diets supplemented with Lactobacillus cultures. Poultry science, 79(6): 886-891. DOI: https://doi.org/10.1093/ps/79.6.886.

Kabir S (2009). The role of probiotics in the poultry industry. International Journal of Molecular Sciences, 10(8): 3531-3546. DOI: https://doi.org/10.3390/ijms10083531.

Kabir S, Rahman M, Rahman M, Hosain M, Akand M and Das S (2005). Viability of probiotics in balancing intestinal flora and effecting histological changes of crop and caecal tissues of broilers. Biotechnology, 4(4): 325-330. DOI: https://doi.org/10.3923/biotech.2005.325.330.

Leeson S and Summers JD (2005). Commercial Poultry Nutrition, third edition: Nottingham University Press.

Lokapirnasari WP, Dewi AR, Fathinah A, Hidanah S and Harijani N (2017). Effect of probiotic supplementation on organic feed to alternative antibiotic growth promoter on production performance and economics analysis of quail. Veterinary world, 10(12):1508. DOI: https://doi.org/10.14202/vetworld.2017.1508-1514.

Mahfuz S, Nahar M, Mo C, Ganfu Z, Zhongjun L and Hui S (2017). Inclusion of probiotic on chicken performance and immunity: A Review. International Journal of Poultry Science, 16:328-335. DOI: https://doi.org/10.3923/ijps.2017.328.335.

Mellor S (2000). Nutraceuticals-alternatives to antibiotics. World Poultry, 16(2):30-33. Available at: https://www.cabdirect.org/cabdirect/abstract/20001413878.

Mi J, Chen X and Liao X (2019). Screening of single or combined administration of 9 probiotics to reduce ammonia emissions from laying hens. Poultry science, 98(9): 3977-3988. DOI: https://doi.org/10.3382/ps/pez138.

Mousavi A, Ali SM, Mahmoodzadeh Hosseini H and Mirhosseini SA (2018). A Review of Dietary Probiotics in Poultry. Journal of Applied Biotechnology Reports, 5(2):48-54. DOI: https://doi.org/10.1079/WPS19850008.

Nursiwi A, Utami R, Andriani M and Sari AP (2015). Fermentasi Whey Limbah Keju Untuk Produksi Kefiran Oleh Kefir Grains. Jurnal Teknologi Hasil Pertanian, 8(1):37-45. DOI: https://doi.org/10.20961/jthp.v0i0.12794.

Park J, Jeong J, Lee S and Kim I (2016). Effect of dietary supplementation with a probiotic (Enterococcus faecium) on production performance, excreta microflora, ammonia emission, and nutrient utilization in ISA brown laying hens. Poultry science, 95(12): 2829-2835. DOI: https://doi.org/10.3382/ps/pew241.

Pezzuolo A, Sartori C, Vigato E and Guercini S (2019). Effect of litter treatment with probiotic bacteria on ammonia reduction in commercial broiler farm. Jelgava, Latvia University of Life Sciences and Technologies Faculty of Engineering.

Pradikta RW, Sjofjan O and Djunaidi IH (2018). Evaluasi penambahan probiotik (Lactobacillus sp) cair dan padat dalam pakan terhadap penampilan produksi ayam petelur. Jurnal Ilmu-Ilmu Peternakan, 28(3): 203-212. DOI: https://doi.org/10.21776/ub.jiip.2018.028.03.03.

Prasetyo B, Kustiawan E (2012). Pemanfaatan Whey Fermentasi Sebagai "Funtional Feed" Dalam Meningkatkan Performans Ayam Broiler. Jurnal Ilmiah Inovasi, 12(1): 84-88. DOI: https://doi.org/10.25047/jii.v12i1.331.

Sathya C and Muragian P (2015). Effect of dietary supplementation of probiotic and Curculigo orchioides rhizome powder on egg quality parameters and biochemical composition of Japanese quail (Coturnix coturnix japonica). International Journal Of Advances in Pharmacy Biology and Chemistry, 4(1):162-170. Available at: http://www.ijapbc.com/files/23-4128.pdf.

Sugiharto S (2016). Role of nutraceuticals in gut health and growth performance of poultry. Journal of the Saudi Society of Agricultural Sciences, 15(2):99-111. DOI: https://doi.org/10.1016/j.jssas.2014.06.001.

Taklimi SMSM, Lotfollahian H, Shahne AZ, Mirzaei F and Alinejad A (2012). Study on efficacy of probiotic in broiler chickens diet. Agricultural Sciences 3(01):5. DOI: https://doi.org/10.4236/as.2012.31002.

Toghyani M, kazem Mosavi S, Modaresi M and Landy N (2015). Evaluation of kefir as a potential probiotic on growth performance, serum biochemistry and immune responses in broiler chicks. Animal Nutrition, 1(4): 305-309. DOI: https://doi.org/10.1016/j.aninu.2015.11.010.

Watson RR, Collier RJ and Preedy VR (2017). Dairy in Human Health and Disease across the Lifespan: Academic Press.

Yaman H, Ulukanli Z, Elmali M and Unal Y (2006). The effect of a fermented probiotic, the kefir, on intestinal flora of poultry domesticated geese (Anser anser). Revue de médecine vétérinaire, 157(7): 379-386. Available at: https://www.revmedvet.com/2006/RMV157_379_386.pdf.

Youssef AW, Hassan H, Ali H and Mohamed M (2013). Effect of probiotics, prebiotics and organic acids on layer performance and egg quality. Asian Journal Poultry Science, 7(2):65-74. DOI: https://doi.org/10.3923/ajpsaj.2013.65.74.

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A Novel Mutation in the Promoter Region of Avian Uncoupling Protein3 Associated with Feed Efficiency and Body Composition Traits in Broiler Chicken

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ABSTRACT

The Avian Uncoupling Protein (avUCP) belongs to the mitochondrial anion transporter family. It has a pivotal homeostatic mechanism that associated with energy regulation and lipid metabolism. The avUCP considered as a candidate gene for chicken growth-related traits according to its predominant expression is in skeletal muscle. To address genetic distance pattern of UCP3 between mammalian and avian species, sequence similarity analysis using the protein alignment of UCP3 identified the high amino acid identity between the species and complementarily detected two protein conserved regions which are known as the ADP/ATP transporter translocase and the Mitochondria-carrier. Likewise, for mutation detection, samples were genotyped, afterward PCR-SSCP method implemented. In addition, association analysis was performed for investigating single nucleotide polymorphism within the UCP3 gene relating to the given economic traits. A detected polymorphic site, on the promoter region of UCP3 (-40 T/A substitution), has displayed significant influences on the Feed Conversion Rate (FCR), Residual Feed Intake (RFI), Average Daily Gain (ADG), and Carcass Weight (CW%). In the case that, birds with genotype AA had better FCR, ADG, RFI as compared to the genotype BB and birds with genotype AA revealed a higher CW% as compared to the genotype BB. According to the obtained results from the in-silico survey, Myoblast determination protein (MyoD) was predicted as a best-matched transcription factor with a consensus sequence harboring the -40 T/A -novel SNP- in the promoter region of UCP3, where might be responsible for phenotypic variation between two genotypes. In conclusion, the result suggests important roles for UCP3 polymorphism in feed efficiency and growth traits which is better to be used in broiler chicken breeding programs.

Key words: Association analysis, Avian uncoupling protein3, Body composition traits, Broiler chicken, Feed efficiency, Novel mutation

INTRODUCTION

Mitochondria are the primary site of cellular energy production, generating the most of the cell's supply of ATP by using the energy derived from the oxidation of nutrients to create an electrochemical gradient across the mitochondrial inner membrane through proton pumping from the matrix (Lodish et al., 2000; Kolath et al., 2006). Actually the electron transport chain is composed of 83 subunits, 70 of which are encoded by the nuclear and the rest 13 encoded by the mitochondrial genome (Lodish et al., 2000). The nuclear-encoded proteins (uncoupling proteins 2 and 3) can uncouple oxidative phosphorylation and electron transport by transporting protons back into the mitochondrial matrix due to increasing the permeability of the inner mitochondrial membrane (Garlid et al., 2000; Criscuolo et al., 2006; Jastroch et al., 2010). A Survey conducted by Raimbault et al, in 2001 has discovered a mitochondrial Uncoupling Protein (UCP) homolog in the avian lineage for the first time, which called as Avian Uncoupling Protein (avUCP), also they have been highlighted that avUCP is sharing more than 70% amino acid homology with both mammalian UCP2 and 3 (Raimbault et al., 2001). However, most of the recent attention has been devoted to the important contribution of UCPs to non-shivering thermogenesis, thyroid hormone control, and cold-induced gene expression (Raimbault et al., 2001; Masaaki et al., 2002; Pawade et al., 2005; Rey et al., 2010). Whereas, regulation of UCP3 expression appeared to be related with Free Fatty Acid (FFA) utilization and free radical metabolism (Jian-Guo et al., 2005; Murata et al., 2013;

Moazeni et al., 2016). Therefore, avUCP can act as similar to mammalian UCPs, which have been strongly inducing during cold-exposure states (Raimbault et al., 2001; Masaaki et al., 2002; Rey et al., 2010; Nguyen et al., 2015; Lin et al., 2017).

Additionally, the vast investigation indicated that this protein may play a major role in body energy expenditure, particularly in skeletal muscle (Bailey et al., 1998; Boss et al., 1998; Raimbault et al., 2001; Abe et al., 2006; Liu et al., 2007). In addition, consecutive pieces of literature were implemented to characterize the gene structure and detect variants in the UCP3, paid particular attention to alteration in energy expenditure, growth, obesity, and body mass index in Human (Mutombo et al., 2013; Brondani et al., 2014; An et al., 2018). Also, UCP3 variation analysis in animal genetic fields, collectively, elucidated significant differentiation on feed efficiency and performance traits in farm animals (Choi et al., 2006; Kolath et al., 2006; Liu et al., 2007; Murata et al., 2013; Moazeni et al., 2016; Jin et al., 2018). Therefore, running lines of evidence which have justified the localized expression of avUCP in skeletal muscle in birds and its biological function of decreasing mitochondrial membrane potential and mediating proton leaks, made it a suitable candidate gene related to fat metabolism and production traits in the chicken (Masaaki et al., 2002; Abe et al., 2006; Rey et al., 2010; Murata et al., 2013).

As a result of any alteration in promoter regions that can change the activity of encoded protein so it will modify energy expenditure by affecting on the coupling level of oxidative phosphorylation, thereby providing variations in body weight and abdominal fat (Liu et al., 2007; Cieslak et al., 2009; Jastroch et al., 2010; Murata et al., 2013). Since, more accumulation of fat could decrease the feed conversion rate and meat quality in meat-typed chicken, therefore, rapid growth and lower fat deposition have required a great deal of interest concordantly in recent and following years because of its prominent issues in animal breeding (Sharma et al., 2008; An et al., 2018; Jin et al., 2018). In summary, due to a broad perspective appeared from previous studies, UCP3 has a crucial impact on growth traits and feed efficiency in many species. Besides, efficient genomic variations have been thought ordinarily of as a key factor for selection strategies, genetic improvement and ultimately having soared in the chicken meat industry, so the UCP3 can be a candidate gene for chicken production performance. Accordingly, the aim of the present study was to examine the association of a novel polymorphism in the promoter region of the UCP3 gene with feed efficiency and body composition traits in broiler chickens.

MATERIAL AND METHODS

Phenotypic measurements

The experiment carried out using Iranian commercial lines of Arian broiler chicken including two dam and sire lines. All lines primarily selected for growth traits in sire lines and reproductive traits in dam lines, for 19 successive generations and finally, a total of 253 day-old chickens selected at generation 19 to rear on the floor with *ad libitum* access to feed and water. The chicks fed on commercial corn-soybean diets in accordance with the national research council requirements containing 2745 kcal/kg Metabolizable Energy (ME), 14.3 % Crude Protein (CP) and 2920 kcal/kg ME, 13.3% CP respectively in dam and sire lines.

At the age of 21 days, all chicks weighed individually by digital scale, then transferred to the individual cages and grown for another 21 days recording for traits including Feed Conversion Ratio (FCR), Average Daily Gain (ADG), Residual Feed Intake (RFI), and Daily feed Intake (DFI) in all birds through 21 days as follows: The FCR calculated by dividing the mass of input (feed intake) by the mass of output (meat production) for measuring the efficiency of converting food into the desired output (Willems et al., 2013).

Next, the RFI also calculated as a difference between actual and predicted feed intakes based on body weight and growth rate. In the end, once the trial accomplished, the DFI calculated from offered and refused food by birds in each cage, then the ADG captured for the same period of rearing (Zhen-qiang et al., 2014). After fasting for 12 hours on 42th days, sample birds weighed individually, then, transferred to the slaughter-house and sacrificed by manual exsanguination using ethical guidelines according to animal welfare legislation, afterward, body composition traits such as Final Body Weight (FBW), Drumstick Weight (DW), Carcass Weight (CW), Breast Muscle Weight (BMW), Abdominal Fat Weight (AFW), Hand Weight (HNDWT) and Back Weight (BAKWT) recorded by digital scale as well.

DNA sequencing, Polymorphism Chain Reaction-Single-strand conformation polymorphism and population screening for single-nucleotide polymorphisms

Required DNA extracted from whole blood using modified salting-out procedure (Miller et al., 1988). Four

sets of primers (table 2) designed based on the sequence of UCP3 gene given from the GenBank database. Amplification of the UCP gene was performed in the volume of 20 µl containing 100 ng of genomic DNA, 1× PCR buffer, 1 unit of Taq DNA polymerase (Cinagene, Iran), 2.5 mM dNTPs, 10 ρ mol of each primer and 2 mM MgCl2. PCR condition was set in three min initial denaturation at 94 ºC, 35 cycles of 45s denaturing at 94 ºC, 45s annealing at 62 ºC and 45s elongation at 72 ºC. Amplification product was assessed on a 1.5% agarose gel electrophoresis and purified using a QIAquick Gel extraction micro centrifuge and vacuum (QIAGEN, USA) according to the manufacturer's instructions. To increase the reliability of measures, direct sequencing was performed on both strands in samples of each line by applying the primer sets using the 3730XL DNA analyzer (Applied Biosystem (ABI), USA). Single Strand Conformation Polymorphism (SSCP) method was used to evaluate the frequency of the SNPs in the promoter region of the gene at the population level because it is a simple and sensitive technique for mutation, detection and genotyping (Hayashi 1992). The PCR pair primer of 5' GAG CGG GAT TTG ATT CTG TGC 3' and 5' GAA GGT GCA GAG GTC AGC GAT 3' was designed in both forward and reverse strands to amplify a 230-bp of UCP's promoter region harboring the substitution. The PCR condition was the same as the method mentioned above except adding 5% Ddimethyl Sulfoxide (DMSO). Then, 10µl PCR product mixed with 8µl loading buffer (98% Formamide, 0.025% Bromophenol blue, 0.025% Xylene Cyanol, 10 mM Ethylenediamine Tetraacetic Acid (EDTA). The mixture, then denatured at 95°C for five min, and placed in to an icebox for five min, and in the next stage, electrophoresed for 8 to 12 h at 200 Von a 6% polyacrylamide gel with 10% glycerol. The silver staining method implemented to visualize the DNA patterns on the gel. The samples producing different SSCP patterns on the gel, finally DNA segments sequenced to confirm the causative SNP.

Statistical model and analysis method

One of the most well-known statistical model for using in association analyses is GLM (Fang et al., 2009). So that the association between the UCP mutations with growth and body composition traits analyzed by GLM procedure (SAS, version 9.4). The fitted model contained fixed effects of genotype (G with 2 levels), sex (S with 2 levels), and line (L with 4 levels) and random effect of sire (SI) nested within the lines SI (L), as follows:

$$y = \mu + G + S + L + SI(L) + e.$$

where y is the response variable, µ is the population mean, and e is unknown random error. Significant differences between least square means of the different genotypes were calculated using a LSMEANS contrast procedure (Wang and Goonewardene, 2004). The significance threshold defined at $P<0.05$ level.

Table 1. Four sets (the sets number 1 to 4) of designed primers used for sequencing the avian uncoupling protein gene in Arian bird samples and set 5 used for single-strand conformation polymorphism analysis

Gene	Primer set	Sequences of designed primer	Length of product
UCP3	1	F 5´-GAGCGGGATTTGATTCTGTGC-3´ R 5´-GGTGGGTTGGTCCTTCATTGG-3´	946
	2	F 5´-TGGGACCAATCATGTCAGTGG-3´ R 5´-ATGGATGGAGCTACGGACACC-3´	876
	3	F 5´-GATAGTGGTGAGGAAGGTAGG-3´ R 5´-AAAGCAGCCACGAAGTGACAG-3´	734
	4	F 5´-CATCAAAGGACACACTGCTGC-3´ R 5´-AGGAATACCCGGACTCACCAC-3´	801
	5	F 5´-GAGCGGGATTTGATTCTGTGC-3´ R 5´-GAAGGTGCAGAGGTCAGCGAT-3´	230

Primer sets designed in Tarbiat modares laboratory based on the sequence of UCP3 gene with accession number AF433170.2 from the GenBank, USD. UCP3: uncoupling protein3; F: forward; R: reverse

Bioinformatics studies

To address genetic similarity of UCP3 between mammalian and avian species, sequence similarity analysis used the protein alignment of UCP3. Moreover, Evolutionary relationship assessed by measuring pairwise distance between species, since several sources of molecular information have been undertaken to illustrate evolutionary relationships, protein sequences of chicken and 14 other species extracted from NCBI database. Then, phylogeny and molecular evolutionary analyses conducted by using MEGA in order that, genetic distances derived from protein weight matrix by using BLOSUM algorithm for homology searches (Tamura et al., 2011). Then phylogenetic tree drown using both character-based and distance-based procedure presented in maximum likelihood model. For the reason, achieving better interpretation, promoter and transcription factor prediction performed by different online software including EPD, BBCU and TFBIND (Matys et al., 2003; Deaton and Bird 2011; Gaudry and Campbell, 2017; Lizio etal., 2017). The TFBIND online software uses weight matrix in

transcription factor (TF) database. Since this database uses all sequences related to TATA-Box, initiator region, CCAAT-Box, and GC-box it can obtain more purified and reliable results (Matys et al., 2003; Kumar et al., 2017).

Ethical approval

All bird's experiment performed according to standard animal welfare and approved by the Committee of Animal Science of Tarbiat Modares University, Iran. All experimental protocols applied in accordance with relevant legislation and recommendations by this committee.

RESULTS

Sequence analysis, Single Nucleotide Polymorphism detection and genotyping

The first set of analyses examined whether variation can be discovered in the interested gene or not. Direct sequencing of the UCP3 gene in sample birds discoverd five novel polymorphic sites including, a substitution of -40 T/A in 5' Untranslated Region (5' UTR), a silent mutation of 1833 A/G in fourth exon, and also the rest of mutant-sites detected in third intron and 3' UTR , but We for further exanination just foucesd on a novel polymorphic site in promoter region or 5' UTR. Due to the importance of the mutations which occur in the promoter region of the genes, further analyses particularly conducted to examin -40 T/A polymorphic site. As figure showes, -40 T/A substitution produced two different SSCP pattern on gel, namely A and B across the population (Figure 1).

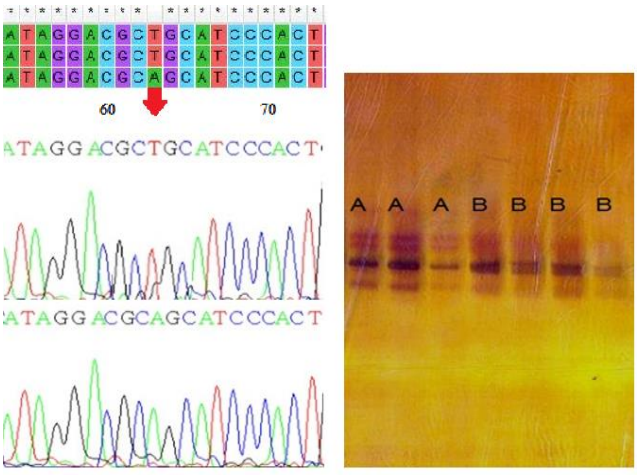

Figure 1. Electrograph of 230 base-pair sequenced segment of uncoupling protein3 gene carrying a novel single nucleotiede polymorphism in Arian lines

Association of avian uncoupling protein3 -40 T/A polymorphism with feed efficiency and body composition traits

The genotypes of -40 T/A in upstream site of the UCP3 included in the genetic association analysis. Hardy-Weinberg Equilibrium test (HWE) for this locus indicated that this site is out of HWE. Because allelic frequency in this locus indicated that, allele A with a frequency of 0.67 was more prevalent than allele B with frequency of 0.33 in the trail population. There were significant associations between this polymorphism and all feed efficiency traits including FCR, ADG and RFI (P = 0.001) except DFI. So that chickens with genotype AA indicated better FCR and RFI than genotype BB, and also chickens with genotype AA displayed higher ADG than genotype BB. Likewise, -40 T/A substitution was significantly associated with carcass weight percentage of samples (P = 0.05) so that chickens with genotype AA revealed a higher CW% than the birds with genotype BB. However, tiny differences in other composition traits (CW, DW, BMV, AFW and AFW %) did not show any association (Table 2).

Table 2. Least square means ± SE for feed efficiency and body composition traits among genotypes in the uncoupling protein3 in broiler chickens considering the effects of -40 T/A substitution

Trait	Genotype		P-value
	AA	BB	
FCR g:g	1.925±0.023	2.149±0.04	0.000***
RFI (g)	1.421±0.006	8.854±2.62	0.017**
DFI (g)	165.8±1.951	164.8±3.462	0.827
ADG (g/d)	86.298±0.898	77.485±1.593	0.000***
CW (g)	1661.9±10.987	1626±26.692	0.513
DW (g)	473.99±5.43	473.62±8.883	0.674
BMW (g)	550.14±3.286	533.45±9.628	0.236
AFW(g)	22.616±0.862	26.625±1.544	0.113
CW%	69.157±0.337	66.834±0.934	0.053*
AFW%	0.911±0.037	1.08±0.62	0.111

SE: standard error; FCR: feed conversion ratio in the interval; RFI: residual feed intake from 21 to 42 days of age; DFI: daily feed intake; ADG: average daily gain; CW: carcass weight; DW: drumstick weight; BMV: breast muscle weight; AFW: abdominal fat weight

Bioinformatics' results

In this study promoter prediction performed by TFBIND and the logical results presented in table.3. The predicted binding sites and transcription factors including: Myoblast determination protein (MyoD with M00184 code), Specificity Protein 1 (SP1 with M00008 code), and Nuclear factor I (NF1 with M00193 code) corresponded to

the consensus sequences of promoter region of UCP3. Amazingly, MyoD was exactly covering the region harboring novel single nucleotide polymorphism. The Schematic figure of matched transcription factor with binding sites in promoter region of uncoupling protein gene has designed in figure 3. To address genetic distance pattern of UCP3 between mammalian and avian species, alignment of protein sequences in UCP3s identified high amino acid identity between the species and complementarily detected two protein conserved regions which are known as the ADP/ATP transporter translocase and the Mitochondria-carrier. The output of genetic distance and revolution relationship between mentioned species displayed in a figure of rooted phylogenetic tree using maximum likelihood method that indicates common ancestor of UCP3 in all species (Figure 4).

Table 3. Predicted transcription factors and binding sites in promoter region of uncoupling protein3 gene of broiler chicken by TFBIND online software

Code	Transcription factors	Score	Consensus sequence of binding sites
M00008	SP1	0.823046	GRGGCRGGGWGTTCCCCCCC
M00184	Myo D	0.781616	NNCANCTGNYTCCCACTGCC
M00193	NF1	0.741892	NNTTGGCNNNNNNCCNNNTCTATCCGTGAGCCAGGA

SP1: Specificity Protein 1; Myo D: Myoblast determination protein; NF1: Nuclear factor I

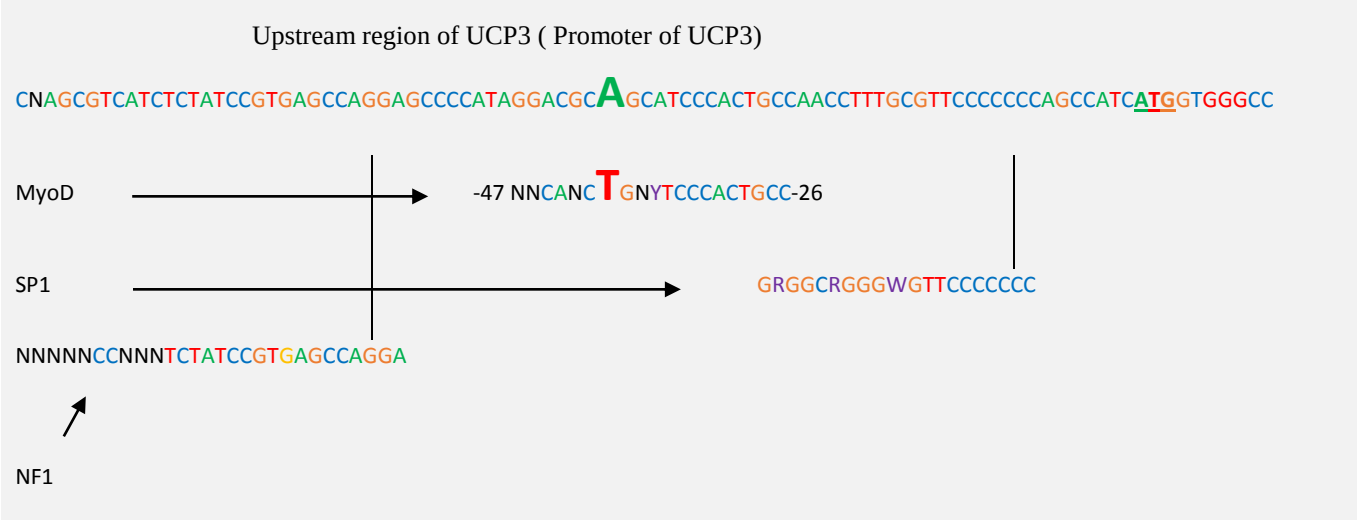

Figure 3. Schematic figure of predicted binding sites in uncoupling protein gene by MyoD with the logo of MyoD in Bioinformatics analysis. UCP3: Uncoupling protein3; SP1: Specificity Protein 1; Myo D: Myoblast determination protein; NF1: Nuclear factor I

DISCUSSION

The candidate gene approach has been known as a significant tool to improve the selection methods by finding behind the curtain association between genotype variation in certain gene and phenotype of interest (Liu et al., 2007; Moazeni et al., 2016; Jin et al., 2018). Thus, by discovering the contribution of UCP3 in the regulation of energy metabolism, it is concluded that it may affect the production traits and promote the performance of meat-type poultry. As a consequence, the polymorphism of UCP3 can be used in genetic improvement if genotypic and allelic frequencies are determined. Since the confirmation of such effects requires a considerable amount of multilateral studies, and there is still insufficient information on the function of this gene, so, to pave the path for finding this information, this study was designed.

A basic objective of our research was to estimate the allelic and genotypic frequency of UCP3 polymorphism of the -40 T/A substitution in the promoter region in distinct genotypes. Hardy-Weinberg Equilibrium test (HWE) can be used for searching how the alleles within one locus are in equilibrium, this test for polymorphic locus in upstream of UCP3 in our samples indicated that it is out of HWE, because certain selection strategies for reproduction and production traits in both dam and sire lines of Arian chickens affected the allelic frequencies during the past years Arian population.

To detect the association of UCP3 variants, according to the result of frequency association analyses that presented in table 3, the pattern of genotype AA showed maximum frequency (0.7312%) and the pattern of genotype BB indicated minimum frequency (0.2688%). Moreover, genotype AA produced better performance for most of feed efficiency traits except DFI than genotype BB. Although there were no significant differences between the two genotypic patterns in body composition traits, except CW% ($p < 0.053$), that figured out chickens with AA genotype had higher CW% compared to chickens with BB genotype pattern. On average, body composition traits in chickens with genotype AA were superior to those with BB genotype. Also, Sharma et al. in 2008 suggested that polymorphism in the avUCP is associated with feed conversion and body weight in commercial broiler lines which approves our findings in the present study (Sharma et al., 2008).

Moreover, in the human field, some supportive investigations have indicated common mutations on promoter sites of UCP3 are associated with childhood obesity, energy expenditure, body weight alteration, and BMI (Mutombo et al., 2013; Brondani et al., 2014).

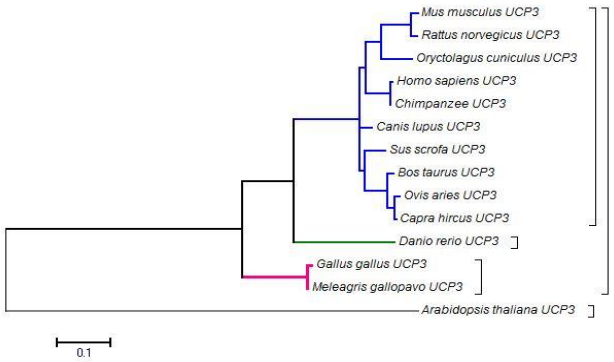

Figure 4. Molecular Phylogenetic analysis of the uncoupling protein3 in 14 different species by using protein sequences

Almost every research conducted on UCP3 recommended some promotion in feed efficiency and growth performance traits in chicken associated with UCP3 polymorphism in chicken (Nguyen et al., 2015; Moazeni et al., 2016; Jin et al., 2018), in Japanese quail (Murata et al., 2013), in New Zealand Romney lambs (An et al., 2018), and in Angus' steers (Kolath et al., 2006). Furthermore, recent studies suggest that proton uncoupling action of UCP3 in mitochondria can permit heat production and regulate energy metabolism in beige adipose tissue in pigs (Cieslak et al., 2009; Lin et al., 2017).

As usual, the less abdominal fat in chickens enhances the quality and market acceptability of meat production. In addition, emerging evidence report that lipid metabolism to be controlled by UCP3, and it has been found to be associated with fat distribution and accumulation of abdominal fat in avian species (Liu et al., 2007; Murata et al., 2013). Conversely, the data structure in our research did not reveal a significant influence on abdominal fat weight between different genotypes carrying the novel SNP. This contrasting result may come from the fact that different determinants including hormonal levels, nutrition, genotypes, and even data structure are responsible for phenotypic plasticity (De-Jong and Bijma 2002; Johnsson et al., 2018). The strategy of using UCP3 polymorphism as a DNA marker would increase body weight, improve production quality and decrease abdominal fat next will cause low production costs in the poultry industry (Liu et al., 2007; Moazeni et al., 2016; An et al., 2018; Jin et al., 2018). The existence of some alteration in the promoter region makes biological sense which confirms regulatory sequences should be variable. Since gene promoters are critical role players in gene regulation, so they ordinarily receive signals from different sources to down and up-regulate the level of transcription, which mainly determines gene expression (Rey et al., 2010; Hoffmann et al., 2013; Murata et al., 2013). For the reason, that transcription start sites and surrounding regulatory elements normally are often in the upstream region of gene sequence. Therefore, we deduced that if the novel change occurs in the allelic level by substitution of T to A in promoter region finally will cause significant variation in phenotype in birds which are harboring different types of variants. On the other hand, the UCP3 has been shown to involve in the regulation of some biological process of energy metabolism so it is most likely to have an effect on pathways related to growth performance (An et al., 2018; Jin et al., 2018).

On the other side, due to the sequence identity, mitochondrial proteins were grouped into the core UCP family and phylogenetic deduction classifies avUCP as a UCPs orthologue (Jastroch et al., 2010; Tamura et al., 2011; Pardi and Gascuel, 2016). Besides, it should be considered that relatively little information is available concerning regulatory mechanisms of UCP in avian species, especially about promoter binding sites and other controlling elements (Jian-Guo et al., 2005; Lizio et al., 2017). Notwithstanding the fact that existing some differences in the number of exons and the genomic region

that the gene is located on, our assessment in the field of sequence alignment of UCP3 from chicken and other species has demonstrated a relatively uniform structure that displays conserved composition of amino acids in UCP3 between studied species which means UCP3 is under purifying natural selection, suggesting that it's functions has not changed severely during evolution. The amino acid similarity above 70% previously endorsed by Raimbault et al. (2001). The conserved regions in uncoupling protein encoded by UCP3s in different species, known as the ADP/ATP transporter translocase and the Mito_carr with slight variation in the state of their genomic region (Gaudry and Campbell, 2017; Lin et al., 2017).

In the current study, -40 T/A SNP in UCP3 located in promoter region, and since a possible explanation for this might be that the promoter is the most important region to gene transcription, because, in initial stage of gene transcription, specific transcriptional factors should bind to the promoter site, so any mutations or indels, particularly in binding sites can alter the pattern of transcription and finally change the phenotype (Lu and Sack, 2008; Deaton and Bird 2011; De-Luis et al., 2012).

Bioinformatics available tools have provided the opportunity to predict certain parameters based on the genomic information of other organisms. In this regard, we used in silico analyses for prediction of probable binding sites in the promoter region and transcription factors, using the humane and mouse genomic information. The output of prediction represents that -40 T/A located in the probable consensus binding motifs in the promoter region and might combine with MyoD which qualified in this study. (Bailey et al., 1998; Solanes et al., 2000; Hoffmann et al., 2013). If this hypothesis becomes true so the significant changes in the mentioned traits will become more justified. According to text mining further transcription factors have been found related to UCP3 such as Coup-TFII, MyoD, Sp1, PPAR-alpha, and the others (Lu and Sack, 2008; Hoffmann et al., 2013). As biology is mysterious and complicated we guess other regulators have been located out of our eyes. To support this attitude, we point to study which determined that the product of UCP3 can be governed by a complex interaction between chicken ovalbumin upstream promoter transcription factor II (Coup-TFII) and other transcription factors including PPARalpha, MyoD, and histone acetyltransferase p300.

Bailey et al. (1998) and Solanes et al. (2000) already underlined that when MyoD as a promoter activator of UCP3, co-transfects, then other factors will contribute and can cause a significant induction. Additionally, extra findings obtained from previous studies have established that any alteration in the promoter region and binding sites can counteract the attachment of MyoD, PPAR, and other transcription factors so that ultimately transcription level can be attenuated (Lu and Sack, 2008; Nguyen et al., 2015).

As this novel SNP is located exactly in the predicted consensus region mentioned in table 3. It can be concluded it was able to alter the transcription level. Finally, it is deduced that excavated association between UCP3's SNP and phenotypes in broiler lines most likely produced by underlying linkage disequilibrium between this SNP and causal gene which modulates the expression of the UCP3 in our chicken samples. Although, it should be noted the significant associations obtained in this study needs to be confirmed by increasing the sample size in further complementary studies and it might be a distinct application of this novel SNP which could be benefitted by further studies.

CONCLUSION

This study investigated the association between the sequence variant in the avUCP with feed efficiency and body composition traits in Arian broiler lines. The result identified new mutations in the structure of the gene. Association analysis indicated that a mutation in the promoter region was significantly associated with feed efficiency traits and carcass weight% in Arian broiler lines. Indeed, because of the location of the mutation in the promoter region and the vicinity of the mutation with some important transcription factors, in particular, MyoD, indicate that -40 T/A will be a valuable SNP for application in poultry breeding strategies by complementary study supports.

DECLARATIONS

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Authors' contributions

Ali Akbar Masoudi designed the experiments and guided the team until the end, after all, edited and approved the final manuscript. Rasoul Vaez Torshizi

performed statistical analysis and revised the manuscript. Mojtaba Darzi Niamami performed laboratory experiments and analyzed obtained data. Peymaneh Davoodi conducted bioinformatics analysis, writing and drafting manuscript. Finally, all authors read and approved the final manuscript and they consent to be published on JWPR.

Competition interests

The authors have declared that they have no competing interests

REFERENCES

Abe T, Mujahid A, Sato K, Akiba Y and Toyomizu M (2006). Possible role of avian uncoupling protein in down-regulating mitochondrial superoxide production in skeletal muscle of fasted chickens. Federation of European Biochemical Societies Letters. 580: 4815-4822. DOI:https://doi.org/10.1016/j.febslet.2006.07.070

An Q, Zhou H, Hu J, Luo Y and Hickford JGH (2018). Sequence and haplotypes variation of the ovine uncoupling protein-1 gene (ucp1) and their association with growth and carcass traits in new zealand romney lambs. Genes. 9: 189-201. DOI:https://doi.org/10.3390/genes9040189

Azzu V, Jastroch M, Divakaruni AS, and Brand MD (2010). The regulation and turnover of mitochondrial uncoupling proteins. Biochimica et Biophysica Acta. 1797: 785-791. DOI:https://doi.org/10.1016/j.bbabio.2010.02.035

Bailey P, Sartorelli V, Hamamori Y and Muscat GE (1998). The orphan nuclear receptor, coup-tf ii, inhibits myogenesis by post-transcriptional regulation of myod function: Coup-tf ii directly interacts with p300 and myod. Nucleic Acids Research. 26: 5501-5510. DOI:https://doi.org/10.1093/nar/26.23.5501

Boss O, Samec S, Kuhne F, Bijlenga P, Assimacopoulos JF, Seydoux J, Giacobino JP et al (1998). Uncoupling protein-3 expression in rodent skeletal muscle is modulated by food intake but not by changes in environmental temperature. Journal of Biological Chemistry. 273: 5-8. https://doi.org/10.1074/jbc.273.1.5

Brondani LA, Assmann TS, De-Souza BM, Bouças AP, Canani LH and Crispim D (2014). Meta-analysis reveals the association of common variants in the uncoupling protein (ucp) 1–3 genes with body mass index variability. PLOS ONE. 9: 96411-96421 DOI:https://doi.org/10.1371/journal.pone.0096411

Choi CH, Cho BW, Jeon GJ and Lee HK (2006). Identification of novel snps with effect on economic traits in uncoupling protein gene of korean native chicken. Asian-Australian Journal of Animal Science. 19: 1065-1070. DOI:https://doi.org/10.5713/ajas.2006.1065

Cieslak J, Nowacka-Woszuk J, Bartz M, Fijak-Nowak H, Grzes M, Szydlowski M and Switonski M (2009). Association studies on the porcine retn, ucp1, ucp3 and adrb3 genes polymorphism with fatness traits. Meat Science. 83: 551-554. DOI:https://doi.org/10.1016/j.meatsci.2009.07.001

De-Jong G and Bijma P (2002). Selection and phenotypic plasticity in evolutionary biology and animal breeding. Livestock Production Science. 78: 195-214. DOI:https://doi.org/10.1016/S0301-6226(02)00096-9

De-Luis DA, Aller R, Izaola O, Gonzalez-Sagrado M and Conde R (2012). Association of -55c/t polymorphism of ucp3 gene with fat distribution, cardiovascular risk factors and adipocytokines in patients with type 2 diabetes mellitus. Journal of Endocrinology Investigation. 35: 625-633. DOI:https://doi.org/10.3275/7908

Deaton AM and Bird A (2011). Cpg islands and the regulation of transcription. Genes and Developments. 25: 1010-1022. DOI:https://doi.org/10.1101/gad.2037511

Fang M, Nie Q, Luo C, Zhang D and Zhang X (2009). Associations of ghsr gene polymorphisms with chicken growth and carcass traits. Molecular Biology Reports. 37: 423-428 DOI:https://doi.org/10.1080/10495398.2013.803478

Garlid KD, Jaburek M, Jezek P and Varecha M (2000). How do uncoupling proteins uncouple? Biochimica et Biophysica Acta. 1459: 383-389. DOI:https://doi.org/10.1016/s0005-2728(00)00175-4

Gaudry MJ and Campbell KL (2017). Evolution of ucp1 transcriptional regulatory elements across the mammalian phylogeny. Frontiers in physiology. 8: 670-692. DOI:https://doi.org/10.3389/fphys.2017.00670

Hayashi K (1992). Pcr-sscp: A method for detection of mutations. Genetic, Analysis, Techniques and Applications. 9: 73-79. DOI:https://doi.org/10.1016/1050-3862(92).90001-I

Hoffmann C, Zimmermann A, Hinney A, Volckmar AL, Jarrett HW, Fromme T and Klingenspor M (2013). A novel sp1/sp3 dependent intronic enhancer governing transcription of the ucp3 gene in brown adipocytes. PLOS ONE. 8: e83426-e83426. DOI:https://doi.org/10.1371/journal.pone.0083426

Jian-Guo Z, Hui L, Yu-Xiang W and He M (2005). The expression characterization of chicken uncoupling protein gene. Asian-Australas Journal of Animal Science. 18: 1552-1556. DOI:https://doi.org/10.5713/ajas.2005.1552

Jin S, Yang L, He T, Fan X, Wang Y, Ge K and Geng Z (2018). Polymorphisms in the uncoupling protein 3 gene and their associations with feed efficiency in chickens. Asian-Australian Journal of Animal Science. 31: 1401-1406. DOI:https://doi.org/10.5713/ajas.18.0217

Johnsson M, Henriksen R, Höglund A, Fogelholm J, Jensen P and Wright D (2018). Genetical genomics of growth in a chicken model. BioMed Central Genetics. 19: 72-84. DOI:https://doi.org/10.1186/s12864-018-4441-3

Kolath WH, Kerley MS, Golden JW and Keisler DH (2006). The relationship between mitochondrial function and residual feed intake in angus steers. Journal of Animal Science. 84: 861-865. DOI:https://doi.org/10.2527/2006.844861x%J

Kumar S, Ambrosini G and Bucher P (2017). Snp2tfbs - a database of regulatory snps affecting predicted transcription factor binding site affinity. Nucleic Acids Research. 45: 39-44. DOI:https://doi.org/10.1093/nar/gkw1064

Ledesma A, De-Lacoba MG and Rial E (2002). The mitochondrial uncoupling proteins. Genome biology. 3: 3015-3024. DOI:https://doi.org/10.1186/gb-2002-3-12-reviews3015

Lin J, Cao C, Tao C, Ye R, Dong M, Zheng Q, Wang C et al (2017). Cold adaptation in pigs depends on ucp3 in beige adipocytes. Journal of Molecular and Cellular Biology. 9: 364-375. DOI:https://doi.org/10.1093/jmcb/mjx018

Liu S, Wang SZ, Li ZH and Li H (2007). Association of single nucleotide polymorphism of chicken uncoupling protein gene with muscle and fatness traits. Journal of Animal Breeding Genetics. 124: 230-235. DOI:https://doi.org/10.1111/j.1439-0388.2007.00654.x

Lizio M, Deviatiiarov R, Nagai H, Galan L, Arner E, Itoh M, Lassmann T et al (2017). Systematic analysis of transcription start sites in

avian development. PLOS Biology. 15: 2002887-2002910. https://doi.org/10.1371/journal.pbio.2002887

Lu Z and Sack MN (2008). Atf-1 is a hypoxia-responsive transcriptional activator of skeletal muscle mitochondrial-uncoupling protein 3. Journal of Biological Chemistry. 283: 23410-23418. DOI:https://doi.org/10.1074/jbc.M801236200

Masaaki T, Masatoshi U, Shinichi S, Yoshinori S, Kan S and Yukio A (2002). Cold-induced mitochondrial uncoupling and expression of chicken ucp and ant mrna in chicken skeletal muscle. Federation of European Biochemical Societies Letters. 529: 313-318. DOI:https://doi.org/10.1016/S0014-5793(02)03395-1

Matys V, Fricke E, Geffers R, Gossling E, Haubrock M, Hehl R, Hornischer K et al (2003). Transfac: Transcriptional regulation, from patterns to profiles. Nucleic Acids Research. 31: 374-378. DOI:https://doi.org/10.1093/nar/gkg108

Miller SA, Dykes DD and Polesky HF (1988). A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Research. 16: 1215-1215. DOI:https://doi.org/10.1093/nar/16.3.1215

Moazeni SM, Mohammadabadi M, Sadeghi M, Moradi-Shahrbabak H, Esmailizadeh-Koshkoieh A and Bordbar F (2016). Association between ucp gene polymorphisms and growth, breeding value of growth and reproductive traits in mazandaran indigenous chicken. Open Journal of Animal Sciences. 6: 1-8 DOI:https://doi.org/10.4236/ojas.2016.61001

Murata S, Kawabe K, Taura S, Shimogiri T and Okamoto S (2013). Relationship between abdominal fat content and avian uncoupling protein gene expression in skeletal muscle of japanese quail coturnix japonica. British Poultry Science. 54: 42-51. DOI:https://doi.org/10.1080/00071668.2013.764398

Mutombo PB, Yamasaki M and Shiwaku K (2013). Ucp2 i/d modulated change in bmi during a lifestyle modification intervention study in japanese subjects. Genetic Test of Molecular Biomarkers. 17: 16-20. DOI:https://doi.org/10.1089/gtmb.2012.0229

Myers SA, Wang SC and Muscat GE (2006). The chicken ovalbumin upstream promoter-transcription factors modulate genes and pathways involved in skeletal muscle cell metabolism. Journal of Biological Chemistry. 281: 24149-24160. DOI:https://doi.org/10.1074/jbc.M601941200

Nguyen P, Greene E, Ishola P, Huff G, Donoghue A, Bottje W and Dridi S (2015). Chronic mild cold conditioning modulates the expression of hypothalamic neuropeptide and intermediary metabolic-related genes and improves growth performances in young chicks. PLOS ONE. 10: 142319-142341 DOI:https://doi.org/10.1371/journal.pone.0142319

Pardi F and Gascuel O (2016). Distance-based methods in phylogenetics. Encyclopedia of Evolutionary Biology. 1: 458-465. Available at:https://hal-lirmm.ccsd.cnrs.fr/lirmm-01386569

Pawade T, Ho PW, Kwok KH, Chu AC, Ho SL and Ramsden DB (2005). Uncoupling proteins: Targets of endocrine disruptors? Mol Cell Endocrinol. 244: 79-86. DOI:https://doi.org/10.1016/j.mce.2005.06.008

Raimbault S, Dridi S, Denjean F, Lachuer J, Couplan E, Bouillaud F, Bordas A et al (2001). An uncoupling protein homologue putatively involved in facultative muscle thermogenesis in birds. Biochemistry Journal. 353: 441-444. DOI:https://doi.org/10.1042/0264-6021:3530441

Rey B, Roussel D, Romestaing C, Belouze M, Rouanet JL, Desplanches D, Sibille B et al (2010). Up-regulation of avian uncoupling protein in cold-acclimated and hyperthyroid ducklings prevents reactive oxygen species production by skeletal muscle mitochondria. BioMed Central Physiology. 10: 1186-1191. DOI:https://doi.org/10.1186/1472-6793-10-5

Sharma P, Bottje W, and Okimoto R (2008). Polymorphisms in uncoupling protein, melanocortin 3 receptor, melanocortin 4 receptor, and pro-opiomelanocortin genes and association with production traits in a commercial broiler line. Poultry Science. 87: 2073-2086. DOI:https://doi.org/10.3382/ps.2008-00060

Solanes G, Pedraza N, Iglesias R, Giralt M, and Villarroya F (2000). The human uncoupling protein-3 gene promoter requires myod and is induced by retinoic acid in muscle cells. Federation of American Societies for Experimental Biology Journal. 14: 2141-2143. DOI:https://doi.org/10.1096/fj.00-0363fje

Tamura K, Peterson D, Peterson N, Stecher G, Nei M and Kumar S (2011). Mega5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular Biology Evolution. 28: 2731-2739. DOI:https://doi.org/10.1093/molbev/msr121

Tompa M, Li N, Bailey TL, Church GM, De-Moor B, Eskin E, Favorov AV et al (2005). Assessing computational tools for the discovery of transcription factor binding sites. Nature Biotechnology. 23: 137-144. DOI:https://doi.org/10.1038/nbt1053

Villarroya F, Iglesias R and Giralt M (2007). Ppars in the control of uncoupling proteins gene expression. Peroxisome Proliferator-Activated Receptors (PPAR) Research 20: 74364-74386. DOI:https://doi.org/10.1155/2007/74364

Wang LA and Goonewardene Z (2004). The use of mixed models in the analysis of animal experiments with repeated measures data. Canadian Journal of Animal Science. 84: 1-11. DOI:https://doi.org/10.4141/A03-123

Willems OW, Miller SP and Wood BJ (2013). Aspects of selection for feed efficiency in meat producing poultry. World's Poultry Science Journal. 69: 77-88. DOI:https://10.1017/S004393391300007X

Zhen-qiang Z, Jie C, Yan Z, Cong-liang J, De-xiang Z and Xi-quan Z (2014). Determination of residual feed intake and its associations with single nucleotide polymorphism in chickens. Journal of Integrative Agriculture. 13: 148-157. DOI:https://doi.org/10.1016/S2095-3119(13)60383-4

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Response of Laying Quail to a Diet Enriched with Cocoa Pods Fermented by *Pleurotus ostreatus*

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ABSTRACT

Improving the quality of cocoa pods through fermentation with *Pleurotus ostreatus* reduced its crude fiber content, especially for lignin and cellulose. Fermentation of cocoa pods product increased their utilization in poultry diet. An experiment was conducted to determine the effect of feeding cocoa pods waste fermented by *Pleurotus ostreatus* on the laying performance and egg quality of quail. This experiment employed completely randomized design with five dietary treatments: 0%, 5%, 10%, 15% and 20% cocoa pods fermented by *Pleurotus ostreatus* in the diets and four replications. 200 laying quail (7 weeks of age) were randomly allocated into 5 treatments (10 birds per treatment) and 4 replications. Diets included iso nitrogen 20% and iso metabolism energy 2800 kcal/kg. The results of the experiment indicated that feed intake, hen-day egg production, egg weight, egg mass production, and feed conversion were not affected but egg cholesterol was affected by increasing cocoa pods fermented products in the diet. In this experiment, a diet which utilized 15% cocoa pods fermented using *Pleurotus ostreatus* maintained the laying performance of quail and reduced egg yolk cholesterol (20.30%).

Key words: Cocoa pods, Egg quality, laying Quail, Performance, *Pleurotus ostreatus*

INTRODUCTION

Laying quail (*Coturnix coturnix japonica)* is a domesticated bird that has been widely farmed because of high egg production. High egg production is clearly influenced by the quality of the feed. Providing of high quality and continuous feed, especially for poultry, still has problems, namely difficulty in obtaining feed ingredients that do not compete with human needs and expensive feed prices that are still imported. The reason of high feed costs, especially in Indonesia, was related to imported feed ingredients which cause high prices. Expensive feed costs can be overcome by the use of alternative feed, one of them which can be used as alternative feed namely cocoa pods.

Indonesia ranks third in the world among cocoa producing countries with a production of 659.8 thousand tons with a plantation area of 1730 thousand hectares in 2017. West Sumatra is a cocoa producing provinces with a production of 52.2 thousand tons with an area of plantation reaching 158.9 thousand hectares on 2017 (Nuraini et al., 2019a). According to Amrullah (2012) in area of one hectare productive cocoa can produce fresh cocoa pods at a yield of approximately 5 tons/ha/year. Cocoa pods consist of 75% pods, 2% placenta and 23% seeds (Nuraini et al., 2019a); therefore it can be estimated that the cocoa pod production was 38,628 thousand tons in 2017.

Cocoa pods contain 11.71% crude protein, 11.80% fat, 34.95% nitrogen free extract, crude fiber 32.12% (cellulose 22.11% and lignin 23.14%) tannins 0.11% and theobromine 0.19% (Nuraini et al., 2019a). However, the cocoa pods can not be used as animal feed directly, due to several constraints, including the crude fiber content, especially lignin and cellulose, which are sufficiently high and the presence of alkaloid substances, namely theobromine. Theobromine is a harmless alkaloid that can be damaged by heating or drying. According to Nuraini et al. (2019a), the use of cocoa pods in broiler rations is limited, and only can be used up to the level of 5%.

One means of improving the nutritional quality of the cocoa pod is by reducing the content of crude fiber (especially lignin and cellulose) through fermentation with *Pleurotus ostreatus*. *Pleurotus ostreatus* is a white rot fungus that is able to degrade lignin. *Pleurotus ostreatus* produces extracellular ligninase enzymes i.e. manganese peroxidase (Mnp), lignin peroxidase (Lip) and laccase (Sekan et al., 2019). Cellulase (Fernadez-Fueyo et al.,

2016; Yuanzheng and Hyun-Jae, 2017) amylase and protease enzymes (Ergun and Urek, 2017) are also produced by *Pleurotus ostreatus.* Fermentation with *Pleorotus ostreatus* also produce lovastatin. Lovastatin can reduce cholesterol (Ramakrishnan et al., 2017). Another advantage of fermentation with *Pleorotus ostreatus* is the presence of lovastatin compounds that can inhibit the formation of cholesterol. The mechanism of action of lovastatin can inhibit the action of the HMG-CoA reductase enzyme which acts to synthesize mevalonate (mevalonate is needed for cholesterol synthesis) such that cholesterol production is reduced (Haslinger-Loffler, 2008).

Fermentation is influenced by several factors including the composition of the substrate, inoculum dose and length of fermentation (Nuraini et al., 2019b). Our previous research reported 8% inoculum dosage and 9 days of incubation with a substrate composition of 80% cocoa pods and 20% tofu waste mixture fermented with *Pleurotus ostreatus.* The crude protein increased from 13.21% to 21.68%, the crude fiber content decreased from 24.46% to 16.24% (% reduction of crude fiber 33.61%), the lignin content decreased from 19.66% to 15.01% (% lignin reduction 23.66%), the cellulose content decreased from 18.93% to 13.79% (% cellulose reduction 27.16%), nitrogen retention 67.16%, digestibility of crude fiber 53.81%, crude fat content 1.73%, calcium 0.14%, phosphorus 0.9%, metabolic energy 2786.7 kcal/kg, content of glycine 0.98%, methionine 0.42%, and mevastatin 65 mg/kg (Nuraini et al., 2019a).

Increasing crude protein, decreasing crude fiber of cocoa pods by fermentation with *Pleurotus ostreatus* is expected to increase the use of cocoa pods in quail diet. The use of cocoa pods fermented by *Pleurotus ostreatus* on quail laying has not been determined. The purpose of this research was to determine the effect of feeding cocoa pods waste fermented by *Pleurotus ostreatus* on the laying performance and egg quality of quail.

MATERIALS AND METHODS

Ethical approval

The present research was approved by the Animal Ethic Committee of the Universitas Andalas Padang Indonesia (No 456/KEP/FK/2019).

Materials

The material used in this study was cocoa pods fermented with *Pleurotus ostreatus*, corn, bran, 126 concentrate, bone flour, top mix, $CaCO_3$, and fish oil. The quail used in this study were the strain *Coturnix-coturnix japonica* layer phase of 200 birds at 7 weeks of age with 40% egg production. The cages used are battery cages with 20 units, each unit measuring (45x40x40 cm). Feed ingredients, food content (%) and metabolic energy (kcal/kg) of the ingredients composing rations (as feed) presented in table 1.

Table 1. Nutrient content and energy metabolism of feedstuff (as feed) of laying quail

Ingredient	CP (%)	Lipid (%)	Crude Fiber (%)	Ca (%)	P (%)	ME[c] (Kcal)	Methionine (%)	Lysine (%)
Concentrate 126[b]	38.00	4.00	8.00	5.50	1.00	2910.00	1.00	1.76
Corn meal[a]	8.20	2.66	2.90	0.38	0.19	3300.00	-	0.20
Rice Bran[a]	9.50	5.09	14.84	0.69	0.26	1630.00	0.27	0.67
Fish Oil	-	100.00	-	-	-	8600.00	-	-
CPF[a]	19.04	1.52	14.26	0.12	0.08	2447.56	0.42	0.98
Bone Meal[a]	-	-	-	24.00	12.00	-	-	-
Caco3[a]	-	-	-	40.00	-	-	-	-
Top mix	-	-	-	5.38	1.44	-	0.30	0.30

[a]Nuraini et al. (2018);[b] PT. Charoen Pokphan (Consist of fish meal, soybean meal, dicalcium phosphate, NaCl, niacin, trace mineral and antioxidant); [c]Scott et al. (1982); CPF: Cocoa pod fermented with *Pleurotus ostreatus.* Top mix from PT Medion (Consist of 10kg: vitamin A=12.000.000 IU, vitamin D3=2.000.000 IU, vitamin E= 8.000.000 IU, vitamin B1= 2.000mg, vitamin B2= 5.000mg, niacin= 40.000, methionin= 30.000 mg, lysine= 30.000, manganese= 120.000mg, iron= 20.000mg, iodin= 200mg, zinc= 100.000mg, cobalt= 200mg, copper= 4.000mg)

Fermented Cocoa Pods with *Pleurotus ostreatus*

The 500 grams substrate consisted of 80% (400 grams) cocoa pods and 20% (100 gram) tofu waste with 35 ml of mineral solution added. The mineral composition consists of $MgSO_47H_2O$ (2.5 g), $FeSO4_7H_2O$ (1 g), KH_2PO_4 (0.01 g), $ZnSO_44H_2O$ (1 g), $MnSO_44H_2O$ (0.01 g), thiamine hydrochlorine (0.1225 g) and urea (50 g). Then, pods were sterilized in an autoclave (temperature of 121^0C for 15 minutes), and inoculated with *Pleurotus ostreatus* as much as 8% of the substrate dry material. The substrate was stirred until homogeneous and flattened to a thickness of 3 cm and incubated for 9 days (Nuraini et al., 2019a).

Experimental design

This research was conducted in a Completely Randomized Design (CRD) method with 5 treatments (Cocoa pods fermented with *Pleurotus ostreatus*/ CPF) and 4 replications, with the following treatments: 0%, 5%, 10%, 15% and 20% CPF in the diet. There were 10 laying quails per unit of experiment. The nutrient content and metabolizable energy content of the diets are shown in table 1. The composition of the treatment diet and the content of the treatment diet presented in table 2

Table 2. Composition of diet and nutrient content of quail diet

Ingredient (%)	Treatment				
	A	B	C	D	E
Corn Meal	45.50	44.50	43.50	42.50	41.50
Consentrat 126	41.00	39.00	37.00	35.50	33.75
Rice Bran	10.00	8.00	5.75	3.00	0.50
Fish Oil	1.00	1.00	1.00	1.00	1.00
CPF	0.00	5.00	10.00	15.00	20.00
Bone meal	1.00	1.00	1.00	1.00	1.00
Top Mix	0.50	0.50	0.50	0.50	0.50
CaCO3	1.00	1.00	1.25	1.50	1.75
Total	100.00	100.00	100.00	100.00	100.00
Crude Protein (%)	20.26	20.18	20.08	20.12	20.09
Crude Fiber (%)	6.08	6.31	6.50	6.66	6.83
Fat (%)	4.36	4.23	4.08	3.93	3.78
Ca (%)	3.16	3.04	3.02	3.02	3.01
Available P	0.65	0.63	0.60	0.58	0.56
ME (kcal/kg)	2943.60	2942.18	2936.68	2937.58	2935.29
Methionine (%)	0.44	0.43	0.43	0.43	0.42
Lysine (%)	0.93	0.92	0.92	0.92	0.92

Parameter measurements

The effect of using fermented cocoa pods with *Pleorotus ostreatus* on quail laying includes: feed consumption (g/head/day), hen day egg production (%), egg mass (g/head/day), egg weight (g/grain), feed conversion, egg yolk fat (%) and egg yolk cholesterol (mg/100g).

Data analysis

All of the data were statistically analyzed by one-way analysis of variance in the Completely Randomized Design (CRD). Significant differences between treatments were determined using Duncan's multiple range test, with a $p < 0.05$ considered to be significant.

RESULTS

The effects of treatments on the production performance of laying quails are provided in table 3.

Feed consumption

The feed consumption of laying quail was not affected ($p < 0.05$) by the levels of CPF present in the diet. Utilization of CPF until 15% CPF was similar with feed consumption in the control. Increasing utilization of CPF until 20% decreased ($p > 0.05$) feed consumption. Feed consumption in the A treatment (control) was 21.72 g/head/day, and feed consumption decreased in the 20% CPF treatment group by 21.52 g/head/day.

Hen-day egg production

The levels of CPF in the diet affected ($p < 0.05$) the hen-day egg production of laying quails. Increasing CPF levels until 15% was similar with 0% CPF, but increasing CPF until 20% decreased ($p < 0.05$) hen-day egg production. Hen-day egg production in the control group was 61.00% and hen-day egg production in the 15% CPF treatment group was 59.84% and in the 20% CPF treatment group was 58.17%.

Egg mass production

The egg mass production of laying quail was affected ($p < 0.05$) by the levels of CPF in the diet. Increasing fermented product levels until 15% CPF in the diet was similar to that in the 0% CPF/control group on egg mass production. The egg mass production in the control group was 6.33 g/head/day and was still similar to the 15% CPF treatment group (6.16 g/head/day) but decreased to 5.94 g/bird/day in the 20% CPF treatment group.

Feed conversion

The feed conversion ratio of laying quail was affected ($p < 0.05$) by the levels of CPF in the diet. Increasing CPF levels until 15% were still similar to the control group, but the 20% CPF treatment group increased feed conversion. The feed conversion in the control was 3.43, which was similar to the 15% CPF treatment group (3.50) but increased to 3.63 in the 20% CPF treatment group. The effects of utilization CPF in the diet on the egg quality of laying quail are illustrated in table 4.

Table 3. Average production performance of quail aged 7-11 weeks

Treatment	Feed Consumption (g/head/day)	Hen Day Egg Production (%)	Egg Mass (g/head/day)	Feed Conversion
A (0% CPF)	21.72^{a}	61.00^{a}	6.33^{a}	3.43^{b}
B (5% CPF)	21.71^{a}	60.92^{a}	6.31^{a}	3.44^{b}
C (10% CPF)	21.70^{a}	60.83^{a}	6.27^{a}	3.47^{b}
D (15% CPF)	21.58^{ab}	60.03^{a}	6.16^{a}	3.50^{b}
E (20% CPF)	21.52^{b}	59.27^{b}	5.94^{b}	3.63^{a}
SE	0.05	0.53	0.08	0.04

^{a,b}: Superscript difference in the same column was affected significantly ($p < 0.05$)

Table 4. Effect of utilization of cocoa pod fermented in laying quail on the quality of egg

Treatment	Egg Weight (g/egg)	Egg Yolk Cholesterol (mg/ 100g)	Egg Yolk Fat (%)
A (0% CPF)	10.42	877.38[a]	28.59[a]
B (5% CPF)	10.20	843.81[a]	28.47[a]
C (10% CPF)	10.25	789.85[b]	28.27[a]
D (15% CPF)	10.25	744.70[b]	28.21[ab]
E (20% CPF)	10.26	701.00[b]	27.98[b]
SE	0.14	16.45	0.40

[a,b]:Superscript difference in the same column affected significantly ($p < 0.01$)

Egg weight

The egg weight of laying quail was not significantly affected ($p > 0.05$) by utilization of CPF in the diet. The egg weight in the control treatment (10.42 g/egg) was similar to that in the 20% CPF group (10.26 g/egg).

Egg cholesterol

Inclusion of CPF in the diet of quails significantly decreased ($p < 0.05$) the egg cholesterol content in a concentration-dependent manner. Increasing the amount of CPF decreased the egg cholesterol content. The egg cholesterol in the 0% CPF treatment group (877.38 mg/100 g) was decreased compared to that in the 20% CPF treatment group (701.00 mg/100g).

Egg yolk fat

The egg yolk fat of laying quail was affected ($p < 0.05$) by the levels of CPF in the diet. The egg yolk fat in the control was 28.50% and decreased to 27.98% in the 20% CPF treatment group.

DISCUSSION

The effect of the utilization CPF in the diet on laying quail performance is shown in table 3. Feed consumption that were not significantly different between treatment A with treatments B, C, and D showed that the utilization of cocoa pods fermented with *Pleurotus ostreatus* palatable (preferred) by quail, despite a reduction in the utilization of corn by 8.79%, reduction in concentrate 17.68% and bran reduction of 95%. Treatments B, C and D contain CPF and contain little (Corn, concentrate and rice bran) but still have the same palatability as treatment A which does not contain CPF, but contains more corn, concentrate and rice bran.

The similar palatability between treatment A and treatments B, C and D showed that the quality of the diet in treatments B, C, and D did not differ with the quality of treatment A, causing the fermentation process to break down complex food substances into the simple ones to improve feed quality and improve digestibility. According to Nuraini et al. (2019b) fermented products can produce a preferred flavor and have several vitamins such that livestock are preferred compared to the original product.

The decrease in feed consumption in treatment E caused by the high amount of CPF in the diet which caused high crude fiber content treatment E (20% CPF), i.e 6.83%. High crude fiber in the diet will have negative effects on growth and disrupt quail productivity, according to Ridla et al. (2019), high crude fiber causes poultry to feel full quickly such that it can reduce consumption, because the crude fiber is voluminous. Poultry cannot use crude fiber as an energy source; crude fiber is needed in small quantities as bulk, which is to expedite fecal expenditure. According to Nuraini et al. (2019a), the factors that influenced feed consumption in poultry are crude fiber content in feed, feed quality, palatability and taste of feed.

The feed consumption of laying quail (age of 7-11 weeks) in treatment D (15% CPF) in the diet is 21.58 g/head/day. This feed consumption was similar to that observed by Nuraini et al. (2017b), who showed that the feed consumption of quails (age 7-11 weeks) in a range of 21.20-22.03 g/bird/day and higher than the results of a study by Khairani et al. (2016), who found that the feed consumption of quail was 17.64-20.52 g/bird/day when fed 18% crude protein.

Hen-day egg production was not significantly different between treatments A with treatments B, C, and D due to the feed consumption, which was also the same in treatments A with B, C, and D. The same feed consumption showed that the food substances that were utilized to produce eggs were the same such that hen-day egg production was the same. Reduction in the use of corn, bran and concentrates in the B, C and D treatments results in a decrease in the amino acid content of lysine and methionine. However, a mixture of cocoa pod and tofu waste fermented with *Pleurotus ostreatus* is able to cover up the deficiency of the amino acids lysine and methionine. The amino acid contents of lysine and methionine in fermented product with *Pleurotus ostreatus* were 0.98% lysine and 0.42% methionine. According to Nuraini et al. (2017a) reported feed fermented with microorganisms had higher amino acid content than the original feed, as these amino acids are produced by microorganism.

Fermentation of cocoa pod with *Pleurotus ostreatus* can improve nutrition better than the original product, which increases crude protein content, decreases crude

fiber content (cellulose, hemicellulose and lignin), increases digestibility of crude fiber and nitrogen retention and decreases theobromine antinutrient compounds contained in the cocoa pod. *Pleurotus ostreatus* fungi in fermentation can degrade lignin because it produces extracellular ligninolytic enzymes such as laccase, lignin peroxidase and manganese peroxidase (Fernadez-Fueyo, 2016; Nuraini et al., 2017a). In addition cellulase enzymes and amylase enzymes are also produced by *Pleorotus ostreatus* such that the crude fiber in the cocoa pod dcrease (Yuanzheng dan Hyun-Jae, 2017). Nuraini et al. (2019a) found that cocoa pods and tofu waste mixture with *Pleurotus ostreatus* decreased crude fiber by 45.34%, decreased lignin by 32.34%, cellulose by 35.12%, cellulose enzyme activity by 3.32 U/ml, nitrogen retention by 67.16% and crude fiber digestibility by 53.81%.

The decrease in hen-day egg production and egg mass in treatment E was caused by feed consumption, which also decreased in treatment E. Low feed consumption showed that less food was digested and absorbed for production. Indreswari (2016) states that egg production is determined by feed consumption. High crude fiber content in treatment E also results in decreased hen-day egg production. Next the level of crude fiber becomes so overly high, and the digestion of nutrients and, the value of productive energy decreases, thereby slowing growth and interfering with productivity. Effects of crude fiber that cannot be digested can carry digested food substances out through feces; thus poultry livestock production and growth may not be optimal. Hen-day egg production of quails aged 7-11 weeks at treatment D (15% CPF) in the diet was 59.83%. This research was lower than that of Nuraini et al. (2017b), who reported hen day egg production in laying quails for the 7-12 weeks layer period of 70.45% and also lower than the research finding by Indraswari (2019) who reported HDEP of quail aged 10- 14 weeks at 68.70%.

In terms of feed conversion, feed conversion that was not significantly different between treatment A with treatment B, C and D was due to the same feed consumption and egg mass in treatments A with, B, C and D. According Khairani et al. (2016), the feed conversion ratio is the ratio between feed intakes in producing a number of eggs. Feed conversion can indicate the production coefficient; a smaller value indicates more efficient use of feed to produce eggs. According to Khairani et al. (2016), who observed that feed conversion is influenced by feed consumption and egg mass such that if there is an increase between them, the feed conversion values will remain balanced. The feed conversion of laying quail rations at the age of 7-11 weeks in treatment D (15% CPF) was determined to be in the ratio is 3.67.

The effect of utilization of CPF in laying quail on the quality off egg presented in table 4. The effect of utilization of CPF in the diet on egg weight did not differ notably, ranging from 10.20-10.31 g/egg. According to Nuraini et al. (2017b) laying quails aged 7-15 weeks have an egg weight ranging from 10-12 g /egg. This result is similar to the results obtained by Al-Daraji et al. (20) who reported that the average egg weight of quail (*Coturnix-coturnic japonica*) ranged from 9.40-11.13 g/egg when fed 6% linseed in the diet.

Increasing CPF in quail rations can reduce egg yolk cholesterol. Utilization of CPF up to the level of 20% decreased by 20.30% compared to treatment A without the addition of CPF. Cocoa pods fermented with *Pleurotus ostreatus* contain lovastatin compounds. Ramakrishnan et al. (2017) stated that lovastatin compounds can inhibit the formation of cholesterol. The mechanism of action of lovastatin can inhibit the action of the HMG-CoA reductase enzyme, which acts to synthesize mevalonate where mevalonate is needed for cholesterol synthesis, thus cholesterol production is reduced. Cholesterol levels obtained in this study are lower than the finding of the Ukachukwu et al. (2017) that quail egg yolk has cholesterol level higher than chicken egg yolk. In 1 g of the quail egg yolk have the total cholesterol, HDL and LDL concentrations (6.79; 3.95; and 1.80 respectively) were significantly higher compared to their concentrations (4.03; 1.84 and 0.40 respectively) in 1 g of chicken egg yolk.

The utilization of CPF in the diet can reduce the fat content found in quail egg yolks. The low content of egg yolk fat in treatment E compared to treatments A, B, C and D is related to the use of fermented cocoa pod which is mostly used in treatment E, which is 20% (more CPF in the diet is associated with lower egg yolk fat in the quail content), because of the fermented product content of lovastatin. In this research egg yolk obtained using a mixture of cocoa pods and tofu waste, fermented with *Pleurotus ostreatus* to the level of 20% in the quail diet was 27.98%. Egg yolk fat can be affected by crude fiber in the diet. The crude fiber content increased from treatment A (0% CPF) to treatment E (20% CPF) in the quail diet, but this increase was still within the specified limits.

CONCLUSION

It can be concluded that the utilization of a mixture of cocoa pods and tofu waste which is fermented by

Pleurotus ostreatus at the level of 15% in diet of laying quail can effect on feed consumption, egg production, egg mass and feed conversion, egg yolk cholesterol and egg yolk fat.

DECLARATIONS

Author`s contribution

Nuraini contributed on created the idea, designed the experiment (fermentation and utilization CPF to quail), analyzed data and wrote this article. Yuliaty Shafan Nur contribution on preparing fermented products and checked the written article. Ade Djulardi contributed on utilization CPF to quail and assisted in revision of article. All authors confirmed the final revised form of article for publishing in this journal.

Competing interests

The authors declared that they have no competing interests.

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REFERENCES

Ergun SO and Urek RO (2017). Production of ligninolytic enzymes by solid state fermentation using Pleurotus ostreatus. Annals of Agrarian Sciences, 15(2): 273-277. DOI: https://doi.org/10.1016/j.aasci.2017.04.003

Fernandez-Fueyo E, Ruiz-Duerias FJ, Lopez Lucendo and Perez-Buanda M (2016). A secretomic view of woody and non woody lignocellulose degradatin by Pleurotuas osteratus. Biotechnol Biofuels, 9:49. DOI: https://doi.org/10.1186/s13068-016-0462-9

Haslinger-Loffler B (2008). Multiple effects of HMG-CoA reductase inhibitors (statin) besides their lipid lowering function. Kidney International, 74: 553-555. DOI: http://dx.doi.org/10.18502/kls.v4i11.3874

Indreswari R, Ratriyanto A and Nugroho T (2016). Performance of japanese quail (Coturnix coturnix japonica Temminck and Schlegel 1849) feed hatchery waste meal. The UGM annual scientifific conference life sciences, pp. 281-287. DOI: http://dx.doi.org/10.18502/kls.v4i11.3874.

Khairani, Sumiati and Wiryawan KG (2016). Egg production and quality of quails fed diets with varying levels of methionine and choline chloride. Media peternakan, 39(1): 34-39. DOI: http://10.5398/medpet.2016.39.1.34.

Nuraini, Djulardi A and Trisna A (2017a). Palm oil sludge fermented by using lignocellulolytic fungi as poultry diet. Internatonal Journal of Poultry Science, 16 (1): 6-10. DOI: http://dx.doi.org/10.3923/ijps.2017.6.10.

Nuraini, Djulardi A and Trisna A (2019b). Palm kernel cake fermented with *Lentinus edodes* in the diet of quail. International journal of poultry science, 18(8):387-392. DOI: https://doi.org/10.3923/ijps.2019.387.392.

Nuraini, Mirzah and Djulardi A (2017b). Marigold flower extract as a feed additive in the poultry diet: Effects on laying quail performance and egg quality. International Journal of Poultry Science, 16 (1): 11-15. DOI: https://scialert.net/fulltext/?doi=ijps.2017.11.15.

Nuraini, Yuliaty SN and Djulardi A (2019a). Cocoa pods with different nitrogen sources fermented by using *Pleurotus ostreatus* as poultry feed. International Journal of Poultry Science, 18 (7): 328-333. DOI: https://scialert.net/fulltext/?doi=ijps.2019.328.333.

Ramakrishnan M, Dubey C, Tulasi V and Kislai P (2017). Investigation of lovastatin,the anti-hypercholesterolemia drug molecule from three oyster mushroom species. International Journal of Biomedical and Clinical Sciences, 2(4): 26-31. DOI: http://creativecommons.org/licenses/by/4.0.

Ridla M, Imran A, Jayanegara A, Hermana W and Tarman A (2019). Influence of hemicell addition on diets containing different level of crude fiber on performance of laying hens. Bulletin of Animal Science, 43(3): 179-183. DOI: http://10,21059/buletinpeternak.v43i3.44618.

Scott ML, Nesheim MC and Young RJ (1982). Nutrition of The Chicken Fourth Ed. Published by M. L. Scott and Associates, Ithaca, New York. Available at: https://www.amazon.com/Scotts-Nutrition-Chicken-v-4/dp/0969560044

Sekan AS, Myronycheva OS, Karlsson O, Gryganskyi AP and Blume Y (2019). Green potential of *Pleurotus* spp. in biotechnology. Peer J, DOI: https://doi.org/10.7717/peerj.6664. eCollection 2019.

Ukachukwu UG, Ozougwu VE and Nwankwo NE (2017). A comparative study on the total cholesterol, triacylglycerides and lipid concentrations of quail and chicken eggs. International Journal of Research in Pharmacy and Biosciences, 4 (10): 11-16.

Wu Y and Hyun-Jae S (2017). Cellulase from the fruiting bodies and mycelia of edible mushrooms: A review. Journal of Mushroomvol, 14(4): 127-135. DOI: https://doi.org/10.14480/JM.2016.14.4.127

Yuanzheng W and Hyun-Jae S (2017). Cellulase from the fruiting bodies and mycelia of edible mushrooms: A review. Journal of Mushroomvol 17(3). Doi: https://doi.org/10.14480/JM.2016.14.4.127.

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Determination of the Appropriate Ratio of Rice Bran to Cassava Leaf Meal Mixture as an Inoculum of *Rhizopus Oligosporus* in Broiler Chicken Ration

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ABSTRACT

Rice bran (RB) was used as a medium for the fermentation process because it contained complete nutrients necessary for the growth of microbes. The ability to induce a fermentation substrate by microbes dependent on the availability of an inducer in the medium. The enzyme activity would increase when a suitable inducer was in the medium. In order to increase protease and cellulase activities of *Rhizopus oligosporus* in processing Cassava Leaf Meal (CLM), it was necessary to add CLM in medium producing an inoculum *Rhizopus oligosporus*. This study was conducted in a completely randomized design with 4 treatments and 5 replications. The treatments were ratios of RB to CLM as follows: 100:0% (A), 90:10% (B), 80:20% (C), and 70:30% (D). The observed variables were protease activity, cellulase activity, dry matter content, organic matter content and the total colony of *Rhizopus oligosporus* numerically. The different ratios of RB to CLM significantly affected protease and cellulase activities as well as the content of dry matter and organic matter. The addition of 10% CLM to the RB medium increased protease and cellulase activities and reduced dry matter and organic matter contents. However, when more than 10% CLM was added, it reduced the protease and cellulase activities and increased dry matter and organic matter content. The highest total colony of *Rhizopus oligosporus* was numerically in treatment B (90:10% of RB to CLM ratio). The appropriate ratio of RB to CLM mixture as the inoculum of *Rhizopus oligosporus*, based on their enzyme activities, dry matter, and organic matter contents, and the total colony was 90:10% (treatment B).

Key words: Cassava leaf meal, Enzyme activity, Inoculum, *Rhizopus oligosporus,* Rice bran.

INTRODUCTION

Cassava leaf flour (CLM) has the potential to be used as an alternative feed ingredient. Judging from its availability, CLM is quite widely available. Cassava leaf flour is obtained as waste material when harvesting cassava roots from cassava plants. Cassava production in West Sumatra reaches 201,833 tons, in Indonesia 19,341,233 tons (Statistics of Indonesia, 2018).This cassava plant was cultivated on an area of 792,952 Hectare (Ha) or 0.8 million Ha (Statistics of Indonesia, 2018). The production of fresh cassava leaves per Ha was between 7 to 15 tons by Sudaryanto et al., 1982 in Yuniza et al., (2016). From the area of cassava plantation and cassava leaf production per hectare, the availability of fresh cassava leaves as animal feed was plentiful, ranging from 5.6 to 12 million tons or about 1 to 2 million tons under dry conditions per year. If only 1 million tons of dried cassava leaves was used as chicken feed, this amount could be estimated to feed up to 274 million laying hens per year when the cassava leaves were included 8% in the ration.

The potential of CLM as an alternative feed ingredient in terms of nutritional content. The nutritional content of CLM is as follows: dry matter (DM) 25.30%, crude protein (CP) 25.1%, crude fiber (CF) 11.4%, crude fat 12.7%, nitrogen retention 46 , 1% and 9.1% ash (Iheukwumere et al., 2008). On the other hand, CLM has a limiting factor as alternative feed ingredients. The use of CLM in broiler chicken rations can only be used up to 5% because utilization of up to 10 and 15% can reduce the efficiency of feed use (Wyllie and Chamanga, 1979;

Melesse et al. 2018). The use of CLM in broiler chicken rations is still limited due to high CF, tannins, HCN, alkalis, low digestibility (Ravindran et al., 1986) and deficiency of amino acids containing sulfur, especially methionine (Eggum, 1970). Therefore, the use of CLM was still limited. In order to further increase the amount of CLM in poultry rations, the physical, chemical and biological fermentation must be carried out.

The biological fermentation by using microbes is a process of the activity of microorganisms or microbes that can produce the product whose texture, flavor, smell and nutrient quality change better than that of the raw material (Mirnawati et al., 2019a; Mirnawati et al., 2019b; Dewi et al., 2019; Mirnawati et al., 2018; Mirrnawati et al., 2017; Adrizal et al., 2017; Mirnawati et al., 2013; Rizal et al., 2013; Rizal et al., 2012; Mirnawati et al., 2012; Aisjah and Abun, 2012; and Mirnawati et al., 2010). According to Sugiharto (2019), solid-fermentation using fungi could be a simple method to improve the nutrient qualities of cassava pulp and thus increased the production of such a cheap agro-industrial by-product in chicken rations. According to Han et al. (2003), *Rhizopus oligosporus* produced protease, lipase, alpha-amylase, glutaminase, and alpha-galactosidase and (Dewi, 2015) found that *Rhizopus oligosporus* also produces cellulase. The presence of these enzymes were expected to reduce the anti-nutrients and toxins contained in CLM and also increase their nutritional values (Sumiati et al., 2011), thus increasing the use of CLM in broiler chicken rations.

To increase the productivity of *Rhizopus oligosporus* in producing hydrolytic enzymes (protease and cellulase) and to increase the enzyme activities, it was necessary to produce *Rhizopus oligosporus* starter or inoculum from Rice Bran (RB) and CLM mixture as an inducer. Therefore, the ability of hydrolytic enzymes for the degradation of CP and CF of CLM supposed to increase. The addition of CLM to the inoculum as an inducer should accelerate the adaptation phase and increase the productivity of *Rhizopus oligosporus* hydrolytic enzymes. *Rhizopus oligosporus* would be able to synthesize the necessary enzymes required for metabolism according to the availability of inducer in the starter (Kurnia, 2010; Purkan et al., 2016). The addition of CLM as a substrate in the production of *Rhizopus oligosporus* inoculums could accelerate the adaptation phase (Soeprijanto et al., 2008 and Zakaria et al., 2013).

Therefore, the purpose of this study was to investigate the appropriate ratio of RB to CLM mixture that will be utilized for producing inoculum of *Rhizopus oligosporus*.

MATERIALS AND METHODS

Experimental design

This experiment was conducted in a complete randomized design with 4 treatments and 5 replications. The treatments were the combination ratio of RB to CLM including 100:0% (A), 90:10% (B), 80:20% (C), and 70:30% (D). These combinations of RB and CLM mixtures were fermented by using *Rhizopus oligosporus* at the dose of one test tube for 100 gram (g) dry matter and 2-day fermentation length.

Measured variables

These measured variables wereprotease and cellulase activities as well as dry matter and organic matter contents, and the numericalcalculation ofthe total colony of *Rhizopus oligosporus* in each treatment.

Data analysis

All the data were analyzed by analysis of variance of complete randomized design. The differences among treatment means were detected by Duncan's Multiple Range Test or (DMRT) according to Steel and Torrie (1980).

Preparation of Cassava Leaf Meal sample

After harvesting the tuber roots, cassava leaves were taken from two-third of the entire leaves from the an teriorside. Cassava leaves were dried under the sun and then milled in a two mm screen.

Propagation of fungi

Eight g of Potato Dextrose Agar or (PDA) was weighed and placed into the glass cup and added with 200 milliliter (ml) of distilled water and heated on the hotplate with constant stirring until it was limpid. This PDA solution was poured into 30 test tubes (each test tube contained 5 ml solution) and covered with cotton and aluminum foil, then sterilized by using an autoclave at 2 atmosphere (atm.) pressure and 121^0C for 15 minutes. Other inoculation pieces of equipment (one needle, one mask, one glove) were also sterilized. They were chilled in laminar flow while UV light was turned on a tilted position until solid. *Rhizopus oligosporus* was inoculated by using sterile needle against tilted PDA medium in a sterile environment. Then, the inoculated PDA medium was sealed in test tubes with cotton and aluminum foil and incubated for 36 hours in an incubation container in flowing air under facultative anaerobic condition.

The starters were weighted according to the treatments [ratios of RB to CLM were 100:0% (A), 90:10% (B), 80:20% (C) and 70:30% (D)]. To each starter was added 65 ml of water (until the moisture content was 45%) and homogenized, and then these starters and minerals were prepared according to Brook et al. (1969). The composition of nutrients included Urea 7.5 g, MgSO4 7 H2O 0.375 g, FeSO4 7 H2O 0.15 mg, ZnSO4 7 H2O 0.15 mg, MnSO4 4 H2O 0.15 mg, KH2PO 1.5 g, and thiamine hydrochloride 18.75 mg, which had dissolved in 100 ml equates. The mixture was used, 5 ml in 100 g subtract were sterilized using autoclave at 2 atm. pressure, temperature 121^0c for 15 minutes, and chilled in laminar flow. *Rhizopus oligosporus* in the PDA medium was inoculated in laminar flow into each starter and then was incubated in facultative anaerobe over a period of 36 hours.

Determination of Enzyme Activity

Crude enzyme extract

10 g of inoculum sample was weighed and then immersed in 90 ml of 0.05 M phosphate buffer at pH 7 in 250 ml of Erlenmeyer and inserted into the incubator shaker for 2 hours at 27^0C at 100 round per minute (rpm). Afterwards, the solution was filtered and the filtrate was taken out, and then was centrifuged at a speed of 5000 rpm for 15 minutes, thus the supernatant was taken out as a crude enzyme extract for the purposes of analyzing enzyme activity by the following methods.

Protease activity

One percent casein solution was pipette up to 2.5 ml and phosphate buffer (0.1 M pH 7) was pipetted up to 1.5 ml and then placed into a test tube and then mixed by using a vortex, then was incubated in a water bath at 37^0C for 10 minutes. One ml of crude enzyme extract was added and then incubated for 10 minutes in a water bath at 50 ^{0}C. To obtain the blank, enzyme activity was stopped by adding 5 ml of 20% TCA solution, then mixed by using a vortex and then refrigerated for 30 minutes to agglomerate the protein. On the other hand, the sample was centrifuged at 5000 rpm for 15 minutes at 4^0C, then filtered and the supernatant was taken. Furthermore, two ml of supernatant was pipetted into the test tube and 0.5 ml of NaOH up to 5 ml and folinciocalteu reagent was added to 0.5 ml and then allowed to stand for 10 minutes. Furthermore, the absorbance was measured using a spectrophotometer at a wavelength of 650 nm. According to Henriette (1993**),** the calculation is done with the following equation;

Activity of protease enzyme (U/ml) = (Y x a)/b x 1/t

Y = Absorbance of sample

a= the a value of the regression curve Y: a + bx

b= value b of the regression curve Y: a + bx

t= incubation time

Casein regression curve = y = 0.031x + 1.136R^2 = 0.986

Cellulase activity

One ml crude enzyme extract was pipetted and added one ml (0.5 ml CMC + 10 ml phosphate buffer) and then poured into the microtube, and incubated for 30 minutes at 40^0C in a water bath shaker. Then one ml of this mixture solution was taken out and poured into the test tube, and then one ml of Nelson AB (25 ml Nelson A and one ml Nelson B)was added. Nelson A was consisted of 2.5 g of Na2CO3, 2 g of NaHCO3, 2 g of NaSO4, 2.5 g of KNaCaH4O6. 4H2O and 100 ml of distilled water. Nelson B was consisted of 75 g of COSO4 and one drop of H2SO4. Thus, the mixture was heated in boiling water for 20 minutes, and after the solution got cold, one ml of phosphor-molybdate and seven ml of distilled water were added. The absorbance was measured by using a spectrophotometer Simadzu 1800 from Kyoto, Japan at a wavelength of 575 nm (Nelson, 1994). The calculation is done with the following equation;

Cellulase activity (U / ml) = (X × P × 1000) / (T× M)

X = the result of standard curve conversion

P = Dilution

T = Time

BM = Molecular weight of glucose

Glucose standard curve =y = 0.045x - 0.010 R^2 = 0.994

Calculation of Total Colonies

The total colonies were calculated by the Pour Plate Method (Putri and Kurnia, 2018). The counting of the colonies had tube sterile from the beginning to the end. All equipments were moistened with 70% alcohol. In addition, the process was carried out near a flame to reduce or prevent microbial contaminants to the equipment and media. The calculation of whole colony was startedwith the productionof physiological solutions. In the first step, oneg of sample was mixed with nine ml of distilled water, then it was homogenized. One ml of this solution was taken with a micropipette and added to nine ml of distilled water to obtain a 1:10 dilution. The solution of 1:10 homogenized dilutions was taken as much as one ml and then poured into a test tube with nine ml of distilled water to made a 2:10 dilution, and the same procedure was used

to prepare the solution until 10:10 dilutions. In the next process, media plate count agar was made using Potato Dextrose Agar (PDA) solution. The PDA solution was sterilized together with Petri dishes and other equipment by using an autoclave at a pressure of 2 atm., 121 ^{0}C for 15 minutes. Then, 0.1 ml of the physiological solution from the dilution tube (10-6) was pipetted into a sterile Petri dish, after which the PDA solution was poured under warm conditions. It was also placed on a flat table and gently shaken like the number eight. This procedure was also repeated for physiological solutions resulting from dilutions of 10-8 and 10-10. All planted Petri dishes were labeled and sealed with stretch film. All Petri dishes were incubated for 24 hours in an incubator jar. After the incubation, the fungi colonies were observed and counted using a colony counter. Total colonies were calculated according to Standard Plate Count (SPC). Several colonies that had been assembled from a large collection of colonies counted as one colony, and a row or chain of colonies considered as a combined row were also counted as a colony. The amount per gram colony was determined using the equation according to Sukmawati and Hardianti (2018).

$$N = n \times \frac{1}{fP}$$

N = total colony per gram of sample (CFU/g);

n = Number of colonies counted in the colony counter

*f*P =Dilution factor of the sample (initial dilution × amount of colonies grown)

RESULT

The effect of RB and CLM ratios in the production of *Rhizopus oligosporus* inoculum on their protease and cellulase activities, total colony, and dry matter and organic matter contents were shown in table 1. The effect of ratios and RB to CLM for producing inoculum of *Rhizopus oligosporus* on protease activity was statistically significant ($p < 0.05$). The highest protease activity was found in treatment B (9.84 U/ml), in which it was not different from treatment C (9.90 U/ml), and treatment D (9.79 U/ml) but it was different from treatment A (9.13 U/ml)

The effect of the ratio of RB and CLM to produce the inoculum *Rhizopus oligosporus* on cellulase activity was statistically significant ($p < 0.05$) (table 1). The highest activity of cellulase was at treatment B (1.50 U/ml). It was not different compared to treatment C (1.51 U/ml), D (1.52 U/ml), but it was different from treatment A (1.38 U/ml). The ratio of RB to CLM for assembling the inoculum of *Rhizopus oligosporus* significantly affected the dry matter content ($p < 0.05$). The lowest dry matter content was found in treatment B (91.63%). It was different from treatments A (92.67%), C (92.59%) and D (92.94%). Meanwhile, treatments A, C, and D were not different. The effect of the ratio of RB and CLM to produce an inoculum of *Rhizopus oligosporus* on the content organic matter was statistically significant ($p < 0.05$). The lowest organic matter content was found in treatment B (76.48%), but it was not different compared to treatment C (77.66%). Whereas, this treatment was different from treatments A (78.55) and D (79.00%).

Table 1. The effects of the ratios of Rice bran and Cassava leaf meal for producing *Rhizopus oligosporus* starter, on their protease and cellulase activities, and the content of dry matter, organic matter, and the numerical form of total colony, in the Non-Ruminant Nutrition Laboratory, at the Faculty of Animal Science, Andalas University, Padang, Indonesia.

Ratios of	**Measured Variables**				
RB:CLM	**Protease activity (U/ml)**	**Cellulase activity (U/ml)**	**Dry matter (%)**	**Organic matter (%)**	**Total colony (CFU/g)**
A (100:0)	9.13^b	1.38^b	92.67^a	78.55^a	0.77x10^{10}
B (90:10)	9.84^a	1.50^a	91.63^b	76.48^b	3.00x10^{10}
C (80:20)	9.80^a	1.51^a	92.59^a	77.66^b	2.08 x10^{10}
D (70:30)	9.79^a	1.52^a	92.94^a	79.00^a	1.03x10^{10}
Standar error	0.02	0.01	0.17	0.30	

$^{a, b}$ = Different superscripts at the same columns indicates significantly different effects ($p < 0.05$). U/ml = Unit/ milliliters (%): percentage. (CFU/g) = colony forming unit/ gram. RB:CLM = Rice bran: Cassava leaf meal.

DISCUSSION

The activity of protease

The highest protease activity was founded by the addition of cassava leaves as a source of protein up to 10%. Although increasing the addition of cassava leaves up to 20% and 30% did not significantly alter the activity of the protease, it decreased numerically since the addition of 10% inducer reached optimal. Thus, if the addition of the inducer was increased, it did not increase the protease activity, but reduced it. The protease activity increased to an optimal level according to the inducer contained in the substrate in the starter. According to Kurnia (2010), the protease activity increased to optimal level depending on the inducer contained in the substrate in the starter.

The mechanism of increasing protease activity in accordance with the available inducer to the optimal point was that cassava leaves would bind to the repressor protein as an available inducer so that the repressor protein underwent allosteric changes that could change its shape and cause the repressor to cease the ability to bind to the operator. As a result, the RNA polymerase could copy the genes required for cassava leaf degradation so that the *Rhizopus oligosporus* could synthesize the enzymes necessary to degrade the available inducers. In this case, cassava leaves were used as a source of protein because the abundant crude protein content was 21.59%.

The table 1 indicated that the increase in the addition of cassava leaves (inducer) was directly proportional to the high production of the enzyme protease. This stage lasted until the optimal inducer concentration, which was the addition of 10% of cassava leaves with an enzyme activity of 9.84 (U/ml). The addition of 20% and 30% of cassava leaves did not significantly increase the protease activity, but instead decreased it numerically since the addition of cassava leaves as an inducer had exceeded the optimal concentration. This excessive inducer concentration caused saturation of the protease enzyme productivity, thus inhibited the formation of enzyme complexes on the substrate (Purkan et al., 2016).

The cellulase activity

Cellulase activity increased to the optimum at the time of addition of 10% cassava leaves as an inducer, then there was no significant increase on addition of 20% and 30% of cassava leaves. This happened because at the point of addition of 10% of cassava leaves had reached the optimal point of adding inducers, increasing the addition of inducers (cassava leaves) did not increase enzymatic productivity. According to Kurnia (2010), the activity of cellulase enzymes increased to the optimum point according to the inducers available in the substrate in the starter. The mechanism of increasing cellulase activity by adding an inducer of cassava leaves to the optimal point was that cassava leaves as an inducer will bind the repressor protein so that the repressor protein undergoes allosteric changes which can change its shape and cause the repressor can no longer bind to the operator. As a result, the RNA polymerase could copy the genes needed for cassava leaf degradation so that the *Rhizopus oligosporus* could synthesize the enzymes needed for metabolism. In this case, cassava leaves are used as fiber inducers because of the high CF content of 14.59%.

In the table, it can be seen that the increase in the concentration of the inducer is directly proportional to the high activity of cellulase. This lasted until the optimum point of adding 10% inducer (cassava leaves) with enzyme activity (1.50 U / mL), then at an increase of 20% and 30%, there was no significant increase in cellulase activity. This happens because, the addition of an inducer of more than 10% causes saturation in the productivity of cellulase enzymes, because the concentration of the inducer is too large to inhibit the formation of enzyme complexes on the substrate, so that enzyme production does not run optimally (Purkan et al., 2016).

Dry matter content

The low dry matter content at treatment B was due to the high protease activity in this treatment, in which it hydrolyzed the protein of the substrate. Thus, the dry matter content of the substrate was reduced. The decrease in dry matter after fermentation was an indicator of the success of fermentation, as the retaining of dry matter in the fermentation process was affected by the use of nutrients from the substrate (dry matter) by microbes as a source of carbon, nitrogen and minerals, and the release of CO2 and energy in the form of heat that evaporated with water particles. According to Astuti et al. (2017), the fermentation process could result in a reduction in the amount of dry matter. The water molecule was formed through a catabolic process that remodeled complex compounds into simpler materials

Organic matter content

The high and low content of organic matter in the treatment was caused by microbial activity in the fermentation process, which caused the breakdown of the substrate content, whereby microorganisms could easily

digest organic matter. The fermentation of organic substances in the form of glucose, alcohol, and amino acids led to changes that affecting the nutritional value. According to Astuti et al. (2017), the fermentation process carried out by microorganisms, so that the carbohydrates were converted into alcohol, organic acids, water, and CO_2. The use of tofu waste in addition to nitrogen sources was also a source of carbohydrates for microbes used in the fermentation, which causes an increase in water content and leaded to the loss of organic matter.

Total colony

The total colonies of *Rhizopus oligosporus* in treatment A ($0.77x10^{10}$ CFU / g), B ($3.00x10^{10}$ CFU / g), C ($2.08x10^{10}$ CFU / g), and D ($1.03x10^{10}$ CFU / g) differ numerically. The highest total of *Rhizopus oligosporu*s colonies in treatment B was due to the availability of inducers needed to synthesize enzymes by *Rhizopus oligosporus*, so that the entire colony became stable and continued to grow (Kurnia, 2010). Conversely, too much inducer concentration inhibited the formation of substrate enzyme complexes, so that enzyme production was not optimal. It also disrupted the stability and growth of the colony (Purkan et al., 2016).

CONCLUSION

The best ratio of rice bran and cassava leaf meal for producing the inoculum of *Rhizopus oligosporus,* based on the enzyme activities, the dry matter and the content of organic matter, and the whole colony was 90% rice bran and 10% cassava leaves (B treatment).

DECLARATIONS

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Author's contribution

Annisa, Yose Rizal, Mirnawati, Irfan Suliansyah and Amri Bakhtiar participated in design, experimental procedure, writing, revised, and reviewing the final edition of manuscript.

Competing interests

The authors have declared that no competing interest exists.

Consent to publish

The authors guarantee that this work has not been published elsewhere and any person named as a coauthor of this study is aware of the facts and has agreed to be named.

REFERENCES

Adrizal, Heryandi Y, Amizar R and Mahata ME (2017). Evaluation of pineapple [Ananas comosus (L.) Merr] waste fermented using different local microorganism solutions as poultry feed. Pakistan Journal Nutrition, 16: 84-89. DOI: http://10.3923/pjn.2017.84.89

Aisjah T (2012). Bioprocess of winged bean seeds (psophocarpus tetragonolobus (l) dc) by *Rhizopus oligosporus* to improve of pure protein content and decreased of cyanide. Journal of Animal Science (Jurnal Ilmu Ternak) Universitas Padjadjaran12(1): 35-40. DOI: https://doi.org/10.24198/jit.v12i1.5135

Astuti T, Rofiq MN and Nurhaita N (2017). Evaluation of the content of dry matter, organic matter and crude protein of palm fronds fermentation with the addition of carbohydrate sources. Journal of Animal Husbandry (Journal Peternakan), 14 (2): 42–47. DOI: https://org/10.24014/jupet.v14i2.4247

Brook EJ, Stantion WR and Bridge AW (1969). Fermentation methods for protein enrichment of cassava. *Biotechnology and Bioengineering* 11: 1271-1284. DOI: https://doi.org/10.1002/bit.260110620

Dewi S (2015). Isolation and identification of cellulolytic thermophilic fungi and examination of its cellulase enzyme activity. Thesis. Faculty of Agricultural Technology. Gadjah Mada University. Yogyakarta. Google Scholar, Available at: http://etd.repository.ugm.ac.id/index.php?mod=penelitian_detail&sub=PenelitianDetail&act=view&typ=html&buku_id=87475&obyek_id=4

Dewi YL, Yuniza A, Nuraini, Sayuti K and Mahata ME (2019). Fermentation of Sargassum binderi Seaweed for Lowering Alginate Content of Feed in Laying Hens. Journal of World's Poultry Research. 9(3): 147-153 DOI: https://dx.doi.org/10.36380/jwpr.2019.18

Han BZ, Ma Y, Rombouts FM and Nout MR (2003). Effects of temperature and relative humidity on growth and enzyme production by action mucorelegans and *Rhizopus oligosporus* during sufupehtze preparation. Food Chemistry.. 81 (1): 27-34. DOI: https://doi.org/10.1016/S0308-8146(02)00347-3

Henriette C, Zinebi S, Aumaitre MF, Petitdemange E and Petitdemange H (1993). Protease and lipase production by a strain of Setaria marcessen. Journal of Industrial Microbiology, 12(2): 129-135. DOI: https://doi.org/10.1007/BF01569913

Iheukwumere FC, Ndubuisi EC and Mazi EA (2008). Performance, Nutrient utilization and organ characteristics of broilers fed cassava leaf meal (Manihot esculenta Crantz). Pakistan Journal of Nutrition, 7 (13): 13-16. DOI: https://10.3923/pjn.2008.13.16

Kurnia DR (2010). Study of lipase enzyme activity from *Aspergillus niger* as a biocatalyst in the glycerolysis process to produce monoacylglycerol (Studi aktivitas enzim lipase dari *Aspergillus niger* sebagai biokatalis dalam proses gliserolisis untuk menghasilkan monoasilgliserol). Doctoral dissertation, Universitas Diponegoro, Semarang. Available at: http://eprints.undip.ac.id/36573/

Melesse A, Masebo M and Abebe A (2018). The substitution effect of noug seed (*Guizotia abyssinica*) cake with cassava leaf (*Manihot*

Escutulata Crantz.) meal on feed intake, growth performance, and carcass traits in broiler chickens. Journal of Animal Husbandry and Dairy Science, 2(2): 1-9. Available at: https://www.sryahwapublications.com/journal-of-animal-husbandry-and-dairy-science/pdf/v2-i2/1.pdf

Mirnawati, Ciptaan G and Ferawati (2017). The effect of Mananolytic fungi and humic acid dosage to improve the nutrient content and quality of fermented palm kernel cake. International journal of Chemistry Technology Research, 10(2): 56-61. Available at: http://www.sphinxsai.com/2017/ch_vol10_no2/1/(56-61)V10N2CT.pdf

Mirnawati, Ciptaan G and Ferawati (2019a). Improving the quality and nutrient content of palm kernel cake through fermentation with *Bacillus subtilis*. International Journal of Animal and Veterinary Sciences, 31(7). Available at: http://www.lrrd.org/lrrd31/7/mirna31098.html

Mirnawati, Ciptaan G and Ferawati (2019b). The Effect of *Bacillus subtilis* Inoculum Doses and Fermentation Time on Enzyme Activity of Fermented Palm Kernel Cake. Journal of World's Poultry Research 9(4): 211-216. DOI: https://dx.doi.org/10.36380/jwpr.2019.26

Mirnawati, Djulardi A and Ciptaan G (2018). Utilization of fermented palm kernel cake with *Sclerotium rolfsii* in broiler ration. International Journal of Poultry Science. 17(7): 342-347. DOI: http://10.3923/ijps.2018.342.347

Mirnawati, Djulardi A and Marlida Y (2013). Improving the quality of palm kernel cake fermented by *Eupenicillium javanicum* as poultry ration. Pakistan Journal of Nutrition, 12 (12): 1085-1088. DOI: http://10.3923/pjn.2013.1085.1088.

Mirnawati, Kompiang IP and Latif SA (2012). Effect of substrate composition and inoculums dosage to improve quality of palm kernel cake fermented by *Aspergillus niger*. Pakistan Journal of Nutrition, 11(5): 434-438. DOI: http://10.3923/pjn. 2012. 434.438.

Mirnawati RY, Marlida Y and Kompiang IP (2010). The role of humic acid in palm kernel cake fermented by *Aspergillus niger* for poultry ration. Pakistan Journal of Nutrition, 9 (2): 182-185. DOI: http://10.3923/pjn.2010.182.185

Nelson N (1994). A. Photometric Adaption of Somogyi Method for Determination of Glucose. The Journal of Biological Chemistry, ., 153-375. Available at: http://www.jbc.org

Pooja NS and Padmaja G (2014). Pretreatment techniques to enhance the enzymatic degradability of agricultural and processing residues of cassava. Journal of Microbiology and Biotechnology Research, 4(1): 57-67. Available at: http://scholarsresearchlibrary.com/JM

Priadi DO, Permana DR, Dona SE, Hartati S and Sudarmonowati EN (2009). Selection of Indonesia cassava (*Maniho tesculenta* Crantz) genotype as source of β-carotene. Journal of Biological Diversity, 10 (1): 6-11. DOI: https://10.13057/biodiv/d100102

Purkan P, Baktir A and Sayyidah AR (2016). Produksi Enzim Kitinase dari *Aspergillus Niger* Menggunakan Limbah Cangkang Rajungan sebagai Induser (Production of Chitinase Enzymes from *Aspergillus niger* using rajungan shell as waste inducer). Journal Kimia Riset, 1 (1): 34-41. DOI: https://org/10.20473/jkr.v1i1.2440

Putri AM and Kurnia P (2018). Identification of coliform bacteria and total microbes in dung-dung ice around Muhammadiyah University, Surakarta campus. Indonesian Nutrition Media (Media Gizi Indonesia), 12: 41-48. DOI: 10.20473/mgi.vl3il.41-48

Ravindran V, Kornegay ET, Rajaguru ASB, Potter LM and Cherry JA (1986). Cassava leaf meal as a replacement for coconut oil meal in broiler diets. Poultry Science, 65: 1720–1727. DOI: https://org/10.3382/ps.0651720

Rizal Y, Nuraini, Mirnawati and Mahata ME (2013). Comparisons of nutrients contents and nutritional values of palm kernel cake fermented by using different fungi. Pakistan Journal of Nutrition, 12 (10): 943-948. DOI: https://10.3923/pjn.2013.943.948.

Rizal Y, Mahata ME, Joli I and Wu G (2012). Improving the nutrient quality of juice wastes mixture through fermentation by using *Trichoderma viridae* for poultry diet. Pakistan Journal of Nutrition, 11 (3): 203-207. DOI: 3923/pjn.2012.203-207

Soeprijanto, Ratnaningsih T and Prasetyaningrum (2008). Bioconversion of cellulose from corn cob waste to glucose using *Aspergillus niger* mushroom . Purification Journal (Jurnal Purifikasi), 9(2):105-114. DOI: https://doi.org/10.12962/j25983806.v9.i2.146; Available at: https://purifikasi.id/index.php/purifikasi/article/view/146

Statistics Indonesia (BPS - Badan Pusat Statistik) (2018). Available at: https://www.bps.go.id/

Steel RGD and Torrie JH (1980). Principle and Procedures Statistics: A Biometrical Approach. 2nd Edn., McGraw Hill Book Co., New York. Available at: https://www.scirp.org/(S(i43dyn45teexjx455qlt3d2q))/reference/ReferencesPapers.aspx?ReferenceID=383208 Google Scholar

Sugiharto S (2019). A review on fungal fermented cassava pulp of as a cheap alternative feedstuff in poultry ration. Journal of World's Poultry Research, 9(1): 01-06. DOI: https://dx.doi.org/10.36380/jwpr.2019.0

Sukmawati and Hardianti F (2018). Analysis of Microbial Total Plate Count (TPC) in Snapper Salted Fish in Sorong City, West Papua. Jurnal Biodjati, 3(1):72-78. Available at: http://journal.uinsgd.ac.id/indx.php/biodjati

Sumiati, Farhanuddin, Hermana W, Sudarman A, Istichomah N and Setiyono A (2011). Broiler chicken performance given jatropha seed meal rations (*Jatrophacurcas l.*) Fermented using *Rhizopus oligosporus*. Animal Husbandry Media, 34 (2): 117-125. DOI or Available at: DOI: https://doi.org/10.5398/medpet.2011.34.2.117. Google Scholar

Wyllie D and Chamanga PJ (1979). Cassava Leaf Meals in Broiler Diets. Tropical Animal Production. 4(3): 232-240. Available at: http://www.fao.org/WAICENT/FaoInfo/Agricult/aga/AGAP/FRG/TAP43/4_3_4.PDF

Yuniza A, Nova TD, Angga WA, Annisa and Rizal Y (2016). Effects of the combinations of cassava leaf meal and palm kernel cake mixture fermented by *Bacillus amyloliquefaciens* on the alteration of their dry matter, crude protein, crude fiber, and crude lipid contents. Pakistan Journal of Nutrition, 15:1049-1054. DOI: https://10.3923/pjn.2016.1049.1054

Zakaria Y, Novita CI and Samadi S (2013). The effectiveness of fermentation with different substrate sources on the quality of rice straw. Jurnal Agripet, 13(1): 22-25. DOI: https://doi.org/10.17969/agripet.v13i1.548

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Characterization of Renal Inflammatory Cytokines and Related Nephropathy in Experimentally Infected Broiler Chickens with Velogenic Newcastle Disease Virus

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ABSTRACT

Velogenic Newcastle disease virus (VNDV) shows systemic dissemination and it affects the histological structure and function of the kidneys. Present study is an novel attempt to correlate the changes in renal biochemical functions and gene expression of different inflammatory cytokines with related renal pathology induced by VNDV in experimentally infected broilers in different ages. One hundred broiler chickens were divided into 4 groups: group 1 served as control (non-infected group), and groups 2, 3 and 4 were inoculated with 10^6 EID_{50} of NDV on the 10^{th}, 20^{th} and 30^{th} days of age, respectively. Serum samples were collected to evaluate uric acid and creatinine levels. Kidney specimens were collected for virus detection using real time RT-PCR, evaluation of gene expression of IL-6 and IFN-γ as well as histopathological, histochemical and immunohictochemical analysis. The highest concentration of uric acid and creatinine profile were found in Group 4 (12.06 ± 1.25 and 2.16 ± 0.12, respectively) on 7 dpi. IL-6 and IFN-γ were significantly elevated at the 7^{th} dpi in Group 4 compared to other infected groups. The renal histopathological lesions included tubulointerstitial nephritis and glomerulopathy with expression of virus antigen in different areas of renal tissue. histopathology was the hallmark of VNDV infection in broiler chickens; their severity was related to the increased expression of inflammatory cytokines genes (IL-6 and IFN-γ) and virus antigen residence in renal tissue.

Key words: IL-6, IFN-γ, Immunohistochemistry, Nephropathy, Velogenic NDV

INTRODUCTION

Newcastle disease (ND) is widespread disease and it affects several species of birds and causes severe losses in the poultry sector. Newcastle disease can drastically limit the amount of dietary protein and also damage the microeconomy of poultry farms (Cattoli et al., 2011; Sultan etal 2020).

Non-virulent NDVs have the typical a virulent cleavage motif, ^{112}R/G-R/ K-Q-G-R^{116}, with a leucine (L^{117}) at the N terminus of the F protein after cleavage (F1), which is only susceptible to trypsin-like enzymes that were limited to specific tissues such as those in respiratory and digestive tracts, causing localized infection. On the other hand, the virulent NDVs have a virulent cleavage motif, ^{112}R/K-R-Q-R/K-R^{116}, and phenylalanine (F^{117}) at the N terminus of F1, which enables them to infect the host leading to systemic dissemination of virulent strains of NDV in different host tissues by acquiring susceptibility to furin or other ubiquitous intracellular host cell proteases (Nagai, 1993; De Leeuw et al., 2003). For many decades, ND has been facing problems and losses due to outbreaks of the disease despite the research and efforts made to have control and possible eradication. The wide variation of clinical signs and lesions, in addition to absence of pathognomonic clinical signs and lesions, make diagnosis very difficult (Okorie-Kanu et al., 2016).

Renal lesions induced by NDV have been reported in several avian species as in turkeys in which the presence of viral antigen was observed in a number of parenchymatous organs and not just at mucosal surfaces, reflecting systemic spread (Piacenti et al., 2006). However, few literatures were interested in renal lesions in

NDV infections, and limited studies have addressed the relationship between histopathological alterations in chicken kidney tissues and NDV replication (El-Bahrawy et al., 2017).

Among avian species, chickens showed more susceptibility and renal histopathological alterations as previously stated in a comparative study with waterfowl which presented more prominent lesions. The pathological lesions were characterized by proliferations of the ectopic lymphoid tissues and tubulointerstitial nephritis with detection of viral nucleoprotein in degenerated renal tubules (Anis et al., 2012).

Present study aimed to investigate the gene expression of inflammatory cytokines genes and renal pathology induced by NDV infection in broiler chickens receiving no NDV vaccine during rearing, from an early to a late phase of infection and to detect the viral antigens residence in the renal tissue.

MATERIALS AND METHODS

Virus

Velogenic NDV genotype VII (NDV-B7-RLQP-CH-EG-12) local strain isolated from Kafr El-Sheikh Governorate, Egypt was kindly provided by National Laboratory for Veterinary Control on Poultry Production, Animal Health Research Institute. Phylogenetically, the virus was characterized as velogenic VII which isolated by sequencing of F gene partially around the cleavage site and the sequence was deposited in Gen Bank with Accession Number KM288609.

Infectivity titration in Embryonated chicken egg

The NDV virus suspension was titrated on 9th and 10th days of inoculation. 10 fold serial dilutions of the virus in saline containing antibiotic were prepared. Virus suspension was inoculated in 5 embryos for each dilution via allantoic sac (0.1 ml per egg). The inoculated embryos were incubated at 37°^{C} and candled twice a day for 6 days. On first day, the dead embryos were considered nonspecific deaths. Haemagglutination (HA) was applied to the allantoic fluid of inoculated chicken embryos to detect the HA-positive eggs. It was carried out according to the standard method described by Council (1971). For detection of haemagglutination in embryonic fluid, 10% of the washed chicken red blood cell suspension in saline were used. One drop of the fluid was mixed with one drop of blood suspension on glass slide and results were recorded within 2 minutes. The harvested allantoic fluid was used to determine the egg infectious dose 50 (EID_{50}) according to Reed and Muench (1938) as 10^6 EID_{50} to be used for infection of the chickens.

Experimental design

One hundred day old broiler chicks (Ross) were obtained from commercial poultry company. Chicks were kept in different (separated) rooms and were given water and feed *ad libitum.* All chicks were vaccinated against avian influenza H5N1 and infectious bursal disease virus on 10 days old and no vaccination for NDV was used. The birds were then randomly divided into four groups (25 per each) as follows: Control Non-infected (Group 1), infected group of 10-day old chicks (Group 2), infected group of 20-day old chicks (Group 3) and infected group of 30-day old chicks (group 4). The infected groups were inoculated intraocular with a dose 10^6 EID_{50} of NDV (in a total volume of 0.1 ml). Three chickens from each group were sacrificed on the 1st, 3rd, 5th and 7th days after inoculation (dpi).

Serum biochemical analysis

Serum was obtained by collecting blood samples without anticoagulant in a sterile test tube and kept for biochemical analysis. Serum uric and creatinine were determined in serum samples calorimetrically using commercial diagnostic kits (Stanbio-laboratory,USA). supplied by Spectrum Diagnostics Company and measured according to the manufacturer's instructions (Tiffany et al., 1972; Bowers and Wong, 1980).

Real time RT-PCR for detection of virulent Newcastle disease

PCR kit (Qiagen, Inc. Valencia CA, Cat. no. 204443) with specific primers and probes designed by Wise *et al.*(33) supplied from Metabion (Germany). Primers were (F+4839) 5′- TCC GGA GGA TAC AAG GGT CT-3′, (F-4939) 5′- AGC TGT TGC AAC CCC AAG-3′ and probe was (F+4894) 5′- [FAM] AAG CGT TTC TGT CTC CTT CCT CCA[TAMRA] -3′.

RNA was extracted from tissue samples using RNeasy minikit (Qiagen, Germany, according to the manufacturer's recommendations). NDV specific primers were utilized in a 25µl reaction containing 12.5 µl of Quantitect probe rt-PCR master mix (Qiagen, Germany, GmbH), 0.5 µl of each primer of 50pmol concentration, 0.125 µl of the specific probe, 4.125 µl of RNase Free Water, 0.25 µl of QuantiTect RT Mix and 7µl of RNA extract. The reaction was recorded by a Stratagene MX3005P real time PCR machine (Stratagene Co, USA). The reserve transcription step was performed at 50˚C for

30 min followed by 15 min at 95˚C as primary denaturation, while the cycle protocol was 40 cycles of three successive steps; 94˚C for 30 sec., 52˚C for 30 sec and 72˚C for 10 sec.

Profiling of cytokine gene expression

Total RNA was extracted from kidneys' tissue from infected groups at 7 dpi using an RNeasy Mini RNA Purification Kit and RNase-Free DNase Kit (QIAGEN) for RNA purification according to the manufacturer's instructions. The expression of different cytokines was quantified by reverse transcription real-time PCR (RT-PCR) using relative quantification. The primers and probes used in this study have been described previously: IL-6, IFN-γ and 28srRNA (13) and have been described in table 1.

Quantitative real-time RT-PCR was performed using Quantitect probe RT-PCR (QIAGEN) according to the manufacturer's recommendations. Real-Time RT-PCR was carried out using a 7500 Real-time PCR System (Applied Biosystems). PCR conditions were the same for each cytokine gene, as follows: 30 min at 50 C, 95 _C for 15 min, followed by 40 cycles of 95 _C for 15 sand 60 _C for 1 min.

Statistical analysis for fold changes in cytokine levels were determined by the ΔΔCt method (Livak and Schmittgen, 2001), using 28S ribosomal RNA as the endogenous reference gene to normalize the level of the target gene expression.

Histopathological and histochemical examination

Samples of kidneys were cut into 1 cm × 1 cm × 0.5 cm and fixed in neutral buffered formalin 10%. 5µm sections were prepared and the slides were stained with Haematoxylin and Eosin (H&E) for histopathological examination using Olympus BX43 light microscope and captured using Olympus DP27 camera linked to Cellsens dimensions software (Olympus). Periodic acid-Schiff (PAS) and Masson's trichrome (MTC) staining were performed on selected tissue sections (Bancroft, 2013).

Immunohistochemistry

For immunohistochemistry, Paraffin blocks were collected at the 7th dpi from different groups. NDV hyperimmune serum was prepared by series injection in rabbits (Samiullah et al., 2006). Antibody purification was performed using Magne™ Protein G Beads for Antibody Purification according to the manufacturer's instructions. Tissue sections were obtained on Poly-L-Lysine coated slides, deparaffinized and rehydrated. Antigen retrieval step was performed by heat induction and blocking of non-specific protein binding and endogenous peroxide was followed by overnight incubation in primary antibody (Rabbit anti NDV Ig previously mentioned) then incubated with horse radish peroxidase–conjugated goat polyclonal secondary antibody to rabbit Ig (SM802 EnVision™ FLEX /HRP). Colour was developed with 3, 32 - Diaminobenzidine (DAB) substrate (DM827 EnVision™ FLEX DAB+ Chromogen) (Burns et al., 2005).

Table 1. Real-time quantitative RT-PCR probes and primers

RNA target		Probe/primer sequence (5′- 3′)	Accession number
28S	Probe	(VIC)-AGGACCGCTACGGACCTCCACCA-(TAMRA)	X59733
	F	GGCGAAGCCAGAGGAAACT	
	R	GACGACCGATTTGCACGTC	
IL-6	Probe	(FAM)-AGGAGAAATGCCTGACGAAGCTCTCCA-(TAMRA)	AJ250838
	F	GCTCGCCGGCTTCGA	
	R	GGTAGGTCTGAAAGGCGAACAG	
IFN-γ	Probe	(FAM)-TGGCCAAGCTCCCGATGAACGA-(TAMRA)	Y07922
	F	GTGAAGAAGGTGAAAGATATCATGGA	
	R	GCTTTGCGCTGGATTCTCA	

Table 2. Serum biochemistry profile of uric acid and creatinine of broiler chickens from different experimental groups at 1, 3, 5 and 7dpi

Groups	Uric acid (mg/dl)				Creatinine (mg/dl)			
	1dpi	3dpi	5dpi	7dpi	1dpi	3dpi	5dpi	7dpi
Group 1	2.63 ± 0.28^{a}	2.83 ± 0.12^{a}	2.66 ± 0.17^{a}	2.93 ± 0.33^{a}	0.14 ± 0.01^{a}	0.15 ± 0.00^{a}	0.16 ± 0.03^{a}	0.17 ± 0.01^{a}
Group 2	3.33 ± 0.16^{a}	4.10 ± 0.32^{b}	4.16 ± 0.44^{a}	4.90 ± 0.20^{a}	0.70 ± 0.05^{b}	1.00 ± 0.15^{b}	0.80 ± 0.05^{b}	1.03 ± 0.08^{b}
Group 3	5.16 ± 0.38^{b}	5.13 ± 0.18^{b}	6.83 ± 0.60^{b}	7.33 ± 0.33^{b}	0.76 ± 0.08^{b}	1.20 ± 0.05^{b}	1.63 ± 0.17^{c}	1.86 ± 0.08^{c}
Group 4	7.73 ± 0.73^{c}	8.16 ± 0.60^{c}	9.33 ± 0.72^{c}	12.06 ± 1.25^{c}	1.26 ± 0.06^{c}	1.56 ± 0.14^{c}	1.90 ± 0.05^{c}	2.16 ± 0.12^{d}

Values expressed as means ± Standard Error. Different superscripts a, b and c indicate a significant different between values within the same column. Group 1 (control uninfected group), Group 2 (infected at 10 days old), Group 3 (infected at 20 days old) and Group 4 (infected at 30 days old). Significant values at $p \leq 0.05$.

Scoring system

Scoring system for the renal lesions of NDV-infected-tissues was done according to Hussein et al. (2018) with some modification. Briefly, it was as follows: 0 = no changes; 1 = few inflammatory cells infiltration in interstitial tissue; 2 = inflammatory cells infiltration and focal degeneration in renal tubules; 3 = inflammatory cells together with foci of necrobiotic changes in renal tubular epithelium and/or glomeruopathy; 4 = inflammatory cells, necrobiotic changes of the medullary epithelium and renal glomeruopathy.

Statistical analysis

Statistical analyses were performed using one-way factorial analysis of variance (ANOVA). Statistical significance was defined as ($p \leq 0.05$) using SPSS 17.

RESULTS

Serum biochemical profile

The mean values of serum uric acid and creatinine were summarized in table 2 and figures 1 and 2. The mean values of serum uric acid was significantly ($p \leq 0.05$) increased in the infected groups of 20 and 30 day old chicks at 1, 3, 5 and 7 dpi compared with the control non-infected group as well as infected group at 10 days old (figure 1). The mean values of serum creatinine were significantly increased ($p \leq 0.05$) with advancing age at 1 and 3 dpi with no significant difference between the infected groups of 10 and 20-days old and the highest significant levels were detected in chickens of the 30-day old group at 1 and 3 dpi. The serum creatinine level was significantly increased at 5 and 7 dpi in all infected groups and the values significantly increased with advancing age (figure 2).

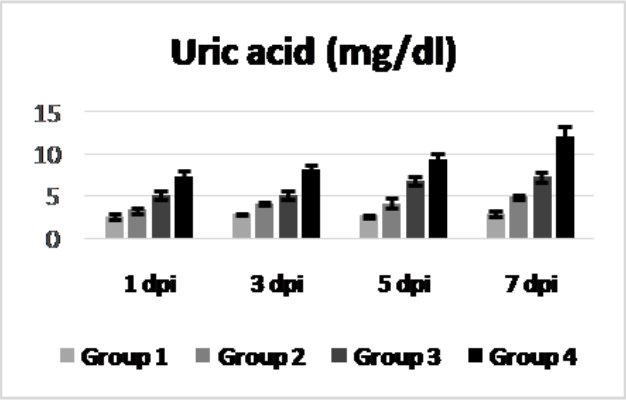

Figure 1. Serum uric acid profile of control and infected broiler chickens at 1, 3, 5 and 7 dpi

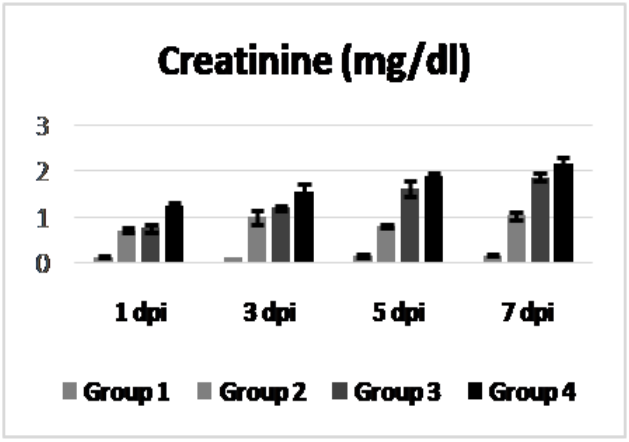

Figure 2. Serum creatinine profile of control and infected broiler chickens at 1, 3, 5 and 7 dpi

Real-Time PCR and cytokine mRNA gene expression

The PCR result confirmed the highest detection for VND in kidneys' tissues collected at 7 dpi was in group 4. (30 days-old) in comparison with groups 2 and 3 (10 and 20 days-old). The gene expression of IL6 was significantly increased in Group 4 compared with groups 2 and 3. Meanwhile, no significant difference in gene expression of IFN-γ was detected in the infected 10-20-day-old chickens compared with the 30-day old group that conversely showed a significant higher expression of IFN-γ (figure 3).

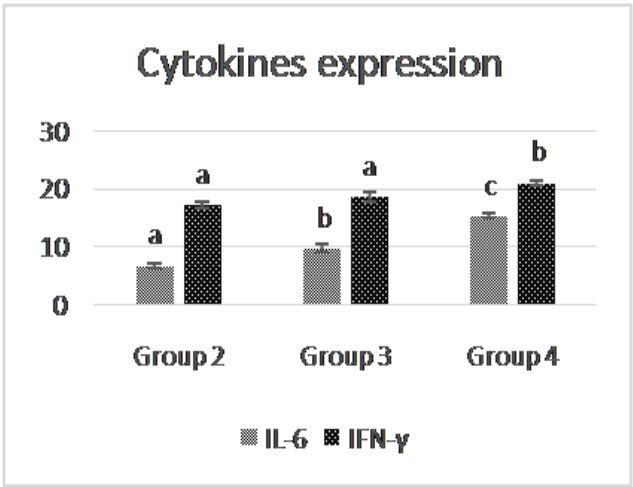

Figure 3. Relative expression of cytokines in the kidneys of infected broiler chickens from different infected groups at 7dpi for IL-6 and IFN-γ. Error bars represent the standard error of the mean (n = 3). Significant values at $p \leq 0.05$. a, b and c above the error bar indicate a significant different between values within the same data series.

Histopathology and histochemistry

Group 1 showed normal histological structure in all sacrifices which present cortical areas composed of closely packed nephrons and medullary cones (medullary tracts) containing collecting tubules, looped tubules of the mammalian nephrons, ureteral branches and a network of blood capillaries in interstitial connective tissue. At 1 dpi all infected groups presented vascular changes characterized by perivascular oedema and haemorrhage with congestion of peritubular capillary sinuses. Group 2 and group 3 showed at 1 and 3 dpi mild to moderate necrobiotic changes. Meanwhile, Group 4 showed moderate necrobiotic changes admixed to focal interstitial nephritis at 1 dpi characterized by mononuclear and heterophilic cells infiltration (figure 4a).

At 5 dpi, the inflammatory reaction was evident in all the infected groups with variable severity. Group 2 showed focal pattern of interstitial nephritis, while groups 3 and 4 exhibited multifocal to diffuse interstitial nephritis (figure 4b) mixed with glomerular changes. At 7 dpi, group 2 showed mild histoapthological alterations in which renal tubular epithelium showed necrosis with focal interstitial nephritis (figure 4c). Severe necrosis of tubular epithelium developed in groups 3 and 4 mixed with diffuse cortical interstitial nephritis (figure 4d and e). Moreover, some examined sections from group 4 tended to show more severe lesions by extending the inflammatory reaction into the medullary cones (figure 4f) with mild cortical interstitial fibrosis.

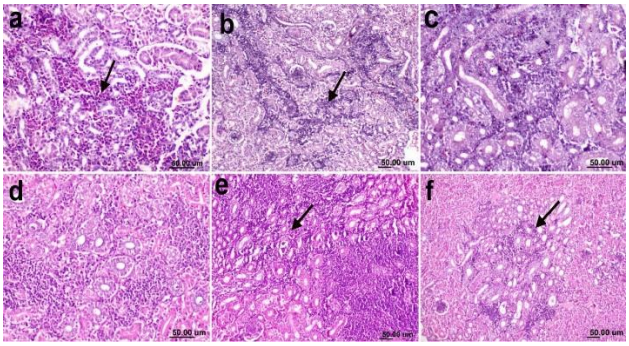

Figure 4. Histological kidney sections stained by H & E from infected broiler chickens**. a**: Group 4 at 1 dpi showing heterophilic interstitial nephritis (arrow). **b**: Group 3 at 5 dpi showing multifocal mononuclear cell aggregation (arrow). (**c**) Group 2 at 7 dpi showing mild mononuclear cells infiltration. **d**: Group 3 at 7 dpi showing diffuse mononuclear cells infiltration. **e**: Group 4 at 7 dpi showing diffuse mononuclear cells infiltration the renal cortex and necrosis of renal tubular epithelium (arrow). **f**: Group 4 at 7 dpi showing diffuse mononuclear cells infiltration in the medullary cone (arrow).

At 7 dpi, kidneys from group 2 exhibited membranous glomerulopathy affecting few glomeruli. The lesion was characterized by thickening of glomeular basement membrane (figure 5a), whereas group 3 at 5 and 7 dpi presented moderate number of glomerular changes characterized by membranous and proliferative glomerulopathy manifested by large sized glomeruli and hypercellularity due to the increase in the mesangial cells (figure 5b). Moving to group 4, glomerular changes started earlier at 3 dpi with membranous glomerulopathy, and many glomeruli showed proliferative glomerulopathy at 5 dpi (figure 5c). Moreover, atrophy of glomerular capillary tuft (figure 5d) was observed in some glomeruli in group 4 at 7dpi, whereas other glomeruli showed membranous glomerulopathy with mononuclear cells infiltration in the interstitial tissue (figure 5e).

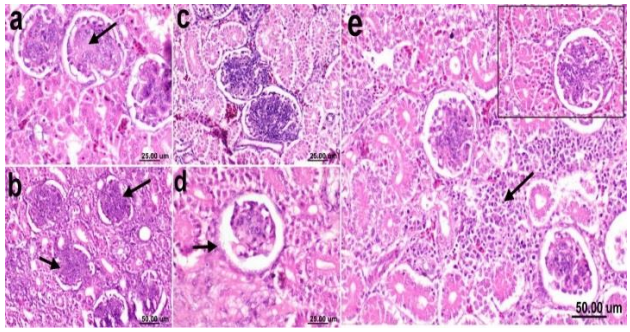

Figure 5. Histological kidney sections stained by H & E from infected broiler chickens**. a**: Group 2 at 7 dpi showing membranous glomerulopathy with thickening of glomerular basement membrane (arrow). **b**: Group 3 at 7 dpi showing proliferativglomerulopathy characterized by enlarged glomeruli with glomerular hypercellularity (arrow). **c**: Group 4 at 5 dpi showing proliferative glomerulopathy that showed both glomerular hypercellurity and thickening of glomerular basement membrane. **d**: Group 4 at 7 dpi showing atrophy of glomerular capillary.

The mild peritubular and interstitial fibrosis were detected in group 4 and was confirmed by MTC stain (figure 6a) by which the periglomerular deposition of delicate collagen fibers was noticed (figure 6b and c). Thickening of the glomerular capillary wall in different infected groups was positively stained by PAS stain. Also, the renal tubules showed destruction and loss of continuation of the apical brush border of the proximal convoluted tubules with decreased PAS staining affinity of apical borders of tubular epithelium (figure 6d and e).

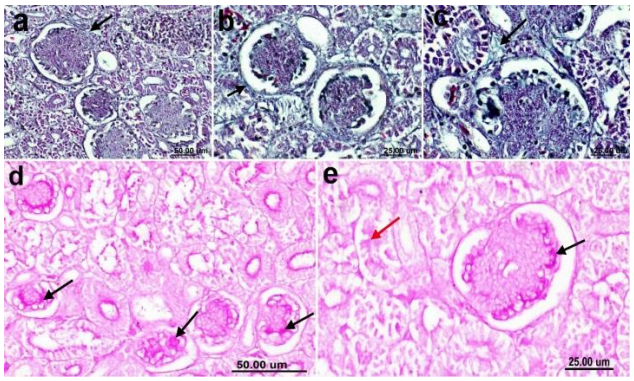

Figure 6. Masson's Trichrom stained sections of infected broiler chickens from group 4 at 7 dpi. **a**: peritubular and interstitial fibrosis (arrow). **b** and **c**: showing periglomerular deposition of delicate collagen bundles (arrow). **d**: Periodic acid-Schiff stained sections of chicken from group 2 at 7 dpi highlighting the thickening of the glomerular capillary walls (arrows). **e**: PAS stained section from group 4 at 7 dpi illustrating the thickening of the glomerular capillary tuft (black arrow) with loss of continuation of the apical brush border of the proximal convoluted tubules (red arrow).

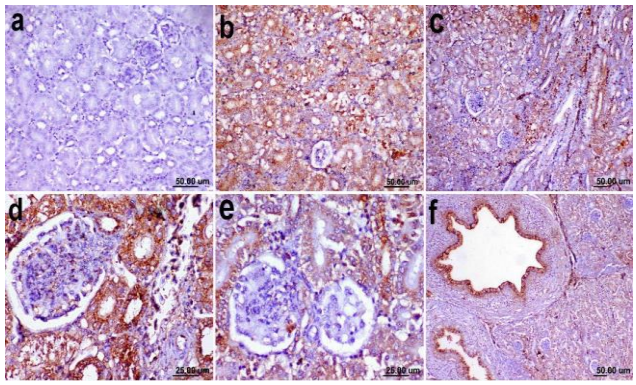

Figure 7. Immunohistochemical reaction of broiler chickens from different experimental groups at 7 dpi; **a**: Group 1 showed negative peroxidase reaction for NDV. **b**: Positive expression of the viral antigen lining epithelium of the degenerated tubules. **c**: Viral expression was detected in the medullary cones of the infected birds. **d** and **e**: Positive IHC for NDV was detected in the glomerular capillary tuft. **f**: Major ureter branches showed positive expression of the NDV in pseudostratified columnar epithelial lining.

Immunohistochemistry

Group 1 showed negative peroxidase reaction for NDV antigen (figure 7a). At 7 dpi, expression of viral antigen was detected in several areas of the renal tissue; renal tubular epithelium d showed strong positive reaction of NDV antigen especially in the degenerated tubular epithelium (figure 7b). Medullary cone showed positive IHC reaction for NDV (figure 7c). Glomeruli of different infected groups exhibited positive expression of viral antigen in the capillary tuft (figures 7d and 7e). Some major ureter branches showed positive expression of the viral antigen in pseudostratified columnar epithelial lining (figure 7f).

Histopathological lesions score

The results of histopathological lesion score are summarized in table 3. All infected groups showed increased lesion severity ($p \leq 0.05$) in all infected sacrifices when compared to group 1 (control group) which exhibited normal histological structure. Group 4 showed the highest lesions scoring in all sacrifices compared to other infected groups and a significant difference between it and the other infected groups (Groups 2 and 3) at 1 and 5 dpi recording 1.73 and 2.4, respectively. At 7 dpi, the highest lesion scores were observed in all infected groups and no significant difference ($p \leq 0.05$) was detected between group 3 and group 4 which scored 2.66 and 2.73, respectively (table 3).

Table 3. Histopathological lesions score induced by NDV in the renal tissues of broiler chickens from different experimental groups at 1,3,5 and 7dpi

Groups	1dpi	3dpi	5dpi	7dpi
Group 1	00.00± 0.00	00.00± 0.00	00.00± 0.00	00.00± 0.00
Group 2	1.00 ± 0.29 [a]	1.00 ± 0.13[a]	1.60± 0.19 [a]	2.06 ± 0.66 [a]
Group 3	1.06± 0.20[a]	1.46± 0.16 [b]	1.86± 0.27 [a]	2.66 ± 0.33 [b]
Group 4	1.73 ± 0.20[b]	1.80± 0.17[b]	2.40± 0.16[b]	2.73 ± 0.00[b]

Values expressed as means ± Standard Error

DISCUSSION

As for NDV affecting broilers in developed and developing countries, massive economic losses occurred due to outbreaks in many localities (Cattoli et al., 2011). Until now, it has not been clear whether there is a specific host-cell protease responsible for cleavage of the different F0 proteins. Briefly, there is a correlation between virulence or pathogenicity and a high content of basic amino acid residues at the F0 cleavage site (De Leeuw et

al., 2003) which allows systemic distribution of virulent strains of NDV.

Serum uric acid and creatinine levels are used mainly as markers of renal functions and indicators to different histopathological alterations of kidney (Sanders et al., 1980). Serum uric acid level is the primary marker of renal functions in avian species (Tully et al., 2009). Increased serum levels of these parameters in the infected groups occurred due to severe kidney pathology that was detected in all infected groups. In addition, there was a correlation between increased serum level and the severity of renal pathology). (table 2 and figures 1, 2, 4 &5). These results confirmed that the developed nephropathy induced by NDV severely affects the kidney functions as confirmed by Najafi et al. (2014). Renal tubules necrobiotic changes may lead to hyperuricemia, as Okorie-Kanu et al. (2016) stated that over 80% of nitrogen excreted by birds are in the form of uric acid through tubular secretion. Meanwhile, Echols et al. (2006) mentioned that blood uric acid levels are mildly affected by the birds' hydration status, but they reflect the functional capacity of the renal proximal tubules. Loss of renal function combined with water deprivation during the peak of clinical signs may enhance the effect (El-Bahrawy et al., 2017).

IL-6 is a pro-inflammatory cytokine involved in acute-phase responses, immune regulation and hematopoiesis (Hirano, 1998). IL-6 plays a role in the activation of B and T lymphocytes, induction of macrophage production and development. Moreover, it acts synergistically with granulocyte-macrophage colony stimulating factor (GMCSF) (Wigley and Kaiser, 2003).

Using real-time RT-PCR of RNA isolated from NDV-infected kidneys, we demonstrated that the virulence of NDV was increased in the older age indicating the increased rate of viral dissemination and replication in kidney tissue with advancing age. Thus, it interpreted the age related increase in inflammatory cytokines in renal tissue with subsequent severe nephropathy and inflammatory reaction that were detected in older ages of infected chickens. This result was agree with Rue et al. (2011) who mentioned that virulent strain of NDV is capable of rapid and strong releasing of IFN-γ and IL-6. Moreover, Alexopoulou et al. (2001) characterized IL-6 as an early innate response due to viruses. Furthermore, IFN-γ activate macrophage and modulat the immune system (De Mayer and De Mayer-Guinard, 1998).

In our results, we early observe heterophils in renal intersitium in all infected groups. This explained by Anis et al. (2012) who stated that avian heterophils are highly phagocytic and capable of a broad spectrum of antimicrobial activity, and they form the first line of cellular defense against invading pathogens. Additionally, in the present results, severe lesions occurred in kidney observed at a late phase of infection (5 and 7 dpi), and these were in agreement with previous report by Wen et al. (2016) who mentioned that nephritis developed as the virus invaded respiratory tract, replicated inside mucosal epithelial cell of the upper respiratory tract and digestive tract and then, right after infection, the virus spread through blood circulation to kidney. Lately, it has been observed that severe interstitial nephritis observed in our study was similar to Anis et al. (2012) who detected tubulointerstitial nephritis by the sixth dpi with associated NDV-NP–immunopositive staining in the degenerated renal tubules. The severe damage occurring in the kidney may be attributed to the direct effect of the viral replication in the renal tubular epithelial, which was confirmed by expression of the viral antigen in degenerated tubular epithelium by immunohistochemistry and confirmed by the increased gene expression of inflammatory cytokines in kidneys of infected chickens. Moreover, Vascular changes that occurred in the present study may be due to the adjacent focal inflammation as vessels adjacent to primary areas of infection can show some degrees of hyaline degeneration, which most likely due to intense exudation of proteins (Cattoli et al., 2011).

The distribution of renal lesion in the present study was different depending on the age of infected chickens with increased distribution of renal glomerular, tubular and interstitial pathology with advancing age to involve both cortex and medullary cones. The susceptibility of the renal cortex and the medulla to NDV infection in birds are unknown as the pH is variable (Echols, 2006). Furthermore, species also varied in the susceptibility of renal infection. Anis et al. (2012) concluded that, in a comparison study between chickens and ducks, Tubulointerstitial nephritis was noticed only in chickens and the lesions dramatically developed at the late stage and did not subside until the 10^{th} dpi. In addition to the previously mentioned factors, age appeared to affect susceptibility to infection as older birds in our study showed more severe lesions compared with younger birds. This was confirmed by a previous related study on the age factor affecting severity of infection in immune organs. This result was in agreement with Mohammed et al. (2019) and Mousa et al. (2019) who concluded that the older chickens are more susceptible to infection with more developed histopathological alteration and more expression of viral antigen in the exposed tissues.

The glomerular changes, including membranoproliferative lesions, occurred in association with viral disease that proposes an immune complex–mediated form of glomerulonephritis as we detected NDV antigen in glomerular tuft by immunohistochemistry. Membranoproliferative glomerular lesions were seen with neoplasia, autoimmune disorders, and persistent infections or other diseases producing prolonged antigenic stimulation including viral and bacterial infections (Maxie and Prescott, 1993; Wilson et al., 2010). Maxie and Prescott (1993) added that the glomeruli variably respond to the damage by cellular proliferation or thickening of the basement membrane.

In our study, viral antigen was expressed in several areas of infected kidneys, similar results obtained by El-Bahrawy et al. (2017) who stated that most immunostaining for NDV- NP was distributed in a multifocal pattern in the intact and vacuolated epithelium of proximal and distal tubules, in infiltrating macrophages, in some cells of reptilian-type glomeruli in the cortex, and in medullary cones mainly in the tubular epithelium and the infiltrating mononuclear cells. Moreover, Susta et al. (2011) stated that several epithelial cells in the distal tubules of the kidneys had positive signal for ND.

CONCLUSION

The present study showed an age-related difference in Newcastle disease virus replication and dissemination into renal tissue. Such difference is correlated with gene expression of inflammatory cytokines with subsequent increase of the severity of nephropathy with advancing age that dramatically affects renal function and reflects the health status of broiler chickens. These results highlight the importance of NDV as one of the diseases that could contribute to kidney affection in broiler chickens under field condition. Glomerulopathy developed in the infected birds showed a new feature of NDV which was increased with age.

DCLARATIONS

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Competing interests

The authors declare that they have no competing interests.

Ethical approval

This experimental protocol was approved by Institutional Animal Care and Use Committee (IACUC), Cairo University, Egypt (Approval number, CU/II/F/65/17).

Authors' contributions

Mohamed R. Mousa, Faten F. Mohammed, Kawkab A. Ahmed: Data collection and study design planning. Histopathological, histochemical and immune-histochemical analysis. Fatma Amer Abdel reheem: Real-Time PCR and cytokine mRNA gene expression. Ayman H. El-deeb: study design planning and virus infectivity titration. All authors: Data interpretation, final manuscript writing, editing and revising.

REFERENCES

Alexopoulou L, Holt AC, Medzhitov R and Flavell RA (2001). Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature, 413: 732. DOI: https://www.nature.com/articles/35099560.

Anis Z, Morita T, Azuma K, Ito H, Ito T and Shimada A (2012). Comparative Study on the Pathogenesis of the Generated 9a5b Newcastle Disease Virus Mutant Isolate Between Chickens and Waterfowl. Veterinary Pathology, 50: 638–647. DOI: https://doi.org/10.1177/0300985812467470.

Bancroft JD (2013). Histochemical techniques. Butterworth-Heinemann. Available at: https://www.elsevier.com/books/histochemical-techniques/bancroft/978-0-407-00033-9.

Bowers LD and Wong ET (1980) Kinetic serum creatinine assays. II. A critical evaluation and review. Clinical chemistry, 26: 555–561. DOI: https://www.ncbi.nlm.nih.gov/pubmed/7020989.

Burns Robert (2005). Immunochemical protocols. Vol. 295. Totowa, NJ: Humana Press. Available at: https://www.springer.com/gp/book/9781588292742.

Cattoli G, Susta L, Terregino C and Brown C (2011). Newcastle disease a review of field recognition and current methods of laboratory detection. Journal of Veterinary Diagnostic Investigation, 23: 637–656. DOI: https://doi.org/10.1177/1040638711407887.

Council NR (1971). Methods for Examining Poultry Biologics and for Identifying and Quantifying Avian Pathogens: Report of the Subcommittee on Avian Diseases, Committee on Animal Health, Agricultural Board, National Research Council. National Academy of Sciences. Available at: https://ucdavis.on.worldcat.org/discovery

De Mayer EM and De Mayer-Guinard J (1998). Interferons. In: AW Thomson (editor). The Cytokine Handbook 3rd edition. Academic Press, San Diego, 491–515.

Echols MS (2006). Evaluating and Treating the kidneys. Clinical Avian Medicine, 2: 451–492.

El-Bahrawy A, Zaid A, Sunden Y, Sakurai M, Ito H, Ito T and Morita T (2017). Pathogenesis of Renal Lesions in Chickens After Experimental Infection With 9a5b Newcastle Disease Virus Mutant Isolate. Veterinary Pathology, 54: 94–98. DOI: https://doi.org/10.1177/0300985816655852.

Hirano T (1998). Interleukin 6. In: AE Thomson (editor). The Cytokine Handbook 3rd edition. Academic Press, San Diego, 197–227. Available at: http://www.med.osaka u.ac.jp/pub/molonc/www/old/Eindex.html.

Hussein EA, Hair-Bejo M, Adamu L, Omar AR, Arshad SS, Elmutaz AA and Aini I (2018). Scoring System for Lesions Induced by Different Strains of Newcastle Disease Virus in Chicken.

Veterinary Medicine International, 1–9. DOI: https://doi.org/10.1155/2018/9296520.

Kaiser P, Rothwell L, Galyov EE, Barrow PA, Burnside J and Wigley P (2000). Differential cytokine expression in avian cells in response to invasion by Salmonella typhimurium, Salmonella enteritidis and Salmonella gallinarum. Microbiology, 146: 3217–3226. DOI: https://doi.org/10.1099/00221287-146-12-3217.

de Leeuw OS, Hartog L, Koch G and Peeters BPH (2003). Effect of fusion protein cleavage site mutations on virulence of Newcastle disease virus: Non-virulent cleavage site mutants revert to virulence after one passage in chicken brain. Journal of General Virology, 84: 475–484. DOI: https://doi.org/10.1099/vir.0.18714-0.

Livak KJ and Schmittgen TD (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. Methods, 25: 402–408. DOI: https://doi.org/10.1006/meth.2001.1262.

Maxie MG and Prescott JF (1993). The urinary system. In: Pathology of domestic animals. K. V. F. Jubb, P. C. Kennedy, and N. Palmer, eds. Academic Press, New York, 2: 478–481. available at: https://www.elsevier.com/books/jubb-kennedy-and-palmers-pathology-of-domestic-animals-volume-3/9780702053191.

Mohammed FF, Mousa MR, Khalefa HS, El-Deeb AH and Ahmed KA (2019). New insights on neuropathological lesions progression with special emphasis on residence of velogenic Newcastle disease viral antigen in the nervous system of experimentally infected broiler chickens. Exploratory Animal and Medical Research, 9(2): 145-157. Avaliable at: www.animalmedicalresearch.org.

Mousa MR, Mohammed FF, Khalefah HS, El-deeb AH and Ahmed KA (2019). Comparative Serological, Histopathological and Immunohistochemical Evaluation of Immune Status of Broiler Chickens Experimentally Infected with Velogenic Newcastle Disease Virus in Different Ages. International Journal of Veterinary Science, 8: 143–150. Available at :www.ijvets.com

Nagai Y (1993). Protease-dependent virus tropism and pathogenicity. Trends in Microbiology, 1: 81–87. DOI: https://doi.org/10.1016/0966-842X(93)90112-5.

Najafi H, Ghalyanchi-Langeroudi A, Hashemzadeh M, Madadgar O and Karimi V (2014). Comparison of Laboratory Parameters in the Serum of SPF Chickens Infected with Different Iranian Genotypes of Infectious Bronchitis Virus. Iranian Journal of Virology, 8: 1–6. Avialable at: http://journal.isv.org.ir/article-1-163-en.html.

Okorie-Kanu CO, Okorie-Kanu OJ, and Okoye JOA (2016). Blood biochemistry responses of ducks infected with a velogenic Newcastle disease virus. Comparative Clinical Pathology. Comparative Clinical Pathology, 25: 681–688. Avialable at: https://link.springer.com/article/10.1007/s00580-013-1870-x.

Piacenti A M, King DJ, Seal BS, Zhang J and Brown CC (2006). Pathogenesis of Newcastle disease in commercial and specific pathogen-free turkeys experimentally infected with isolates of different virulence. Veterinary pathology, 43: 168–78. DOI: https://doi.org/10.1354/vp.43-2-168

Reed LJ and Muench H (1938). A simple method of estimating fifty per cent endpoints. American journal of epidemiology, 27: 493–497. DOI: https://doi.org/10.1093/oxfordjournals.aje.a118408

Rue CA, Susta L, Cornax I, Brown CC, Kapczynski DR, Suarez DL, King DJ, Miller PJ and Afonso CL (2011). Virulent Newcastle disease virus elicits a strong innate immune response in chickens. Journal of General Virology, 92: 931–939. DOI: https://doi.org/10.1099/vir.0.025486-0

Samiullah M, Rizvi F, Anjum AD and Shah MFA (2006). Rising hyperimmune serum against avian paramyxovirus (APMV-1) and pigeon paramyxovirus (PPMV-1) in rabbits and their cross-reactivity. Pakistan Journal of Biological Sciences, 9: 2184–2186. DOI: 10.3923/pjbs.2006.2184.2186

Sanders GTB, Pasman AJ and Hoek FJ (1980). Determination of uric acid with uricase and peroxidase. Clinica Chimica Acta, 101: 299–303. DOI: https://doi.org/10.1016/0009-8981(80)90257-0

Sultan HA, Shaimaa T, Wael Kl, Karim SZ, Mohamed AK, Sameh AA and Kang SC (2020). Protective efficacy of the Newcastle disease virus genotype VII–matched vaccine in commercial layers. Poultry Science, 99:1275–1286. DOI: https://doi.org/10.1016/j.psj.2019.10.063.

Susta L, Miller PJ, Afonso CL and Brown CC (2011). Clinicopathological characterization in poultry of three strains of Newcastle disease virus isolated from recent outbreaks. Veterinary pathology, 48: 349–360. DOI: https://doi.org/10.1177/0300985810375806

Tiffany TO, Jansen JM, Burtis CA, Overton JB and Scott CD (1972). Enzymatic kinetic rate and end-point analyses of substrate, by use of a GeMSAEC fast analyzer. Clinical Chemistry, 18: 829–840. DOI: https://doi.org/10.1093/clinchem/18.8.829

Tully TN, Dorrestein GM and Jones AK (2009). Handbook of avian medicine. Elsevier/Saunders. https://www.elsevier.com/books/handbook-of-avian-medicine/9780702028748

Wen G, Hu X, Zhao K, Wang H and Zhang Z (2016). Molecular basis for the thermostability of Newcastle disease virus. Scientific Reports, 6: 1–9. DOI: https://www.nature.com/articles/srep22492

Wigley P and Kaiser P (2003). Avian cytokines in health and disease. Brazilian Journal of Poultry Science, 5: 1–14. DOI: https://doi.org/10.1590/S1516-635X2003000100001

Wilson FD, Wills RW, Senties-Cue CG, Maslin WR, Stayer PA and Magee D (2010). High Incidence of Glomerulonephritis Associated with Inclusion Body Hepatitis in Broiler Chickens: Routine Histopathology and Histomorphometric Studies. Avian Diseases, 54: 975–980. DOI: https://doi.org/10.1637/9050-090709-Reg.1

Wise MG, Suarez DL, Seal BS, Pedersen JC, Senne DA, King DJ, Kapczynski DR and Spackman E (2004). Development of a real-time reverse-transcription PCR for detection of Newcastle disease virus RNA in clinical samples. Journal of Clinical Microbiology, 42: 329–338. DOI: https://jcm.asm.org/content/42/1/329

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Effect of Nutritional Supplements of Fermented and Unfermented Betel Nutshell Waste in Performance of Broiler Chickens

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ABSTRACT

An experiment was conducted to see the broiler's performance fed with fermented and unfermented betel nutshell. Harvesting betel nut seed leaved peel as a waste that contains some nutrients and active ingredients. This waste was useful for poultry feed, to maintain poultry performance and to reduce cholesterol. The aim of this research was to fermented betel nutshell waste with indigenous microorganism from a vegetable waste mixture that produced cellulase to reduce their crude fiber before feeding to the broiler. The nutrient profile and metabolizable energy of fermented betel nutshell in dry matter basis showed 15.96 % water, 84.04 % dry matter, 23.69 % crude fiber, 10.39 % crude protein, 0.31 % crude fat, 2.60% calcium, 1.26 % phosphorus, and 1360,44 kcal/kg. In this experiment betel nutshell waste from a local farmer in Indonesia, and 80 birds from the broiler strain CP 707 from Charoen Pockphand were used. The experiment was conducted in a completely randomized design with different levels of fermented betel nutshell waste (0, 2, 4, and 6%) and 6 % of unfermented of betel nutshell waste in the broiler ration. Each treatment was repeated four times. The measured values included daily feed intake, daily weight gain, feed conversion, body weight, carcass content, abdominal fat pads, lipoprotein profile (total cholesterol, low-density lipoprotein (LDL), and high-density lipoprotein (HDL) in serum of broiler chikens. The results indicated that fermented betel nutshell waste significantly increased daily feed intake, daily body weight gain, carcass content, and decreased abdominal fat pad, total cholesterol, HDL, and LDL of broiler chickens, however did not affect the feed conversion. The fermentation of betel nutshell waste with indigenous microorganism from vegetable waste mixture could be used up to 6 % in the broiler ration and had a positive effect on performance, blood serum lipoprotein, and carcass quality of broiler chickens.

Key words: Betel nutshell waste, Broiler, Crude fiber, Fermented betel nutshell waste, Performance

INTRODUCTION

The broiler is one of the potential livestock to meet the demand for animal protein in Indonesian society. The consumption of chicken meat increases from year to year. According to Livestock and Animal Health Statistics (2018), broiler meat production on 2016 was 1.905.497 tons, on 2017 it was 2.046.794 tons and on 2018 it was even 2.144.013 tons. The high consumption of broiler meat in Indonesia due to the price of broiler meat was cheaper than local chicken meat and beef. The poultry industry needed food availability on a continuous basis.

Indonesia still imports some poultry feeds such as corn, soybean meal, and fish flour. According to ID Indonesia (2015), Indonesia was the country in the Southeast Asia region that bought soybean meal in the largest amount, in the 2013/2014 period, the top four soybean meal importers in Southeast Asia were Indonesia (4 million tons), Vietnam (3.3 million tons), Thailand (2.7 million tons), and Malaysia (1.6 million tons). On 2017 corn was imported from Indonesia at 360.355 tons, and it reached to 481.471 tons on 2018 (Situmorang, 2018). Import feed increased feed costs in the poultry industry, so Indonesia should look foralternative feeds to increase the availability of poultry feed and reduce imported feed. In addition to the diet that could reduce fat and cholesterol in broiler meat, which was important to the mixture in broiler diet, as consumers with limited fat intake were considering consuming broiler meat, the increasing growth rate of broilers followed by increasing fat deposition (Tumova and Teimouri, 2010). Furthermore, excess fat in poultry was one of the main problems faced by the poultry industry (Zhou et al., 2006).

Agriculture wastes could be used as unconventional poultry feeds, and it still contains active compounds like catechin, anthocyanin, lycopene to reduce fat. Harvesting

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of seed betel nut will produce betel nutshell waste yielded up to 76% of the weight of fresh betel nuts (Mahata et al., 2018). It is reported that areca nut production in Indonesia reached 1,100.35 tons in 2016 (Badan Pusat Statistik Indonesia, 2016). The high production of betel nut follows with high betel nutshell waste and is available throughout the year.

Proximate analysis and energy metabolism indicated that betel nut shell waste had contained 65.41% water, 34.59% dry matter, 2.22% crude protein, 0.15% crude fat, 47.02% crude fiber, 0.28% calcium, 0.36% phosphorus, and 2.495 kcal/kg energy metabolism besides that betel nutshell waste contained 1.466% catechin (Mahata et al., 2018), The high crude fiber in betel nutshell waste was an inhibiting factor to be used in poultry ration. The poultry's digestive tract does not produce enzymes (cellulose, hemicellulose, and lignocellulose) to hydrolize crude fiber, therefore the utilization of betel nutshell in poultry ration would be limited and needed processing before feeding to poultry. To overcome the high crude fiber content in betel nutshell waste, it could be fermented with Indigenous Microorganisms (IMO) which had the required enzymes to degrade the crude fiber. Adrizal et al. (2017) reported several IMO from the vegetable waste mixture, such as bamboo sprout, fruit waste mixture, rice water waste, banana corm to reduce the crude fiber. The best IMO was obtained from bamboo sprout to reduce the crude fiber in the pineapple (*Ananas comosus* L. Merr.) waste, also it was founded that the crude fiber in the pineapple waste decreased from 24% to 17.16% after fermentation. Furthermore, Mahata et al. (2018) stated that the obtained IMO from the vegetable waste mixture was the best from other IMO (bamboo sprout, fruit waste mix, rice water waste and banana corm) to degrade the crude fiber in the betel nutshell waste. Crude fiber and dry matter content of fermented betel nutshell waste with IMO from vegetable waste mixture was 12.27% dry matter and 25.95% crude fiber (Mahata et al., 2019). In addition, fermented betel nutshell waste contained some chemical active compounds and fatty acids such as 0.174% catechins, 354.520 mg/100g tannins, 0.663% laurate, 34.745% miristate, 55.116% palmitate, 0.527% stearate, 3.731% oleic, and 0.618% linoleate (Mahata et al., 2018).

Catechin contained in betel nutshell waste is a phytochemical substance that is naturally produced and contains flavonoid compounds. Catechin potentially improved carcass and meat quality of broiler through reducing cholesterol and fat. According to Kara et al. (2016a), catechins had effective antioxidant, hypoglycemic and hypocholesterolemic properties, and also potentially increased meat quality and had no negative effects on quail performance. Some researchers reported that the administration of catechins reduced total cholesterol, and it did not affect total protein levels in the blood serum of poultry (Abdo et al., 2010; Ariana et al., 2011; Kara et al., 2016b). Furthermore, Tang et al. (2002) indicated that the catechin could reduce fat in broiler meat. Also Yunarto et al. (2015) reported that the administration of extracted catechin with ethyl acetate from gambier (*Uncaria gambir* Roxb.) leaf would reduce cholesterol in the blood serum of rat by inhibiting HMG-CoA reductase activity to synthesis mevalonate from HMG-CoA in cells, so the synthesis of total cholesterol and LDL would decrease, while HDL would increase. There is no published study about the effect of fermented betel nutshell waste with obtained IMO from the vegetable waste mixture on the performance and blood profile of broiler chickens, and so the present research was decided to carry out the effectiveness.

MATERIALS AND METHODS

Experimental site

The experiment was performed at the Universitas Andalas farm, Limau Manis Campus, West Sumatra Province, Indonesia. The area is located at an altitude of ± 255 meters above the sea level and approximately 15 kilometer from Padang, the capital city of West Sumatra Province (Website Universitas Andalas, 2019).

Dietary treatment

The feed consumed in this experiment was formulated according to the standards for broiler feed. The feeds in the diet were corn, commercial feed (bravo 511), rice bran, palm oil, bone meal, Fermented Betel Nutshell Waste (FBNSW) and Unfermented Betel Nutshell Waste (UBNSW). The five treatment rations used in this study were formulated to contain approximately 20% crude protein, and 2900 kcal/kg metabolic energy. The nutrient percentage and metabolic energy (kcal/kg) contained in the experiment ration are indicated in table 1.

Experimental design and treatments

Completely Randomized Design (CRD) with five treatments of FBNSW and UBNSW (0, 3, 4, and 6% FBNSW, and 6% UBNSW) were used in this study. Each treatment was repeated four times. Twenty pens were used for the 80 day-old chickens, and four chickens in each pen were randomly assigned.

Table 1. Food compositions, nutrient content, and metabolic energy of experimental ration in broiler chickens

Feed compositions (%)	FBNSW and UBNSW treatments in ration				
	0% FBNSW	2% FBNSW	4% FBNSW	6% FBNSW	6% UBNSW
Corn	30.00	29.50	29.00	28.50	26.50
Soybean meal	20.00	20.00	20.00	20.00	21.00
Coco nut oil	2.00	2.25	2.50	2.75	3.00
Rice bran	11.50	9.75	8.00	6.25	7.00
Fermented betel nutshell waste	0.00	2.00	4.00	6.00	0.00
Unfermented betel nutshell waste	0.00	0.00	0.00	0.00	6.00
Bone meal	1.50	1.50	1.50	1.50	1.50
Bravo 511 (commercial feed)	35.00	35.00	35.00	35.00	35.00
Total	100.00	100.00	100.00	100.00	100.00
Nutrient content and energy metabolismof experiment ration					
Crude protein (%)	20.72	20.72	20.71	20.71	20.55
Crude fat (%)	6.47	6.60	6.74	6.87	6.74
Crude fiber (%)	5.44	5.66	5.89	6.11	8.04
Calcium (%)	0.90	0.95	0.99	1.05	0.91
Phosphorus (available) (%)	0.52	0.54	0.56	0.58	0.36
Energy metabolism(kcal/kg)	2.930.45	2.933.88	2.937.32	2.940.75	2.925.42
Catechin (g/kg ration)	0.00	0.03	0.06	0.10	0.88

FBNSW = Fermented Betel Nutshell Waste, UBNSW = Unfermented Betel Nutshell Waste. g = gram, kcal = kilo calorie, kg = kilogram

Rearing condition

The chicken cages, food and drinking equipment were cleaned and disinfected before the day-old chickens arrived. The lighting for each cage was 100-watts. Food and drinking water were given *ad libitum*. Day-old chickens (Charoen Pokphand 707, commercial broiler strains) were purchased from poultry shop in Padang City, West Sumatra Province, Indonesia.

Measured characteristics

Daily feed intake was measured by calculating the difference between delivered and denied food which were recorded for a trial period (28 days) in the morning. The average daily feed intake per bird was calculated by dividing the total amount of feed provided and refusals for a trial period (28 days). Afterwards the feed intake was divided for 28 days (trial period). Daily weight gain was calculated as the difference between the mean final and mean initial body weights divided by the number of experimental days (28 days). Feed conversion: It was determined by dividing the average daily feed intake with a mean daily weight gain. Body weight at the end of the study (28 days) was weighed. Twelve hours before weighing, the chickens were fasted. One bird was taken randomly from each pen for measuring the body weight. The percentage carcass fraction was measured at the end of the experiment, and one broiler was randomly selected from each replication. The broiler was slaughtered and weighed after 12 hours starvation. Exception of lung and kidney, the viscera, head, shank, trachea, heart, liver, gizzard, and skin of slaughter broiler were eviscerated and expressed as carcass weight (gram). Furthermore, carcass percentage was calculated by dividing the carcass weight (gram) by the body weight (gram), and then multiplying by 100%. The abdominal fat pad percentage was obtained by comparing abdominal fat pad weight with body weight and then multiplying by 100%. Total cholesterol of broiler blood serum was calculated by Elitech group (2012) method. Up to 10 microliter (µl) of broiler blood serum was pipetted and then poured in the test tube, and 1000 µl cholesterol reagent was added to the test tube, further mixed and incubated for 10 minutes. Afterwards, it calculated by a photometer. The High-Density Lipoprotein (HDL) in the broiler blood serum was calculated by using the Elitech group (2012) method. Broiler blood serum was pipetted up to 250 µl, then the reagent of HDL up to 500 µl added afterwards centrifuged for 10 minutes at the speed of 2500 rpm. Centrifuge results (supernatant) pipetted up to 100 µl was added to the cholesterol reagent up to 1000 µl mixed, and incubated for 10 minutes, afterwards, it was calculated by using a photometer. The Low-Density Lipoprotein (LDL) levels were determined by using Friedewald et al. (1972) formula. LDL = Total cholesterol – HDL – 1/5 Trigliseride.

Statistical Analysis

All data were analyzed by analysis of variance (ANOVA). Duncan's multiple range tests were used to determine the differences between the treatment agents (Stell and Torrie, 1995).

RESULTS AND DISCUSSION

The inclusion of FBNSW and UBNSW in broiler ration influenced daily feed intake and daily weight gain significantly ($p < 0.05$), while feed conversion did not affect ($p > 0.05$) (Table 2). The daily feed intake and daily weight gain of broilers consumed 2, 4 and 6% of FBNSW in ratio greater than 0% FBNSW, and 6% UBNSW. These results indicated that the diets which contained 2, 4, and 6% FBNSW were more palatable by broiler compare with diets contained 0% FBNSW and 6% UBNSW. Increasing FBNSW content in broiler ration made the ration color a little darker than ration with 0% FBNSW and 6% UBNSW, while the color of the ration with 6% UBNSW resembled the color of ration with 0% FBNSW, so that the palatability of both rations (0% FBNSW and 6% UBNSW) were the same. The changing in ration color caused by increasing the FBNSW utilization in the ration did not affect the palatability of the broiler, although the ration's color was slightly darker.

Table 2. Average daily feed intake, daily weight gain, and feed conversion of broiler chicken fed fermented betel nutshell waste and unfermented betel nutshell waste for 4 weeks

Treatments	Daily feed intake (gram/bird/d)	Daily weight gain (gram/bird/d)	Feed conversion
0% FBNSW	81.32[b]	51.24[b]	1.59
2% FBNSW	85.83[a]	55.40[a]	1.55
4% FBNSW	85.83[a]	54.86[a]	1.57
6% FBNSW	85.89[a]	54.34[a]	1.58
6% UBNSW	82.02[b]	49.53[b]	1.66
SE	0.67	0.73	0.001

SE= Standard error, FBNSW= Fermented Betel Nutshell Waste, UBNSW = Unfermented Betel Nutshell Waste. [a, b, c, d] = Means in a row that are not followed by the same letters are significantly different at ($p < 0.05$). g = gram, d = day

According to Sulasmi et al. (2013), the ration's colors with brownish-yellow was more palatable than ration with dark color. The reduction in daily feed intake of broiler on ration contained 6% UBNSW as the crude fiber content was high compared to ration contained FBNSW. Fermentation treatment will increase the various of nutrients digestibility such as organic matter, nitrogen, amino acids, fiber, and calcium, and also increase feedstuff palatability (Canibe and Jensen, 2012; Shahowna et al., 2013). In this experiment, it was found that the average range of daily feed intake of broiler (4-weeks-old) was 81.31 to 85.89 g/bird/d. These results were almost the same with the experiment reported by Selle et al. (2019), where the average of broiler's daily feed intake of broiler chickens fed 28-days with sorghum for was between 83.36 and 89.25 (g/bird/d). The daily weight gain of the broiler specified according to feed consumption. A ration with 2, 4, and 6% FBNSW had an effect on increasing daily weight gain of the broiler and was above 0 % FBNSW and 6% UBNSW. According to Uzer et al. (2013) the increase in body weight gain was closely related to the feed consumption. A ration with 6% UBNSW reduced the daily weight gain of the broiler as it contained a high proportion of crude fiber compared to other rations. According to Kras et al. (2013), high crude fiber in poultry rations was not recommended as it had a negative effects on nutrient utilization, low body weight gain, and bad conversion rate. In addition, due to the low daily weight gain of the broiler chickens, the present study was suspected to contain 6% UBNSW with high tannin contain. According to Mahata et al. (2018), the tannin content in UBNSW was 456.59 mg/100, while in FBNSW was 354.52 mg/100g. Tannins inhibited the process of protein digestion, while protein is a substance needed for growth and muscle building. Cook (2000), indicated that tannin bound to proteins and reduced metabolic protein, disrupting growth. The average daily weight gain of a 4-week-old broiler in this study ranged from 49.53 to 55.40 g/bird/d. The results of this study were almost the same as those reported by Selle et al. (2019), where the average daily weight gain of broiler fed sorghum for 28 days was between 54.25 and 55.56 g/bird/d. Increasing daily feed intake and daily weight gain of broiler which consumed 2, 4 and 6% of FBNSW in the ration did not match the feed conversion, and the ration efficiency for all treatments (0, 2, 4, 6% FBNSW and 6% UBNSW) were the same, because the nutritional substances in all of the treatments for the normal life of the broiler is sufficient. In this experiment, protein and energy were adjusted to iso-protein and iso-energy for all rations. The feed conversion index increased when the ratio between the amount of energy in the ration and protein content was technically adjusted (Mookiah et al., 2014). Furthermore, Andriyanto et al. (2015) stated, the only factor that affected the value of feed conversion was the nutrition quality. The average feed conversion of a 4-week-old broilers in this study ranged from 1.55 to 1.66. The results of the present study were almost the same as those reported by Zampiga et al. (2018), where the average feed conversion of broiler feed conversion was between 1.494 and 1.524 for 33 days.

The inclusion of FBNSW and UBNSW in the broiler ration significantly affected body weight, percentage of abdominal fat pads, and percentage of carcass ($p > 0.05$) (Table 3). The body weights of the broilers, that consumed

rations with 2, 4, and 6% of FBNSW were higher than the body weight of broilers, consumed 0% FBNSW and 6% UFBNSW. The high body weight of the broiler, which consumed 2, 4, and 6% of FBNSW, was associated with high daily feed intake and daily weight gain in these treatments so that nutrient uptake to grow and produce high body weight was met. In addition, the digestibility of FBNSW in the ration was higher than the rations contained UBNSW in terms of fermentation efficiency, and the acceleration of digestive enzymes in the broiler digestive tract on FBNSW was optimum to better utilize nutrients from broilers for growth.

Table 3. Average body weight, abdominal fat pad percentage, and carcass percentage of broiler chicken fed fermented betel nutshell waste and unfermented betel nutshell waste for 4 weeks,

Treatments	Body weight (gram/bird)	Abdominal fat pad percentage (%)	Carcass percentage (%)
0% FBNSW	1204.00^{a}	1.58^{a}	70.39^{b}
2% FBNSW	1361.50^{c}	1.38^{a}	71.90^{a}
4% FBNSW	1315.75^{bc}	0.95^{b}	72.37^{a}
6% FBNSW	1248.75^{b}	0.93^{b}	70.64^{b}
6% UBNSW	1162.50^{a}	0.90^{b}	69.28^{c}
SE	24.23	1.15	0.31

SE= Standard error, FBNSW= Fermented Betel Nutshell Waste, UBNSW = Unfermented Betel Nutshell Waste. $^{a, b, c, d}$= Means in a row that are not followed by the same letters are significantly different at ($p < 0.05$).

Table 4. Average lipoprotein (total cholesterol, LDL and HDL) of broiler chicken fed fermented betel nutshell waste and unfermented betel nutshell waste for 4 weeks

Treatments	Total cholesterol (mg/dl)	Low-density lipoprotein (mg/dl)	High-density lipoprotein (mg/dl)
0% FBNSW	160.50^{a}	120.25^{a}	30.62^{b}
2% FBNSW	149.50^{b}	109.25^{b}	30.55^{b}
4% FBNSW	143.75^{b}	105.75^{b}	28.00^{b}
6% FBNSW	125.25^{c}	87.25^{c}	29.00^{b}
6% UBNSW	153.00^{b}	109.25^{b}	33.75^{a}
SE	2.14	1.97	0.90

SE= standard error, FBNSW= Fermented Betel Nutshell Waste, UBNSW = Unfermented Betel Nutshell Waste. $^{a, b, c, d}$= Means in a row that are not followed by the same letters are significantly different at ($p < 0.05$).

According to Sari and Purwadaria (2004) generally, all fermented end-products typically contained compounds that are simpler and more digestible than the original ingredients. The low body weight of the broiler in treatment 6% UBNSW was caused by a higher tannin content (456.59 mg/100g) than the treatments 2, 4, and 6 in which FBNSW was 354.52 mg/100g (Mahata et al., 2018). Tannin could not be digested by poultry because the digestive tract of poultry did not produce tannase enzyme to hydrolyze tannins, and tannin bound the protein in UBNSW. In addition, tannin affected the reduction of feed consumption. Anita et al. (2012) reported that the feed consumption of broiler chickens decreased up to 4.5% after feeding on aged tea leaves flour, as the tea leaves contained tannin the body weight of broiler chickens depended on the amount of protein consumed for meat production in its growth process. Although the body weight of the broiler in treatment 6% UBNSW was lower than treatment 2, 4, and 6% FBNSW ,the body weight was the same asin treatment 0% FBNSW in the ration. Inclusion of FBNSW and UBNSW in ration reduced the abdominal fat pad percentage of the broiler, due to FBNSW and UBNSWcontain catechin compounds. Catechin was a flavonoid class drived from polyphenol compound that could inhibit the formation and accumulation of fat by condensing catechin with bile salts as a fat solvent in the digestive tract of poultry. Therefore, the absorption process of fat in the digestive tract was disrupted and reduced, so that the production of abdominal fat was small. According to Koo and Cho (2004), catechin compounds contained in tea could reduce the fat content. The average percentage of broiler's abdominal fat pad in this study was between 0.90 and 1.58%. The percentage of broiler carcasses in treatments 2 and 4% of FBNSWwas higher than in the treatments 0 and 2% of FBNSW, and 6% of UBNSW (Table 3). The percentage of carcasses is closely related to body weight and percentage of abdominal fat pads. In the present study, the body-weight of broiler was also higher in 2, 4, and 6% FBNSW treatments than in the 0% FBNSW and 6% UBNSW treatments.

The inclusion of FBNSW and UBNSW in the broiler ration significantly affected total cholesterol, LDL, and HDL ($p < 0.05$) (Table 4). The total cholesterol in broiler blood serum was lower in the 6% FBNSW and 6% UBNSW treatments than in the 0, 2 and 4% FBNSW treatments in ration due to catechin content in FBNSW and UBNSW. According to Khalaji et al. (2011), revealed a significant reduction in total cholesterol in the broiler blood serum after feeding catechin containing green tea extract. The average total cholesterol level in blood serum was ranged from 125.25 to 160.50 mg/dL. The decreasing in total cholesterol content in broiler blood serum was also followed by a decrease in LDL due to the presence of tanase enzymes produced by IMO during the fermentation

process, and it reduced tannins in FBNSW. According to Velayutham et al. (2008), catechins reduce the production of apolipoprotein B, the main component of LDL, so that in this experiment, LDL decreased in broiler blood serum. As the total cholesterol decreased and affected the lower HDL synthesis in the liver of broiler chickens, the HDL levels in the blood serum of broiler chickens treated with 2, 4 and 6% also reduced.

CONCLUSION

The fermentation of betel nutshell waste by indigenous microorganism from vegetable waste mixture could be used up to 6 % in broiler ration which had a positive effect on performance, blood serum lipoprotein, and carcass quality of broiler chicken

DECLARATION

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REFERENCES

Abdo ZMA, Hassan RA, El-Salam AA and Helmy SA (2010). Effect of adding green tea and its aqueous extract as natural antioxidants to laying hen diet on productive, reproductive performance and egg quality during storage and its content ofcholesterol. Egypt Poultry Science, 30(4): 1121-1149.

AdrizalY, Amizar R and Mahata ME (2017). Evaluation of pineapple (*Ananascomosus*) (L.)Merr.) Waste fermented using different local microrganism solutions as poultry feed. Pakistan journal of nutrition, 16: 84-89. DOI: https://10.3923/pjn.2017.84.89.

Andriyanto AS, Satyaningtijas R, Yufiadri R, Wulandari VM, Darwin and Siburian SNA (2015). Performance and digestibility of broiler chicken feed fed with testosterone with multilevel doses. Acta Veterinaria Indonesiana, 3(1): 29-37. Available at: https://journal.ipb.ac.id/index.php/actavetindones/article/view/11191.

Anita WY, Astuti I and Suharto (2012). Effect of inclusion old tea leaf flour in the ration on the performance and abdominal fat percentage of broiler. Tropical Animal Husbandry, 1 (1): 1-6. Available at: https:// https://docplayer.info/34409279-Pengaruh-pemberian-tepung-daun-teh-tua-dalam ransum-terhadap-performan-dan-persentase-lemak-abdominal-ayam-broiler.html.

Ariana M, Samie A, Edriss M A and Jahanian R (2011). Effects of powder and extract form of green tea and marigold, and ±-tocopheryl acetate on performance, egg quality and egg yolk cholesterol levels of laying hens in late phase of production. Journal of Medicinal Plants Research, 5: 2710-2716. Available at: http://www.academicjournals.org/JMPR.

Badan pusat statistik indonesia (2016). Forestry production statistics. Publication Number: 05320.1706. Catalog: 5601005. P. 51. Available at: https://www.bps.go.id/publication/2017/11/27/dc0ea537e5f36f7ed3ac6c2d/statistik-produksi-kehutanan-2016.html.

Canibe N and Jensen BB (2012).Fermented liquid feed-Microbial and nutritional aspects and impact on enteric diseases in pigs. Animal Feed Science and Technology, 173: (1-2), 17-40. DOI: https://org/10.1016/j.anifeedsci.2011.12.021.

Cook ME (2000). The interplay between modern management practices and the chicken: how immune esponse and the physiological mechanism for growth and efficeincy have adapted over time. Where do we go from here? In: Biotechnology in the Feed Industry. Proceedings of Altech's 16th. Annual Symposium pp. 97-109. Available at: https://www.tib.eu/en/search/id/BLCP%3ACN067452536/The-interplay-between-modern-management-practices/.

Elitech group (2012). Clinical System. Reagents and Instruments. Available at: http://www.elitchgroup.com/corporate/home.

Friedewald WT, Levy RI and Fredrickson DS (1972). Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. Clinical Chemistry, 18: 499-502.

Investor Daily (ID) Indonesia (2015). Southeast Asia Soybean Meal Imports 23 Million Tons. Available at: https://investor.id/archive/2020-impor-bungkil-kedelai-asia-tenggara-23-juta-ton.

Kara K, Şentürk M, Guclu B K, Sariozkan S and Eren M (2016a). Effect of catechins on fattening performance, meat quality, some antioxidant and blood parameters and fattening costs in Japanese quail (Coturnix coturnix japonica), British Poultry Science, 57(4): 522-530. DOI: https://10.1080/00071668.2016.1174977.

Kara K, Güçlü BK, Şentürk M and Konca Y (2016b). Influence of catechin (flavan-3-ol) addition to breeder quail (*Coturnix coturnix japonica*) diets on productivity, reproductive performance, egg quality and yolk oxidative stability. Journal of Applied Animal Research, 44(1): 436-441. DOI: https://10.1080/09712119.2015.1091337.

Khalaji S, Zaghari M, Hatami KH, Hedari-Dastjerdi S, Lotfi L and Nazarian H (2011). Black cumin seeds, Artemisia leaves (Artemisia sieberi), and Camellia L. plant extract as phytogenic products in broiler diets and their effects on performance, blood constituents, immunity, and cecal microbial population. Poultry Science, 90: 2500–2510. DOI: https://10.3382/ps.2011-01393.

Koo MWL and Cho HC (2004). Pharmacological effects of green tea on thegastrointestinal system. European Journal of Pharmacology, 500: 177-185. DOI: https://10.1016/j.ejphar.2004.07.023.

Kras RV, Kessler AM, Ribeiro AML, Henn J, Santos II, Halfen DP and Bockor L (2013). Effect of dietary fiber and genetic strain on the performance and energy balance of broiler chickens. Brazilian Journal of Poultry Science, 15(1):15-20. DOI: https://org/10.1590/s1516-635x2013000100003.

Livestock and animal health statistics (2018). The ministry of agriculture the directorate general of animal husbandry and animal health. Indonesia. Available at:http://ditjenpkh.pertanian.go.id/userfiles/File/BukuStatistik_2018_Final_ebook.pdf?time=1543210844103.

Mahata ME, Rizal Y and Ardi (2018). Processing and utilization of Areca catechu L. waste as feed for poultry additives. Higher Education Competency Grant, Institute of Research and Community Service Andalas University, Available at: http://repo.unand.ac.id/21655/.

Mahata ME, Ardi and Rizal Y (2019). Selection of local microorganism solutions as crude fiber digester in *Areca catechu* L. Peel waste for producing poultry feed additives, International Journal of Poultry Science, 18 (2): 58-62. DOI: https://doi: 10.3923/ijps.2019.58.62.

Mookiah S, Sieo CC, Ramasamy K, Abdullah N and Ho YW (2014). Effect of dietary prebiotic, probiotic and synbiotics on performance, caecal bacterial populations and caecal fermentation concentrations of broiler chicken. Journal Science Food Agriculture, 94(2):341-348. DOI: https://doi.org/10.1002/jsfa.6365.

Sari L and Purwadaria T (2004). Evaluate the effect of mutans *Aspergillus niger*to the nutritive value of fermentation at coconut meal and karnel palm meal. Biodiversitas, 5(2):48-51. DOI: https://10.13057/biodiv/d050202.

Selle PH, Truong HH, Khoddami A, MossAF, Roberts TH and Liu SY (2019). The impacts of hammer-mill screen size and grain particle size on the performance of broilerchickens offered diets based on two red sorghum varieties, British Poultry Science, 60(3): 209-218. DOI: https://10.1080/00071668.2016.1257777.

Shahowna EM, Mahala AG, Mokhtar AM, Amasaib EO and Attaelmnan B (2013). Evaluation of nutritive value of sugar cane bagasse fermented with poultry litter as animal feed. African Journal of Food Science and Technology, 4(5):106-109. Available at: http://www.interesjournals.org/AJFST.

Situmorang AP (2018). Central Bureau of statistics noted that Indonesia imports corn to 72.710 ton in September 2018, Senin, 15 October 2018. 14:25. Available at: https://www.merdeka.com/uang/bps-catat-indonesia-impor-jagung-hingga-72710-ton-di-september-2018.html

Stell RGD and Torrie JH (1995). Statistical principles and procedures of a biometric approach. 2nd edition, translated by Bambang Sumatri. PT. Gramedia Pustaka Utama, Jakarta, Indonesia. Available at: https://onesearch.id/Record/IOS3774.JAKPU000000000005505.

Sulasmi, Sapsuha Y and Saelan E (2013). Effect of adding different types of legume leaf flour on consumption, body weight gain, and conversion of broiler rations. Agribusiness and Fisheries Journal, 6 (1): 10-16. DOI: https://doi.org/10.29239/j.agrikan.6.1.10-16.

Tang SZ, Kerry JP, Sheeban D and Buckley DJ (2002). Antioxidative mechanisms of tea catechins in chicken meat sysyems. Food Chemistry, 76: 45-51. DOI: https://10.1016/S0308-8146 (01)00248-5.

Tumova E and Teimouri A (2010). Fat deposition in the broiler chicken: a review. Scientia Agriculturae Bohemica, (41): 121-128. DOI: https://10.3382/ps/pex184.

Utami MMD, Pantaya D and Agus A (2018). Addition of Garlic Extract in Ration to Reduce Cholesterol Level of Broiler. Journal of Physics Conference Series, 953 (1): 012124. DOI: https://10.1088/1742-6596/953/1/012124.

Uzer F, Iriyanti N and Roesdiyanto (2013). The use of functional feed in the ration on feed consumption and body weight gain of the broiler. Journal of Animal Husbandry Scientific, (1): 282-288. Available at: http://id.portalgaruda.org/?ref=browse&mod=viewarticle&article=121574.

Velayutham P, Babu A and Liu D (2008). Green tea catechins and cardiovascular health: An up dare. Current Medicinal Chemistry, 15 (18): 1840-50. DOI: https://10.2174/092986708785132979.

Website Universita Andalas (2019). Available at: https://www.unand.ac.id/id/tentang-unand/selayang-pandang/lokasi-kampus.html.

YunartoN, Elya B and Konadi L (2015). Potential ethyl fraction of gambir leaf extract (Uncaria Gambir Roxb) as Anti-hyperlipidemia. Journal of Pharmacy, I5 (1): 1-10. DOI: https://10.22435/jki.v5i1.4080.1-10.

Zampiga M, Laghi L, Petracci M, Zhu C, Meluzzi A, Dridi S and Sirri F (2018). Effect of dietary arginine to lysine ratios on productive performance, meat quality, plasma and muscle metabolomics profile in fast-growing broiler chickens.Journal of animal science and biotechnology, 9:79.5-14. DOI: https://10.1186/S40104-018-0294-5.

Zhou H, Deeb N, Evock-Clover CM, Ashwell CM and Lamont J (2006). Genome-Wide Linkage Analysis to Identify Chromosomal Regions Affecting Phenotypic Traits in the Chicken. II. Body Composition. Poultry Science, 85(10):1712–1721. DOI: https://10.1093/ps/85.10.1712.

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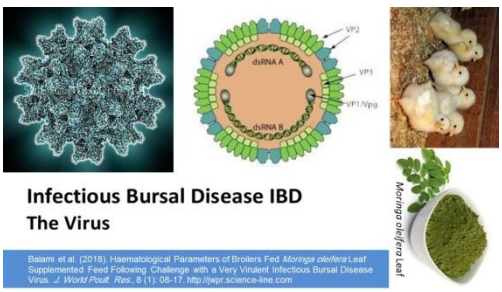

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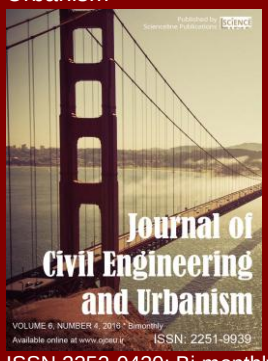

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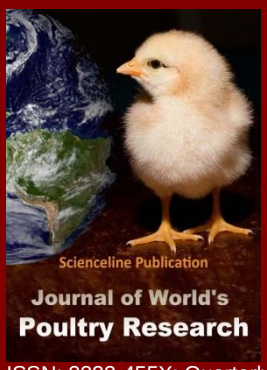

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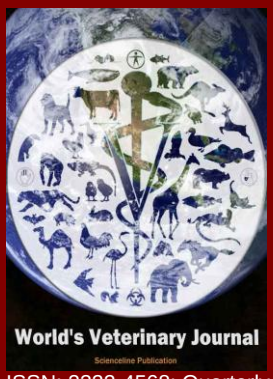

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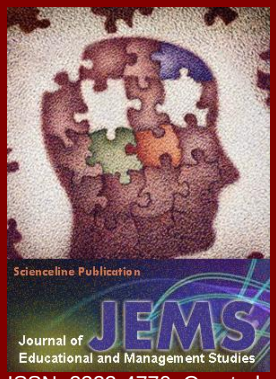

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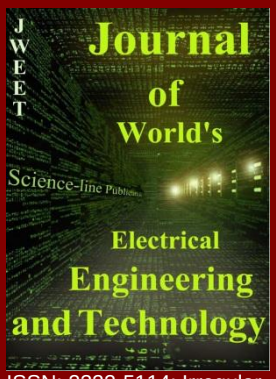

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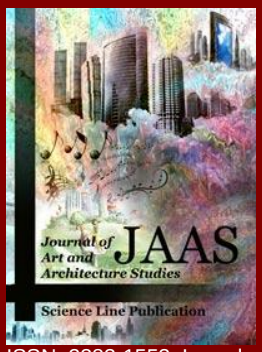

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