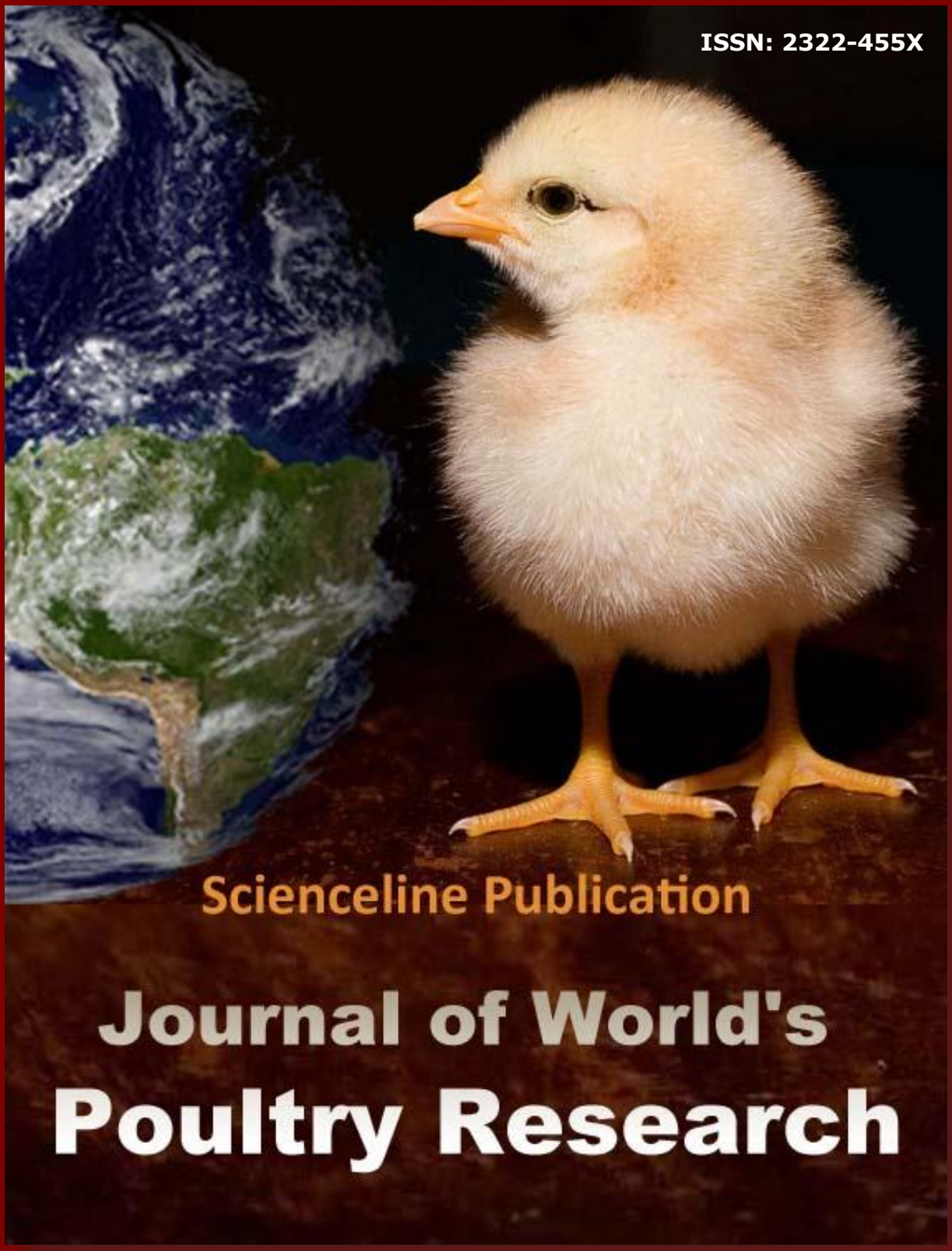
In the Name of God



An international peer-reviewed journal which publishes in electronic format

Volume 10, Issue 3, September 2020

Journal of World's Poultry Research

J. World Poult. Res. 10 (3): 407-544; September 25, 2020

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Research Paper

Genomic Analysis Reveals Strong Signatures of Selection in Guangxi Three-Yellow Chicken in China.

Liao Y, Sun J, Huang Y, Wei F, Mo G, Zellmer L and Liao DJ.
J. World Poult. Res. 10(3): 407-428, 2020; pii: S2322455X2000048-10
DOI: https://dx.doi.org/10.36380/jwpr.2020.48

ABSTRACT: Much like other indigenous domesticated animals, Guangxi Three-yellow chickens (GX-TYC) in China have experienced strong selective pressure, and show specific phenotypic changes in physiology, morphology and behavior. To identify genomic footprints or selection signatures left by artificial selection during domestication of GX-TYC, the whole genomes of 12 GX-TYC hens were sequenced to executed selective sweep analyses and gene functional enrichment analysis (Gene Ontology and Kyoto Encyclopedia of Genes and Genome pathways). A total of 10.13 million single nucleotide polymorphisms and 842,236 insertion/deletion polymorphisms (Indels) were found. Forty-six windows showed a Z score of heterozygosity (ZHp) lower than -5, which potentially were considered to be positively selected regions. Gene annotation identified 55 genes in these regions. Selection signatures were found mainly on the SSC5, SSC8, SSC23 and SSCZ. GO and KEGG analyses revealed that these genes were related to growth, immune responses as well as carbohydrate, lipid and amino acid metabolisms. In addition, two genes, fructose-1,6-bisphosphatase 1 and fructose-1,6-bisphosphatase 2 were enriched into four signaling pathways, three of which are involved in carbohydrate metabolism and insulin signaling. SHC3, FANCC and PTCH1, in combination with FB1 and FBP2, were clustered together in a region of chromosome Z, and thus might have been selected together. The results have uncovered some genetic footprints of chicken domestication, providing not only an important resource for further improvements of fowl breeding, but also a useful framework for future studies on the genetics of domestic chickens as well as on the phenotypic variations and certain diseases of chickens.
Key words: Chicken; Selective sweeps; Single nucleotide polymorphism; Whole genome resequencing

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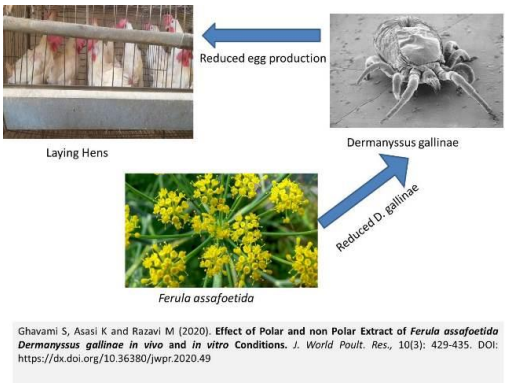
Research Paper

Effect of Polar and non Polar Extract of *Ferula assafoetida Dermanyssus gallinae in vivo* and *in vitro* Conditions.



Ghavami S, Asasi K and Razavi M.
J. World Poult. Res. 10(3): 429-435, 2020; pii: S2322455X2000049-10
DOI: https://dx.doi.org/10.36380/jwpr.2020.49

ABSTRACT: *Dermanyssus gallinae* is one of the most common arthropods in layers that affects the quality and quantity of egg production. Although there are different synthetic compounds against this mite, but despite these compounds, drug resistance and the presence of these compounds and synthetic compounds in meat and eggs makes that the use of alternative methods, as well as increased use of herbal extracts and essential oils. In the present study, the N-Hexane and Ethanol extracts of *Ferula assafoetida* were used. GC-MS analysis revealed the constituents of the two extracts. The lethal properties of the extracts were determined by contact toxicity. In this field study, ethanolic extract of *Ferula assafoetida* was sprayed on laying hens that infected with red mite. The most available compounds of the Ethanol extract and N-hexan extract of *Ferula assafoetida* were Diethylpyridine and Aurapten respectively in this study. The LC50 of Ethanol extract of *Ferula assafoetida* was 16 µg/cm^3 and *in vitro* study determined that ethanolic extract of *Ferula assafoetida* has been able to reduce the red mite population. This study indicated Ethanol extract of *Ferula assafoetida* could use as a substitute compound against red mite.
Keywords: Polar and nonpolar extract, *Ferula assafoetida*, Red mite

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Research Paper

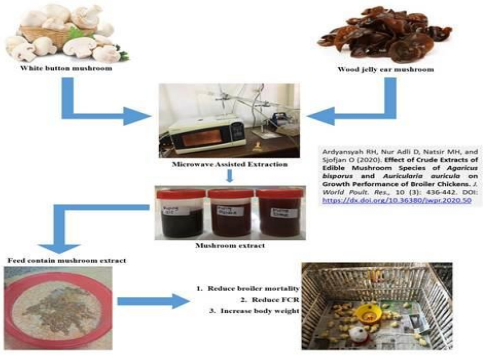
Effect of Crude Extracts of Edible Mushroom Species of Agaricus bisporus and Auricularia auricula on Growth Performance of Broiler Chickens.

Ardyansyah RH, Nur Adli D, Natsir MH, and Sjofjan O.
J. World Poult. Res. 10(3): 436-442, 2020; pii: S2322455X2000050-10
DOI: https://dx.doi.org/10.36380/jwpr.2020.50

ABSTRACT: The current study aimed to evaluate the effect of *Agaricus bisporus* and *Auricularia auricula* crude extracts as feed additives on the growth performance of broiler chickens. The samples included 240 one-day-old chicks randomly

divided into 8 dietary treatments, each treatment consisted of 3 replicates with 10 chicks per replicate. The dietary treatment groups were control group (T0), basal diet + zinc bacitracin (T1), basal diets + 0.4% *Agaricus bisporus* extract (T2), basal diet + 0.8% *Agaricus bisporus* extract (T3), basal diet + 1.2% *Agaricus bisporus* extract (T4), basal diet + 0.4% *Auricularia auricula* extract (T5), basal diet + 0.8% *Auricularia auricula* extract (T6), and basal diet + 1.2% *Auricularia auricula* extract (T7). The measured variables included feed intake, body weight gain, feed conversion ratio, and production index. In addition, the study aimed to evaluate the reducing sugars level, antioxidant IC_{50}, and antimicrobial efficacy of mushroom extracts prepared using three different solvents (i.e., water, ethanol, and methanol). The findings indicated that methanolic extract contained higher reducing sugars and had better antimicrobial efficacy. The results of experimental research revealed that mushrooms crude extracts had no significant effects on the growth performance of broiler chickens.

Keywords: Antibiotic, Broiler performance, Extract, Mushroom



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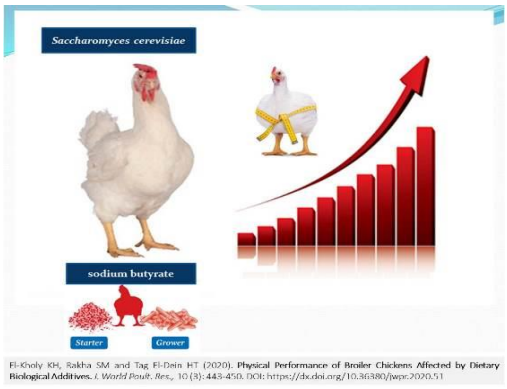
Research Paper

Physical Performance of Broiler Chickens Affected by Dietary Biological Additives.

El-Kholy KH, Rakha SM and Tag El-Dein HT.
J. World Poult. Res. 10(3): 443-450, 2020; pii: S2322455X2000051-10
DOI: https://dx.doi.org/10.36380/jwpr.2020.51



ABSTRACT: The current study aimed to evaluate the effects of *Saccharomyces cerevisiae* yeast as a probiotic, compared to sodium butyrate as an organic acid on the productive performance of broiler chickens, with special attention to their economic efficiency. Therefore, 270 of one-day-old Hubbard broiler chickens were divided into 5 groups. The first group included chickens receiving basal ration without any treatment (and considered as a control group). The second group was composed of chickens treated with 0.2 g SB/kg, the third group embraced chickens treated with 0.3 g SB/kg, the fourth group included chickens treated with 0.2 g SC/kg, and the fifth group consisted of chickens treated with 0.3 g SC/kg. The obtained results showed that administration of sodium butyrate or yeast showed a significant improvement of final body weight (BW), body weight change, feed conversion ratio and performance index from third to fifth weeks of age. Nevertheless, all treated groups showed an insignificant effect in feed intake, compared to control group. Furthermore, the dietary addition of *Saccharomyces cerevisiae* was detected more profitable than sodium butyrate addition. Accordingly, it can be concluded that sodium butyrate and yeast can be successively used as a natural substitute for antibiotic growth-promoting agents in the broiler chickens. Yeast can be considered as the most important alternative followed by sodium butyrate.

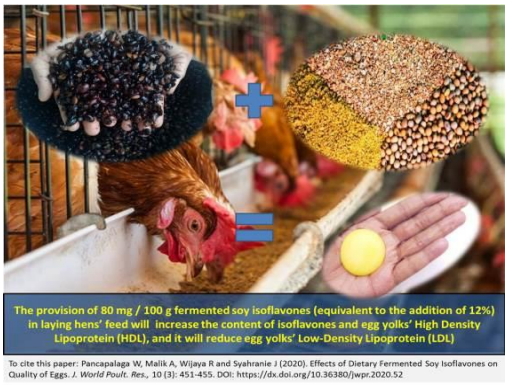
Keywords: Carcass, Economic efficiency, Hubbard; Productive, Sodium Butyrate, Yeast.

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

Research Paper

Effects of Dietary Fermented Soy Isoflavones on Quality of Eggs.

Pancapalaga W, Malik A, Wijaya R and Syahranie J.
J. World Poult. Res. 10(3): 451-455, 2020; pii: S2322455X2000052-10
DOI: https://dx.doi.org/10.36380/jwpr.2020.52



ABSTRACT: The present study aimed to examine the effects of fermented soy isoflavones on the poultry feed towards the quality of eggs. A total of 100 Isa Brown chickens aged 32 weeks were divided into 4 groups and 5 replicates, including T0 (control feed without the provision of fermented soy isoflavones), T1 (feed with 4% of fermented soy isoflavones), T2 (feed with 8% of fermented soy isoflavones), and T3 (feed with 12% of fermented soy isoflavones). The treatments were given for 10 weeks. The observed chemical qualities of eggs included HDL, LDL, isoflavones in egg yolks, physical quality (e.g., their weight and eggshell thickness). All the data were analyzed by using analysis of variance. The results showed that the administration of fermented soy isoflavones in poultry feed had no significant effect on egg weight and eggshell thickness ($p > 0.05$), but had a significant effect on HDL, LDL, and isoflavones in egg yolk ($p < 0.01$). Based on the results, it can be concluded that 80 mg/100 g of the fermented soy isoflavones (equivalent to the addition of 12%) in laying hens' feed would increase the content of isoflavones and egg yolks' HDL, reduce egg yolks' LDL, and lead to no significant change in the weight and thickness of eggshells.

Keywords: Feed, Fermented, Isoflavones, laying hens, Soybean

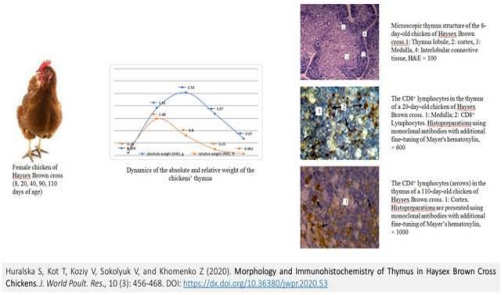
[Full text-PDF] [XML] [Crossref Metadata]

Research Paper

Morphology and Immunohistochemistry of Thymus in Haysex Brown Cross Chickens.

Huralska S, Kot T, Koziy V, Sokolyuk V, and Khomenko Z.

J. World Poult. Res. 10(3): 456-468, 2020; pii: S2322455X2000053-10
DOI: https://dx.doi.org/10.36380/jwpr.2020.53



Huralska S, Kot T, Koziy V, Sokolyuk V, and Khomenko Z (2020). Morphology and Immunohistochemistry of Thymus in Haysex Brown Cross Chickens. *J. World Poult. Res.*, 10 (3): 456-468. DOI: https://dx.doi.org/10.36380/jwpr.2020.53

ABSTRACT: Thymus plays an important role in the development and regulation of immune responses and other physiological processes. The present study aimed to examine the morphological and immunohistochemical changes of the thymus in Haysex Brown cross chickens with regard to their age. The morphofunctional studies of thymus were performed to determine and analyze age-related changes in anatomical (absolute and relative thymus mass), histological (area of the thymus cortex and medulla, area of connective tissue base, cortex index, number of thymic corpuscles in the lobule), and immunohistochemical (subpopulations of lymphocytes with surface markers CD4+ and CD8+ and their differentiation status) indexes. The study demonstrated that maximum morphological development of thymus could be observed in 20 and 40-day-old chickens. Up to 20 days of age, thymus mass increased proportionally with the chicken body weight. The results indicated that 40-day-old chickens had 1.46 times increase in the cortex index, compared to 20-day-old birds and 1.82 times compared to 8-day-old birds. An increase in the density and number of T-lymphocytes with surface markers (CD4+) and was evident through aging. Regarding aging-associated alternations, the differentiation index (CD4+: CD8+ ratio) reached 1.26±0.09, 1.52±0.25, and 1.56±0.23 in 40, 90, and 110-day-old chickens, respectively. The histological and cell parameters of the thymus in clinically healthy chickens can be used as indicators of normal functioning and to diagnose immunodeficiency in birds.
Keywords: CD8 Positive Lymphocytes, CD4 Positive Lymphocytes, Chickens, Cortex Index, Thymus, Immunohistochemical Characteristics

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Research Paper

The Efficacy of Synbiotic Application in Broiler Chicken Diets, Alone or in Combination with Antibiotic Growth Promoters on Zootechnical Parameters.

Syed B, Wein S and Ruangapanit Y.

J. World Poult. Res. 10(3): 469-479, 2020; pii: S2322455X2000054-10
DOI: https://dx.doi.org/10.36380/jwpr.2020.54

Syed B, Wein S and Ruangapanit Y (2020). **The Efficacy of Synbiotic Application in Broiler Chicken Diets, Alone or in Combination with Antibiotic Growth Promoters on Zootechnical Parameters.** *J. World Poult. Res.*, 10 (3): 469-479. DOI: https://dx.doi.org/10.36380/jwpr.2020.54

ABSTRACT: In recent years, probiotics and synbiotics have gained considerable interest in poultry feeding as an alternative to antibiotics due to antibiotic resistance concerns. The objective of this dual study was to evaluate the efficacy of synbiotic supplementation alone or in combination with different Antibiotic Growth Promoters (AGPs), compared to the untreated control group of broiler chickens production performance. In the first experiment, a total of 1260 one-day-old male Ross 308 broiler chickens were randomly assigned to 7 diet treatments, with 6 replicates per diet treatment and 30 birds per replicate over a 42-day period. The diet treatments included a control diet based on corn-soybean without additives (T1), and the diet treatment with bacitracin (BMD 100 ppm, T2), colistin (10 ppm, T3), synbiotic (PoultryStar me, 0.5 kg/t, T4), a combination of synbiotic (0.5 kg/t) and bacitracin (60 ppm, T5), synbiotic (0.5 kg/t) and colistin (5 ppm, T6), synbiotic (0.5 kg/t), bacitracin (60 ppm), and colistin (5 ppm, T7). During the critical period of rearing from hatch to day 10, the synbiotic supplementation resulted in a significantly higher body weight gain than its combination with bacitracin. No other dietary treatment showed a remarkable improvement in the body weight gain, feed intake, or feed conversion ratio, compared to the only synbiotic application (T4) during the entire trial period. The tendency towards an improved feed conversion ratio was observed during the use of symbiotic (T4, 1.87), compared to the control group (T1, 1.93) during the entire trial period. Compared with the control group (T1, 2.78%), broiler mortality was also lower in the synbiotic group (T4, 1.11%). In the second experiment, a total of 1500 one-day-old male Ross 308 broiler chickens were randomly assigned to 4 diet treatments; with 15 replicates per diet treatment, and 25 birds per replicate over a 42-day period. The dietary treatments included a control group diet based on corn-soybean without additives (T1), and the treatment diets with bacitracin (BMD 1000 ppm, T2), synbiotic (PoultryStar me, 0.5 kg/t, T3), and a combination of synbiotic (0.5 kg/t) plus bacitracin (BMD 1000 ppm T4). Birds fed antibiotic or synbiotic alone or in a combination had numerically a higher body weight and an average daily gain than the control group. There was a tendency of improvement in the feed conversion ratio during the age of 1-24 days, and throughout the experimental period. The evaluated synbiotic could serve as an effective alternative to AGPs, such as bacitracin and colistin in broiler chicken diets, especially during the first crucial period. The synbiotic can serve this purpose without combining it with AGPs, such as colistin or bacitracin.
Keywords: Antibiotic growth promoter, Broilers, Performance, Synbiotic

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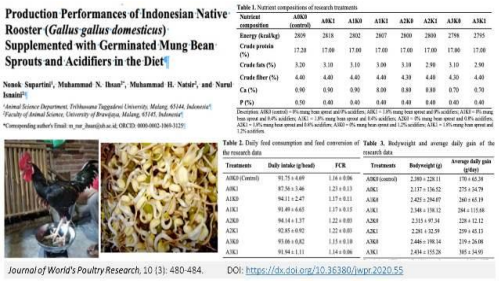
Research Paper

Production Performances of Indonesian Native Rooster (*Gallus gallus domesticus*) Supplemented with Germinated Mung Bean Sprouts and Acidifiers in the Diet.



Supartini N, Ihsan MN, Natsir MH, and Isnaini N.

J. World Poult. Res. 10(3): 480-484, 2020; pii: S2322455X2000055-10
DOI: https://dx.doi.org/10.36380/jwpr.2020.55

ABSTRACT: The research aimed to analyze the production performances of the Indonesian native rooster (*Gallus gallus domesticus*) fed germinated mung bean sprouts and acidifier supplementation in the diet. A total of 24 roosters aged 12 months with an average body weight of 2.29 ± 0.23 kg were used for the research subject. The diet was composed of a basic diet supplemented with 48-hours germinated mung bean sprouts and acidifier, with a basic no supplement diet as a control group. The research was conducted as an *in vivo* factorial randomized block design with different amounts of germinated mung bean sprouts (0% and 1.8%) and acidifiers (0%, 0.4%, 0.80%, and 1.20%) as the research treatment. Each treatment was performed in triplicate, and the observed production performances include Daily Intake (DI) of feed, Feed Consumption Ratio (FCR), Average Daily Gain (ADG), and Body Weight (BW). All data were analyzed using ANOVA (analysis of variance) and then tested by Tukey's test to determine significant differences. The results showed that the supplementation of mung bean sprouts and acidifiers did not give any differences from DI, FCR, ADG, and BW of *Gallus gallus domesticus*. However, the supplementation of germinated mung bean sprouts and acidifiers in the present research showed better overall production performances compared to the control group. The best production performance of the treatments was found at 1.8% germinated mung bean sprout and 1.2% acidifier additive based on the FCR (1.14±0.06) with DI at 91.94±1.11 gram (g)/head, ADG at 305.33±34.93 g/day, and final BW found after 30 days at 2,434.67±155.28 g. It has been concluded that the germinated mung bean sprout and acidifiers supplementation increases the production performance of *Gallus gallus domesticus*, with longer and higher supplement levels being suggested.
Keywords: Mung bean sprouts, Native chicken, Poultry diet, Production performances

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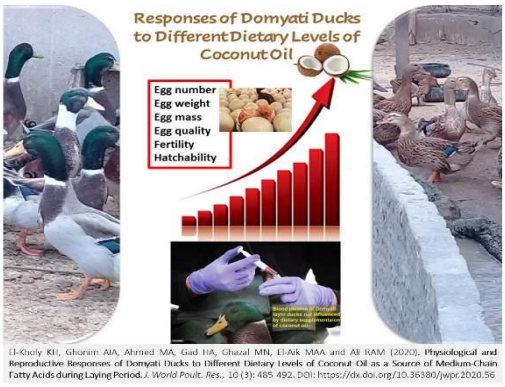
Research Paper

Physiological and Reproductive Responses of Domyati Ducks to Different Dietary Levels of Coconut Oil as a Source of Medium-Chain Fatty Acids during Laying Period.



El-Kholy KH, Ghonim AIA, Ahmed MA, Gad HA, Ghazal MN, El-Aik MAA and Ali RAM.

J. World Poult. Res. 10(3): 485-492, 2020; pii: S2322455X2000056-10
DOI: https://dx.doi.org/10.36380/jwpr.2020.56

ABSTRACT: The objective of this study was to determine the optimal level of coconut oil (CO) supplementation in the diet to enhance the performance of Domyati ducks. A total number of 300 Domyati ducks (240 females and 60 males) aged 25-week-old were randomly assigned to 4 experimental groups of three replicates, each replicate included 5 males and 20 females of Domyati ducks. The groups received CO at 0, 1.0, 1.5, and 2.0% during the experimental period. The results indicated that egg weight, egg number, and egg mass significantly increased in treated groups, compared to the control group. Furthermore, fertility and hatchability percentages were superior in 1.0, and 1.5% CO groups, compared to other experimental groups. Low-density lipoprotein in ducks that received CO was significantly lower than that of the control group. It is concluded that the inclusion of CO at a 1.5% level could be enough and useful for improving the reproductive and physiological performance of Domyati ducks.
Keywords: Blood, Coconut oil, Ducks, Egg, hatchability, Laying period

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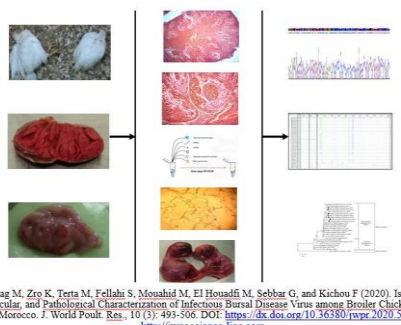
Research Paper

Isolation, Molecular, and Pathological Characterization of Infectious Bursal Disease Virus among Broiler Chickens in Morocco.



Cheggag M, Zro K, Terta M, Fellahi S, Mouahid M, El Houadfi M, Sebbar G, and Kichou F.

J. World Poult. Res. 10(3): 493-506, 2020; pii: S2322455X2000057-10

DOI: https://dx.doi.org/10.36380/jwpr.2020.57

ABSTRACT: Infectious bursal disease (IBD) is a contagious viral disease of young chickens that causes immunosuppression, mortality, and growth retardation. This pathology has severely affected the Moroccan poultry industry. The objective of the present study was the isolation, molecular characterization, and histopathology examinations of infectious bursal disease virus collected from 49 suspected farms from different regions of the country from 2013 to 2016. The real-time PCR results indicated that 41 out of 49 farm cases were found positive with a prevalence of 41% for classical virulent IBD virus (IBDV) and 59% for very virulent IBDV (vvIBDV). Pathological examinations showed the presence of two types of lesions, including acute to subacute lesions with a prevalence of 86%, and the sub-chronic to chronic lesions with a prevalence of 14%. The nucleotide and deduced amino acid sequences for the hypervariable region of VP2 for 7 vvIBDVs were compared to worldwide IBDV isolates and the findings suggested that it belonged to a group of very virulent strains. Phylogenetically, all the Moroccan vvIBDV field isolates were grouped in the same cluster with Malaysian and European vvIBDV isolates. This report demonstrated the continuous circulation of vvIBDV in commercial poultry farms in Morocco since 2013.
Keywords: Histological investigations, Infectious bursal disease virus, Phylogeny, Morocco, Virulent infectious bursal disease virus, VP2

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

Research Paper

Circulating Antibodies against Avian Influenza and Newcastle Disease in Semi-captive Peacocks in Southwestern Guatemala.

Castillo-Grijalva M, Guerra-Centeno D, Talgi Y, Valdez-Sandoval C, Lepe-López M and Santizo B.

J. World Poult. Res. 10(3): 507-512, 2020; pii: S2322455X2000058-10
DOI: https://dx.doi.org/10.36380/jwpr.2020.58

ABSTRACT: Avian Influenza and Newcastle disease are the two most important diseases of poultry and are globally considered as threats to public health and economy. There is little information published about these diseases in peacocks and other common backyard poultry in Guatemala. Therefore, an exploratory serosurvey was conducted to determine the presence of circulating antibodies to Avian Influenza (AI) and Newcastle Disease (ND) viruses in a semi-captive population of peacocks in southwestern Guatemala. Additionally, the circulation of antibodies to these pathogens in backyard chickens, ducks, and turkeys from a neighboring community was explored. Blood samples were obtained from 48 peacocks, 30 chickens, 6 ducks, and 4 turkeys. The samples were processed in the Regional Reference Laboratory for Animal Health, at the Veterinary Medicine Faculty, University of San Carlos of Guatemala, located in Guatemala City. Antibodies against AI virus were investigated by Agar Gel Immunodiffusion, and antibodies against ND virus were examined using Hemagglutination Inhibition. No antibodies against AI virus were detected. Most of the samples (97.7%) were negative for antibodies against ND virus, except for two turkeys that carried low antibody titers. The findings of the present study indicate that no virulent strains of AI or ND viruses were circulating in the investigated site.
Keywords: Avian influenza, Epidemiology, Newcastle disease, Serology, Zoonoses

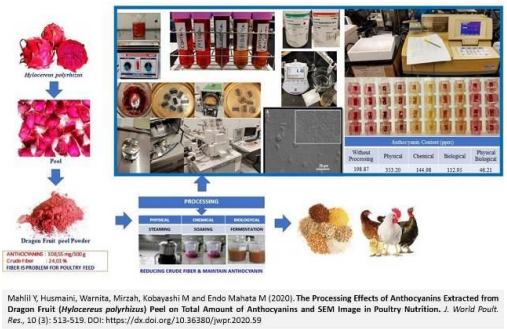
[Full text-PDF] [XML] [Crossref Metadata]

Research Paper

The Processing Effects of Anthocyanins Extracted from Dragon Fruit (Hylocereus polyrhizus) Peel on Total Amount of Anthocyanins and SEM Image in Poultry Nutrition.

Mahlil Y, Husmaini, Warnita, Mirzah, Kobayashi M and Endo Mahata M.

J. World Poult. Res. 10(3): 513-519, 2020; pii: S2322455X2000059-10
DOI: https://dx.doi.org/10.36380/jwpr.2020.59



ABSTRACT: The purpose of present study was to know the effects of different processing of anthocyanin content and scan electron microscope image of anthocyanin of dragon fruit peel in poultry diet. The experiment was performed in a completely randomized design with different processing like untreated dragon fruit peel or control, physical, chemical, biological, and physical-biological, and each treatment was replicated 4 times. Variables measured were total amount of anthocyanin and anthocyanin image of dragon fruit peel. The results indicated that physical treatment significantly increased anthocyanin content of dragon fruit peel. Furthermore, treated dragon fruit peel with chemical, biological, and combination of physical-biological significantly reduced anthocyanin content. The image of anthocyanin from each treated processing revealed that control image was similar to physical treatment, and it was different from other treatments. The physical treatment was the best method to increase anthocyanin content, and did not change the image of anthocyanin from dragon fruit peel.
Keywords: Anthocyanins, Dragon fruit peel, Processing, Scanning electron microscope, Spectrophotometry

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

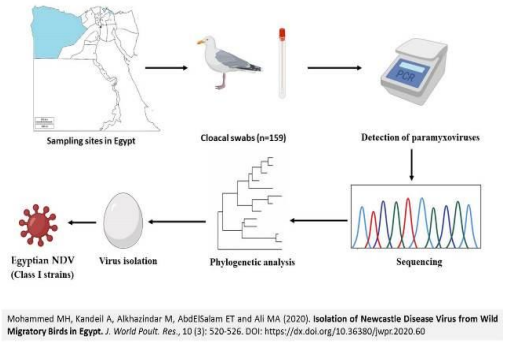
Research Paper

Isolation of Newcastle Disease Virus from Wild Migratory Birds in Egypt.

Mohammed MH, Kandeil A,

Alkhazindar M, AbdElSalam ET and Ali MA.

J. World Poult. Res. 10(3): 520-526, 2020; pii: S2322455X2000060-10
DOI: https://dx.doi.org/10.36380/jwpr.2020.60



Mohammed MH, Kandeil A, Alkhazindar M, AbdElSalam ET and Ali MA (2020). **Isolation of Newcastle Disease Virus from Wild Migratory Birds in Egypt.** *J. World Poult. Res.*, 10 (3): 520-526. DOI: https://dx.doi.org/10.36380/jwpr.2020.60

ABSTRACT: Surveillance studies for Newcastle disease virus (NDV) are critical to monitor the potential spreading of these viruses among wild birds as well as domestic poultry. This study was conducted to determine the incidence of NDV in wild birds in Egypt in 2016. Out of 159 collected samples from eight different species of wild birds, six (3.77%) samples were positive for paramyxoviruses by semi-nested RT-PCR assay based on the RNA-dependent RNA polymerase gene. Of six positive samples, four NDVs were successfully isolated in 11-day-old specific-pathogen-free embryonated hens' eggs. Partial sequences of the fusion gene of the four isolates were amplified using RT-PCR. Phylogenetic analysis of partial sequences of RNA-dependent RNA polymerase gene and fusion genes indicated that the detected NDV viruses in wild birds in Egypt are related to class I NDVs strains. Four Egyptian NDV isolates from wild birds exhibited sequence motif of 111GERQER↓LVG119 at the cleavage site as lentogenic virus in wild birds. Continuous active surveillance may help better monitoring of NDVs circulating in wild birds before newly emerging viruses in domestic poultry.
Keywords: Egypt, Fusion protein, Newcastle disease virus, Wild birds

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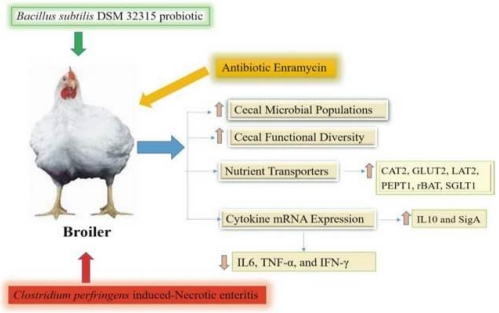
Research Paper

Effects of *Bacillus subtilis* DSM 32315 on Immunity, Nutrient Transporters and Functional Diversity of Cecal Microbiome of Broiler Chickens in *Necrotic Enteritis* Challenge.

Bodinga BM, Hayat Kh, Liu X, Zhou J, Yang X, Ismaila A, Soomro RN, Ren Zh, Zhang W and Yang X.

J. World Poult. Res. 10(3): 527-544, 2020; pii: S2322455X2000061-10
DOI: https://dx.doi.org/10.36380/jwpr.2020.61



Bodinga BM, Hayat Kh, Liu X, Zhou J, Yang X, Ismaila A, Soomro RN, Ren Zh, Zhang W and Yang X (2020). **Effects of Bacillus subtilis DSM 32315 on Immunity, Nutrient Transporters and Functional Diversity of Cecal Microbiome of Broiler Chickens in Necrotic Enteritis Challenge.** *J. World Poult. Res.*, 10 (3): 527-544. DOI: https://dx.doi.org/10.36380/jwpr.2020.61

ABSTRACT: This study was conducted to determine the effects of *Bacillus subtilis* DSM 32315 probiotic and antibiotic enramycin in broiler chickens with *Clostridium perfringens* induced-Necrotic enteritis on cecal microbial populations, functional diversity, nutrients transporters and cytokines mRNA expression. Day-old broilers (n= 360), Arbor Acre were randomly assigned to three dietary treatments such as control, basal diet fed-group only; antibiotic, basal diet plus enramycin 5 mg/kg; and probiotic group, basal diet plus *Bacillus subtilis* 2 x10^9 CFU/g. Antibiotic and probiotic fed groups was challenged with *Clostridium perfringens* at day1, and from day 14 to day 21. The results of present study showed that broiler chickens supplemented with antibiotic and probiotic significantly exhibited higher abundance of gut beneficial bacteria at the 21 and 35 days of age, while upregulated the expression of anti-inflammatory cytokine enterleukin-10 and secretory immunoglobulin-A. Expression of proinflammatory cytokines interleukin-6 tumor necrosis factor alpha, and interferon gamma were downregulated. Nutrient transporters of Peptide transporter-1, L amino transporter-2 and Cationic amino acid transporter-2 were upregulated in supplemented groups. More so, glucose transporter-2 Sodium glucose transporter-1, Solute carrier family 3, member 1, carbohydrates and vitamin metabolism cofactor enriched in probiotic fed-group, while control group exhibited up-regulation in interleukin-6, tumor necrosis factor alpha, and interferon gamma. Overall, supplementation of *Bacillus subtilis* DMS 32315 reduced the negative impact of necrotic enteritis in broiler chickens, and enhanced the gut-microbial community.
Keywords: Antibiotic growth promoter, *Bacillus subtilis*, *Clostridium perfringens,* Immune response, probiotic

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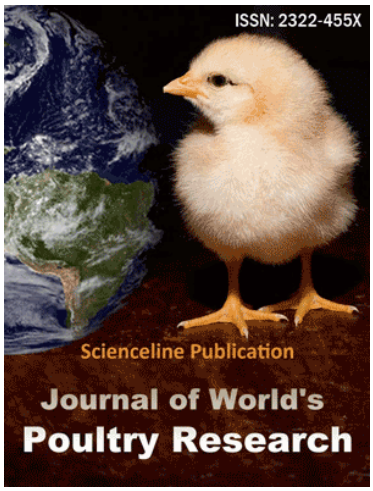
Journal of World's Poultry Research



ISSN: 2322-455X

Frequency: Quarterly

Current Issue: 2020, Vol: 10, Issue: 3 (September 25)

Publisher: SCIENCELINE

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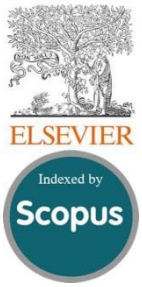

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Journal of World's Poultry Research

2020, Scienceline Publication

J. World Poult. Res. 10(3): 407-428, September 25, 2020

Research Paper, PII: S2322455X2000048-10

DOI: https://dx.doi.org/10.36380/jwpr.2020.48

Genomic Analysis Reveals Strong Signatures of Selection in Guangxi Three-Yellow Chicken in China

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Received: 29 Jun. 2020
Accepted: 20 Aug. 2020

ABSTRACT

Much like other indigenous domesticated animals, Guangxi Three-yellow chickens (GX-TYC) in China have experienced strong selective pressure, and show specific phenotypic changes in physiology, morphology and behavior. To identify genomic footprints or selection signatures left by artificial selection during domestication of GX-TYC, the whole genomes of 12 GX-TYC hens were sequenced to executed selective sweep analyses and gene functional enrichment analysis (Gene Ontology and Kyoto Encyclopedia of Genes and Genome pathways). A total of 10.13 million single nucleotide polymorphisms and 842,236 insertion/deletion polymorphisms (Indels) were found. Forty-six windows showed a Z score of heterozygosity (ZHp) lower than -5, which potentially were considered to be positively selected regions. Gene annotation identified 55 genes in these regions. Selection signatures were found mainly on the SSC5, SSC8, SSC23 and SSCZ. GO and KEGG analyses revealed that these genes were related to growth, immune responses as well as carbohydrate, lipid and amino acid metabolisms. In addition, two genes, fructose-1,6-bisphosphatase 1 and fructose-1,6-bisphosphatase 2 were enriched into four signaling pathways, three of which are involved in carbohydrate metabolism and insulin signaling. SHC3, FANCC and PTCH1, in combination with FB1 and FBP2, were clustered together in a region of chromosome Z, and thus might have been selected together. The results have uncovered some genetic footprints of chicken domestication, providing not only an important resource for further improvements of fowl breeding, but also a useful framework for future studies on the genetics of domestic chickens as well as on the phenotypic variations and certain diseases of chickens.

Key words: Chicken; Selective sweeps; Single nucleotide polymorphism; Whole genome resequencing

INTRODUCTION

Three-yellow chicken (TYC) is internationally well known for its desirable meat quality including juiciness, flavor and tenderness. They were named for their yellow feather, yellow beak and yellow feet. Three-yellow chicken is not a particular species, but rather is a collective name for those chicken breeds with these three yellow traits, including Huxu, Qingyuan, Xinghua, Huaixiang, Wenchang and Yangshan chickens in the Guangdong province, Pudong chicken in Shanghai, Xiaoshan chicken in the Zhejiang province, etc. (Zheng et al.1989). Guangxi three-yellow chicken (GX-TYC), a breed that has been intensively selected both naturally and artificially, is mainly distributed in Yulin, Beiliu, Bobai, Cenxi counties or cities in the Guangxi province as a typical traditional breed locally. Because of its aforementioned meat quality, GX-TYC has been widely used in the development of many special lines of yellow-feather broilers in China (Wei et al. 2019). For future breeding efforts to develop better breeds for the broiler industry, a better understanding of the GX-TYC domestication and identify genetic components obtained from various selections that are likely the consequence of GX-TYC domestication are needed. For these purposes, the whole genome sequencing approach to explore favorable alleles, candidate mutations or single nucleotide polymorphisms (SNPs), and insertions/deletions (Indel) of TX-TYC were used, and the resulting data were reported herein.

MATERIALS AND METHODS

Ethical approval

All animal procedures used in this study were carried out in accordance with the Guide for Care and Use of Laboratory Animals (8th edition, released by the National

Research Council, USA) and were approved by the Institutional Animal Care and Use Committee (IACUC) of Guangxi Institute of Animal Science.

Sequencing of the Guangxi Three-yellow chicken genome

Twelve GX-TYC hens raised at Chunmao Farming Co. Ltd. of Guangxi, China, were used in this study. Blood samples were collected from the wing vein using standard venipuncture. Genomic DNA was isolated from the blood samples with a bloodGen Mini Kit (Cwbiotech., China), and it was assessed for purity and quality using NanoDrop and gel electrophoresis. A pair-end library with insert sizes varying from 250 to 300 base-pairs (bp) was constructed and sequenced with the Illumina Hiseq 2000/2500 platform by BerryGenomics Biotechnology Co., Ltd., Beijing, China. Raw reads contained some interference information, including the adapter, low quality paired reads and unidentified nucleotides. Clean reads were obtained by removing this interference information (Li et al., 2010), and were mapped onto the chicken reference genome (*Gallus gallus*, Galgal 14.78) using the BWA software (Li and Durbin, 2009).

Single nucleotide polymorphisms and insertion/deletion polymorphisms Calling

After the alignment, SNP and InDel calling using a Bayesian approach implemented in the package SAMtools were performed. The 'mpileup' command was used to identify SNPs and InDels with the parameters as '-m 2 -F 0.002 -d 1000'. The identified SNPs were filtered with more stringent parameters, i.e., coverage depth ≥ 4, and Root Mean Square (RMS) mapping quality ≥ 20, to obtain high quality SNPs, which were annotated using the Ensembl gene sets (http://www.ensembl.org/biomart/). The SNPs and InDels in gene regions were annotated using the ANNOVAR annotation tool (Wang et al., 2010).

Selective sweep analysis

Selective sweep screen was performed with the sequenced DNA pools. Allele counts at each SNP position were used to detect signatures of selection in 200-Kb sliding windows with a step size of 50% overlapping for the genome sequences of GX-TYC. At each detected SNP position, the sums of major and minor alleles (n_{MAJ} and n_{MIN}) were determined, and then the corresponding heterozygosity score were calculated using the following formula: $Hp=2\sum n_{MAJ}\sum n_{MIN}/ (\sum n_{MAJ}+\sum n_{MIN})^2$. Individual Hp was then Z-transformed to a standard normal distribution as follows: $ZHp=(Hp-\mu Hp)/\sigma Hp$. A threshold of ZHp≤-5 was set for putative selective sweeps because windows below it ended the distribution (Rubin et al., 2012).

Analysis of functional enrichment

Functional enrichment analysis of Gene Ontology (GO), as well as Kyoto Encyclopedia of Genes and Genome (KEGG) pathways were performed using "Benjamini-corrected modified Fisher's exact test" in the DAVID web server (Huang et al., 2009). Genes were mapped onto their respective human orthologs. *P* values that indicated the significance of the overlap between various gene sets were corrected with Benjamini-Hochberg false discovery rate (FDR). Only were terms with a *P* value less than 0.05 considered significant, and were listed. The GO categories "biological processes", "molecular function" and "cellular component" were used in these analyses.

RESULTS AND DISCUSSION

Data production and short read alignment

Sequencing of the GX-TYC genome generated a total of 35.85 Gbs of paired-end DNA sequences, of which 35.58 (99.25%) Gbs of high quality paired-end reads were mapped onto the chicken reference genome assembly (*Gallus_gallus*, Galgal 4.78) with 33.66-fold sequence depth using Burrows-Wheeler-Alignment tool (BWA). Several categories of genetic variation, including SNPs and Indels were identified between the uniquely mapped reads and the reference genome.

Single nucleotide polymorphisms and insertion/deletion polymorphisms Identification

Mapping the sequencing reads to the reference genome revealed about 0.13 million SNPs, which exceeded the findings reported in the literature (Wong et al., 2004; Fan et al., 2013). A total of 4,332,562 (43%) SNPs located in genic regions, of which 125,732 were coding ones that leaded to 37,045 nonsynonymous nucleotide substitutions (291 stop gains, 47 stop losses and 36,707 being non-synonymous) detected in a total of 5,839 genes (Figure 1 and supplementary table 1). Identification of 842,236 small Indel polymorphisms ranging from 1 to 50 bps in length (Supplementary table 2) was done, which tended to be detected with a greater frequency than their longer counterparts. About 43% of the Indels were in genic regions, similar to the distribution of SNPs, of which 1613 located in coding sequences (Figure 1).

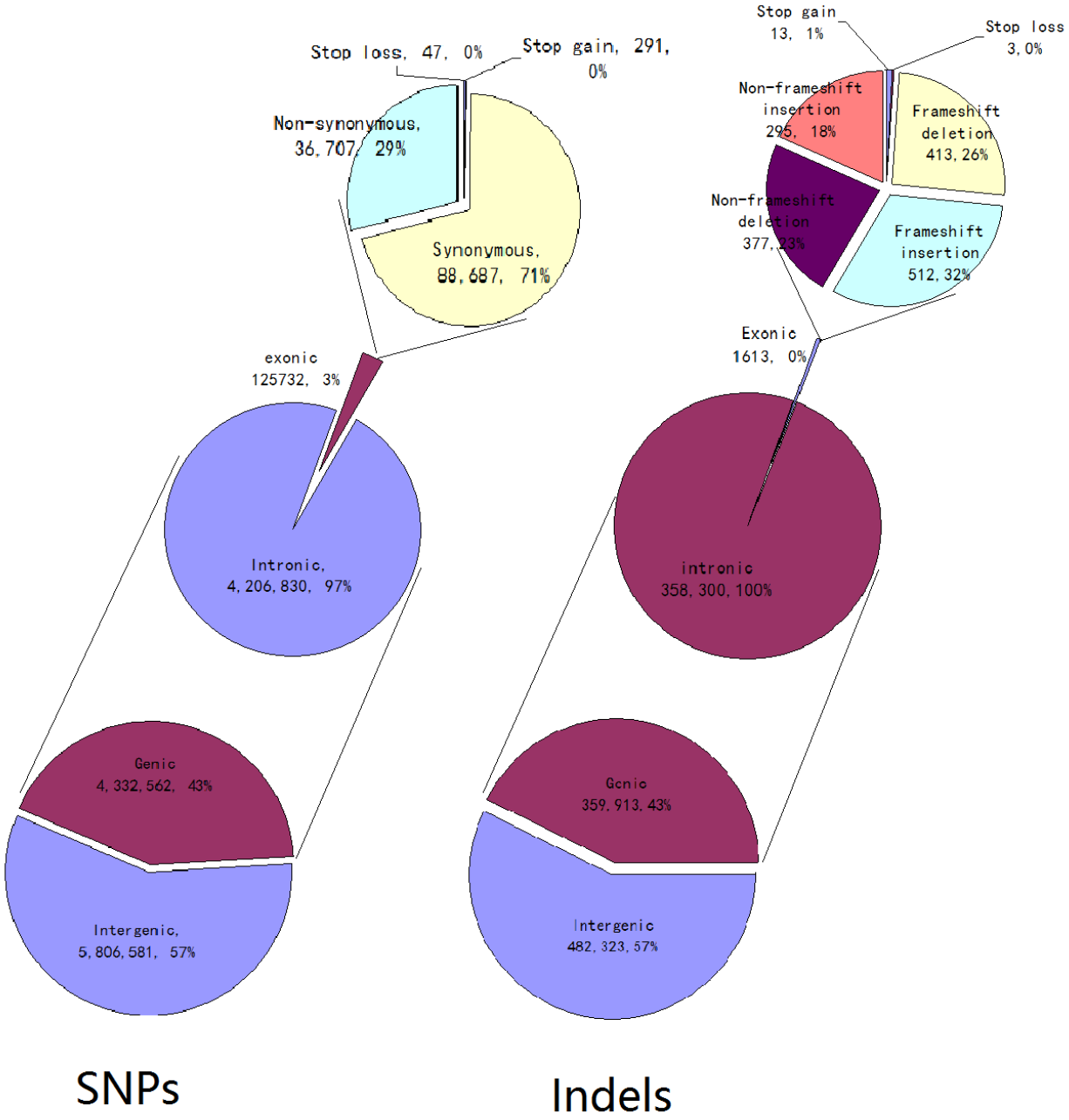

Figure 1. Annotation and distribution of single nucleotide polymorphisms and insertion/deletion polymorphisms

Potential independent signatures of selection in guangxi three-yellow chicken

Domestic animals were excellent models for genetic studies of phenotypic evolution (Andersson, 2001). They evolved genetic adaptations to new environments and were subjected to long-term artificial selections (Rubin et al., 2010). As a result of this process, marks in the proximity of genes influencing breed-defining traits were reduced levels of variability, and showed specific selection signature, including high population differentiation, greatly reduced variation, temporary increase in linkage disequilibrium, skewed allele frequency, and long-ranged haplotype homozygosity (Kaplan et al., 1989; Fay and Wu 2000; Kim and Stephan 2002; Kim and Nielsen 2004; Pollinger et al., 2005; Smith and Haigh, 2007). Selective sweep drew much attention, and a number of statistical tests, mostly based on summed statistics such as the tests by Lewontin and Krakauer (1973), Li et al. (1985), Tajima

(1989), McDonald and Kreitman (1991), Fu and Li (1993), Fu (1997), Fay and Wu (2000) and Sabeti et al. (2002). Recently, the commonly used method was H-based heterozygosity of SNPs and Fst-based genetic diversification (Rubin et al., 2012). To accurately detect the genomic footprints left by selection in the GX-TYC, a selective sweep screen was performed by searching for genomic regions with high degrees of fixation. The pooled heterozygosity Hp was calculated, in sliding 200-Kb windows crossing the chromosomes from sequence reads that correspond to the most and least frequently observed alleles at all SNP positions. The distribution of observed Hp values and the Z transformations of Hp and ZHp were marked in the Figure 2. The putative sweeps on those reaching a ZHp score of -5 or less were mainly described, as they are in the lower end of the distribution. In the genome-wide screen, only about 0.45% of windows (n=46) showed a Z score of heterozygosity (ZHp) lower than -5 (Figure 2 and supplementary table 3). Striking selection signatures were mainly found on the SSC5, SSC8 and SSCZ regions (Figure 2), while some windows that did not reach the significance threshold may have contributed significantly to chicken domestication. The strongest signature of selection (ZHp = -17.158) was observed at 2.20 to 2.24 Mbs on the chromosome 5, which included two genes, for instance SLC6A5 (Solute Carrier family 6, member 5) and NELL1 (neural EGFL like 1). The SLC6A5 gene encodes a sodium- and chloride-dependent glycine neurotransmitter transporter, which is an important glycoprotein for scavenging extracellular glycine in glycine-mediated neurotransmission. Mutation in this gene can cause hyperekplexia. The neural EGFL like (NELL) gene encoded a cytoplasmic protein that contained epidermal growth factor (EGF) -like repeats. The protein may be involved in cell growth regulation and differentiation in a variety of tissues, including heart muscle, skeletal muscle and blood vessels, and may promote osteoblast cell differentiation and terminal mineralization (Bokui et al., 2008). The NELL1 gene was identified in a selective sweep in broilers (Elferink et al., 2012). The biological functions of NELL1 may be related to the selection on the muskuloskeletal integrity in modern broiler chickens. Bone integrity was likely to be co-selected with growth rate and meat yield, as the skeleton of modern broilers needed to support a heavier weight (Zhou et al., 2007). The second convincing signature of selection (ZHp = -14.043) occurred on the sex chromosome Z that harbored the death-associated Protein Kinase 1 (DAPK1), cathepsin L2 (CTSL2), fructose-1,6-bisphosphatase 2 (FBP2) and fructose-1,6-bisphosphatase 1 (FBP1). Death-associated Protein Kinase 1 gene is a calmodulin-dependent serine-threonine kinase involved in a variety of cell signaling pathways that regulate cell survival, apoptosis and autophagy. Cathepsin L2, a lysosomal cysteine proteinase, has been shown to be particularly powerful in degrading myofibrillar components in post-mortem autolysis. In fish muscles, CTSL2 exhibits heat-stability on 50 to 60℃, and can degrade surimi protein during the manufacturing of silver carp surimi products (Li et al., 2008). fructose-1,6-bisphosphatase 1 that acts as a rate-limiting enzyme in gluconeogenesis, catalyzes the hydrolysis of fructose 1,6-bisphosphate to fructose 6-phosphate, and inorganic phosphate in the presence of divalent cations, and mediates in gluconeogenesis and carbohydrate biosynthesis. fructose-1,6-bisphosphatase deficiency is associated with hypoglycemia and metabolic acidosis. FBP1 and FBP2 are two important paralogs. Although there is a strong selective signature on chromosome 8, it was impossible to annotated any genes on it.

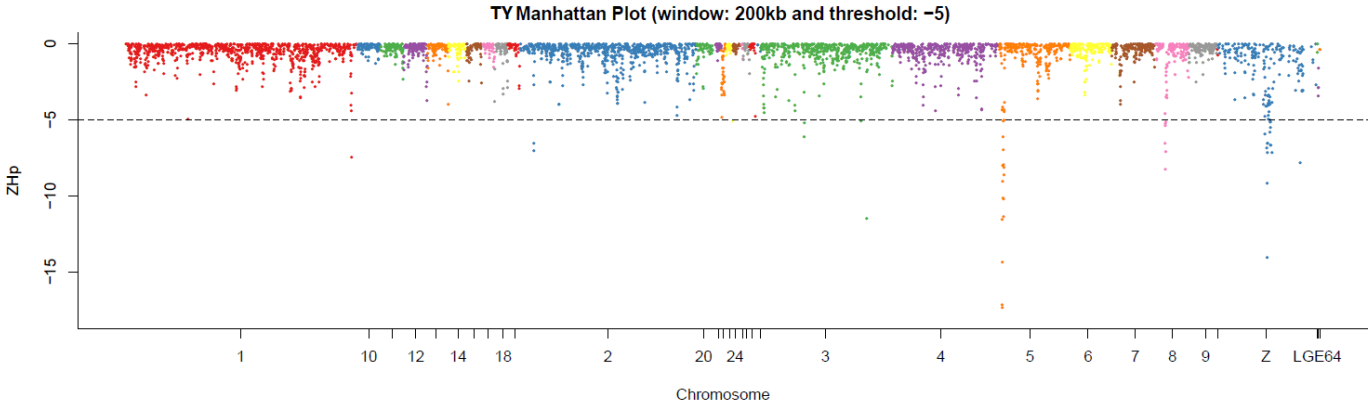

Figure 2. Genome-wide Z score of heterozygosity (ZHp) distribution. The Y axis is ZHp and the X axis shows positions of windows along each chromosome. Dotted lines indicate the thresholds with ZHp = -5.

Gene Ontology and Kyoto Encyclopedia of Genes and Genome pathways analyses

A total of 55 genes were identified in the regions that were considered to be positively selected (Supplementary table 3). Analysis of gene enrichment within this set of genes showed that, in biological-process (BP), significant enrichment for genes was primarily concentrated on the acid and anion transport, the hexos and monosaccharide metabolisms, the mesonephric development, and the defense response, whereas in cellular-component (CC) enrichment was potentially in cell periphery, plasma membrane and interleukin-28 receptor complex. In molecular-function (MF), enrichment was mainly concentrated on several sugar phosphatase activities and on rRNA (cytosine) methyltransferase activity (Figure 3 and Supplementary table 4). As gene enrichment analysis may yield high false-positive rates (Pavlidis et al., 2012), additional functional and physiological experiments were needed to verify the contribution of these genes to these processes. KEGG analysis identified eight pathways retaining a statistical significance (P<0.05), i.e. Hedehog signaling pathway (3 genes, P=0.0017), pentose phosphate pathway (2 genes, P=0.0059), fructose and mannose metabolism (2 genes, P=0.012), valine, leucine and isoleucine degradation (2 genes, P=0.020), insulin signaling pathway (3 genes, P=0.022), Fanconi anemia pathway (2 genes, P=0.026), glycolysis/gluconeogenesis (2 genes, P=0.028), as well as synthesis and degradation of ketone bodies (1 gene, P=0.049) (Figure 4, table 1 and Supplementary table 5). Most of these pathways were related to carbohydrate, lipid and amino acid metabolisms, while some were involved in processing genetic information and environmental information (Table 1).

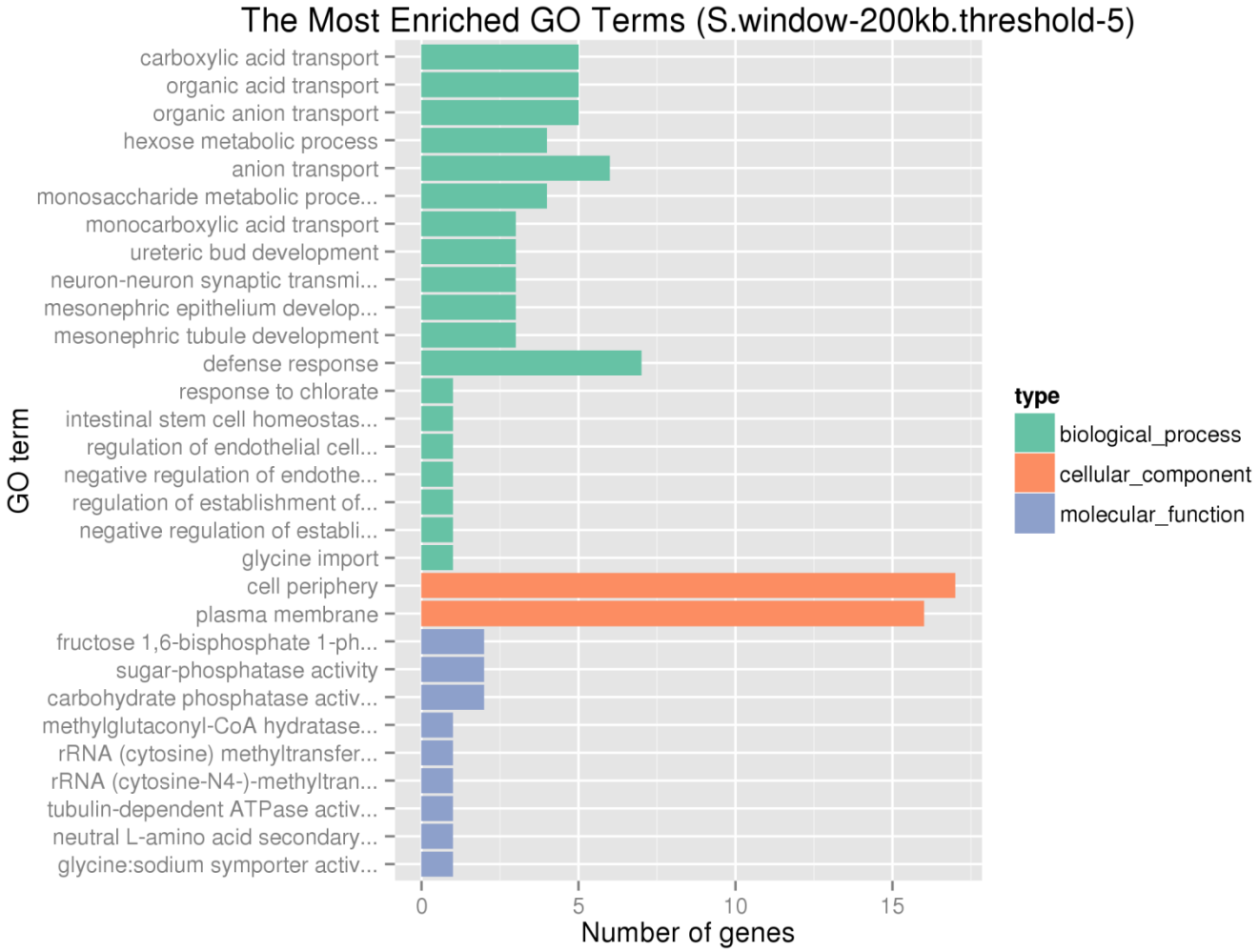

Figure 3. The most enriched gene ontology terms within significant selection of genes on Guangxi Three-yellow chicken of the present study.

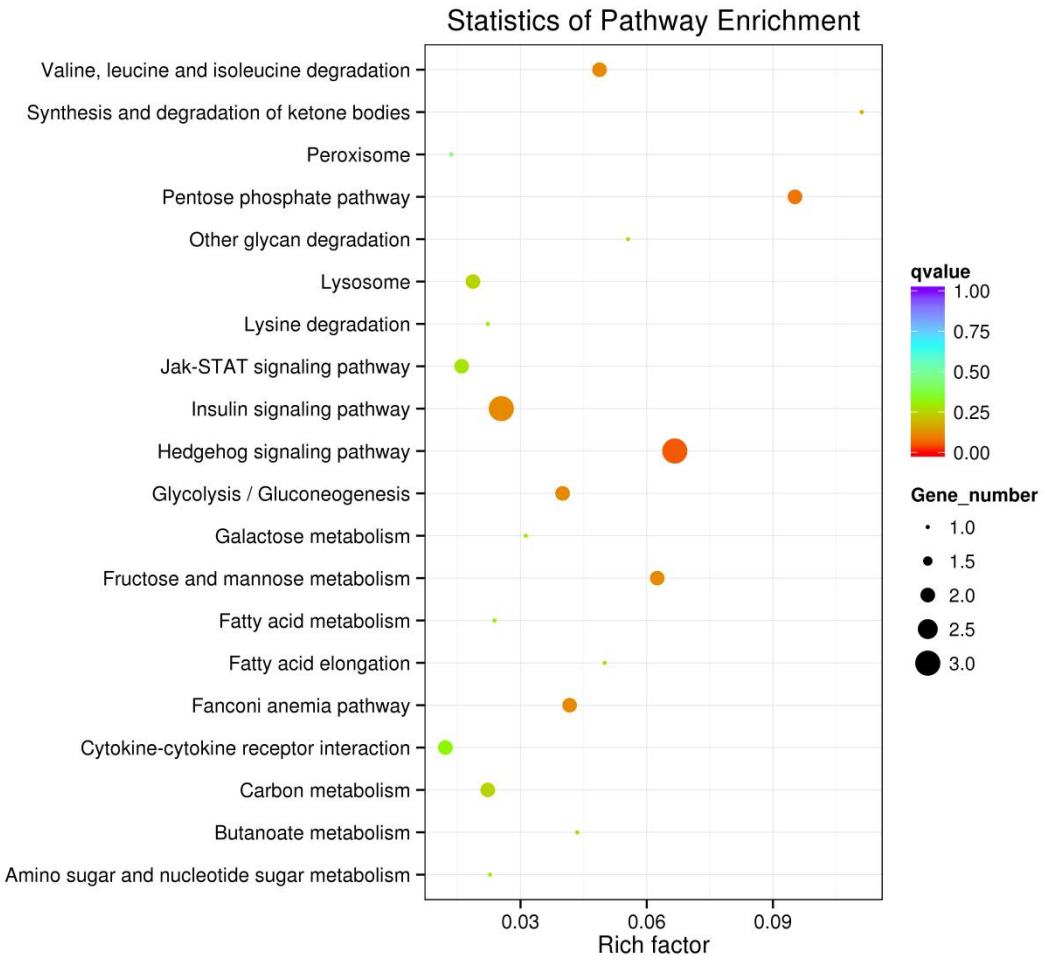

Figure 4. The 20 most enriched Kyoto Encyclopedia of Genes and Genome pathways within significant selection of genes on Guangxi three-yellow chicken in the present study.

Table 1. Results of Kyoto encyclopedia of genes and genome pathways analysis

ID	KEGG Term	Gene	P-Value
Environmental information processing			
Gga 04340	Hedgehog signaling pathway	GAS1, Novel, PTCH1	1.78E-03
Carbohydrate metabolism			
Gga 00030	Pentose phosphate pathway	FBP1, FBP2	5.90E-03
Gga 00051	Fructose and mannose metabolism	FBP1, FBP2	1.26E-02
Gga 00010	Glycolysis / Gluconeogenesis	FBP1, FBP2	2.82E-02
Amino acid metabolism			
Gga 00280	Valine, leucine and isoleucine degradation	HMGCL, AUH	1.97E-02
Organismal systems-Endocrine systems			
Gga 04910	Insulin signaling pathway	FBP1, FBP2, SHC2	2.29E-02
Genetic information processing			
Gga 03460	Fanconi anemia pathway	FANCF, FANCC	2.62E-02
Lipid metabolism			
Gga 00072	Synthesis and degradation of ketone bodies	HMGCL	4.93E-02

Three genes, i.e. the growth arrest-specific gene-1 (GAS1), Novel and protein patched homolog-1 (PTCH1), were enriched on the Hedgehog (Hh) signaling pathway that has many roles in development, cell proliferation, tissue patterning and stem cell maintenance. As a putative tumor suppressor gene (Del et al., 1992; Del et al., 1994; Atsumi et al., 2014), Growth arrest-specific 1(GAS1) inhibits cell replication by blocking the entry into the S

phase of the cell cycle (Del et al., 1992). Protein patched homolog 1 (PTCH1) was a member of the patched gene family, and was the receptor for sonic hedgehog (SHH), which was a secreted molecule implicated in the formation of embryonic structures, and in tumorigenesis (Carpenter et al., 1998). PTCH1 prevented cells from growing and dividing in the absence of SHH, thus it was considered as a tumor suppressor (Villavicencio et al., 2000), although it stoped suppressing cell proliferation in the presence of SHH.Fanconi anemia group F (FANCF) and Fanconi anemia group C (FANCC) belonged to the Fanconi anemia (FA) family, which contained of 22 genes whose protein products form a complex to participate in the efficient repair of damaged DNA (Nepal et al., 2017; Nalepa and Clapp, 2018; Tsui and Crismani, 2019). FANCF stabilized the FANCC/FANCE sub complex and the FANCA/FANCG subcomplex, and locked the whole FA core complex in a conformation that was essential for DNA repair (Leveille et al., 2004), suggesting its important role in maintaining the cell's genomic integrity (Medhurst et al., 2001). FANCF-deficient mice found with no germ cells in the seminiferous tubules, and no or almost no primordial follicles in the ovaries (Bakker et al., 2012). As a mitochondrial enzyme, 3-Hydroxymethyl-3-Methylglutaryl-CoA Lyase（HMGCL） was involved in the valine, leucine and isoleucine degradation and synthesis as well as in the degradation of ketone bodies. When glucose is not available, such as during fasting, ketones are the compounds used for energy by certain organs and tissues, particularly the brain. In human, HMGCL deficiency, often as an autosomal recessive mitochondrial disease (Lin et al., 2009), usually presented with acute episodes of vomiting, hypotonia, hypoketotic, hypoglycemia metabolic acidosis and hyperammonemia in infancy. In the valine, leucine and isoleucine degradation pathways, 3-methylglutaconyl-CoA hydratase (AUH) was another selected gene encoding a bifunctional mitochondrial protein that had both RNA-binding and hydratase activities. The protein can catalyze the transformation of 3-methylglutaconyl-CoA to 3-hydroxy-3-methyl-glutaryl-CoA, and binds AU-rich elements found in the 3'-untranslated regions of rapidly decaying mRNAs. Decreased levels of AUH also leaded to a slower cell growth. Reduced or elevated levels of AUH can lead to defects in mitochondrial translation, ultimately leading to changes in decreased RNA stability as well as in the mitochondrial morphology, biogenesis and respiratory function (Mack et al., 2006). FBP1 and FBP2 were enriched in pentose phosphate pathway, in fructose and mannose metabolism, in glycolysis/gluconeogenesis, and in insulin signaling pathway that regulates carbohydrate metabolism and endocrine systems. The pentose phosphate pathway is a glucose metabolism process that produces reduced Nicotinamide Adenine Dinucleotide Phosphate and pentoses, which is an essential part of histidine and purine/pyrimidine biosynthesis nucleotides. Glycolysis/gluconeogenesis is the process of converting glucose to pyruvate and producing small amounts of ATP (energy) and NADH (reducing power). FBPs ultimately control the rate of gluconeogenesis, whereas the insulin signaling pathway is responsible for regulation of glucose and lipid metabolism, besides many other functions such as regulation of cell proliferation in response to mitogens. Src homology 2 domain containing-transforming protein 2 (SHC2), as a substrate of insulin receptor, can activate the RAS/MAPK pathway independently of IRS-1 (Taha and Klip, 1999; Ferguson et al., 2014). Of the ten genes enriched in the aforementioned pathways, FBP1, FBP2, SHC3, FANCC and PTCH1 were located on the 41.2 to 43.3 region of chromosome Z, which might be integrally chained due to selected certain particular genes, with FBP1 and FBP2 being the most likely objectives and the others likely being jointly selected. Within the selective sweeps in all of the domestic chickens in the present and other studies (Rubin et al., 2010), some of the genes were also found to be associated with domestication traits in chickens and other farmed animals, which reinforced their important roles in chicken domestication. For instance, BCDO2 was found to be associated with the yellow skin (Eriksson et al., 2008). However, this gene in GX-TYC was not detected. ESRP2 is associated with chicken abdominal fat contents (Zhang et al., 2012), and NELL1 was identified in a selective sweep in the broilers (Elferink et al., 2012). In the present study, NELL1 gene was found to undergo a strong selection in GX-TYC, which verified GX-TYC as a broiler, thus conforming to the long-term breeding purpose of GX-TYC and confirming that the present approach and the resulting data were reliable.

CONCLUSION

In summary, herein a whole genome map of Single nucleotide polymorphisms (SNPs), insertion/deletion polymorphisms (InDels) of Guangxi Three-Yellow chicken **(**GX-TYC) were presented and some genetic footprints of its domestication were uncovered. These data provide important resources for further improvements of fowl breeding and for future studies on the molecular mechanisms of chicken phenotypic variations and certain diseases.

DECLARATIONS

Consent to publish

All authors agree to publish this manuscript.

Availability of data and materials

All data have been presented in the manuscript as figures and tables and as the supplementary data. There is no additional data and materials.

Competing interests

All authors claim that there is no completing interest concerned.

Funding

This work was supported by Guangxi Provincial Natural Science Foundation of China (NO. 2013GXNSFDA019013) to Dr. Yuying Liao. Guangxi Special Fund for Specially-invited Expert.

Authors' contributions

YL drafted the manuscript. YL and DJL formulated the concepts. JS, YH, FW and GM analyzed the data and prepared the figures and tables. LZ performed English editing of the manuscript. DJL finalized the manuscript.

Acknowledgements

We would like to think Dr. Fred Bogott at the Medical Center, Austin of Minnesota for his excellent English editing of the manuscript.

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Supplementary Data

Supplementary Table 1. Summary and annotation of single nucleotide polymorphisms in Guangxi three-yellow chickens

Category		Number of SNPs
Upstream		140,563
Exonic	Stop gain	291
	Stop loss	47
	Synonymous	88,687
	Non-synonymous	36,707
	Unknowns	0
Intronic		3,938,603
Splicing		387
Downstream		122,273
Upstream/downstream		5,004
Intergenic		5,806,581
Total		10,139,143

Supplementary Table 2. Summary and annotation of Indels in Guangxi three-yellow chickens

Category		Number of Indels
Upstream		10632
Exonic	Stop gain	13
	Stop loss	3
	Frameshift deletion	413
	Frameshift insertion	512
	Non-frameshift deletion	377
	Non-frameshift insertion	295
Intronic		335096
Splicing		175
Downstream		11948
Upstream/Downstream		418
Intergenic		482323
Insertion		380249
Deletion		461987
Het Rate (%)		0.643
Total		842236

Supplementary Table 3. Detail information of single nucleotide polymorphisms loci with ZHp ≤ -5

Ensembl Gene ID	ZHp	CHROM	Start	Gene
ENSGALG00000003958	-11.572	5	2129860	PRMT3
ENSGALG00000003908	-17.158	5	2204843	SLC6A5
ENSGALG00000003777	-17.158	5	2243366	NELL1
ENSGALG00000003748	-9.0503	5	2760128	ANO5
ENSGALG00000003660	-10.144	5	2946165	FANCF
ENSGALG00000003655	-10.144	5	2976276	GAS2
ENSGALG00000003648	-6.1477	5	3060193	SVIP
ENSGALG00000013311	-6.9695	5	3278760	ANO3
ENSGALG00000013304	-11.351	5	3407884	SLC5A12
ENSGALG00000023904	-11.351	5	3505528	FIBIN
ENSGALG00000013297	-11.351	5	3530348	BBOX1
ENSGALG00000012194	-10.236	5	3627318	CCDC34
ENSGALG00000012191	-8.6053	5	3651822	LGR4
ENSGALG00000012170	-8.6053	5	3717817	LIN7B
ENSGALG00000012163	-8.6053	5	3757392	BDNF
ENSGALG00000012162	-8.6053	5	3783967	Novel
ENSGALG00000012160	-8.137	5	3878424	KIF18A
ENSGALG00000012153	-5.0344	5	3921314	METTL15
ENSGALG00000004112		23	5552378	FUCA1
ENSGALG00000004120		23	5558027	CNR2
ENSGALG00000003971		23	5518731	TCEB3
ENSGALG00000004002		23	5540404	LYPLA2
ENSGALG00000004047		23	5545884	GALE
ENSGALG00000003936	-5.0735	23	5514567	PPT1
ENSGALG00000004268		23	5696766	NIPAL3
ENSGALG00000004155		23	5586447	MYOM3
ENSGALG00000003879		23	5493121	MFSD2A
ENSGALG00000004141		23	5581470	LBFABP
ENSGALG00000004057		23	5548630	HMGCL

ENSGALG00000004122		23	5565578	PNRC2
ENSGALG00000004249		23	5643857	GRHL3
ENSGALG00000003986		23	5536714	PITHD1
ENSGALG00000004133		23	5572421	SRSF10
ENSGALG00000004231		23	5625795	IFNLR1
ENSGALG00000004221		23	5618293	IL22RA1
ENSGALG00000003912		23	5502015	CAP1
ENSGALG00000017658	-6.8264	Z	40920596	GAS1
ENSGALG00000026583	-6.8264	Z	40920611	Novel
ENSGALG00000012608	-14.043	Z	41139762	DAPK1
ENSGALG00000012610	-14.043	Z	41267496	CTSL2
ENSGALG00000012612	-14.043	Z	41282076	FBP2
ENSGALG00000012613	-14.043	Z	41306732	FBP1
ENSGALG00000012615	-7.1684	Z	41350384	C9orf3
ENSGALG00000012618	-7.1684	Z	41521328	FANCC
ENSGALG00000012620	-6.5791	Z	41632373	PTCH1
ENSGALG00000010683	-5.8349	Z	43300329	S1PR3
ENSGALG00000010688	-5.8349	Z	43311888	SHC3
ENSGALG00000010694	-5.8349	Z	43414992	SECISBP2
ENSGALG00000010697	-5.8349	Z	43446316	SEMA4D
ENSGALG00000005323	-5.1417	Z	43855798	DIRAS2
ENSGALG00000015216	-5.1608	Z	43939409	SYK
ENSGALG00000015213	-5.1608	Z	43980705	TPPP2
ENSGALG00000021843	-6.6507	Z	44067508	AUH
ENSGALG00000015209	-6.683	Z	44205924	NFIL3
ENSGALG00000000151	-7.1704	Z	45148296	ADAMTS19

Supplementary Table 4. Gene functional enrichment analysis of genes significant selection in Guangxi three-yellow chickens

GO_accession	Term_type	Description	N	P Value
GO:0042132	MF	fructose 1,6-bisphosphate 1-phosphatase activity	2	9.74E-06
GO:0046942	BP	carboxylic acid transport	5	1.72E-04
GO:0015849	BP	organic acid transport	5	1.77E-04
GO:0050308	MF	sugar-phosphatase activity	2	4.43E-04
GO:0019203	MF	carbohydrate phosphatase activity	2	5.40E-04
GO:0015711	BP	organic anion transport	5	6.52E-04
GO:0019318	BP	hexose metabolic process	4	7.37E-04
GO:0071944	CC	cell periphery	17	7.53E-04
GO:0006820	BP	anion transport	6	8.51E-04
GO:0005996	BP	monosaccharide metabolic process	4	1.12E-03
GO:0005886	CC	plasma membrane	16	1.48E-03
GO:0015718	BP	monocarboxylic acid transport	3	2.05E-03
GO:0001657	BP	ureteric bud development	3	2.60E-03
GO:0007270	BP	neuron-neuron synaptic transmission	3	2.65E-03
GO:0072163	BP	mesonephric epithelium development	3	2.69E-03
GO:0072164	BP	mesonephric tubule development	3	2.69E-03
GO:0006952	BP	defense response	7	2.71E-03
GO:0004490	MF	methylglutaconyl-CoA hydratase activity	1	3.15E-03
GO:0010157	BP	response to chlorate	1	3.15E-03
GO:0016434	MF	rRNA (cytosine) methyltransferase activity	1	3.15E-03
GO:0036335	BP	intestinal stem cell homeostasis	1	3.15E-03
GO:0071424	MF	rRNA (cytosine-N4-)-methyltransferase activity	1	3.15E-03
GO:0070463	MF	tubulin-dependent ATPase activity	1	3.15E-03
GO:1901550	BP	regulation of endothelial cell development	1	3.15E-03
GO:1901551	BP	negative regulation of endothelial cell development	1	3.15E-03
GO:1903140	BP	regulation of establishment of endothelial barrier	1	3.15E-03
GO:1903141	BP	negative regulation of establishment of endothelial barrier	1	3.15E-03
GO:0005294	MF	neutral L-amino acid secondary active transmembrane transporter activity	1	3.15E-03
GO:0015375	MF	glycine:sodium symporter activity	1	3.15E-03
GO:0036233	BP	glycine import	1	3.15E-03
GO:1990379	BP	lipid transport across blood brain barrier	1	3.20E-03
GO:0001823	BP	mesonephros development	3	3.21E-03
GO:0046854	BP	phosphatidylinositol phosphorylation	2	3.51E-03
GO:0032002	CC	interleukin-28 receptor complex	1	3.54E-03
GO:0046834	BP	lipid phosphorylation	2	4.02E-03
GO:0050747	BP	positive regulation of lipoprotein metabolic process	1	4.18E-03
GO:1903061	BP	positive regulation of protein lipidation	1	4.18E-03
GO:0006836	BP	neurotransmitter transport	3	4.30E-03
GO:0005548	MF	phospholipid transporter activity	2	4.53E-03
GO:0003978	MF	UDP-glucose 4-epimerase activity	1	4.53E-03
GO:0005169	MF	neurotrophin TRKB receptor binding	1	5.14E-03
GO:0061193	BP	taste bud development	1	5.14E-03

GO:0045668	BP	negative regulation of osteoblast differentiation	2	5.15E-03
GO:0061005	BP	cell differentiation involved in kidney development	2	5.53E-03
GO:0051234	BP	establishment of localization	18	5.94E-03
GO:0021997	BP	neural plate axis specification	1	6.29E-03
GO:0097108	MF	hedgehog family protein binding	1	6.29E-03
GO:2001013	BP	epithelial cell proliferation involved in renal tubule morphogenesis	1	6.29E-03
GO:0010693	BP	negative regulation of alkaline phosphatase activity	1	6.29E-03
GO:1900220	BP	semaphorin-plexin signaling pathway involved in bone trabecula morphogenesis	1	6.29E-03
GO:0071226	BP	cellular response to molecule of fungal origin	1	6.29E-03
GO:0004300	MF	enoyl-CoA hydratase activity	1	6.29E-03
GO:0060995	BP	cell-cell signaling involved in kidney development	1	6.29E-03
GO:0061289	BP	Wnt signaling pathway involved in kidney development	1	6.29E-03
GO:0061290	BP	canonical Wnt signaling pathway involved in metanephric kidney development	1	6.29E-03
GO:0072204	BP	cell-cell signaling involved in metanephros development	1	6.29E-03
GO:0045329	BP	carnitine biosynthetic process	1	6.29E-03
GO:0005119	MF	smoothened binding	1	6.31E-03
GO:0005901	CC	caveola	2	6.33E-03
GO:0015908	BP	fatty acid transport	2	6.35E-03
GO:0045121	CC	membrane raft	3	6.51E-03
GO:0072073	BP	kidney epithelium development	3	6.53E-03
GO:0044853	CC	plasma membrane raft	2	6.67E-03
GO:0004560	MF	alpha-L-fucosidase activity	1	6.72E-03
GO:0006004	BP	fucose metabolic process	1	6.72E-03
GO:0015928	MF	fucosidase activity	1	6.72E-03
GO:0015850	BP	organic hydroxy compound transport	3	6.81E-03
GO:0008474	MF	palmitoyl-(protein) hydrolase activity	1	6.91E-03
GO:0098599	MF	palmitoyl hydrolase activity	1	6.91E-03
GO:0007042	BP	lysosomal lumen acidification	1	7.03E-03
GO:1903070	BP	negative regulation of ER-associated ubiquitin-dependent protein catabolic process	1	7.32E-03
GO:1903059	BP	regulation of protein lipidation	1	7.32E-03
GO:0070475	BP	rRNA base methylation	1	7.66E-03
GO:0019388	BP	galactose catabolic process	1	7.67E-03
GO:0016049	BP	cell growth	4	7.89E-03
GO:0004888	MF	transmembrane signaling receptor activity	7	7.92E-03
GO:0046717	BP	acid secretion	2	7.98E-03
GO:0032229	BP	negative regulation of synaptic transmission, GABAergic	1	8.42E-03
GO:0007166	BP	cell surface receptor signaling pathway	13	8.58E-03
GO:0007267	BP	cell-cell signaling	6	8.83E-03
GO:0044425	CC	membrane part	19	8.87E-03
GO:0007611	BP	learning or memory	3	9.03E-03
GO:0060012	BP	synaptic transmission, glycinergic	1	9.42E-03
GO:0008469	MF	histone-arginine N-methyltransferase activity	1	9.42E-03
GO:0019919	BP	peptidyl-arginine methylation, to asymmetrical-dimethyl arginine	1	9.42E-03

GO:0035242	MF	protein-arginine omega-N asymmetric methyltransferase activity	1	9.42E-03
GO:0035247	BP	peptidyl-arginine omega-N-methylation	1	9.42E-03
GO:0045602	BP	negative regulation of endothelial cell differentiation	1	9.42E-03
GO:0097025	CC	MPP7-DLG1-LIN7 complex	1	9.42E-03
GO:0002238	BP	response to molecule of fungal origin	1	9.42E-03
GO:0097016	MF	L27 domain binding	1	9.42E-03
GO:0009957	BP	epidermal cell fate specification	1	9.44E-03
GO:0002351	BP	serotonin production involved in inflammatory response	1	9.48E-03
GO:0002442	BP	serotonin secretion involved in inflammatory response	1	9.48E-03
GO:0002554	BP	serotonin secretion by platelet	1	9.48E-03
GO:0006578	BP	amino-acid betaine biosynthetic process	1	9.49E-03
GO:0045926	BP	negative regulation of growth	3	9.54E-03
GO:0006094	BP	gluconeogenesis	2	9.60E-03
GO:0042806	MF	fucose binding	1	9.87E-03
GO:0042015	MF	interleukin-20 binding	1	9.96E-03
GO:0048549	BP	positive regulation of pinocytosis	1	1.00E-02
GO:0002084	BP	protein depalmitoylation	1	1.00E-02
GO:0098734	BP	macromolecule depalmitoylation	1	1.00E-02
GO:0006810	BP	transport	17	1.01E-02
GO:0035751	BP	regulation of lysosomal lumen pH	1	1.02E-02
GO:0040007	BP	growth	6	1.04E-02
GO:0031324	BP	negative regulation of cellular metabolic process	10	1.04E-02
GO:2000027	BP	regulation of organ morphogenesis	3	1.07E-02
GO:1903069	BP	regulation of ER-associated ubiquitin-dependent protein catabolic process	1	1.09E-02
GO:0090237	BP	regulation of arachidonic acid secretion	1	1.10E-02
GO:0071286	BP	cellular response to magnesium ion	1	1.10E-02
GO:0004419	MF	hydroxymethylglutaryl-CoA lyase activity	1	1.12E-02
GO:0016833	MF	oxo-acid-lyase activity	1	1.12E-02
GO:0019319	BP	hexose biosynthetic process	2	1.14E-02
GO:0032429	BP	regulation of phospholipase A2 activity	1	1.19E-02
GO:0007412	BP	axon target recognition	1	1.21E-02
GO:0008589	BP	regulation of smoothened signaling pathway	2	1.25E-02
GO:0043313	BP	regulation of neutrophil degranulation	1	1.25E-02
GO:1902563	BP	regulation of neutrophil activation	1	1.25E-02
GO:0016273	MF	arginine N-methyltransferase activity	1	1.25E-02
GO:0016274	MF	protein-arginine N-methyltransferase activity	1	1.25E-02
GO:0035246	BP	peptidyl-arginine N-methylation	1	1.25E-02
GO:0005828	CC	kinetochore microtubule	1	1.25E-02
GO:0017128	MF	phospholipid scramblase activity	1	1.25E-02
GO:0061588	BP	calcium activated phospholipid scrambling	1	1.25E-02
GO:0061590	BP	calcium activated phosphatidylcholine scrambling	1	1.25E-02
GO:0061591	BP	calcium activated galactosylceramide scrambling	1	1.25E-02
GO:0030279	BP	negative regulation of ossification	2	1.25E-02
GO:0008170	MF	N-methyltransferase activity	2	1.27E-02

GO:0034969	BP	histone arginine methylation	1	1.29E-02
GO:0050890	BP	cognition	3	1.29E-02
GO:0032009	CC	early phagosome	1	1.30E-02
GO:0001658	BP	branching involved in ureteric bud morphogenesis	2	1.30E-02
GO:0006002	BP	fructose 6-phosphate metabolic process	1	1.31E-02
GO:0046364	BP	monosaccharide biosynthetic process	2	1.31E-02
GO:0004949	MF	cannabinoid receptor activity	1	1.32E-02
GO:0038023	MF	signaling receptor activity	7	1.35E-02
GO:0000835	CC	ER ubiquitin ligase complex	1	1.36E-02
GO:0000836	CC	Hrd1p ubiquitin ligase complex	1	1.36E-02
GO:0005113	MF	patched binding	1	1.37E-02
GO:0015187	MF	glycine transmembrane transporter activity	1	1.38E-02
GO:0048511	BP	rhythmic process	3	1.39E-02
GO:0019320	BP	hexose catabolic process	1	1.39E-02
GO:0046849	BP	bone remodeling	2	1.41E-02
GO:0031982	CC	vesicle	14	1.42E-02
GO:0071345	BP	cellular response to cytokine stimulus	4	1.42E-02
GO:0001649	BP	osteoblast differentiation	3	1.47E-02
GO:0060675	BP	ureteric bud morphogenesis	2	1.53E-02
GO:0007406	BP	negative regulation of neuroblast proliferation	1	1.53E-02
GO:0005319	MF	lipid transporter activity	2	1.54E-02
GO:0015293	MF	symporter activity	2	1.55E-02
GO:0097484	BP	dendrite extension	1	1.56E-02
GO:0032368	BP	regulation of lipid transport	2	1.57E-02
GO:0010692	BP	regulation of alkaline phosphatase activity	1	1.57E-02
GO:0016051	BP	carbohydrate biosynthetic process	3	1.58E-02
GO:0071702	BP	organic substance transport	10	1.58E-02
GO:0030812	BP	negative regulation of nucleotide catabolic process	1	1.59E-02
GO:0045820	BP	negative regulation of glycolytic process	1	1.59E-02
GO:0051195	BP	negative regulation of cofactor metabolic process	1	1.59E-02
GO:0051198	BP	negative regulation of coenzyme metabolic process	1	1.59E-02
GO:0072171	BP	mesonephric tubule morphogenesis	2	1.60E-02
GO:0018216	BP	peptidyl-arginine methylation	1	1.60E-02
GO:0006869	BP	lipid transport	3	1.61E-02
GO:0042159	BP	lipoprotein catabolic process	1	1.63E-02
GO:0048548	BP	regulation of pinocytosis	1	1.64E-02
GO:0015816	BP	glycine transport	1	1.69E-02
GO:0016208	MF	AMP binding	1	1.71E-02
GO:0072203	BP	cell proliferation involved in metanephros development	1	1.71E-02
GO:0045056	BP	transcytosis	1	1.73E-02
GO:0032026	BP	response to magnesium ion	1	1.73E-02
GO:0006811	BP	ion transport	8	1.74E-02
GO:0016139	BP	glycoside catabolic process	1	1.76E-02
GO:0060896	BP	neural plate pattern specification	1	1.77E-02

GO:0044724	BP	single-organism carbohydrate catabolic process	2	1.77E-02
GO:0038036	MF	sphingosine-1-phosphate receptor activity	1	1.79E-02
GO:0010629	BP	negative regulation of gene expression	7	1.80E-02
GO:0044723	BP	single-organism carbohydrate metabolic process	5	1.89E-02
GO:0001820	BP	serotonin secretion	1	1.92E-02
GO:0033008	BP	positive regulation of mast cell activation involved in immune response	1	1.93E-02
GO:0043306	BP	positive regulation of mast cell degranulation	1	1.93E-02
GO:0032928	BP	regulation of superoxide anion generation	1	1.94E-02
GO:0016052	BP	carbohydrate catabolic process	2	1.95E-02
GO:0038171	BP	cannabinoid signaling pathway	1	1.97E-02
GO:0009892	BP	negative regulation of metabolic process	10	1.97E-02
GO:0072078	BP	nephron tubule morphogenesis	2	1.98E-02
GO:0032940	BP	secretion by cell	5	2.00E-02
GO:0034097	BP	response to cytokine	4	2.01E-02
GO:0017121	BP	phospholipid scrambling	1	2.02E-02
GO:0072088	BP	nephron epithelium morphogenesis	2	2.04E-02
GO:0061333	BP	renal tubule morphogenesis	2	2.09E-02
GO:0000153	CC	cytoplasmic ubiquitin ligase complex	1	2.09E-02
GO:0098542	BP	defense response to other organism	3	2.09E-02
GO:0072028	BP	nephron morphogenesis	2	2.10E-02
GO:0016137	BP	glycoside metabolic process	1	2.11E-02
GO:0046365	BP	monosaccharide catabolic process	1	2.11E-02
GO:0007269	BP	neurotransmitter secretion	2	2.12E-02
GO:0005167	MF	neurotrophin TRK receptor binding	1	2.13E-02
GO:0002281	BP	macrophage activation involved in immune response	1	2.18E-02
GO:0046668	BP	regulation of retinal cell programmed cell death	1	2.19E-02
GO:0009437	BP	carnitine metabolic process	1	2.20E-02
GO:0051010	MF	microtubule plus-end binding	1	2.21E-02
GO:0002576	BP	platelet degranulation	1	2.22E-02
GO:0008509	MF	anion transmembrane transporter activity	3	2.23E-02
GO:0033005	BP	positive regulation of mast cell activation	1	2.24E-02
GO:0017075	MF	syntaxin-1 binding	1	2.25E-02
GO:0072282	BP	metanephric nephron tubule morphogenesis	1	2.26E-02
GO:0001558	BP	regulation of cell growth	3	2.27E-02
GO:0050746	BP	regulation of lipoprotein metabolic process	1	2.28E-02
GO:0016857	MF	racemase and epimerase activity, acting on carbohydrates and derivatives	1	2.28E-02
GO:0010876	BP	lipid localization	3	2.30E-02
GO:0010605	BP	negative regulation of macromolecule metabolic process	9	2.32E-02
GO:0007035	BP	vacuolar acidification	1	2.33E-02
GO:0003376	BP	sphingosine-1-phosphate signaling pathway	1	2.41E-02
GO:0005283	MF	sodium:amino acid symporter activity	1	2.49E-02
GO:0005229	MF	intracellular calcium activated chloride channel activity	1	2.50E-02
GO:0004896	MF	cytokine receptor activity	2	2.51E-02
GO:0006577	BP	amino-acid betaine metabolic process	1	2.52E-02

GO:0018195	BP	peptidyl-arginine modification	1	2.55E-02
GO:0002888	BP	positive regulation of myeloid leukocyte mediated immunity	1	2.56E-02
GO:0031988	CC	membrane-bounded vesicle	13	2.58E-02
GO:0040008	BP	regulation of growth	4	2.62E-02
GO:0002009	BP	morphogenesis of an epithelium	4	2.64E-02
GO:0001656	BP	metanephros development	2	2.65E-02
GO:0015129	MF	lactate transmembrane transporter activity	1	2.67E-02
GO:0015727	BP	lactate transport	1	2.67E-02
GO:0035873	BP	lactate transmembrane transport	1	2.67E-02
GO:0031430	CC	M band	1	2.68E-02
GO:0014047	BP	glutamate secretion	1	2.69E-02
GO:0043090	BP	amino acid import	1	2.71E-02
GO:0043092	BP	L-amino acid import	1	2.71E-02
GO:0090520	BP	sphingolipid mediated signaling pathway	1	2.72E-02
GO:0072080	BP	nephron tubule development	2	2.75E-02
GO:0042157	BP	lipoprotein metabolic process	2	2.77E-02
GO:0002251	BP	organ or tissue specific immune response	1	2.79E-02
GO:0002385	BP	mucosal immune response	1	2.79E-02
GO:0032303	BP	regulation of icosanoid secretion	1	2.81E-02
GO:0060856	BP	establishment of blood-brain barrier	1	2.81E-02
GO:0045978	BP	negative regulation of nucleoside metabolic process	1	2.83E-02
GO:0006837	BP	serotonin transport	1	2.85E-02
GO:0019370	BP	leukotriene biosynthetic process	1	2.86E-02
GO:0048588	BP	developmental cell growth	2	2.86E-02
GO:0045125	MF	bioactive lipid receptor activity	1	2.86E-02
GO:0043302	BP	positive regulation of leukocyte degranulation	1	2.86E-02
GO:0006865	BP	amino acid transport	2	2.87E-02
GO:0072173	BP	metanephric tubule morphogenesis	1	2.88E-02
GO:2000310	BP	regulation of N-methyl-D-aspartate selective glutamate receptor activity	1	2.88E-02
GO:0046666	BP	retinal cell programmed cell death	1	2.90E-02
GO:0001843	BP	neural tube closure	2	2.90E-02
GO:0001822	BP	kidney development	3	2.91E-02
GO:0030856	BP	regulation of epithelial cell differentiation	2	2.92E-02
GO:0005165	MF	neurotrophin receptor binding	1	2.94E-02
GO:0060606	BP	tube closure	2	2.96E-02
GO:0060993	BP	kidney morphogenesis	2	2.99E-02
GO:0061029	BP	eyelid development in camera-type eye	1	2.99E-02
GO:0061326	BP	renal tubule development	2	3.01E-02
GO:0006012	BP	galactose metabolic process	1	3.01E-02
GO:0032269	BP	negative regulation of cellular protein metabolic process	5	3.01E-02
GO:0046903	BP	secretion	5	3.01E-02
GO:0031330	BP	negative regulation of cellular catabolic process	2	3.03E-02
GO:0010875	BP	positive regulation of cholesterol efflux	1	3.06E-02
GO:0043240	CC	Fanconi anaemia nuclear complex	1	3.06E-02

GO:0048589	BP	developmental growth	4	3.08E-02
GO:0008150	BP	biological_process	46	3.09E-02
GO:0008649	MF	rRNA methyltransferase activity	1	3.10E-02
GO:0060831	BP	smoothened signaling pathway involved in dorsal/ventral neural tube patterning	1	3.11E-02
GO:0044459	CC	plasma membrane part	8	3.12E-02
GO:0090330	BP	regulation of platelet aggregation	1	3.12E-02
GO:0040015	BP	negative regulation of multicellular organism growth	1	3.13E-02
GO:0004872	MF	receptor activity	7	3.14E-02
GO:0048672	BP	positive regulation of collateral sprouting	1	3.17E-02
GO:0014020	BP	primary neural tube formation	2	3.19E-02
GO:0002283	BP	neutrophil activation involved in immune response	1	3.19E-02
GO:0043312	BP	neutrophil degranulation	1	3.19E-02
GO:0016021	CC	integral component of membrane	14	3.23E-02
GO:0005416	MF	cation:amino acid symporter activity	1	3.23E-02
GO:0045667	BP	regulation of osteoblast differentiation	2	3.24E-02
GO:0008757	MF	S-adenosylmethionine-dependent methyltransferase activity	2	3.25E-02
GO:0045579	BP	positive regulation of B cell differentiation	1	3.27E-02
GO:1902578	BP	single-organism localization	14	3.28E-02
GO:0042742	BP	defense response to bacterium	2	3.28E-02
GO:0006907	BP	pinocytosis	1	3.32E-02
GO:0060080	BP	regulation of inhibitory postsynaptic membrane potential	1	3.32E-02
GO:0016192	BP	vesicle-mediated transport	6	3.33E-02
GO:0072001	BP	renal system development	3	3.35E-02
GO:0001840	BP	neural plate development	1	3.37E-02
GO:0048025	BP	negative regulation of mRNA splicing, via spliceosome	1	3.38E-02
GO:0061430	BP	bone trabecula morphogenesis	1	3.41E-02
GO:0043524	BP	negative regulation of neuron apoptotic process	2	3.42E-02
GO:0060429	BP	epithelium development	6	3.44E-02
GO:1903307	BP	positive regulation of regulated secretory pathway	1	3.48E-02
GO:0044712	BP	single-organism catabolic process	5	3.48E-02
GO:0048771	BP	tissue remodeling	2	3.49E-02
GO:0006691	BP	leukotriene metabolic process	1	3.49E-02
GO:0043586	BP	tongue development	1	3.51E-02
GO:0030308	BP	negative regulation of cell growth	2	3.52E-02
GO:0072009	BP	nephron epithelium development	2	3.52E-02
GO:0072661	BP	protein targeting to plasma membrane	1	3.56E-02
GO:0048523	BP	negative regulation of cellular process	14	3.58E-02
GO:0090322	BP	regulation of superoxide metabolic process	1	3.59E-02
GO:0046488	BP	phosphatidylinositol metabolic process	2	3.60E-02
GO:0034122	BP	negative regulation of toll-like receptor signaling pathway	1	3.61E-02
GO:0008574	MF	ATP-dependent microtubule motor activity, plus-end-directed	1	3.64E-02
GO:0031224	CC	intrinsic component of membrane	14	3.65E-02
GO:0016500	MF	protein-hormone receptor activity	1	3.67E-02
GO:0032373	BP	positive regulation of sterol transport	1	3.67E-02

GO:0032376	BP	positive regulation of cholesterol transport	1	3.67E-02
GO:0046943	MF	carboxylic acid transmembrane transporter activity	2	3.78E-02
GO:0072111	BP	cell proliferation involved in kidney development	1	3.78E-02
GO:1900449	BP	regulation of glutamate receptor signaling pathway	1	3.80E-02
GO:0050482	BP	arachidonic acid secretion	1	3.80E-02
GO:1903963	BP	arachidonate transport	1	3.80E-02
GO:0050829	BP	defense response to Gram-negative bacterium	1	3.80E-02
GO:2000191	BP	regulation of fatty acid transport	1	3.82E-02
GO:0045934	BP	negative regulation of nucleobase-containing compound metabolic process	6	3.83E-02
GO:0005342	MF	organic acid transmembrane transporter activity	2	3.85E-02
GO:0090179	BP	planar cell polarity pathway involved in neural tube closure	1	3.85E-02
GO:0001505	BP	regulation of neurotransmitter levels	2	3.85E-02
GO:0050691	BP	regulation of defense response to virus by host	1	3.85E-02
GO:0060627	BP	regulation of vesicle-mediated transport	3	3.85E-02
GO:0006629	BP	lipid metabolic process	6	3.86E-02
GO:0016854	MF	racemase and epimerase activity	1	3.87E-02
GO:0001841	BP	neural tube formation	2	3.87E-02
GO:0045780	BP	positive regulation of bone resorption	1	3.88E-02
GO:0046852	BP	positive regulation of bone remodeling	1	3.88E-02
GO:0051248	BP	negative regulation of protein metabolic process	5	3.91E-02
GO:0072202	BP	cell differentiation involved in metanephros development	1	3.95E-02
GO:0046641	BP	positive regulation of alpha-beta T cell proliferation	1	3.99E-02
GO:0005975	BP	carbohydrate metabolic process	5	4.00E-02
GO:0045601	BP	regulation of endothelial cell differentiation	1	4.01E-02
GO:0051172	BP	negative regulation of nitrogen compound metabolic process	6	4.04E-02
GO:0060628	BP	regulation of ER to Golgi vesicle-mediated transport	1	4.05E-02
GO:0072224	BP	metanephric glomerulus development	1	4.07E-02
GO:0043304	BP	regulation of mast cell degranulation	1	4.11E-02
GO:0090178	BP	regulation of establishment of planar polarity involved in neural tube closure	1	4.15E-02
GO:0031167	BP	rRNA methylation	1	4.18E-02
GO:0007398	BP	ectoderm development	1	4.18E-02
GO:0007224	BP	smoothened signaling pathway	2	4.21E-02
GO:0044765	BP	single-organism transport	13	4.24E-02
GO:0061436	BP	establishment of skin barrier	1	4.24E-02
GO:0007625	BP	grooming behavior	1	4.24E-02
GO:0072330	BP	monocarboxylic acid biosynthetic process	2	4.25E-02
GO:0060037	BP	pharyngeal system development	1	4.25E-02
GO:0031672	CC	A band	1	4.29E-02
GO:0070062	CC	extracellular exosome	11	4.29E-02
GO:1903561	CC	extracellular vesicle	11	4.29E-02
GO:1901215	BP	negative regulation of neuron death	2	4.29E-02
GO:0043931	BP	ossification involved in bone maturation	1	4.30E-02
GO:0070977	BP	bone maturation	1	4.30E-02
GO:0043230	CC	extracellular organelle	11	4.30E-02

GO:0065010	CC	extracellular membrane-bounded organelle	11	4.30E-02
GO:0008158	MF	hedgehog receptor activity	1	4.31E-02
GO:0045087	BP	innate immune response	3	4.38E-02
GO:0033006	BP	regulation of mast cell activation involved in immune response	1	4.42E-02
GO:0051649	BP	establishment of localization in cell	9	4.42E-02
GO:0031579	BP	membrane raft organization	1	4.45E-02
GO:0000154	BP	rRNA modification	1	4.48E-02
GO:0090177	BP	establishment of planar polarity involved in neural tube closure	1	4.51E-02
GO:0033561	BP	regulation of water loss via skin	1	4.54E-02
GO:0033119	BP	negative regulation of RNA splicing	1	4.55E-02
GO:0008514	MF	organic anion transmembrane transporter activity	2	4.56E-02
GO:0006006	BP	glucose metabolic process	2	4.56E-02
GO:0009895	BP	negative regulation of catabolic process	2	4.57E-02
GO:0015095	MF	magnesium ion transmembrane transporter activity	1	4.57E-02
GO:0004871	MF	signal transducer activity	7	4.58E-02
GO:0003854	MF	3-beta-hydroxy-delta5-steroid dehydrogenase activity	1	4.62E-02
GO:0072006	BP	nephron development	2	4.62E-02
GO:2000647	BP	negative regulation of stem cell proliferation	1	4.67E-02
GO:0001655	BP	urogenital system development	3	4.67E-02
GO:0051181	BP	cofactor transport	1	4.70E-02
GO:0060562	BP	epithelial tube morphogenesis	3	4.70E-02
GO:0007623	BP	circadian rhythm	2	4.71E-02
GO:0015693	BP	magnesium ion transport	1	4.73E-02
GO:2000178	BP	negative regulation of neural precursor cell proliferation	1	4.75E-02
GO:0016358	BP	dendrite development	2	4.76E-02
GO:0010874	BP	regulation of cholesterol efflux	1	4.78E-02
GO:0034105	BP	positive regulation of tissue remodeling	1	4.79E-02
GO:0042249	BP	establishment of planar polarity of embryonic epithelium	1	4.81E-02
GO:0004683	MF	calmodulin-dependent protein kinase activity	1	4.82E-02
GO:0044700	BP	single organism signaling	18	4.85E-02
GO:0048670	BP	regulation of collateral sprouting	1	4.85E-02
GO:0033630	BP	positive regulation of cell adhesion mediated by integrin	1	4.85E-02
GO:0032228	BP	regulation of synaptic transmission, GABAergic	1	4.86E-02
GO:0000184	BP	nuclear-transcribed mRNA catabolic process, nonsense-mediated decay	1	4.87E-02
GO:0002446	BP	neutrophil mediated immunity	1	4.88E-02
GO:0023052	BP	signaling	18	4.90E-02
GO:0042554	BP	superoxide anion generation	1	4.94E-02
GO:0008038	BP	neuron recognition	1	4.95E-02
GO:0009620	BP	response to fungus	1	5.03E-02
GO:0071526	BP	semaphorin-plexin signaling pathway	1	5.05E-02
GO:0005215	MF	transporter activity	7	5.07E-02
GO:0006110	BP	regulation of glycolytic process	1	5.08E-02

N: The enrichment number of genes; MF: molecular function; BP: biological process; CC: cellular component

Supplementary Table 5. KEGG pathway analysis of genes showing significant selection in TY chickens

Term	ID	Input number	Background number	P-Value	Hyperlink
Hedgehog signaling pathway	gga04340	3	45	1.78E-03	http://www.genome.jp/kegg-bin/show_pathway?gga04340/gga:770168%09red/gga:395806%09red
Pentose phosphate pathway	gga00030	2	21	5.90E-03	http://www.genome.jp/kegg-bin/show_pathway?gga00030/gga:395218%09red/gga:395217%09red
Fructose and mannose metabolism	gga00051	2	32	1.26E-02	http://www.genome.jp/kegg-bin/show_pathway?gga00051/gga:395218%09red/gga:395217%09red
Valine, leucine and isoleucine degradation	gga00280	2	41	1.97E-02	http://www.genome.jp/kegg-bin/show_pathway?gga00280/gga:396316%09red/gga:427269%09red
Insulin signaling pathway	gga04910	3	118	2.29E-02	http://www.genome.jp/kegg-bin/show_pathway?gga04910/gga:431265%09red/gga:395218%09red/gga:395217%09red
Fanconi anemia pathway	gga03460	2	48	2.62E-02	http://www.genome.jp/kegg-bin/show_pathway?gga03460/gga:427468%09red/gga:101750641%09red
Glycolysis / Gluconeogenesis	gga00010	2	50	2.82E-02	http://www.genome.jp/kegg-bin/show_pathway?gga00010/gga:395218%09red/gga:395217%09red
Synthesis and degradation of ketone bodies	gga00072	1	9	4.93E-02	http://www.genome.jp/kegg-bin/show_pathway?gga00072/gga:396316%09red
Carbon metabolism	gga01200	2	90	7.84E-02	http://www.genome.jp/kegg-bin/show_pathway?gga01200/gga:395218%09red/gga:395217%09red
Other glycan degradation	gga00511	1	18	9.17E-02	http://www.genome.jp/kegg-bin/show_pathway?gga00511/gga:419687%09red
Fatty acid elongation	gga00062	1	20	1.01E-01	http://www.genome.jp/kegg-bin/show_pathway?gga00062/gga:419681%09red
Lysosome	gga04142	2	107	1.05E-01	http://www.genome.jp/kegg-bin/show_pathway?gga04142/gga:419681%09red/gga:427466%09red
Butanoate metabolism	gga00650	1	23	1.14E-01	http://www.genome.jp/kegg-bin/show_pathway?gga00650/gga:396316%09red
Jak-STAT signaling pathway	gga04630	2	125	1.34E-01	http://www.genome.jp/kegg-bin/show_pathway?gga04630/gga:419692%09red/gga:419694%09red
Galactose metabolism	gga00052	1	32	1.54E-01	http://www.genome.jp/kegg-bin/show_pathway?gga00052/gga:419686%09red
Fatty acid metabolism	gga01212	1	42	1.96E-01	http://www.genome.jp/kegg-bin/show_pathway?gga01212/gga:419681%09red
Amino sugar and nucleotide sugar metabolism	gga00520	1	44	2.04E-01	http://www.genome.jp/kegg-bin/show_pathway?gga00520/gga:419686%09red
Cytokine-cytokine receptor interaction	gga04060	2	165	2.06E-01	http://www.genome.jp/kegg-bin/show_pathway?gga04060/gga:419692%09red/gga:419694%09red
Lysine degradation	gga00310	1	45	2.08E-01	http://www.genome.jp/kegg-bin/show_pathway?gga00310/gga:426932%09red
Peroxisome	gga04146	1	74	3.17E-01	http://www.genome.jp/kegg-bin/show_pathway?gga04146/gga:396316%09red
ErbB signaling pathway	gga04012	1	77	3.27E-01	http://www.genome.jp/kegg-bin/show_pathway?gga04012/gga:431265%09red
Glycerophospholipid metabolism	gga00564	1	86	3.57E-01	http://www.genome.jp/kegg-bin/show_pathway?gga00564/gga:419685%09red
Neuroactive ligand-receptor interaction	gga04080	2	261	3.85E-01	http://www.genome.jp/kegg-bin/show_pathway?gga04080/gga:431264%09red/gga:428232%09red
Spliceosome	gga03040	1	105	4.17E-01	http://www.genome.jp/kegg-bin/show_pathway?gga03040/gga:419689%09red
Metabolic pathways	gga01100	6	1049	4.44E-01	http://www.genome.jp/kegg-bin/show_pathway?gga01100/gga:395217%09red/gga:419681%09red/gga:395218%09red/gga:419686%09red/gga:427269%09red/gga:396316%09red
Phagosome	gga04145	1	127	4.79E-01	http://www.genome.jp/kegg-bin/show_pathway?gga04145/gga:427466%09red
Protein processing in endoplasmic reticulum	gga04141	1	146	5.27E-01	http://www.genome.jp/kegg-bin/show_pathway?gga04141/gga:771022%09red
Focal adhesion	gga04510	1	184	6.11E-01	http://www.genome.jp/kegg-bin/show_pathway?gga04510/gga:431265%09red
MAPK signaling pathway	gga04010	1	214	6.67E-01	http://www.genome.jp/kegg-bin/show_pathway?gga04010/gga:396186%09red

JWPR
Journal of World's Poultry Research

2020, Scienceline Publication

J. World Poult. Res. 10(3): 429-435, September 25, 2020

Research Paper, PII: S2322455X2000049-10
License: CC BY 4.0

DOI: https://dx.doi.org/10.36380/jwpr.2020.49

Effect of Polar and non Polar Extract of *Ferula assafoetida* *Dermanyssus gallinae in vivo* and *in vitro* Conditions

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Received: 29 Jun. 2020
Accepted: 16 Aug. 2020

ABSTRACT

Dermanyssus gallinae is one of the most common arthropods in layers that affects the quality and quantity of egg production. Although there are different synthetic compounds against this mite, but despite these compounds, drug resistance and the presence of these compounds and synthetic compounds in meat and eggs makes that the use of alternative methods, as well as increased use of herbal extracts and essential oils. In the present study, the N-Hexane and Ethanol extracts of *Ferula assafoetida* were used. GC-MS analysis revealed the constituents of the two extracts. The lethal properties of the extracts were determined by contact toxicity. In this field study, ethanolic extract of *Ferula assafoetida* was sprayed on laying hens that infected with red mite. The most available compounds of the Ethanol extract and N-hexan extract of *Ferula assafoetida* were Diethylpyridine and Aurapten respectively in this study. The LC50 of Ethanol extract of *Ferula assafoetida* was 16 µg/cm^3 and *in vitro* study determined that ethanolic extract of *Ferula assafoetida* has been able to reduce the red mite population. This study indicated Ethanol extract of *Ferula assafoetida* could use as a substitute compound against red mite.

Key words: Polar and nonpolar extract, *Ferula assafoetida*, Red mite

INTRODUCTION

Dermanyssus gallinae (red mite) is one of the most important arthropods, especially in laying hens all over the world (Sparagano et al., 2013; Kim et al., 2016; Tabari et al., 2017; Kim et al., 2018) that affects the quality and quantity of egg production, irritation, anemia and, even in the case of severe contamination, causes the death of the bird (George et al., 2009; Spargano et al., 2013). *D. gallinae* plays an important role in the transmission of rickettsial, viral and bacterial pathogens and occasionally causes skin irritation in humans (George et al., 2009; Na et al., 2011; Spargano et al., 2013).

There are various chemical combinations including organophosphates, pyrethroids and carbamates to counteract this mite. The continuous applications of these compounds have increased the resistance to these compounds in this mite (Marangi et al., 2009; George et al., 2009; Tabari et al., 2015). In addition, the chemical residues of these compounds in meat, eggs and the environment are among the limitations of the use of these compounds (Dalton et al., 2001). Because of this, it increases the importance of using alternative methods such as extracts and essential oils of plants of *Ferula assafoetida* to control red mite. The acaricidal properties of many plant extracts and essential oil have been reported against *D.gallinae* (Kim et al., 2004, 2007; George et al., 2009; Tabari et al., 2015; Nechita et al., 2015; Masoumi et al., 2016; Kim et al., 2016, 2018)

The genus of Ferula belong to the family of Apiaceae that distributed the Mediterranean area and central Asia including Iran and Afghanistan (Bagheri et al., 2010). *F. assafoetida* is traditionally use for the treatment disease including parasitic disease (Iranshahi et al., 2011). The compounds of this plant are 40-46% resin, 25% gum, 10-17 % volatile oil and 1.5 10% ash. The resin consist of ferulic acid esters, free ferulicacid ,umblliferone and coumarine (Iranshahi et al., 2011). The sesquiterpenes and sesquiterpene coumarins are the most compound of the genus Ferula (Pimenov et al., 1982). The essential oils of *F. assafoetida* are strong ovicides and larvicides of mosquitoes (Muturi et al., 2018). anthelmintic (Kakar et al., 2013; Upadhyay et al., 2017) antiprotozoal activity (El Deeb et al., 2012; Bafghi et al., 2014; Barati et al., 2014)

are a characteristic of this plant The acaricidal activity of *F. assafoetida* has not been investigated against *D. gallinae.* The purpose of this study was to investigate the acaricidal activity of polar (Ethanol) and nonpolar (N-hexan) extracts of *F. assafoetida* on red mite under *in vivo* and *in vitro* conditions.

MATERIALS AND METHODS

Mites source

Between October 2018 and may 2019 *D. gallinae* samples were collected from a laying poultry farm in Amol, Iran. The mites were placed in dark containers under 25 ºC and humidity of 55% transferred to the laboratory in Science and Technology Park of sari, Iran

Essential oil extraction and GC-MS analysis

The aerial parts of *F. assafoetida* were collected in Mashhad city, Iran and dried on 25ºC. The aerial parts of *F. assafoetida* were ground mechanically using a commercialelectric mill. To provide Extract mill plant was macerated with ethanol and N-hexan in soxhlet apparatus and subsequently, the extract was filtered and solvent was evaporated by using a rotary evaporator and the acquired extract was dried in desiccator (Sonar et al., 2016). The polar extract was obtained by Ethanol solvent. However, the non-polar extract was obtained by N-hexan solvent. To analyze and identify the constituents of the extract Gas chromatography coupled to mass spectrometry (model Shimadzu-QP5050A, Japan) was used. In this study, gas chromatography Agilent-6890 model equipped with DB-5 column with a length of 40 m, an inner diameter of 0.18 mm to 0.25 mm thick layer of stationary phase are used. The column heat program was adjusted from 60 to 210 °C with a gradient of 5 °C/min. The injection chamber temperature was 280 °C and the used detector temperature was 270 °C. Helium gas was used as carrier gas and its speed was 0.9 mm/min and fission ratio of 1 to 43. The injection rate was 0.1 µl of sample and the source ionization temperature was 230 °C. The electron ionizatin mode and the ionization energy were 70 ev. A series of normal alkanes were also injected under the same conditions to calculate the retention index inhibition index. The sample retention index was calculated using a computer program.

Finally, the essential oil components were identified by comparing the mass spectra obtained with the standard mass spectra in the Wiley 2000 electronic library in Labsolution GC/MS software and computing the standard inhibition index and comparing them with the standard numbers in the references (Shibamoto, 1987; Adams, 2001).

Contact toxicity

Contact toxicity assay was done according to the method described by Tabari et al. (2015). In this study, treatment groups, control group of solvents, negative control and standard group were considered. 50 mites were added to all studied groups. The number of dead mites were recorded during 24, 48 and 72 hours after spraying the extract, and then the concentration of Lethal Concentration 50 (LC50) were calculated. Two replicates were carried out for all tested groups of mite.

Experimental groups

In the studied groups, the different concentrations were prepared (0.5, 1, 2, 4, 8, 16, 32, 64, 128 µg/cm^3). For dilution of polar extract from Ethanol and for nonpolar extract of N- hexane solvent used. Polar extract diluted in 50 µl Ethanol and non polar extract diluted in 50 µl N-hexane, the Wattemn's paper was then embedded with dilutions after three minutes, the paper dried and the paper was loaded on to the plate, about 50 mites were added to each plate. The number of red mite that were lost during 24, 48, and 72 hours after treatment were counted. Negative control group without any treatment was placed with 50 red mites on filter papers at the bottom of the plate. In Ethanol solvent control group, the filter paper was smeared with 50 µl of ethanol solvent and after two minutes, the filter paper was dried and placed on the bottom of the plate and then 50 red mites added to the plate. In N-hexan solvent control group, the filter paper was smeared with 50 µl of N-hexan solvent and after 2 minutes, the filter paper was dried and placed on the bottom of the plate and then 50 red mites added to the plate. In standard group, the filter paper impregnated with 50 µl of diluted cypermethrin solution and then dried at the bottom of the plate, about 50 red mites were added to the plate.

In vitro experiment

Preparation nest

The research was conducted in one of the laying farms of Amol city in may 2019**.** Each study group contained 20 laying hens (at 40 weeks of age) that reared in cage system (5 laying hens in each cage). Ventilation, lighting and temperature controlled on the basis of breed recommendations (LSL catalog, 2018).

Treatments

In the treatment groups, the ethanolic extracts of *F. assafoetida* was sprayed on 20 LSL laying hens at 40 weeks of age based on the LC50 concentration obtained *in vivo* studies. The negative control group (without conflict with the red mite and untreated), positive control group (involved with the red mite untreated), standard (involved with red mite and treatment with Cypermethrin Mahan Chemical Company) was considered. Two replicates were considered for all study groups. In order to create red mite contamination, contaminated fields were collected from laying farm of Amol city, Iran and then in each group about 2000 experimental red mite were generated. According to the LC50 concentration indicated in the *in vivo* studies, Ethanolic extract of *F. assafoetida* and cypermethrin toxin were sprayed on the bird's body and repeated for one week more. 24 hours after each spray, the number of dead mites on the floor of each cage was measured and counted using adhesive paper traps.

Statistical analysis

The mortality rates of mites were analyzed using a one-way ANOVA in SPSS software (version 16). Values of $P \leq 0.05$ were considered significant.

RESULTS

GC-MS analysis

The major constituents of the extract are shown in tables 1 and 2. The most available compounds in Ethanol extract were Diethylpyridine (23.54%), Aurapten (15.58%), Coumarin (5.11%) respectively and N-hexan extract of *Ferula assafoetida* were Aurapten (16.39%), Lutidine (7.36%) and Ergosten (4.87) respectively.

Table 1. The constituents of Ethanolic extract of *Ferula assafoetida*

Compound	Retention index	Peak area
Coumarin	41.65	5.11
Quinolinium	48.37	1.68
Benzene	48.76	1.96
Ethylene	48.98	2.1
Methoxyindole	49.82	7.19
Phenol	50.11	2.37
Naphthalenone	50.33	3.1
Benzopyran	50.62	2.93
Aurapten	51.16	15.58
Diethylpyridine	51.63	23.54
Costol	52.48	2.63
Lavandulol	53.92	3.72

Table 2. The constituents of N-hexan extract of *Ferula assafoetida*

Compound	Retention index	Peak area
Geranyl	33.467	3.62
a.-Selinene	33.99	3.78
Farnesal	34.81	1.07
Myristoleate	36.9	1.2
Oleic acid	37.03	1.85
Decanone	39.4	1.56
Farnesol	41.89	1.17
Phenyl ethanone	47.45	1.89
Oelsauere	48.15	2.06
Dehydrogingerdione	48.33	1.84
Formamide	48.83	1.27
Isothiocyanate	49	3.69
Trienoic acid	49.54	1.97
Dimethoxyindole	49.76	3.04
Benzenedicarboxylic acid	50.12	2.7
Xanthene	50.42	2.9
Aurapten	51.35	16.39
Lutidine	51.7	7.36
Ergosten	52.89	4.87
Quinoline	53.21	3.91
Squalene	55.91	1.32

Contact toxicity

Generally in all studied timings (After 24 hours, 48 hours and 72 hours), the results indicated that the responses to treatments were the extraction method and dose dependent in contact toxicity assay, Ethanol extracts of *Ferula assafoetida* was effective than N-hexan extract on red mite ($P \leq 0.05$). The LC50 was 16 $\mu g/cm^3$ for Ethanol extracts (Table 3).

***In vitro* study**

The ethanolic extract of *F. assafoetida* was effective on red mite under field conditions and the differences between groups were significant ($P \leq 0.05$). After first spray, the number of dead red mite was not significant between positive and negative control ($P > 0.05$) but mean mortality rates of mites in treatment group with *F.assafoetida* extract and standard groups significantly higher than the control groups ($P \leq 0.05$). After the second spray, the number of dead red mites in treatment group with *F. assafotida* and standard group were significantly higher than the control groups ($P \leq 0.05$, Table 4).

Table 3. Comparison of lethal effect polar and nonpolar extracts of *Ferula assafoetida* on red mite at different times

Treatment	After 24 hours	After 48 hours	After 72 hours	Total
N-hexan *F. assafoetida* 128 µg/cm^3	1^a	1.33±0.33^a	1.33±0.33^a	1.22±0.14^a
N-hexan *F. assafoetida* 64 µg/cm^3	0^a	0.33±0.33^a	0.33±0.33^a	0.22±0.14^a
N-hexan *F. assafoetida* 32 µg/cm^3	0.33±0.33^a	0.33±0.33^a	0.33±0.33^a	0.33±0.16^a
N-hexan *F. assafoetida* 16 µg/cm^3	0^a	0.33±0.33^a	0.33±0.33^a	0.22±0.14^a
N-hexan *F. assafoetida* 8 µg/cm^3	1±0.57^a	1±0.57^a	1±0.57^a	1±0.28^a
N-hexan *F. assafoetida* 4 µg/cm^3	1^a	1^a	1^a	1^a
N-hexan *F. assafoetida* 2 µg/cm^3	0^a	0^a	0^a	0^a
N-hexan *F. assafoetida* 1 µg/cm^3	0.66±0.33^a	0.66±0.33^a	0.66±0.33^a	0.66±0.16^a
N-hexan *F.assafoetidaa* 0.5 µg/cm^3	0^a	0^a	0^a	0^a
Ethanol *F. assafoetida* 128µg/cm^3	41±0.57^f	41.33±0.66^f	41.33±0.66^f	41.22±0.32^g
Ethanol *F. assafoetida* 64µg/cm^3	35^e	41±0.57^f	41±0.57^f	39±1^g
Ethanol *F. assafoetida* 32µg/cm^3	25±0.57^d	33.33±1.6^e	33.33±1.6^e	30.55±1.55^f
Ethanol *F. assafoetida* 16µg/cm^3	16.66±1.66^c	24.66±0.88^d	24.66±0.88^d	22±1.46^d
Ethanol *F. assafoetida* 8µg/cm^3	8.33±1.66^b	17.66±1.45^c	17.66±1.45^c	14.55±1.73^c
Ethanol *F. assafoetida* 4µg/cm^3	3.33±1.66^a	10^b	10.33±0.33^b	7.88±1.24^b
Ethanol *F. assafoetida* 2µg/cm^3	0.33±0.33^a	0.33±0.33^a	0.66±0.33^a	0.44±0.24^a
Ethanol *F. assafoetida* 1µg/cm^3	0^a	1^a	1^a	0.66±0.16^a
Ethanol *F. assafoetida* 0.5µg/cm^3	0^a	0.33±0.33^a	0.33±0.33^a	0.22±0.14^a
Standard	45.33±0.33^g	46.33±0.33^a	46.33±0.33^a	46.11±0.26^h
N-hexan control	1±0.57^a	2^a	2^a	1.66±0.23^a
Ethanol control	0.33±0.33^a	2^a	2^a	1.44±0.29^a
Pasitive control	0.33±0.33^a	1.66±0.33^a	2^a	1.33±0.28^a

Non-anonymous latin letters in each column indicate significant difference ($P \leq 0.05$).

Table 4. Comparison of lethality of ethanolic extract of *Ferula assafoetida* on red mite under field conditions

Treatment	Number of dead red mite after first spray	Number of dead red mite after second spray
Ethanolic Extract of *F. assafoetida*	30±2.3^b	40±1.52^c
Standard	60±3.46^c	50±1.15^d
Negative control	0^a	0^a
Positive control	5±1.15^a	7±0.57^b

Non-anonymous Latin letters in each column indicates significant difference ($P \leq 0.05$)

DISCUSSION

F. assafoetida is a well-known traditional plant with anti-parasitc properties. Anti-parasitic activity of this plant against leishmania (Bafghi et al. 2014, Gholami et al. 2013), cestode (Farhadi et al. 2016) culex (Muturi et al. 2018) Giardia (Nazer et al., 2019) and mosquito (Evergetis et al., 2012) has been demonstrated. Despite there are extensive reports concerning excellent insecticidal activity af *F. asafoetida*, there was no study to evaluate its acaricidal activity on *D. gallinae*. The present study is one the first *in vitro* and *in vivo* acaricidal studies of *F. assafoetida* against *D. gallinae*. In present study it was obtained that ethanolic extract was effective on *D. gallinae in vitro* and *in vivo* but N-Hexan extract had no significant effect on *D. gallinae*. This could be due to such active compounds in the ethanolic extract of *F. assafoetidae*.

The GC-MS analyses indicated that Ethanolic extract of *F. assafoetidae* was Diethylpyridine (23.54%) but N-Hexan extract of *F. assafoetidae* was Aurapten (16.39%). The anti parasitic effect of Diethylpyridine has been demonstrated against leshmania (Abdala et al., 2002). Generally, the major components of plant materials play the main role to determine the biological properties, but this point should not be ignored that the potential of the major compositions may be regulated by other minor components and the biological activites of plant materials are on account of synergistic/antagonistic interactions of all constitutes (Szcepanik et al., 2012).

El-Razek et al. (2001) and Pimenov et al. (1982) and Iranshahi et al. (2012) have reported that the most constituent compounds of *F. assafoetida* are sesquiterpenes and sesquiterpene coumarins. Numerous factors like geographic origin, seasonality, method of oil extraction, year of harvest and even storage conditions could affect the composition of essential oils, so the results from different toxicity studies might not always be adequate (Chalchat et al., 2007).

According to the present study ethanolic extract of *F. assafoetidae* was effective on red mite and LC50 was obtained 16 µg/cm^3. However N-Hexan extract of *F. assafoetidae* was not effective on red mite. In a similar study the aquatic and ethanolic extracts of Conocarpus erectus, regarding relative toxic and repellency properties, were used as botanical, safe acaricide and repellent agent for control of *D. gallinae* in avicultures (Rajabpour et al., 2018). The similar results were reported about Ethanol extract of *Syzygium cumini* and indicated the most efficient acaricidal activity against *Tetranychus urticae* followed by hexane extract, ether and ethyl acetate extracts (Afify et al., 2011). The similar studies reported Ethanolic extracts of *Veratrum album* and *Tanacetum parthenium* could be useful to control *Tetranychus urticae* populations on vegetable plants grown through Integrated Pest Management and organic systems of agriculture (Yildirim et al., 2012).

Present results shows the ethanol extract of *F. assafoetidae* was effective on red mite *in vitro* condition. Some studies reported that the neem seed extract acaricide has positive against red mite *in vitro* coundition (Abdel-ghafar et al. 2008; Locher et al., 2010). Similar findings were reported on garlic (*Allium sativum*) extract for controlling red mite infestation in a layer farm in Babol, North of Iran (Faghihzade et al., 2014).

CONCLUSION

Present findings indicated that the ethanolic extract of *Ferula assafoetidae* was effective on red mite and reduced the red mite population *in vivo* and *in vitro* condition but N-Hexan extract of *Ferula assafoetidae* was not effective on red mite, due to active components like Diethylpyridine and LC50 of ethanolic extract that was indicated 16 µg/cm3.

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JWPR
Journal of World's Poultry Research

2020, Scienceline Publication

J. World Poult. Res. 10(3): 436-442, September 25, 2020

Research Paper, PII: S2322455X2000050-10
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DOI: https://dx.doi.org/10.36380/jwpr.2020.50

Effect of Crude Extracts of Edible Mushroom Species of *Agaricus bisporus* and *Auricularia auricula* on Growth Performance of Broiler Chickens

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Received: 23 Jul. 2020
Accepted: 02 Sept. 2020

ABSTRACT

The current study aimed to evaluate the effect of *Agaricus bisporus* and *Auricularia auricula* crude extracts as feed additives on the growth performance of broiler chickens. The samples included 240 one-day-old chicks randomly divided into 8 dietary treatments, each treatment consisted of 3 replicates with 10 chicks per replicate. The dietary treatment groups were control group (T0), basal diet + zinc bacitracin (T1), basal diets + 0.4% *Agaricus bisporus* extract (T2), basal diet + 0.8% *Agaricus bisporus* extract (T3), basal diet + 1.2% *Agaricus bisporus* extract (T4), basal diet + 0.4% *Auricularia auricula* extract (T5), basal diet + 0.8% *Auricularia auricula* extract (T6), and basal diet + 1.2% *Auricularia auricula* extract (T7). The measured variables included feed intake, body weight gain, feed conversion ratio, and production index. In addition, the study aimed to evaluate the reducing sugars level, antioxidant IC_{50}, and antimicrobial efficacy of mushroom extracts prepared using three different solvents (i.e., water, ethanol, and methanol). The findings indicated that methanolic extract contained higher reducing sugars and had better antimicrobial efficacy. The results of experimental research revealed that mushrooms crude extracts had no significant effects on the growth performance of broiler chickens.

Keywords: Antibiotic, Broiler performance, Extract, Mushroom

INTRODUCTION

Zinc bacitracin is one of the antibiotic growth promoters (AGP) that is usually used in the poultry industry (Sarmah et al., 2006). The AGP residues in animal products, such as meat or eggs, have negative effects on human health and immunity mainly due to the development of bacterial resistance. Therefore, it is of utmost importance to seek alternatives to AGP (Suresh et al., 2017).

Agaricus bisporus and *Auricularia auricular* are two types of mushroom species cultivated in the subtropical climate, such as Indonesia. They contain different polysaccharide compounds, including β-glucan, which has a significant pharmacological effect on activating innate immunity by macrophage cell activation (Minato and Abe, 2013). Chae et al. (2006) reported that broilers fed with β-glucan at 0.04% level had a significant increase in CD8+ cells at 42 days of age and a relative increase in CD4+ cells.

Lee and Kim (2005) reported that *Auricularia auricular* contains 8.86% glucan, out of which 0.31% is α-glucan and 8.55% is β-glucan. On the other hand, *Agaricus bisporus* is made of 7.19% glucan, including 0.60% α-glucan and 6.59% β-glucan. Zeng et al. (2012) reported that *Auricularia auricula* extract contains a heteropolysaccharide composed of various mono-saccharides, such as galactose, mannose, glucose, arabinose, and rhamnose. *Agaricus bisporus* extracted and semi-purified using hot water and 65% ethanol showed a higher polysaccharide content of up to 74.4%, encompassing 63.8% glucan content, 5.6% α-glucan and 58.2% in β-glucan (Kozarski et al., 2011).

Sulfated polysaccharides of *Auricularia auricula* extracted using 95% ethanol could enhance the immunity of white roman chickens (Nguyen et al., 2012). Another study demonstrated that extract from shiitake mushroom increased the total population of *Bifidobacteria* from 7.47 to 8.67 $\log^{10}$ CFU and reduced *Salmonella* counts from 5.98 to 5.81 $\log^{10}$ CFU (Willis et al., 2009). *Bifidobacteria* are known to improve animal production, gut morphology, and health. Another study conducted by Willis et al.

To cite this paper: Ardyansyah RH, Nur Adli D, Natsir MH, and Sjofjan O (2020). Effect of Crude Extracts of Edible Mushroom Species of *Agaricus bisporus* and *Auricularia auricula* on Growth Performance of Broiler Chickens. *J. World Poult. Res.*, 10 (3): 436-442. DOI: https://dx.doi.org/10.36380/jwpr.2020.50

(2013) indicated that 5% oyster mushroom supplementation in broiler diets resulted in higher body weight than other mushroom types. The supplementation of *Agaricus bisporus* mushroom in turkey diets significantly increased body weight and weight gain. Moreover, it reduced feed conversion ratio (FCR) but had an insignificant effect on feed intake (Giannenas et al., 2011). With this background in mind, the present study aimed to evaluate the effects of *Agaricus bisporus* and *Auricularia auricular* crude extracts prepared using microwave-assisted extractor (MAE) on broiler performance.

MATERIALS AND METHODS

Ethical approval

The *in vivo* trials were approved by the Animal Care and Use Committee of Brawijaya University (certificate number: 065-KEP-UB-2020).

Mushroom preparation and extraction

Agaricus bisporus and *Auricularia auricula* were purchased from a local mushroom farmer in the Singosari sub-district, Malang District, East Java, Indonesia. The mushrooms were segmented into the fragments of 0.2-0.3 cm in size, then 100 g of each mushroom was added into a 500 mL flask and mixed and soaked with different solvents (200 mL of distilled water, ethanol 96%, and methanol 70%) for 24 h. In the next step, the solvents were evaporated using modified microwave heating as described by Purwanto et al. (2010). The modified MAE was used during the experiment at 50 °C for 15 minutes.

Determination of reducing sugars, antioxidant IC_{50}, and antimicrobial efficacy of mushrooms (Experiment 1)

The disk diffusion method was performed to investigate the antimicrobial activity of *Agaricus bisporus* and *Auricularia auricula* crude extracts using nutrient agar medium (Merck KGaA, 64271 Darmstadt, Germany). The bacteria (*Salmonella Typhimurium*, *Escherichia coli*, and *Lactobacillus aureus*) were provided and cultured in nutrient agar at 37 °C for 24 hours at the Department of Plant Pest and Diseases, Brawijaya University. Afterward, *Agaricus bisporus* and *Auricularia auricula* crude extracts (in 50% concentration) were placed in the cylinders. Finally, the plates were incubated at 37 °C for 24 hours, then the diameter of the inhibition zone was measured using calipers.

Lane-Eynon method was performed to determine the concentration of reducing sugar of *Agaricus bisporus* and *Auricularia auricula* crude extracts (Afriza and Ismanilda, 2019). Approximately 5 g mushroom crude extract was added to 25 mL of distilled water and 1 mL of HCl 37%. The sample was then heated for 15 min at 80°C. Following that Na_2CO_3 10% was added until greenish, and total volume was made to 125 ml by adding distilled water, then the final solution was stirred, and filtered. The filtrate was immediately transferred into the burette. Furthermore, 5 mL of Fehling A and Fehling B were mixed at the ratio of 50:50. The mixture of Fehling A and Fehling B was then added to 7.5 mL of the solution and the solution was boiled later. The color indicator was used by adding 75 µL of methylene blue. Finally, the solution was titrated until the blue color disappeared. The reducing sugars was calculated as follows:

$$\text{Reducing sugar} = \frac{\text{Dilution volume}}{\text{Titrated volume}} \times \frac{100}{\text{Sample weight}} \times 0.001$$

The antioxidant activity was performed according to (Osawa and Namiki, 1981). Briefly, 20 mg/mL of mushroom crude extract was dissolved in 4 mL of 95% (w/v) ethanol and mixed with linoleic acid (2.51%, v/v) in 99.5% (w/v) ethanol (4.1 mL), 0.05 M phosphate buffer pH 7.0 (8 mL), and distilled water (3.9 mL) and kept in screwcap containers at 40 °C in the dark. Then, 0.1 mL of this solution was then added to 9.7 mL of 75% (v/v) ethanol and 0.1 mL of 30% (w/v) ammonium thiocyanate. Precisely 3 min after the addition of 0.1 mL of 20 mM ferrous chloride in 3.5% (v/v) hydrochloric acid to the reaction mixture, the absorbance at 500 nm of the resulting red solution was measured, and it was measured again every 24 h until the absorbance of the control reached the maximum value. The percentage inhibition of linoleic acid peroxidation was calculated as follows:

Inhibition (%) = 100 - [(absorbance increases of the sample/absorbance increases of the control) × 100].

Effect of mushroom-supplemented diet on broiler growth performance (Experiment 2)

In this experiment, methanol was used as a solvent to extract the mushroom samples. To conduct the study, 240 one-day-old chicks (unsexed, average body weight of 37.6 ± 2.90 g/chick), strain Lohman Grade Platinum, were purchased from Multibreeder Adirama Company in Indonesia. The samples were randomly divided into eight dietary treatments with three replicates in each treatment and 10 chicks in each pen with the dimensions of 1 x 1.2 x 0.8 m^3. All chicks were kept in the floor pens equipped

with hanging feeder, drinker, and rice husk as litter. Feed and water were offered *ad libitum*. During 10 days, all chicks got into the brooding phase with controlled room temperature of 26-35 °C and humidity of about 65-75%. The mushroom crude extract was diluted with distilled water in a ratio of 1:2, then sprayed on basal diets.

The experiment was performed for 35 days. The basal diet was formulated for the starter (1-21 days) and the finisher phase (22-35 days). Ingredients and chemical composition of starter and finisher diets are presented in Table 1. The treatment groups included basal diet without mushroom extract as the control group (T0), basal diet with 0.2% of zinc bacitracin as AGP-treated group (T1), and basal diet containing 0.4%, 0.8%, and 1.2% of *Agaricus bisporus* crude extract formed groups T2, T3, and T4, respectively. Furthermore, basal diet with 0.4%, 0.8%, and 1.2% of *Auricularia auricular* crude extract defined groups T5, T6, and T7, respectively. Feed was prepared every day and given twice a day in the morning and afternoon.

Table 1. Ingredients and chemical composition of basal diets[a]

Ingredients (%)	Starter diet	Finisher diet
Yellow corn	55.16	49.75
Soybean meal	23.34	23.25
Rice bran	0.00	5.00
Fish meal	10.00	10.00
Meat bone meal	5.00	5.00
Palm kernel meal	3.00	3.00
Salt	0.25	0.25
DL-Methionine	0.25	0.25
Palm oil	2.07	2.93
Premix[c]	0.93	0.57
Chemical composition		
Dry matter (%)	86.58	86.49
Crude Protein (%)[b]	23.09	20.93
Ether extract (%)[b]	6.07	5.25
Crude fiber (%)[b]	3.55	5.25
Ash (%)[b]	6.14	4.92

[a]Based on Animal Feed Science and Technology Laboratory, Animal Science Faculty, Brawijaya University; [b]Based on 100 % dry matter; [c]Composition/10 kg: vitamin A=12.000.000IU, vitamin D3= 2.000.000IU, vitamin E=8.000.000, vitamin B1= 2.000mg, vitamin B2=5.000mg, niacin=40.000mg, methionine=30.000mg, lysine=30.000mg, manganese=120.000mg, iron=20.000mg, iodine= 200mg, zinc=100.000mg, cobalt=200mg, copper=4.000mg

The broilers were individually weighed weekly and body weight gain (BWG) was determined. The feed intake was weekly calculated as the difference between feed offered to broiler and remaining feed. The FCR was determined by feed intake divided by BWG of the broiler during the experiment. Mortalities were recorded per pen from the beginning until the end of the experiment. The broiler production index was calculated as follows:

$$\text{Production index (PI)} = \frac{100 - \text{mortality (\%)} \times \text{BWG (kg)}}{\text{FCR} \times \text{days of rearing}} \times 100$$

Statistical analysis

Collected data in experiment 1 were analyzed descriptively while the collected data in experiment 2 were analyzed using one-way ANOVA. P-value less than 0.05 was considered statistically significant. The means of treatments were compared by Duncan multiple range test and additional orthogonal contrast test significance were considered up to 10% (0.10) in the F test in function of specificity and singularity comparisons. All data were tabulated and analyzed using Minitab® 18.1 (Minitab Inc., USA).

RESULTS

Reducing sugars, antioxidant IC_{50}, and antimicrobial activity

The concentration of reducing sugars and IC_{50} values for the antioxidant activity of *Agaricus bisporus* and *Auricularia auricular* crude extracts are shown in Table 2. Crude extracts prepared using methanol solvent had higher level of reducing sugar in comparison with other solvents. Mushrooms extracts prepared using ethanol showed the lowest antioxidant activity compared to other solvents.

Table 2. The effects of mushrooms crude extracts prepared by using different solvents on reducing sugar and antioxidant IC_{50}

Mushroom Species	Solvents	Reducing Sugars (%)	Antioxidant IC_{50} (mg/mL)
Agaricus bisporus	Water	0.020	100.7
	Ethanol	0.016	112.5
	Methanol	0.075	96.6
Auricularia auricular	Water	0.011	81.8
	Ethanol	0.014	102.0
	Methanol	0.024	88.7

The results of the antimicrobial sensitivity test are shown in Table 3. *Agaricus bisporus* extract prepared using methanol solvent showed a wider clear zone against *Salmonella* (0.75 mm), *Escherichia coli* (0.73 mm), and lactic acid bacteria (0.62 mm) compared to water or ethanol solvent. *Auricularia auricular* extract using ethanol showed a wider clear zone against *Salmonella* (0.63 mm) while *Auricularia auricular* extracts using methanol solvent showed a wider inhibition zone against *Escherichia coli* (0.82 mm) and lactic acid bacteria (0.71

mm). Therefore, the methanolic extracts of *Agaricus bisporus* and *Auricularia auricula* were more effective to inhibit the growth of pathogenic bacteria.

Table 3. The effects of mushrooms crude extracts prepared by using different solvents on bacteria inhibition zone (mm)

Mushroom species	Solvents	*Salmonella*	*E. coli*	Lactic acid bacteria
Agaricus bisporus	Water	0.49	0.45	0.46
	Ethanol	0.46	0.42	0.52
	Methanol	0.75	0.73	0.62
Auricularia auricula	Water	0.52	0.56	0.55
	Ethanol	0.63	0.61	0.58
	Methanol	0.52	0.82	0.71

Growth performance

Based on the analysis of variance, there were no significant differences among treatments in terms of broiler performance ($p > 0.05$, Table 4). The control group showed a better production index than the AGP-treated group (369.8 versus 358.3). Both mushroom crude extracts could be replaced with zinc bacitracin in broiler diets. Based on the obtained results of contrast orthogonal test (Table 5), the addition of *Agaricus bisporus* and *Auricularia auricula* crude extracts in broiler diets showed no significant differences with regard to feed intake, BWG, FCR, and production index of broilers ($p > 0.05$). The comparison of T6 with T7 showed that 0.8% *Auricularia auricula* crude extract could reduce feed intake ($p = 0.086$, 3403 versus 3491 g/chick) and FCR ($p = 0.018$, 1.59 versus 1.66). However, 1.2% *Auricularia auricula* crude extract showed the side effect of β-glucan contained in both edible mushroom crude extracts on broiler performance by increasing feed intake and FCR as well as reducing BWG and production index of broilers. The comparison of T3 with T4 showed that 0.8% of *Agaricus bisporus* crude extract addition could significantly reduce FCR with increasing BWG and similar feed intake ($p = 0.047$).

Meanwhile, 0.8% of *Agaricus bisporus* crude extract significantly increased the production index while comparing T3 with T4 ($p = 0.046$). The addition of *Agaricus bisporus* and *Auricularia auricular* crude extracts up to 1.2% showed the side effects of β-glucan that contained in both edible mushroom species. *Agaricus bisporus* and *Auricularia auricular* crude extracts at 0.8% could lead to better broiler performance, compared to those in the control and AGP-treated group.

DISCUSSION

Antioxidant IC_{50} and antimicrobial activity

Major polysaccharide in *Agaricus bisporus* and *Auricularia auricular* is β-glucan, a pathogenic associated molecular pattern (PAMP), which can stimulate and improve activity and maturity of macrophages and dendritic cells (Muta, 2006). In addition, it is reported that β-glucan could inhibit pathogenic bacteria development (Lee et al., 2020). The current study showed that *Agaricus bisporus* and *Auricularia auricular* crude extracts could inhibit the growth of pathogenic and non-pathogenic bacteria. Farzaneh et al. (2018) reported hydrolyzed *Agaricus bisporus* and *Terfezia claveryi* effectively inhibited pathogenic bacteria development. While non-blanched *Agaricus bisporus* inhibited *Bacillus cereus*, *Escherichia coli*, and *Listeria monocytogenes*. Cai et al. (2015) reported that the ethanolic extract of *Auricularia auricula* had effective antimicrobial activity on *Staphylococcus aureus* and *Escherichia coli* with no antimicrobial activity against *Bacillus subtilis*. β-glucan contained in both mushroom crude extracts could improve antimicrobial activity through enhancing bacteriocins secretion by lactic acid bacteria (Perez et al., 2014). Bacteriocins are natural antimicrobial agents against pathogenic bacteria metabolism (Santos et al., 2017).

Wang et al. (2002) reported that microwave heating with high pressure could improve the dispersion of β-glucan in water without polymers degradation. The findings of a study conducted by Zeng et al. (2012) indicated that *Auricularia auricular* extraction using microwave improved antioxidant activity against 2,2-azinobis-3-ethylbenzthiazoline-6-sulfonate, 1,1-diphenyl-2-picryl hydrazyl radical (DPPH), superoxide, and hydroxyl radical. Öztürk et al. (2011) found that methanolic extract of *Agaricus bisporus* had lower Fe^{3+} compounds (59.87 mg/kg) than hexane solvent (206.20 mg/kg). The ferric ion (Fe^{3+}) is used as an indicator of antioxidant activity in the samples, which is characterized by the reduction of Fe^{3+} to Fe^{2+} (Kozarski et al., 2011).

Growth performance

The β-glucan content of both edible mushroom species has differences in terms of β- and α-chain. *Auricularia auricula* contains a β- chain higher than *Agaricus bisporus* (Lee and Kim, 2005). Supplementation of purified β-glucan 0.04% could improve the nutrient digestibility of weanling pig and affect the average daily BWG (Hahn et al., 2006). Giannenas et al. (2010) reported that 2% *Agaricus bisporus* in broiler diets could

significantly improve broiler performance parameters, including BWG and FCR. It was also indicated that the water-soluble polysaccharide of *Agaricus bisporus* could enhance broiler performance. Another study on β-glucan supplementation in broiler diets showed no significant differences among treatments with β-glucan, *Bacillus subtilis*, or combination of β-glucan plus *Bacillus subtilis* on broiler performance (Zhang et al., 2012). Giannenas et al. (2010) found that the addition of *Agaricus bisporus* to broiler diets at 20 g/kg diet increased the colonization of *Lactobacilli* spp. in ileum and caecum. Whereas, *Agaricus bisporus* did not affect small intestine development (Giannenas et al., 2010). In this regard, gut development could affect host performance, health, and nutrient absorption. In the previous study performed by Giannenas et al. (2011), it was reported that the inclusion of *Agaricus bisporus* on turkey diets significantly improved the villus height of the small intestine, also reduced the FCR. Phenolic compounds contained in *Agaricus bisporus* was 402 mg GAE/100g of dry weight (Keles et al., 2011) while *Auricularia auricula* contained phenolic compound in 3.76 mg/100g dry weight (Sikram et al. 2016). The methanolic extract of *Agaricus bisporus* contained phenolic content at about 85.45 µg PE/mg extract (Öztürk et al., 2011), and *Auricularia auricular* water extract contained higher phenolic compounds (2.90 mg GAE/g dry matter) than ethanol or diethyl ether solvent (Boonsong et al., 2016). Polyphenol dietary treatments could enhance barrier function of small intestine by gut microbiota breakdown complex polyphenols into short-chain fatty acids. However, short-chain fatty acids could improve the recovery rate of epithelial cells leading to tight junction protein assembly in epithelium and increased intestinal permeability (Zhu, 2018). Increased intestinal permeability could affect intestinal morphometric (villus and crypt) and improve nutrient utilization and absorption resulting in the improvement of immunity status and body weight of broiler (Yamauchi, 2002). Broiler mortality correlates with the innate immunity of broiler. Intestinal mucosa not only functions in nutrient absorption but also prevents pathogenic bacteria invasion. On the other hand, goblet cells in intestinal mucosa produce mucus to bind and eliminate pathogenic bacteria and could prevent physical and chemical injuries to the intestinal mucosa. Immunoglobulin A (IgA) secretion in intestinal mucosa was to maintain intestinal mucosa stable and also protect it against pathogenic bacteria. Zhang et al. (2008) reported 50 ppm β-(1,3) (1,6)-glucan from *Saccharomyces cerevisiae* in broiler diets significantly increased secretory IgA and IgG in broilers blood serum.

Table 4. The effects of *Agaricus bisporus* and *Auricularia auricula* crude extracts on the growth performance of broiler chickens at 35 days of age[a]

Treatments[b]	FI[c] (g/chick)	BWG[c] (g/chick)	FCR[c]	PI[c]
T0	3517 ± 62.5	2133 ± 45.9	1.65 ± 0.04	369.8 ± 15.8
T1	3483 ± 30.0	2126 ± 4.0	1.64 ± 0.01	358.3 ± 21.5
T2	3433 ± 68.0	2117 ± 46.9	1.62 ± 0.02	348.1 ± 21.1
T3	3498 ± 22.1	2178 ± 38.5	1.61 ± 0.03	387.7 ± 15.2
T4	3451 ± 30.2	2070 ± 26.7	1.67 ± 0.01	342.6 ± 14.8
T5	3476 ± 18.0	2126 ± 54.7	1.64 ± 0.04	371.5 ± 17.6
T6	3403 ± 128.8	2144 ± 131.5	1.59 ± 0.05	374.4 ± 52.4
T7	3491 ± 33.2	2101 ± 91.6	1.66 ± 0.06	361.6 ± 28.2

[a]data are expressed as mean ± standard deviation. [b]T0: control group, T1: Zinc bacitracin group, T2: *Agaricus bisporus* crude extract (0.4%), T3: *Agaricus bisporus* crude extract (0.8%), T4: *Agaricus bisporus* crude extract (1.2%), T5: *Auricularia auricular* crude extract (0.4%), T6: *Auricularia auricular* crude extract (0.8%), T7: *Auricularia auricular* crude extract (1.2%). [c]FI: Feed Intake, BWG: Body Weight Gain, FCR: Feed conversion ratio, PI: Production Index of broiler

Table 5. Orthogonal contrast between the performance of treated broiler chickens with crude extracts of edible mushroom species of *Agaricus bisporus* and *Auricularia auricula*[a]

Set contrast	FI[c]	BWG[c]	FCR[c]	PI[c]
T0 versus T1-T7	0.153	0.812	0.433	0.700
T1 versus T2-T7	0.525	0.944	0.723	0.715
T2-T4 versus T5-T7	0.891	0.954	0.887	0.440
T2 versus T3-T4	0.341	0.885	0.557	0.365
T3 versus T4	0.349	0.057	0.047	0.046
T5 versus T6-T7	0.496	0.950	0.692	0.854
T6 versus T7	0.086	0.435	0.018	0.553

[a] *P*-value of orthogonal contrast test. FI: Feed Intake, BWG: Body Weight Gain, FCR: Feed conversion ratio, PI: Production Index of broiler

CONCLUSION

The methanolic extracts of *Agaricus bisporus* and *Auricularia auricular* had higher concentrations of reducing sugar and also showed a higher capacity to inhibit the growth of pathogenic bacteria and lactic acid bacteria. In addition, 0.8% inclusion level of both mushroom species crude extracts in broiler diets showed the optimum level of broiler performance. *Agaricus bisporus* and *Auricularia auricular* crude extracts could replace antibiotic growth promoters on the broiler diet.

DECLARATIONS

Authors' contributions

Reynaldy H. Ardyansyah worked on the field trial, formulation, data collection, statistical analysis, and writing. Muhammad Halim Natsir created the idea, and designed the study. Osfar Sjofjan performed experiment 1. Danung Nur Adli revised the manuscript grammatically.

Competing interests

The authors have declared no competing interest.

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JWPR
Journal of World's Poultry Research

2020, Scienceline Publication

J. World Poult. Res. 10(3): 443-450, September 25, 2020

Research Paper, PII: S2322455X2000051-10

DOI: https://dx.doi.org/10.36380/jwpr.2020.51

Physical Performance of Broiler Chickens Affected by Dietary Biological Additives

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Received: 12 Jul. 2020
Accepted: 27 Aug. 2020

ABSTRACT

The current study aimed to evaluate the effects of *Saccharomyces cerevisiae* yeast as a probiotic, compared to sodium butyrate as an organic acid on the productive performance of broiler chickens, with special attention to their economic efficiency. Therefore, 270 of one-day-old Hubbard broiler chickens were divided into 5 groups. The first group included chickens receiving basal ration without any treatment (and considered as a control group). The second group was composed of chickens treated with 0.2 g SB/kg, the third group embraced chickens treated with 0.3 g SB/kg, the fourth group included chickens treated with 0.2 g SC/kg, and the fifth group consisted of chickens treated with 0.3 g SC/kg. The obtained results showed that administration of sodium butyrate or yeast showed a significant improvement of final body weight (BW), body weight change, feed conversion ratio and performance index from third to fifth weeks of age. Nevertheless, all treated groups showed an insignificant effect in feed intake, compared to control group. Furthermore, the dietary addition of *Saccharomyces cerevisiae* was detected more profitable than sodium butyrate addition. Accordingly, it can be concluded that sodium butyrate and yeast can be successively used as a natural substitute for antibiotic growth-promoting agents in the broiler chickens. Yeast can be considered as the most important alternative followed by sodium butyrate.

Keywords: Carcass, Economic efficiency, Hubbard; Productive, Sodium Butyrate, Yeast.

Abbreviations: SC: Saccharomyces cerevisiae; SB: sodium butyrate; BW: body weight; BWC: bodyweight change; FI: feed intake; FCR: feed conversion ratio; PI: performance index

INTRODUCTION

The poultry industry is now considered as one of the most important industries related to food security for peoples worldwide. One of the most important pillars of this industry is nutrition, which accounts for 75 to 80% of the industry's costs (El-Kholy et al., 2018). So, the concern was to devise strategies that support this pillar, and contribute to its development (Eltazi et al., 2014). Nowadays, one of the most important strategies is feed additives, because it is of the utmost importance in the poultry production (El-Kholy et al., 2019; Omar, 2020). Feed additives are aimed primarily for improving the physical performance (PR) of birds such as increasing body weight (BW), BW change and improving feed conversion ratios. In addition, they play a vital role in improving the productive and economic efficiency of poultry farms (Omar, 2020). In recent times, use of probiotics and organic acids were considered as natural feed additives used for broiler production. One of the probiotics is *Saccharomyces cerevisiae* (SC) which is produced from malted grains fermentation, also known as "baker's yeast", and it is most widely used as dietary growth promoters for animals (Gao et al., 2008; Omar, 2020). Moreover, SC is a rich source of protein, vitamin B complex, trace minerals and many other useful factors (Sun et al., 2020). Sodium butyrate (SB) is a recently used organic acid in broiler chickens' diet for realizing optimum performance (Awaad et al., 2019; Lan et al., 2020). It is rapidly absorbed to provide energy for the epithelial cells (Lan et al., 2020), and promote sodium and water absorption (Friedel and Levine, 1992). It increased the epithelial cell growth and the proliferation index in the intestinal crypts (Lan et al., 2020), also it had a trophic effect on the gut mucosa. Addition of these acidifiers broiler's diet enhanced nutrient utilization, growth and feed efficiency (Lan et al., 2020). Also, SB improved the balance of the intestinal microflora which led to a positive

impact on the host's health (Candela et al., 2010). In poultry production, dietary acidifier addition did not gain as much attention as in swine production (Dehghani-Tafti and Jahanian, 2016). The previous studies on SC and SB in broiler chickens mainly focused on their phenotypic effects individually. So the aim of the present work was to study the effects of dietary administration of SC in comparison to SB on the growth performance in broiler chickens by estimation of both phenotypic changes in BW, BW change, feed intake, feed conversion ratio, relative growth rate and performance index, and economic efficiency.

MATERIALS AND METHODS

Ethical approval

The current study protocol used in this study was endorsed by the Animal Care and Use Committee of Damietta University, Damietta, Egypt.

Materials

The current experiment was conducted at a private commercial poultry farm under supervision of Poultry Production Department, Faculty of Agriculture, Damietta University, Damietta Governorate, Egypt. The period of study extended from twenty sixth of February, 2019 till second of March, 2019 to demonstrate the effects of dietary addition of probiotic (*Saccharomycis cerevisiae*; "SC") and organic acids (Sodium Butyrate, "SB") on some productive efficiencies of broiler chickens.

Birds and experimental design

Total of 270 one day-old Hubbard broiler chickens with an initial body weight (IBW, g) of 47.43 ± 0.16 gram supplied by commercial hatchery (El-Aml Hatching Company, Damietta) were used in this study. Chickens were individually weighed and assigned randomly to 5 equal experimental groups of 54 birds in each. Chickens of each group were subdivided into 3 replicates of 18 birds in each, and housed in floor pens. All birds were kept under the same managerial conditions. Feed and water were offered *ad libitum* throughout the experimental period (1 to 5 weeks of age). The first experimental group was fed with control diet while, the other four groups were fed with the basal diet with 0.2 and 0.3 g for each of SC and SB per kilogram body weight. For each treatment group, both levels of SC and SB were added to the basal diet, and subsequently mixed and stirred with a mixer. The birds were fed on starter and finisher ration according to NRC (1994). The basal diets composition are tabulated in Table 1.

Table 1. Composition and calculated analysis of starter and finisher diets

Ingredients	Starter (%)	Finisher (%)
Yellow corn	56.0	59.9
Soy bean meal	28.3	25.5
Corn gluten meal	10.0	08.5
Vegetable oil	01.5	02.5
DI-Calcium phosphate	01.7	01.7
Limestone	01.8	01.3
L-lysine	00.1	00.0
Salt (NaCl)	00.3	00.3
Vitamins and Minerals (Premix*)	00.3	00.3
Total	100.0	100.0
Calculated analysis,**		
Crude protein, (CP, %)	23.06	21.10
Metabolizable energy, (Kcal/Kg)	3010	3106
Ether extract, (EE, %)	2.773	2.846
Crude fiber, (CF, %)	3.554	3.409
Calcium, (%)	1.143	0.949
Available phosphorus, (%)	0.469	0.463
Lysine, (%)	1.148	0.981
Methionine, (%)	0.55	0.52
Methionine + Cystine, (%)	0.855	0.789

*The premix at 0.30 of the diet supplies, the following per kg of the diet: A, 1000 I.U., Vit D3 2000 I.U., Vit E, 10 mg, Vit K, 1 mg, Vit B1, 5 mg, Vit B2, 5 mg, Vit B6, 1.5 mg, Vit B12, 0.01 mg, folic acid 0.35 mg, Biotin, 0.05 mg, Pantothenic acid 10 mg, Niacin 30 mg, Coline 250 mg, Fe, 30 mg, Zn, 50 mg, Cu, 4 mg and Se, 0.1 mg. **According to NRC, 1994.

Management

The birds were housed in a clean and well ventilated farm that was previously disinfected and prepared for receiving birds for the experiment. Birds were randomly housed in trial pens (2 m×2.10 m×3 m) with stocking density of 15/m2. The ambient temperature during brooding was 35 °C ± 1 at one-day-old of age, and gradually decreased to 25 °C ± 1 on day 21, and then kept constant. The birds were subjected to light schedule similar to commercial condition; 23 hours light from one-day-old birds until seventh day to give them enough time to find out feed and water, followed by 20 hours light from eighth day to the end of the experiment (35 days of age). Broiler chickens were vaccinated with mix (Infectious Bronchitis "IB" Ma5 + Newcastle clone 30) on day 7, and Gumboro D78 vaccine at 14 days of age, and they were replicated at 21 days of age. Finally, the birds were vaccinated against Newcastle (live clone 30) at 28 days of age.

Performance parameters

The averages of body weight (BW, g), body weight change (BWC, g), feed intake (FI, g) and feed conversion ratio (FCR) which were evaluated according to the method described as follow: Average body weight (BW, g): The chickens were weighted individually at the beginning of the experiment, afterward chickens were weekly weighted and the live body weight change was taken; Body weight change (BWC, g): it was calculated as differences between two successive weights; Body weight change: W2 - W1; Where: W1 is the weight at any week, and W2 is the weight at the next week; Feed intake (FI, g): it was calculated by difference between the weight of the offered feed/week and the remained part, and then divided by the birds number in each group to measure the weekly FI per bird; FCR: it was calculated by dividing the amount of feed consumed (g) during the week by the gain in weight (g) during the same week; Performance index (PI): it was calculated by adopting the below formula proposed by Bird (1955); PI: Body weight gain (g) × FCR.

Partial budget analysis

The economics of feeding diet inclusion with SC and SB were calculated on the basis of overall cost of inputs, i.e. the cost of chickens, feeds, labor, medicines and other miscellaneous cost. Final live weight of the bird was considered for calculating the gross return per bird and net profit per bird.

Statistical analysis

All data were expressed as mean ± standard error (SE) by one-way ANOVA with dietary treated addition as the main factor using statistical software of SPSS Version 25 (IBM SPSS, 2017) which used the general linear model (GLM) procedure based on the following model:

Y_{ij}: $\mu + T_i + e_{ij}$; where, Yij: Observation of the j_{th} chickens in the treatment i; μ: Overall mean; T_i: Effect of the treatments (i,: 1, 2, 3, 4 and 5); e_{ij}: Random error component. A probability of $P \leq 0.05$ was required for statements of significance.

RESEALTS

Performance parameters

Effects of dietary biological addition on average body weight of broiler chickens:

Average body weight (BW) of broiler chickens as affected by SC and SB addition are presented in table 2. The IBW of all chicken groups was nearly similar to each other (47.53, 47.37, 47.53, 47.37 and 47.33 gram) indicating that birds were randomly distributed into the experimental treatments. During the starter period (0 to 2 weeks), dietary treatments did not affect ($P \geq 0.05$) chickens' body weight. At the end of 3 weeks of ages, BW for chickens only treated with SC groups (T4 and T5) significantly ($P \leq 0.01$) increased in comparison with the other groups. However, during the finisher (4 to 5 weeks) periods, dietary addition of SC and SB, significantly ($P \leq 0.05$) increased the live BW. At the end of 5 weeks of age (marketing age), BW for groups fed with diets of T2, T3, T4 and T5 was higher (0.1, 8.4, 14.7 and 23.5 percent, respectively) as compared to those fed with control basal diet. Also, results reported in this study clearly indicated that, addition of SC had the significantly ($P \leq 0.01$) highest FBW as compared to SB in all experimental period except of 1 to 2 weeks which had no significant ($P \geq 0.05$) differences.

Effect of dietary biological addition on average body weight change of broiler chicks

Chickens treated with SC or SB (0.2 or 0.3 g/kg) showed a significant increase ($P \leq 0.05$) in the BWC in comparison with the control chickens for all experimental periods, except for the first and the second period (Table 3). Within the treated chickens, chickens treated with SC (0.3 g/kg) showed the highest BWC followed by chickens treated with SC (0.2 g/kg) then chickens treated with SB from third to fifth weeks.

Effects of dietary biological addition on feed intake of broiler chicks

The effect of dietary addition of SC and SB on the FI of broiler chickens, as seen in table 4, showed an insignificant ($P \geq 0.05$) effect through the different weeks of age.

Effects of dietary biological addition on feed conversion ratio of broiler

Feed conversion ratio (FCR) was significantly ($P \leq 0.01$) improved from the third week until the end of experiment, and overall FCR at fifth week of age, and the value was being greater for T1 and T2 than T3, T4 and T5 (Table 5). A significant improvement in FCR was recorded in the treated groups with SC or SB as compared to the untreated group. Within the treated chickens, chickens treated with SC (0.3 g/kg) showed the lowest FCR followed by chickens treated with SC (0.2 g/kg), then chickens treated with SB at the third, fifth and 1 to 5 weeks of age. In general, high level addition of SC showed the best FCR compared to other treated groups.

Effects of dietary biological addition on performance index of broiler

Chickens treated with SC or SB (0.2 or 0.3 g/kg) showed a significant ($P \leq 0.01$) increase in the PI in comparison with the control chickens (T1) for all experimental period except for the first and second period (Table 7). Also, PI showed an insignificant difference ($P \geq 0.05$) among treatments in the first and second period. Within the treated chickens, chickens treated with SC (0.3 g/kg) showed the highest PI followed by chickens treated with SC (0.2 g/kg), then chickens treated with SB at third, fourth, fifth and 1 to 5 weeks of age. In general, high level addition of SC (T5) showed the best PI in compared to other treated groups.

Partial budget analysis

Data concerning economical evaluation are summarized in table 8. The highest net revenue, economic efficiency and relative economic efficiency were obtained for T5 group followed by T4, T3, T2 compared to the lowest values which were detected in T1 (control group).

Table 2. Body weight of broiler chicks as affected by dietary addition of yeast and sodium butyrate during the experimental periods

Periods (Age/wk)	Control (T1)	Sodium Butyrate (SB, g/kg)		Yeast (SC, g/kg)		Sig.
		0.2 (T2)	0.3 (T3)	0.2 (T4)	0.3 (T5)	
Initial-Body Weight (BW)	47.53±0.50	47.37±0.50	47.53±0.50	47.37±0.17	47.33±0.37	NS
On 1st wk	146.80±0.71	146.60±2.63	147.53±1.99	148.20±3.95	155.60±1.60	NS
On 2nd wk	353.63±3.19	365.83±2.92	361.43±5.78	359.67±0.44	358.20±3.86	NS
On 3rd wk	680.67^{a}±5.29	702.43^{a}±2.50	697.87^{a}±8.08	749.70^{b}±13.84	802.30^{c}±2.15	**
On 4th wk	1111.53^{a}±0.09	1259.27^{c}±11.79	1212.03^{b}±0.42	1227.87^{bc}±19.48	1340.63^{d}±9.69	**
On 5th wk (Final BW)	1501.37^{a}±1.02	1678.67^{bc}±21.33	1628.00^{b}±32.08	1722.00^{c}±22.00	1854.67^{d}±22.88	**

[a,b,c, d]Means within the raw with different superscripts are significantly different (P≤0.05). Sig: significant; NS: non-significant; **: (P≤0.01).

Table 3. Body weight change (of broiler chicks as affected by dietary addition of yeast and sodium butyrate during the experimental periods

Periods (Age/wk)	Control (T1)	Sodium Butyrate (SB, g/kg)		Yeast (SC, g/kg)		Sig.
		0.2 (T2)	0.3 (T3)	0.2 (T4)	0.3 (T5)	
On 1st wk	99.26±0.37	99.23±2.89	100.00±2.00	100.83±3.79	108.27±1.23	NS
On 2nd wk	206.83±3.88	219.23±5.47	213.90±3.79	211.47±3.79	202.60±2.33	NS
On 3rd wk	327.03^{a}±3.58	336.60^{a}±5.41	336.43^{a}±2.30	390.03^{b}±13.58	444.10^{c}±2.82	**
On 4th wk	430.87^{a}±5.38	556.83^{d}±11.12	514.17^{c}±8.35	478.17^{b}±5.65	494.13^{d}±5.58	**
On 5th wk	389.83^{a}±0.94	419.40^{a}±10.51	415.97^{a}±32.19	494.13^{b}±5.58	514.03^{b}±20.30	**
1-5 wk	1453.82^{a}±20.00	1631.29^{bc}±21.80	1580.47^{b}±32.26	1674.63^{c}±21.83	1763.13^{d}±23.08	**

[a,b,c, d]Means within the raw with different superscripts are significantly different (P≤0.05). Sig: significant; NS: non-significant; **: (P≤0.01)

Table 4. Feed intake of broiler chicks as affected by dietary addition of yeast and sodium butyrate during the experimental periods

Periods (Age/wk)	Control (T1)	Sodium Butyrate (SB, g/kg)		Yeast (SC, g/kg)		Sig.
		0.2 (T2)	0.3 (T3)	0.2 (T4)	0.3 (T5)	
On 1st wk	159.33±3.05	155.53±2.10	155.60±2.12	157.60±3.45	158.77±4.72	NS
On 2nd wk	400.80±9.01	405.37±7.82	394.10±2.63	402.13±5.49	407.87±4.84	NS
On 3rd wk	648.60±7.64	652.13±4.02	637.77±19.27	663.00±4.22	653.63±4.96	NS
On 4th wk	907.47±19.31	895.23±30.51	881.37±26.63	907.13±37.28	909.10±20.65	NS
On 5th wk	1054.33±54.17	984.67±15.76	913.47±33.73	986.00±2.87	921.67±46.52	NS
1-5 wk	3170.53±39.65	3092.93±32.23	2982.30±81.52	3115.87±45.68	3051.03±35.60	NS

Sig: significant; NS: non-significant. Feed intake unit: g/chicks

Table 5. Feed conversion rate of broiler chicks as affected by dietary addition of yeast and sodium butyrate during the experimental periods

Periods (Age/wk)	Control (T1)	Sodium Butyrate (SB, g/kg)		Yeast (SC, g/kg)		Sig.
		0.2 (T2)	0.3 (T3)	0.2 (T4)	0.3 (T5)	
On 1st wk	1.61±0.03	1.57±0.04	1.56±0.05	1.57±0.05	1.47±0.05	NS
On 2nd wk	1.94±0.07	1.85±0.03	1.84±0.04	1.90±0.06	2.01±0.02	NS
On 3rd wk	1.98^{c}±0.02	1.94^{c}±0.04	1.90^{b}±0.06	1.70^{ab}±0.05	1.47^{a}±0.01	**
On 4th wk	2.11^{c}±0.05	1.61^{a}±0.08	1.71^{a}±0.04	1.90^{b}±0.06	1.69^{a}±0.05	**
On 5th wk	2.70^{c}±0.14	2.35^{bc}±0.09	2.23^{ab}±0.24	1.99^{ab}±0.02	1.80^{a}±0.10	**
1-5 wk	2.07^{c}±0.03	1.86^{b}±0.03	1.85^{b}±0.09	1.81^{ab}±0.01	1.69^{a}±0.02	**

^{a,b,c} Means within the raw with different superscripts are significantly different (P≤0.05). Sig: significant; NS: non-significant; **: (P≤0.01).

Table 6. Performance index of broiler chicks as affected by dietary addition of yeast and sodium butyrate during the experimental periods

Periods (Age/wk)	Control (T1)	Sodium Butyrate (SB, g/kg)		Yeast (SC, g/kg)		Sig.
		0.2 (T2)	0.3 (T3)	0.2 (T4)	0.3 (T5)	
On 1st wk	9.15±0.11	9.36±0.43	9.50±0.41	9.50±0.54	10.63±0.41	NS
On 2nd wk	18.28±0.78	19.79±0.41	19.64±0.76	18.93±0.56	17.80±0.34	NS
On 3rd wk	34.33^{a}±0.64	36.27^{a}±0.94	36.91^{a}±1.55	44.14^{b}±2.17	54.51^{c}±0.36	**
On 4th wk	52.82^{a}±1.14	78.65^{cd}±4.64	70.79^{bc}±1.55	64.82^{b}±0.95	79.49^{d}±2.81	**
On 5th wk	55.81^{a}±2.92	71.65^{ab}±3.61	74.93^{b}±9.28	86.30^{bc}±1.62	104.02^{c}±7.07	**
1-5 wk	34.08^{a}±0.42	43.14^{b}±1.46	42.35^{b}±2.63	44.74^{b}±0.57	53.29^{c}±1.77	**

^{a,b,c}Means within the raw with different superscripts are significantly different (P≤0.05). Sig: significant; NS = non-significant; **: (P ≤ 0.01)

Table 7. Partial budget analysis of broiler chicks as affected by dietary addition of yeast and sodium butyrate during 1-35 days

Periods	Control (T1)	Sodium Butyrate (SB, g/kg)		Yeast (SC, g/kg)	
		0.2 (T2)	0.3 (T3)	0.2 (T4)	0.3 (T5)
Body weight change (kg)	1.45	1.63	1.58	1.67	1.76
Price/kg body weight (LE)[1]	24.00	24.00	24.00	24.00	24.00
Selling price (LE/chick)	34.8	39.12	37.92	40.08	42.24
Total feed intake/chick(kg)	3.17	3.09	2.98	3.12	3.05
Price/kg diet (LE)	5.12	5.55	5.56	5.33	5.34
Total feed cost /chick (LE)	16.23	17.15	16.57	16.63	16.29
Net revenue (LE) of each chick[2]	9.57	12.97	12.35	14.45	16.95
Economic efficiency[3]	58.96	75.63	74.53	86.89	104.05
Relative economic efficiency[4]	100.00	128.27	126.41	147.37	176.48

[1]The price was calculated on the base of ingredients price through the experimental period; LE: Egyptian pound. [2]Net revenue of each chick = [Selling price/chick – (Total feed cost/chick + 9 Considering each chick coasted 9 LE included rent, labor costs, total veterinary management costs, mortality, all managerial efforts, etc.)]. [3]Economic efficiency: (net revenue/ total feed coast/chicks) × 100. [4]Relative economic efficiency of the control, assuming that the relative economic efficiency of the control: 100.

DISCUSSION

In order to determine the productive effect of dietary addition of either SC or SB in broiler chickens was performed as natural and environmentally friendly alternatives. It was found that the birds treated with either SB or SC had a significant improvement effect on the broiler chickens' growth during all experimental period except for the first and second period. The insignificant differences in the results obtained during the first and second periods could be explained as the functionality of intestines of day-old chickens, and the activity of the digestive enzymes were not sufficiently developed (Ravindran, 2003). All these results were in agreement with findings of Sun et al. (2020) and Omar (2020) in broiler chickens. In contrary to the present results, no negative effect on BW, BWC and FCR were detected when broiler chickens had either supplements of SC (Eltazi et al., 2014; Devi et al., 2019) or organic acid (Denli et al., 2003; Lan et al., 2020). This might be due to kind and concentration of *Saccharomyces*. The result for FCR was in agreement with Mulatu et al. (2019) who

reported a significant improvement in feed per gain ratio. In addition, many authors reported significant and better FCR on either SC (Mulatu et al., 2019; Sun et al., 2020) or SB addition in the diet of commercial broiler chickens (Awaad et al, 2019; Lan et al., 2020). In contrary to this result, Devi et al. (2019) and Al-Khalaifa et al. (2019) illustrated that dietary addition of SC had no impact on FCR.

In addition, Aghazadeh et al. (2012) recorded that dietary SB supplementation during the study period of 35 days had no effect on average FCR. These different results may be due to the available contents of SC or SB, animal age, health status and environment hygiene. The relative growth rate (RGR) result could be speculated that this was due to growth-stimulating effect of SC in broiler chickens as mentioned in the effects of SC in final BW and BWC (Tables 2 and 3). Higher PI was observed in group T5 which might be due to the effect of SC in final BW, and better FCR as compared to the other treatment groups in the current study. Comparable findings were discovered by Devi et al. (2019) stating the improvement in PI due to probiotic addition. The insignificant differences for FI in the present study were in line with the finding of Eltazi et al. (2014); Devi et al. (2019); Al-Khalaifa et al. (2019) for SC and Awaad et al. (2019) for SB. But these results disagreed with those obtained by Hernandez et al. (2013) who found that dietary addition of SC or SB, respectively, increased significantly the FI of broiler chickens.

In addition, the current study became in confrontation with those of Mulatu et al. (2019) who showed the lowest fed intake in all chickens received probiotic, and highest in the untreated group. So, the dietary addition of either SB or SC did not affect the FI of broiler chickens, but they increased the body weight gain significantly indicating an improved feed efficiency. This improvement in PR of broiler chickens in treated groups compared to control may be related to SC constituent with many metabolites like peptides, organic acids oligosaccharides, organic acids and flavor possibly some unidentified growth factors which were proposed to deliver useful responses in poultry production (Gao et al., 2008; Sun et al., 2020). These results also agreed with Markovic et al. (2009) demonstrating that dietary addition of SC improved broiler PR as SC improved the intestinal mucosal aspects, and produced new epithelial cells in the intestinal crypts, and migrated along the villi to the top. For instance, the significant ($P \leq 0.01$) increase of PR in SB groups as compared to the control one, may be discussed from the point of view that SB associated with direct or indirect modulation of insulin-like growth factor (GF) production by insulin which can be related to impulse of muscle development (Mátis et al., 2019). The present results indicated an improvement in the net profit for chickens fed with diets containing of either SC or SB compared to those fed with diets with no additive (control). The present study was in agreement with those of Mulatu et al. (2019) and Devi et al. (2019) who indicated that the ration containing SC addition was potentially profitable than untreated one (control). Therefore, T5 appeared to be cost-effective in economic parameters used in the study. In the identical pattern, Omar (2020) verified that the supplementation of dried brewer's yeast as 0.4 % for broiler breeds changed into more economically profitable than 0.2% and untreated. Also, Mátis et al. (2019) illustrated that dietary addition of sodium butyrate as organic acid gave the best economic profits compared to the control group (untreated) on broiler production.

CONCLUSION

From this trial, it was concluded that sodium butyrate can be used as a growth promoter in chickens as it improves the final body weight, body weight change, feed conversion ratio and relative growth rate by increasing intestinal absorption surface. But when sodium butyrate compared to yeast, yeast achieve the highest improvements. Also, the dietary addition of yeast and sodium butyrate for broiler breed was more economic than the control group.

DECLARATIONS

Acknowledgements

The authors gratefully acknowledge the excellent assistance of Prof. Dr. Ahmed Abdel-Khalek (Chief Researcher at Animal Production Research Institute, Cairo Egypt) for his kind provide the feed additives in the current study.

Competing interests

The authors declare that they have no conflict of interest.

Authors contribution

K.H.E., S.M.R. and T.H.T. developed the concept of the manuscript. K.H.E. wrote the manuscript. All authors checked and confirmed the final revised manuscript.

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2020, Scienceline Publication

J. World Poult. Res. 10(3): 451-455 September 25, 2020

Research Paper, PII: S2322455X2000052-10
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DOI: https://dx.doi.org/10.36380/jwpr.2020.52

Effects of Dietary Fermented Soy Isoflavones on Egg Quality of Laying Hens

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Received: 09 Jul. 2020
Accepted: 28 Aug. 2020

ABSTRACT

The present study aimed to examine the effects of fermented soy isoflavones on the poultry feed towards the quality of eggs. A total of 100 Isa Brown chickens aged 32 weeks were divided into 4 groups and 5 replicates, including T0 (control feed without the provision of fermented soy isoflavones), T1 (feed with 4% of fermented soy isoflavones), T2 (feed with 8% of fermented soy isoflavones), and T3 (feed with 12% of fermented soy isoflavones). The treatments were given for 10 weeks. The observed chemical qualities of eggs included HDL, LDL, isoflavones in egg yolks, physical quality (e.g., their weight and eggshell thickness). All the data were analyzed by using analysis of variance. The results showed that the administration of fermented soy isoflavones in poultry feed had no significant effect on egg weight and eggshell thickness ($p>0.05$), but had a significant effect on HDL, LDL, and isoflavones in egg yolk ($p<0.01$). Based on the results, it can be concluded that 80 mg/100 g of the fermented soy isoflavones (equivalent to the addition of 12%) in laying hens' feed would increase the content of isoflavones and egg yolks' HDL, reduce egg yolks' LDL, and lead to no significant change in the weight and thickness of eggshells.

Keywords: Feed, Fermented, Isoflavones, laying hens, Soybean

INTRODUCTION

The isoflavone content is found in many vegetables, fruits, grains and nuts, and especially in soybeans (Yousef et al., 2004). Soybeans and most soy products contain large amounts of isoflavone genistein and daidzein (Helen Kim et al., 1998). Typically, isoflavone content is around 0.2-1.6 mg /100 g of the balanced diet. According to Izumi et al. (2000), in soy and their processed products, isoflavone is in the form of glycoside, while fermented soy isoflavone is in the form of aglycone which is more quickly absorbed in the small intestine. Furthermore, Messina (2010) declared that soy and its processed products contain phytoestrogens as it is healthy food components which may prevent certain types of cancers, reduce the risk of osteoporosis, decrease plasma cholesterol, act as an antioxidant agent and may increase immunity for both humans and livestock.

Research on soy isoflavone for poultry has been extensively carried out by some researchers, such as the provision of isoflavone in laying hens that were reported by Abdelghani et al. (2019), Malik et al. (2019), Lu et al. (2017); to quail by Akdemir and Sahin (2009); and to mojosari ducks (anas javanica) by Jayanti et al. (2017) and Saputro et al. (2018).

Performance of laying hens with isoflavone added at 59 weeks showed an increased in egg production (Lu et al., 2017), likewise Abdelghani et al. (2019) reported that egg yolk color and Haugh units increased, and lipoprotein cholesterol levels dropped, but added isoflavone to the diet of hens aged 80 weeks was not be able to improve egg quality (Kusumaningrum et al., 2018).

Isoflavones have been proven as compounds that have the ability to influence enzymes in the liver in the process of lipid metabolism (Yilmaz et al., 2008). Consumption of soy isoflavone at a dose of 5 mg / kg will reduce lipid peroxidation in vivo, and increase Low-Density Lipoprotein (LDL) resistance (Wiseman H et al., 2000). Isoflavones will be hydrolyzed by beta-glucosidase, and produce aglycone, daidzein, genistein and glycitein

To cite this paper: Pancapalaga W, Malik A, Wijaya R and Syahranie J (2020). Effects of Dietary Fermented Soy Isoflavones on Quality of Eggs. *J. World Poult. Res.*, 10 (3): 451-455. DOI: https://dx.doi.org/10.36380/jwpr.2020.52

which will then be absorbed and bounded with glucuronic acid. Then, it reaches the enterohepatic cycle, and are secreted through the gallbladder (Malik et al., 2019).

Processing and fermentation of Soybean are known to affect the performance of isoflavones. Soy sauce by-product sauce is a fermentation process of soybean that produces a source of Isoflavones in the feed for poultry (Susanti, 2006).

Present study aimed to examine the effects of fermented soy isoflavones on Isa Brown's feed towards the quality of eggs.

MATERIALS AND METHODS

One hundred of 32-week-old Isa brown strain were used in the current study. Laying hens were reared in closed house experimental-farm in University of Muhammadiyah Malang, Indonesia (Figure 1).

Figure 1. The conditions of study area

Feed treatments were given for 12 weeks starting from the age of 20 weeks to 32 weeks. During that time, all of the laying hens were given Gumboro vaccine and Newcastle Disease (ND) vaccine. They were also clinically examined routinely during the study. The feed ingredients for the research were as follows: fish meal, meat and bone meal, Corn Gluten Meal (CGM), Destillers Dried Grains with Solubles (DDGS), corn, rice bran, oil, grit, amino acid lysine, methionine and fermented soy Isoflavone (Malik et al., 2019). The study used an experimental method with a Completely Randomized Design (CRD). The treatments were divided into 4 groups (each group contained 25 hens), with 5 replicates (each replicate contained 5 hens). They were T0 (control feed without the provision of fermented soy isoflavones), T1 (feed with 4% fermented soy isoflavones), T2 (feed with 8% fermented soy isoflavones) and T3 (feed with 12% fermented soy isoflavones). The composition and nutrient content of the treatment diets are presented in table 1. The feed was given measuredly in the form of mash and drinking water was prepared *ad libitum.* The diets were balanced and used according to the treatments (120 grams/head/day).

Soybeans fermentation process

Soybeans fermentation process was using Malik et al. (2019) method. Firstly, provides 1 kg of black soybean (*Glycine soja (L) Merrit*) purchased at the Malang market, Indonesia sorting was done with the aim of selecting good and dense soybeans. Secondly, washing was aimed to remove impurities that were attached or mixed in the soybeans. The next was boiling the beans for 1 hour. It was aimed to soften the soybeans, and make it easier to peel the skin was stripped, and then, the soybeans were allowed to get cool to the temperature of ± 30℃. Furthermore, the yeast inoculum was sprinkled in a ratio of 1:2 (0.5% of the number of soybeans) on the cooled and dried soybeans' surface to let them be fermented. After being fermented, the second boiling process was done at 100℃ for 20-30 minutes, so that the soybeans became soft. After that, they were being cooled for the last filtering. The result of the filter pulp was used as a fermented soy isoflavone feed.

Measurement of observed variables

Measurement of the egg yolk High-Density Lipoprotein (HDL) and Low-density lipoprotein (LDL) levels was made by using the enzymatic-calorimetry method (Bursteinetal, 1970). On the other hand, the egg yolk isoflavone levels were measured using High-Performance Liquid Chromatography (HPLC) that is according to a modified procedure by Harborne (1992). The eggshell's thickness was measured using a micrometer, and measurements were made on the blunt end, middle and sharp end of the eggs, then the measurements were being averaged (Kul and Seker, 2004). In addition, the eggs' weight measurement was done by weighing the eggs' weight every day using a digital scale with units (g).

Statistical analysis

The collected data were analyzed using Analysis of Variance (ANOVA), and F test at 5% level and continued with the Least Significant Difference (LSD) (Steel and Torrie, 1991).

Table 1. Composition and nutrition content of the treatment diets in laying hens, strain isa Brown aged 32 weeks

Feed ingredients %	TO (0%) *	T1 (4%) *	T2 (8%) *	T3 (12%) *
Rice Bran	15.65	15.80	15.40	15.50
Corn	58.5	57.6	55.4	55.2
Fish oil	0.20	0.90	0.90	0.90
Destillers Dried Grains with Solubles	5.65	3.1	3.5	2.0
Corn Gluten Meal	4.4	4.0	3.1	2.0
Meat and Bone Meal	2.5	2.0	1.0	0.7
Fishmeal	10	10	10	10
Grit	2.9	2.4	2.5	1.5
Lysine	0.1	0.1	0.1	0.1
Methionine	0.1	0.1	0.1	0.1
Fermented soy Isoflavone	0.2	4.0	8.0	12.0
Nutrient Content **				
Crude Protein (%)	17.03	17.02	17.05	17.04
Crude Fat (%)	4.09	4.59	4.54	4.47
Crude Fiber (%)	2.71	2.85	2.93	3.07
Cacium (%)	1.80	1.60	1.59	1.22
Phosphorus Total (%)	0.52	0.51	0.52	0.51
Natrium (%)	0.05	0.05	0.05	0.05
Energy Metabolism (Kcal/kg)	3060.39	3000.00	3020.75	3002.00

*T0: Treatment 0 is 100 kg feed without fermented soy isoflavone. T1 (4%): Treatment 1 is 100 kg feed added with 4kg fermented soy isoflavone. T2 (8%): Treatment 2 is 100 kg feed added with 8 kg fermented soy isoflavone. T3 (12%): Treatment 3 is 100 kg feed added with 12kg fermented soy isoflavone. **Results of the laboratory analysis on the nutrition of each food

RESULTS

Data on the results of average eggs' weight, eggshell's thickness, HDL, LDL and Isoflavones in egg yolks can be seen in table 2.

Egg yolks' isoflavone

According to table 2, the results showed that the provision of fermented soy isoflavones in the feed had a significant effect ($P < 0.01$) on the content of egg yolks' isoflavone. The content of isoflavone in the egg yolk increased from 0.164 mg / g (T0) to 0.267 mg / g (T3). This increase reached 62%.

HDL in the egg yolks

Table 2 shows that the provision of fermented soy isoflavones in the feed had a remarkable effect ($P < 0.01$) on HDL in the egg yolks. High Density Lipoprotein or as known as HDL's level in the egg yolks without being given fermented soy isoflavone to the birds was 42.5 mg/dl. However, after being given 12% (T3) of fermented soy isoflavone in the feed, the HDL level in the egg yolks became 130.4 mg / dl.

LDL in the egg yolks

LDL level in the egg yolks decreased by approximately 48.9% after being given fermented soy isoflavone to the birds. This means that the provision of fermented soy isoflavone in feed had a notable effect ($P < 0.05$) on LDL in the egg yolks.

Eggs' weight and eggshell's thickness

The eggs' weight and eggshell's thickness slightly changed after isoflavone was added to the feed. Therefore, the provision of fermented soy isoflavones in the feed had no important effect ($P > 0.05$) on the eggs' weight and eggshell's thickness.

Table 2. The effect of diets with different levels of fermented soy isoflavones on egg quality of layer hens aged 32 weeks

Variable	TO (0%)	T1 (4%)	T2 (8%)	T3 (12%)
Isoflavon (mg/100 g)	0.16^{b}	0.25^{a}	0.24^{a}	0.26^{a}
High-Density Lipoprotein (mg/dl)	42.50^{b}	125.50^{a}	116.80^{a}	130.40^{a}
Low-density lipoprotein (mg/dl)	6.85^{a}	5.08^{ab}	5.16^{ab}	3.50^{b}
Eggs' weight (g)	59.93^{a}	60.57^{a}	61.57^{a}	62.40^{a}

Different superscript letters on the same row show significant differences ($P<0.05$). *T0: Treatment 0 is 100 kg feed without fermented soy isoflavone. T1 (4%): Treatment 1 is 100 kg feed added with 4kg fermented soy isoflavone. T2 (8%): Treatment 2 is 100 kg feed added with 8 kg fermented soy isoflavone. T3 (12%): Treatment 3 is 100 kg feed added with 12kg fermented soy isoflavone.

DISCUSSION

The provision of fermented soy isoflavones in Isa Brown would increase the content of isoflavones and HDL in egg yolks. The results of present study were similar to the findings of Cai et al. (2013) on quails' eggs, Malik et al. (2019) on the blood of laying hens and et al (2018) on Mojosari Ducks' eggs. Isoflavones from fermented soybeans can increase functional components such as aglycone and active peptide which are very beneficial for health (Jiang et al., 2014). Whereas, aglycone isoflavone had a faster absorption rate in greater amounts than glucoside (Izumi et al., 2000). In addition, according to Saitoh et al. (2001), isoflavone in the blood turned into a soluble form of conjugate, so that it would facilitate the

transfer of soy isoflavones into egg yolks. Therefore, adding high concentration of isoflavones in laying hens would affect the concentration of cholesterol in the egg yolk produced.

On the other hand, the results of current study found a decrease in LDL content of egg yolk. This was in line with the LDL found in ducks' eggs (Saputro et al., 2018; Abdelghani et al., 2019) and the blood in laying hens (Malik et al., 2019). Potter et al. (1998) suggested that isoflavones in the feed have estrogenic effects that cause a decrease in LDL, and an increase in HDL's concentrations which may be the result of stimulation of bile acid excretion. Furthermore, this estrogenic effect can inhibit the absorption of cholesterol which causes inhibition of VLDL formation that consequently leads to decrease of LDL levels.

Meanwhile, according to Saitoh et al. (2001), Fermented soy isoflavones in animals' feed had hypocholesterolemia effects. Isoflavones are plant-derived sterols (phytosterols) which can inhibit the absorption of cholesterol when are consumed. These phytosterols compete and replace the position of cholesterol in micelles. Because of those mechanism, the cholesterol absorbed by the intestine would be reduced. Moreover, when VLDL is inhibited, LDL levels fall.

Eggs' weight and eggshell's thickness in this study showed a slight change compared to the control group (without provision of isoflavones). The results of present study were different from those reported by Lu et al. (2017) who found that providing isoflavones in the final quails' diet will increase eggs' weight and eggshell's thickness. Chicken eggs taken from laying hens aged 32 weeks. Current study used laying hens with a productive period of 32 weeks, so that the absorption of calcium and phosphor was not for the formation of thick eggshells, but for the formation of bones that are still productive. This was reinforced by Gjorgovska et al. (2016) who states that isoflavones are effective supplements for increasing body weight, body length and calcium content in bones during the final period.

CONCLUSION

The provision of 80 mg / 100 g fermented soy isoflavones (equivalent to the addition of 12%) in laying hens' feed will increase the content of isoflavones and egg yolks' High Density Lipoprotein (HDL), and it will reduce egg yolks' Low-Density Lipoprotein (LDL), but not change the weight and thickness of eggshells much.

DECLARATIONS

Acknowledgements

The researchers would like to thank the Rector of the University of Muhammadiyah Malang, Indonesia (Jln. Raya Tlogomas 246, Malang 65144) and the Directorate of Research and Community Service of University of Muhammadiyah Malang for facilitating this research.

Competing interests

The researchers state that there are no conflicts of financial interests or conflicts with other people or organizations that can affect or create bias in the contents of this paper.

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JWPR
Journal of World's Poultry Research

2020, Scienceline Publication

J. World Poult. Res. 10(3): 456-468, September 25, 2020

Research Paper, PII: S2322455X2000053-10

DOI: https://dx.doi.org/10.36380/jwpr.2020.53

Morphology and Immunohistochemistry of Thymus in Haysex Brown Cross Chickens

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Received: 03 Aug. 2020
Accepted: 09 Sept. 2020

ABSTRACT

Thymus plays an important role in the development and regulation of immune responses and other physiological processes. The present study aimed to examine the morphological and immunohistochemical changes of the thymus in Haysex Brown cross chickens with regard to their age. The morphofunctional studies of thymus were performed to determine and analyze age-related changes in anatomical (absolute and relative thymus mass), histological (area of the thymus cortex and medulla, area of connective tissue base, cortex index, number of thymic corpuscles in the lobule), and immunohistochemical (subpopulations of lymphocytes with surface markers $CD4^+$ and $CD8^+$ and their differentiation status) indexes. The study demonstrated that maximum morphological development of thymus could be observed in 20 and 40-day-old chickens. Up to 20 days of age, thymus mass increased proportionally with the chicken body weight. The results indicated that 40-day-old chickens had 1.46 times increase in the cortex index, compared to 20-day-old birds and 1.82 times compared to 8-day-old birds. An increase in the density and number of T-lymphocytes with surface markers ($CD4^+$) and was evident through aging. Regarding aging-associated alternations, the differentiation index ($CD4^+$: $CD8^+$ ratio) reached 1.26±0.09, 1.52±0.25, and 1.56±0.23 in 40, 90, and 110-day-old chickens, respectively. The histological and cell parameters of the thymus in clinically healthy chickens can be used as indicators of normal functioning and to diagnose immunodeficiency in birds.

Keywords: CD8 Positive Lymphocytes, CD4 Positive Lymphocytes, Chickens, Cortex Index, Thymus, Immunohistochemical Characteristics

INTRODUCTION

The poultry industry is one of the promising areas of agriculture (Sharma, 1999). However, the resistance of birds to various diseases depends on their immune system status. One of the main organs of the immune system is thymus. The thymus is the central organ of the immune system. It is responsible for the maturation and differentiation of T-lymphocytes. T-cells are the main providers of the cellular immunity response (Kannan et al., 2015; Ali, 2017). Thymus in the early stages of ontogenesis controls and directs the structural and functional maturation of immunocompetent cells, and it ensures the safety and feasibility of immunological reactions in the later stages (Rezzani et al., 2008). The thymus, in addition to its role as primary lymphoid tissue, functions as a secondary lymphoid organ and is directly involved in the formation of immune competence in poultry (Rieker et al., 1995; Song et al., 2012; Mullakaev et al., 2013; Treesh et al., 2014).

Among all lymphoid organs, the thymus is the most histologically heterogeneous one. It contains both lymphoid and epithelial components, which makes it unique among all organs of the immune system (Senapati et al., 2015). Moreover, the thymus is rich in the number of cells with various neuroendocrine functions (Oubre et al., 2004). An investigation into the thymus functions of productive birds can contribute to the evaluation of their stress level and diet appropriateness. The chicken's maturation is accompanied by the increases of cytokine-like molecules responsible for the restoration of immune homeostasis in post-stress (Franchini et al., 2004). Both the excess and the lack of certain nutrients in the chickens may increase oxidative stress with characteristic changes

in the expression of cytokines in the thymus cells (Wang et al., 2016; Li et al., 2018).

Therefore, this study investigated the morphological changes in the thymus of Haysex Brown cross chickens at different ages, which can be considered an important issue for the reassessment of the feeding and growing methods and act as the protocol of preventive treatments in industrial poultry farming.

MATERIALS AND METHODS

Ethical approval

All animal experiments were conducted in accordance with the Law of Ukraine "On the Protection of Animals from Brutal Treatment" and the recommendations of the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes.

Animals and study design

The sample in this study was composed of 70 female chickens of Haysex Brown cross, aged 1 day. They were selected and raised in the conditions of the chicken farm "Solotvinsk Poultry Factory" Berdychiv district, Zhytomyr region Ukraine. The preventive vaccination was not provided for the tested birds. The bodyweight of birds was determined on the scales PS 6000/C/2. At different ages (i.e., 8, 20, 40, 90, and 110 days of age), the chickens (14 chickens per each age group) were euthanized by acute bleeding after ether anesthesia. After slaughtering, the thymus was detached and weighed on laboratory scales PS 1000/C/2.

For histological studies, each thymus was divided into the smaller tissue specimens and they were fixed in 10-12% aqueous solution of neutral formalin and Carnoy's fluid. After fixation, tissue specimens were washed with running tap water for 24-48 hours. In the next step, they were dehydrated in ethyl alcohol with increasing concentrations to 40, 70, 96, and 100%. Afterwards, the tissue specimens were embedded in paraffin blocks. Histological sections with a thickness of 5-8 µm were produced on a Luge scrotom MS–2. The sections were stained with hematoxylin and eosin and Van Gieson (Horalskyi et al., 2011).

To further the study, morphometric methods were used to obtain objective data about the structural organization of the thymus in chickens. Morphometric analysis was carried out using light microscopes MBS-10 (Russia), Micros MC-50 (Austria). The ratio of cortex and medulla area of the thymus and connective tissue framework was calculated using an ocular grid (square mesh panels) mounted in the eyepiece of the microscope. The percentage of the desired indicator was determined by calculating the ratio of the occupied and total area. Therefore, it was necessary to count the squares occupied by the examined structures in all chickens at the same magnification. The cortex index (CI) was defined as the ratio of cortex and medulla areas. The number of thymic corpuscles in the slice (at magnification × 100) was counted in 15 fields of view (Horalskyi et al., 2011).

Immunohistochemical study was performed at the pathological laboratory, CSD Health Care, Kiev, Ukraine. The $CD4^+$ (T helper) and $CD8^+$ (T cytotoxic) cells in paraffin sections were counted using monoclonal antibodies and visualization system (all reagents were of the "DAKO" firm, Denmark). Briefly, the studied tissue specimens were fixed in a 10% solution of buffering neutral formalin (Fixx Shandon, USA) for 24 hours. After dehydration, the tissue specimens were embedded in pure paraffin wax with polymer additives (Richard-Allan Scientific, USA) at a temperature not more than 60 °C. The tissue specimens were cut with a thickness of 5 µm from paraffin blocks on a rotational scrotom Microm HM325 (Carl Zeiss, Germany), which were mounted on glass slides (Menzel, Germany) and then stained with hematoxylin and eosin (Kaltek, Italy). For further immunohistochemical studies, paraffin sections were placed on adhesive glass (Super Frost Plus, Menzel, Germany). The study was performed on deparaffined and rehydration slices.

To visualize the tissue antigens, the thermal section processing method in Target Retrieval Solution High pH (DAKO, Denmark) was employed through exposing the treatment in PT Modul (Dako, Denmark) for 32 min at 98-99 °C. The primary antibodies were applied after blocking non-specific protein binding by protein block (Diagnostic Biosystems, USA) and endogenous peroxidase activity by peroxidase block (Diagnostic Biosystems, USA). The DAKO EnVision FLEX+ detection system (DAKO, Denmark) was used to visualize primary antibodies. To visualize the histological structure of the studied tissues, the treated tissues were additionally stained with Meyer's hematoxylin (DAKO, Denmark) for 1–3 min. After that, the stained sections were placed in Eukitt® medium (Germany). To gain a better understanding, samples were subjected to a light microscope (Olympus AX70, Japan) with a digital camcorder (Olympus DP50, Japan) connected to a personal computer. This procedure aimed to determine the content, location, and number of

lymphocyte subpopulations (per relative unit area with the magnification ×400). Furthermore, qualitative marker expression was assessed in 10 randomly selected fields of histological section view with the magnification of ×400, ×600, ×800, and ×1000. Differentiation or immunoregulative index (ID) was determined as the quantitative ratio of lymphocytes with the antigenic determinants ($CD4^+$: $CD8^+$). The microphotography of histological specimens was performed using a digital camera embedded in a Primo Star microscope (Carl Zeiss, Germany) and connected to a personal computer.

Statistical analysis

For data analysis, variational-statistical methods were performed using the Statistic 6.0 program (StatSoft Inc., USA) with regard to the peculiarities of statistical methods in biomedical studies (Horalskyi et al., 2011). P-value less than 0.05 was considered statistically significant.

RESULTS

Anatomical studies

The results of organometric investigations are indicated in Figure 1. As can be seen, the absolute weight (AW) of the thymus of chickens progressively increases as the chickens grow to 40 days of age. During 8-20 days of age, the AW of the thymus increases 18.4 times ($p < 0.05$), and from 20 to 40 days of age, thymus AW increases 1.32 times ($p < 0.05$). Beginning at the age of 40 days, there is a rectilinear decrease in thymus AW by 3.78 times in 110-day-old chickens (Figure 1).

The indicator of the relative weight of the thymus reached its maximum value in 20-day-old chickens. Over the next 20 days, the thymus AW increased, however, the thymus RW index decreased almost twice as much during this period ($p < 0.05$). Therefore, the thymus acquires maximum morphological development in chickens by 20 and 40 days of age. By the age of 20 days, its weight increased in proportion to body weight. From 20 to 40 days of age, the rate of growth of the thymus decreases with a further morphological involution of this organ.

Histological research

The histological examination of the chicken's thymus revealed that the thymus is divided into the lobules of different sizes and shapes. Each of its lobules is surrounded by a thin connective tissue capsule made of collagen fibers. The capsule gives the separation membranes deep into the organ parenchyma. There was a differentiated cortex in each part of the thymus, massively filled with lymphocytes and medulla, with a much smaller number of lymphocytes (Figure 2). The medulla contained thymic corpuscles in the form of concentric accumulation of thickened epithelial cells.

The lobular structure of the thymus was expressed in 8-day-old chickens. In the cortex, lymphocytes were massively deposited, and reticuloepithelial cells were observed in some places. Lymphocytes and epithelial cells were observed in the medulla and thymic corpuscles represented by unicellular forms.

According to the results of morphometric examinations of 9-day-old chickens, the cortical area of the thymus was 35.51±0.31% and the medulla area was 55.58±0.34%, accordingly the CI was estimated 0.65±0.02. The connective tissue base occupied a small area and amounted to 8.91±0.38%. The number of thymic corpuscles in the lobule was 4.5 ± 0.06 pieces (Table 1).

In the thymus of 20-day-old chickens, the cortex was shaped by tightly located lymphocytes, which in certain lobules formed wide layers of the cortex. The cortical area and CI in this age group were relatively small. In the medulla of 20-day-old chickens, there was the chaotic placement of the thymic corpuscles. Their number in one particle was 5.42 ± 0.05 pieces. In the medulla lobules, the processes of epithelial cells formed the mesh in the loops of which there were located lymphocytes and other cells (Figure 3).

At 20 days of age, the cortical area of the chickens' thymus increased to 1.16 times ($p < 0.05$), compared to the previous age group. Moreover, and the medulla area decreased to 1.09 times and the CI was 0.81 ± 0.01 ($p < 0.05$).

At 40 days of age, the cortical area of the thymic lobules of clinically healthy chickens increased significantly in comparison with the 20-day-old chickens and reached 48.84 ± 0.74% ($p < 0.05$). The cortical area reduced to 35.36 ± 0.36% ($p < 0.05$) and CI was 1.18 ± 0.01 ($p < 0.0501$). The number of thymus calves increased and it was 6–9 pieces in each lobe with an average of 7.5 ± 0.12 pieces (Table 1). The thymic corpuscles differed in size and shape. Some of them were optically dense, small, and homogeneous, whereas others were stratified and large (Figure 4). The microscopic structure of the thymus of 90-day-old chickens was similar to those of 40-day-old chickens. According to microscopic results, the cortex contained loose thymocytes, and there was a significant number of thymus bodies in the medulla (Figure 5). Their average number in the organ lobules was 9.43 ± 0.13 pieces ($p < 0.05$, table 1).

110-day-old chickens have the interlobular structure of the thymus which changed slightly compared to the previous age groups. The cortex consisted of loosely located thymocytes, medulla contained thymic corpuscles, the number of which was the largest, compared to all the other age groups, and amounted to 9.56 ± 0.05 pieces (Figure 6). The area of cortex decreased significantly (42.57 ± 0.89%, $p < 0.05$) with the medulla area reaching 31.26 ± 0.17%. The CI was measured 1.56 ± 0.23 ($p < 0.05$, table 1).

Therefore, histological studies showed that in the thymus of 40-day-old chickens, the cortex of the lobules forms wide layers and consists of tightly placed lymphocytes. As a result, there is a significant increase of CI in 1.46 and 1.82 times in 20 and 8-day-old chickens respectively. Such histological parameters of the chicken's thymus in this age characterize it as a morphologically mature organ.

Immunohistochemical studies

In the thymus of an 8-day-old chicken, the subpopulations of lymphocytes with markers $CD8^+$ and $CD4^+$ are seldomly located in the medulla and cortex of the thymus lobules. The cytomorphometric analysis revealed that the number of T-helper cells in chickens aged 8 days was 15.28 ± 1.18 pieces, and accordingly T-cytotoxic cells amounted to13.61 ± 1.21 pieces. The differentiation index (ID) was 1.13 ± 0.27 (Table 2).

In the medulla of 20-day-old chickens, thymus $CD8^+$ lymphocytes are located mostly solitary around the thymic corpuscles. In some cases, they form clusters in the form of "wedding rings" (Figure 7). Lymphocytes expressing the $CD4^+$ marker are found in organ lobules both in medulla and cortex. There are more lymphocytes with markers $CD4^+$ in medulla than in the cortex.

The number of such cells in the thymus of the chickens of this age increased in 2.48 times, compared to the previous age group, $CD8^+$ in 1.5 times. The localization and location of T-lymphocytes with $CD8^+$ markers in the thymus of 40-day-old chickens was almost at the same level as in 20-day-old chickens. These cells are located loosely in the medulla and form clusters around the thymic corpuscles. Their loose content was observed in the cortex and in the interlobular connective tissue. Lymphocytes with $CD4^+$ markers are located loosely and diffusely in both cortex and medulla and form focal clusters (Figure 8).

According to the results of cytoimmunohistochemical analysis, the number of lymphocytes expressing $CD4^+$ antigen markers was increased in 40-day-old chickens, compared to the previous age group. At the same time the number of lymphocytes with $CD8^+$ markers ($p < 0.05$) increases 1.53 times (Table 2), and the ID of 40-day chickens was 1.26 ± 0.09.

The majority of lymphocytes with $CD8^+$ markers are individually located around the lobules of the thymus in almost all chickens aged 90 and 110 days. Furthermore, they often form separate areas of round or elongated shape in the medulla of the thymus lobules or they may be found in the form of uniformly spaced single cells (Figure 9). They were also found around the thymic corpuscles.

Comparing the thymus of 90- and 110-day-old chickens with those in the previous age group, no significant difference was observed regarding the localization of T-lymphocytes with $CD4^+$ markers (Figure 10). The number of lymphocytes with markers $CD4^+$ in the thymus of 90-day chickens significantly increased in 4.95 pieces, compared to 40-day chickens ($p < 0.05$). The number of lymphocytes with $CD8^+$ markers remained almost unchanged. The ID of chickens was 1.52 ± 0.25 and 1.56 ± 0.125 when chickens aged 90 and 110 days, respectively ($p < 0.05$, Table 2).

Therefore, the immunohistochemical analysis indicated that there is a significant increase (1.53 times) of T-cytotoxic cells with the surface marker $CD8^+$ in the thymus of 40-day chickens while comparing with the 20-day bird and 2.29 times increase compared to the 8-day-old chickens. Lymphocytes expressing $CD4^+$ surface markers also increased in 1.04 and 2.58 times, respectively. Such an increase in the number of lymphocytes with markers $CD8^+$ and $CD4^+$ correlates with changes in the thymus mass and may indicate the most pronounced functional activity of this organ at the relevant age period.

Table 1. Morphometric parameters of structural components in the chickens' thymus (n = 14)

Chicken age (day)	Area (%)			Number of thymus bodies in a lobule	Cortex index
	Cortex	Medulla	Connective tissue base		
8	35.51 ± 0.31	55.58±0.34	8.91±0.38	4.5±0.06	0.65±0.02
20	41.06 ± 0.18*	50.89±0.34*	8.05±0.41	5.42±0.05*	0.81±0.01*
40	48.84±0.74*	35.36±0.36*	15.8±0.82*	7.5±0.12*	1.18±0.01*
90	52.79±0.31*	30.84±0.48*	16.37±0.47	9.43±0.13*	1.43±0.02*
110	42.57±0.89*	31.26±0.17	26.17±0.96*	9.56±0.05	1.56±0.23*

* p<0.05 in relation to the previous age group; Data are presented as Mean ± Standard Error of Mean

Table 2. The number of lymphocyte subpopulations in the thymus of chickens at different ages

Age (day)	$CD4^+$	$CD8^+$	differentiation index
	Units area (x400), pcs.		
8	15.28±1.18	13.61±1.21	1.13±0.27
20	37.94±0.69*	20.44±1.03*	1.86±0.29*
40	39.44±1.59	31.28±1.51*	1.26±0.09*
90	44.39±2.02*	29.11±1.07	1.52±0.25*
110	45.56±1.42	29.28±1.41	1.56±0.15

*p<0.05; Data are presented as Mean ± Standard Error of Mean

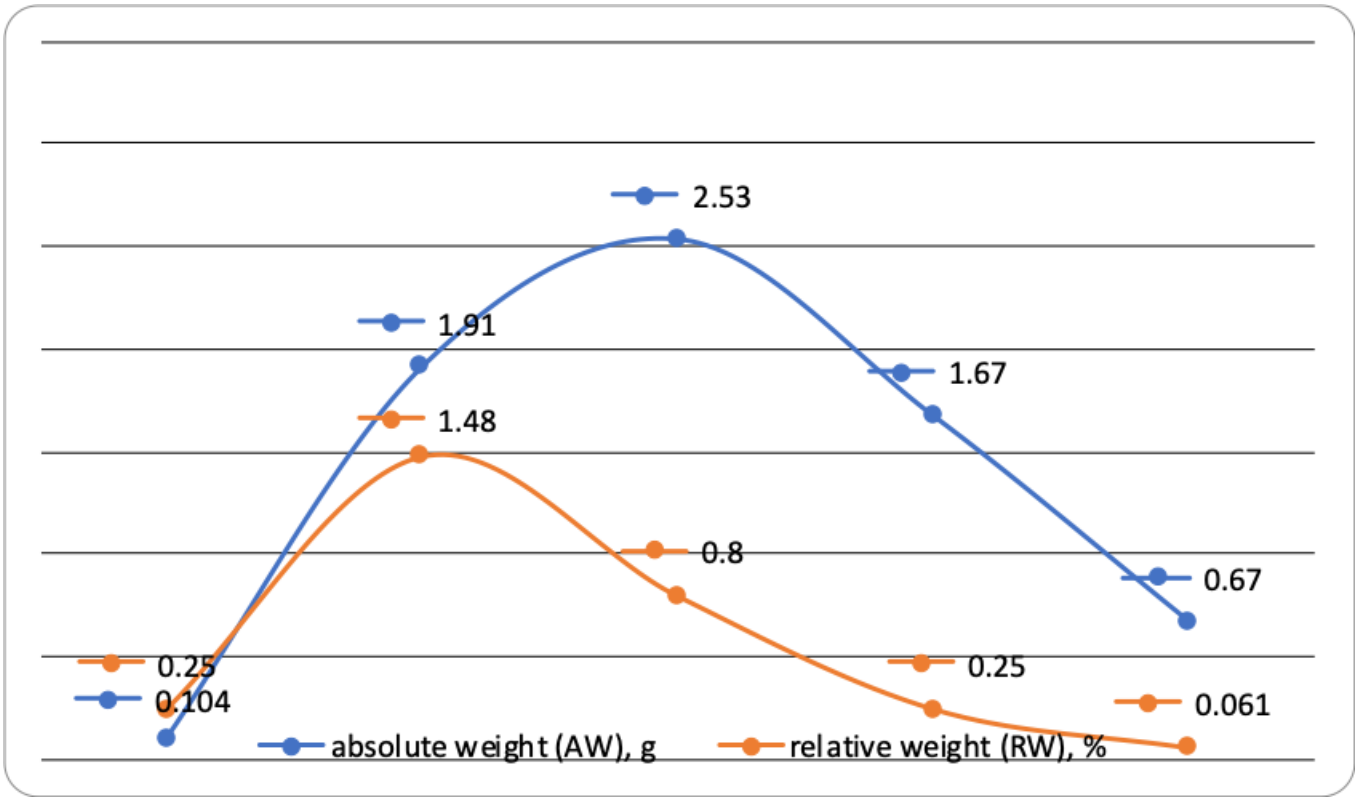

Figure 1. Dynamics of the absolute and relative weight of the chickens' thymus

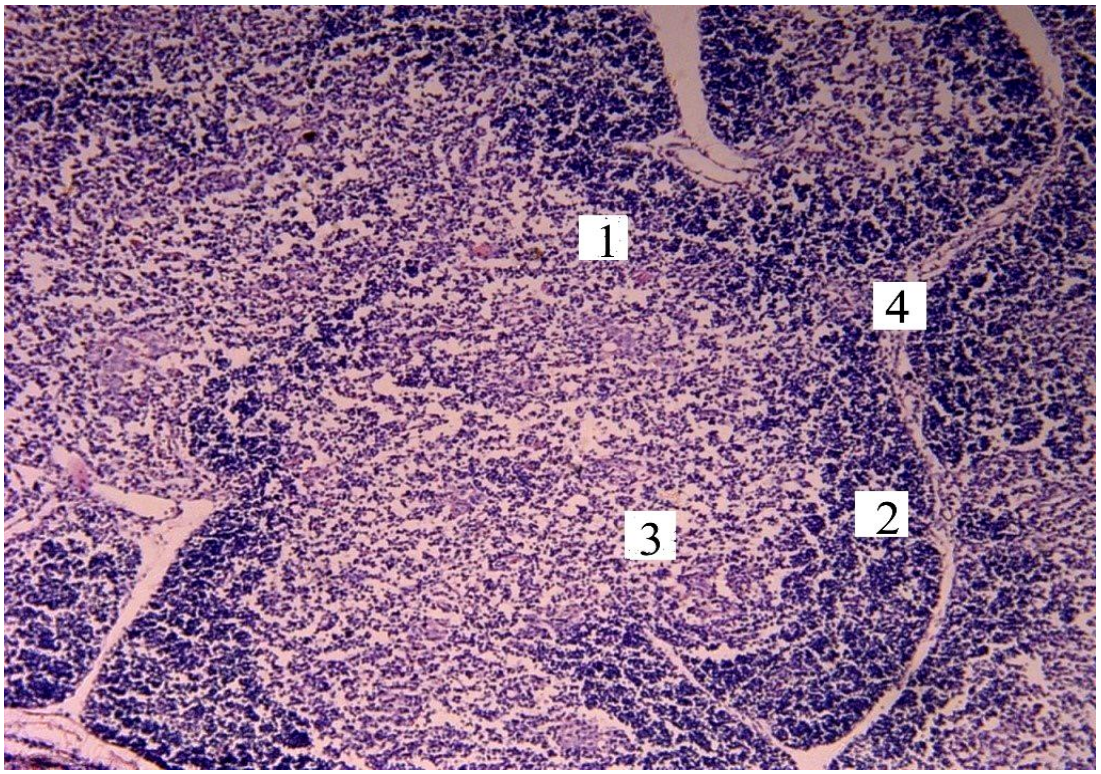

Figure 2. Microscopic thymus structure of the 8-day-old chicken of Haysex Brown cross. 1: Thymus lobule, 2: cortex, 3: Medulla, 4: Interlobular connective tissue, H&E × 100

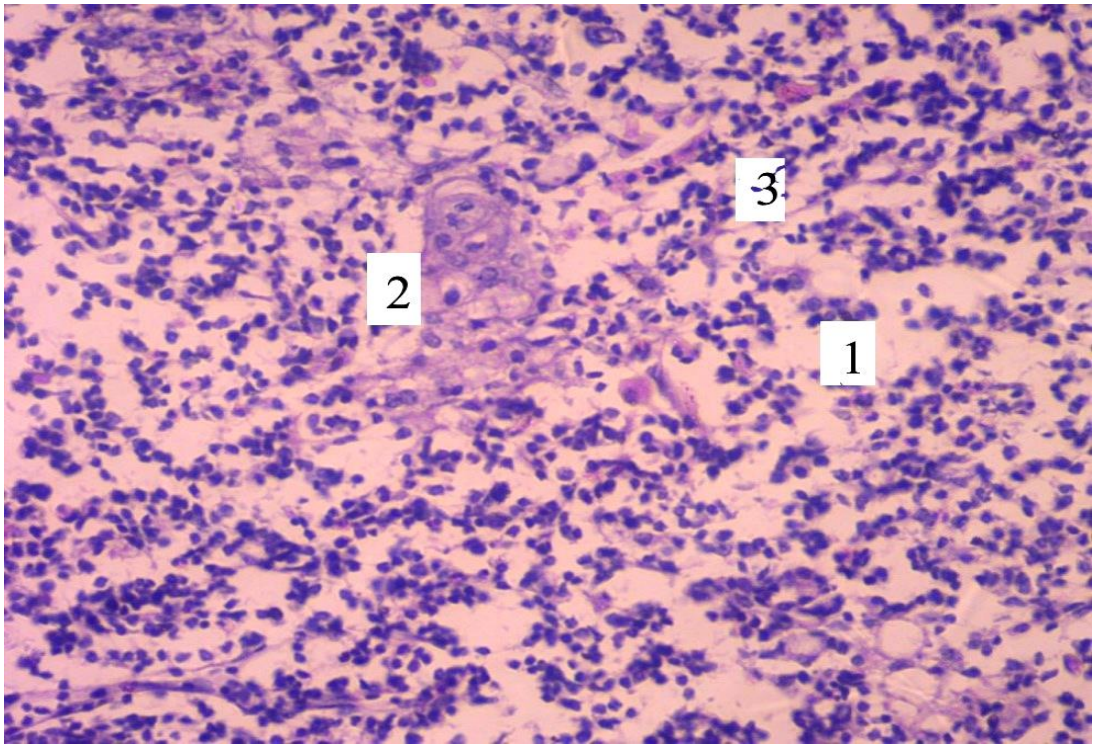

Figure 3. Microscopic thymus structure of a 20-day-old chicken of Haysex Brown cross.1: Medulla, 2: Thymic corpuscles, 3: Growing up epitheliocytes, H&E ×400

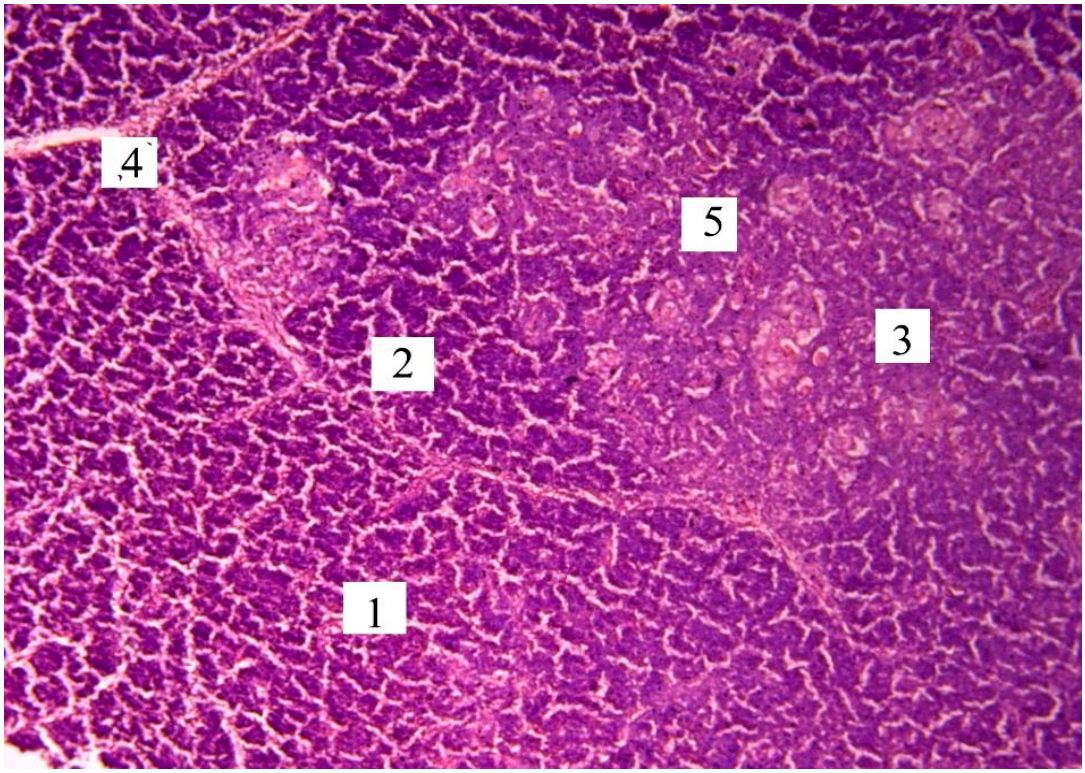

Figure 4. Microscopic thymus structure of a 40-day-old chicken of Haysex Brown cross (1: thymic lobule, 2: cortex, 3: medulla, 4: interlobular connective tissue, 5: thymic corpuscles. H&E x 100)

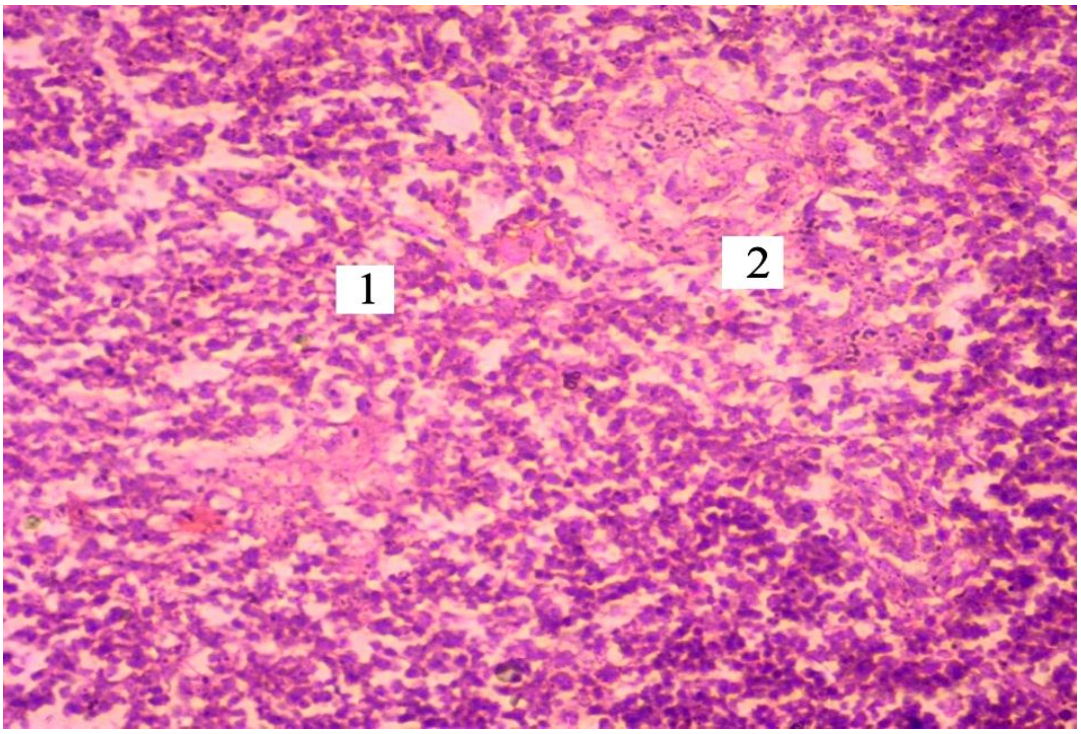

Figure 5. Microscopic structure of the thymus of the 90-day-old chicken of Haysex Brown cross.1: Medulla; 2: Thymic corpuscles, H&E ×400

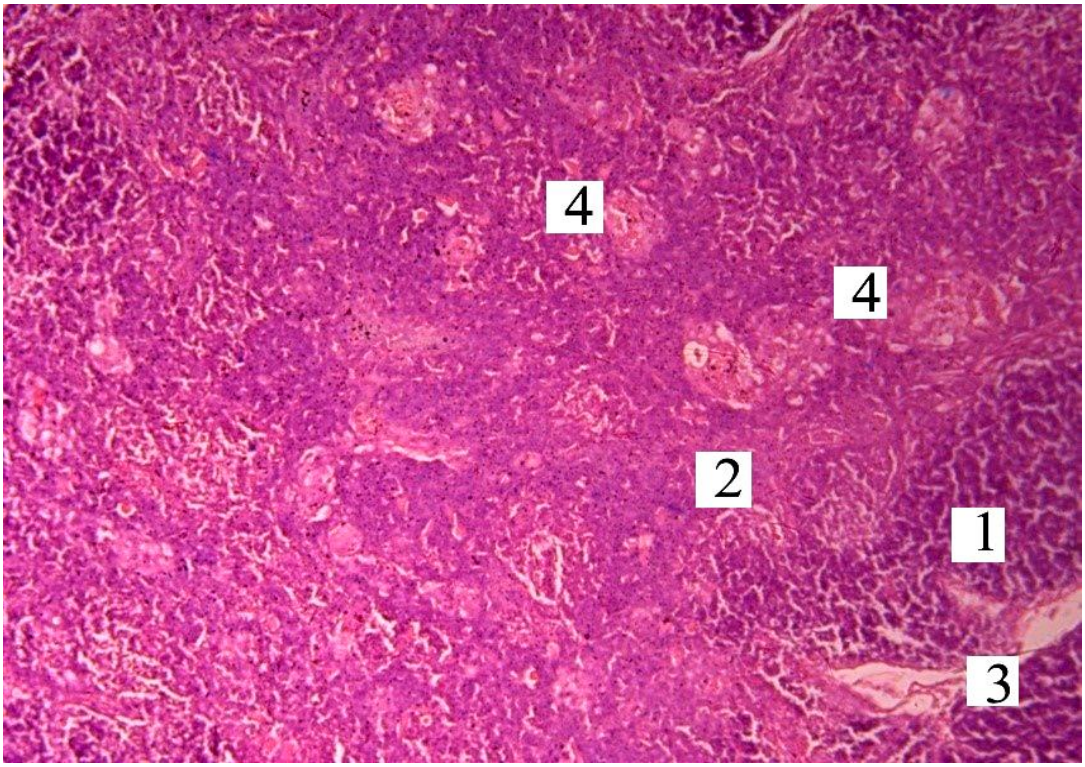

Figure 6. Microscopic thymus structure of a 110- day-old chicken of Haysex Brown cross (1: Cortex, 2: Medulla, 3: Interlobular connective tissue, 4: Thymic corpuscles, H&E ×100)

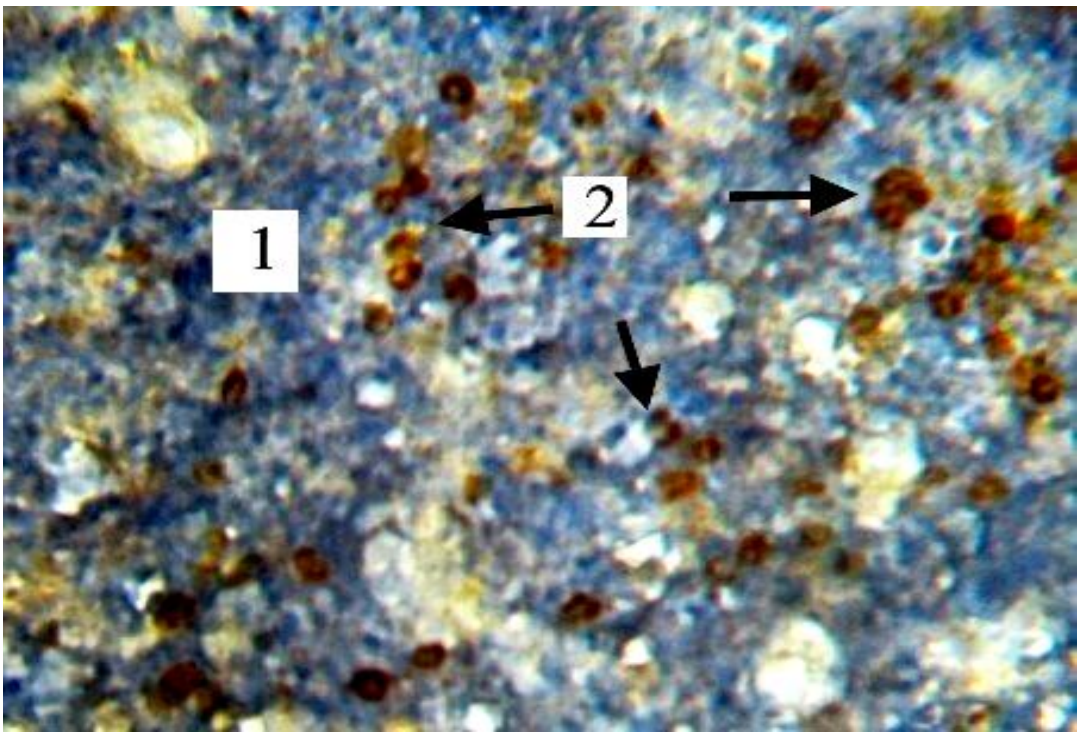

Figure 7. The $CD8^+$ lymphocytes in the thymus of a 20-day-old chicken of Haysex Brown cross.1: Medulla; 2: $CD8^+$Lymphocytes. Histopreparations using monoclonal antibodies with additional fine-tuning of Mayer's hematoxylin, ×600

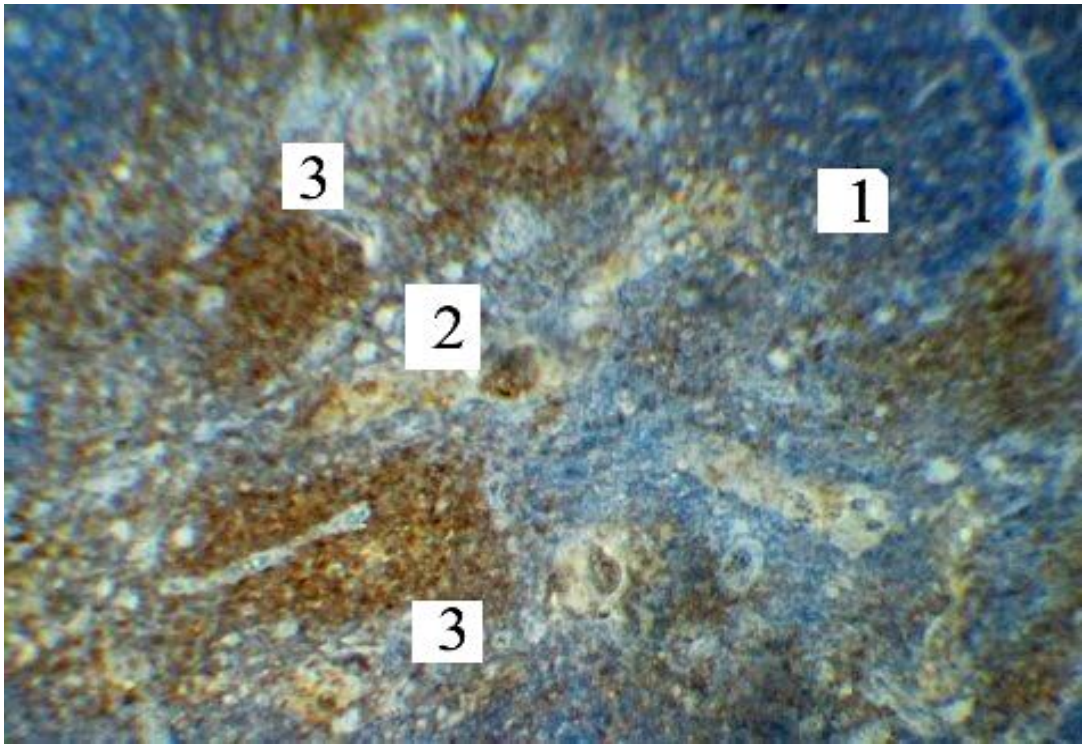

Figure 8. $CD4^+$ lymphocytes in the thymus of a 20-day-old chicken of Haysex Brown cross. 1: Cortex, 2: Medulla, 3: Focal clusters of $CD4^+$ lymphocytes. Histopreparations using monoclonal antibodies with additional fine-tuning by Mayer's hematoxylin, × 100

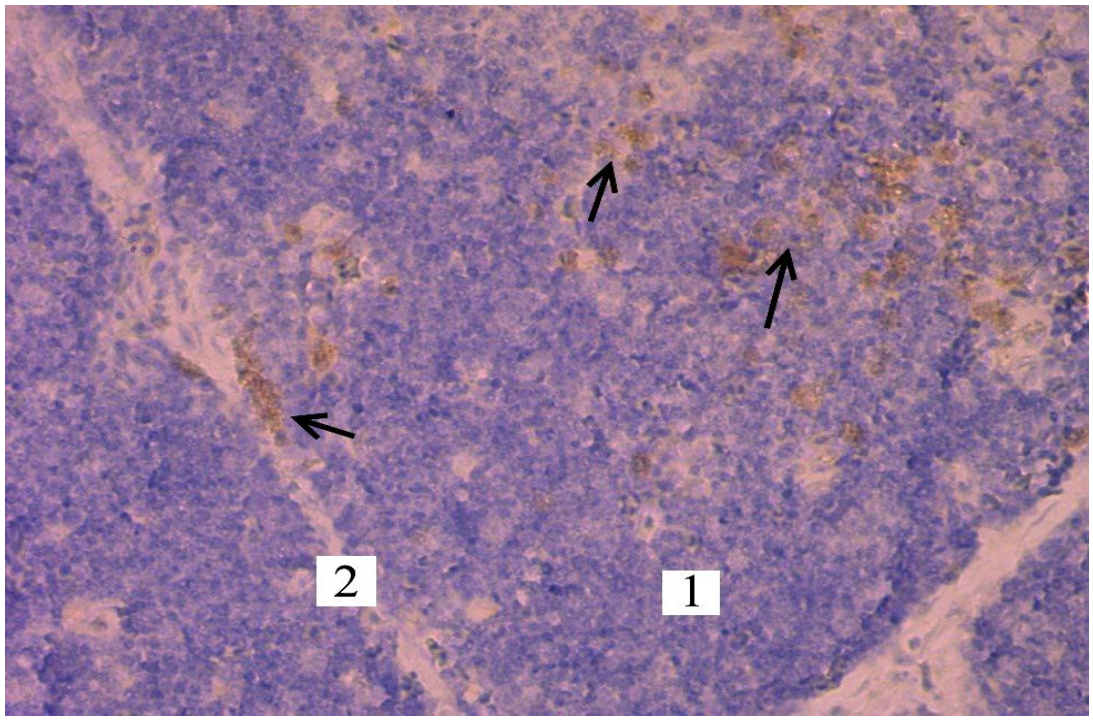

Figure 9. The $CD8^+$ lymphocytes (arrows) in the thymus of a 90-day-old chicken of Haysex Brown cross 1: Thymic lobule, 2: Interlobular connective tissue. Histopreparation are presented using monoclonal antibodies with additional fine-tuning of Mayer's hematoxylin ×400

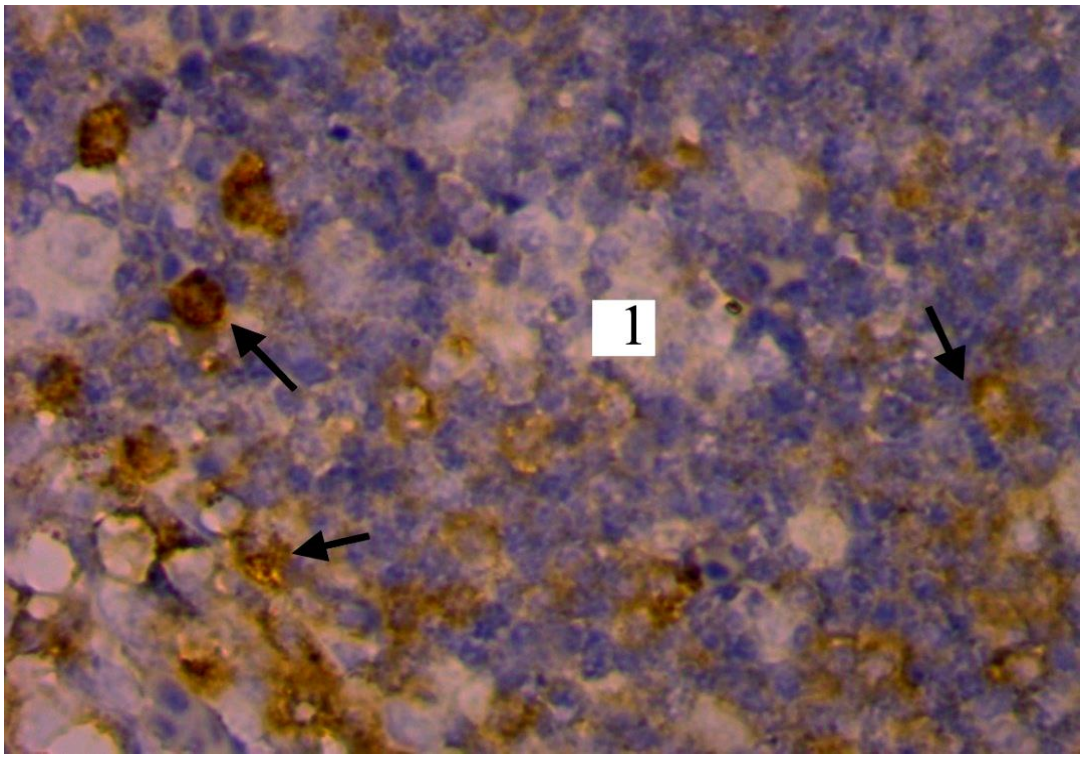

Figure 10. The $CD4^+$ lymphocytes (arrows) in the thymus of a 110-day-old chicken of Haysex Brown cross. 1: Cortex. Histopreparations are presented using monoclonal antibodies with additional fine-tuning of Mayer's hematoxylin, ×1000

DISCUSSION

Considering the evaluation of the avian immune system, there is a need to pay great concerted attention to the condition of the organs involved in their immune system (e.g., thymus, cloacal bursa, and spleen). Despite a number of studies addressing the morphology of lymphoid organs, there are still questions about the dynamics of the development of immunocompetent organs in the postnatal ontogeny and the timing of their involution onset. Understanding the morphological features of the thymus and their changes are important for the functional evaluation of the immune system (Pearse, 2006).

It has been found that with the growth and development of young animals, the absolute and relative weight of the thymus increases (Bellamy, 1982), and these indicators significantly decrease with the puberty, especially in the case of the onset of sexual activity of the bird (Ciriaco et al., 2003). At the time, an involution of the thymus mass and cortical area develops and the boundary between the cortex and medulla matter is shrunk. There is a change in the cellular composition of the thymus where cortex becomes thinner and involution takes its place (Haseeb et al., 2014). Age involution is characterized by a decrease in the amount of lymphoid tissue and the production of T-lymphocytes. However, the total content of circulating T-lymphocytes is maintained at the achieved level since this population of T-lymphocytes represents long-living cells with no requirement of a permanent restoration (Rezzani et al., 2008; Hui, 2012). This leads to the changes in cortex and medulla ratio and proliferation of connective stroma and adipose tissues (Haseeb et al., 2014). One of the reasons for the early onset of organ involution may be the stress factors (Hussan et al., 2009). According to some authors, the degree of its development and morphological condition of thymus significantly affects body's resistance to infections (Treesh et al., 2014).

According to present study, the growth and development of the thymus continue gradually during the first 90 days of birds' life. However, the growth and development of the relative weight of the thymus in broiler chickens take place during the first seven weeks (Tarek, 2012). According to present data, the relative weight of the thymus at 40 days of age is around 0.80 ± 0.03%, which is probably due to the individual characteristics of chickens' development, maintenance conditions, and breeding properties.

The obtained results of the current study addressing the morphological characteristics of the thymus on cellular and tissue levels are supported by many authors (Akter et al., 2006; Haseeb et al., 2014; Senapati et al., 2015; Ali et al., 2017; Kanasiya et al., 2018). It was found that 8-day-old chickens have already fully formed the structural elements of the thymus. Thus, 8-day-old chickens have partly expressed structure with the CI of 0.65±0.02. The dense arrangement of lymphocytes was established in the cortex of thymic corpuscles and reticulospinal cells were found in some places. Lymphocytes and epithelial cells were also observed in the medulla of thymic lobules, and thymic corpuscles were presented by the unicellular forms. These findings confirm the previous studies conducted by many authors. Akter et al. (2006), Khan et al. (2014), Biben (2015), and Ali et al. (2017) described the lobe structure of the thymus by differentiating cortex (densely packed with lymphocytes) and medulla (with a fewer number of lymphocytes) in each lobule.

The present results were also in line with the data obtained by Biben's (2015) indicating that 3-week-old chickens have epithelial cells in thymus medulla at various stages of maturation and apoptosis. This means a normal physiological process testified about the activation of the processes in the immune system. Thymic corpuscles are well-developed and clearly defined which feature the physiological processes of cell death (Biben, 2015). The findings of the present study showed the chaotic placement of thymic corpuscles in the thymus medulla of 20-day chickens. Their number in one slice was 5.42 ± 0.05 pieces. In the same vein, Soad et al. (2014) observed a significant increase in thymic corpuscles in 6- and 8-week-old chickens. Khalil et al. (2003) also reported the growth of thymic corpuscles as birds grow older. The obtained results of the present study demonstrated the growth of thymic corpuscles among chicken aged up to 90 days.

T-system of birds takes part in forming the immune response and regulates its duration (Rezzani et al., 2008). Although an increased number of CD4+, CD8+ T cells were observed in peripheral blood during virus infections, there is a dearth of research on their role, function, and biological significance (Cui et al., 2004; Nascimbeni et al., 2004). Bridle et al. (2006) identified an increase of CD4 and CD8 T-cells population in the 8-week-old chickens. In present study, it was observed that the ID of the thymus of 8-day-old chickens was 1.13±0.27 indicating the existence of immune defense cells at the end of the prenatal development. However, the thymus ID of 20-day-old chickens had the highest value among the investigated age groups reaching 1.86±0.29.

Erf et al. (1998) observed an increase in the ratio of CD4:CD8 in chickens thymus with age. This ratio was reported 1.20 and 2.30 for 2-week-old and 7-week-old chickens, respectively. Similar changes in CD4:CD8 ratio of chicken's thymus was observed in a study conducted by Kannan et al. (2017). The authors of this study reported an increase of the ratio to 0.67 and 0.72 for 4 and 8 week-old chickens respectively. According to the findings of the present research, the ID of 40-day-old chickens was reported 1.26±0.09, whereas, this estimation was 1.52±0.25 and 1.56±0.23 in the 90 and 110-day-old chickens, respectively.

Accordingly, the obtained results of the current study were indicative of CD4:CD8 ratio growth during the bird's aging process. In addition, it was found that the growth of T lymphocytes quantity ($CD8^+$ and $CD4^+$) can be observed in older chickens, in particular those at 20- and 40-days of age. In this period, there was a significant increase in the absolute weight of the thymus and cortical area. Thus, an investigation into the composition of the lymphocyte markers $CD4^+$, $CD8^+$ in the thymus of healthy chickens is promising since it enriches the existing literature about cytoarchitectonics, growth, and development of the thymus in chickens during the postnatal period of ontogenesis.

CONCLUSION

Thymus acquires maximum morphological development in 40-day-old chickens. During this period, there was an increase in the number of lymphocytes with $CD8^+$ and $CD4^+$ markers. The findings correlate with the changes in thymus mass, cortical area, and the cortex index indicating the highest level of expressed functional activity of this organ during this period. The data can yield new insights into developing preventive vaccination schemes.

DECLARATIONS

Author's contributions

Svitlana Huralska and Tetiana Kot created the idea and designed the study, and wrote the draft of manuscript. Vasyl Sokolyuk and Zoriana Khomenko collected data and performed the statistical analysis. Vasyl Koziy collected the information and revised the manuscript. All

authors checked and confirmed the final version of the manuscript.

Competing interests

The authors have declared no competing interest.

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JWPR
Journal of World's Poultry Research

2020, Scienceline Publication

J. World Poult. Res. 10(3): 469-479, September 25, 2020

Research Paper, PII: S2322455X2000054-10

DOI: https://dx.doi.org/10.36380/jwpr.2020.54

The Efficacy of Synbiotic Application in Broiler Chicken Diets, Alone or in Combination with Antibiotic Growth Promoters on Zootechnical Parameters

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Received: 27 Apr. 2020
Accepted: 09 Aug. 2020

ABSTRACT

In recent years, probiotics and synbiotics have gained considerable interest in poultry feeding as an alternative to antibiotics due to antibiotic resistance concerns. The objective of this dual study was to evaluate the efficacy of synbiotic supplementation alone or in combination with different Antibiotic Growth Promoters (AGPs), compared to the untreated control group of broiler chickens production performance. In the first experiment, a total of 1260 one-day-old male Ross 308 broiler chickens were randomly assigned to 7 diet treatments, with 6 replicates per diet treatment and 30 birds per replicate over a 42-day period. The diet treatments included a control diet based on corn-soybean without additives (T1), and the diet treatment with bacitracin (BMD 100 ppm, T2), colistin (10 ppm, T3), synbiotic (PoultryStar me, 0.5 kg/t, T4), a combination of synbiotic (0.5 kg/t) and bacitracin (60 ppm, T5), synbiotic (0.5 kg/t) and colistin (5 ppm, T6), synbiotic (0.5 kg/t), bacitracin (60 ppm), and colistin (5 ppm, T7). During the critical period of rearing from hatch to day 10, the synbiotic supplementation resulted in a significantly higher body weight gain than its combination with bacitracin. No other dietary treatment showed a remarkable improvement in the body weight gain, feed intake, or feed conversion ratio, compared to the only synbiotic application (T4) during the entire trial period. The tendency towards an improved feed conversion ratio was observed during the use of symbiotic (T4, 1.87), compared to the control group (T1, 1.93) during the entire trial period. Compared with the control group (T1, 2.78%), broiler mortality was also lower in the synbiotic group (T4, 1.11%). In the second experiment, a total of 1500 one-day-old male Ross 308 broiler chickens were randomly assigned to 4 diet treatments; with 15 replicates per diet treatment, and 25 birds per replicate over a 42-day period. The dietary treatments included a control group diet based on corn-soybean without additives (T1), and the treatment diets with bacitracin (BMD 1000 ppm, T2), synbiotic (PoultryStar me, 0.5 kg/t, T3), and a combination of synbiotic (0.5 kg/t) plus bacitracin (BMD 1000 ppm T4). Birds fed antibiotic or synbiotic alone or in a combination had numerically a higher body weight and an average daily gain than the control group. There was a tendency of improvement in the feed conversion ratio during the age of 1-24 days, and throughout the experimental period. The evaluated synbiotic could serve as an effective alternative to AGPs, such as bacitracin and colistin in broiler chicken diets, especially during the first crucial period. The synbiotic can serve this purpose without combining it with AGPs, such as colistin or bacitracin.

Keywords: Antibiotic growth promoter, Broilers, Performance, Synbiotic

INTRODUCTION

In view of the apprehensions of antibiotic resistance, probiotics have gained considerable interest in the poultry industry as alternatives to antibiotics (Gustafson and Bowen, 1997). Presently, this class of feed additives is largely used as an alternative to Antibiotic Growth Promoters (AGP) in poultry feeding. The main impetus that has catalyzed the use of these probiotic feed additives is the worldwide ban on the use of AGPs in the diets of food animals. The alternatives to AGPs should ideally possess the same beneficial effects as AGPs do possess when they are supplemented in the diet of food animals.

Despite the incredulous mechanism of action of the feed antibiotics (Huyghebaert et al., 2011), it is generally believed that the AGPs depict some antibacterial activities, which reduces the incidence and severity of subclinical infections, and decreases the microbial consumption of nutrients, thus improving the absorption of nutrients (Snyder and Wostmann, 1987; Brennan et al., 2003). The subsequent effect of all these activities leads to a better

performance of the animal. The foundation of this explanation lies in the fact that AGPs do not exert growth-promoting effects in germ-free animals. The prevailing practice of the industry to feed livestock with sub-therapeutic doses of antibiotics is unlikely to have a growth inhibitory effect on the resident bacteria (Niewold, 2007). However, when antibiotics were added to the broiler diets at the levels below minimum inhibitory concentration, a clear shift in the intestinal microbiota was observed which at least partly explains the effects of AGPs (Pedroso et al., 2006; Wise and Siragusa, 2007).

Shifts in intestinal microbiota likewise affected the intestinal wall morphology and induced immune reactions which may promote the host animals' growth by affecting their energy expenses (Teirlynck et al., 2009). Thus, AGP-alternatives such as probiotics as the hypothetical AGP mode of action should also have modulatory effects on intestinal microbiota and immune system. Probiotics are live microorganisms that should be viable when they are administered in the livestock diets; in order to exert their beneficial effects on an improved intestinal function, intestinal microbiota balance, host immune responses, and the overall host health (FAO and WHO joint working group, 2002).

Dietary probiotics contribute to establish and maintain a beneficial intestinal microbiota, which may enhance the colonization resistance to pathogens, and strengthen the immune responses, leading to an improved growth performance (Dhama et al., 2011; Yang et al., 2012; Mountzouris, 2014; Mountzouris et al., 2015). The path of considering the gastrointestinal tract of food animals as the real complexity of anatomical system playing digestive, absorptive, metabolic, immunological, and endocrinological roles has progressed a lot in the last three decades (Oviedo-Rondon, 2019), the reason why the asseveration gut health became collectively important for the researchers and the livestock industry (Kogut et al. 2017).

The supplementation of probiotics and prebiotics has shown promising results in controlling bacterial infections in poultry by positively influencing the gut microbiota (Mead, 2000). Probiotics competitively excluded pathogenic microbes (Nava et al., 2005) and can be effective by stimulating the immune responses (Koenen et al., 2004), producing antibacterial substances, and stimulating digestive enzymes secretion (Saarela et al., 2000). Synergistic effects could be achieved through so-called synbiotics, a combination of probiotics and prebiotics (Roberfroid, 1998). The combined supplementation of poultry diets with probiotics and prebiotics (synbiotic) has been reported to be more effective than a single supplementation and in some cases even congruous with antibiotic treatments as reported in several studies and reviews (Gaggia et al., 2010; Gadde et al., 2017; Tayeri et al., 2018). Improvements in feed efficiency in broiler chickens as a result of synbiotic supplementation have been attributed to their potential modulatory effect on gastro-intestinal microbial colonization (Brugaletta et al., 2020). Prebiotics are indigestible carbohydrates supplemented frequently in combination with probiotics, which could stimulate the growth of useful bacteria in the intestines of the host (Lee et al., 2016). Prebiotic supplementation was shown to mimic the attachment sites of the pathogens, decreasing the adherence of pathogenic bacteria to the intestinal wall, and increasing specific beneficial bacteria (Ija and Tivey, 1998). Therefore, it draws a great interest to evaluate the effects of a synbiotic on the broiler chicken's performance.

The synbiotic product (PoultryStar me, Biomin Holding GmbH, Austria) evaluated in previous studies contained probiotic bacterial strains of *Enterococcus*, *Bifidobacterium*, *Pedicoccus*, and *Lactobacillus* species and a prebiotic fructooligosaccharide (Babazadeh et al., 2011).

Given the growth-promoting and immune-modulatory roles of AGPs (Niewold, 2007; Kogut and Swaggerty, 2012; Mountzouris, 2014), the performance response of the broilers to synbiotic products, when experimentally supplemented with AGPs in different combinations had been largely unknown. It was not clear whether there were additive effects due to the combination of AGPs and synbiotics.

The aim of these two experimental trials was therefore to evaluate the effect of dietary inclusion of a specific multi-species poultry synbiotic product alone or in different combinations with Bacitracin and/or Colistin, which are used as AGPs on the performance parameters in broiler chickens.

MATERIALS AND METHODS

Ethical approval

All procedures were performed in compliance with relevant laws and institutional guidelines. All animal experiments comply with the ARRIVE guidelines and were carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments.

First experiment

Animals and bird husbandry

A 42-day broiler feeding trial was conducted at the Poultry Research and Development Center of Kasetsart University in Kamphangsaen, Nakhon Pathom, Thailand with a total of 1261 day-old male Ross 308 broiler chickens (with an average body weight of 45 grams at 6-hours post-hatching), according to the prevailing institutional ethical norms. The chickens were weighed individually and assigned to seven treatment groups, each comprising of 6 replicates (n = 30 chickens on the first day). All chickens were raised in floor pens with rice husk as the litter material. Each compartment was equipped with manual feeders and bell-shaped drinkers without nipples. Feed (starter mash from day 1 to 10, grower mash from day 11 to 24, finisher mash 1 from day 25 to 35, and finisher mash 2 from day 36 to 42), and water were offered *ad libitum.* The lighting program was 23-hours light, and 1-hour dark period during the study. The chickens were housed in the evaporative cooling system during the experimental period. The chickens were vaccinated against Newcastle Disease (ND live B1) and Infectious Bronchitis on day 7, Infectious Bursal disease on day 14, and against Newcastle disease (La Sota strain) and Infectious Bronchitis on day 21 again. The temperature was maintained around 32 to 34°C for the first week, and then reduced weekly from 34°C to 25°C. The clinical observations regarding the animal health status, as well as the temperature, humidity, ventilation, and lighting of the trial house, were recorded daily during the experimental period.

Experimental diets and treatments

The trial chickens were randomly assigned to 7 dietary treatments. Each treatment consisted of 6 replications with 30 chickens per replication using a completely randomized design to minimize the effects of group compartments.

All experimental diets were based on the corn-soybean meal. The dietary treatments are presented in tables 1 and 2.

The ingredients and the chemical composition of the experimental diets are presented in table 3, and the nutrient composition of the experimental diets (proximate analysis) is presented in table 4. The synbiotic product used in the present study was obtained from Biomin Holding GmbH, Getzersdorf, Austria, and was included in the diet according to the manufacturer's recommendation. The multi-species product symbiotic (PoultryStar® me) contained probiotic bacterial strains of *Enterococcus*, *Pediococcus*, *Bifidobacterium,* and *Lactobacillus* species as well as a prebiotic fructooligosaccharide.

All the diets were analyzed (AOAC, 2016) for Dry Matter (DM, method 934.01), crude protein (method 988.05), Crude Fiber (method 962.09, CF, Foss Fiber Cap 2021 Fiber Analysis System, Foss Analytical, Hilleroed, Denmark), and crude fat (petroleum ether extraction; method 920.39). Feed samples of each experimental diet prepared for the trial were collected per phase and group immediately after blending and mixing.

Performance parameters measurement

Chicken live weight was recorded individually for each pen on the days 0, 10, twenty-four, and thirty-five, and per each group on the day forty-two. Body Weight Gain (BWG) was calculated per each group. Furthermore, Feed Intake (FI) was measured for the respective periods in combination with body weight measurements. Hence, the average of FI was determined for the respective periods per each group. Feed Conversion Ratio (FCR) for the respective periods was calculated for each group as the mortality-adjusted ratio between FI and BWG.

Second experiment

Animals and birds' husbandry

One thousand five hundred, one day old, male Ross 308 broiler chicks were divided into 4 dietary treatment groups. Each treatment comprised of fifteen replications with twenty-five chickens per replication, and the housing conditions were identical as in the first experimental trial.

Experimental diets and treatments

All diets were corn-soybean meal, formulated to meet the nutritional requirements recommended by Ross

308 nutrition specification guide as in the first experiment. The trial began when the birds were one-day-old, and it was finalized when they were forty-two days old. The chickens were divided into 4 dietary treatment groups. Each treatment consisted of fifteen replications with twenty-five birds per replication, followed by a fully randomized design to minimize the effects of group compartments. The dietary treatments are presented in table 2.

The ingredients and the chemical composition of the experimental diets are presented in table 5, and the nutritional composition of the experimental diets (proximate analysis) is presented in table 6. The synbiotic product used in the present study was similar to the first trial, obtained from Biomin Holding GmbH, Getzersdorf, Austria, and was included in the diet as recommended by the manufacturer. The multi-species product symbiotic (PoultryStar® me) contained probiotic bacterial strains of *Enterococcus*, *Pediococcus*, *Bifidobacterium*, and *Lactobacillus* species, and a prebiotic fructooligosaccharide. All other details of the conditions and practices related to the preparations, mixing, and application procedures of the experimental diets were similar to those of the first trial.

Measurement of performance parameters

The procedures for measuring the performance parameters were the same as described for the first experiment.

Statistical analysis

The pens were the experimental units, and all data were pooled per pen, unless specified different and expressed as the mean, and pooled the Standard Error of Means (SEM). The data were subjected to a one-way analysis of variance (Statistical Package for Social Sciences, SPSS version 10.1) with the diets as the factor, and it was found to be significant. The means were separated by Duncan's new multiple range test at $p < 0.05$.

Table 1. Description of the Dietary treatments applied to Ross 308 broiler chickens

Treatment groups	Description
T1= Negative control (NC)	No additives in feed
T2 = Positive control (PC) 1 (AGP1)	Bacitracin (100 ppm* active ingredient).
T3 = Positive control (PC) 2 (AGP2)	Colistin (10 ppm active ingredient)
T4 = Synbiotic	Synbiotic (PoultryStar me 0.5 kg/ton of feed)
T5 = Synbiotic + PC 1 (PS**+AGP1***)	Synbiotic 0.5 kg/ton feed + Bacitracin (60 ppm active ingredient)
T6 = Synbiotic + PC 2 (PS+AGP2****)	Synbiotic 0.5 kg/ton feed + Colistin (5 ppm active ingredient)
T7 = Synbiotic + PC 1 + PC2 (PS+AGPs)	Synbiotic 0.5 kg/ton feed + Bacitracin (60 ppm active ingredient) + Colistin (5 ppm active ingredient)

*ppm: parts per million, **PoultryStar me, *** Antibiotic Growth Promoter 1 (Bacitracin), **** Antibiotic Growth Promoter 2 (Colistin)

Table 2. Description of the Dietary treatments applied to Ross 308 broiler chickens

Treatment groups	Description
T1= Negative control (NC)	No additives in feed
T2 = Positive control (PC) 1 (AGP)	Bacitracin (BMD*** 10% 1000 ppm*).
T3 = Synbiotic	PoultryStar 0.5 kg/t**** of feed
T4 = Synbiotic with AGP**	Poultry Star 0.5 kg / t + Bacitracin (BMD 10% 1000 ppm)

*ppm: parts per million, **Antibiotic Growth Promoter Bacitracin, *** Bacitracin methylene di-salicylate, **** kilogram per ton

Table 3. Ingredient composition and calculated analysis of experimental diets fed to the Ross 308 broiler chickens during the 42-day trial in the facility of Kasetsart University.

Ingredients	Unit	Starter (Day 1-10)	Grower (Day 11-24)	Finisher 1 (Day 25-35)	Finisher 2 (Day 36-42)
Corn	%	53.40	57.17	61.69	61.69
Soybean meal (46 % CP)	%	30.78	25.94	19.77	19.77
Full fat soybean (35.5 % CP)	%	12.00	13.50	15.00	15.00
Rice bran oil	%	0.50	0.50	1.24	1.24
MDCP (16.9 % Ca, 21.6 % P)	%	0.52	0.33	0.09	0.09
Limestone (38.7 % Ca)	%	0.96	0.87	0.72	0.72
Salt	%	0.41	0.41	0.39	0.39
Sodium bicarbonate (27 % Na)	%	0.05	0.05	-	-
Choline chloride (60 %)	%	0.04	0.03	0.04	0.04
Premix	%	0.60	0.60	0.60	0.60
L-Lysine	%	0.28	0.22	0.19	0.19
DL-Methionine	%	0.26	0.22	0.20	0.20
L-Threonine	%	0.13	0.09	0.06	0.06
Salinomycin (66 ppm)	%	0.05	0.05	0.05	-
Lutanox	%	0.02	0.02	0.02	0.02
Phytase	%	0.01	0.01	0.01	0.01
Total	%	100.00	100.00	100.00	100.00
Calculated analysis					
ME for poultry	kcal/kg	3053	3100	3200	3200
Protein	%	23.00	21.50	19.50	19.50
Fat	%	5.02	5.41	6.54	6.54
Fiber	%	4.00	3.90	3.72	3.72
Digestible Lysine (Poultry)	%	1.28	1.15	1.02	1.02
Digestible Methionine (Poultry)	%	0.51	0.47	0.43	0.43
Digestible Threonine (Poultry)	%	0.88	0.79	0.70	0.70
Lysine	%	1.44	1.30	1.17	1.17
Methionine + Cysteine	%	0.92	0.87	0.80	0.80
Methionine	%	0.61	0.55	0.50	0.50
Threonine	%	0.97	0.88	0.78	0.78
Calcium	%	0.80	0.72	0.61	0.61
Total phosphorus	%	0.60	0.55	0.48	0.48
Avail. Phosphorus (poultry)	%	0.33	0.29	0.24	0.24
Choline	%	1700	1600	1500	1500
Sodium	%	0.19	0.19	0.17	0.17
Salt	%	0.45	0.45	0.42	0.42

ME = Metabolizable Energy, MDCP = Mono-Dicalcium Phosphate.

Table 4. Nutrient composition of experimental diets (proximate analysis) fed to the Ross 308 broiler chickens during the 42-day trial in the facility of Kasetsart University.

Item	Period			
	Starter	Grower	Finisher1	Finisher2
Protein (%)	22.31	20.54	18.25	18.68
Fiber (%)	4.39	4.66	4.08	3.87
Fat (%)	6.01	6.26	5.49	6.08
Ash (%)	4.6	4.69	3.87	3.84
Calcium (%)	0.79	0.8	0.63	0.57
Phosphorus (%)	0.41	0.43	0.34	0.33
GE (kcal/kg)	4.657.52	4.684.73	4.530.39	4.603.69

GE = Gross Energy

RESULTS

First experiment's results

All chickens were healthy during the experimental period, and there was no mortality during the most critical period from the hatch to day 10. The outcome depicted that the synbiotic supplementation in the broiler diet resulted in a significantly higher Body Weight Gain (BWG) than its combination with bacitracin ($p<0.05$) during the hatch to day 10 (Table 7). Additionally, the treatment groups T3 (colistin alone) and T6 (colistin with synbiotic) resulted in a significantly better ($p<0.05$) BWG

during this period compared to the control group (Table 7). None of the other treatments improved BWG, FI, or FCR significantly compared to the only synbiotic application (T4) during the entire experimental period from the hatch to day 42 (Table 8). An improved FCR ($p = 0.0756$) of 1.86 was observed in the bacitracin group (T2), 1.87 in the symbiotic group (T4), and 1.83 in the synbiotic-AGPs combination group (T7), respectively compared to the control group (T1, 1.93), and other treatment groups during the entire trial period (Table 8). No mortality was observed in the colistin-synbiotic combination group (T6) during the entire trial period. However, remarkably low mortality of 1.11% occurred in the bacitracin group (T2), the synbiotic group (T4), and the synbiotic-AGPs combination group (T7), respectively during the entire trial period compared to the control group (T1, 2.78%) and other treatment groups (Table 8).

Second experiment's results

The birds were healthy throughout the entire experimental trial. The crude protein contents in the mixed feeds corresponded to the calculated values. The amount of crude fat, crude fiber, Calcium (Ca), and phosphorous (P) in the experimental diets also was confirmed well by the calculated values (Table 5). Although no significant differences between the dietary treatments regarding zootechnical parameters were observed, the birds fed only with AGP or synbiotic and AGP in combination with synbiotic had a numerically higher body weight and average daily BWG than the non-supplemented control groups ($p = 0.2500$). This led to a tendency to improve FCR between the age of 1 to twenty-four days old, and throughout the experimental period of 1 to forty-two days (Tables 9 and 10).

Table 5. Ingredient composition and calculated analysis (%) of the second experimental diets fed to the Ross 308 broiler chickens during the 42-day trial in the facility of Kasetsart University.

Ingredient	Unit	Starter	Grower	Finisher
Corn	%	54.75	59.52	64.30
Soybean oil	%	1.92	1.72	1.50
Soybean Meal 48 %	%	30.65	24.48	18.25
Full fat Soybean	%	8.00	10.00	12.00
Calcium carbonate	%	1.45	1.33	1.22
MCP-22	%	1.79	1.60	1.44
Salt	%	0.36	0.36	0.36
DL-Methionine	%	0.34	0.30	0.26
L-Lysine	%	0.25	0.23	0.23
Threonine	%	0.09	0.07	0.04
Choline Chloride 60%	%	0.06	0.06	0.05
Antioxidant	%	0.01	0.01	0.01
Toxin Binder	%	0.15	0.15	0.15
Premix (vitamin + mineral)	%	0.18	0.18	0.18
Total	%	100.00	100.00	100.00
Calculated analysis				
ME for Poultry	kcal/kg	3100.00	3150.00	3200.00
Protein	%	23.00	21.00	19.00
Moisture	%	10.92	10.97	11.03
Fat	%	5.93	6.24	6.53
Fiber	%	3.15	3.20	3.26
Ash	%	5.80	5.31	4.86
Ca	%	0.96	0.87	0.79
Total P	%	0.77	0.71	0.65
P avail	%	0.48	0.44	0.40
Salt	%	0.36	0.35	0.35
Lysine	%	1.44	1.29	1.16
Methionine	%	0.67	0.61	0.55
Methionine + Cysteine	%	1.08	0.99	0.91
Threonine	%	0.97	0.88	0.78
Tryptophan	%	0.28	0.25	0.22
Arginine	%	1.54	1.39	1.24
Choline Chloride	mg/kg	1700.00	1600.00	1500.00

ME = Metabolizable Energy, MCP = Monocalcium Phosphate 22% feed grade

Table 6. Nutrient composition of the second experimental diets (proximate analysis) fed to the Ross 308 broiler chickens during the 42-day trial in the facility of Kasetsart University.

Nutrient (%) / Starter	Treatment 1	Treatment 2	Treatment 3	Treatment 4
Moisture	11.60	11.29	11.48	11.34
Protein	21.93	21.48	21.93	22.31
Fat	5.98	5.70	5.48	5.52
Fiber	2.38	2.44	2.40	2.39
Ash	5.81	5.77	5.72	5.69
Calcium	1.01	0.97	1.00	1.00
Phosphorus	0.80	0.74	0.78	0.77
GE (kcal/kg)	4092.82	4106.91	4174.68	4187.77
Grower				
Moisture	11.44	11.29	10.76	11.18
Protein	19.77	19.78	20.10	19.72
Fat	5.96	6.08	6.13	6.01
Fiber	2.03	2.06	2.15	1.95
Ash	6.23	6.32	6.31	6.16
Calcium	0.89	0.91	0.88	0.92
Phosphorus	0.71	0.74	0.73	0.71
GE (kcal/kg)	4147.89	4180.21	4238.82	4230.72
Finisher				
Moisture	12.01	12.02	11.75	11.70
Protein	18.90	18.78	18.98	18.67
Fat	6.70	6.65	6.40	6.52
Fiber	2.06	2.22	2.02	2.12
Ash	4.82	4.83	4.89	4.94
Calcium	0.80	0.82	0.82	0.79
Phosphorus	0.63	0.63	0.64	0.61
GE (kcal/kg)	4373.33	4385.00	4253.81	4218.67

GE = Gross Energy, ME = Metabolizable Energy

DISCUSSION

Presently, probiotics are largely used as alternatives to antibiotic growth promoters (AGP) in the modern poultry nutrition due to concerns of antibiotic resistance, and the ban imposed on the usage of AGPs in the diets of food animals. Beneficial effects of single or multi-species probiotics on the zootechnical performance of broiler chickens were increasingly documented in the scientific literature (Applegate et al., 2010; Fuentes et al., 2013; Zhang and Kim, 2014; Gadde et al., 2017; Tayeri et al., 2018). The data strongly suggested an improvement in health throughout the experimental period and the complete absence of mortality during the most critical period of day 0 to 10 (Table 7). This is in concordance with studies by Pelicano et al. (2004), and Takahashi et al. (2005), in which the use of different growth promoters in the early phase of rearing led to no differences in the viability and mortality rates of the broiler chickens. The present results indicated that the synbiotic supplementation in the diets of broiler chickens resulted in a significantly higher BWG than the combination with bacitracin ($p < 0.05$) during the first days of the post-hatch brooding period, considered the most critical phase of rearing from hatch to day 10 (Table 7). Probiotics are known to contribute towards the establishment and maintenance of a beneficial intestinal microbiota, which could enhance the colonization resistance to pathogens, and immune response improvements resulting in improved growth performance (Mountzouris, 2014; Mountzouris et al., 2015; Kogut et al. 2017; Baldwin et al., 2018; Oviedo-Rondon, 2019; Brugaletta et al., 2020).

The body weight gain was significantly better ($p < 0.05$) in T3 (colistin alone) and T6 (colistin with synbiotic) treatment groups compared to the control groups during this critical period from hatch to day 10 of age (Table 7). Synergistic effects were observed by feeding synbiotics, which are a combination of probiotics and prebiotics (Roberfroid, 1998; Gaggia et al., 2010; Gadde et al., 2017; Tayeri et al., 2018). There was not any significant improvement in BWG, FI, or FCR in any other group compared to the only synbiotic application (T4) during the entire study period from the hatching day to the day forty-second (Table 8).

An improved FCR ($p=0.0756$) of 1.86 was observed in the bacitracin group (T2), 1.87 in the synbiotic group

(T4), and 1.83 in the synbiotic-AGPs combination group (T7), respectively compared to the control group (T1, 1.93), and other treatment groups in the study (Table 8). No mortality was recorded in the colistin-synbiotic combination group (T6) during the entire trial period. In the bacitracin group (T2), the synbiotic group (T4), and the synbiotic-AGPs combination group (T7), however, a very low bird mortality rate of 1.11% occurred compared to the control group (T1, 2.78%), and other treatment groups (Table 8). Chickens in the second experimental trial were also healthy during the entire study. Although no significant differences among the dietary treatments regarding zootechnical parameters were observed, birds fed with AGP or synbiotic alone, and their combination, had a numerically higher body weight and average daily BWG than that of the control groups (p=0.2500). This improvement in the BWG in these treatment groups tended to improve FCR in the chickens aged 1 to twenty-four days, and 1 to forty-two days old throughout the experimental period (Tables 9 and 10). No significant differences in body weight, FI, FCR, and mortality among the synbiotic, colistin, and bacitracin groups alone or in combination with each other revealed that AGP could be replaced by synbiotics without loss of zootechnical performance.

Table 7. Effect of the combination of synbiotics with antibiotic growth promoters on the production performance of broiler chickens from day of hatch to day 10.

Treatment groups[1]	BWG (g/bird)	FI (g/bird)	FCR	Mortality (%)
T1	192.656 [ab]	263.472	1.36	0.00
T2	193.094 [ab]	272.611	1.41	0.00
T3	199.133[a]	271.389	1.36	0.00
T4	198.461[a]	270.217	1.36	0.00
T5	186.050[b]	272.361	1.46	0.00
T6	199.678[a]	267.611	1.34	0.00
T7	193.189 [ab]	265.583	1.37	0.00
p -value	0.0337	0.6047	0.1492	0.00
SEM	1.2663	1.5107	0.0129	0.00

[a,b] Means with dissimilar letters in a column varied significantly ($p < 0.05$) [1] T1= No additives in feed, T2 = Bacitracin, T3 = Colistin, T4 = Synbiotic (PoultryStar® me), T5 = Synbiotic + Bacitracin, T6 = Synbiotic + Colistin, T7 = Synbiotic + Bacitracin + Colistin. BWG = Body Weight Gain, FI = Feed Intake, FCR = Feed Conversion Ratio, SEM = Standard Error of Means

Table 8. Effect of the combination of synbiotics with antibiotic growth promoters on the production performance of broiler chickens from the first day to day 42.

Treatment groups[1]	BWG (g/bird)	FI (g/bird)	FCR	Mortality (%)
T1	2872.50	5547.01	1.93	2.78
T2	2921.17	5435.59	1.86	1.11
T3	2871.02	5540.05	1.93	1.67
T4	2923.90	5479.02	1.87	1.11
T5	2883.07	5412.70	1.88	1.67
T6	2893.12	5444.65	1.88	0.00
T7	2972.42	5453.08	1.83	1.11
p-value	0.7736	0.6241	0.0756	0.4758
SEM	18.1249	22.4774	0.0096	2.2160

[1] T1= No additives in feed, T2 = Bacitracin, T3 = Colistin, T4 = Synbiotic (PoultryStar® me), T5 = Synbiotic + Bacitracin, T6 = Synbiotic + Colistin, T7 = Synbiotic + Bacitracin + Colistin. BWG = Body Weight Gain, FI = Feed Intake, FCR = Feed Conversion Ratio, SEM = Standard Error of Means.

Table 9. Effect of dietary treatments on growth performance of Ross 308 broiler chickens from day 1 to day 24, fed in the facility of Kasetsart University.

Treatment	Feed intake (g)	Body weight (g)	ADG (g/bird/day)	FCR	% Livability
NC	1720.573	1013.843	42.243	1.698	99.733
AGP	1717.280	1021.277	42.553	1.682	100.000
PS	1721.093	1021.189	42.550	1.687	99.733
PS + AGP	1721.107	1020.827	42.534	1.686	99.733
p-value	0.9818	0.8752	0.8752	0.6611	0.8013
SEM	3.7512	3.7136	0.1547	0.0047	0.1135

NC = negative control, no additives in feed, PS = PoultryStar® me, AGP = antibiotic growth promoters, Bacitracin, ADG = average daily weight gain, FCR = feed conversation ratio, SEM = Standard Error of the mean, g = gram

Table 10. Effect of dietary treatments on growth performance of Ross 308 broiler chickens from day 1 to day 42, fed in the facility of Kasetsart University.

Treatment	Feed intake (g)	Body weight (g)	ADG (g/bird/day)	FCR	Livability (%)
NC	4740.160	2536.299	61.403	1.841	99.200
AGPs	4722.093	2572.408	62.196	1.811	99.733
PS	4724.067	2548.600	61.697	1.824	99.467
PS + AGPs	4751.960	2565.667	62.103	1.824	99.733
p -value	0.8165	0.3723	0.3732	0.2500	0.6368
SEM	12.4302	20.4605	0.4876	0.0115	0.1672

NC = negative control, no additives in feed, PS = PoultryStar® me, AGP = antibiotic growth promoters, Bacitracin, ADG = average daily weight gain, FCR = feed conversation ratio, SEM = Standard Error of the mean, g = gram

CONCLUSION

Overall, the results of these two experiments under the controlled conditions proved that the evaluated synbiotic (PoultryStar® me) could serve as a replacement and an effective alternative to the Antibiotic Growth Promoters (AGPs), such as bacitracin and colistin in the broiler diets. With careful evaluation and the right preventive programs, the synbiotic can serve this purpose without being combined with AGP's. Hence, the replacement could be cost-effective and bring more value to broiler chicken producers.

DECLARATIONS

Authors' contribution

The experimental studies were conceived and designed by Basharat Syed and Yuwares Ruangapanit in consultation with Silvia Wein. Yuwares Ruangapanit supervised the experimental trials, collection of data, and its analysis. Silvia Wein reviewed the statistical analysis. The manuscript was written and drafted by Basharat Syed. All authors read and approved the final manuscript for submission and publication.

Competing interests

The authors declare that they have no competing interests.

Acknowledgements

The authors acknowledge the financial support for the experimental studies extended by BIOMIN Holding GmbH, Austria, and the Poultry Research and Development Center of Kasetsart University in Kamphangsaen, Nakhon Pathom, Thailand for the technical support.

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JWPR
Journal of World's Poultry Research

2020, Scienceline Publication

J. World Poult. Res. 10(3): 480-484, September 25, 2020

Research Paper, PII: S2322455X2000055-10

DOI: https://dx.doi.org/10.36380/jwpr.2020.55

Production Performances of Indonesian Native Rooster (*Gallus gallus domesticus*) Supplemented with Germinated Mung Bean Sprouts and Acidifiers in the Diet

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Received: 06 Jul. 2020
Accepted: 25 Aug. 2020

ABSTRACT

The research aimed to analyze the production performances of the Indonesian native rooster (*Gallus gallus domesticus*) fed germinated mung bean sprouts and acidifier supplementation in the diet. A total of 24 roosters aged 12 months with an average body weight of 2.29 ± 0.23 kg were used for the research subject. The diet was composed of a basic diet supplemented with 48-hours germinated mung bean sprouts and acidifier, with a basic no supplement diet as a control group. The research was conducted as an *in vivo* factorial randomized block design with different amounts of germinated mung bean sprouts (0% and 1.8%) and acidifiers (0%, 0.4%, 0.80%, and 1.20%) as the research treatment. Each treatment was performed in triplicate, and the observed production performances include Daily Intake (DI) of feed, Feed Consumption Ratio (FCR), Average Daily Gain (ADG), and Body Weight (BW). All data were analyzed using ANOVA (analysis of variance) and then tested by Tukey's test to determine significant differences. The results showed that the supplementation of mung bean sprouts and acidifiers did not give any differences from DI, FCR, ADG, and BW of *Gallus gallus domesticus*. However, the supplementation of germinated mung bean sprouts and acidifiers in the present research showed better overall production performances compared to the control group. The best production performance of the treatments was found at 1.8% germinated mung bean sprout and 1.2% acidifier additive based on the FCR (1.14±0.06) with DI at 91.94±1.11 gram (g)/head, ADG at 305.33±34.93 g/day, and final BW found after 30 days at 2,434.67±155.28 g. It has been concluded that the germinated mung bean sprout and acidifiers supplementation increases the production performance of *Gallus gallus domesticus*, with longer and higher supplement levels being suggested.

Key words: Mung bean sprouts, Native chicken, Poultry diet, Production performances

INTRODUCTION

The feed diet provides a source of energy and nutrition required for poultry to live, grow, and reproduce well (Bell and Weaver, 2002). Mustafa et al., (2017) stated that the nutritional value in feed played an important role in determining production performance in poultry, which contributed up to 70% of production performances in native chickens. However, nutritional optimization value in native chicken feed was still underdeveloped due to the lower FCR compared to broiler chickens, even though the feed adaptability of native chickens would provide various approaches to feed optimization by using various alternative feed supplements (Henuk, 2013).

The important factors that to consider when choosing alternative feeds are that they are abundant, inexpensive, of good nutritional value and non-competitive for human consumption in order to achieve optimal native chickens farming (Ahmadani, 2015). One of the abundant and inexpensive alternative feedstuffs in Indonesia is the mung bean sprout (Purwono and Hartono, 2005). Mung bean sprouts are known to be high in protein and multiple vitamins, but they also contained nutritional inhibitory compounds that could be eliminated by certain treatments, such as submersion, germination, and heat. One of the nutritional inhibitors was trypsin inhibitor in the form of tannin or polyphenol, which suppresses protein digestibility. However, the nutritional inhibitory activity of the compound would be reduced during germination (Anggrahini, 2007). In the germination period, some starch content in the mung bean was metabolized into maltose, which was catalyzed by amylase enzyme (Huang et al., 2014), while the protein molecules were converted

into amino acids. Research by Anggrahini (2007) revealed that mung bean sprout contained 24% lysine, 19% threonine, 29% alanine, and 7% phenylalanine, several fatty acids, and minerals.

However, most dietary supplements contained synthetic compounds that could have adverse effects to the chickens (Iji and Tivey, 1998), such as microbial retention, while their residual compounds were also harmful to human health (Pavlovic et al., 2005). Also, the increased public concern about the emergence of antibiotic-resistant strains prompted the exploitation of alternate growth promoters for antibiotics (Yadav et al., 2016). Therefore, the use of natural feed additives was preferred to increase feed efficiency (Huang et al., 2014).

Few alternatives were available for feed additives, such as probiotics, prebiotics, phytochemicals, enzymes, and organic acids. Among these alternatives, the organic acids, also known as acidifiers, had become widely known as the compound played an important role in the intestinal health in animals (Natsir, 2008). The potential of acidifiers in the livestock feed industry has been known for decades for their preservative and nutritional properties (Partanen and Morz, 1999). Fernandez et al. (2006) stated that organic acids were cell metabolites with low toxicity and were beneficially used as feed additives. In addition, research by Soltan (2008) revealed that supplementing organic acid as a feed additive could effectively increase nutrient absorption. Rukmana (2003) added that using alternative feed supplements for native chickens should not only meet the energy and protein requirements but also be high in vitamin E to support the reproduction of the chickens. In the present research investigated the effect of germinated mung bean sprout supplement and acidifier on the production performance of *Gallus gallus domesticus*, a native breed of chicken spread on Indonesia and Malaysia.

MATERIALS AND METHODS

The research was conducted *in vivo* on 24 *Gallus gallus domesticus* at the age of 12 months with an average body weight of 2.29 ± 0.23 kg obtained in Malang, Indonesia. The overall picture of *Gallus gallus domesticus* in the present research is shown in figure 1. The mung bean sprouts were germinated for 48 hours to be used as a dietary supplement along with an acidifier. The basal diet was formulated with yellow maize, bran meal, palm oil, meat bone meal, soybean meal and minerals. The acidifier used in this study consisted of fumaric acid, formic acid, propionic acid, citric, and lactic acid. Proximate analysis performed to examine the nutritional value of the feed, which included energy, crude protein, crude fats, crude fiber, calcium, and phosphor (Table 1).

The *Gallus gallus domesticus* in the present research was reared in a grouped pens model with the area off 100 × 170 cm, each group containing three chickens. The pens were equipped with drinking and feeding gallons. The *Gallus gallus domesticus* was initially adapted with the basic feed for 10 days, gradually switched to the treatment diets for 6 days, and then fed completely with diet treatments of up to 100 g/head/day for 30 days. The present research treatments were basic feed with different additional amounts of germinated mung bean sprout (K_0 = 0%; K_1 = 1.8%) and acidifiers (A_0 = 0.00%; A_1 = 0.40%; A_2 = 0.80%; A_3 = 1.20%). Each treatment was performed in triplicate, and the observed production performances included feed Daily Intake (DI), Feed Consumption Ratio (FCR), Average Daily Gain (ADG), and Body Weight (BW). The research was designed in a factorial randomized design, and all data were analyzed using ANOVA, followed by Tukey's test to determine any significant differences.

Table 1. Nutrient compositions of research treatments

Nutrient composition	A0K0 (control)	A0K1	A1K0	A1K1	A2K0	A2K1	A3K0	A3K1
Energy (kcal/kg)	2809	2818	2802	2807	2800	2800	2798	2795
Crude protein (%)	17.20	17.00	17.00	17.00	17.00	17.00	17.00	17.00
Crude fats (%)	3.20	3.10	3.10	3.00	3.10	2.90	3.10	2.90
Crude fiber (%)	4.40	4.40	4.40	4.40	4.30	4.40	4.30	4.40
Ca (%)	0.90	0.90	0.90	8.00	0.80	0.80	0.70	0.70
P (%)	0.50	0.40	0.40	0.40	0.40	0.40	0.40	0.40

Description: A0K0 (control) = 0% mung bean sprout and 0% acidifiers; A0K1 = 1.8% mung bean sprout and 0% acidifiers; A1K0 = 0% mung bean sprout and 0.4% acidifiers; A1K1 = 1.8% mung bean sprout and 0.4% acidifiers; A2K0 = 0% mung bean sprout and 0.8% acidifiers; A2K1 = 1.8% mung bean sprout and 0.8% acidifiers; A3K0 = 0% mung bean sprout and 1.2% acidifiers; A3K1 = 1.8% mung bean sprout and 1.2% acidifiers.

Figure 1. *Gallus gallus domesticus* used in present research

RESULTS

The proximate analysis was performed to examine the nutritional value of the basic feed (control/no supplement) as well as the research treatments. The result of the proximate analysis is presented in table 1.

The research data on feed consumption and feed conversion are presented in table 2. These data revealed that germinated mung bean sprout and acidifiers supplementation could increase the DI and lower the FCR compared to the control group, even though the increase was not significant ($p > 0.05$). Moreover, it can be seen that in 1.8% mung bean sprout and 1.2% acidifier additive (A3K1), the treatment showed the lowest FCR (1.14 ± 0.06), which indicates the best feed efficiency.

The BW and ADG of *Gallus gallus domesticus* in the present study are presented in table 3. The results revealed that the BW and ADG of *Gallus gallus domesticus* were increased along with germinated mung bean sprout. The highest BW and ADG in the present research were found for A3K0 and A3K1, respectively, with a total of mung bean sprout and acidifier supplementation showed better BW and ADG compared to the control group. The higher BW and ADG indicate that the mung bean sprout and acidifier supplement could provide better nutrient absorption, especially protein, which promotes tissue development, although note that the protein content of all treatments is relatively similar (Table 1).

Table 2. Daily feed consumption and feed conversion of the research data

Treatments	Daily intake (g/head)	FCR
A0K0 (Control)	91.75 ± 4.69	1.16 ± 0.06
A0K1	87.56 ± 3.46	1.23 ± 0.13
A1K0	94.11 ± 2.47	1.17 ± 0.11
A1K1	91.49 ± 6.65	1.17 ± 0.15
A2K0	94.14 ± 1.37	1.22 ± 0.03
A2K1	92.85 ± 0.92	1.22 ± 0.03
A3K0	93.06 ± 0,82	1.15 ± 0.10
A3K1	91.94 ± 1.11	1.14 ± 0.06

Description: A0K0 (control) = 0% mung bean sprout and 0% acidifiers; A0K1 = 1.8% mung bean sprout and 0% acidifiers; A1K0 = 0% mung bean sprout and 0.4% acidifiers; A1K1 = 1.8% mung bean sprout and 0.4% acidifiers; A2K0 = 0% mung bean sprout and 0.8% acidifiers; A2K1 = 1.8% mung bean sprout and 0.8% acidifiers; A3K0 = 0% mung bean sprout and 1.2% acidifiers; A3K1 = 1.8% mung bean sprout and 1.2% acidifiers.

Table 3. Bodyweight and average daily gain of the research data

Treatments	Bodyweight (g)	Average daily gain (g/day)
A0K0 (control)	2.380 ± 228.11	170 ± 65.38
A0K1	2.137 ± 136.52	275 ± 34.79
A1K0	2.425 ± 294.07	260 ± 65.19
A1K1	2.348 ± 138.12	284 ± 115.68
A2K0	2.315 ± 97.34	228 ± 12.12
A2K1	2.281 ± 32.59	259 ± 45.13
A3K0	2.446 ± 198.14	219 ± 26.08
A3K1	2.434 ± 155.28	305 ± 34.93

Description: A0K0 (control) = 0% mung bean sprout and 0% acidifiers; A0K1 = 1.8% mung bean sprout and 0% acidifiers; A1K0 = 0% mung bean sprout and 0.4% acidifiers; A1K1 = 1.8% mung bean sprout and 0.4% acidifiers; A2K0 = 0% mung bean sprout and 0.8% acidifiers; A2K1 = 1.8% mung bean sprout and 0.8% acidifiers; A3K0 = 0% mung bean sprout and 1.2% acidifiers; A3K1 = 1.8% mung bean sprout and 1.2% acidifiers.

DISCUSSION

The increased DI with better FCR found in the present research was due to the rich vitamin content of the mung bean sprouts. Stephens (2018) stated that germinating of mung bean would increase the vitamin content, and after two days of germination would reach the maximum vitamin content while improving the palatability, which directly affects the feed intake. Research by Troszynska et al. (2004) on the legume seeds germination also revealed that germination would improve the mung bean palatability. The palatability of feedstuffs is a response of

the nervous and taste bud system towards the flavour experienced by the animal (Lamichchane et al., 2018), while Mansoub and Nezhady (2011) added that the nutritional value of the feed also had a positive response to feed intake.

In table 2, it can be seen that 1.8% of mung bean sprout and 1.2% acidifier additive gave the best FCR. The FCR indicates the total amount of feed that is required to gain one kilogram of body weight, which indicates that supplementing both feeds could optimize the production performances of *Gallus gallus domesticus*. Lamichchane et al., (2018) stated that nutrients availability in feed plays a vital role in maintaining energy balance, promoting body growth, and immunity, as well as providing antioxidant and repairing damaged tissue. In addition to the mung bean sprouts, acidifiers also contributed to the production performances of the chickens. Brown and Southern (1985) explained that the citric acid content in the acidifier would provide the intestinal environment with a hydrogen ions donor, which helped maintain the pH of the intestinal lumen. The condition thus increases nutrient absorption in the animal intestine (Deepa et al., 2011). Similar results were also shown by Natsir (2008), that stated that acidifier supplementation in the diet could maintain digestive pH, which is essential for the protein absorption of chickens.

Widodo (2002) mentioned that a higher nutrient absorption indicated better digestibility of the feed. The feed digestibility was then reflected in the FCR of the animal, with a lower FCR value indicating more efficient feed consumption (Rasyaf, 2006). Lacy and Vest (2000) stated that FCR was affected by several factors, such as genetic, feed quality, animal health, temperature, sanitation, ventilation, medication, and rearing management. The FCR is one of the indicators for determining the production performances, as it correlates within the BW and ADG of the animal (Rasyaf, 2006). The protein compounds in the feed are essential for chicken metabolism and body growth (Widodo, 2002). It is widely known that protein and energy, along with other micronutrients such as vitamins and minerals, are the main nutrients that affect chicken production performance. Research by Purwono and Hartono (2005) showed that mung bean contained several vitamins, such as niacin, riboflavin, and folic acid, which will be increased after germination.

The increased BW and ADG were also affected by acidifier supplementation, as Lückstädt and Mellor (2011) indicated that acidifiers play an important role in digestibility and nutrient absorption by maintaining the pH of the digestive system, which inhibits the growth of pathogenic bacteria, such as *Escherichia coli* and *Salmonella species*, which would negatively affect product performance. Even though the supplementation of germinated mung bean sprout and acidifier positively affect production performance, the insignificant difference between DI, FCR, BW, and ADG were found in the present research, which was due to nature of the chicken breed. Native roosters, such as *Gallus gallus domesticus*, are known to have slower body growth than broiler chickens. Although the nutritional quality is an important factor, the age and strain of chickens also play an important role in the production performance (Amrullah, 2004). The slower growth of *Gallus gallus domesticus* then suggests a higher and longer supplement of mung bean sprout and acidifier in *Gallus gallus domesticus*.

CONCLUSION

It was concluded that mung bean sprouts and acidifiers supplementation increase the production performances of *Gallus gallus domesticus*, although the supplement amounts in the present study did not indicate any significant differences. The best result was shown with 1.8% mung bean sprout and 1.2% acidifier additive, which had an FCR of 1.14 ± 0.06, a DI of 91.94 ± 1.11 g/head, an ADG of 305.33 ± 34.93 g/day, and a final BW after 30 days of 2,434.67 ± 155.28 g.

DECLARATIONS

Competing interests

The authors declare that they have no competing interests.

Author's contributions

Nonok Supartini and Muhammad Nur Ihsan designed the research. Nonok Supartini and Muhammad Halim Natsir performed the research and analyzed the data. Nonok Supartini wrote the manuscript. Muhammad Nur Ihsan, Muhammad Halim Natsir, and Nurul Isnaini participated in the revision of the manuscript. All authors have read and approved the final version of the manuscript.

Acknowledgements

The authors would like to acknowledge the assistance of Mr Aldyon Restu Azkarahman, colleagues, as well as special work by the technical staff. The authors also appreciate the rector and the dean of the Faculty of Animal Science, who gave them opportunities and

financial support for the research through Penelitian Hibah Guru Besar and Doktor Universitas Brawijaya Tahun 2019 program.

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2020, Scienceline Publication

J. World Poult. Res. 10(3): 485-492, September 25, 2020

Research Paper, PII: S2322455X2000056-10

DOI: https://dx.doi.org/10.36380/jwpr.2020.56

Physiological and Reproductive Responses of Domyati Ducks to Different Dietary Levels of Coconut Oil as a Source of Medium-Chain Fatty Acids during Laying Period

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Received: 07 Jul. 2020
Accepted: 20 Aug. 2020

ABSTRACT

The objective of this study was to determine the optimal level of coconut oil (CO) supplementation in the diet to enhance the performance of Domyati ducks. A total number of 300 Domyati ducks (240 females and 60 males) aged 25-week-old were randomly assigned to 4 experimental groups of three replicates, each replicate included 5 males and 20 females of Domyati ducks. The groups received CO at 0, 1.0, 1.5, and 2.0% during the experimental period. The results indicated that egg weight, egg number, and egg mass significantly increased in treated groups, compared to the control group. Furthermore, fertility and hatchability percentages were superior in 1.0, and 1.5% CO groups, compared to other experimental groups. Low-density lipoprotein in ducks that received CO was significantly lower than that of the control group. It is concluded that the inclusion of CO at a 1.5% level could be enough and useful for improving the reproductive and physiological performance of Domyati ducks.

Keywords: Blood, Coconut oil, Ducks, Egg, hatchability, Laying period

INTRODUCTION

Ducks are the second common strain of poultry in the world and can be considered a potential source of dietary protein for humans (Ali et al., 2018). Domyati ducks are a local breed in Egypt and their meat is more favorable to the Egyptian consumers. However, the relatively high content of fat and cholesterol in meat and eggs may limit their consumption (Pagala and Nur, 2010; Aziz et al., 2012). Consumer anxiety may be eliminated by the production of duck eggs with low cholesterol levels by feeding ducks on diets containing medium-chain fatty acids (MCFA) (Li et al., 2018).

Coconut oil (CO) is considered a highly saturated oil, about 60% of its total fatty acid composition is MCFA with a chain length of 6 to 12 carbon atoms (Bhatnagar et al., 2009). CO is composed primarily of short-chain fatty acids and MCFA; lauric acid (12:0) comprises approximately 46.5% of the fatty acid content (Dauqan et al., 2011). The MCFA are directly absorbed into the portal circulation without any re-esterification in intestinal cells (Ferreira et al., 2012). The MCFAs are partly independent of the carnitine transport mechanism into the mitochondria of the liver and are rapidly and are exclusively oxidized for the production of energy (Rubin et al., 2000). However, most diets commonly contain long-chain fatty acids (LCFA) that are incorporated into chylomicrons after absorption in the small intestine where they are re-esterified and then enter the blood-stream (Ferreira et al., 2012). Most LCFA are stored in the adipose tissue (Rego Costa et al., 2012). While, MCFAs are associated with reduced fat deposition and improved serum lipid profiles in humans and rats (Han et al., 2003; Takeuchi et al., 2006).

However, few researches have been conducted to study the effects of MCFA on broiler chickens, excepting that it reduces weight gain (Santos et al., 2008). In laying hens, Lee et al. (2015) found that chickens that received MCFA had greater egg protein quality (larger Haugh units), stronger egg-shells, and higher content of calcium, and reduced *Escherichia coli* count. Several studies showed that most of fatty acids in CO are potential as antibacterial (Bergsson et al., 2001), antiviral (Bartolotta et al., 2001), and immune-stimulant agents (Witcher et al.,

To cite this paper: El-Kholy KH, Ghonim AIA, Ahmed MA, Gad HA, Ghazal MN, El-Aik MAA and Ali RAM (2020). Physiological and Reproductive Responses of Domyati Ducks to Different Dietary Levels of Coconut Oil as a Source of Medium-Chain Fatty Acids during Laying Period. *J. World Poult. Res.*, 10 (3): 485-492. DOI: https://dx.doi.org/10.36380/jwpr.2020.56

1996; El-Kholy et al., 2014 and 2018), which are important to fight infection. Also, MCFA inhibits the production of lipases by the bacterium (Dierick et al., 2002). As lipases are needed to allow the bacteria to attach to the intestinal wall, this process will be prohibited and the bacteria will be washed out. The immune system requires antioxidants to produce and maintain the balance of immune cells, to protect cell membranes from reactive oxygen species, and to fight microorganisms causing disease (Tugiyanti1 et al., 2016).

In addition, CO could improve fat digestion and performance values during the coccidiosis infection in broilers chickens (Adams et al., 1996). Few studies are available on the effects of CO as a source of MCFA on the physiological and reproductive parameters of Domyati ducks. Also, no studies have shown whether it is beneficial to other sources of energy for local duck breeds, especially during the laying period. Moreover, appropriate inclusion level of CO in laying duck's diet is not definitely known. Therefore, the current study was conducted to investigate the effect of diets containing different CO levels on some physiological and reproductive parameters and some egg quality traits of Domyati ducks during the laying period.

MATERIALS AND METHODS

Ethical approval

The current study protocol used in this study was approved by the Animal Care and Use Committee of Damietta University, Damietta, Egypt.

Study design

The current experimental work was carried out at El–Serw Water Fowl Research Station, Animal Production Research Institute. Agricultural Research Center, Ministry of Agriculture, Egypt; to evaluate the impact of adding different levels of CO on reproductive performance of Domyati duck diets during the first 3 months of egg production season (25-36 weeks of age). A total number of 300 Domyati ducks (240 females and 60 males), 25 weeks old, were weighed and randomly distributed into four experimental groups, each group contained 75 ducks. Each group was also subdivided equally into three replicates of 20 females and 5 males each. Ducks within each group were fed with different diets; G1was fed the basal diet and served as the control group, the other three groups (G2, G3, and G4) were fed the basal diet supplemented with different levels of CO (1.0, 1.5, 2.0%/kg feed, respectively).

Fatty acids composition in CO is presented in Table 1. Diets listed in Table 2 were formulated to be iso-caloric and iso-nitrogenous according to NRC (1994) and were offered in mash form throughout the experimental period. Ducks of each replicate were reared in a house (2.3 ducks/m^2) with windows and received additional artificial light to provide 17 h light and 7 h dark daily. Ducks in all treatments were reared under similar hygienic and management conditions.

Table 1. Fatty acids composition of coconut oil[1]

Common name	Percentage (%)
Caproic acid (C 6:0)	0.4 - 0.6
Caprylic acid (C 8:0)	4.6 – 10
Capric acid (C 10:0)	5.0 - 8.0
Lauric acid (C 12:0)	45.1 - 53.2
Myristic acid (C 14:0)	16.8 - 21.
Palmitic acid (C 16:0)	7.5 – 10
Stearic acid (C 18:0)	2.0 - 4.0
Oleic acid (C 18:1)	5.0 - 10.0
Linoleic acid (C 18:2)	1.0 - 2.5
Other (C 18:3 C 24:1)	< 0.5

[1] According to Rossell (1985).

Table 2. Ingredients and chemical analysis of experimental diets

Ingredients (%)	Treatments (coconut oil levels, %)			
	0.0 (G_1)	1.0 (G_2)	1.5 (G_3)	2.0 (G_4)
Yellow corn	65.05	63.10	62.13	60.93
Soybean meal	23.91	26.31	27.51	27.63
Gluten corn	2.30	0.85	0.12	0.00
Coconut oil	0.00	1.00	1.50	2.00
Wheat bran	0.00	0.00	0.00	0.70
Calcium carbonate	6.15	6.15	6.15	6.15
Dicalcium phosphate	1.75	1.75	1.75	1.75
Lysine	0.00	0.00	0.00	0.00
DL.-Methionine	0.09	0.09	0.09	0.09
Vitamin and mineral premix*	0.30	0.30	0.30	0.30
NaCl	0.35	0.35	0.35	0.35
Sodium Bicarbonate	0.10	0.10	0.10	0.10
Calculated analysis				
Crude protein (%)	17.000	17.000	17.000	17.000
ME (Kcal/Kg)	2,800	2,800	2,800	2,800
Lysine (%)	0.75	0.75	0.75	0.75
Crude fiber (%)	3.15	3.10	3.28	3.33
Methionine (%)	0.35	0.35	0.35	0.35
Methionine +Cystine (%)	0.60	0.60	0.60	0.60
Threonine (%)	0.27	0.27	0.27	0.27
Calcium (%)	2.80	2.80	2.80	2.80
Available Phosphorus (%)	0.45	0.45	0.45	0.45
Chlorine (%)	0.22	0.22	0.22	0.22
Sodium (%)	0.17	0.17	0.17	0.17

*Vit+Min premix: Provided per kilogram of the diet Vit. A: 6000 IU, Vit. E (dl-α-) Tocopherylacetate: 10 IU, menadione: 2.5 mg, Vit. D3: 2000 ICU, riboflavin: 2.5 mg, calcium Pantothenate: 10 mg, nicotinic acid :12 mg, Choline chloride:300 mg, Vit. B12: 4µg, Vit. B 6: 5 mg, thiamine: 3 mg, folic acid: 0.50 mg, and biotin: 0.02 mg. Trace mineral (mg/ kg of diet: Mn: 80 mg, Zn: 60 mg, Fe: 35 mg, Cu: 8 mg and Se: 0.1 mg).

Productive performance

The number of eggs laid (EN) was daily recorded and also eggs were weighed from 25 to 36 weeks of age. The EN was calculated per duck for 4 weeks as follows:

EN per duck=Total EN per replicate / Number of ducks at house.

Egg weight (EW) was recorded for each replicate. Egg mass (EM) was calculated by multiplying EN by EW. The EM was expressed per duck throughout the experimental period. Feed consumption (FC) of each replicate was weekly recorded; it was then averaged and expressed in gram/duck/4 weeks. Feed conversion ratio (FCR) for egg production was also calculated during the same periods.

Egg quality

At 33rd weeks of age, a total number of 60 eggs (15 from each treatment) were randomly taken to determine egg quality traits. During two successive days per each week during the 33rd to 36th weeks of age, all eggs laid by ducks of each treatment were collected and individually subjected to the following measurements and estimations. Egg was broken and the yolk was separated from albumen. Egg yolk, albumen, and shell (with its membranes) were separately weighted. Relative weights of each component (to the whole EW) were then calculated. Shell thickness was measured at the broad, narrow and the middle ends, using a micrometer. The average shell thickness for all regions was calculated. The egg shape index was calculated according to the following formula:

Egg shape index = (Egg width/ Egg length) × 100

Egg fertility and hatchability percentages

A total of 300 eggs were collected from each treatment during the 34th - 36th weeks of age to determine fertility and hatchability percentages. They were randomly divided into three equal replicates. Fertility percentage was determined on the 10th day of incubation. Hatchability percentage was determined at the end of the incubation period.

Plasma analysis

At the end of the experimental period (36th week), three ducks from each treatment group were randomly taken for blood sampling through wing vein. Blood samples were collected in heparinized test tubes and centrifuged at 3500 rpm for 15 minutes to obtain blood plasma. Plasma samples were stored at –20 °C until analysis to determine total protein (TP) and albumin (Alb) levels. The TP and Alb were determined using commercial kits supplied by Randox (Randox Laboratories Ltd, Crumlin, Co, Antrim, UK) according to Henry et al. (1974). Globulin (Glb) concentration was estimated by subtracting the values of Alb from the corresponding values of TP. Also, the plasma was assayed for total cholesterol, triglycerides, high-density lipoprotein (HDL), and low-density lipoprotein (LDL) using standard protocol methods (Vogel and Vogel, 1997). The radioimmunoassay method was used for the determination of triiodothyronine (T_3) and thyroxin hormone (T_4) using commercial RIA kits (Medical Technology, USA). Plasma samples were analyzed for concentrations of aspartate transaminase (AST) and alanine transaminase (ALT), phosphorous, and calcium using commercial kits (Linear Chemicals, Barcelona, Spain) according to the manufacturer procedure.

Statistical analysis

Data were subjected to one-way ANOVA using the general linear model (GLM) procedure of SAS software (SAS, 2004) based on the following model:

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

where:

Y_{ij} = An observation; μ = Overall mean; Ti = Effect of treatments (i = 1, 2, 3 and 4); and e_{ij} = Random error component assumed to be normally distributed. Differences between the treatment groups were considered statistically different at $p \leq 0.05$. The significant differences among treatments were determined by Duncan's multiple range test (Duncan, 1955).

RESULTS AND DISCUSSION

Productive performance

As shown in Table 3, EN significantly ($p \leq 0.05$) increased in the G2 group compared to G1, G3, and G4 at the first experimental period (25-28 weeks of age). While G3 recorded the highest EN during the other experimental periods and at the overall period (25-36 weeks of age). EW significantly ($p \leq 0.05$) decreased in the G2 group compared to G1, G3, and G4 in the first experimental period. On the other hand, ducks in G3 recorded the highest EW at both the second experimental period (29-32 weeks of age) and the overall period (Table 3). The results of the analysis indicated that the use of various dietary CO levels significantly ($p \leq 0.05$) affected the EM at all experimental periods. Also, the dietary CO addition significantly ($p \leq 0.05$) improved FCR at all experimental periods compared to the control group. As shown in Table 3, Domyati ducks that received the diet containing 1.5% CO (G3) had significantly ($p \leq 0.05$) better productive performance parameters, except FC, compared to other

groups (G1, G2, and G4) during the overall laying period. Accordingly, 1.5% CO in the diet could be considered suitable for Domyati laying ducks.

The FC values were not significantly affected by feeding different levels of CO during 29-32 weeks of age and the whole period from 25-36 weeks of age (Table 3). The group fed diet containing 1.0% CO recorded significantly higher value of FC from 25-28 weeks of age, whereas, ducks receiving 1.5% CO had significantly higher value of FC during 33- 36 weeks of age compared to the other treatments group.

The improvement of EM may be due to the higher EN and EW associated with the diet containing 1.5% CO. These results are in agreement with Wang et al. (2009) and Lee et al. (2015) who reported that dietary supplementation of MCFA improved egg production in the laying hens. In contrary, Klementavičiūtė et al. (2016) reported that laying hens fed the diet supplemented with MCFA showed a low egg production rate. These differences may be due mainly to different levels of CO and differences in bird species. Furthermore, it is proposed that CO can lead to a better condition of digestion and absorption so that the ration becomes more efficient to produce eggs as mentioned by Hanczakowska et al. (2013). These effects may be attributed to a healthier and more stable gut environment created by the MCFA. Also, the antimicrobial activity of MCFA (Ferreira et al., 2012) diminishes intestinal infection pressure and improves intestinal morphology, resulting in better digestive and absorptive capacity (Batovska et al., 2009).

Table 3. Effects of supplementing coconut oil to the basal diet on productive performance of Domyati ducks during laying period.

Periods (weeks of age)		Treatments (Coconut oil levels)				SEM	p-value
		G1 (0%)	G2 (1.0%)	G3 (1.5%)	G4 (2.0%)		
Egg number/ duck	25-28	6.8^{b}	8.5^{a}	7.7 ^{b}	7.1^{b}	0.12	0.0001
	29-32	11.4^{d}	15.9^{b}	17.2^{a}	12.2^{c}	0.11	0.0001
	33-36	15.2^{d}	17.7^{b}	18.2^{a}	16.0^{c}	0.08	0.0001
	25-36	33.5^{d}	41.3^{b}	42.2^{a}	35.3^{c}	0.15	0.0001
Egg weight (g)	25-28	60.4^{a}	57.6^{b}	61.5^{a}	61.1^{a}	0.57	0.0047
	29-32	62.5^{c}	62.3 ^{c}	68.6^{a}	63.2^{c}	0.11	0.0001
	33-36	67.2^{d}	72.5 ^{a}	70.5^{b}	68.3^{c}	0.11	0.0001
	25-36	63.2^{d}	66.2 ^{b}	68.5^{a}	64.5^{c}	0.08	0.0001
Egg mass (g) / duck	25-28	412.7^{c}	487.7^{a}	434.6^{b}	433.7^{b}	6.35	0.0002
	29-32	712.1^{d}	988.5^{b}	1177.6^{a}	773.5^{c}	6.0	0.0001
	33-36	1019.0^{c}	1281.4^{a}	1283.1^{a}	1092.8^{b}	6.0	0.0001
	25-36	2115.1^{d}	2732.7^{b}	2904.4^{a}	2275.9^{c}	11.42	0.0001
FC (g) / duck	25-28	4247.0^{b}	4325.0^{a}	4225.0^{d}	4233.0^{c}	1.73	0.0001
	29-32	5070.0	5166.7	5035.0	5028.0	82.6	0.6341
	33-36	5007.0^{c}	5012.0^{c}	5067.0^{a}	5035.0^{b}	1.67	0.0001
	25-36	14324.0	14503.7	14327.3	14296.0	82.67	0.3337
FCR (g feed/ g egg mass) /duck	25-28	10.3^{a}	8.9^{c}	9.7^{b}	9.7^{b}	0.14	0.0006
	29-32	7.1^{a}	5.2^{c}	4.3^{d}	6.5^{b}	0.09	0.0001
	33-36	4.9^{a}	3.9^{c}	4.0^{c}	4.6^{b}	0.03	0.0001
	25-36	6.8^{a}	5.3^{c}	4.9^{d}	6.3^{b}	0.08	0.0001

$^{a,b,c,\ d}$ means within rows with different superscripts are significantly different ($p \leq 0.05$). SEM: standard error mean.

Egg quality traits

The results of feeding with different levels of CO and their effects on egg quality traits are shown in Table 4. The group fed the diet containing 2.0% CO (G4) recorded higher values of shell thickness. It was found that with increasing CO levels in the diet, shell thickness increased. However, there were insignificant differences in shell weight (%) for ducks that received 2.0% CO (G4) compared to the control group (G1).

These results are in agreement with Świątkiewicz et al. (2010) and Klementavičiūtė et al. (2016) who found that the addition of MCFA had a positive influence on eggshell characteristics including egg-shell weight as percentage, density, and breaking strength. This influence can probably be attributed to the increased availability of Ca and P, due to a decrease in pH in the upper part of the intestinal tract and the stimulating effect of fatty acids on the villus height, which was observed in broilers by Hanczakowska et al. (2013).

Hence, maintaining a good quality shell throughout the production cycle is of importance for the egg consumers and producers in terms of health and economics (Hughes et al., 1986). Also, eggshell porosity is of concern during embryonic development in the breeding

industry (Reynard and Savory, 1999). The most commonly used indicators of Ca metabolism in laying hens are shell quality assessment parameters (Gordon and Roland, 1998). Data presented in Table 4 also revealed that different levels of CO in the diet had no significant effects on EW, absolute and percentage weight of yolk and albumin, absolute shell weight, and egg shape index. These results were in agreement with the findings of Klementavičiūtė et al. (2016) who indicated that the inclusion of MCFA in birds' diet reduced egg and yolk weight, but the difference with the control group was not significant. Also, Danicke and Halle (2002) demonstrated that the yolk and albumen weights were not significantly affected by different sources or inclusion levels of lipids.

Table 4. Effects of supplementing coconut oil to the basal diet on egg quality traits of Domyati layer ducks

Items	Treatments (Coconut oil levels)				SEM	p-value
	G1 (0%)	G2 (1.0%)	G3 (1.5%)	G4 (2.0%)		
Egg weight (g)	70.00	71.80	71.60	69.80	2.18	0.8743
Shell weight (g)	9.20	8.80	9.60	9.80	0.31	0.1446
Shell weight (%)	13.14^{ab}	12.26^{b}	13.41^{a}	14.08^{a}	0.01	0.0076
Shell thickness (mm)	0.29^{c}	0.30^{bc}	0.31^{ab}	0.32^{a}	0.01	0.0133
Yolk weight (g)	23.20	24.60	24.00	24.60	0.90	0.6618
Yolk weight (%)	33.25	34.26	33.54	35.18	0.01	0.4953
Albumin weight (g)	37.60	38.40	38.00	35.40	1.47	0.4977
Albumin weight (%)	53.61	53.48	53.05	50.74	0.01	0.1834
Egg shape index	0.78	0.78	0.79	0.79	0.02	0.9367

$^{a,b,c,\ d}$ means within rows with different superscripts are significantly different ($p \leq 0.05$). SEM: standard error mean.

Reproductive traits

There were significant differences in fertility percentages among the treatment group as shown in Table 5. Also, two CO levels (1.0 and 1.5%) significantly increased ($p \leq 0.05$) hatchability percentage of set and fertile eggs as compared to both control and group fed 2.0% CO in the diet. This supports the previous findings that the incorporation of CO-derived MCFA into hen's diet is readily utilized by the embryos (Ding and Lilburn, 1997). It is clear from the results that, inclusion levels of 1.0 and 1.5% CO in the diet could be considered suitable for hatching eggs, where, the groups fed diet contained 1.0 and 1.5% CO showed a good quality of shell thickness and high percentages of hatchability as compared to the other treatment groups. It was found that the hatchability of thick-shelled eggs is higher than that of thin-shelled eggs (Narushin's and Romanov, 2002). It was reported that reduction in egg-shell quality decreases hatchability and is associated with the weakening of the embryos (Peebles et al., 1987). In contrary, Yamak et al. (2016) and Ergun and Yamak (2017) indicated that differences in hatching rates of eggs with different shell thicknesses were not statistically significant.

Blood plasma parameters

Some blood plasma parameters of Domyati layer ducks were influenced by diets supplemented with different levels of CO (Table 6). However, no significant differences in plasma T_3, T_4, P, Ca, ALT, AST, TP, Alb, and Glb values were observed among the treatment groups. The ALT and AST values were 20.2-23.9 and 75.7-87.0 U/l throughout the whole experimental period, respectively. These results are in agreement with those of Ali et al. (2018) who found that ALT and AST values varied between 20.2 to 24.3 U/l and 83.3 to 88.1 U/l, respectively. Regarding the effect of dietary CO on lipid profile, no significant differences were observed in plasma cholesterol and triglyceride (Table 6). However, the groups fed with the diet containing 2.0% CO recorded significantly ($p \leq 0.05$) higher and lower values of HDL and LDL, respectively, as compared to 1.0% CO and control group. Moreover, no significant differences were observed between G1 and G2 in HDL and LDL values.

Wang et al. (2015) showed that with increasing CO levels, serum levels of total cholesterol, LDL, and LDL/HLDL linearly decreased. In general, usage of CO at some levels is a popular concept that is believed to increase the productive and reproductive performance of birds. Similarly, the administration of some levels for long periods is believed to be more effective without considering the other adverse effects on the birds. Previous studies have shown some benefits such as immune modulation and anti-inflammatory effects. Besides efficacy, these supplements also have to be safe for the animals, consumers of products, and the environment.

Table 5. Effects of coconut oil supplemented to basal diet on fertility and hatchability traits of Domyati duck eggs.

Items	Treatments (Coconut oil levels %)				SEM	p-value
	G1 (0%)	G2 (1.0%)	G3 (1.5%)	G4 (2.0%)		
Fertility (%)	93.0[b]	96.0[a]	96.0[a]	94.0[b]	0.58	0.0139
Hatchability of set eggs (%)	71.0[c]	76.0[a]	77.0[a]	73.0[b]	0.58	0.0003
Hatchability of fertile eggs (%)	75.4[b]	79.2[a]	80.2[a]	76.7[b]	0.61	0.0018

[a,b,c, d] means within rows with different superscripts are significantly different (p≤0.05). SEM: standard error mean.

Table 6. Effects of supplementing coconut oil to the basal diet on some blood plasma parameters of Domyati ducks during laying period

Items	Treatments (Coconut oil levels %)				SEM	p-value
	G1 (0%)	G2 (1.0%)	G3 (1.5%)	G4 (2.0%)		
T_3 (ng/ml)	3.30	3.20	3.30	3.20	0.22	0.9804
T_4 (ng/ml)	20.20	18.70	18.40	19.10	1.17	0.7132
Pi	5.70	5.80	6.00	6.20	0.25	0.4348
Ca	21.60	20.60	22.60	21.80	1.18	0.6065
ALT (U/l)	23.60	22.20	21.50	21.90	1.99	0.8930
AST (U/l)	84.20	87.00[c]	82.80	75.70	4.83	0.4395
Triglyceride (mg/dl)	117.5	123.2	133.0	129.4	6.73	0.4302
Cholesterol (mg/dl)	175.6	175.9	178.1	170.1	6.33	0.8305
HDL (mg/dl)	60.60[b]	60.80[b]	67.70[a]	67.90[a]	1.87	0.0336
LDL (mg/dl)	91.50[a]	90.40[a]	83.80[ab]	76.30[b]	3.49	0.0507
Total protein (TP, g/dl)	5.14	5.73	5.80	5.77	0.25	0.3618
Albumin (Alb, g/dl)	3.06	3.50	3.50	3.60	0.25	0.1095
Globulin (Glb, g/dl)	2.13	2.28	2.21	2.13	0.13	0.8307

[a,b,c, d] means within rows with different superscripts are significantly different (p≤0.05). SEM: standard error mean.

CONCLUSION

Inclusion of coconut oil at a level of 1.5%/kg feed to diet can improve the physiological and reproductive performance of local Domyati ducks during the laying period.

Competing interests

The authors declare that they have no conflict of interest.

Authors' contributions

Khaled H. El-Kholy designed the proposal of this study. Aymen I. Ghonim; Mahmoud A. Atef.; Hoda A. Gad.; Mervat N. Ghazal; Mosad A. El-Aik and Reham A. Ali developed the concept for the manuscript. Khaled H. El-Kholy and Ayman I. Ghonim wrote the manuscript.

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JWPR
Journal of World's Poultry Research

2020, Scienceline Publication

J. World Poult. Res. 10(3): 493-506, September 25, 2020

Research Paper, PII: S2322455X2000057-10

DOI: https://dx.doi.org/10.36380/jwpr.2020.57

Isolation, Molecular, and Pathological Characterization of Infectious Bursal Disease Virus among Broiler Chickens in Morocco

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Received: 20 Jul. 2020
Accepted: 24 Aug. 2020

ABSTRACT

Infectious bursal disease (IBD) is a contagious viral disease of young chickens that causes immunosuppression, mortality, and growth retardation. This pathology has severely affected the Moroccan poultry industry. The objective of the present study was the isolation, molecular characterization, and histopathology examinations of infectious bursal disease virus collected from 49 suspected farms from different regions of the country from 2013 to 2016. The real-time PCR results indicated that 41 out of 49 farm cases were found positive with a prevalence of 41% for classical virulent IBD virus (IBDV) and 59% for very virulent IBDV (vvIBDV). Pathological examinations showed the presence of two types of lesions, including acute to subacute lesions with a prevalence of 86%, and the sub-chronic to chronic lesions with a prevalence of 14%. The nucleotide and deduced amino acid sequences for the hypervariable region of VP2 for 7 vvIBDVs were compared to worldwide IBDV isolates and the findings suggested that it belonged to a group of very virulent strains. Phylogenetically, all the Moroccan vvIBDV field isolates were grouped in the same cluster with Malaysian and European vvIBDV isolates. This report demonstrated the continuous circulation of vvIBDV in commercial poultry farms in Morocco since 2013.

Keywords: Histological investigations, Infectious bursal disease virus, Phylogeny, Morocco, Virulent infectious bursal disease virus, VP2

INTRODUCTION

Infectious bursal disease (IBD) is an acute and highly contagious viral and immunosuppressive disease of young chickens imposing severe economic losses to the poultry industry (Sassia et al., 2017). Infectious bursal disease virus (IBDV), member of the genus *Avibirnavirus* in family *Birnaviridae*, is a non-enveloped, icosahedral virus with a diameter of 60-65 nm (Nick et al., 1976; Özel and Gelderblom, 1985; Mosley et al., 2017; Pikula et al., 2018). There are two serotypes of IBDV, namely serotypes 1 and 2; the strains of serotype 1 can also be categorized as classical virulent IBDV (cvIBDV), antigenic variant IBDV (avIBDV), attenuated IBDV (atIBDV), and very virulent IBDV (vvIBDV, Jeon et al., 2008; Tsai et al., 2012; Cheggag et al., 2018). The IBDV possesses a bisegmented genome, namely segments A and B. Segment A encodes two open reading frames for the nonstructural protein, including structural protein VP5 (17 kDa) and the polyprotein pVP2-VP4-VP3 (109 kDa). In contrast, segment B encodes for the RNA-dependent RNA polymerase VP1 (90 kDa). The hypervariable region (HVR) displaying high amino acid variability of the VP2 antigens of IBDV is located on the amino acid residues 206-350 (Bayliss et al., 1990). This region is responsible for antigenic tissue-culture adaptation, variation, and is partly in charge of viral virulence (Escaffre et al., 2013). The clinical or subclinical prevalence of IBD is found in chickens during their first three weeks of age leading to immunosuppression.

The phase of chickens' immunosuppression is associated with their age (Sellaoui et al., 2012). The macroscopic characteristic lesions related to this disease consist of enlargement, orange discoloration of kidneys, and dehydration of the muscles with ecchymotic hemorrhages (Brugere-Picoux, 2015). The IBDV spreads in the bursa of Fabricius (BF) by deteriorating its lymphoid structure, and cause different lesion types depending on the virulence of the strains and immune status of the affected chicken (Jungbaeck and Nutolo, 2001; Juranová et al., 2001). These lesions generally induced immunosuppression resulting in the appearance of secondary infections and weak production performances. The principal characteristics of lesions in the bursa are the cystic formation of follicles, follicular atrophy, bursal hemorrhage, and mild to severe lymphoid depletion in follicles (Mazariegos et al., 1990; Madej et al., 2013). This infection was first described in 1962 in the USA (Cosgrove, 1962) and since then it has been known as one of the most economical important pathologies affecting the poultry industry worldwide. The emergence of various IBD forms in the USA or vvIBDV forms in Europe, Africa, Asia, and other countries has induced severe problems for IBD control and prevention since instances of classical IBDV strains was reported in vaccinated chickens or chicks with maternal antibodies levels for which they were supposed to be IBD resistant (Chettle et al., 1989; Van Den Berg et al., 1991; Snyder 2007; Kasanga et al., 2007). In Morocco, the IBD was determined for the first time in poultry flocks in 1977 (Tahiri et al., 2011). This pathology has become endemic since the high mortality and introduction of very virulent strains of IBDV in 1991 (Bouzoubaa et al., 1992; Kichou et al., 1999). Therefore, the current study was conducted to isolate and characterize the pathological and molecular aspects of the existing IBDV strains affecting Moroccan poultry flocks. It further aimed to describe the sequencing and phylogenetic analysis of IBDV isolates from 2013 to 2016.

MATERIALS AND METHODS

Animals

The autopsied samples were selected among dead broilers suspected of the infectious bursal disease. These broilers were from farms located in five different regions of Morocco (Souss-Massa, Fes-Meknes, Casablanca-Settat, Oriental and Draa Tafilalet). Broilers were monitored by the veterinarian in farms. During the observation, the chickens were given access to water and fed *ad libitum*, and then cared in case of emergency. For confidentiality reasons. It was not possible to provide any data about the vaccines and the vaccination program adopted under supervising of specialist veterinarians in all studied farms.

Sampling

This study was conducted on 49 pools of bursa Fabricius collected from broiler chicken farms. The investigated samples were suspected of IBDV during the period of 2013 to 2016. Lesions reported in broilers suspected of IBDV are located primarily in the bursa of Fabricius and spleen and included hypertrophy and hemorrhage. The presence of petechiae and muscle hemorrhage were also noted in all cases. The bursal samples, taken after autopsy from broilers, were fixed in a 10% neutral buffered formalin for histological examination, and others were frozen at -80 °C for isolation and molecular analysis.

Histopathology

The 10% NBF-fixed bursa Fabricius from 43 farm-cases were subjected to histopathological examination. They were processed in accordance with paraffin embedding standard methods. In this regard, five-µ thick sections were prepared, stained with the hematoxylin and eosin (H&E), and examined under the light microscope (ZEISS SIGMA, Germany) for histopathological evaluation.

The investigated characteristic changes of infectious bursal disease included depletion, necrosis, hemorrhage, infiltration by inflammatory cells and/or fibrin deposit in the lymphoid follicle, Edema, congestion, hemorrhage, infiltration by inflammatory cells, fibrin deposit and/or fibrosis in the interfollicular space, presence of caseous material on the surface epithelium, as well as folding, thickening, and/or hyperplasia of the surface epithelium with or without the formation of pseudo-cysts. Microscopic lesions were recorded, and the lesion scores of 1 to 3 were assigned to the samples based on the degree of lesion severity (1: mild, 2: severe, 3: very severe). A mean lesion score (MLS) was determined for each farm-case for comparison purposes.

Development of chicken embryo fibroblast cell line

The 9- to 11-day-old embryonated chicken eggs, belonging to the production unit of the Society for Veterinarians Biological and Pharmaceutical Production (Biopharma, Morocco) were harvested, and the embryos were taken out. The appendages and viscera were

discarded after proper washing. In the next step, the remainder body was chopped in fine cuts by scissors, kept into specific Erlenmeyer with 50 ml of trypsin EDTA (37 °C), and stirred for 5 minutes at low speed (100 rpm). The supernatant was removed, and then 50mL trypsin was added to pieces in the same Erlenmeyer, and stirred for 5 minutes (this operation was repeated twice). Trypsinized tissue was filtered with a gauze pad, and the recovered volume was tarnished in a conical tube (50 ml). After the addition of 10% donor calf serum, the filtrate was centrifuged at 1200 rpm for 10 minutes and the supernatant was discarded. The pellet was dissolved by adding 1mL of growth medium. Afterward, cells were transferred in culture flask, and the growth media was added later (Sahare et al., 2015).

Virus isolation

The virus isolation was performed using seven suspensions of BF recognized as vvIBDV. The primary chicken embryo fibroblast (CEF) cell line was passaged when the cultured cells reached confluency of 70-80%. The normal and confluent appearance of the monolayer of CEF cells after 24 hours of subculturing was used for the infection with 0.5mL of filtered field IBDV. The virus suspension was overlaid uniformly over the monolayer and incubated at 37 °C for 1 hour. One flask was kept as un-inoculated control. The 5mL of maintenance medium was added to each flask after a 1-hour incubation. Flasks were incubated at 37 °C in 5% CO2 for 48 hours. The cell monolayer was examined twice daily under an inverted microscope for cytopathic effects (CPEs, Sahare et al. 2015). The embryonated chicken eggs were cultivated using 0.2 mL of virus suspension inoculated in 9- to 11-day-old embryonated chicken eggs by the chorioallantoic membrane route. The eggs were incubated for 5 days in the incubator (Sahare et al., 2015). The embryonated chicken eggs were chilled on the fifth day of post-inoculation. Lesions on the embryo were observed, and then the appendages and viscera of the embryo were removed and mixed with the allantoic fluid harvested and arranged in the conical tube (50 ml). Afterward, the mixture was centrifuged at 12 000 rpm for 10 minutes and stored at -80 °C. The IBDV challenge strain (Biopharma, Morocco) was used as a reference strain for this isolation study.

Purity of isolates

It was necessary to evaluate the purity of the seven vvIBDV isolates by real-time RT-PCR before sequencing. To this end, nucleic acids extracted from the isolates were tested for the presence of others avian viruses , including infectious bronchitis virus (IBV, Meir et al., 2010), Newcastle disease virus (NDV, Wise, et al., 2004), and avian influenza virus (AIV, Spackman et al., 2002).

Real-time PCR

Viral RNA was extracted from 150 µl of suspension of the vvIBDV isolates on chicken embryo fibroblast cell line and embryonated chicken eggs using the Nucleospin RNA Virus Extraction kit (Machery-Nagel, Germany) following the manufacturer's instructions. The extracted RNA was eluted in 50 µl of nuclease-free water and stored at -80°C until use. The real-time PCR (RT-PCR) targeted VP5/VP2 overlapping region of segment A using the specific primers and probes (Tomás et al., 2012) designed by Bioneer, Korea, as described by Gonzalo et al. (2012). It could detect and discriminate vvIBDV strains from non-vvIBDV strains. The RT-PCR protocol was described by Cheggag et al. (2018).

Complementary DNA synthesis for VP2-HVR

In the first step, cDNA was synthesized by the Tetro cDNA Synthesis kit (Bioline, Germany) and specific primers for the amplification of VP2-HVR at 97°C for 5 minutes according to the manufacturer's instructions. In the second step, the 5x RT buffer, RT plus RT inhibitor were added and the RT was performed at 25°C for 10 minutes, then 45°C for 60 minutes followed by 85°C for 5 minutes. The synthesized cDNAs were used as templates for PCR.

PCR nucleotide sequencing of VP2-HVR

The IBDV VP2-HVR was detected by PCR using the primer pair 743-F (5´-GCCCAGAGTCTACACCAT-3´) and 743-R (5´-CCCGGATTATGTCTTTGA-3´), as suggested previously by Nwagbo et al. (2016). The PCR was performed on Verity thermocycler (Applied Biosystems) using HS MyTaq DNA polymerase, Bioline kit (Bioline, United Kingdom), following thermocycling conditions of 95°C for 120 seconds, 35 cycles of 47°C for 30 seconds, 72°C for 30 seconds, and 72°C for 3 minutes. The PCR products were visualized following electrophoresis on a 1% agarose gel stained with ethidium bromide.

The PCR products were purified and sequenced at Molecular Biology and Functional Genomics Platform of National Centre for Scientific and Technical Research (Morocco) on an ABI 3130xl Genetic Analyzer, 16 capillary sequencers (Applied Biosystems, California, USA). The sequences were submitted to the GenBank

database, National Center for Biotechnology Information (NCBI), and were assigned the accession numbers as shown in Table 1.

Phylogenetic analysis

The VP2 gene sequences of 7 isolates were corrected and compared with the reference sequence available in the public database (http://www.ncbi.nlm.nih.gov/) and with available sequences deposited in GenBank (http://www.ncbi.nlm.nih.gov/Genbank) using nucleotide Basic Local Alignment Search Tool (http://www.ncbi.nlm.nih.gov/BLAST/) for nucleotide (blastn), and for an amino acid (blastp). The nucleotides and amino acids sequences were edited and aligned using the BioEdit software package and MEGA Version 6.1 (MEGA: Molecular Evolutionary Genetics Analysis). The relationship between the strains was analyzed by phylogenetic tree using MEGA program. The neighbor-joining methods were used for analysis and the bootstraps were calculated with 1000 replications (Fessehaie et al., 2002; Tamura et al., 2007; Tamura et al., 2011).

Statistical analysis

The results of RT-PCR Viral RNA of suspension of the vvIBDV isolates on the Chicken Embryo Fibroblast Cell Line and Embryonated Chicken Eggs were analyzed using the IBM SPSS software, version 25. P-value less than 0.05 was considered significant. The chi-square test was used to investigate the correlation between RT-PCR results of the two different techniques used for IBDV isolation. In addition, the correlation between the severity (MLS) and type of histological changes in birds and the pathogenicity of identified IBDV from field samples was evaluated using Fisher's exact and Pearson chi-square tests.

Ethical approval

The experiment was carried out after the agreement of the Scientific and Technical Committee of BIOPHARMA taking into account the rules of ethics and animal welfare implemented by the BIOPHARMA laboratory in accordance with the norms and standards of the OIE manual. BIOPHARMA has an animal house, its premises and procedures are periodically inspected by a joint commission of the Ministry of Agriculture and Health of Morocco. Any animal presenting suffering or discomfort was euthanized according to the procedures implemented by the laboratory of Biopharma.

Table 1. Description of infectious bursal disease virus isolates included in the present study

Virus isolate	flock type	Date of collection	Sample type	Origine	Age of bird (days)	GenBank accession no
IBDV8VP2MOROCCO2015	Broiler	2015	Bursa	Témara	26	MN241434
IBDV11VP2MOROCCO2014	Broiler	2014	Bursa	Témara	26	MN241437
IBDV27VP2MOROCCO2015	Broiler	2015	Bursa	El Jadida	NA*	MN241433
IBDV28VP2MOROCCO2015	Broiler	2015	Bursa	El Jadida	NA*	MN241438
IBDV30VP2MOROCCO2015	Broiler	2015	Bursa	El Jadida	NA*	MN241439
IBDV41VP2MOROCCO2015	Broiler	2015	Bursa	Tit Milil	25	MN241435
IBDV40VP2MOROCCO2015	Broiler	2015	Bursa	Tit Milil	29	MN241436

* not available

RESULTS

Real-time PCR

The RT-PCR results indicated that 41 out of 49 suspected flocks were found positive with a prevalence of 41% for non-virulent cvIBDV and 59% for vvIBDV. The Threshold Cycle (Ct) value for RT-PCR for seven isolates on CEF ranged from 16.31 to 25.44, and for those on embryonated chicken eggs were in the range of 16.13 to 27.63.

Histopathological changes

The results of the histopathological examination showed that bursas from the 35 cases of broiler chicken farms in the current study were carriers of characteristic bursal lesions that can be attributed to IBD viruses (vaccine or wild viruses). With regard to the nature of lesions and the inflammatory phase detected, two categories of affected bursas were identified, including bursas with acute to sub-acute lesions and bursas with chronic lesions.

Acute to sub-acute lesions in bursa of Fabricius

Lesions in this group of bursas included depletion, necrosis, hemorrhage, infiltration by inflammatory cells, and/or fibrin deposit in the lymphoid follicle as well as edema, congestion, hemorrhage and/or infiltration by heterophilic inflammatory cells in the inter-follicular space (Figure 1A). Among the investigated cases, 32 samples were classified in this first category. The obtained results of MLS within this category were suggestive of three subgroups of bursas. To clarify, subgroup 1 of bursas with an MLS < 2 represented 28.6 % of cases. The subgroup 2 of bursas with MLS range of 2-3, and a subgroup 3 with an MLS > 3 represented in this same category 37.1% and 25.7% of cases, respectively (i.e., a total 91.4% of cases with acute to subacute bursal lesions).

Sub-chronic to chronic lesions in bursa of Fabricius

Bursal lesions classified as chronic included interstitial thickening, lymphoid depletion, fibrosis, and folding of the surface epithelium with or without a formation of pseudocysts (Figure 1B). Bursa from all 3 cases in this category had an MLS < 2. Among all 35 RT-PCR IBDV positive farm-cases, 32 cases had acute to sub-acute bursal changes while only three indicated chronic changes. Within the category of farm-cases with acute bursal lesions, characterized hypervirulent and low pathogenicity IBD viruses were identified with a similar trend among different MLS sub-groups (Table 2) with a slightly higher percentage for vvIBDV, compared to subgroup cases with an MLS > 3 (6/32). However, statistical analysis (Fisher's exact test statistic; $p \geq 0.05$) did not show any correlation between the severity of lesions (MLS) and the pathogenicity of IBDVs characterized by RT-PCR (Table 3).

Virus isolation

The IBDV has the property of causing a specific and characteristic cytopathogenic effect (CPEs) on CEF cells. The CEF cells were examined under the microscope for CPEs (Figure 2A) indicated the presence of IBDV while checking negative controls (Figure 2B) that were free of CPEs. Concerning viral isolation in embryonated chicken eggs, the presence of IBDV was revealed by embryos with hemorrhagic traces throughout the body of the embryo, edema, vessel congestion, growth retardation, and sometimes with a greenish liver (Figure 3).

Comparison between the results of two IBDV isolation techniques

The results of Table 4 tabulating the statistical analyses indicate no correlation between the two IBDV isolation techniques ($p > 0.05$).

Purity of isolates.

No positive results were obtained with any of the other avian viruses, such as NDV, IBV, and IA using RT-PCR, therefore, the purity of IBDV isolates was confirmed.

Phylogenetic analysis

Sequencing was performed on the HVRs of VP2 gene of seven IBDV isolates. The nucleotide and deduced amino acid sequences (Table 5) of these IBDV isolates were blasted and compared with the reference strain sequences retrieved from GenBank from different regions of the world. Table 5 summarizes the classical and very virulent reference strains used in the present study. The phylogenetic tree revealed that the Moroccan strains were clustered into genogroup 3 regrouped very virulent strains (Figure 4). The first genogroup comprised the vvIBDV strains from different countries and formed a common branch with Moroccan strains characterized in the present study and those recently isolated by Drissi Touzani et al. (2019). However, they were clearly clustered into two distinct clusters within the group. The second genogroup included all classical IBDV strains.

Molecular characterization of Moroccan infectious bursal disease virus

The deduced amino acid sequence of the HVR (positions 222 to 428 of the VP2 protein) was determined for each of the isolates, and compared to well-characterized classical virulent IBDV (D78, Faragher 52/70, 2512 Winterfield, Lukertand LC 75) and vvIBDV, which was used to construct a phylogenetic tree with results similar to those obtained with the nucleotide sequences (Figure 4). The sequence identity among the Moroccan vvIBDV isolates fluctuated from 96.2% to 100%. Seven Moroccan IBDV contained the genetic signature of vvIBDVs strains, specifically, A222, I242; I256, I294, S29. It is important to mention that heterogeneous variations were also detected in these isolates.

The deduced amino acid sequence of the HVR was determined for each of the isolates and compared to well-characterized classical virulent IBDV isolates (F52/70 [CAA7518]), African vvIBDV isolates, vvIBDV isolates from other countries, and Moroccan vvIBDV isolates.

Four Moroccan IBDV isolates (i.e., MN241433, MN241434, MN241436, and MN241437) contained the genetic signature of vvIBDVs, specifically, A222, I256, I294, and S299 (Figure 5). A single point mutation, resulting in a single amino acid change, was distinguished in Moroccan IBDV isolates, named MN241439, a nucleotide change resulted in a (G) at position 225 (V→G); 238 (T→P); 249 (Q→H) and 353 (A→T). There were also variations in these seven isolates at amino acid 222 (A→T), in two strains of MN241433 and MN241438.

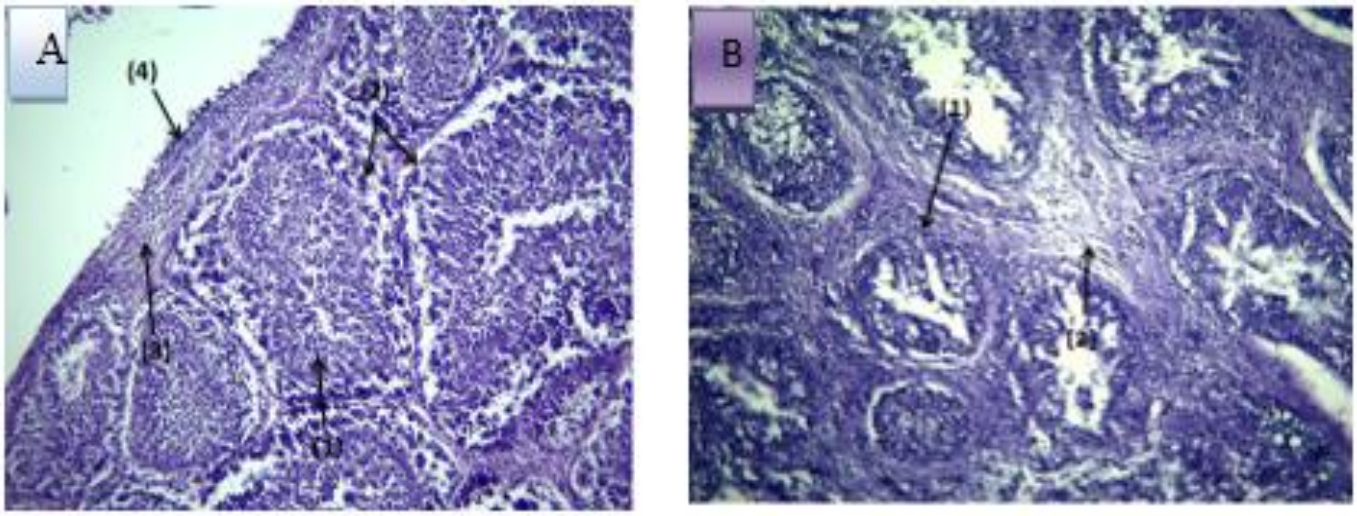

Figure 1. Histological section of the bursa of Fabricius of broiler chicken. Hematoxylin and Eosin. Bar: 168 µm. **A:** Acute to subacute lesions. Discrete lymphoid depletion (1) and slight oedema in parafollicular areas of the lymphoid follicles (2). Moderate infiltration of the surface subepithelial chorion by inflammatory cells (3) and desquamation of the surface epithelium (4). **B:** sub-chronic to chronic. Atrophy of lymphoid follicles with very marked lymphocyte depletion (1). Very marked thickening of the interfollicular spaces by severe fibrosis (2).

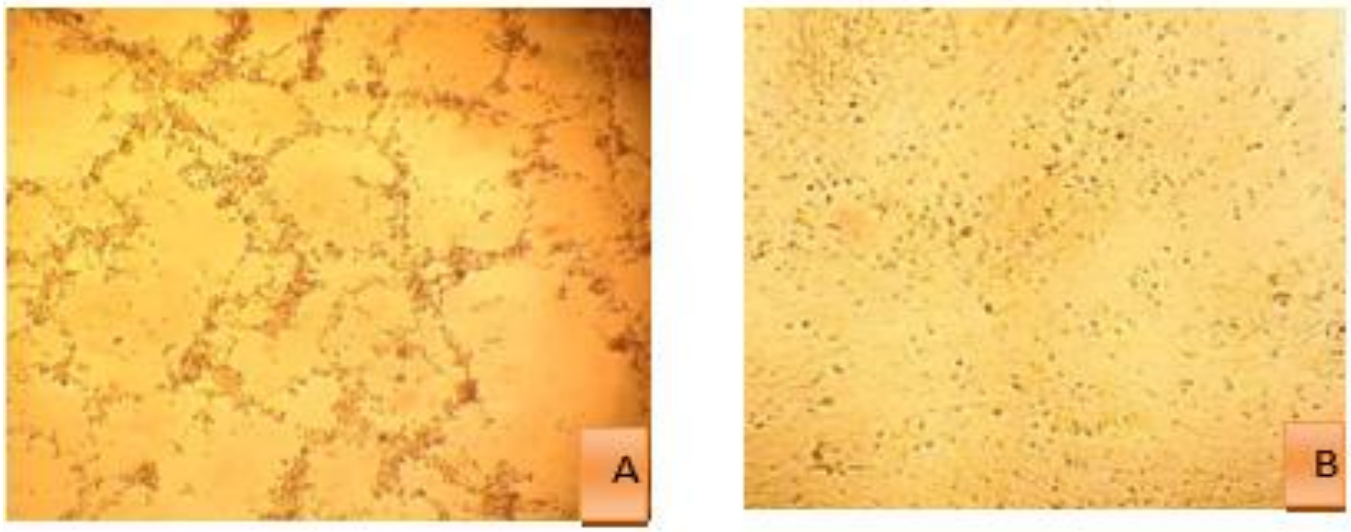

Figure 2. Adaptation of very virulent infectious bursal disease virus in primary chicken embryo fibroblast cells showing cytopathic effects after 48 hours of transfection. **A:** Primary chicken embryo fibroblast cells infected with very virulent infectious bursal disease virus showing prominent cytopathic effects. **B:** Chicken embryo Fibroblast cell line with 90% confluency.

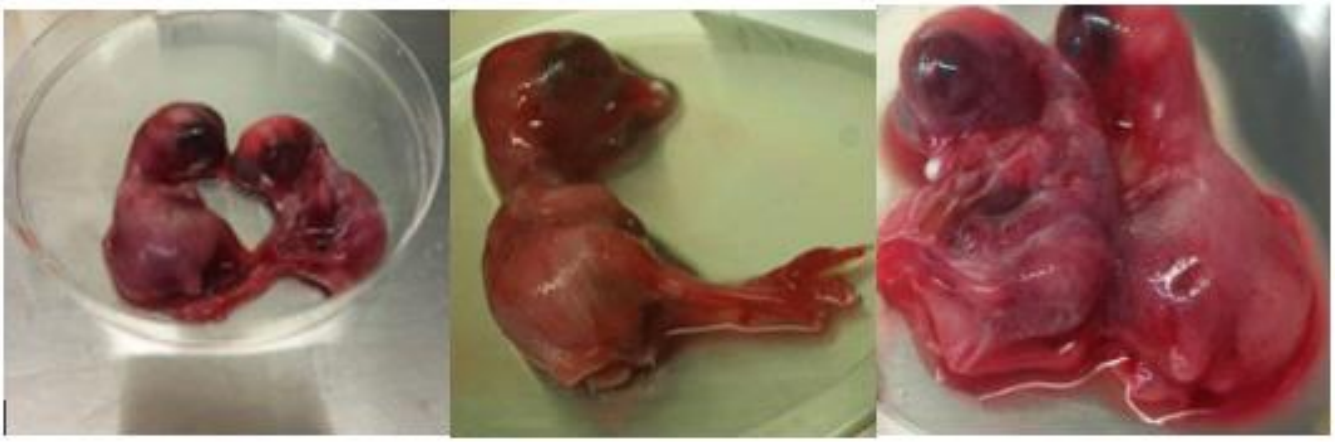

Figure 3. Lesions induced in chicken embryos infected with very virulent infectious bursal disease virus

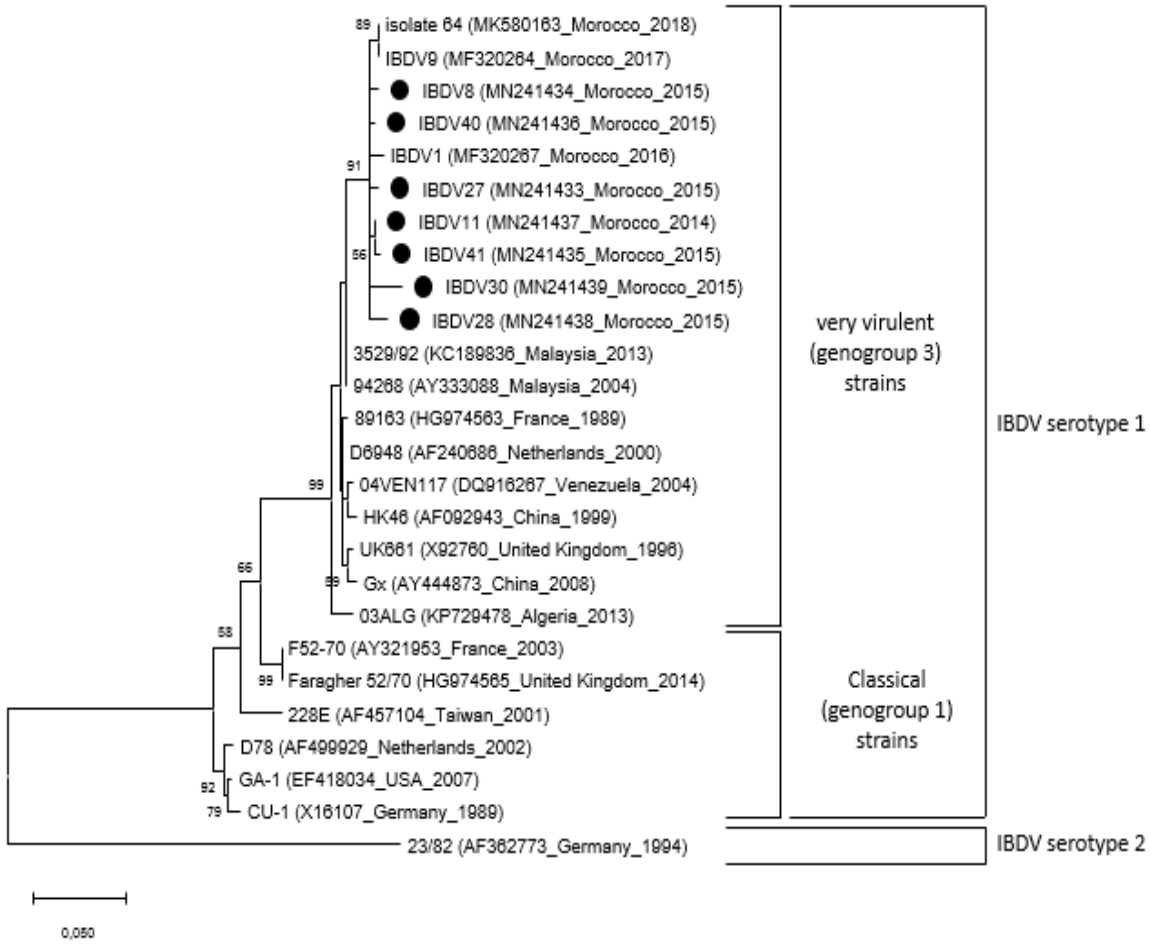

Figure 4. Phylogenetic relationships between some very virulent (genogroup 3) strains of infectious bursal disease virus. The nucleotide sequence encoding the hypervariable region of the structural proteinVP2 protein was used as a phylogenetic marker. The analysis was performed in Mega using the neighbor-joining method with 1000 bootstrap replicates. Only bootstrap values greater than 50 are shown. Four classical strains of infectious bursal disease virus serotype 1 and one strain of IBDV serotype 2 were used as outgroups. The countries of origin of the strains and the GenBank accession numbers for structural protein VP2 sequences are given in brackets after the strain names. Moroccan strains presented in this paper are shown in bold.

Figure 5. Amino acid sequence comparison of 35 infectious bursal disease virus strains. IBDV strains used for comparison include classical virulent IBDV isolates (F52/70 [CAA7518]), African very virulent infectious bursal disease virus isolates, very virulent infectious bursal disease virus isolates from other countries (USA, Asia), and Moroccan vvIBDV isolates

Table 2. Comparison between the results of the histopathological examination and real-time PCR from field samples of broiler chickens

Real-time PCR results	Histopathological lesions							
	Aigue to sub-aigue mean lesion score (MLS)				sub-chronique to chronique mean lesion score (MLS)			Total
	< 2	02 to 03	> 3	S/total	< 2	02 to 03	> 3	
Cases with very virulent infectious bursal disease virus	5	5	6	16	2	-	-	18
Cases with non-very virulent infectious bursal disease virus	5	8	3	16	1	-	-	17
S/total	10 (28.6 %)	13 (37.1%)	9 (25.7%)	32 (91.4%)	3 (8.6%)	-	-	35 (100%)

MLS: Mean lesion score.

Table 3. The correlation between the results of the histopathological examination and real-time PCR

Statical tests	Chi-square tests			
	Value	Degree of freedom (ddl)	Asymptotic signification (bilateral)	Significative. Exact (bilateral)
khi-carre of Pearson	4.738	2	0.094	0.145
Rapport of vraisemblance	4.005	2	0.135	0.159
Fisher's Exact Test	3.99	-	0.296	0.159
N of valid observations		43		

Table 4. The correlation between the two infectious bursal disease virus isolation techniques

			Correlation	
			rRT-PCT CTs of isolates by CEF	**rRT-PCT CTs of isolates by Embryonated Chicken Eggs**
Rho of Spearman	rRT-PCT CTs of isolates by CEF	correlation coefficient	1	-0.536
		Signficative. (bilateral) (p)	.	0.215
		N	7	7
	rRT-PCT CTs of isolates by Embryonated Chicken Eggs	correlation coefficient	-0.536	1
		Signficative. (bilateral) (p)	0.215	.
		N	7	7

Table 5. Infectious bursal disease virus strains used in the present study

Name	Origin	Molecular type	Year of isolation	Genbank accession numbers
isolate 64	Morocco	Very virulent	2018	MK580163
IBDV9	Morocco	Very virulent	2017	MF320264
IBDV1	Morocco	Very virulent	2016	MF320267
3529/92	Malaysia	Very virulent	2013	KC189836
94268	Malaysia	Very virulent	2004	AY333088
89163	France	Very virulent	1986	HG974563
D6948	Netherlands	Very virulent	2000	AF240686
04VEN117	Venezuela	Very virulent	2004	DQ916267
HK46	China	Very virulent	1999	AF092943
UK661	United Kingdom	Very virulent	1996	X92760
Gx	China	Very virulent	2008	AY444873
03ALG	Algeria	Very virulent	2013	KP729478
F52-70	France	Classical	2003	AY321953
Faragher 52/70	United Kingdom	Classical Vaccine strain	2015	HG974565
228E	Taiwan	Classical	2001	AF457104
D78	Netherlands	Classical	2002	AF499929
GA-1	USA	Classical Vaccine strain	2007	EF418034
CU-1	Germany	Classical	1989	X16107
23/82	Germany	Apathogenic serotype 2	1994	AF362773

DISCUSSION

Despite the wide use of vaccines, IBD is still presenting a serious economic threat to the poultry industry in Morocco. The histological lesions caused by IBD differ in terms of severity and virulence of field IBDV strains. The vvIBDV strains induce characteristic bursal lesions with the practically total destruction of the bursal tissue more often with severe hemorrhage and/or lymphoid necrosis (Helmboldt and Garner, 1964; Cheville, 1967; Ley et al., 1983). However, the cvIBDV strains appeared to cause lesions that are somewhat difficult to differentiate from those attributed to other viruses (Jackwood and Saif, 1987). The changes found in the bursa of birds suspected of IBD in this study were mostly of acute to sub-acute types (62.85 of the cases with an MLS > 2). A total of 35 case-farms were considered suffering from severe IBD infection of which 32 case-farms were in the acute phase, and 3 case-farms were in the chronic phase. Lesions in bursas with acute to sub-acute lesions included depletion, necrosis, hemorrhage, infiltration by inflammatory cells and/or fibrin deposit in the lymphoid follicle as well as edema, congestion, hemorrhage and/or infiltration by heterophilic inflammatory cells in the inter-follicular space which constitutes a hallmark of acute IBDV infections (Gimeno and Isabel, 2013). In this category, a sub-group 1 of bursas with an MLS < 2 represented 28.6% of cases which could be linked to an adverse effect of live vaccine viruses. Indeed, it was described that invasive intermediate and intermediate plus or hot vaccine strains retained a non-

negligible immunosuppressive effect on the bursa (Gimeno and Isabel, 2013; Müller et al., 2012). The subgroup 2 of the bursas included 3.62.8% of cases with severe MLS ≥ 2). This severity of changes can be attributed with more certainty to the effects of the wild IBD virus. However, all changes reported in the findings of the current study could not be attributed to vvIBDV since no correlation ($p > 0.05$) was found between lesion severity and IBDV type identified by RT-PCR in field samples. Therefore, these results could only be suggestive of the challenges related to diagnosing IBD and its virulence by histopathology (Chai et al., 1999). This means that IBDV lesions can be caused by factors other than the virus strain, such as the immune status of affected chickens (Mouahid, 2006). Indeed, wild strains of IBDV were recognized to cause two forms of the disease. A clinical form was related to classical strains and hypervirulent strains causing macroscopic and histological lesions in BF with increasing degree of severity (Gimeno and Isabel, 2013; Van Den Berg, 2000), and a sub-clinical form due to the variants of IBDV. These late strains were shown to be pathogenic in broiler flocks vaccinated with conventional strains causing severe immunosuppression (Saif and Eterradossi, 2008) which would increase the susceptibility of the birds to other diseases (Ramahefarisoa, 2011; Müller et al., 2012), such as Newcastle disease, colibacillosis (Ezeibe et al., 2013), Marek's disease, and infectious anemia (Saif, 1991). Moreover, these variants were shown to cause significant damage to the bursa (Gimeno and Isabel, 2013). Bursal lesions classified as chronic in the current study included interstitial thickening, lymphoid depletion, fibrosis, and folding of the surface epithelium with or no formation of pseudocysts. Bursa from all three cases in current category had an MLS ≥ 2 which may be considered quite severe (Gimeno and Isabel, 2013) and may be linked to hypervirulent IBDV.

Moreover, the time between the start of infection and sampling (phase of infection) was one of the most important factors which influenced the level of success in IBDV detection during an infection. As a result, it is important to sample the birds in the acute phase of infection. Thus, The IBDV can be isolated from the infected broilers during the period of 1-2 weeks (Sjaak, 2006). Severe outbreaks of IBD occurred in Morocco from 1992 to 1996 which affected 56.20% of farms practicing vaccination against IBD (Bouzoubaa K. et al., 1992; Jaouzi, 1996). In 1999, Moroccan IBDV strains were isolated and characterized for the first time. They were shown to cause high mortalities in young Specific-Pathogen-Free chickens associated with severe macroscopic and microscopic changes of Fabricius bursa (Kichou et al., 1999). In addition, in a more recent work, the hypervirulent pathogenic strain of the IBD virus was also isolated in the country (Tahiri et al., 2011). On the basis of clinical history and histopathological examination, all broiler farms investigated in the present study were diagnosed with IBD although they were vaccinated against IBD. It should be noted that it was not possible to collect information about the type of vaccines due to confidentiality issues. Different types of IBD vaccines are commonly marketed in Morocco among which were the first-generation vaccines (live and inactivated vaccines) and the second generation (immune complex vaccines and vectorized vaccines HVT-VP2; Gimeno and Isabel, 2013).

Despite vaccination in the investigated farms, the occurrence of IBD can be explained by a set of factors, including a poor estimate of the optimal vaccination time in relation to the level of maternal anti-IBDV antibodies (Ramahefarisoa, 2011). This issue could raise the relevance of serological monitoring which made it possible to know the immune status of the flock to deduce the right time to vaccination (Boumdine, 2009). Heterogeneity of maternal antibody levels in the same vaccinated flock (Ramahefarisoa, 2011) and defective conservation and application of vaccines were other factors that can be highly incriminated in the Moroccan conditions. Moreover, the vaccine strain may not provide satisfactory protection against wild viruses due to the antigenic variability of IBDVs (Arada, 2010). Intermediate and intermediate plus or hot vaccines may not provide complete protection against infection with vvIBDV or by antigenic variants (Müller et al., 2012). However, each suspicion of antigenic variation in the field should be subjected to the isolation of the responsible viral strain and the investigation of its pathogenicity and the protection conferred by conventional vaccine strains (Etienne, 2002). No correlation was found between the techniques used for virus isolation, namely embryonated chicken eggs and primary chicken embryo fibroblast cell. The reason is that embryonated chicken eggs as a VI technique is recommended for field strains, such as the vvIBDV strain because they fail to grow or poorly grow in cell culture, and their adaptation to cell culture results in genetic changes and a loss of pathogenicity that is similar to primary chicken embryo fibroblast cell line (Sjaak, 2006). Therefore, sequencing in the current study was performed on the isolates in embryonated chicken eggs.

The molecular characterization of vvIBDV in the diverse regions of the world was very important for understanding the trends in the evolution, spread, and field status of IBDV for effective control of IBD in chickens. In the current study, the genetic characterization of 7 vvIBDV isolates was carried out by sequencing and analysis of the HVR of the VP2 gene. The HVR, spanning amino acid residues 211 to 350, was a major conformational and neutralizing antigenic domain. Since the greatest differences among serotype-1 strains of IBDV occurred in this part of the genome, the nucleotide and deduced amino acid sequences of this region were widely used for diagnozing and typing as a variant, classic, or very virulent (Jackwood and Sommer, 1999).

The nucleotide sequence spanning the HVR of 7 vvIBDV isolated in this study were compared to the genome sequences available in PubMed (Classical IBDV strains [D78, Faragher 52/70, 2512 Winterfield, Lukertand LC 75]) and vvIBDV strains isolated in different countries. Generally, the IBDV strains were grouped within one of three major genogroups, namely genogroup 1 (predominantly classical), genogroup 2 (predominantly variant), and genogroup 3 (predominantly vvIBDV pathotype or vvIBDV reassortant, Van Den Berg et al., 2004). Based on the results illustrated in the phylogenetic tree, the IBDV strains isolated in this study were classified in genogroup 3 (predominantly vvIBDV). Overall, the nucleotide sequence similarity of the VP2 gene among Moroccan isolates was between 96.2% and 100% (Drissi Touzani et al., 2019). The deduced amino acid sequence of the HVR of VP2 was determined and compared to different strains of vvIBDVs and a vaccine strain (Faragher 52/70). The analyzed HVR included 134 amino acid residues from positions 220 to 354 of the VP2 protein. The phylogenetic tree revealed that all the Moroccan vvIBDV field isolates were grouped in the same cluster with vvIBDV from Nigerian and Ethiopian isolates, which may indicate their ancestral relationships. It was not unexpected to discover that the Moroccan IBDV isolates were phylogenetically close to isolates from Africa due to exchanges between the African countries. It is possible that the vvIBDV strains were introduced into Morocco from neighboring countries. In addition, it is also likely that the vvIBDV has come from a more distant source due to the annual importation of huge numbers of chickens from all over the world to Morocco. The amino acid characteristic of vvIBDVs (222A, 256I, 294I and 299S; Brown et al., 1994 ; Eterradossi et al., 1999) were detected in the four analyzed vvIBDVs (i.e., MN241433, MN241434, MN241436, and MN241437). Mutations were observed in the conserved regions/positions considered exclusive to vvIBDVs. Two strains of MN241433 and MN241438 contained a substitution mutation in amino acid 222 (A→T) indicating that alanine at this position was not a unique characteristic of vvIBDVs (Parede, 2003; Jackwood and Sommer-Wagner, 2007). The HVR of VP2 contains two major and two minor hydrophilic regions (Azad et al., 1987; Van Den Berg et al., 1996). The hydrophilic peaks are located at amino acids 210 to 225 (peak A), amino acids 247-254 (minor peak 1), amino acids 281-292 (minor peak 2), and amino acids 312 to 324 (peak B, Azad et al., 1987; Van Den Berg et al., 1996). These regions are located at the outer part or projection domain of the viral capsid (Coulibaly et al., 2005). In addition to amino acid sequence differences between the studied strains and vaccine strain Faragher 52/70, two substitution mutations were found in hydrophilic regions in a key epitope in the VP2 capsid at positions of single point mutations. This issue induced single amino acid changes which were detected in MN241439 isolates, firstly, a nucleotide change resulted in a (G) at position 225 (V→G) 1 in the major hydrophilic peak region A, and secondly, 249 (Q→H) 1 in the minor hydrophilic peak region 1. The antigenicity of IBDV depended on the structural conformation of the major hydrophilic peaks A and B of the VP2 HVR variable region (Schnitzler et al., 1993), so changes in one or both of them could respectively lead to the emergence of either an antigenically variant serotype 1 strain or of a new serotype (Van Den Berg and Meulemans, 1991; Mardassi et al., 2004; Jackwood and Sommer-Wagner, 2011). The substitution mutation in the minor hydrophilic peak at a position of 248 to 252 was considered to influence IBDV antigenicity, as well (Eterradossi et al., 1999). Furthermore, there were two changes located outside the previously described hydrophilic regions of VP2: 238 (T→P) and 353 (A→T). These substitution mutations, influencing the antigenicity of IBDV, made the prediction of changes in IBDV antigenicity highly unreliable in case the sequence data is the only factor to consider (Durairaj et al., 2011).

CONCLUSION

In conclusion, the current study and previous reports indicated a very virulent infectious bursal disease virus as the main cause of substantial economic losses in the poultry industry. The RT-PCR and histopathological data confirmed that the hyper-virulent form of infectious bursal disease virus continued to cause serious problems for

Moroccan chicken breeders despite the vaccination. The seven very virulent infectious bursal disease virus clustered phylogenetically with very virulent infectious bursal disease virus from Africa (Nigeria and Ethiopia). Hypervariable region VP2 sequences were responsible for the determination of antigenicity and pathogenicity of the infectious bursal disease virus. The mutations in Hypervariable region were noticed in our isolates, especially in major hydrophilic peak region A and in the minor hydrophilic peak region 1. Therefore, the amino acid changes in this hydrophic region could affect these characteristics of very virulent infectious bursal disease virus strains and the control of the disease in the future.

DECLARATIONS

Funding

This study was conducted within the framework of a collaborative agreement between Institute of Agronomy and Veterinary Medicine, Rabat, Morocco and Biopharma, Rabat, Morocco.

Competing interests

All authors declare no competing interest.

Consent to publish

Not applicable.

Availability of data and materials

The manuscript contains all datasets generated and/or analyzed in the current study.

Authors' contributions

The conceptualization of this paper was carried out by Maryame CHEGGAG, Khalil ZRO, Mohamed MOUAHID, Mohammed EL HOUADFI, Ghizlane SEBBAR, Siham FELLAHI, and Faouzi KICHOU. The formal analysis was performed by Maryame CHEGGAG, Khalil ZRO, Meriam Tarta, Ghizlane SEBBAR, Siham FELLAHI, and Faouzi KICHOU. Maryame CHEGGAG wrote the first draft of the manuscript. Faouzi KICHOU, Ghizlane SEBBAR, Meriam Tarta, and Siham Fellahi edited the manuscript prior to the submission. All authors read and approved the final manuscript.

Acknowledgments

The authors express their great thanks to sanitary mandated veterinarians supervising poultry farms in different regions in Morocco, who collaborated in sample collection and case submission.

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JWPR
Journal of World's Poultry Research

2020, Scienceline Publication

J. World Poult. Res. 10(3): 507-512, September 25, 2020

Research Paper, PII: S2322455X2000058-10

DOI: https://dx.doi.org/10.36380/jwpr.2020.58

Circulating Antibodies against Avian Influenza and Newcastle Disease in Semi-Captive Peacocks in Southwestern Guatemala

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Received: 01 Jul. 2020
Accepted: 15 Aug. 2020

ABSTRACT

Avian Influenza and Newcastle disease are the two most important diseases of poultry and are globally considered as threats to public health and economy. There is little information published about these diseases in peacocks and other common backyard poultry in Guatemala. Therefore, an exploratory serosurvey was conducted to determine the presence of circulating antibodies to Avian Influenza (AI) and Newcastle Disease (ND) viruses in a semi-captive population of peacocks in southwestern Guatemala. Additionally, the circulation of antibodies to these pathogens in backyard chickens, ducks, and turkeys from a neighboring community was explored. Blood samples were obtained from 48 peacocks, 30 chickens, 6 ducks, and 4 turkeys. The samples were processed in the Regional Reference Laboratory for Animal Health, at the Veterinary Medicine Faculty, University of San Carlos of Guatemala, located in Guatemala City. Antibodies against AI virus were investigated by Agar Gel Immunodiffusion, and antibodies against ND virus were examined using Hemagglutination Inhibition. No antibodies against AI virus were detected. Most of the samples (97.7%) were negative for antibodies against ND virus, except for two turkeys that carried low antibody titers. The findings of the present study indicate that no virulent strains of AI or ND viruses were circulating in the investigated site.

Keywords: Avian influenza, Epidemiology, Newcastle disease, Serology, Zoonoses

INTRODUCTION

Avian Influenza (AI) and Newcastle Disease (ND) are the most important diseases of poultry (Alexander, 2000; Capua and Marangon, 2006), and are considered global threats to public health and economy (Wong and Yuen, 2006). AI viruses have been a common cause of epidemics and pandemics (De Jong and Hien, 2006; Alexander, 2007; Peiris et al., 2007; Monto and Fukuda, 2020), and their ability to mutate and become more pathogenic or more capable to invade other host species makes them relevant to animal and public health.

Due to this mutation capacity, numerous subtypes of influenza A virus have evolved. On the other hand, Newcastle disease is one of the threats to farm economy and poultry production, not only because it causes economic losses due to mortality, but also because many countries have sanitary barriers that prohibit importations from countries where the disease is endemic (Miller and Koch, 2013). Both AI and ND are known to affect a wide variety of hosts (Stallknecht and Shane, 1998; Ito and Kawaoka, 2000; Baigent and McCauley, 2003; Swayne and King, 2003). These pathogens have already been found in Guatemala in some avian species in certain areas of the country (Gonzalez-Reiche et al., 2016; Mérida et al., 2016; Gonzalez-Reiche et al., 2017). However, published information about AI or ND in peacocks or backyard fowl species in southwestern Guatemala is practically inexistent.

In response to this gap of knowledge, the presence of circulating antibodies against AI and ND viruses was investigated in a semi-captive population of peacocks in southwestern Guatemala. Additionally, the circulation of antibodies against these pathogens was explored in backyard chickens, ducks, and turkeys from a neighboring community.

MATERIALS AND METHODS

Study site

A population of 94 peacocks (*Pavo cristatus*) kept in semi-captive conditions in an amusement park located at Retalhuleu department, in southwestern Guatemala (coordinates 14°35'41" N 91°36'42" W) was studied. The study site was a fenced area of 128 thousand square meters surrounded by small villages and hamlets where peasants strongly depend on family agriculture and animal raising to survive (Figure 1). In the park facilities, peacocks roamed freely among visitors (one million visitors a year), and frequently came into the contact with wild birds. To explore the antibody circulation in the surroundings, a sample of backyard fowl was also studied in households of the neighboring community, San Martín Zapotitlán, Retalhuleu, Guatemala.

Species and sampling

A sample of 48 peacock individuals was calculated and randomly sampled based on 50% prevalence, 95% confidence interval, and 0.1 margin of error. Additionally, thirty chickens (*Gallus gallus*), four turkeys (*Meleagris gallopavo*), and six ducks (*Anas platyrhynchos*) were sampled by convenience. All sampled birds were adults. Three millimeters of blood was taken from the ulnar vein using 3cc syringes with 23g X 1.5" needles, while gently holding the birds by hand. The blood samples were put into test tubes without anticoagulant, centrifuged for four minutes at 3,000 rpm, transferred to plastic straws, and transported to be processed in the Regional Reference Laboratory for Animal Health (Larrsa), at the Veterinary Medicine and Animal Husbandry Faculty, University of San Carlos of Guatemala, in Guatemala City.

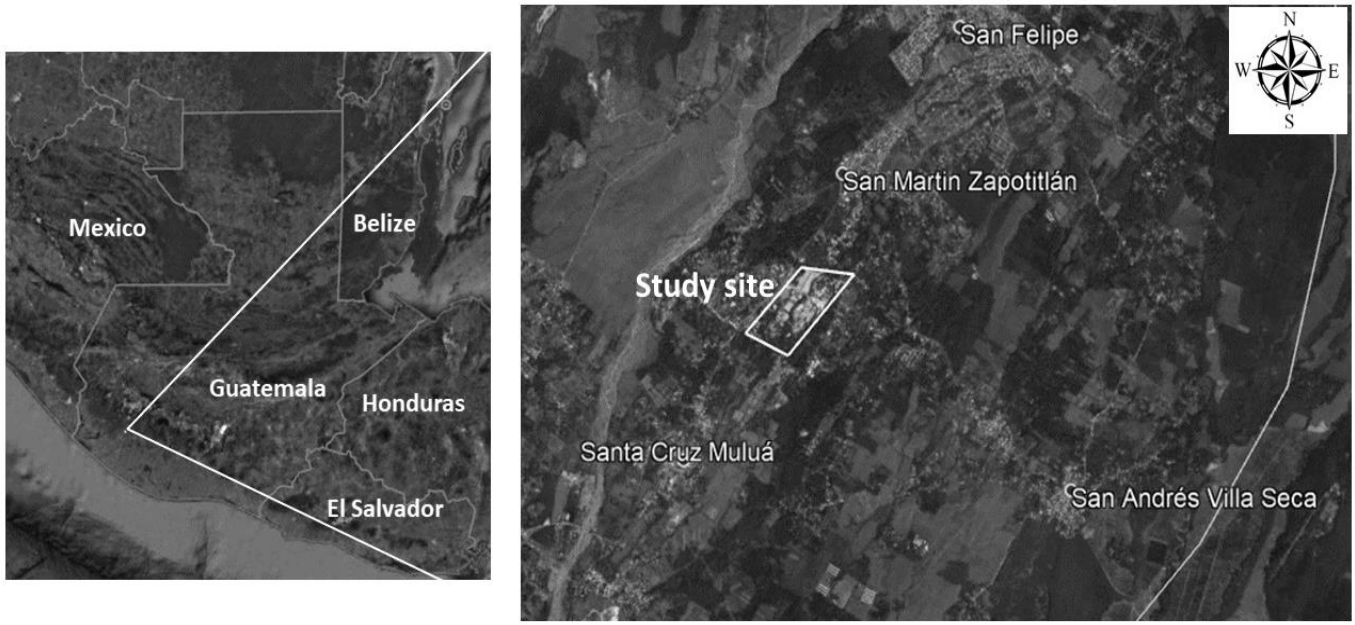

Figure 1. Study site location in Retalhuleu, southwestern Guatemala.

Laboratory procedures

Antibodies against AI virus were studied by agar gel immunodiffusion (GID) tests, performed according to standard procedures (OIE, 2018a), using Type I molecular biology grade agarose (Calbiochem, USA), NaCl and pH 7.2 phosphate buffer (Merk, Germany) and AI antigen (Larrsa, Guatemala).

Antibodies against ND virus were studied by hemagglutination inhibition tests performed according to standard procedures (OIE, 2018b), using a 1% chicken red blood cell solution (Larrsa, Guatemala), local ND antigens (Larrsa, Guatemala) isotonic PBS (Merk, Germany), positive control (Charles Rivers, USA), 10-100µl unichannel micropipettes (Transferpette, Germany), 10-100 µl multichannel micropipettes (Transferpette, Germany), micropipette tips (Transferpette, Germany) V-bottomed microtiter plates (Nunc, Denmark) and an orbital shaker (Barnstead, Germany). HI antibody titers were considered positive if there was inhibition of hemagglutination at serum dilutions of 1/16 (2^4 or $\log_2$ 4, when expressed as the reciprocal, as is customary in Larrsa).

Ethical approval

This research was approved by the Bioethics Committee of the Graduate School, Veterinary Medicine and Animal Husbandry Faculty, University of San Carlos of Guatemala.

RESULTS AND DISCUSSION

No circulating antibodies against AI and ND viruses were found in the sampled peacocks. Antibodies were also not found in the backyard birds of the neighboring community, except for two turkeys that had low antibody titers (Log_2 5 and 6) against ND virus (Table 1).

Table 1. Frequency of positive blood samples to antibodies against Avian Influenza and Newcastle disease in peacocks.

Bird species	Number of samples	Avian Influenza antibodies	Newcastle Disease antibodies
Pavo cristatus	48	0	0
Gallus gallus	30	0	0
Meleagris gallopavo	4	0	2
Anas platyrhynchos	6	0	0
Total	88	0	2

Note: the titers of the positive *Meleagris gallopavo* samples were 5 and 6 (log_2 reciprocal).

Although peacocks are susceptible to some AI and ND virus strains (Munir et al., 2012; Desingu et al., 2016; Umar, 2017; Wajid et al., 2017), and even though the studied population was kept in semi-captive conditions and in frequent contact with people, and wild birds that were possible sources of infection (Rehan et al., 2019), no antibodies were found in the sampled individuals. It was also highly likely that the rest of the population did not have antibodies either, especially considering that the entire flock slept every night at the same roosting site, and therefore, if the population were susceptible, any outbreak of AI or ND would have readily spread due to disease ecology determinants (Beldomenico and Begon, 2010).

The absence of antibodies against AI virus observed in the sampled peacocks in the present study was consistent with previous studies in the USA by Hollamby et al. (2003) and in Hong Kong by Ellis et al. (2004), but inconsistent with studies in the Kingdom of Saudi Arabia by Ismail et al. (2010) and in Iraq by Rashid et al. (2017). On the other hand, the absence of antibodies against ND virus was consistent with the findings of Ibitoye et al. (2013) in Nigeria, and inconsistent with the findings of Vijayarani et al. (2010), Khulape et al. (2014) and Desingu et al. (2016) in India; Sadiq et al. (2011) in Nigeria; Chumbe et al. (2015) in Peru and Munir et al. (2012) and Mustafa et al. (2015) in Pakistan.

The first interesting consideration when interpreting our findings was that GID test is a screening assessment that detects antibodies against all subtypes of AI virus (Jenson, 2014). In that sense, there was not only the absence of antibodies against the previously isolated subtypes in Guatemala, but also against all subtypes. The second consideration would be the cause underlying the absence of antibodies in almost all the sampled birds (including the backyard poultry from the neighboring community). Failure to find antibodies could mean that past epizootic outbreaks have swept birds −as has been previously reported in Guatemala (Lepe-López et al., 2020)– and have left no immune history. It could also mean that the circulating virus strains were not pathogenic enough to stimulate the production of antibodies in the studied birds. It has also been suggested that in backyard poultry, a lack of immune response could be the result of infectious bursal disease, chronic aflatoxicosis, or vitamin A deficiency (Awan et al., 1994). However, this was unlikely to be the underlying cause, at least in the case of the peacocks, because the population had veterinary care and adequate nutritional management.

The lack of evidence of the circulation of virulent strains of AI and ND viruses in the studied landscape is epidemiologically noticeable considering that in Guatemala, more than 26 million backyard chickens are being raised –and probably being trade– in practically all the country (Ministry of Agriculture, Livestock and Food, Guatemala, 2015). In fact, antibodies against AI and ND viruses have been detected in some backyard chicken populations (Aguilar-Miller et al., 2016; Aquino-Sagastume et al., 2016; Mérida-Ruiz et al., 2016) as well as in commercial poultry (Lee et al., 2004) and in wild birds (Gonzalez-Reiche et al., 2012; Gonzalez-Reiche et al., 2016). On the other hand, 758 species of birds are distributed in Guatemala, and at least 240 are migratory (Eisermann and Avendaño, 2018). This host diversity seems to be accompanied by a virus diversity because recently, 19 Influenzavirus A subtypes were isolated from migratory ducks in Guatemala, including the H7N3 subtype (Gonzalez-Reiche et al., 2017).

It is important to notice that, depending on the virus strain and the avian host species, some low pathogenicity AI viruses are unable to infect hosts stimulating only low or imperceptible immune responses (Alexander et al., 1978; Alexander et al., 1986). This differential species-dependent immune response was also observed for the ND

virus (Eze et al., 2014). During an H5N1 AI outbreak in a natural park in Hong Kong, several species of birds resulted infected, but peacocks were among the non-affected species (Ellis et al., 2004).

The absence of antibodies in the backyard poultry of the neighboring community found in the present study could mean that no virulent strains of AI or ND viruses were circulating in the landscape. In a backyard poultry national Serosurvey for AI and ND in Oman, the bird seroprevalence was 37.5% and 42.1% respectively, and the flock seroprevalence was 84% and 90% respectively (Al Shekaili et al., 2015) but this massive seroprevalences seemed unlikely for Guatemala where previous data suggest a patchy distribution of these pathogens in the backyard poultry population.

On the other hand, the finding of two seropositive turkey individuals to antibodies against ND virus in the present study is rather difficult to explain, considering that all the other sampled specimens did not show circulating antibodies against ND virus. These two antibody-carrier turkeys could have been recently added to the population by the peasants.

Considering the evidence found in the present study, more research needs to be done to establish the distribution pattern of Influenza and Newcastle viruses in the avian host populations throughout the country, mainly, at the human-animal interface (Chaudhry et al., 2020). This would enable the design of sound intervention policies to assure commercial and backyard poultry productivity and public health.

CONCLUSION

Considering that the population was not recently-established and based on the absence of antibodies and on the fact that no significant mortality was observed in recent years, the results indicate that no AI or ND viruses are circulating in the studied population of peacocks.

DECLARATIONS

Acknowledgements

The researchers want to thank Dr. Karen Calderón for collaborating in the laboratory procedures.

Competing interests

The authors have declared that no competing interest exists.

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Author's contribution

MC-G conception of the idea, drafting the manuscript and field sampling DG-C conception of the idea, drafting and editing the manuscript YT field sampling and reviewing the manuscript CV-S and ML-L conception of the idea and reviewing the manuscript BS laboratory procedures, and reviewing the manuscript. All authors checked and confirmed the final version of the article.

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JWPR
Journal of World's Poultry Research

2020, Scienceline Publication
J. World Poult. Res. 10(3): 513-519, September 25, 2020

Research Paper, PII: S2322455X2000059-10
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DOI: https://dx.doi.org/10.36380/jwpr.2020.59

The Processing Effects of Anthocyanins Extracted from Dragon Fruit (*Hylocereus polyrhizus*) Peel on Total Amount of Anthocyanins and SEM Image in Poultry Nutrition

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Received: 07 Jul. 2020
Accepted: 21 Aug. 2020

ABSTRACT

The purpose of present study was to know the effects of different processing of anthocyanin content and scan electron microscope image of anthocyanin of dragon fruit peel in poultry diet. The experiment was performed in a completely randomized design with different processing like untreated dragon fruit peel or control, physical, chemical, biological, and physical-biological, and each treatment was replicated 4 times. Variables measured were total amount of anthocyanin and anthocyanin image of dragon fruit peel. The results indicated that physical treatment significantly increased anthocyanin content of dragon fruit peel. Furthermore, treated dragon fruit peel with chemical, biological, and combination of physical-biological significantly reduced anthocyanin content. The image of anthocyanin from each treated processing revealed that control image was similar to physical treatment, and it was different from other treatments. The physical treatment was the best method to increase anthocyanin content, and did not change the image of anthocyanin from dragon fruit peel.

Keywords: Anthocyanins, Dragon fruit peel, Processing, Scanning electron microscope, Spectrophotometry

INTRODUCTION

Dragon fruit is a fruit from cactus plant which have many different species such as white dragon fruit (*Hylocereus undatus*), red dragon fruit (*Hylocereus polyrhizus*), super red dragon fruit (*Hylocereus contaricensis*), and yellow dragon fruit (*Selenicereus megalanthus*). The most popular dragon fruit in Indonesia is red dragon fruit (*Hylocereus polyrhizus*), this dragon fruit is used by people to make a juice, cake, syrup, and jam, where all these processings will produce dragon fruit peel waste. There is no report about total production of dragon fruit in Indonesia, but Mahata et al. (2015) and Mahlil et al. (2018a) reported that processing and utilization of the fresh dragon fruit (*Hylocereus polyrhizus*) would produce dragon fruit peel waste as much as 22% from the whole fruit. In Indonesia, the dragon fruit peel waste is potentially as poultry feed for lowering cholesterol, and as antioxidant (Mahata et al., 2015; Mahlil et al., 2018a). The phytochemical in dragon fruit peel such as anthocyanins, beta-carotene, and lycopene were reported as antioxidants, lowering the blood cholesterol, natural dyes, and anti-cancer (Wu et al., 2006; Charoensiri et al., 2009). According to Lewis and Rader (2005) and Anggraeni (2010), the mode of action of anthocyanin in reducing of LDL cholesterol in mice and humans was by inhibiting of 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase enzyme for producing mevalonate from HMG-CoA compound in cell. In addition, anthocyanin was reported to increase the activity of lecithin cholesterol acyltransferase to convert free cholesterol to hydrophobic cholesterol ester, then the cholesterol ester binds the lipoprotein core to produce new HDL, this reaction increased the blood plasma HDL in humans and mice. Fang (2014), reported that Anthocyanin compounds can be absorbed from the stomach or intestines. Anthocyanin can bind to proteins, so they can accumulate into egg yolks, and increase egg yolk color (Fayyaz et al., 2016; Iskander et al., 2017).

To cite this paper: Mahlil Y, Husmaini, Warnita, Mirzah, Kobayashi M and Endo Mahata M (2020). The Processing Effects of Anthocyanins Extracted from Dragon Fruit (*Hylocereus polyrhizus*) Peel on Total Amount of Anthocyanins and SEM Image in Poultry Nutrition. *J. World Poult. Res.*, 10 (3): 513-519. DOI: https://dx.doi.org/10.36380/jwpr.2020.59

Previous researches showed that processing of poultry feed by physical methods (steaming, boiling), chemical methods like submerge in acid or base solution, biological method like fermentation with microorganism were performed for improving the quality of poultry feed (Mahlil et al., 2018a). Mahata et al. (2016a) and Mahata et al. (2016b) reported that the processing of tomato waste by physical method (boiled in hot water for eight minutes) to change the structure of trans-lycopene to cis-lycopene in tomato waste showed that tomato waste can be used as an alternative feed for broiler and laying hens. The utilization of boiled tomato wastes can be used as much as 7% in broiler ration, and as much as 12% in laying hens ration (Mahata et al., 2016a; Mahata et al., 2016b). Furthermore, Handayani et al. (2018) mentioned that physical method (steaming and boiling) of tomato waste for 12 minutes was the appropriate process for increasing lycopene and organic matter content, and maintaining the crude protein content. Mahata et al. (2012) reported that the crude fiber content in a juice waste mixture decreased from 17.10 to 12.02 percent after steaming in an autoclave for 30 minutes. Mahlil et al. (2018a) reported that the processing of dragon fruit peels by physical method (steaming for 20 minutes), and chemical method (soaking in acetic acid solution for four hours) decreased the crude fiber content in dragon fruit peel from 24.01 to 19.81 percent, and from 24.01 to 20.39 percent, respectively. Crude fiber content in poultry's feed was decreased by biological method such as fermentation using microorganism (Rizal et al., 2013; Adrizal et al., 2017; Heryandi et al., 2018; Mahlil et al., 2018b)

The problem of dragon fruit peel utilization for laying hen diet was the high value of crude fiber content (24%) (Mahlil et al., 2018). The digestive tract of poultry does not produce cellulase enzyme for degrading fiber, therefore the anthocyanin in dragon fruit peel was not absorbed by poultry maximally (Mahlil et al., 2018). Furthermore, dragon fruit peel waste must be processed by some methods to decrease crude fiber content before it is utilized for laying hens feed.

The processing of dragon fruit peel by some methods like heating and soaking with alkali and acid solution would destroy its anthocyanin structure and quantity, because some factors like pH, temperature, light, metal ion, oxygen, sugar content, and enzyme will affect the anthocyanin stability and quantity (Khazaei et al., 2014; Khoo et al., 2017). In present experiment, the anthocyanin content from dragon fruit peel after treated by all methods (physical, chemical, biological, and combination of physical-biological method) was detected by spectrophotometer. It was also seen the figure of anthocyanin after treated by any possible method (physical, chemical, biological, and combination of physical-biological method) under scan electron microscope (SEM).

MATERIALS AND METHODS

Collection of dragon fruit peel

Dragon fruit peels (Hylocereus polyrhizus) were obtained from dragon fruit field in Payakumbuh City, and also from restaurants and various juice counters in Padang City, West Sumatra Province, Indonesia.

Experimental design

The experiment was performed in a completely randomized design with different processing like untreated dragon fruit peel or control, physical (steaming at 98°C for 20 minutes), chemical (soaking in 7.5% acetic acid, pH of 4 for duration of four hours), biological (fermentation using local microorganism solution from bamboo sprout with fermentation durations of 13 days, and inoculum dosage 6%), and physical-biological, and each treatment was replicated 4 times. Variables measured in the experiment were anthocyanin contents and anthocyanin image of dragon fruit peel.

Preparation of dragon fruit sample

The sample of dragon fruit peel used in this experiment was obtained from restaurant and juice counters. Furthermore, dragon fruit peel was treated by some methods such as physical (steaming for 20 minutes), chemical method (soaking in acetic acid solution for four hours) (Mahlil et al., 2018a), biological method with fermentation utilizing microorganism-containing solution from local bamboo sprout like *Lactobacillus*, *Streptococcus*, *Azotobacter*, *Azospirilium* and fungi (*Fusarium* and *Trichoderma*) with fermentation duration of 13 days, and inoculum dosage of 6%, and combination of the physical and the biological processing methods (Mahlil et al., 2018b). After processing by each method, the dragon fruit peel was dried in oven with temperature of 60° C until getting dry, and then it was blended to mash form.

Anthocyanin extraction

Anthocyanin extraction was prepared by maceration method by using distilled water solvent (Lapornik et al.,

2005). Two gram of dragon fruit peel mash sample from each different method processing (steaming 20 minutes, soaking in acetic acid solution for four hours, fermentation utilizing microorganism-containing solution from bamboo sprout, combination of the physical and the biological) were macerated in Erlenmeyer flask volume 250 ml, and the surface of Erlenmeyer was covered with aluminum foil, and then they were placed at a hot plate for extraction process (Lapornik et al., 2005). Furthermore, samples were centrifuged at a cold centrifuge (4 ^{o}C) with 8000 rpm, and the supernatant (anthocyanin) of each sample were separated from pellet, and then dried at oven in temperature of 60 $^{\circ}$C.

Calculation of total anthocyanin

Total anthocyanin was calculated by Giusti et al. (2001) method used spectrophotometer (UV-Visible 1700, Shimadzu). As much as one ml of anthocyanin extract from each different processing method of dragon fruit peel was diluted into two different buffer solutions. For the first solution, the dragon fruit peel sample was diluted with 0.025 M of Potassium Chloride buffer at pH of 1, and the second solution was diluted with 0.4 M buffer of natrium acetate at pH of 4.5 (Giusti et al., 2001). For determination of anthocyanin content and lambda visible maximum of sample solution in both buffer (Potassium chloride and natrium acetate), the scanning of wave length in 510 nm to 700 nm was arranged in spectrophotometer, and then absorbance was measured (Giusti et al., 2001). Furthermore, total anthocyanin was calculated with mathematical equation below:

Absorbance of solution: [lambda visible maximum (510 nm) – A700 nm] pH1.0 – [lambda visible maximum (510 nm) – 700 nm] pH4.5

Monomeric total of anthocyanin from dry extract of dragon fruit peel after each processing method was calculated as cyaniding-3- glucoside base on mathematical equation below:

$$MAP\ (mg/L) : [(A \times MW \times DF \times 1000) / (\varepsilon) \times 1]$$

A: absorbance of solution; MW: molecular weight; DF: dilution factor; ε: absorptivity molar cyaniding-3-glucoside; 1: thick cuvette (cm); MAP: monomeric anthocyanin pigment

Scanning electron microscope observation of anthocyanin

The anthocyanin sample was attached to the carbon tape, and then put on the metal plate (Echlin, 2009). Furthermore, samples were sputter-coated with gold (50 nm) using a VPS-020 quick coater (ULVAC Inc., Kanagawa, Japan) for observation by SEM (SU-6600, Hitachi High-Technology Corp., Tokyo, Japan) at an acceleration voltage of 1.0 kV (Echlin, 2009).

Statistical analysis

All data were statistically analyzed by a one-way analysis of variance (completely randomized design). The differences between treatments were determined using Duncan's multiple range test, with a 5% significance level ($P < 0.05$) (Steel and Torrie, 1991).

RESULTS AND DISCUSSION

The mean values of the anthocyanin content in dragon fruit peel before and after treated with each method are depicted in table 1. The processing of dragon fruit peel with some methods (control, physical, chemical, biological, and combination of physical biological) affected anthocyanin content significantly ($P<0.05$). Anthocyanin content from physical treatment was significantly different ($P<0.05$), higher than control, chemical, biological, and combination of physical biological treatments. Control treatment was significantly different ($P<0.05$), higher than chemical, biological, and combination physical-biological. Chemical treatment was significantly different ($P<0.05$), higher than biological and combination of physical-biological, and anthocyanin content of biological treatment was significantly different ($P<0.05$), higher than anthocyanin content from combination of physical-biological treatments.

Table 1. The Amount of total anthocyanin (ppm) content in dragon fruit peel (*Hylocereus polyrhizus*) before and after treated by Physical, Chemical, Biological and Physical-Biological methods

Treatment	Replication				Means
	A	B	C	D	
Control	201.54	212.22	187.53	194.20	198.87[a]
Physical	351.70	345.03	373.72	149.49	353.20[b]
Chemical	134.14	147.49	148.82	142.35	144.98[c]
Biological	111.45	112.12	113.45	114.79	112.95[d]
Physical-Biological	46.05	48.72	45.38	44.71	46.21[e]

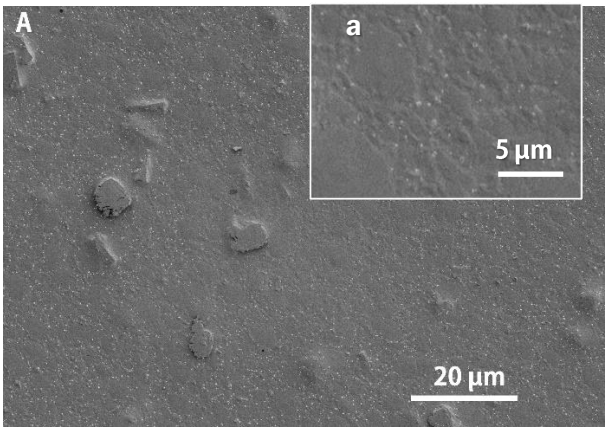
[a-e] Value in the same column with different letters are significantly different *p<0.05

The processing of dragon fruit peel by physical method (steaming at 98^{o}C for 20 minutes) increased anthocyanin content of dragon fruit peel from 198.87 to 353.20 ppm. Furthermore, processing of dragon fruit peel by chemical method (soaking in 7.5% acetic acid, pH of 4 for duration of four hours), biological method (fermentation using local microorganism solution from bamboo sprout with fermentation durations of 13 days, and inoculum dosage 6%), and processing of dragon fruit peel with combination of physical-biological method decreased anthocyanin content from 198.87 to 144.98, and 198.87 to 112.95, and also 198.87 to 46.21 ppm. This research was related with Mulyawanti et al. (2018) indicating that anthocyanin of purple sweet potato slightly increased after steaming processing. Furthermore, fermentation blueberry using *Lactobacillus* reduced total anthocyanins content (29%) compared with fresh blueberry (Nie et al., 2017). It was similar with the findings of Hornedo et al. (2017) indicating that Fermentation process decreased anthocyanin content from strawberry. Wiczkowski et al. (2015) reported that anthocyanin content from red cabbage reduced after fermentation.

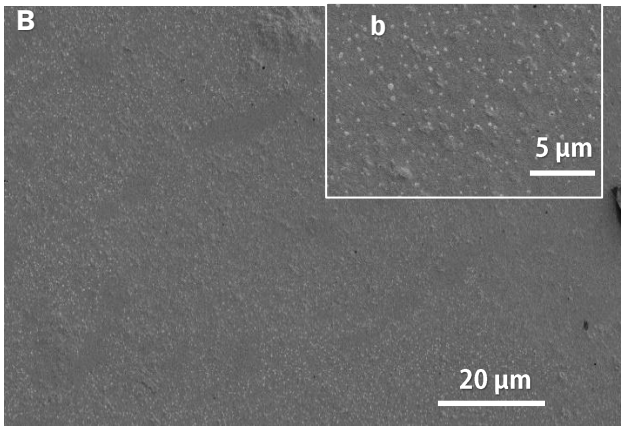
The highest content of anthocyanin in dragon fruit peel which was processed by physical method due to degradation of β-(1,4)-glycoside bond in crude fiber fraction of dragon fruit peel cell by steaming, so that the anthocyanin came out easily from inside of the cell to solution (KCl and natrium acetic) when dragon fruit peel maceration processed. Processing of dragon fruit peel by chemical method caused low level of anthocyanin content in comparing with control and physical method, this condition was correlated with crude fiber content in physical method that indicated more decrease than chemical method, it caused low levels of anthocyanin coming out of cells in chemical method than physical method. The processing of dragon fruit peel via biological method was possible by local microorganism from bamboo sprout, consist of bacteria (*Lactobacillus*, *Streptococcus*, *Azotobacter*, *Azospirilium*) and fungi (*Fusarium* and *Trichoderma*) (Fatoni, 2016). The loss of total anthocyanins content by fermentation was related to the unstable structure of blueberry anthocyanins during fermentation (Nie et al., 2017). This condition was a reason of decreased anthocyanin content of dragon fruit peel in biological method compared to anthocyanin content from dragon fruit without processing method or control, physical methods, and chemical methods in the current experiment. The lowest content of anthocyanin in dragon fruit peel was processed by combination of physical-biological method due to degradation of β-(1,4)-glycoside bonds in crude fiber fraction of dragon fruit peel cells by steaming, so that the anthocyanin came out easily from cells, and easier for microorganism to make anthocyanin structure unstable. Khazaei et al. (2014) reported that the processing by some methods like heat processing and fermentation would destroy anthocyanin structure and quantity, because of some factors like enzyme, pH, and temperature would affect the anthocyanin stability and quantity.

The result of anthocyanins from dragon fruit peel processed by different methods with SEM are presented in figure 1. The image of each anthocyanins from dragon fruit peel after processing by different methods indicated changing on their surface image.

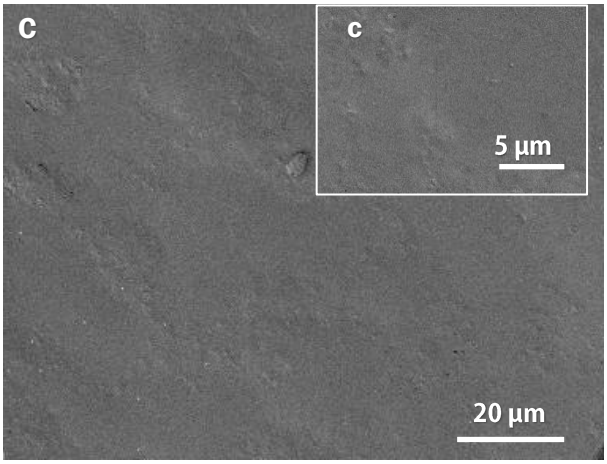
SEM observation showed that anthocyanin image from dragon fruit peel before being treated (See figure 1A and 1a), have many white spots, and after being treated by physical (steaming in boil water at 98^{o}C, at figure 1B and 1b), the white spots increased, then being treated by chemical (soaking in acetic acid solution at pH4, at figure 1C and 1c), white spots decreased, and also being treated by biological (fermentation with local microorganism from bamboo sprout, at figure 1D and 1d), the white spots vanished, and the surface wrinkled, and combination physical-biological method (Figure 1E and 1e), the image showed white spot turned in to black spot. Therefore, anthocyanin image of dragon fruit peel for control was the same with physical, and different from chemical, biological, and combination of physical-biological methods. It means that the anthocyanins of dragon fruit peel treated by physical method was equal with anthocyanin from untreated dragon fruit peel (control), while the image of dragon fruit peel treated by physical method was not destroyed . The anthocyanin of dragon fruit peel processed by other methods (chemical, biological, and combination of physical-biological) showed a different image of anthocyanin in control and physical methods. It means that the processing (chemical, biological, and combination of physical-biological) affected the anthocyanin.



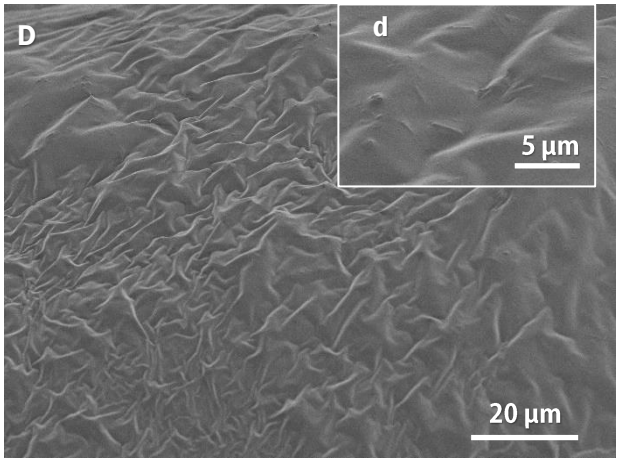
(**A**) before treated



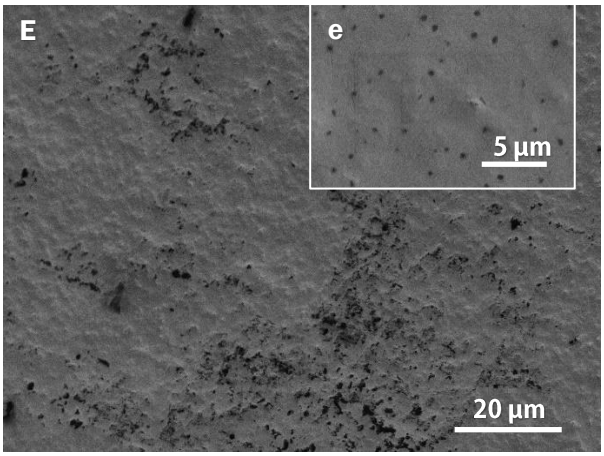
(**B**) after treated by physical



(**C**) after treated by chemical



(**D**) after treated by biological



(**E**) after treated by combination physical-biological method

Figure 1. Scanning Electron Microscope (SEM) image of anthocyanin extract surface

CONCLUSION

Anthocyanin content in dragon fruit peel treated by physical method was higher than anthocyanin of dragon fruit peel treated by chemical, biological, and combination of physical-biological methods, and untreated dragon fruit peel (control). The image of anthocyanin of dragon fruit peel processed by physical method resembled the anthocyanin image of untreated dragon fruit peel, and the anthocyanin image of dragon fruit peel processed by

chemical, biological and combination of physical-biological methods was different compared to anthocyanin image of dragon fruit peel processed by physical method and untreated (control).

DECLARATIONS

Acknowledgments

This study was a part of main research for my PhD program at Universitas Andalas funded by the Ministry of Research, Technology and Higher Education of the Republic of Indonesia through PMDSU No: 059/SP2H1LT/DRPM/IV 2017. We are very grateful to the Rector of Universitas Andalas, and Nara Institute of Science and Technology (NAIST), Japan at Division of Materials Science as Host University for conducting a part of this research, and to the Minister of Research, Technology and Higher Education of the Republic of Indonesia for their support.

Competing interests

The authors declare that they have no competing interests.

Author's contribution

Mahlil wrote the paper, collected data, and performed statistical analysis, Mirzah, Warnita, Husmaini, and Mahata created the idea and designed the study. Kobayashi facilitate laboratory equipment, support on research and writing. Mahlil and Mahata drafted the manuscript and approved the final manuscript.

Consent to publish

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

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JWPR
Journal of World's Poultry Research

2020, Scienceline Publication

J. World Poult. Res. 10(3): 520-526, September 25, 2020

Research Paper, PII: S2322455X2000060-10

DOI: https://dx.doi.org/10.36380/jwpr.2020.60

Isolation of Newcastle Disease Virus from Wild Migratory Birds in Egypt

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Received: 07 Aug. 2020
Accepted: 22 Sept. 2020

ABSTRACT

Surveillance studies for Newcastle disease virus (NDV) are critical to monitor the potential spreading of these viruses among wild birds as well as domestic poultry. This study was conducted to determine the incidence of NDV in wild birds in Egypt in 2016. Out of 159 collected samples from eight different species of wild birds, six (3.77%) samples were positive for paramyxoviruses by semi-nested RT-PCR assay based on the RNA-dependent RNA polymerase gene. Of six positive samples, four NDVs were successfully isolated in 11-day-old specific-pathogen-free embryonated hens' eggs. Partial sequences of the fusion gene of the four isolates were amplified using RT-PCR. Phylogenetic analysis of partial sequences of RNA-dependent RNA polymerase gene and fusion genes indicated that the detected NDV viruses in wild birds in Egypt are related to class I NDVs strains. Four Egyptian NDV isolates from wild birds exhibited sequence motif of ^{111}GERQER↓LVG^{119} at the cleavage site as lentogenic virus in wild birds. Continuous active surveillance may help better monitoring of NDVs circulating in wild birds before newly emerging viruses in domestic poultry.

Keywords: Egypt, Fusion protein, Newcastle disease virus, Wild birds

INTRODUCTION

Newcastle Disease Virus (NDV) is an enveloped RNA virus with a negative single-stranded, non-segmented genome of approximately 15 kb in length which encodes nucleocapsid protein (NP), fusion protein (F), hemagglutinin-neuraminidase protein (HN), matrix protein (M), polymerase protein (L) and phosphoprotein (P) (Swayne and King, 2003). The NDV was reported first time in Newcastle, England in 1926 and was responsible for the four panzootic in the UK (Alexander, et al., 1992). NDV is a member of the genus *Avulavirus*, which belongs to the family *Paramyxoviridae* (ICTV, 2013). NDVs have been classified into two main classes I and II. Class I of NDV comprises only one genotype that has been detected and isolated from wild birds whereas class II includes 18 genotypes that are circulating in domestic and wild birds (Xiao et al., 2012). NDVs can infect at least 240 different domestic and wild bird species. Based on virulence, NDVs are classified into three strains: velogenic strains that cause killer hemorrhagic, neurological disorders, and respiratory illness; mesogenic strains causing infection of respiratory system with limited mortality; and lentogenic strains that cause enteric diseases and not severe respiratory infection (Dortmans et al., 2011).

In Egypt, there have been continuous reports associated with Newcastle disease outbreaks among domestic poultry, which have affected the economy of poultry industries (Shakal et al., 2020). Based on NDV genotypic characterization, genotype II, VI, and VII of class II have been identified in domestic poultry in Egypt (Mohamed et al., 2009; Mohamed et al., 2011; Saad et al., 2017).

The northern coast of Egypt serves as a stopover for migratory birds during the annual migration of birds from Asia and Europe to Africa and vice versa. Accordingly, these migratory birds play an essential role in the transmission of pathogens throughout their migration routes (Alexander, 2007). In addition to migratory birds, Egypt has several species of resident wild birds that live in

close contact with domestic poultry as well as migratory wild birds and might play a role in the spread of newly emerging viruses.

Despite the potential threat posed by wild birds in the evolution of NDV, there is limited information about the genetic profile of NDVs circulating in wild birds in Egypt. Herein, the study detected and characterized the circulating NDVs among wildlife in Egypt.

MATERIAL AND METHODS

Ethical approval

Sampling from birds was approved by the ethical committee of the National Research Centre, Cairo, Egypt (registration number: 16247). All international, national, and institutional regulations on sampling from wild birds were followed.

Samples collection

From October to December 2016, a total of 159 cloacal samples taken from wild birds were individually collected from live bird markets of three northern coastal cities of Egypt [Damietta (n=102), Port Said (n=33), and Matruh (n=24)]. The birds sampled included northern shoveler (n=76), pintail (n=39), laughing dove (n=6), teal (n=31), wigeon (n=2), moorhen (n=1), mallard (n=1) and coot (n=3). The cloacal swabs were collected in 2 ml cryovials containing viral transport medium. Then the samples were transmitted to the laboratory in icebox to keep the temperature of the samples within the viral survival range. The collected samples were kept at -80 ºC for further analysis.

Detection of paramyxoviruses in wild birds

A volume of 140 µl of each collected sample was subjected to viral RNA extraction using the QIAamp Virus RNA Mini Kit (Qiagen, Germany). Extracted viral RNA from each collected sample was subjected to cDNA synthesis using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA). Detection of paramyxoviruses in all collected samples was based on semi-nested PCR targeting the RNA-dependent RNA polymerase (RdRp) gene (Tong et al., 2008). For the first round of semi-nested PCR for detection of paramyxoviruses in wild birds, 2 µl of each cDNA was mixed with 23 µl of 12.5 µl Green GoTaq master mix (Promega, USA), 1 µl of forward primer (PAR-F1, 10 pmol/µl) (5′- GAAGGITATTGTCAIAARNTNTGGAC-3′), 1 µl of reverse primer (PAR-R, 10 pmol/µl) (5′-GCTGAAGTTACIGGITCICCDATRTTNC-3′) and 8.5 µl of RNase free water (Promega, USA) (Tong et al., 2008). After an initial incubation at 95 °C for 1 minute, 40 cycles of amplification were carried out consisting of denaturation at 95 °C for 1 minute, annealing at 48 °C for 1 minute, extension at 72 °C for 1 minute and terminated with a final extension of 72 °C for 7 minutes. The second round of semi-nested PCR was carried out by mixing of 12.5 µl Green GoTaq master mix (Promega, USA), 10 pmol forward (PAR-F2) primer (5′-GTTGCTTCAATGGTTCARGGNGAYAA-3′), 10 pmol reverse primer (PAR-R same reverse primer as round 1) and 8.5 µl RNase free water. The thermal cycling condition for the second round was the same as the first round. PCR products were analyzed by standard agarose gel electrophoresis. The final 561 bp PCR product was gel purified by Gel extraction kit (Qiagen, Germany) then sequenced using the primers of the second round of PCR at the Macrogen sequencing facility (Macrogen, South Korea).

Newcastle disease virus isolation and amplification of partial F gene and phylogenetic analysis

All positive samples for paramyxoviruses were individually inoculated in the allantoic cavities of 11-day-old specific-pathogen-free (SPF) embryonated hens' eggs (Kom Oshem, Egypt) and incubated for three days. A volume of 50 µl of harvested allantoic fluid was individually tested for hemagglutinating activity using 0.5% chicken red blood cells. The positive samples were subjected to viral RNA extraction using QIAamp Viral Mini Kit (Qiagen, Germany) according to the manufacturer's protocol. RT-PCR amplification of partial F gene was carried out in a 25 µl which consist of 10 pmol each of the forward NDV-F primer: 5′-GGAGGATGTTGG CAGCATT-3′ and reverse NDV-F primer: 5′-GTCAACATATACACCTCATC-3′(Pang et al., 2002). PCR reaction was carried out using Phusion master mix (Thermo Scientific, USA) according to the manufacturer's instructions. The final 320 bp PCR product was gel purified by PCR purification kit (Qiagen, Germany) and sequenced with forward and reverse NDV-F primers at the Macrogen sequencing facility (Macrogen, South Korea). Sequence alignment was performed using the BioEdit 7.0 software. The phylogenetic trees of RdRp and F genes were elaborated using the MEGA7 program by applying the neighbor-joining method with Kimura's two-parameter distance model and 1000 bootstrap replicates. Also, maximum-likelihood analysis for NDVs was performed using the MEGA7 program. All

representative sequences from class I and genotypes (I to XVIII) of class II were obtained from GenBank as previously described by Dimitrov et al. (2016) and used to construct the phylogenetic trees for RdRp and F genes to identify the newly characterized NDVs in Egypt. Alignment of the deduced amino acid sequences of the F gene of NDVs isolated from wild birds in Egypt compared with representative class I and class II NDV strains was analyzed using BioEdit 7.0 software.

RESULTS AND DISCUSSION

In Egypt, Newcastle disease has been identified as a major cause of setbacks in the poultry industry. While there are no reports describing the incidence and pathotyping of NDVs in wild birds in Egypt, this study investigated the prevalence and diversity of NDVs circulating in wild birds in live bird markets in different cities of Egypt.

Out of 159 collected samples from eight different species of wild birds, 6 (3.77%) samples were tested positive for paramyxoviruses. According to the location of collection, five (4.90%) samples from Damietta, and one (4.16%) sample from Matruh were positive for paramyxoviruses. No positive sample was detected in Port Said. According to the species of wild birds from which the samples were collected, five (3.35 %) positive samples were detected from the Anseriformes (2 from pintail and 3 from northern shoveler) and one (16.66%) from Columbiformes (laughing dove). No positive sample was detected from coot, mallard, moorhen, teal, and wigeon.

Among six positive samples that were inoculated in SPF eggs, four samples were positive by hemagglutination assay. Positive samples were confirmed as NDV by RT-PCR targeting the RdRp gene. Based on the application of RT-PCR targeting the F gene for NDV isolates, the results revealed 320 bp PCR amplicons.

Huge data sets of NDVs sequences from different regions around the world were used to determine the pathotyping and origin of newly characterized viruses. Phylogenetic trees were constructed using partial sequences from the RdRp and F genes. Representative sequences from class I and genotypes (I to XVIII) of class II were used to build the general trees to identify the newly studied isolates from Egypt. Phylogenetic analysis showed that the isolated viruses during the current study were clustered and closely related to class I NDVs (Figures 1 and 2).

Following the BLAST analysis, the NDVs isolated in this study revealed about 97% similarity to the NDV strains JX07 and DE-R49/99 isolates based on the nucleotide identities.

The amino acid sequences of the F protein proteolytic cleavage site motifs (^{111}GERQER↓LVG^{119}) were identical in the entire isolated NDV strains detected in different wild birds in Egypt (Figure 3). As a result of amino acids present in the cleavage site of F protein, which considers as a fingerprint for NDV pathogenicity, all Egyptian NDV strains detected in wild birds had lentogenic motif at the cleavage site. While the cleavage sites of velogenic and mesogenic strains (R/K-R-Q-R/K-R↓F) were not detected in the obtained sequences (Collins et al., 1993; Wang et al., 2017).

These results are in agreement with several previous studies detecting lentogenic NDVs in wild birds in several countries (Huovilainen et al., 2001; Kim et al., 2007; Jindal et al., 2009). The Egyptian NDVs isolated in this study do not appear to be phylogenetically related to live vaccines used in Egypt such as Lasota, indicating that the source of these viruses is not related to the shedding of the used vaccine strains. Although class I of NDVs was detected in domestic poultry in previous studies (Fan et al., 2015; Dimitrov et al., 2016), it was not wildly circulated like class II and caused effective losses in poultry industries. A previous study detected a virulent strain of class I of NDV during an outbreak recorded in Ireland in 1990 (Alexander et al., 1992).

Further virological and epidemiological studies from a broader host range are needed to determine the pathogenicity and host range of the circulating paramyxoviruses. However, virulent and mesogenic strains of NDVs were not detected in the current study, the presence of them cannot be excluded due to the nature of migration of wild birds across continents. Thus, continuous surveillance for NDV in wild birds is essential for a better understanding of its epidemiology.

In conclusion, this study characterized NDVs circulating in wild birds in Egypt. The close phylogenetic relationship between circulating NDV strains in wild birds and previously detected viruses of class I confirms the role of wild birds in the possibility of cross-species transmission among different hosts. The findings suggest the need for continuous systematic surveillance in wild birds.

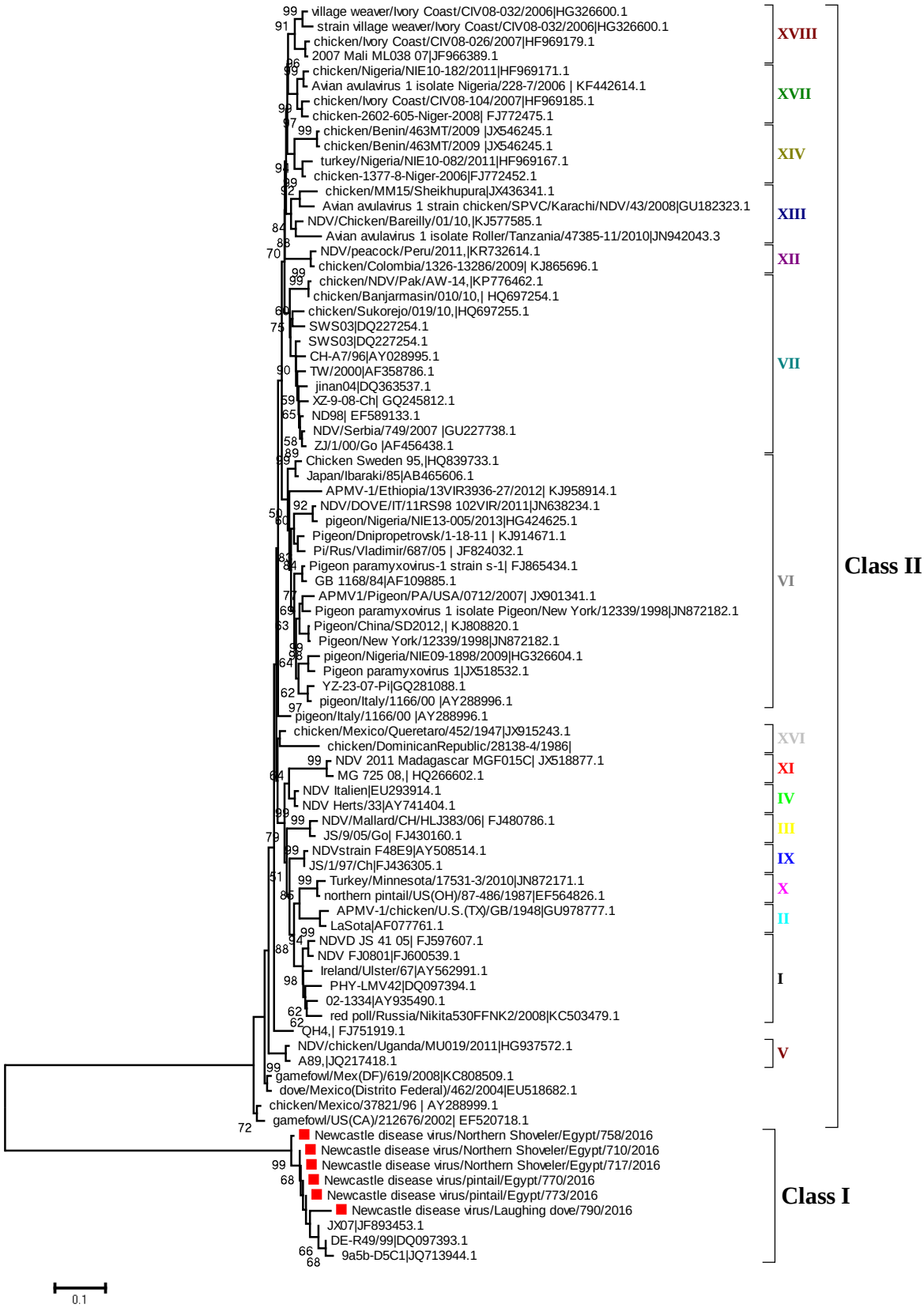

Figure 1. Neighbor-joining phylogenetic tree based on RNA-dependent RNA polymerase (RdRp) gene of Newcastle disease virus isolates from wild birds in Egypt. The wild bird isolates sequenced in the present study are marked with red squares.

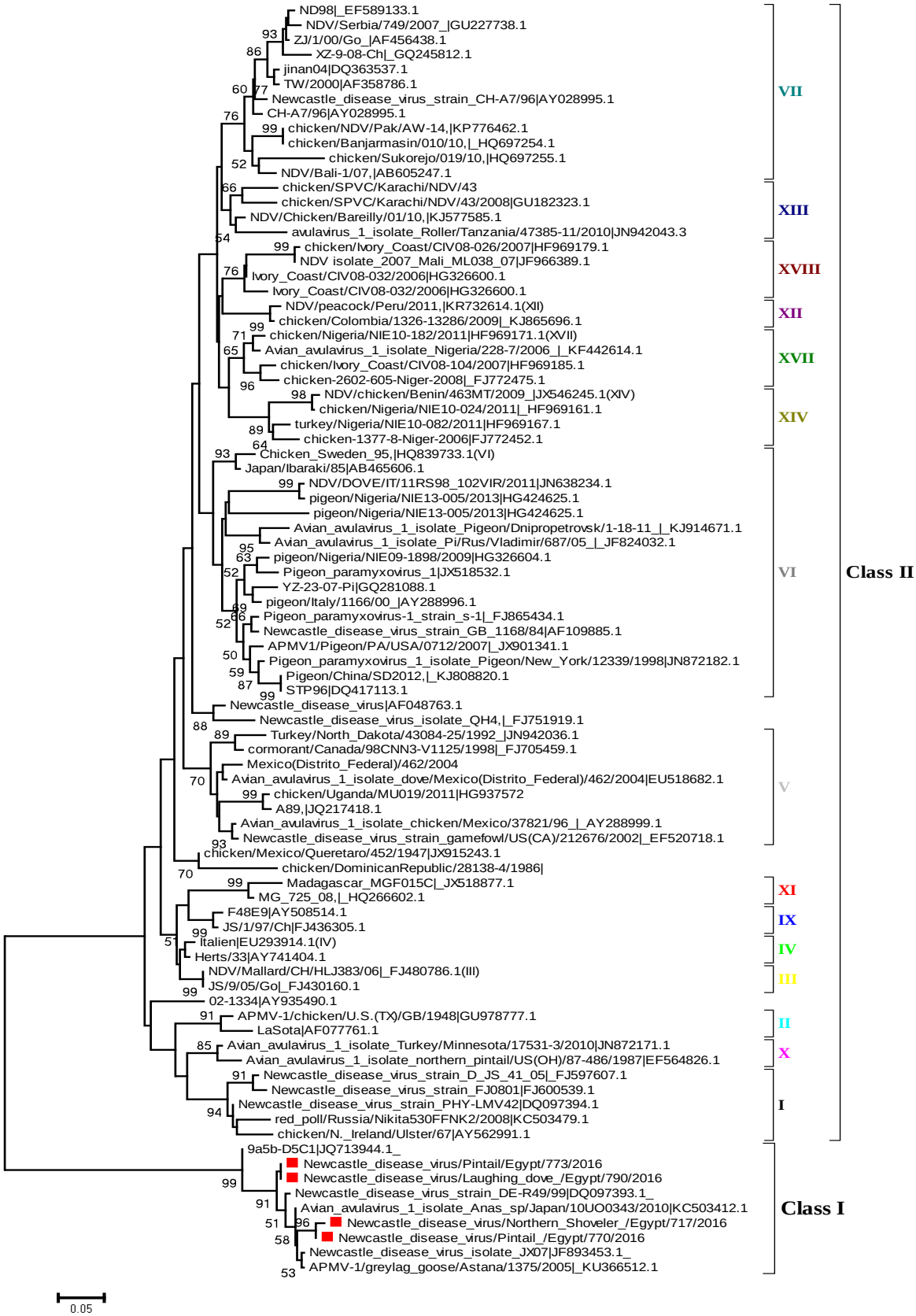

Figure 2. Neighbor-joining phylogenetic tree of Newcastle disease virus isolates based on F gene sequences. The wild bird isolates sequenced in this study are marked with red color.

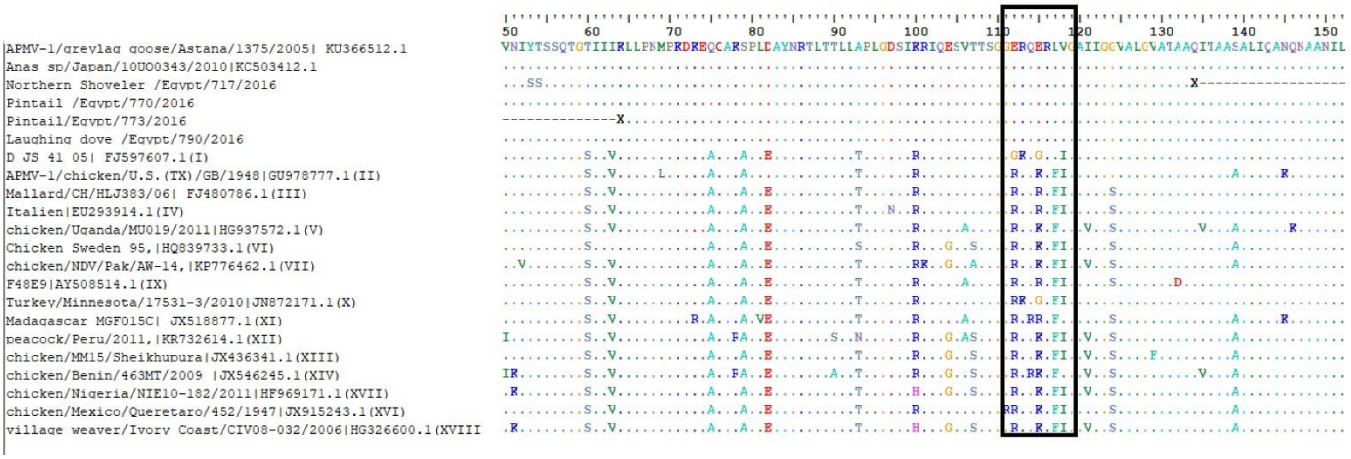

Figure 3. Alignment of the deduced amino acid sequences of the F gene of Newcastle disease viruses (NDV) isolated from wild birds in Egypt compared with class I and class II NDV strains. '.' indicates a site at which the amino acid residue is identical. The F protein cleavage site amino acid sequences of NDV isolates were labeled by a black rectangle.

DECLARATIONS

Acknowledgments

This work was funded by the Science and Technology Development Fund (STDF) in Egypt, under contract number 5175.

Authors' contributions

All authors participated in the design of the study. Mohammed Hasan did all the experimental procedures. Mohammed Hasan and Ahmed Kandeil analyzed the obtained data. All authors participated in writing and reviewing the manuscript.

Competing interests

The authors declare no conflict of interest.

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JWPR
Journal of World's Poultry Research

2020, Scienceline Publication
J. World Poult. Res. 10(3): 527-544, September 25, 2020

Research Paper, PII: S2322455X2000061-10
License: CC BY 4.0

DOI: https://dx.doi.org/10.36380/jwpr.2020.61

Effects of *Bacillus subtilis* DSM 32315 on Immunity, Nutrient Transporters and Functional Diversity of Cecal Microbiome of Broiler Chickens in *Necrotic Enteritis* Challenge

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Received: 12 Aug. 2020
Accepted: 22 Sept. 2020

ABSTRACT

This study was conducted to determine the effects of *Bacillus subtilis* DSM 32315 probiotic and antibiotic enramycin in broiler chickens with *Clostridium perfringens* induced-Necrotic enteritis on cecal microbial populations, functional diversity, nutrients transporters and cytokines mRNA expression. Day-old broilers (n= 360), Arbor Acre were randomly assigned to three dietary treatments such as control, basal diet fed-group only; antibiotic, basal diet plus enramycin 5 mg/kg; and probiotic group, basal diet plus *Bacillus subtilis* 2 x10^9 CFU/g. Antibiotic and probiotic fed groups was challenged with *Clostridium perfringens* at day1, and from day 14 to day 21. The results of present study showed that broiler chickens supplemented with antibiotic and probiotic significantly exhibited higher abundance of gut beneficial bacteria at the 21 and 35 days of age, while upregulated the expression of anti-inflammatory cytokine enterleukin-10 and secretory immunoglobulin-A. Expression of proinflammatory cytokines interleukin-6 tumor necrosis factor alpha, and interferon gamma were downregulated. Nutrient transporters of Peptide transporter-1, L amino transporter-2 and Cationic amino acid transporter-2 were upregulated in supplemented groups. More so, glucose transporter-2 Sodium glucose transporter-1, Solute carrier family 3, member 1, carbohydrates and vitamin metabolism cofactor enriched in probiotic fed-group, while control group exhibited up-regulation in interleukin-6, tumor necrosis factor alpha, and interferon gamma. Overall, supplementation of *Bacillus subtilis* DMS 32315 reduced the negative impact of necrotic enteritis in broiler chickens, and enhanced the gut-microbial community.

Keywords: Antibiotic growth promoter, *Bacillus subtilis*, *Clostridium perfringens*, Immune response, probiotic

INTRODUCTION

Antibiotic growth promoters (AGPs), are substantially used in Agriculture to improve the economic value of animals through increased growth and feed efficiency, while indirectly they are used to control some enteric diseases (Gadde et al., 2017). Mismanagement in the use of AGPs in agricultural animals leaded to drug-resistant bacteria "superbugs", drug residue and its possible negative consequences in the intestinal microbial homeostasis (Bai et al., 2017). Moreover, regulatory agencies and consumer demands on animals produced free from antibiotics mandated agricultural industry to make changes in the use of AGPs in animal production (WHO, 2017). Thus, it led to withdrawal or restrictions of antimicrobial use in poultry production in many parts of the world, this resulted to many consequences, among others are prevalence of enteritis related diseases commonly Necrotic Enteritis (NE) and widespread of ill-defined intestinal dysbacteriosis which is associated with reduced nutrients digestibility in poultry (Latorre et al., 2014). Necrotic enteritis is a serious disease in poultry caused by *Clostridium perfringens*, a gram positive, ubiquitous and anaerobic bacterium, found at a level less than 10^5 CFU/g of the intestinal contents of healthy birds, but disturbances in normal intestinal

Microflora may cause rapid proliferation of *C. perfringens*, increasing bacterial numbers to 10^7 to 10^9

To cite this paper: Bodinga BM, Hayat Kh, Liu X, Zhou J, Yang X, Ismaila A, Soomro RN, Ren Zh, Zhang W and Yang X (2020). Effects of Bacillus subtilis DSM 32315 on Immunity, Nutrient Transporters and Functional Diversity of Cecal Microbiome of Broiler Chickens in Necrotic Enteritis Challenge. *J. World Poult. Res.*, 10 (3): 527-544. DOI: https://dx.doi.org/10.36380/jwpr.2020.61

CFU/g of digesta resulting in the development of clinical NE (Opengart et al., 2013). Similarly, NE was characterized as the 'clinical and subclinical form', the subclinical form of NE does much more harm to animals than the clinical form due to its persistent in the flock without any clinical manifestation, no peak of mortality, and are associated with reduced feed intake and weight gain, and increased feed conversion ratio (Dahiya et al., 2006; Timbermont and Immerseel, 2011). Besides the health risk imposed by NE to animals, it was assessed to cost $ 6 billion loss to the poultry industry annually (Wade and Keyburn, 2015). Thus, it was imperative to explore other alternatives to AGPs that can maintain both productive potentials and gut health of the animals (Bai et al., 2017). Previous studies proved that, among the basic strategies that can be employed to cope with the loss of AGPs, and to control NE in broiler chickens, is the incorporation of probiotic into the animal's diet, which maintains animal health, growth and feed efficiency (Zhao and Kim, 2015; Musa Bodinga et al., 2019). An increase in performance of animals can be related to the changes in the microbial population dynamics of the gastrointestinal tract (GIT) of the animals creating favorable microbial environment as a result of shift in balance between beneficial and harmful microbes (Cao et al., 2012). Beneficial bacteria such as ''*Bacillus, Bifidobacterium, Enterococcus, Lactobacillus, Lactococcus,* and *Streptococcus*'' were commonly used as probiotics in animal production (Simon et al., 2001). Nevertheless, in recent time, *Bacillus* based probiotics are gaining acceptance as potential alternative to AGPs in the poultry industry. Perhaps this could be due to their distinct advantage in the production of naturally synthesized peptides, production of beneficial metabolites, modification of gut microbial structure, and stimulation of various immune modulators (Lee et al., 2010; Sumi et al., 2015). Bacillus spores were resistant to antimicrobial low pH, bile salts, and harsh conditions of the GIT. (Shivaramaiah et al., 2011). The nutrient transport system played a vital role in the small intestine of the birds, where specific nutrients were conveyed by specific transporters found at the brush border of the small intestines for absorption by enterocytes (Shivaramaiah et al., 2011). Monosaccharides (glucose, galactose, fructose and mannose) were mediated at the brush border membrane by Sodium dependent glucose and galactose transporter1 (SGLT1), and exit of glucose across the basolateral membrane was mediated by facilitated transporter Na^+ - independent glucose, galactose and fructose transporter 2 (GLUT2) the cell via glucose transporter-2 (Shivaramaiah et al., 2011). Where peptide transporter-1 was responsible for absorption of the most di and tri-peptides, and functions as obligatory exchangers of cationic amino acids, CAT1 was responsible for the efflux of cationic amino acids (Shivaramaiah et al., 2011). Similarly, the composition of gut microbiota were hypothesized to affect many host functions including nutrients' utilization of gut epithelium and development of gut immune system (Hill et al., 2010). In our previous study, it was discovered that supplementation of *Lactobacillus fermentum* 1.2029 probiotic in *C. perfringens* challenged broiler, modulated toll-like receptors and some cytokines related genes (Cao et al., 2012). Nonetheless, the immunopathology of NE in broiler chickens was not fully understood (Oh and Lillehoj, 2016), but it was believed that, NE infection caused numerous changes to immunological structure of cytokines and toll-like receptors (Collier et al., 2008). Despite the fact that, a number of probiotics which have been extensively studied here and others in the poultry industry, yet there are many probiotics without clearly defining their mode of action. Therefore, the main objective of this study, was to evaluate the effect of *Bacillus subtilis* DSM 32315 probiotic and antibiotic (enramycin) on *Clostridium perfringens*-induced Necrotic enteritis on immunomodulators, nutrient transporters, distribution of bacterial population and functional diversity in broiler chickens.

MATERIAL AND METHODS

Ethical approval

The present study was approved by the Institutional Animal ethics Committee at the Northwest Agriculture and Forestry University (protocol number NWAFAC1008 Yangling, Shaanxi, China).

Animals and Trial Design

Test strain information

Bacillus subtilis DSM 32315 used in this study was provided by Evonik Nutrition and Care GmbH, (Hanau, Germany) throughout the experimental period, the product contained a spray-dried spore forming bacteria at a concentration of 2 x 10^9 CFU/g, while AGP (Enramycin) was obtained from Wuhan Guangtu Technology Coop., Ltd. (China).

A total of 360 one-day-old Arbor Acre mixed sexed chickens were randomly assigned to three treatment groups (each one had ten cages of twelve birds). One group was supplemented with basal diet only; control (CON); The second group was antibiotic group (AB)

supplemented with basal diet supplemented with 5 mg/kg enramycin, challenged with *C. perfringens*; and the third group was probiotic group (PB) supplemented basal diet with 2× 10^9CFU/g *Bacillus subtilis* DSM 32315, challenged with *C. perfringens*. The broiler chickens were kept in a closed, ventilated and wire-floor caged broiler house (100 cm long × 80 cm wide × 50 cm height/cage). The cages had a linear feeder at the front and a nipple drinker at the back to provide feed and water *ad libitum* throughout the experimental period. Thirty-three ^{O}C room temperature was maintained for the first week, and then reduced by 3 ^{O}C per week until it reached 24 ^{O}C. Light was provided 24 hour/day.

***Clostridium perfringens* infection**

The *C. perfringens* challenge was carried out as previously described (Dahiya et al, 2007). *C. perfringens* type-A (CPA) (CVCC2030) was obtained from China Veterinary Culture Collection Center (CVCC52) of China Institute of Veterinary Drug Control (Beijing, China).The bacteria was cultured under anaerobic condition at 37 ^{O}C in peptone yeast broth overnight (Shoemaker and Pierson, 1976). The cultured medium was centrifuged at 6000 x g at 4^{o}C for 10 minutes, and re-suspended in 0.01 M Phosphate Buffered Saline (PBS). The concentration of *C. perfringens* was adjusted to 10^8 CFU/mL, and each chicken in the challenged groups was orally gavaged with 0.5 mL at day 1 of age, and 1mL from day 14 to day 21 of age, once per day, to induce the NE infection.

Tissue sample collection

At each time of sample collection, one bird/pen and ten birds/treatment were randomly selected and euthanized for the intestinal tissue sample collection, and five samples were used for testing out of the ten collection. The intestinal tissue samples were collected at day 7 and day 21 post challenge (PC) (21 and 35 days of age) for cytokines and nutrient transporters mRNA expression and cecal samples were collected at 14, 21 and 35 days of age for qRT-PCR and at 21 and 35 days of age for cecal microbiota composition. The contents of the intestine from duodenum, jejunum and ileum were separated and squeezed out, and segments were rinsed with PBS, and mucosal scrapping from each segment (duodenum, jejunum and ileum) were collected for RNA extraction for cytokines and nutrients transporter genes expression. Cecal luminal contents were also collected for qRT-PCR and microbiota composition. All the samples were collected into an aseptic 2mL tubes and frozen immediately in liquid nitrogen, later stored at −80°C for further analysis.

DNA Extraction and Cecal Bacterial Determination by Absolute qPCR

The genomic bacterial DNA was extracted using 80 ±10 mg of cecal contents using a modified Cetyltrimethylammonium Ammonium Bromide (CTAB) method as described by Minas et al. (2011). However, the total DNA quantity and quality were determined using Nanodrop® ND-2000 spectrophotometer (Thermo scientific, MA, USA), while the quality of the DNA was determined using agarose gel (1%) electrophoresis. All DNA samples were diluted to 30ng/µL and stored in -20°C thereafter. The cecal abundance of *Bifidobacterium bifidum*, Pediococcus, Enterobacter, *Lactobacillus salivarius Escherichia coli*, and *Clostridium perfringens* were determined using SYBR Green I based absolute qPCR contained the specific primers for each gene (Table 1). The qPCR plasmid standard was prepared as previously described by Liu et al. (2017). The concentration of plasmid standard of the aforementioned bacteria was diluted to 20 ng/µL, and then subjected to a serial of tenfold dilutions (10^{-1}~10^{-6}) to obtain the standard curve. The copy number of the diluted plasmid standard obtained were used and calculated as previously describe by Li et al. (2018). The abundance of the aforementioned bacteria in each sample with 30 ng metagenomic DNA where calculated with the equation: X log [(M_{DNA}/M_C) × (C30/30)×D], M_{DNA} represented the weight of total DNA in the sample (ng); M_C represented the weight of the content used (g); C30 represented the copy number of 30ng metagenomic DNA (plug Ct values into the standard curve); where D represented the dilution ratio. The copy number of each bacterial population was calculated from the standard curves, and finally the population of the bacteria was expressed as $\log_{10}$ CFU/g content.

RNA Isolation and Quantitative RT-PCR for relative expression

The total RNA and complementary DNA from the mucosal scrapping were obtained using TRIzol Reagent following manufacturer's instruction (Invitrogen, Carlsbad, CA). Quantity and purity of RNA were determined using a Nanodrop® ND-2000 spectrophotometer (Thermo scientific, MA, USA), and RNA quality was assessed by Agarose gel (1%) electrophoresis, the cDNA was synthesized from the total RNA using cDNA reverse transcription kit (TaKaRa, Dalian, China) according to manufacturer's instructions, as previously described by Li et al. (2018). A summary of the gene sequences forward and reverse primers used for PCR are shown in table 2. The average threshold cycle

values for relative quantification after normalizing with β-actin were used for each target gene using $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Metagenomic sequencing

The total DNA from the cecal samples were extracted using QIAamp DNA Stool Mini Kit (QIAFEN, Germany), according to manufacturer's instructions, and then were eluted with double-distilled water and stored at -20°C for further use. Ultrasonic genomic DNA was randomly disrupted, and purified by agarose gel electrophoresis. The sequencing linker was ligated, and the ligation product was separated by 2% agarose gel electrophoresis. The recovered product of the appropriate size was cut and amplified using qPCR and purified to obtain the final library; the DNA library was constructed and sequenced using illumina Hiseq 2500. To ensure high-quality reads, the raw image data obtained by sequencing were subjected to preliminary mass analysis, and second-generation sequencing data quality filtering were used to remove the low-quality and linker sequences (removal of adapter-containing reads; removal of N-containing reads with a ratio greater than 10%; remove low-quality reads mass value Q ≤ 20 bases account for more than 40 % of the entire read. The final reads (Last HQ Clean Reads) and high-quality sequences were used for subsequent analysis. Default selection parameter: -id 0.90, -id was similar (90% similarity). The software deduplicated all the Scaftigs assembled from each sample. Statistical summarization was carried out and the abundance table of the redundant genes was combined to perform functional abundance analysis. The putative amino acid sequence was aligned from the gene catalogue Kyoto Encyclopedia of Genes and Genomes (KEGG). The database KEGG Pathway divided the biological metabolic pathway into six categories named Metabolism, Genetic Information Processing, Environmental Information Processing, Cellular Processes, Organismal Systems, and Human Diseases; each of which was also classified into B, C, and D levels by the system. Among them, the B class used in this study currently included 43 seeds functions; the C class which was the metabolic pathway map; and the D class was the specific annotation information for each metabolic pathway map. The version of the KEGG database used in the current annotation was Released 76.0, October 1, 2015, and the comparison software used was BLAST+ (version: 2.2.29, parameter: -evalue 1e-5).

Statistical analyses

With the exception of 16S rRNA gene sequencing, all data were analyzed by one-way Analysis of Variance (ANOVA) using the General Linear Model (GLM) Procedure of Statistical Analysis System (SAS) (SAS Institute Inc. Base SAS® 9.4, 2015). Post-hoc Duncan's Multiple Range test was carried out with a P-value < 0.05 as significant. Results were expressed as mean ± Standard Error of the Mean (SEM).

Table 1. Primers for absolute qPCR analysis by 16S rRNA

Target species	Amplicon size (bp)	Sequence (5'–3')[1]	References
Bifidobacterium bifidum	290	F: CCACCGTTACACCGGGAA R: GGGTGGTAATGCCGGATG	Self-designed
Lactobacillus salivarius	108	F: CACCGCTACACATGGAG R: AGCAGTAGGGAATCTTCCA	Self -designed
Pediococcus	341	F: CACCGCTACACATGGAG R: AGCAGTAGGGAATCTTCCA	Heiling et al. (2002)
Enterobacter	198	F: ATTGACGTTACCCGCAGAAGAAGC R: CTCTACGAGACTCAAGCTTGC	Walter et al. (2001)
Escherichia coli	96	F: CGGGTAACGTCAATGAGCAAA R: CATGCCGCGTGTATGAAGAA	Self-designed
Clostridium perfringens	101	F: GCATAACGTTGAAAGATGG R: CCTTGGTAGGCCGTTACCC	Wise and Siragusa (2005)

[1]F: Forward, R: Reverse.

Table 2. Oligonucleotide primers used for quantitative RT-PCR.

Gene	Gene full name	Accession number	Primer Sequence (5'–3')[1]	References
GLUT-2	Glucose transporter-2(SLC2A1)	XM_205209.1	F: CACACTATGGGCGCATGCT R: ATTGTCCCTGGAGGTGTTGGTG	Hayat et al., 2019
SGLT-1	Sodium glucose transporter-1(SLC5A1)	XM_415247	F: AGCATTTCAGCATGGTGTGTCTTC R: GATGCTCCTATCTCAGGGCAGTTC	Hayat et al., 2019
rBAT	Solute carrier family 3, member1(SLC3A1)	XM_426125	F: CCCGCCGTTCAACAAGAG R: AATTAAATCCATCGACTCCTTTGC	Hayat et al., 2019
CAT-2	Cationic amino acid transporer-2 (SLC7A2)	EU360448	F: CAAGTCTTCTCGGCTCTAT R: GTGCCTGCCTCTTACTCA	Su et al., 2014
PepT-1	Peptide transporter1(SLC15A1)	NM_204365.1	F: GGCCCACAGTTCACCAACAG R: CAAAAGAGCAGCAGCAACGA	Su et al., 2014
Y^+LAT-2	y+ L amino acid transporter-2 (SLC7A6)	NM_001005832	F: CCCCTGAGGAGGATCACTGTT R: TTCAGTTGCATTGTGTTTTGGTT	Su et al., 2014
IL-6	Interleukin-6	AJ309540	F: GCTCGCCGGCTTCGA R: GGTAGGTCTGAAAGGCGAACAG	Rajput et al., 2017
IL-10	Interleukin-10	EF554720.1	F: GACCAGCACCAGTCATCAG R: CCGTTCTCATCCATCTTCTCG	Palamidi et al., 2016
IFN- γ	Interferon gamma	NM_205149	F: AGCTCCCGATGAACGAC R: CAGGAGGTCATAAGATGCCA	Palamidi et al., 2016
SigA	Secretary immunoglobulin A		F: GCGGCACACAATTGCACTGA R: GTTAAGGGGTAAGGTGGCCG	Self designed
TNF-α	Tumor Necrosis Factor-alpha	JN942589.1	F: GACATCCTTCAGCATCTCTTCA R: AGGCGCTGTAATCGTTGTCT	Rajput et al., 2017
B-Actin	Beta-Actin	NM_205518.1	F: ATTGTCCACCGCAAATGCTTC R: AAATAAAGCCATGCCAATCTCGTC	Musa Bodinga et al., 2019

[1]F: Forward, R: Reverse.

RESULTS

Data and sequencing

Cecal chicken samples from Control, Antibiotic and Probiotic groups were freshly collected, and sent for sequencing. The total DNA was extracted using QIAamp DNA Stool Mini Kit (QIAFEN, Germany), and was eluted with ddH_2O and stored at -20°C for further use. Ultrasonic genomic DNA in the sample was randomly disrupted, and the target fragment was recovered and purified by agarose gel electrophoresis. The ligation product was separated using 2% agarose gel electrophoresis, and the recovered product of the appropriate size was cut and amplified by PCR, and purified to obtain a final library. The constructed library was sequenced using Illumina HI sequence TM 2500. Illumina, in cooperation, San Diego, CA USA, A total of 776,294,600 higher quality classifiable reads were generated from all samples with the average of 129,328,433 sequence per sample, and a maximum number of sequences of 143,723,136, and a minimum of 122,474,100 (Table 3).

Variation of cecal bacterial microbiome

The sample richness and alpha diversity of the cecal microbiome of boiler chickens are shown in Table 4. Alpha diversity was compared among the three fed-groups at twenty first and thirty fifth days of age respectively. The results revealed that the species richness of Chao1 and Ace indices were highest in AGP and PB, and lowest in CON group at both 21_{st} and 35_{th} days of the age.

Bacterial taxonomic composition

The bacterial compositions at the phylum level are presented in figure 1a and 1b respectively. At 21_{st} day of age, Firmicutes was the dominant microbiota in all the three fed-groups, 81.3%, 80.9% and 81.2% in CON, AB and PB respectively, followed by proteobacteria, Actinobacteria as well as other unclassified group respectively with no significant variation between the groups (Figure 1a). However, at 35_{th} day of age, the dominant microbiota were likewise Firmicutes. Proteobacteria, and Actinobacteria with phylum Firmicutes were having the highest abundance in AB and PB fed-groups than in CON group, 73.3%, 76.5% and 77.3% (Figure 1b), while in addition to the three major phylum detected, 6.8% of Bacteroidetes were detected in PB fed group, nonetheless, observed decreased of 8%, 4.4% and 3.9% of Firmicutes in CON, AB and PB respectively, and increase in the relative taxa abundance of 3.1%, 2.1% and 0.5% of proteobacteria in CON, AB and PB fed-groups respectively (Figure 1b).

Three relative abundance and microbial functions

The B level of Kyoto Encyclopedia of Genes and Genomes (KEGG) and the orthologues variation for important microbial functions of the cecal microbiome of the three groups at 21_{st} and 35_{th} days of age were identified respectively (Figure 2a and 2b). Six orthologues pathways for the relative abundance of genes encoded in KEGG level B were considered such as translation, replication and repair, amino acid metabolism, carbohydrate metabolism, membrane transport, and vitamin metabolism co-factor (Figure 2a). At 21_{st} day of the age, the gut microbiota in CON group was richest in translation, replication and repair pathways, while AB fed-group showed enrichment in amino acid metabolism. It was also observed that gut microbiota was richest in pathways related to carbohydrate metabolism, and membrane transport in PB fed-group. Similarly, at 35_{th} day of age, PB fed-group maintained the highest enrichment in carbohydrate metabolism of cecal microbiota related pathways. However, membrane transport replication and repairs pathways were relatively richer in AB fed-group, while the CON group was richer in translation pathways. (Figure 2). The distribution of functional components of cecal microbiome B level of KEGG classification pathways, each data represents ten chickens for each five samples from each group (a) at 21_{st} day of age; (b) 35 days of age; 21 and 35 days of age (day 7 and 21 post challenged). The three groups were control group (CON); supplemented with basal diet only, antibiotic group (AB); basal diet supplemented with enramycin (5mg/kg) and challenged with *C. perfringens*, probiotic group (PB); basal diet supplemented with *B. subtilis* DSM 32315 ($2x10^9$CFU/g) and challenged with *C. perfringens*.

Cecal microbial populations detected by absolute qPCR

The mean $\log_{10}$ 16S rRNA gene copies/g of the cecal digesta for *Bifidobacterium bifidum, Escherichia coli, Clostridium perfringens, Enterobacter, Lactobacillus salivarius* and *Pediococcus* of broilers were detected at 14, 21 and 35 days of the age which are shown in figure 3. At 14_{th} day of the age, the bacterial abundance of *B. bifidum*, *Enterobacter*, and *L. salivarius* were significantly higher ($P<0.05$) in AB and PB, while relative abundance of *E. coli* was significantly ($P<0.05$) higher in CON group (Figure 3a). However, at 21_{st} day of the age, no significant difference ($P>0.05$) in the populations of *C. perfringens*, *B. bifidum* and Pediococcus, was detected, but the relative abundance of *Enterobacter* and *L. salivarius* were significantly higher in AB and PB group, while relative abundance of *E. coli* was significantly higher in CON and PB fed-group than in their counterpart groups (Figure 3b). Similarly, at 35_{th} day of the age, no significant difference was observed in the cecal abundance in *Pediococcus* and *C. perfringens*, whereby the abundance of *B. bifidum*, *Enterobacter* and *L. salivarius* were significantly higher in AB and PB than in CON fed-group.

Sugars, amino acids and peptides transporters mRNA genes expression

The mRNA expression levels of nutrient transporters (Monosaccharides) such as Glucose transporter- 2 (GLUT-2), Sodium glucose transporter-1 (SGLT-1) and (Solute carrier family 3, member-1 (rBAT) of the intestinal mucosa from duodenum, jejunum and ileum at 21_{st} and 35_{th} days of the age are presented in figure 4. In the duodenum, at 21_{st} day of age, mRNA expressions of GLUT-2 were significantly ($P < 0.05$) higher in PB and AB, while SGLT-1 and rBAT significantly showed high expression in PB than in CON and AB fed-groups (Figure 4a). Similarly, at 35_{th} day of age, PB fed-group showed significantly ($P < 0.05$) higher expression of GLUT-2 and SGLT-1, whereas, rBAT was significantly higher in AB and PB than in CON fed-group (Figure 4b). Likewise, in jejunum, AB and PB showed high expression of GLUT-2 and rBAT, while, at 35_{th} day of age, SGLT-1 and GLUT-2 significantly showed down regulation with the highest expression in PB than in CON and AB fed-groups (Figure 4d). However, in ileum, at 21_{st} and 35_{th} days of age, GLUT-2 and rBAT were significantly ($P<0.05$) expressed in PB than in AB and CON group (Figure 4e).

The amino acids and peptides mRNA genes expression

The amino acid and peptide transporters; Oligopeptide tansporter-1(PepT-1), L amino acid transporter-2 (LAT-2) and Cationic amino acid transporter-2 (CAT-2*)* from duodenum, jejunum and ileum at 21_{st} and 35_{th} days of age are presented in figure 5. Results showed the expression of LAT-2 at 21_{st} and 35_{th} days of age in duodenum, and CAT-2 at 35_{th} days was significantly ($P < 0.05$) higher in AB and PB fed-groups. Also, PB showed significantly ($P<0.05$) higher expression of PepT-1 than in AB, while CON group shows the lowest expression (Figure 5a and 5b). In jejunum, at 21_{st} day of age, groups fed AB and PB significantly ($P<0.05$) showed a higher expression of PepT-1 and LAT-2, while CAT-2 at 21_{st} days of age and PepT-1 at 35_{th} day of age, were significantly ($P < 0.05$) higher in AB than in PB fed-group, with the lowest expression in CON group (Figure

5c). Similarly, expression of CAT-2 was significantly ($P < 0.05$) higher in AB and PB than in CON group (Figure 5d). In Ileum at 21_{st} day of age, only LAT-2 showed substantial difference with AB fed-group having the highest expression (figure 5e), On the other hand, at 35_{th} day of age, LAT-2 and CAT-2 showed significantly ($P < 0.05$) higher expression in AB and PB than in the CON group, while expression of PepT-1 was meaningfully ($P< 0.05$) higher in PB than AB with the lowest expression in CON group (Figure 5f).

The Cytokines mRNA genes Expression

There were also some changes in mRNA expression of proinflammatory cytokines of interleukin-6, tumor necrosis factor alpha and interferon gamma from duodenum, jejunum and ileum of the three groups at 21_{st} and 35_{th} days of the age (Figure 6). In duodenum at 21_{st} day of age (figure 6a), expression of IL-6, TNF-α and IFN-γ, and at 35_{th} day of age (figure 6b), IL-6 and TNF-α were significantly higher compared to AB and PB challenged groups. Also, in jejunum, at 21_{st} day of age (figure 6c), IL-6, TNF-α, IFN-γ and at 35_{th} day of age (figure 6d), TNF-α and IFN-γ were significantly ($P < 0.05$) higher in CON than in AB and PB. Similar trends were also observed in ileum at 21_{st} day of age (figure 6e), and 35_{th} day of age (Figure 6f). The mRNA expression level of anti-inflammatory cytokine of IL-10, and mucosal secretory immunoglobulin A at 21_{st} and 35_{th} days of the age were measured. At 21_{st} day of age, AB and PB fed-group considerably ($P < 0.05$) indicated higher expression of SigA than in CON group (figure 7a), while at 35_{th} day of age, PB fed-group showed significantly ($P < 0.05$) higher expression of IL-10, and SigA than in AB fed-group, and the lowest expression was in CON group (Figure 7b). In jejunum, at 21_{st} day of age, SigA was upregulated in AB than in PB and CON fed-group, whereas expression of IL-10 was meaningfully ($P < 0.05$) upregulated in AB and PB than in CON fed-group (Figure 7c). However, at 35_{th} day of age, IL-10 was upregulated in PB than in CON, while SigA was upregulated in AB than in CON group (Figure 7d). Similarly, in ileum at 21_{st} day of age, AB and PB showed notably ($P < 0.05$) high expression of IL-10 and SigA than in CON (Figure 7e). Similarly, at 35_{th} day of age, expression of SigA in PB fed-group was substantially ($P <0.05$) up-regulated than in AB and CON fed-groups. Nonetheless, the changes in mRNA expression of proinflammatory cytokines of IL-10 in AB and PB were up-regulated than in CON group (Figure 7f).

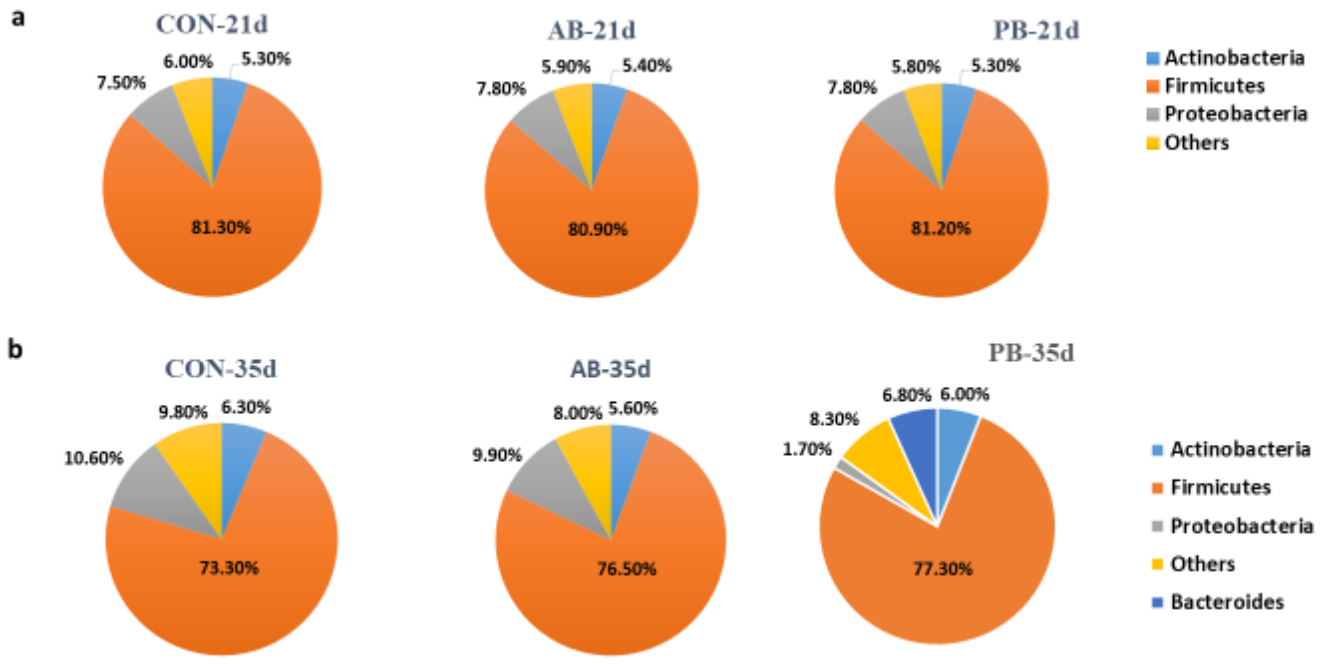

Figure 1. Cecal bacterial composition of broiler chickens. Relative taxa abundance (%), **a:** at 21 days of age and; **b:** at 35 days of age; 21 and 35 days of age means (day 7 and 21 post challenged respectively). The three groups contained: CON: control group supplemented with basal diet only; AB: antibiotic group (enramycin 5mg/kg) challenged with *Clostridium perfringens;* PB: probiotic group contained *Bacillus subtilis* ($2x10^9$ CFU/g) challenged with *Clostridium perfringens*

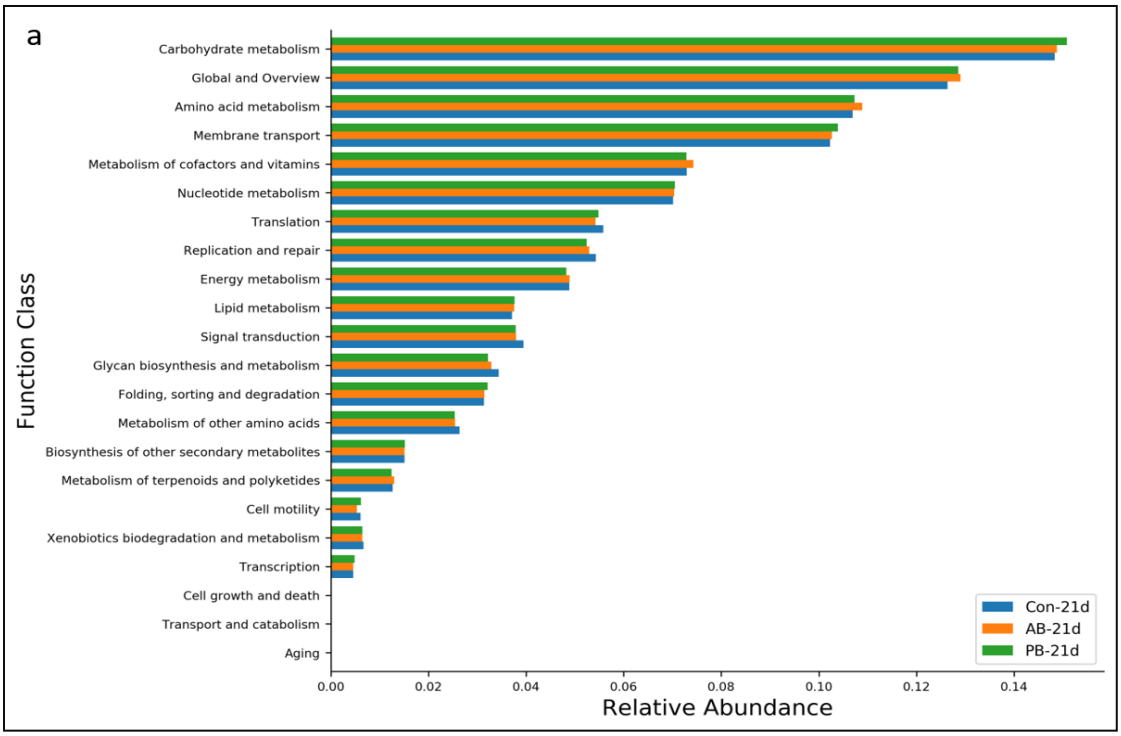

Figure 2a. Distribution of functional components of cecal microbiome B level of Kyoto Encyclopedia of Genes and Genomes (KEGG) classification pathways. Each data represents ten chickens for each five samples from each group; at 21 days of age means (day 7 post challenged). The three groups contained CON: control group supplemented with basal diet only; antibiotic group (AB): basal diet supplemented with (enramycin 5mg/kg) challenged with *Clostridium perfringens*; probiotic group (PB): basal diet supplemented with *Bacillus subtilis* DSM 32315 ($2x10^9$ CFU/g) challenged with *Clostridium perfringens*

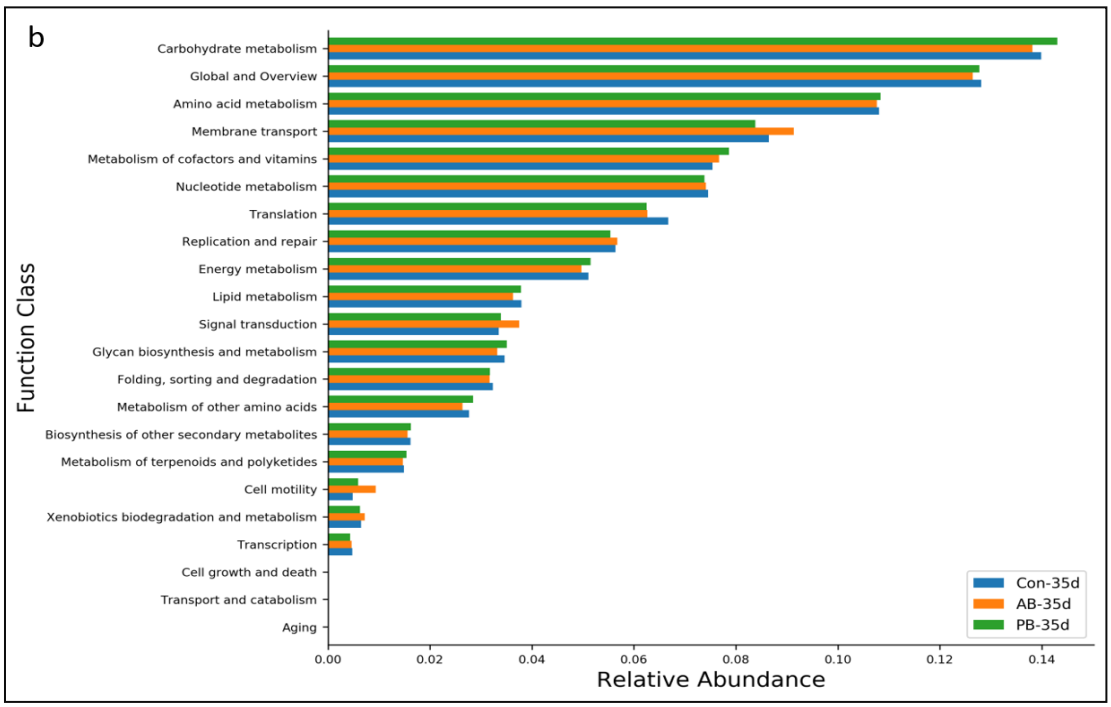

Figure 2b. Distribution of functional components of cecal microbiome B level of Kyoto Encyclopedia of Genes and Genomes (KEGG) classification pathways. Each data represents ten chickens for each five samples from each group; at 35 days of age means (day 21 post challenged). The three groups contained CON: control group supplemented with basal diet only; antibiotic group (AB): basal diet supplemented with (enramycin 5mg/kg) challenged with *Clostridium perfringens*; probiotic group (PB): basal diet supplemented with *Bacillus subtilis* DSM 32315 ($2x10^9$ CFU/g) challenged with *Clostridium perfringens*

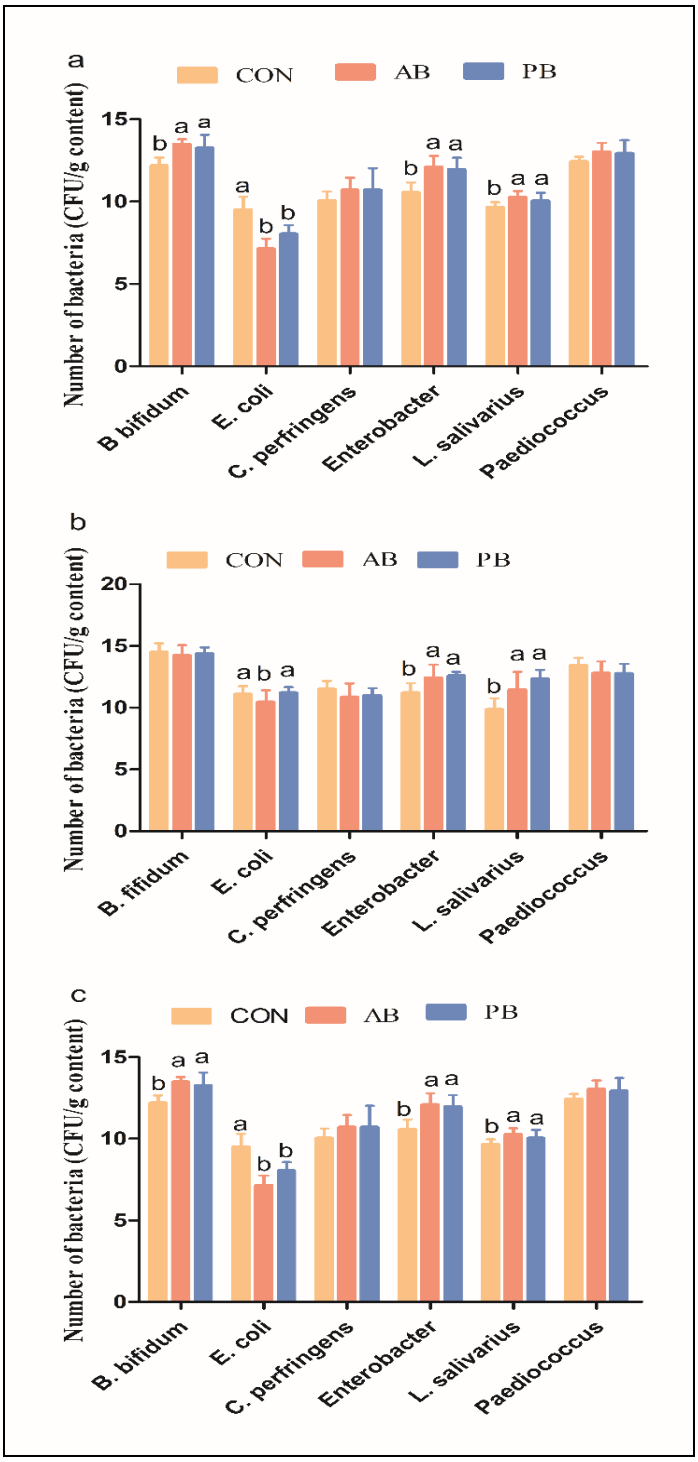

Figure 3. Composition of cecal microbiome number of 16S rRNA gene copies broilers from three groups (a); at 14 days of age; (b); at 21 days of age (c); at 35 days of age**;** 14 days of age (before challenge); 21 days of age (7 days after challenged); and 35 days of age (21 days after challenged). The three groups are CON: control group supplemented with basal diet only; antibiotic group (AB): basal diet supplemented with; (enramycin 5mg/kg) challenged with *Clostridium perfringens;* probiotics group (PB): basal diet supplemented with *Bacillus subtilis* ($2x10^9$ CFU/g) challenged with *Clostridium perfringens*, each data represents five replicates intestinal cecal samples for each group. The error bars indicate the standard error of the mean. Data was presented as log10CFU/g cecal content. a-c Means with different letters are significantly different ($P < 0.05$).

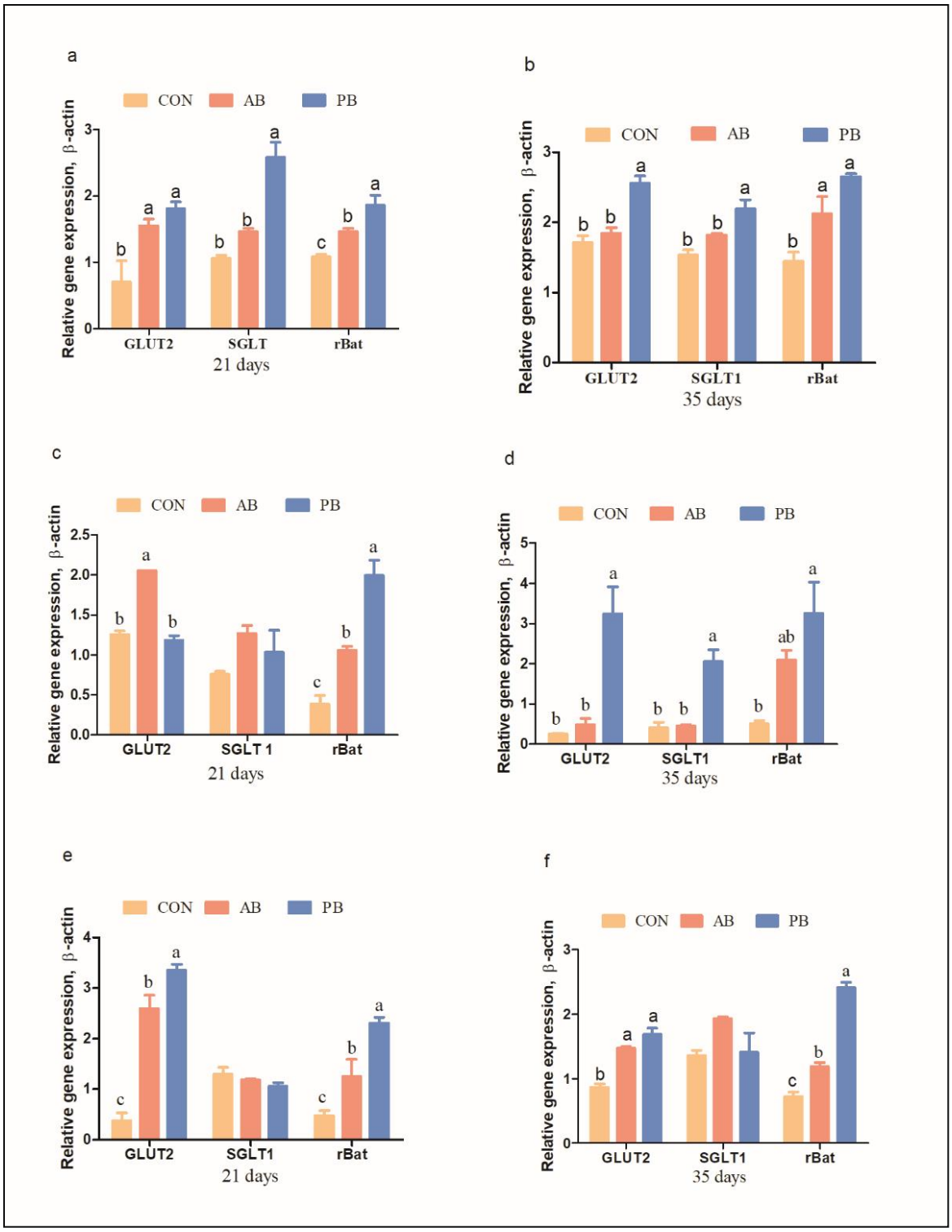

Figure 4. Relative mRNA expression levels of sugars transporters-related genes of the intestinal mucosa of broiler from duodenum, (Figure 4a and 4b) jejunum (Figure 4b and 4c) and ileum (Figure 4E and 4f) at 21 and 35 days of age, 21 and 35 days of age means (d 7 and d 21 post challenged). The three groups contained CON: control group supplemented with basal diet only; antibiotic group (AB): basal diet supplemented with; (enramycin 5mg/kg) challenged with *Clostridium perfringens;* probiotic group (PB): basal diet supplemented with *Bacillus subtilis* ($2x10^9$CFU/g) challenged with *Clostridium perfringens*, GLUT-2= glucose transporter-2, SGLT1= sodium glucose transporter-1, rBAT = Solute carrier family 3, member1 Data are the means ± SEM of five chicks in each group. a-c Means with different letters are significantly different (P <0.05)

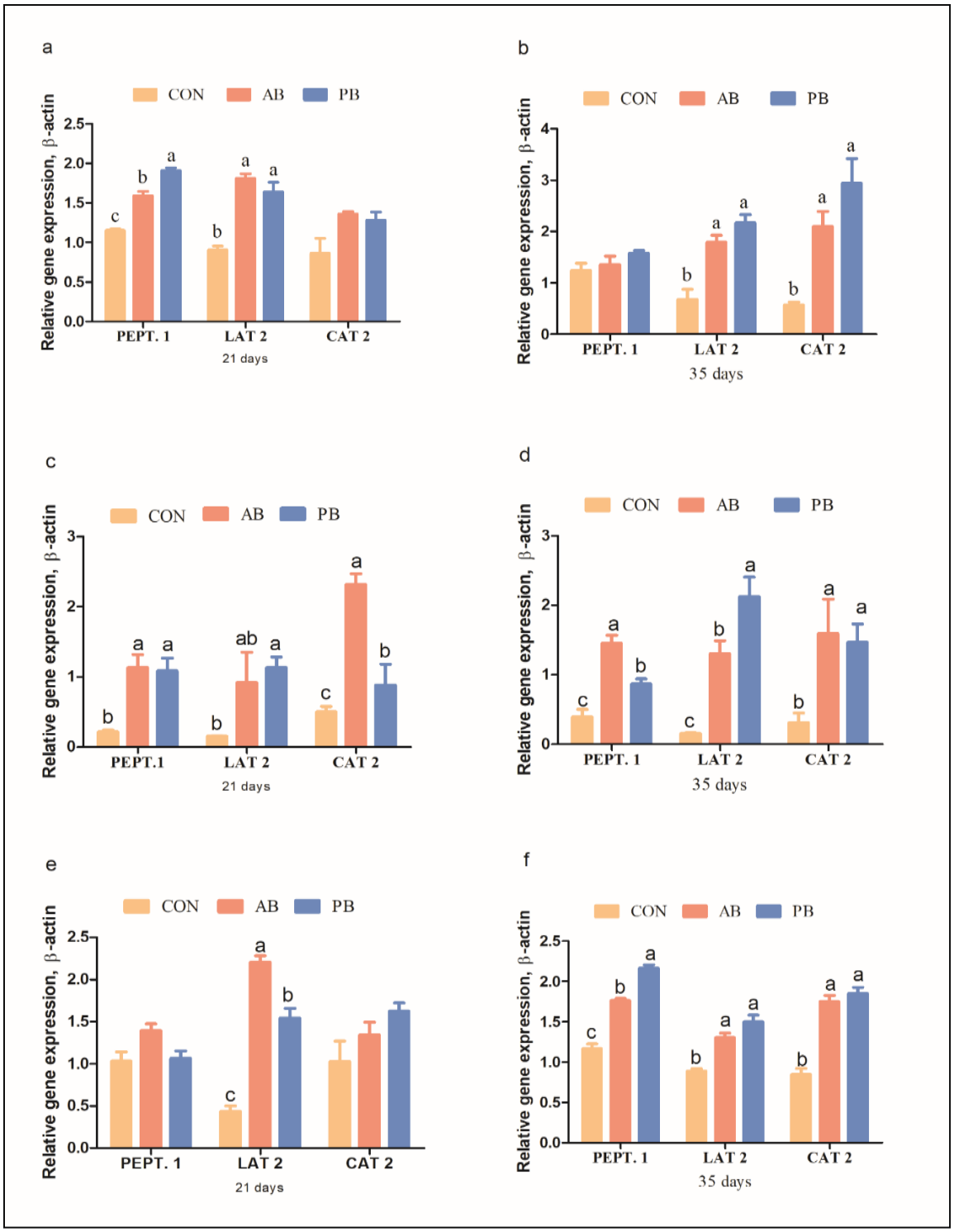

Figure 5. Relative mRNA expression levels of amino acid and peptide transporter related-genes of the intestinal mucosa of broiler from duodenum (Figure 5a and 5b), jejunum (Figure 5c and 5d) and ileum (Figure 5e and 5f) at 21 and 35 days of age, 21 and 35 days of age means (d 7 and d 21 post challenged). The three groups contained CON: control group supplemented with basal diet only; antibiotic group (AB): basal diet supplemented with; (enramycin 5mg/kg) challenged with *Clostridium perfringens;* probiotics group (PB): basal diet supplemented with *Bacillus subtilis* ($2x10^9$ CFU/g) challenged with *Clostridium perfringens*, CAT-2= cationic amino acids transporter-2; PepT-1= peptide transporter-1, Y^+LAT-2 =amino acid transporter-2. Data are the means ± SEM of five chicks in each group. a-c Means with different letters are significantly different ($P < 0.05$).

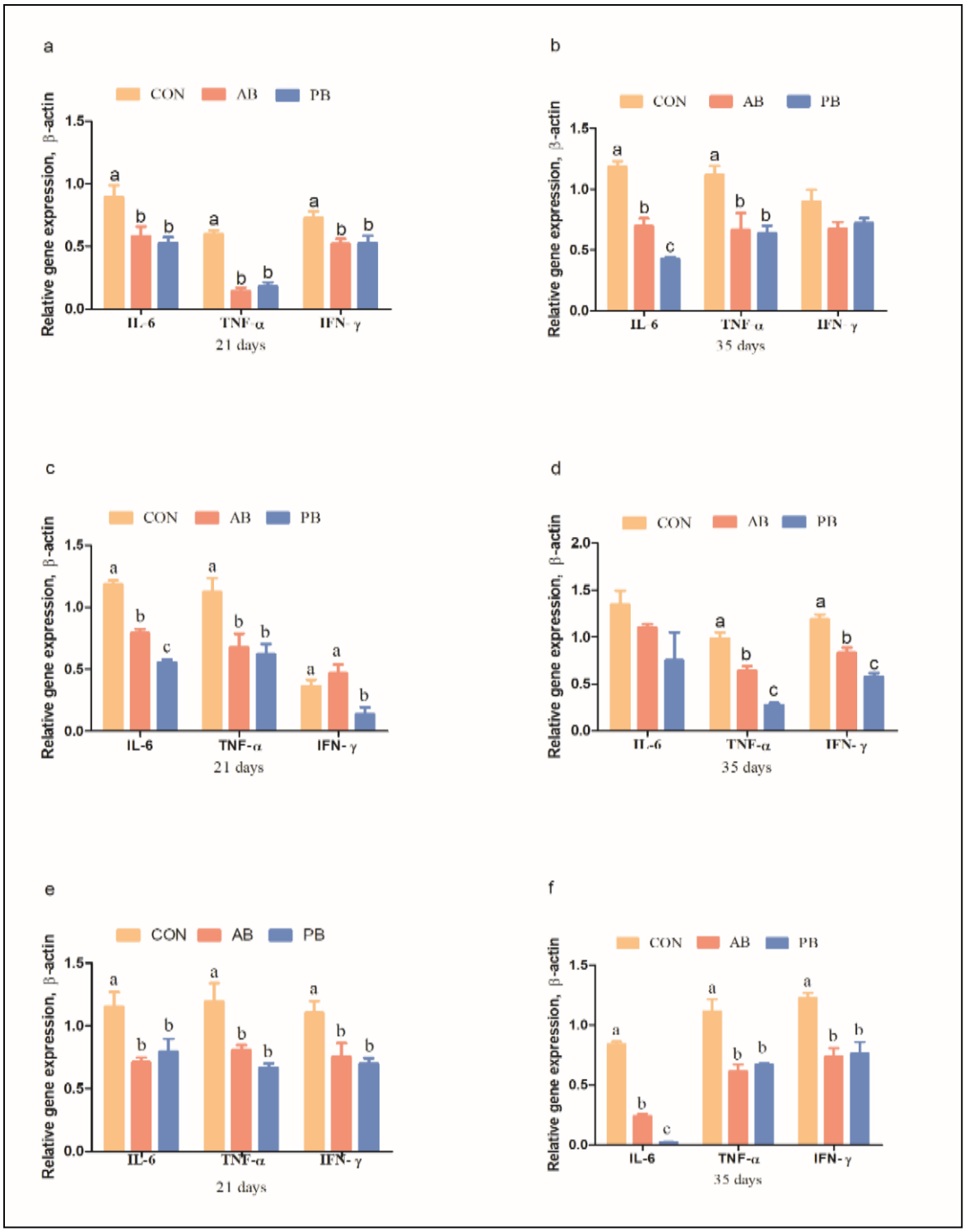

Figure 6. Relative mRNA expression levels of cytokine-related-genes of the intestinal mucosa of broiler from duodenum (Figure 6a and 6b), jejunum (Figure 6c and 6d) and ileum (figure 6e and 8f) at 21 and 35 days of age, 21 and 35 days of age means (d 7 and d 21 post challenged). The three groups are contained CON: control group supplemented with basal diet only; antibiotic group (AB): basal diet supplemented with; (enramycin 5mg/kg) challenged with *Clostridium perfringens;* probiotic group (PB): basal diet supplemented with *Bacillus subtilis* ($2x10^9$ CFU/g) challenged with *Clostridium perfringens*, IL-6= interleukin 6; TNF-α=Tumor necrotic factor alpha; IFN-γ = Interferon gamma. Data are the means ± SEM of five chicks in each group. a-c Means with different letters are significantly different (P <0.05).

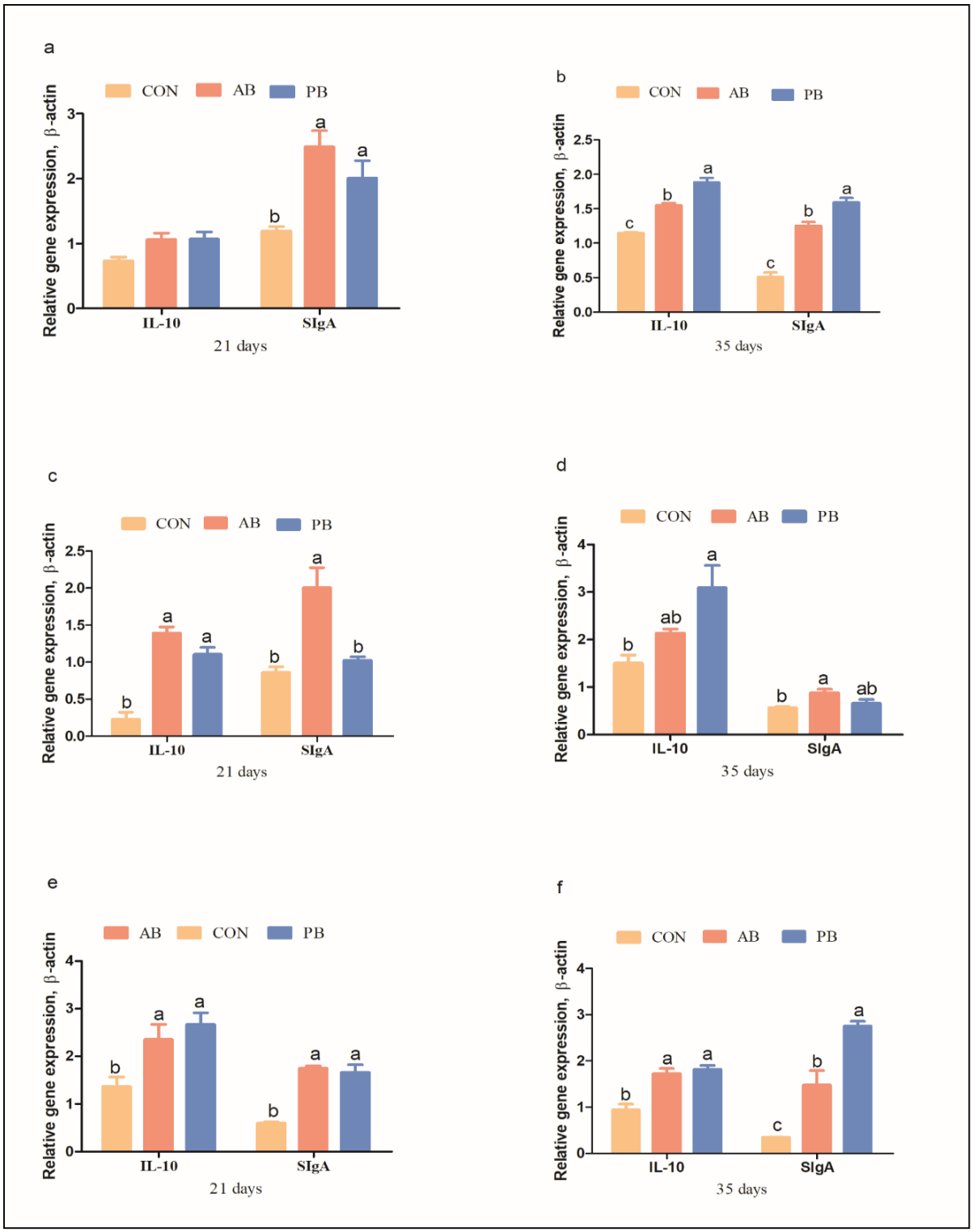

Figure 7. Relative mRNA expression levels of cytokine-related-genes in the intestinal mucosa of broiler from duodenum, (Figure 7a and 7b) jejunum (Figure 7c and 7d) and ileum (Figure 7e and 7f) at 21 and 35 days of age,21 and 35 days of age means (d 7 and d 21 post challenged). The three groups contained; CON: control group supplemented with basal diet only; antibiotic group (AB): basal diet supplemented with (enramycin 5 mg/kg) challenged with *Clostridium perfringens*; probiotic group: (PB): basal diet supplemented with *Bacillus subtilis* ($2x10^9$CFU/g) challenged with *Clostridium perfringens*, IL-10= Interleukin 10; SigA= Secretory immunoglobulin A. Data are the means ± SEM of five chicks in each group. a-c Means with different letters are significantly different (P <0.05).

Table 3. Metagenome Illumina HI Sequencing raw and validated data statistics

Groups	Total length (read)	Total base (bp)	Q20Base (%)	Q30 Base (%)
Control 21d (Raw data)	143723136	21558470400	95.82	90.84
(Validated data)	137267732	20095347393	97.90	93.80
Antibiotic 21d (Raw data)	124568192	18685228800	96.24	91.38
(Validated data)	120150374	17579482737	97.94	93.89
Probiotic 21d (Raw data)	126434430	18965164500	96.12	91.14
(Validated data)	121778776	17822523973	97.87	93.71
Control 35d (Raw data)	133089518	19963427700	96.21	91.18
(Validated data)	128706256	18818324785	97.82	93.60
Antibiotic 35d (Raw data)	126005224	18900783600	96.34	91.85
(Validated data)	120660598	17707723742	98.19	94.49
Probiotic 35d (Raw data)	122474100	18371115000	96.90	92.65
(Validated data)	118742952	17442437811	98.27	94.68

Table 4. Alpha diversity indices of the cecal microbiome of broiler chickens

Groups	Number of reads	Chao1	Ace	Observed species	Shannon	Simpson
CON (21d)	54981879	3837.72	3798.27	3715	4.252	0.86
AB (21d)	63691912	3954.03	3933.65	3880	4.189	0.85
PB (21d)	58508325	3922.09	3898.63	3844	4.205	0.84
CON (35d)	52962927	3994.97	3979.22	3917	4.304	0.83
AB (35d)	63280909	4065.55	4059.32	4002	4.455	0.85
PB (35d)	56346246	4060.63	4026.71	3947	4.309	0.84

CON: control group supplemented with basal diet only; AB: antibiotic group supplemented with basal diet and enramycin (5mg/kg) challenged with *Clostridium perfringens*; PB: probiotics group supplemented with basal diet and *Bacillus subtilis* ($2x10^9$CFU/g) challenged with *Clostridium perfringens*

DISCUSSION

Previous studies showed that pathogenic strains of *C. perfringens* can cause NE that disrupts, and lead to significant shift in the normal structure of gut microbiome in broiler chickens (Stanley et al., 2012; Mountzouris et al., 2015). Similarly, it was revealed that gut microbiome can provide the host with nutrients exchange, modulation of immune system and exclusion of pathogens, which had great impact on metabolic reactions (Binek et al., 2012). It was also revealed that, inclusion of *B. subtilis* strains probiotics in broiler diet could improve gut health, in the production of antimicrobial peptides and alteration of gut microbiome (Knap et al., 2011; Lee et al., 2011). Likewise, the use of in feed antibiotics were believed effective in mediating and decreasing the number of gut pathogenic bacteria in animals and humans (Dibner and Richards, 2005). Infeed antibiotics also proved an effective way to prevent animals from diseases, enhance feed efficiency, and to improve animal performance (Hooge et al., 2003; Kamran et al., 2013). Various species of Bacillus genus were known in producing bacteriocins and antimicrobial peptides (Lee et al., 2011; Cochrane and Vederas, 2014; Park and Kim, 2014). *B. subtilis* SP6 showed an anti *C. perfringens* effect *in vitro* (Teo and Tan, 2005). The results of present study demonstrated that supplementation of *B. subtilis* DSM 32315 at $2x10^9$ and antibiotic, enramycin at the dose of 5 mg/kg relatively altered the gut microbial diversity and functions of broiler chickens at 21_{st} and 35_{th} days of age as compared to the control group. This could be as a result of the early and continuous supplementation of *B. subtilis* probiotic from 1 to 42 days of age for the feeding period, which could probably mediate the beneficial effect of the gut microbiota through competitive exclusion (CE) in chickens by colonization and modulation of pathogens, while restoring the normalcy and integrity of gut ecosystem as well as the effect of the antibiotic administered to the chicken. Previous studies confirmed that several strains of *B. subtilis* have antagonistic activities against *C. perfringens in vitro* (Martaet al., 2014; Cochrane and Vederas, 2016). Similarly, the results of this

study disclosed that AB and PB challenged groups had the highest percentage of population of dominant taxa such as *Firmicutes*, *Proteobacteria* and other bacterial groups at 35 days of age. Likewise, we observed a unique group of "*Bacteroidetes*" (6.8%) in PB fed group, the dominant cecal microbiome composition obtained in this study was contrary to study of Xiao et al. (2017), that reported *Bacteroidetes* as the dominant bacterial group which could be to the fact that intestinal microbiome composition could be affected by many factors like surrounding environment, dietary supplementation, and pathological condition (Xiao et al., 2017). *Firmicutes* and *Bacteroidetes* were important gut microbiota in broiler that function in energy production and metabolism, specifically in microbial fermentation and starch digestion (Shaufi et al., 2015). Bacteroidetes primarily functioned in the formation of potential toxins via putrefaction that leads to increase pH of the intestinal contents which is of beneficial to the gut health against acid-sensitive pathogens (Apajalahti, 2005). Other species such as, *B. salanitronis*an which are inhabitant of chickens' cecum was well known for its ability in aiding further digestion of food by making the nutrients available to the host (Gronow et al., 2011). Also, with regards to 16S rRNA sequences, *B. bifidum*, *E. coli*, *C. perfringens*, *L. salivarius* Enterobacter, and Pediococcus, the cecal contents, revealed significantly ($P < 0.05$) higher relative abundance in AB and PB fed groups, with suggestively higher population of *E. coli* in CON group without any supplementation. The higher relative abundance of *L. salivarius* and *B. bifidum* in PB fed-group could be attributed to their tolerance against *C. perfringens* infection. Alike results were found by Pyoung et al. (2007) who isolated several *Lactobacillus* and *Bifidobacterium* from pig gut that had antagonistic action against *C. perfringens*. Teo and Tan (2007) reported higher amount of *Lactobacillus* and *Bifidobacterium* from ileum of chickens supplemented with *B. subtilis* PB6. Similarly, Gilbert et al. (2008) observed a substantial increase in *Lactobacillu*s abundance in the ilea and cecum of chickens following by *B. subtilis* C-3104 supplementation. Stanley et al. (2014) and Jeong and Kim (2015) reported that genus *Lactobacillus* in chickens' gut was predominant, and did not disturb the following *C. perfringens* infection. However, *Lactobacillus* was associated with reduction of intestinal inflammation and enhanced immunological barrier functions in broilers (Chen et al., 2012). Nevertheless, in this study, statistically no significant difference in the cecal population of *C. perfringens* either in supplemented groups or in the CON group fed basal diet only. This could signify that supplementation of either AB or PB directly or indirectly minimizes the proliferation of gut pathogens. More so, corresponding results were also obtained by Barbosa et al. (2005) and Teo and Tan (2005) who recognized *B. subtilis* and *B. licheniformis* as antagonistic species against *C. perfringens* infections. Likewise, in the present study, six enriched KEGG orthologues markers related to replication and repair, translation, amino acids metabolism, vitamin metabolism cofactor, membrane transport, and carbohydrate metabolism were considered. The CON group indicated enrichment in translation related pathways compared to AB and PB challenged groups. This could be attributed to the fact that AB and PB fed groups were challenged with *C. perfringens* which meant to induce NE, whereas, the subclinical form of the disease is characterized by necrosis, inflammation, pseudo-membrane and mucosal hemorrhages (Wielen, 2000). Increase in carbohydrates metabolism related pathways in PB fed-group could be attributed to the high density of the commensals bacteria in the gut, in the sense that, many gut bacteria can additionally hydrolyze indigestible carbohydrate of polysaccharides, oligosaccharides and disaccharides to their compositional sugars, which later be fermented by gut bacteria, producing short chain fatty acids which can be utilized as energy source by the host (Hooper et al., 2002; Tellez et al., 2006). The high relative abundance of *Lactobacillus spp*. and *Bifidobacterium* from both AB and PB fed-groups could influence carbohydrate metabolism, and release energy into the host.

CONCLUSION

In conclusion, *C. perfringens* challenged broilers, with supplementation of AGP enramycin at the dose of 5 mg/kg or probiotic *B. subtilis* DSM 32315 at $2x10^9$ CFU/kg, during broiler production period of 1 to 42 days of age increased the cecal relative abundance of beneficial microbiome, upregulated the expression of some anti-inflammatory cytokines, and downregulated the expression of some proinflammatory cytokines. Thus, it can cause the reduction of gut pathogens. Similarly, the amino acids and peptides transporter genes expressions were also enhanced in both antibiotic and probiotic fed-groups when compared with the control group. In addition, sugar transporter related genes were upregulated by *B. subtilis* probiotic fed-group compared to antibiotic and control group. The overall results clearly confirmed the potential of *B. subtilis* DSM 32315 probiotic against *C. perfringens* bacteria causing necrotic enteritis infection,

and thus it could be used as an alternative to AGPs from day one to day 42 of production period in broiler chickens.

DECLARATIONS

Consent to publish

All authors have approved to publish the work

Competing interests

The authors declare that they have no conflict of interests.

Author`s contributions

The conceptualization was done by Bello M. Bodinga and XiaoJun Yang. The methodology was designed by Rab N. Soomro, Khawar Hayat, JinHui Zhou and Xinshuai Liu. Validation and formal analysis were performed by Abdullahi Ismaila and Zhouzheng Ren.

Acknowledgments

The authors appreciate the financial support provided by Evonik Nutrition and Care GmbH, (Hanau, Germany). This work was supported by the National Key Research and Development Program of China (2017YFD0500500, 2017YFD0502200) and the Program for Shaanxi Science &Technology (2017TSCXLNY-04-04, 2018ZDCXL-NY-0201,2018ZDXM-NY-051) and the Program for Yangling Agricultural High-tech Industries Demonstration Zone (2018CXY-10).

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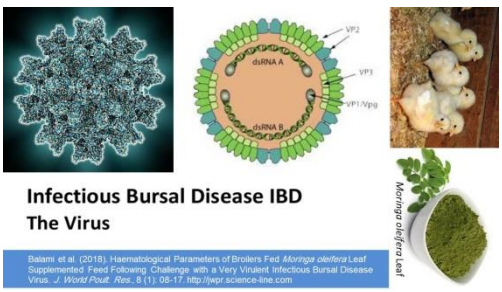

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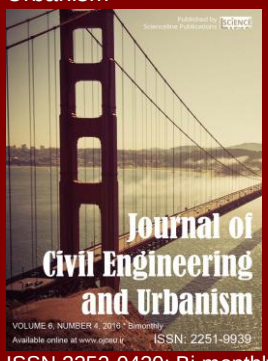

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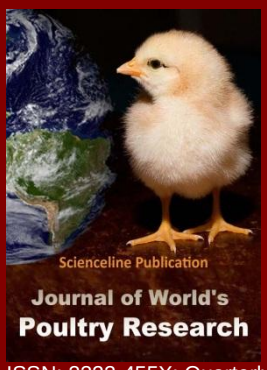

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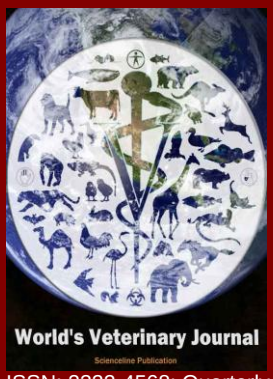

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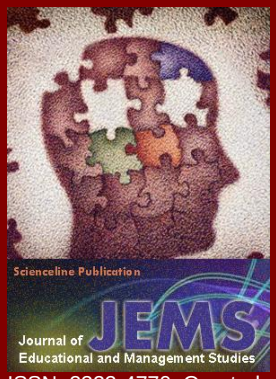

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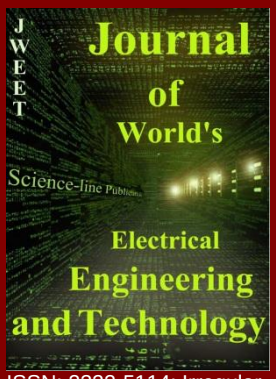

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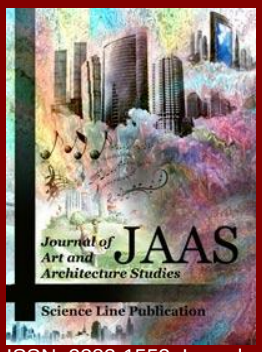

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