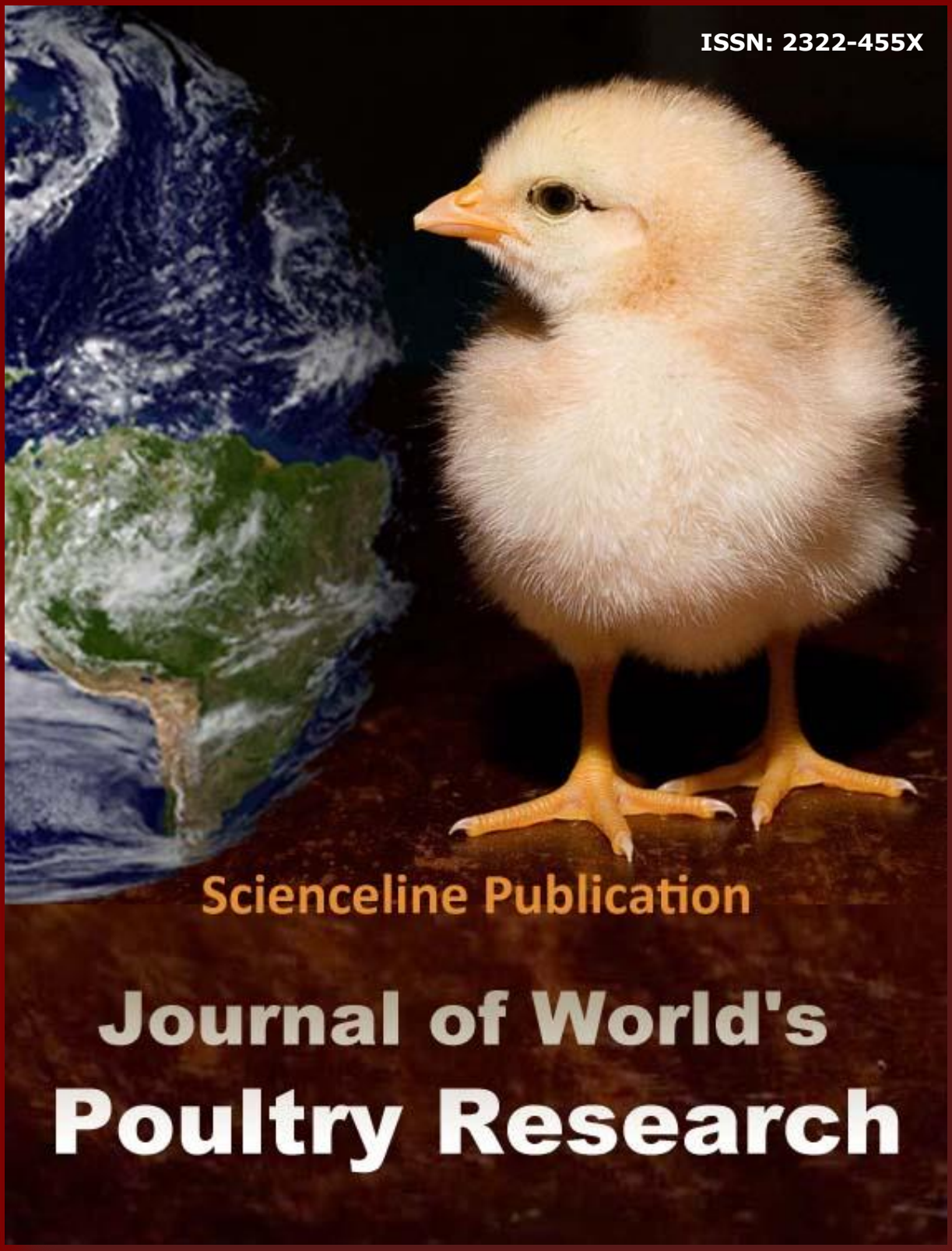
In the Name of God



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Volume 10 (2); June 25, 2020

Research Paper

Functions of *Epimedium* on Regressed Oviduct and Follicles of Force Molted Layer Hens.

Guo Y, Huo Sh , Li Y, Zhang Sh, Wu X, Jiang L, Zhao Q and Xue W.

Guo Y, Huo Sh , Li Y, Zhang Sh, Wu X, Jiang L, Zhao Q and Xue W (2020).
Functions of *Epimedium* on Regressed Oviduct and Follicles of Force Molted Layer Hens.
J. World Poult. Res., 10 (2): 326-335. DOI: https://dx.doi.org/10.36380/jwpr.2020.37

J. World Poult. Res. 10(2): 326-335, 2020; pii: S2322455X2000037-10
DOI: https://dx.doi.org/10.36380/jwpr.2020.37

ABSTRACT: In order to reveal the functions of *Epimedium* (EPI) on the recovery of the reproductive system of laying hens, 120 Hy-Line Brown laying hens (43-week-old) were forced molt through feed withdrawal for 14 days. After the molting period, layer hens were divided into four groups: Dark-control group in a dark environment without any treatment; dark-EPI group in a dark environment and with EPI treatment; light-control group without any treatment under normal illumination (16L: 8D); light- EPI group with EPI treatment under normal illumination (16L: 8D). EPI treatment was maintained for 15 days and each hen received 2 ml of EPI extract (1 g/mL) daily. The rate of egg production was calculated every day. At the end of the experiment, estrogen receptor alpha mRNA, estrogen receptor beta mRNA, and progesterone receptor mRNA in the albumen secreting part and uterus of the oviduct were detected by q-PCR, and the level of serum progesterone, estrogen and luteinizing hormone was measured by enzyme-linked immunosorbent assay. The results showed that EPI effectively improved the laying rate of hens both in dark groups and light groups by promoting the recovery of the oviduct and follicle maturation. In addition, EPI promoted the secretion of estrogen and progesterone both in dark and light groups and improved the expression of estrogen receptor alpha and progesterone receptor in the light group. The results of the experiment provide a good reference for using EPI to improve the development and recovery of the reproductive system of layer hens.

Key words: *Epimedium*, Forced molting, Layer hens, Oviduct

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Research Paper

Influenza A, and Salmonella spp. in Backyard Poultry Eggs in Guatemala City.

Guerra-Centeno D, Díaz-Rodríguez M, Valdez-Sandoval C, Lepe-López M, Álvarez E, Aguilar Ch, Hernández C and Borja J.

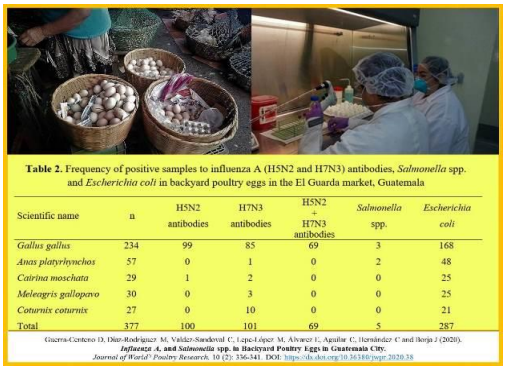

Table 2. Frequency of positive samples to influenza A (H5N2 and H7N3) antibodies, *Salmonella* spp. and *Escherichia coli* in backyard poultry eggs in the El Guarda market, Guatemala

Scientific name	n	H5N2 antibodies	H7N3 antibodies	H5N2 + H7N3 antibodies	*Salmonella* spp.	*Escherichia coli*
Gallus gallus	234	99	85	69	3	168
Anas platyrhynchos	57	0	1	0	2	48
Cairina moschata	29	1	2	0	0	25
Meleagris gallopavo	30	0	3	0	0	25
Coturnix coturnix	27	0	10	0	0	21
Total	377	100	101	69	5	287

Guerra-Centeno D, Díaz-Rodríguez M, Valdez-Sandoval C, Lepe-López M, Álvarez E, Aguilar Ch, Hernández C and Borja J (2020).
***Influenza A*, and *Salmonella* spp. in Backyard Poultry Eggs in Guatemala City.**
Journal of World Poultry Research, 10 (2): 336-341. DOI: https://dx.doi.org/10.36380/jwpr.2020.38

J. World Poult. Res. 10(2): 336-341, 2020; pii: S2322455X2000038-10
DOI: https://dx.doi.org/10.36380/jwpr.2020.38

ABSTRACT: Influenza A and salmonellosis are two of the most relevant zoonotic infectious diseases. Influenza A is one of the main threats to public health worldwide and is considered one of the causative agents of pandemics. Salmonellosis, meanwhile, has been identified by the World Health Organization as one of the four main causes of diarrheal diseases in the world. Poultry is an important source of both influenza A and *Salmonella* spp. but little is known about these potential threats in poultry products in Guatemala. The presence of influenza A virus antibodies and *Salmonella* spp. was studied in backyard poultry eggs sold in the El Guarda market in Guatemala City. 377 backyard poultry eggs were collected throughout seven months and sampled for hemagglutination inhibition test to determine the presence of antibodies to influenza virus A (H5N2 and H7N3) and cultured for *Salmonella* isolation. The eggs of chicken (*Gallus gallus*), turkey (*Meleagris gallopavo*), quail (*Coturnix coturnix*), mallard duck (*Anas platyrhynchos*) and muscovy duck (*Cairina moschata*) were sampled. Twenty-six percent of the eggs carried H5N2 antibodies, 27% carried H7N3 antibodies and 1.3% carried *Salmonella* spp. The presence of *Escherichia coli* inside the sampled eggs was an incidental common finding. These results suggest that backyard poultry eggs sold at markets could be a potential source of influenza A virus and *Salmonella* for the human population. The evidence found in the sampled eggs also shows that these potential pathogens are circulating in backyard poultry populations in Guatemala.

Key words: Food security, One Health, Public health, Zoonosis

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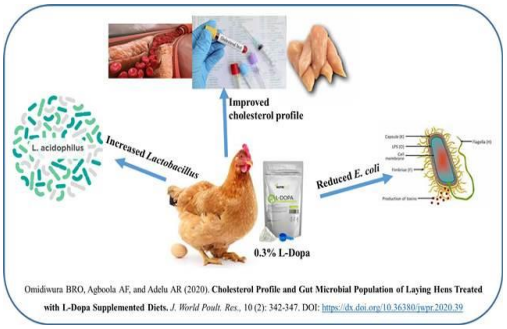
Research Paper

Cholesterol Profile and Gut Microbial Population of Laying Hens Treated with L-Dopa Supplemented Diets.

Omidiwura BRO, Agboola AF, and Adelu AR.

J. World Poult. Res. 10(2): 342-347, 2020; pii: S2322455X2000039-10
DOI: https://dx.doi.org/10.36380/jwpr.2020.39



ABSTRACT: In a 42-day feeding trial, the effect of L-Dopa on the performance, serum cholesterol and intestinal microbial load in laying hens were investigated. One hundred and twenty layers aged 34 weeks old were allocated to five dietary treatments with eight replicates and three birds per each replicate. The birds were fed diets supplemented with graded levels of L-Dopa (0, 0.1, 0.2, 0.3 and 0.4%) in a completely randomized design. Performance parameters were monitored. On the day 42, egg, meat and blood samples were obtained to determine total cholesterol and lipoproteins using standard procedures. The ileal digesta was collected for microbial analysis. The results indicated that inclusion of L-Dopa in the diet did not affect the performance parameters and egg cholesterol profile. Serum cholesterol levels of birds fed the control diet and those on 0.1% and 0.2% L-Dopa supplemented diets were similar, but significantly higher than those fed 0.3% and 0.4% L-Dopa. The levels of cholesterol and low-density lipoproteins in the meat of the layers fed on the control diet were significantly higher than those on 0.1% and 0.3% L-Dopa, but were similar to the birds on other treatment diets. The highest and the lowest population of *Escherichia coli* were found in the birds on the control diet and 0.3% L-Dopa supplemented diet, respectively. The layer hens on L-Dopa supplemented diets had a significantly higher *Lactobacillus* count than those on the control diet. In conclusion, 0.3% L-Dopa inclusion considerably improved the cholesterol profile in the blood and meat, reduced the population of *E. coli*, and effectively increased the population of *Lactobacillus* in the laying hens.

Key words: Cholesterol profile, Layers, Levodopa, Microbial load, Performance

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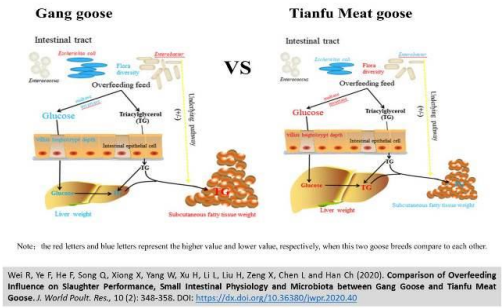
Research Paper

Comparison of Overfeeding Influence on Slaughter Performance, Small Intestinal Physiology and Microbiota between Gang Goose and Tianfu Meat Goose.

Wei R, Ye F, He F, Song Q, Xiong X, Yang W, Xu H, Li L, Liu H, Zeng X, Chen L and Han Ch.

J. World Poult. Res. 10(2): 348-358, 2020; pii: S2322455X2000040-10
DOI: https://dx.doi.org/10.36380/jwpr.2020.40



ABSTRACT: The objective of this research was to compare the influence of overfeeding on slaughter performance and small intestinal physiology and microbiota between Gang goose and Tianfu Meat Goose. Fifty Gang geese were randomly divided into the control group and overfed group, as were fifty Tianfu Meat geese. All geese were slaughtered after 3 weeks of overfeeding. After overeating, the results indicated that the liver weight, villus height to crypt depth ratio in duodenum and ileum, and the activity of invertase and maltase enzymes of Tianfu Meat goose was higher than those of Gang goose. However, the subcutaneous adipose tissue weight of Gang goose was higher than that of Tianfu Meat goose. Moreover, the Enterobacterial Repetitive Intergenic Consensus-Polymerase Chain Reaction (ERIC-PCR) fingerprint indicated that the band number of intestinal bacteria in each small intestinal segment decreased in Gang goose and increased in Tianfu Meat goose. The *Enterobacter* had a higher gene expression in the jejunum of Gang goose than that of Tianfu Meat goose and *Escherichia coli* the gene expression decreased in the ileum of Tianfu Meat goose after overfeeding. In conclusion, Tianfu Meat goose's liver weight, small intestinal digestion and absorption capacity and microbiota homeostasis were much better than those of Gang goose. Therefore, Tianfu Meat goose is more suitable for *foie gras* production. The results of the present study will provide a reference for the use of goose breeding for overfeeding and the relationship between intestinal physiology and the mechanism of goose fatty liver formation.

Key words: Digestive enzyme, Goose, Intestinal flora, Intestinal morphology, Overfeeding

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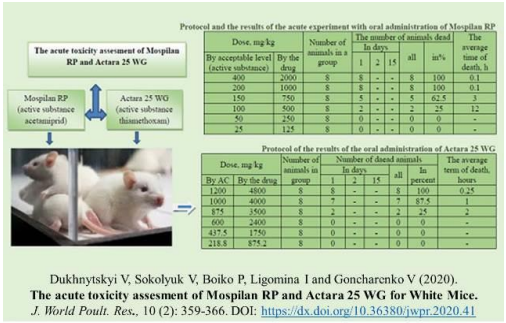
Research Paper

The acute toxicity assesment of Mospilan RP and Actara 25 WG for White Mice.

Dukhnytskyi V, Sokolyuk V, Boiko P, Ligomina I and Goncharenko V.

J. World Poult. Res. 10(2): 359-366, 2020; pii: S2322455X2000041-10
DOI: https://dx.doi.org/10.36380/jwpr.2020.41



Dukhnytskyi V, Sokolyuk V, Boiko P, Ligomina I and Goncharenko V (2020). **The acute toxicity assesment of Mospilan RP and Actara 25 WG for White Mice.** *J. World Poult. Res.*, 10 (2): 359-366. DOI: https://dx.doi.org/10.36380/jwpr.2020.41

ABSTRACT: In this article an attempt was made to present the results of toxicity assessment of insecticides from the group of neonicotinoids, Mospilan RP (active substance acetamiprid) and Actara 25 WG (active substance thiamethoxam) in white mice. The aim was to investigate the acute toxicity of Mospilan RP and Actara 25 WG in white mice. The half-lethal dose (LD50) of Mospilan RP was found to be131.25 ± 34.12 mg/kg Body Weight (BW) per active substance and 656.25 ± 170.6 mg/kg BW per drug, and the Lethal Dose (LD100) was 200 mg/kg BW by the active ingredient (1000 mg/kg BW by drug). The half-life dose (DL50) of Actara 25 WG for white mice was 907.81 ± 24.03 mg/kg BW for the active substance, and 3631.24 ± 96.12 mg/kg BW for the drug; The lethal dose (LD100) was also 1200 mg/kg BW per active ingredient (4800 mg/kg BW per drug). The acute course of poisoning by Mospilan RP and Actara 25 WG in mice was characterized mainly by nervous disorders (lesions of the central and peripheral nervous systems), which is evidenced by the clinical features of poisoning including depression, convulsions, ataxia (impaired movement coordination), tremor and impaired breathing. It has been established that Mospilan RP belongs to the third toxicity class according to the Hygienic classification of substances by skin-resorptive toxicity (DL50> 2000 mg/kg).
Key words: Actara 25 WG insecticides, Acute toxicity, Insecticides toxicity, Mospilan PP, Neonicotinoids, White miced

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Research Paper

Detection of *Campylobacter jejuni* among Commercial Broiler Chickens in East-Coast Malaysia.

Nur-Aziera-Aina CMN, Nur-Syafiqah MN and Zaidah AR.

J. World Poult. Res. 10(2): 367-370, 2020; pii: S2322455X2000042-10
DOI: https://dx.doi.org/10.36380/jwpr.2020.42

Nur-Aziera-Aina CMN, Nur-Syafiqah MN and Zaidah AR (2020). **Detection of Campylobacter jejuni among Commercial Broiler Chickens in East-Coast Malaysia.** *J. World Poult. Res.*, 10 (2): 367-370. DOI: https://dx.doi.org/10.36380/jwpr.2020.42

ABSTRACT: Human campylobacteriosis is associated with contaminated foods and drinks or direct contact with the source of organisms. Broilers are the main reservoir of *Campylobacter* as well as the primary source of meat in many countries. The microorganism load in broilers is converted into the rate of food-related campylobacteriosis. The present study aimed to determine the *Campylobacter jejuni* colonization load in commercial broiler chickens in east coast Malaysia. Cloaca swabs were taken after consent from the owners of selected broiler farms in Kelantan, Malaysia. Swabs were kept in Amie's transport media before being placed in enrichment broth. The DNA was extracted directly from the broth and specific Polymerase Chain Reaction (PCR) was performed, which targeted hippurate hydrolase (hipO) gene of *C. jejuni*. Three broiler farms from different districts in Kelantan were identified. A total of 120 cloacal swabs were analyzed, 20% (24/120) confirmed by PCR-positive for hipO gene when the amplified product with the band size of approximately 344 bases per was visualized on agarose gel. It is concluded that the prevalence of *C. jejuni* colonization among living broiler flocks in Kelantan was at a low level. However, further studies with bigger sample size and involvement of more farms are needed.
Key words: Broiler chicken, *Campylobacter jejuni*, Cloaca swab, Polymerase Chain Reaction, Hippurate hydrolase

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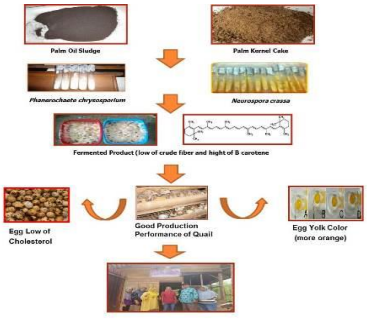
Research Paper

Effect of Dietary Supplementation of Palm Oil Waste Fermented with *Phanerochaete chrysosporium* and *Neurospora crassa* on Performance and Some Egg Characterizes of Laying Japanese Quails.

Nuraini N, Djulardi A, and Yuzaria D.

J. World Poult. Res. 10(2): 371-377, 2020; pii: S2322455X2000043-10



Nuraini N, Djulardi A, and Yuzaria D (2020). **Effect of Dietary Supplementation of Palm Oil Waste Fermented with *Phanerochaete chrysosporium* and *Neurospora crassa* on Performance and Some Egg Characterizes of Laying Japanese Quails.** *J. World Poult. Res.*, 10 (2): 371-377. DOI: https://dx.doi.org/10.36380/jwpr.2020.43

DOI: https://dx.doi.org/10.36380/jwpr.2020.43

ABSTRACT: The aims of present study was to determine the effect of dietary supplementation of palm oil waste fermented with *Phanerochaete chrysosporium* and *Neurospora crassa* (POWF) with ratio 1:1 on performance and some egg characterizes of laying Japanese quails. This experiment used 200 quails (*Coturnix-coturnix japonica*) aged 20 weeks, with 70% egg production. This study used an experimental method with a completely randomized design (CRD) with four treatments (0%, 8%, 16%, and 24% POWF and five replications). The results of this research showed that the use of POWF in the diet was not affected to feed consumption, daily egg production, egg mass, egg weight, and feed conversion. But, a significant difference in blood total cholesterol, LDL, HDL, egg cholesterol, and index egg yolk color was obsereved, and no significant effect on triglycerides and egg yolk fat. In conclusion, feeding the mixture of palm oil waste fermented with *Phanerochaete chrysosporium*, and *Neurospora crassa* with ratio1:1 can be used up to 24% in laying quail rations, without any adverse effect on egg production. It may decrease the blood cholesterol and egg yolk cholesterol.
Key words: Eqq quality, *Neurospora crassa,* Palm oil waste, Performance, *Phanerochaete chrysosporium,* Japanese quail

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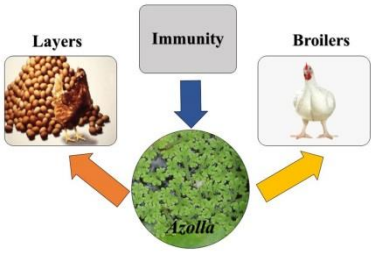
Review

A Review on the Use of *Azolla* Species in Poultry Production.

Abd El-Ghany WA

J. World Poult. Res. 10(2): 378-384, 2020; pii: S2322455X2000044-10
DOI: https://dx.doi.org/10.36380/jwpr.2020.44



Abd El-Ghany WA (2020).
A Review on the Use of *Azolla* Species in Poultry Production.
J. World Poult. Res., 10 (2): 378-384. DOI: https://dx.doi.org/10.36380/jwpr.2020.44

ABSTRACT: Aquatic plants are receiving a lot of attention in nutrition research, which is due to their broad range of uses in animal and human food. *Azolla* is one of the commonly used these floating plants. Currently, different *Azolla* species are used as maintainable feed alternatives for cattle, pigs, poultry, and fish as dried flakes or fresh form. In addition to high protein and essential amino acid content of *Azolla*, the fern is rich in other nutrients such as minerals, vitamins, and pigments. There is a discrepancy in the use of *Azolla* to improve the productivity of poultry. However, most studies have indicated that the incorporation of broiler ration with certain levels of *Azolla* promotes feed intake, body weight gain, feed conversion rate, and general health conditions as *Azolla* comprises growth promoters. In layers, the use of *Azolla* improves productivity in terms of egg quantity and quality as *Azolla* contains pigments, minerals, and essential amino acids. Moreover, *Azolla*s improves carcass traits at processing. Therefore, this review article provides information on *Azolla* plant, its composition, and the significance of its supplementation for different poultry species.
Key words: *Azolla*, Broilers, Immunity, Layers, Performance

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Research Paper

Gallibacterium anatis: Moleculer Detection of Tetracycline Resistance and Virulence Gene.

Sahan Yapicier O and Yaman S.

J. World Poult. Res. 10(2): 385-390, 2020; pii: S2322455X2000045-10
DOI: https://dx.doi.org/10.36380/jwpr.2020.45

Sahan Yapicier O and Yaman S (2020). ***Gallibacterium anatis*: Moleculer Detection of Tetracycline Resistance and Virulence Gene.** *J. World Poult. Res.*, 10 (2): 385-390. DOI: https://dx.doi.org/10.36380/jwpr.2020.45

ABSTRACT: *Gallibacterium anatis* causes infections in the reproductive tract of egg-laying hens and it is associated with increased mortality and decreased egg production. For this study we used singeleplex and multiplex PCR with specific primers to assess the presence of tetracycline resistance (Tcr) (*tet* A, B, C, D, E, G, H, K, L, M, O, S, P, Q and X), virulence [cytotoxic (RTX-like toxin, *gtxA*) and fimbrial (*flfA*)] genes and antibiotic resistance in *G. anatis* isolates. Among the 20 isolates tested, the highest antimicrobial resistance patterns were observed in erythromycin, streptomycin, tilmicosin (100%) followed by colistin sulphate (65%), cephalexin and tulathromycin (50%). Among 20 isolates examined, 10 (50%) carried tetracycline resistance genes, 7 (35%) had *tet*(B), 2 (10%) had *tet*(G), and 1 (5%) had *tet*(A), (D), (M) or (L). Of these *G .anatis* isolates were carried out 6 (30%) *gtxA* but none of *flfA* gene. Based on present results, it is concluded that virulence and Tcr genes could contribute to pathogencity of *G. anatis*, which is a major risk to poultry health.
Key words: Antibiotic resistance*, G. anatis*, Poultry, Virulence genes, Tetracycline resistance genes

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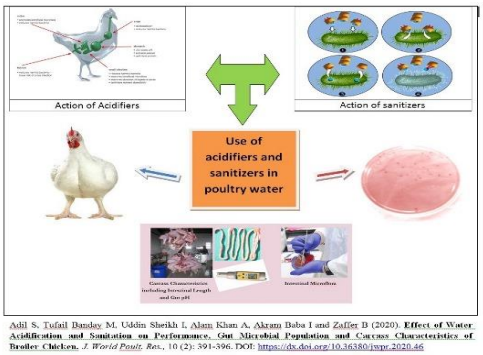
Research Paper

Effect of Water Acidification and Sanitation on Performance, Gut Microbial Population and Carcass Characteristics of Broiler Chicken.

Adil S, Tufail Banday M, Uddin Sheikh I, Alam Khan A, Akram Baba I and Zaffer B.

J. World Poult. Res. 10(2): 391-396, 2020; pii: S2322455X2000046-10
DOI: https://dx.doi.org/10.36380/jwpr.2020.46



ABSTRACT: Management of water is of utmost importance in order to ensure better performance of poultry birds. A study was thus conducted to evaluate the effect of water acidification and sanitation on performance, gut microbial population and carcass characteristics of broiler chicken. A total of 144 broiler chickens aged one-week were utilized for the study up to 6 weeks of age. The birds were distributed into 4 treatment groups of three replicates of 12 chickens. Untreated drinking water was routinely used in the control group (T1). In T2 and T3, acidifier and sanitizer were used in an amount of 1ml and 5ml/20 liters of water, respectively, while in T4 a combination of acidifier and sanitizer was used in similar doses. There was a significant effect of acidification and sanitization of water on body weight gain and feed conversion ratio of broiler chicken. The combination of acidifier and sanitizer (T4) was found to be highly effective in improving chickens' performance, followed by sanitizer alone (T3) and acidifier alone (T2), compared to the control group. There was no significant effect on various carcass characteristics of broiler chicken except for the gut pH and intestinal length. There was a significant effect on the pH value of various intestinal segments in broiler chickens using acidifier treated water (T2) compared to T1 and T3, but no statistical effect was noticed between T2and T4. A similar trend was noticed in the length of intestines of broiler chickens in various treatment groups. There was a significant reduction in Caecal Coliform Count (in all the treatment groups that was used acidifier and sanitizer compared to the control group.
Key words: Acidifier, Broiler chicken, Gut microbiology, Performance, Sanitizer

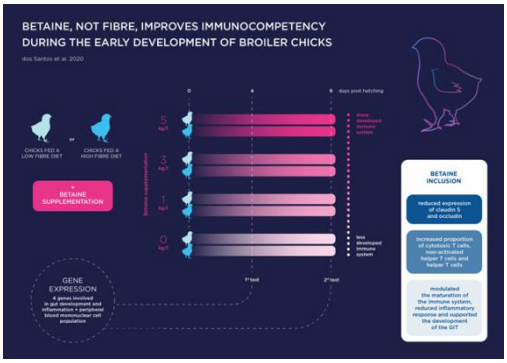
[Full text-PDF] [XML] [Crossref Metadata]

Research Paper

Immune Profile of Broilers between Hatch and 9 Days of Age Fed Diets with Different Betaine and Fibre Concentrations.

Santos TTdos, Baal SCS, Lee SA, Bedford M, Fávaro Jr C and Silva AVFda.

J. World Poult. Res. 10(2): 397-406, 2020; pii: S2322455X2000047-10
DOI: https://dx.doi.org/10.36380/jwpr.2020.47



ABSTRACT: An experiment was designed to determine the immune profile of broiler chickens between hatch and 9 days of age when fed diets with different fibre and betaine concentrations. A total of 240 day-old Cobb 500 male chickens were allocated to 16 cages with 15 chickens each. Treatments were arranged in a 2 x 4 factorial design, with 2 replicate cages per treatment. Treatments consisted of two feed formulations (low and high fibre diets) and four levels of betaine (0, 1, 3 or 5 kg/tonne). Before the start of trial (hatch), 10 broilers were weighed and blood samples were collected by cardiac puncture, then euthanised by cervical dislocation and jejunal samples collected for the determination of gene expression of claudin 1, claudin 5, occludin and interleukin 2 by PCR. Mononuclear cells populations in the blood samples were determined by flow cytometry. On days 4 and 9, five birds/cage (10 birds/treatment) were selected, euthanised and samples taken as per the start of hatch. Gene expression of claudin 1, claudin 5 and occludin was reduced between 4 and 9 days, independent of the group tested, while interleukin tended to increase between hatch and 4 days and decreased thereafter. Betaine inclusion reduced claudin 5 and occludin gene expression and increased CD8- CD28+ and CD8+ CD28+, suggesting it may aid in accelerating maturation of both the gastrointestinal tract and immune system for broilers in the early days post-hatch.
Key words: Betaine; Fibre; Gene expression; Maturation; Immune response

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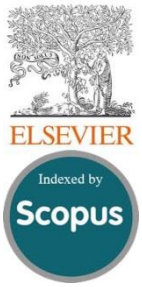

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Functions of *Epimedium* on Regressed Oviduct and Follicles of Force Molted Layer Hens

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ABSTRACT

In order to reveal the functions of *Epimedium* (EPI) on the recovery of the reproductive system of laying hens, 120 Hy-Line Brown laying hens (43-week-old) were forced molt through feed withdrawal for 14 days. After the molting period, layer hens were divided into four groups: Dark-control group in a dark environment without any treatment; dark-EPI group in a dark environment and with EPI treatment; light-control group without any treatment under normal illumination (16L: 8D); light- EPI group with EPI treatment under normal illumination (16L: 8D). EPI treatment was maintained for 15 days and each hen received 2 ml of EPI extract (1 g/mL) daily. The rate of egg production was calculated every day. At the end of the experiment, estrogen receptor alpha mRNA, estrogen receptor beta mRNA, and progesterone receptor mRNA in the albumen secreting part and uterus of the oviduct were detected by q-PCR, and the level of serum progesterone, estrogen and luteinizing hormone was measured by enzyme-linked immunosorbent assay. The results showed that EPI effectively improved the laying rate of hens both in dark groups and light groups by promoting the recovery of the oviduct and follicle maturation. In addition, EPI promoted the secretion of estrogen and progesterone both in dark and light groups and improved the expression of estrogen receptor alpha and progesterone receptor in the light group. The results of the experiment provide a good reference for using EPI to improve the development and recovery of the reproductive system of layer hens.

Key words: *Epimedium*, Forced molting, Layer hens, Oviduct

INTRODUCTION

The development of follicles and oviduct is crucial to the hens' laying abilities. Hen follicle development can be divided into three stages: primary (0.8-2 mm), prehierarchical (2-8 mm), and preovulatory follicles (9-35 mm) (Rangel et al., 2014; Lin et al., 2019). The oviduct of hens develops normally only on the left side. The oviduct of the hen consists of five parts: infundibulum, albumen secreting part, isthmus, uterus, and vagina (Michailidis et al., 2011; Socha et al., 2018), each with unique functions morphologically. The albumen secreting part is the longest part of the oviduct that secretes most of the albumen (Li., 2007), and the uterus is responsible for the formation of eggshell membranes (Socha et al., 2018). Estrogen (E_2), follicle-stimulating hormone (FSH), and luteinizing hormone (LH) are crucial during the development of the reproductive system and the laying period. E_2 is secreted by the membrane cells in the follicle, and its concentrations are the highest in the small yellow follicles (Rangel et al., 2014). E_2 and progesterone (P_4) start the development of the oviduct (Zhao, 2018). The progesterone receptor (PR) is expressed on the fallopian tube and binds to P_4. The activity of E_2 depends on the estrogen receptor (ER) that has two subtypes of estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ) (Miyagawa et al., 2014; Nirmali et al., 2019). FSH is secreted by basophilic cells in the anterior pituitary of birds. FSH can regulate follicle growth and drive the proliferation, growth, and differentiation of granulosa cells (Hunzicker-Dunn and Maizels, 2006; Uhm et al., 2010). Selection of prehierarchical follicles depends on levels of FSH and its receptor expressions (Lin et al., 2011). LH is a major hormone that promotes follicle maturation and excretion. FSH binds to its receptor on follicular granulosa cells, resulting in follicular differentiation, and then follicular membrane cells begin to express the LH receptor. This lays a foundation for the synthesis of E2 and ovulation (Tischkau et al., 2011). P_4 is secreted by follicular granulosa cells and has positive associations with egg production (Wang, 2018).

The laying performance of layer hens is not always at its peak (Perry and Yousef., 2013). Many factors such

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as environmental stress, infectious bronchitis virus, and the aging of hens can lead to a decrease in egg production. In China, the use of hormonal drugs and antiviral western medicine in the production of food animals is not permitted.

One of the solutions for restoring performance and egg quality of laying hens is to induce the molting (Alodan and Mashaly, 1999). The process of molting is accompanied by the degeneration of the oviduct tract (Berry, 2003). Fasting is used as the main method of forced-molting (Han et al., 2019). Since light plays a major role in the development of the hen reproductive system (Liu et al., 2015), avoiding light along with forced molting can be used to accelerate the degradation of the oviduct.

Epimedium (EPI) is one of the most famous resources of Chinese herbal medicine (Zhang et al., 2008; Zhang et al., 2013). It is used to treat animals with reproductive diseases (Zhang et al., 2005). Flavonoids and polysaccharides are the main active components of EPI, which have the functions of enhancing immunity, anti-cancer, and anti-aging in traditional Chinese medicine (Liang et al., 2012). The total flavonoids of EPI have E_2-like effects, which can thicken the endometrium of ovariectomized mice (Zhou et al., 2012). *Epimedium* may protect the testicles from damage by suppressing inflammation and oxidative stress (Cheng et al., 2019). The extraction methods of EPI include alkali extraction, ultrasonic extraction, hot water extraction, microwave extraction, organic solvent extraction, and supercritical fluid extraction (Li et al., 2014).

E_2 controls the growth of the oviduct by regulating cell proliferation, differentiation, and egg cell protein synthesis (Socha et al., 2018). Similarly, EPI can promote the egg-laying performance of laying hens (Huo et al., 2018), but the impact of EPI on hens' reproductive system remains unclear. In this study, the forced molting method was used to degrade the hen's reproductive system, and then hens were treated with EPI to study the functions of EPI on the repairing of oviduct and follicles and secreting of reproductive hormones. Since light has a great influence on the development of the reproductive system of hens, lighting was considered as a variable for comparison.

MATERIALS AND METHODS

Ethical approval

This study was approved by the Experimental Animal Ethics Committee of Hebei Agricultural University (certificate code: 2020013).

Extraction and determination of *Epimedium*

Epimedium koreanum was purchased from the Anguo Oriental Medicine City, Hebei, China. The active components of the EPI were obtained through alcohol extracting by an ultrasonic method (Zhang et al., 2008). First, EPI was crushed into powder, added in 65% ethanol solution at a ratio of 1:30, and then rested at room temperature for 60 min. The effective components of the EPI were extracted twice in the ultrasonic purification device at 60 ℃ for 60 min, filtrated with the filter paper. The filtered solution was centrifuged at 3000 r/min for 10 min and then concentrated to 1 g/mL by a rotary evaporator at 80 ℃, stored at 4 ℃. The quantitative determination of the total flavonoids of EPI was measured by colorimetry at 496 nm, with rutin as the reference and aluminum nitrate as the chromogenic agent (Guo et al., 2019).

Animal grouping and treatment

A total of 120 Hy-Line Brown layer hens at 43 weeks of age were provided by Ding Nong Corporation of Hebei, Baoding, China. Layer hens were weighed before forced molting and raised on the poultry breeding farm of Hebei Agricultural University. They were divided into four equal groups of 30 hens and three replicates for each group. Then all hens were placed in an environment shaded by a dark nylon cover; and received no food for 14 days but they could drink water freely. Forced molting lasted for 14 days until the average body weight of layer hens lost a third and egg production completely ceased. From the 15th day of the experiment, all the hens were allowed to recover with *ad libitum* access to food and water, according to the nutrient suggestions for layer hens (Clark et al., 2019). Dark control (D-CON) group was still kept in a dark feeding environment without any treatment; dark EPI (D-EPI) group was kept in a dark feeding environment and each hen was given 2 mL of EPI extract (1g/mL) by gavage every day; light control (L-CON) group was without EPI treatment under normal illumination (16L: 8D); light-EPI (L-EPI) group received 2 mL EPI extract (1g/mL) per hen by gavage every day, under normal illumination (16L: 8D). During the experiment, egg production was recorded every day, and the laying rate of each group was obtained by dividing the number of eggs laid during the day by the number of hens. The EPI treatment on layer hens was maintained for 15 days. At the end of the experiment, the hens of each group were weighed and then sacrificed with bleeding of the carotid artery. Blood was collected and serum was isolated for P_4, E_2, and LH detection. The oviduct of hens was

taken out for weighing and length measurement. The relative weight of the oviduct to body weight was calculated. The uterus and albumen secreting parts were separated and half of the tissue samples were stored at −80 ℃ for later real-time polymerase chain reaction (q-PCR). The rest tissue fragments were fixed in a 4% paraformaldehyde solution for histomorphology observation.

Histomorphology observation of albumen secreting part and uterus

The albumen secreting part and uterus were fixed in a 4% paraformaldehyde solution overnight and washed by pH 7.4 phosphate buffer saline (PBS). Routine dehydration and paraffin embedding were done. Histological sections of 5 μm thickness were sliced by an automatic slicer for hematoxylin and eosin (H&E) staining. Images were observed via a digital camera (Leica DFC320).

mRNA expressions of estrogen and progesterone receptors in albumen secreting part and uterus

The total RNA was extracted by the Trizol reagent of a commercial RNA assay kit (Invitrogen Co., USA) according to the manufacturer's instructions. Reverse transcription was performed with 25 μl of the reaction mixtures containing 10 μl of total RNA extraction solution, 2 μl Olig (dT) primer, 2 μl RNase inhibitor, 5 μl dNTPs, 5 μl 5× M-MLV buffer and 1 μl M-MLV reverse transcriptase. Before 1 μl M-MLV (100 U) reverse transcriptase was added, other components were incubated at 65 ℃ for 5 min and then placed on ice for 5 min. After 1 μl M-MLV (100 U) reverse transcriptase was added, reverse transcription was conducted at 42 ℃ for 1 h.

Expression of ERα, ERβ, and PR was detected by q-PCR on a fluorescence ration PCR instrument (CFX96 Bio-Rad, CA, USA). The q-PCR reaction was performed with 25 μl of the reaction mixtures containing 12.5 μl of 2 × M5 Hiper SYBR Premix Es Taq (Mei5 Biotechnology, Beijing, China), 2 μl of cDNA, 9.5 μl ddH2O and 0.5 μl of each forward and reverse primer. The primers (Table 1) were designed based on sequences in GenBank using Primer Premier 5.0 software and manufactured by Sangon Biotech Company (Shanghai, China).

They followed the following procedure respectively: 95 ℃ for 3 min; 95 ℃ for 5 s and 60 ℃ for 30 s for 40 cycles. The PCR efficiency was close to 100%, indicating the $2^{-\Delta\Delta CT}$ method was applicable to the calculation of relative gene expression levels (Zhao et al., 2018). Three samples were amplified and the data were normalized to glyceraldehyde phosphate dehydrogenase (GAPDH) expression.

Determination of progesterone, estrogen and luteinizing hormone in serum

P4, E2, and LH were detected by enzyme-linked immunosorbent assay (ELISA) with the Hen Progesterone ELISA Kit (MLBIO, China). The serum was removed from -80 ℃, and the standard, blank diluent, and samples were added to the ELISA Kit. All procedures were conducted according to the manufacturer's protocol. The absorbance was measured at 600 mm, a standard curve was established, and the hormone content of each sample was calculated.

Statistical analysis

All of the experiments were repeated at least three times, and the results were expressed as means ± standard error. Statistical analyses were performed on the SPSS software V22.0 (SPSS Inc., Chicago, IL, USA). All data were analyzed by one-way analysis of variance (ANOVA) to determine the differences among the groups. Mean values were considered significantly different at $p < 0.05$.

Table 1. Primers used for detection of the glyceraldehyde phosphate dehydrogenase (GAPDH), estrogen receptor alpha (ERα), estrogen receptor beta (ERβ) and progesterone receptor (PR) genes by q-PCR

Target gene	Primer sequences (5'-3')	Size (base pair)	GenBank accession number
GAPDH-F	ACGTCGCACTGGATTTCGAG	82	NM_204305
GAPDH-R	TGTCAGCAATGCCAGGGTAC		
ERα-F	GTACGGCTCTACTACACTCAGTTATGC	99	NM_205183.2
ERα-R	GGGCTTGGTGGGACATTGTTCAG		
ERβ-F	CCCTCCCAGCAGCAAACAACTC	148	NM_204794.2
ERβ-R	TCAACATCTCCAGCAGCAAGTCATAC		
PR-F	CCTGGACGGGCTGCTCTACC	89	M37518.1
PR-R	GCGGTTCTTCCTCCTCCTCCTC		

RESULTS

Effects of *Epimedium* on egg-laying rates

On the 14th day of the experiment, egg-laying rates of all four groups were 0% because of the oviduct atrophy and follicular degeneration by forced molting. From the 15th day of experiment with normal feeding, layer hens of L-CON and L-EPI groups began to lay eggs on the 25th day. Laying rates of the L-EPI group were higher than that of the L-CON group from the 25th day to the 30th day. On the 30th day, laying rates of the D-CON group was the lowest, and the D-EPI group had an extremely higher laying rate than the D-CON group ($P < 0.01$). As shown in figure 1, the L-EPI group had the highest rate of laying, followed by L-CON, D-EPI, and D-CON group. The results showed that EPI can improve the laying rate of layer hens both in dark and light environments.

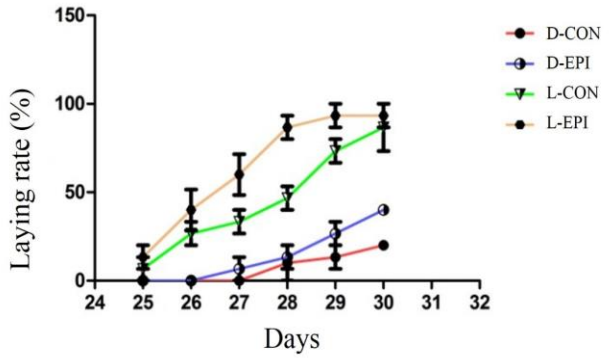

Figure 1. The daily laying rate of laying hens subjected to forced molting and treatment. EPI: *Epimedium*. D-CON: without EPI treatment in a dark environment; D-EPI: with EPI treatment in a dark environment; L-CON: without EPI treatment under normal illumination; L-EPI: with EPI treatment under normal illumination. Two milliliters of EPI extract (1 g/mL) was daily administered each chicken for 15 days.

Effect of *Epimedium* on the development and recovery of follicle and oviduct

On the 30th day, the follicles in the D-CON group grew sluggishly, a large number of small white follicles existed and the oviducts were found still to be atrophied and not to return to normal (Figure 2A). However, large white follicles and small yellow follicles were evident in the D-EPI group, oviduct developed to nearly normal (Figure 2B). In the L-CON and L-EPI groups, follicles growth returned to normal, follicles in different stages of development were present and the oviducts of both groups developed to normal (Figure 2C and 2D). The relative weights of oviduct to body weight of layer hens from the D-CON group were remarkably lower than other groups on the 30th day ($P < 0.01$), as shown in figure 2A. The relative weights of oviduct in the D-EPI group were lower than L-EPI and L-CON group ($P < 0.05$). In addition, the average length of oviduct in the D-CON group was remarkably shorter than that in the other groups ($P < 0.01$, figure 3B) and there was no significant difference in the average length of oviduct among D-EPI, L-CON, and L-EPI group.

Histomorphology of albumen secreting part and uterus of oviduct

On the 30th day of the experiment, it was found that columnar ciliated epithelial cells in the uterus and albumen secreting part were normally arranged in D-EPI, L-CON and L-EPI group, with intact serosa observed, and the cilia in the D-CON group were thinner (Figure 4). In addition, there was corresponding atrophy of the uterus and albumen secreting part in the D-CON group. The D-EPI group had a slight deficit in cilia development compared to the L-CON and L-EPI groups. In the D-EPI, L-CON and L-EPI group, the uterus and albumen secreting part showed normal arrangement of columnar ciliated epithelial cells, while the D-CON group showed dysplasia of cilia (Figure 4).

mRNA expressions of estrogen and progesterone receptors in albumen secreting part and uterus

The results showed that the relative value of ERα mRNA to GAPDH in the D-CON group was the lowest both in the albumen secreting part and uterus ($P < 0.01$), the D-EPI group had no significant difference compared to D-CON group. The expression of ERα mRNA in the L-CON and L-EPI group was higher than that in D-CON and D-EPI group, and the L-EPI group had an extremely higher value compared to L-CON group ($P < 0.05$) (Figure 5 A and D). There was no significant difference in terms of the expression of ERβ mRNA in the albumen secreting part and uterus of the four groups ($P > 0.05$) (Figure 5 B and E). The relative value of PR mRNA in D-CON and D-EPI group had no significant difference both in albumen secreting part and uterus, while expression of PR mRNA in L-CON and L-EPI group were both higher than that in D-CON and D-EPI group, and PR mRNA in L-EPI group was extremely higher than in L-CON group ($P < 0.01$) (Figure 5 C and F).

Effects of *Epimedium* on the level of progesterone, estrogen and luteinizing hormone in serum

The results (Table 2) showed that concentration of P_4 in the serum of D-CON group was significantly lower than that in the other three groups, and P_4 level was higher in L-EPI group compared to D-EPI ($P < 0.05$), but was lower than that of L-CON group ($P < 0.05$). E_2 concentration of serum in the L-CON group and L-EPI group was higher than that in the D-CON group and D-EPI group ($P < 0.01$). The concentration of E_2 in the D-EPI and L-EPI group was higher than that in control groups ($P < 0.05$). The serum concentration of LH in the four groups was not significantly different ($P > 0.05$).

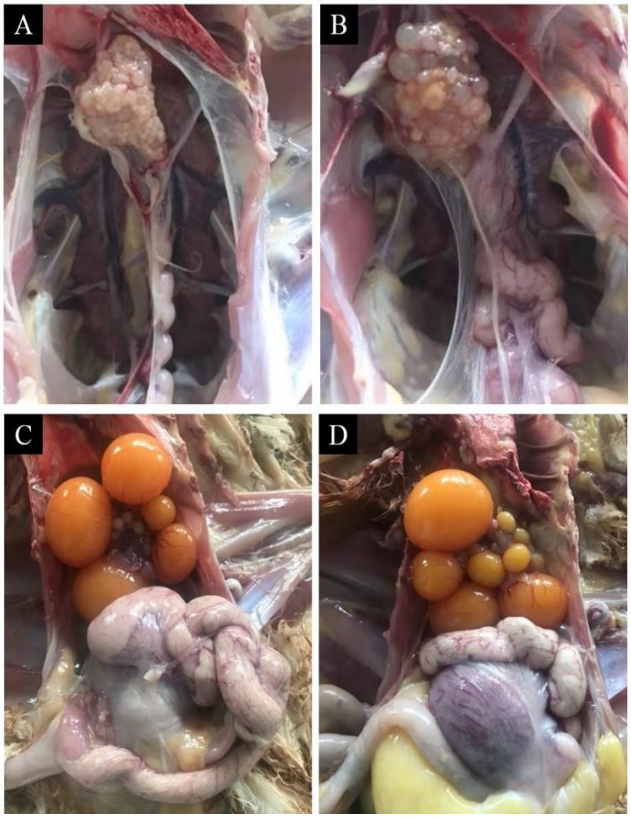

Figure 2. Ovarian follicles and oviducts of laying hens subjected to forced molting and treatment (A: D-CON group; B: D-EPI group; C: L-CON group; D: L-EPI group). EPI: *Epimedium*. D-CON: without EPI treatment in a dark environment; D-EPI: with EPI treatment in a dark environment; L-CON: without EPI treatment under normal illumination; L-EPI: with EPI treatment under normal illumination. Two milliliters of EPI extract (1 g/mL) was daily administered each chicken for 15 days.

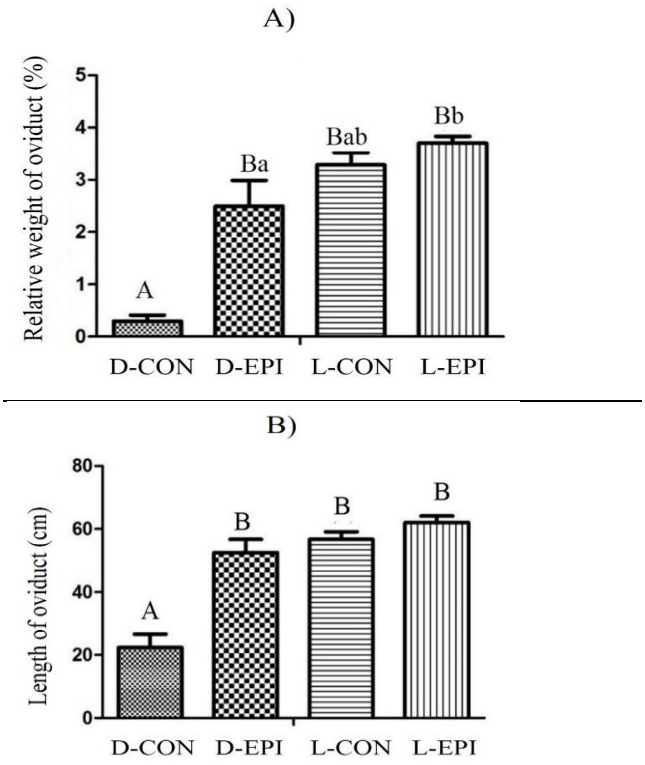

Figure 3. Effects of *Epimedium* on the relative weight and average length of oviduct of laying hens treated in different groups after forced molting. EPI: *Epimedium*. D-CON: without EPI treatment in a dark environment; D-EPI: with EPI treatment in a dark environment; L-CON: without EPI treatment under normal illumination; L-EPI: with EPI treatment under normal illumination. Two milliliters of EPI extract (1 g/mL) was daily administered each chicken for 15 days. $^{a, b, c}$ on the bar means $P < 0.05$ and $^{A, B, C}$ means $P < 0.01$.

Table 2. Serum concentration of the progesterone, estrogen and luteinizing hormone in layer chickens treated in different groups after 14-day forced molting

Parameters \ Treatment groups	D-CON	D-EPI	L-CON	L-EPI
Progesterone (pmol/L)	267.7 ± 38.96^{Aa}	493.65 ± 105.92^{Ab}	728.06 ± 131.04^{Bc}	584.56 ± 26.49^{Ad}
Estrogen (pg/mL)	40.57 ± 5.85^{Aa}	71.74 ± 13.21^{Ab}	136.75 ± 40.77^{Bc}	153.32 ± 29.67^{Bd}
luteinizing hormone (pg/mL)	22.11±4.56	43.93±15.16	47.05±14.27	54.75±9.55

EPI: *Epimedium*. D-CON: without EPI treatment in a dark environment; D-EPI: with EPI treatment in a dark environment; L-CON: without EPI treatment under normal illumination; L-EPI: with EPI treatment under normal illumination. Two milliliters of EPI extract (1 g/mL) was daily administered each chicken for 15 days. $^{a, b, c;}$ Means within a row with different superscripts differ significantly ($P < 0.05$); $^{A, B, C;}$ Means within a column with different superscripts differ highly significantly ($P < 0.01$).

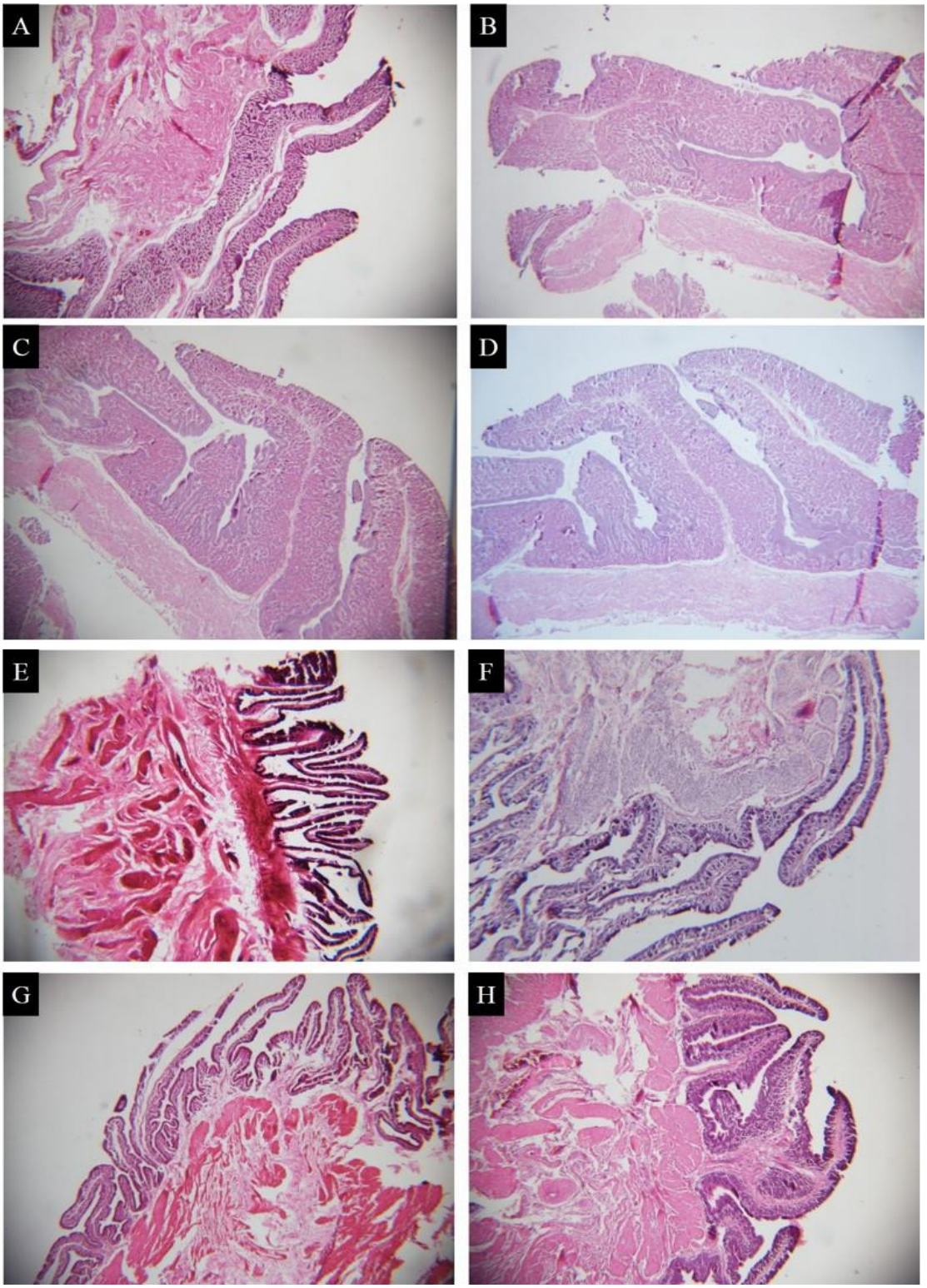

Figure 4. Histomorphology of albumen secreting part and uterus of oviduct of laying chickens treated in different groups after forced molting. A, B, C, and D are albumen secreting part of oviduct in D-CON, D-EPI, L-CON, and L-EPI groups, respectively (H&E, 10×). E, F, G, and H are uterus of the oviduct in D-CON, D-EPI, L-CON and L-EPI group respectively (H&E, 10×). EPI: *Epimedium*. D-CON: without EPI treatment in a dark environment; D-EPI: with EPI treatment in a dark environment; L-CON: without EPI treatment under normal illumination; L-EPI: with EPI treatment under normal illumination. Two milliliters of EPI extract (1 g/mL) was daily administered each chicken for 15 days.

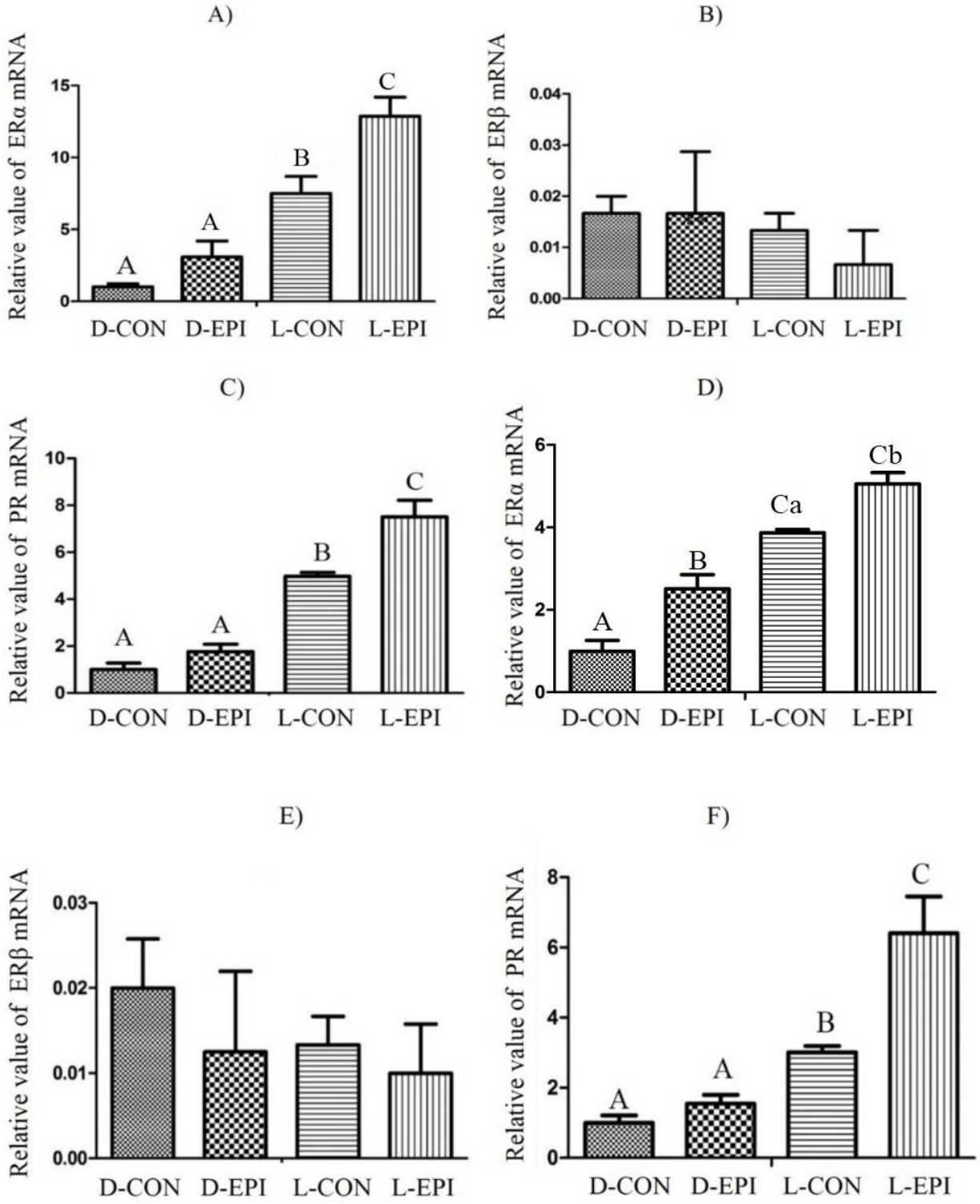

Figure 4. Effects of *Epimedium* on mRNA expression of estrogen receptor alpha (ERα), estrogen receptor beta (ERβ), and progesterone receptor (PR) in albumen secreting part and uterus of laying hens in different treatment groups after forced molting. A, B, C: The expression of ERα, ERβ, and PR mRNA in the albumen secreting part. D, E, F: The expression of ERα, ERβ, and PR mRNA in the uterus. EPI: *Epimedium*. D-CON: without EPI treatment in a dark environment; D-EPI: with EPI treatment in a dark environment; L-CON: without EPI treatment under normal illumination; L-EPI: with EPI treatment under normal illumination. Two milliliters of EPI extract (1 g/mL) was daily administered each chicken for 15 days.
$^{a, b, c}$ on the bar means $P < 0.05$; $^{A, B, C}$ on the bar means $P < 0.01$.

DISCUSSION

In this study, the active components of EPI were extracted by alcohol with an ultrasonic method, a better way for the extraction of the total flavonoids of EPI. Layer hens, whose oviduct and follicles were atrophic after forced molting, were fed with EPI extracts in dark and light environments and the laying rates extremely increased both in the D-EPI group and the L-EPI group. The results demonstrated that EPI extracts have the functions of improving the development of the reproductive system of layer hens, especially in the light environment. Previous studies have revealed that total flavonoids of EPI can cure premature ovarian insufficiency, and the therapeutic targets are ESR1, AR, ESR2, KDR, CYP19A1, and ESRRG (Zhao et al., 2019). EPI has been reported to restore the oxidative damage of mouse testis and maintain sperm motility and has a protective effect on the reproductive function of male mice (Yuan et al., 2014).

The number of eggs a hen lays is determined by the development of follicles in ovary, and the quality of eggs such as the hardness of the shell is determined by the oviduct (Socha et al., 2018). After treatment with EPI, the oviduct of layer hens in the D-EPI group was fully developed, and the follicles included primary follicles and large white follicles and small yellow follicles. Some layers contained preovulatory follicles and mature follicles and began to lay eggs. The oviduct in the D-CON group was severely atrophic, and the follicles were most primary follicles and small white follicles without further development. Only a few layers began to lay eggs. The L-CON and L-EPI groups were raised under the environment of light, which has a great influence on the development of the hen's reproductive system. Therefore, the development of the ovary and oviduct in L-CON and L-EPI groups was very normal. It is worth noting that there were significantly more prehierarchical follicles in the D-EPI and L-EPI groups of layers treated with EPI than in D-CON and L-CON groups.

According to the results of the percentage of oviduct weight to body weight, the length of oviduct, and the rate of egg production, the impact of EPI and light on the development of the hen's reproductive system and the rate of egg production is significant. Light has a bigger effect than EPI; while EPI can build on that.

The biological activity of EPI can be largely mediated through E_2 receptor-mediated pathway or blocked by ER blockers (Zhang et al., 2016). E_2 is closely related to the development of oviducts and ovaries, and P_4 is positively associated with ovulation. The determination of expression of ERα, ERβ, and PR mRNA in the albumen secreting part and the uterus of the oviduct showed that the expression of ERα mRNA was significantly higher under the action of EPI, especially in the light environment. ERβ mRNA was poorly expressed both in dark groups and in light groups. The impact of light on PR mRNA expression appeared to be better than that of EPI, especially when the sample was treated with EPI under light conditions. The results indicated that the impacts of EPI on the expression of ER and PR were not obvious without light; however, it became stronger under the light conditions. From the results, it can be inferred that ERα and PR are the crucial receptors mediating the process of E_2 and P_4 and improving the development of oviduct, and EPI participated in the process. The measurement of E_2 and P_4 in serum showed that EPI improved the secretion of endogenous hormones both in dark and light groups. The impact of light was greater than that of EPI. However, both light and EPI had a small impact on the secretion of LH. It was found that EPI and light could promote the development and proliferation of epithelial cells in the oviduct.

EPI promoted the development of follicles from primary follicles to prehierarchical follicles. Hormones that promote follicular growth and differentiation include FSH and LH, and cytokines include insulin-like growth factor family (IGF) (Roberts et al., 1994), transforming growth factor-β (TGF-β) (Johnson et al., 2004; Woods et al., 2005), epidermal growth factor (EGF) (Onagbesan et al., 1994; Lin et al., 2011), fibroblast growth factor (FGF) (Miyahara et al., 2016), and tumor necrosis factor-α (TNF-α) (Bornstein et al., 2004). The experimental results showed that EPI and light had no significant impacts on LH secretion. Hence, EPI is likely to have a similar effect like FSH or other follicle-promoting cytokines.

CONCLUSIONS

This study may aid the development of treatment drugs for reproductive diseases in layer hens.

EPI has the potential functions to increase egg production by improving the recovery of oviducts and maturation of follicles. EPI could promote the secretion of endogenous E_2 and P_4, especially in light environment. Also, EPI could improve the expression of ERα mRNA and PR mRNA in the oviduct.

DECLARATIONS

Competing interests

The authors have declared that no competing interest exists.

Authors' contributions

Shuying H and Yu G designed research and wrote the paper. Yurong L and Xianjun W analyzed data. Shuang Z and Luying J conceived of the study and participated in its coordination. Qianhui Z and Wenhui X contributed vital new reagents. All authors read and approved the final manuscript.

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Influenza A, and *Salmonella* spp. in Backyard Poultry Eggs in Guatemala City

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ABSTRACT

Influenza A and salmonellosis are two of the most relevant zoonotic infectious diseases. Influenza A is one of the main threats to public health worldwide and is considered one of the causative agents of pandemics. Salmonellosis, meanwhile, has been identified by the World Health Organization as one of the four main causes of diarrheal diseases in the world. Poultry is an important source of both influenza A and *Salmonella* spp. but little is known about these potential threats in poultry products in Guatemala. The presence of influenza A virus antibodies and *Salmonella* spp. was studied in backyard poultry eggs sold in the El Guarda market in Guatemala City. 377 backyard poultry eggs were collected throughout seven months and sampled for hemagglutination inhibition test to determine the presence of antibodies to influenza virus A (H5N2 and H7N3) and cultured for *Salmonella* isolation. The eggs of chicken (*Gallus gallus*), turkey (*Meleagris gallopavo*), quail (*Coturnix coturnix*), mallard duck (*Anas platyrhynchos*) and muscovy duck (*Cairina moschata*) were sampled. Twenty-six percent of the eggs carried H5N2 antibodies, 27% carried H7N3 antibodies and 1.3% carried *Salmonella* spp. The presence of *Escherichia coli* inside the sampled eggs was an incidental common finding. These results suggest that backyard poultry eggs sold at markets could be a potential source of influenza A virus and *Salmonella* for the human population. The evidence found in the sampled eggs also shows that these potential pathogens are circulating in backyard poultry populations in Guatemala.

Key words: Food security, One Health, Public health, Zoonosis

INTRODUCTION

Influenza A and salmonellosis are the most relevant zoonotic infectious diseases (Russell et al., 2014; Lee et al., 2015). Influenza A is one of the main threats to public health worldwide and is considered as one of the highly contagious infectious entities that can cause pandemics at any time (WHO, 2005). Salmonellosis, meanwhile, has been considered by the WHO (2018) as one of the four leading causes of diarrheal diseases in the world. Enteric *Salmonella* causes 1.3 billion cases of gastroenteritis and 3 million deaths worldwide (Bhunia, 2018) and it is, without a doubt, the most widespread foodborne disease in Latin America (Gil and Samartino, 2001). These pathogens are two of the infectious and contagious entities that represent a permanent risk to public health whose study should be a permanent priority to ensure their prevention, control, and eradication.

Sick poultry and its products can be taken to the markets to sell for human consumption. For this reason, markets are considered as reservoirs of diseases such as avian influenza and salmonellosis (Wray et al., 1991; Cardona et al., 2009; Singh et al., 2010). Therefore, it is important to investigate the risk to humans posed by the commercialization of poultry and its products in urban markets where many people come together, who could not only become infected but spread infectious agents.

The eggs of various poultry species that are sold in markets, can be a source of zoonotic pathogens. Influenza A virus of subtype H5N2 has been detected in eggs during disease outbreaks (Cappucci et al., 1985). Highly pathogenic H5N1 influenza virus has been isolated in table eggs after a mutation of a vaccine virus in chickens (Kilany et al., 2010). The eggs have also been considered as the main vehicle for enteric *Salmonella* infection in humans (Telzak et al., 1990; Braden, 2006; Bhunia, 2018).

Published data about the detection of influenzavirus A and *Salmonella* spp. in backyard poultry eggs sold in Guatemalan markets are practically non-existent even

though animal products not only represent a possible source of infection for humans, but also provide information about the circulation of pathogens in the environment. In response to this gap of knowledge, the presence of antibodies against two variants of Influenza A virus (H5N2, H7N3) and *Salmonella* spp was investigated in chicken, duck, turkey and quail eggs that are being sold for human consumption in the El Guarda market –which is popularly considered as the most important place for the trade of animals and their products in Guatemala City. Present findings provide useful public health information and epidemiological data about pathogen circulation in the backyard poultry population that represents almost half of the national poultry farming in Guatemala.

MATERIALS AND METHODS

Study site

El Guarda is a traditional market located in Guatemala City (N 14 ° 36'48.77"; W 90 ° 32'20.08") and is the main center for the sale of domestic and wild animals and their products.

Study design and sample collection

A longitudinal study of exploratory scope was carried out for the present investigation. Seven backyard poultry eggs selling points were located in the El Guarda market and randomly sampled. Eggs were randomly collected from various poultry species from each of these selling points (Table 1). The eggs were collected every week from February to October 2019. Samples were taken from shell and yolk of all the eggs.

Table 1. Number of eggs collected and sampled from each species of backyard poultry in the El Guarda market, Guatemala

Scientific name	Common name	N
Gallus gallus	Chicken	234
Anas platyrhynchos	Mallard duck	57
Cairina moschata	Muscovy duck	29
Meleagris gallopavo	Turkey	30
Coturnix coturnix	Quail	27
Total		377

N: Number of sampled eggs

Sample transportation and laboratory procedures

The samples were transported in padded boxes, made of expanded polystyrene, to the Regional Reference Laboratory of Animal Health (Larrsa), at the Veterinary and Animal Husbandry Faculty, University of San Carlos of Guatemala, in Guatemala City.

Influenzavirus A antibodies were investigated by hemagglutination inhibition tests performed according to standard procedures (OIE, 2018a), using Merial (Italy) H7 antigens, Larrsa (Guatemala) H5 antigens, Merk (Germany) isotonic PBS, Charles Rivers (USA) positive control, Transferpette (Germany) micropipettes, Nunc (Denmark) V-bottomed microtiter plates and a Barnsted (Germany) orbital shaker.

Salmonella spp. isolation was performed according to standard procedures (OIE, 2018b), using Puritan (USA) sterile cotton swabs, Merk (Germany) and Difco (USA) culture media, Difco (USA) peptonated water, a Thermo Scientific (USA) incubator, a Labconco (USA) laminar flow hood and Biometieux (France) API identification kits.

RESULTS

The findings of the present study indicated antibodies against influenza A virus in several sampled eggs. Some eggs carried antibodies against H5N2, some against H7N3 and some against both. Chicken eggs were the most commonly positive eggs to both H5N2 and H7N3 influenza A antibody subtypes. However, the H7N3 variant was also detected in eggs from ducks (both species), turkeys and quails. *Salmonella* spp. was found in chicken and mallard duck eggs. Although this was not an initial objective of this study, *Escherichia coli* organisms were frequently isolated both from the shell and the interior of the sampled eggs. Table 2 shows the frequencies of positive reactors to influenza A antibodies, and the carriers of *Salmonella* and *E. coli* between poultry species and table 3 shows the distribution of positive samples in the sampled egg selling points at the El Guarda market. Figures 1 and 2 show the frequencies of H5N2 and H7N3 antibody titers in the sampled eggs (all species).

Table 2. Frequency of positive samples to influenza A (H5N2 and H7N3) antibodies, *Salmonella* spp. and *Escherichia coli* in backyard poultry eggs in the El Guarda market, Guatemala

Scientific name	N	H5N2 antibodies	H7N3 antibodies	H5N2 + H7N3 antibodies	*Salmonella* spp.	*Escherichia coli*
Gallus gallus	234	99	85	69	3	168
Anas platyrhynchos	57	0	1	0	2	48
Cairina moschata	29	1	2	0	0	25
Meleagris gallopavo	30	0	3	0	0	25
Coturnix coturnix	27	0	10	0	0	21
Total	377	100	101	69	5	287

N: Number of sampled eggs

Table 3. Frequency of positive samples to influenza A (H5N2 and H7N3), *Salmonella* spp. and *E. coli* in backyard poultry eggs in the El Guarda market, Guatemala, according to the selling point.

Selling point	N	H5N2	H7N3	*Salmonella* spp.	*E. coli*
A	89	43	49	2	61
B	50	9	8	0	36
C	85	21	18	0	71
D	28	4	4	0	23
E	60	8	12	3	43
F	57	14	8	0	47
G	8	1	2	0	6

N: Number of sampled eggs

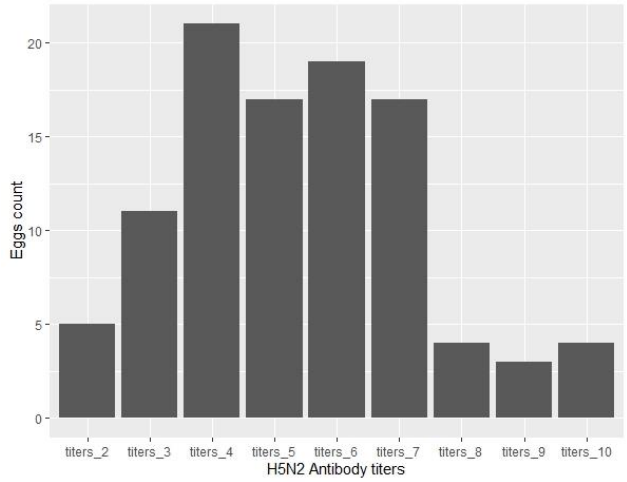

Figure 1. Frequency of logarithmic antibody titers of avian influenza A H5N2 variant in the sampled eggs.

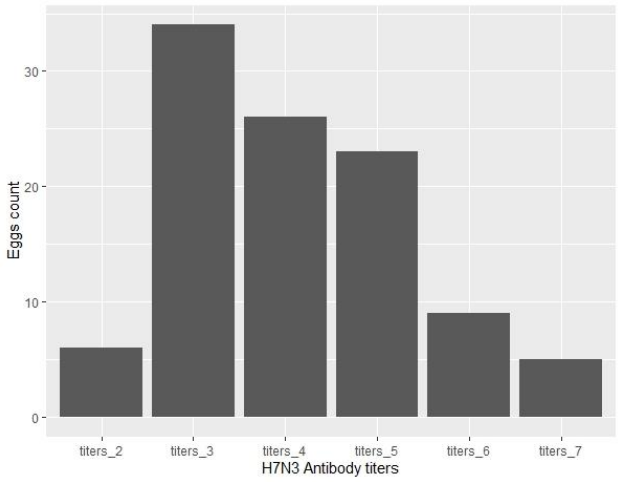

Figure 2. Frequency of logarithmic antibody titers of influenza A virus subtype H7N3 in the sampled eggs.

DISCUSSION

The observed frequencies of influenza A virus subtypes H5N2 and H7N3 antibodies in the sampled chicken eggs (42.3% and 36.3% respectively) are epidemiologically interesting especially considering that they probably come from unvaccinated backyard populations. There is some evidence indicating that Guatemalan peasants do not vaccinate their backyard poultry (Aguilar-Miller et al., 2016; Aquino-Sagastume et al., 2016; Mérida-Ruiz et al., 2016). In recent years the government passed a law for massive vaccination against the H7N3 type, however, this vaccination was intended to cover only those backyard poultry populations inside a 3 km radius around

commercial poultry farms (Ministry of Agriculture, Livestock and Food, Guatemala, 2019). Therefore, the detected blood antibodies would indicate field exposure to influenza viruses and, by extension, the possibility of viral particles passing to the eggs from viremic chickens.

Another important issue to consider was the fact that, in backyard conditions, a hen lays around 30 eggs per year, in batches of about 10 eggs (Sonaiya et al., 1999). This means that if a market vendor wishes to maintain a constant supply of eggs throughout the year, he is forced to purchase eggs coming from several hens from several backyard flocks, from many parts of the country, suggesting that influenza A virus subtypes H5N2 and H7N3 could be ubiquitous in Guatemalan rural landscapes.

On the other hand, the presence of circulating antibodies against influenza virus A in mallard ducks, muscovy ducks, turkeys, and quails is an uncommon finding for Guatemala and perhaps the first published report. These antibodies could be either the result of vaccination response or a challenge with field viruses. Although a H5N1 vaccine designed for chicken induced immunity in ducks and geese (Tian et al., 2005) it is unlikely that the government vaccination program has covered the entire territory of Guatemala.

The findings of the present study also support the previous observations of influenza A subtypes H5N2 and H7N3 variants in both wild and farm avian populations of Guatemala (Gonzalez-Reiche et al., 2012; Gonzalez-Reiche and Perez, 2012; Jarquin et al., 2015; Lee et al., 2015). Although the antibodies found in the eggs may come from vaccinated individuals, there is some evidence of post-vaccination outbreaks in poultry populations (Kilany et al., 2010).

In this study, *Salmonella* was detected in chickens and mallard ducks, but the frequencies were rather low. *Salmonella* had previously been reported in chicken meat in Guatemala (Jarquín et al., 2015). Salmonellosis cases have also been reported in humans in some provinces of the country (Díaz et al., 2015).

An incidental but significant finding of the present study was the overall frequency (76.43%) of *E. coli* isolations from the interior of the sampled eggs. Not only does this indicate a pathogen-permeable egg but also a public health concern because *E. coli* from domestic animal populations use to become multiresistant (Krumperman, 1983; Kojima et al., 2005; Sayah et al., 2005). It is known that antibiotic-resistant *E. coli* present in chicken meat can colonize the human bowel after consumption (Linton et al., 1977). The contamination of foods with bacteria from farm animals has been identified as a relevant problem, especially from the One Health approach (Van den Bogaard and Stobberingh, 2000).

From a public health perspective, the presence of *Salmonella* and *E. coli* and the possible presence of influenza A viruses is relevant in a country like Guatemala, where the consumption of raw eggs with orange juice and the feeding of young children with boiled under-cooked eggs is traditional.

Finally, although 18 influenza A virus subtypes have been found in migratory ducks in Guatemala (Gonzalez-Reiche et al., 2017), the governmental surveillance system is only looking for two subtypes (H5N2 and H7N3). This situation generates a knowledge gap about the subtypes that could be circulating in poultry farm and backyard populations, as well as in poultry products for human consumption.

CONCLUSION

Antibodies against influenza A, subtypes H5N2 and H7N3 were common findings in the eggs of backyard poultry. This means that, under certain conditions, the eggs could also be a source of viral particles for consumers. *Salmonella* spp. was not a frequent finding in this study and *Escherichia coli* (both outside and inside most of the studied eggs) was an incidental finding.

DECLARATIONS

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Competing interests

The authors have declared that no competing interest exists.

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Author's contribution

DG-C conception, design and drafting the manuscript, MD-R conception, design, interpretation of data and reviewing the manuscript, CV-S fund

management, data analysis and reviewing the manuscript, ML-L data analysis and interpretation and reviewing the manuscript, EA, CA, CE and JB sampling, management of samples and reviewing the manuscript.

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Cholesterol Profile and Gut Microbial Population of Laying Hens Treated with L-Dopa Supplemented Diets

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ABSTRACT

In a 42-day feeding trial, the effect of L-Dopa on the performance, serum cholesterol and intestinal microbial load in laying hens were investigated. One hundred and twenty layers aged 34 weeks old were allocated to five dietary treatments with eight replicates and three birds per each replicate. The birds were fed diets supplemented with graded levels of L-Dopa (0, 0.1, 0.2, 0.3 and 0.4%) in a completely randomized design. Performance parameters were monitored. On the day 42, egg, meat and blood samples were obtained to determine total cholesterol and lipoproteins using standard procedures. The ileal digesta was collected for microbial analysis. The results indicated that inclusion of L-Dopa in the diet did not affect the performance parameters and egg cholesterol profile. Serum cholesterol levels of birds fed the control diet and those on 0.1% and 0.2% L-Dopa supplemented diets were similar, but significantly higher than those fed 0.3% and 0.4% L-Dopa. The levels of cholesterol and low-density lipoproteins in the meat of the layers fed on the control diet were significantly higher than those on 0.1% and 0.3% L-Dopa, but were similar to the birds on other treatment diets. The highest and the lowest population of *Escherichia coli* were found in the birds on the control diet and 0.3% L-Dopa supplemented diet, respectively. The layer hens on L-Dopa supplemented diets had a significantly higher *Lactobacillus* count than those on the control diet. In conclusion, 0.3% L-Dopa inclusion considerably improved the cholesterol profile in the blood and meat, reduced the population of *E. coli*, and effectively increased the population of *Lactobacillus* in the laying hens.

Key words: Cholesterol profile, Layers, Levodopa, Microbial load, Performance

INTRODUCTION

Among the anti-nutritional factors in raw velvet beans (*Mucuna pruriens*), L-Dopa (3, 4-dihydroxy-phenylalanine) has been proved to have a marked effect on the growth response in some poultry species (Meites et al., 1977). In birds, L-Dopa is naturally produced through the biosynthesis of L-tyrosine in the presence of tyrosine hydroxylase. Lampariello et al. (2012) reported that supplementation of L-Dopa in the diet increased dopamine levels in the body. Meites et al. (1977) stated that L-Dopa could pass through the blood-brain barrier, where it becomes decarboxylated to dopamine that is effective in stimulating the hypothalamus and hypophysis to release and increase the level of the growth hormone. Administration of L-Dopa could correct the retardation in the reproductive activity related to hypothalamic catecholamine deficiency in aging rats (Quadri et al., 1973).

It was also reported that feeding with substantial levels of L-Dopa significantly increased brain dopamine levels (Wilson and Meier, 1989) which affected the neuroendocrine-gonadal axis, resulting in changes of the body growth and reproductive conditions. The injection of L-Dopa in Japanese quails stimulated testicular and ovarian development as well as the production of follicle-stimulating hormone and luteinizing hormone (Prasad et al., 2007). Also, an intravenous injection of L-Dopa had a lowering effect on the cholesterol levels in the blood and eggs of the poultry (Meier and Wilson, 1998). An earlier study indicated that L-Dopa reduced gastrointestinal motility, and also the activity of lymphocytes (Shultz, 2007). As a pharmacologically active ingredient, the ingestion of large amounts of L-Dopa could be toxic (Pieriset et al., 1980). However, it was reported that levels of 0.1 to 0.4% L-Dopa inclusion had no detrimental effect on broiler hens (Omidiwura et al., 2016; Omidiwura et al., 2017; Omidiwura, 2019). Additionally, Omidiwura (2019) reported that an inclusion in the diet improved serum cholesterol profile and performance of the broiler chickens. It is worthy to note that each region of the gastrointestinal tract developed its special microbial profile, and this community became more complex with

increasing age of the chicken (Gong et al., 2002). Omidiwura et al. (2015) studied the influence of L-Dopa on the gut mucosa integrity of broiler chickens, and found that L-Dopa improved the villus to crypt depth ratio. Regarding the previous findings on the efficacy of L-Dopa in growth and reproductive performances in some poultry species, the present study aimed to investigate the effect of L-Dopa on the performance and intestinal microbial population of laying birds. Furthermore, this study evaluated the impacts of L-Dopa supplements on the cholesterol levels in blood, egg, and meat.

MATERIALS AND METHODS

Ethical approval

The project was in compliance with the policy document of the University of Ibadan, Nigeria, on research ethics and requirements for animal handling.

Study design

The study was conducted at the poultry unit of Teaching and Research Farm, University of Ibadan, Ibadan, Nigeria. One hundred and twenty laying birds (34-week-old) were obtained from the poultry unit of Teaching and Research Farm, University of Ibadan. The birds were tagged, weighed, and randomly allocated to five dietary treatments with eight replicates and three birds per replicate in a completely randomized design. Feed and water were supplied *ad libitum* to the hens in the cages during the study period. Recommended vaccines and other medications were administered, and routine management practices strictly adhered to the six-week study. The proximate composition of treatment diets is shown in table 1. Treatment 1 was the control diet, a basal diet without L-Dopa while treatments 2, 3, 4, and 5 contained the basal diets and L-Dopa at inclusion levels of 0.1%, 0.2%, 0.3%, and 0.4%, respectively. The L-Dopa used in the experiment was a pure extract from *Mucuna pruriens* seed.

Data collection

Feed intake

The feed intake was calculated by deducting the amount of leftover feed from the total feed given.

Feed conversion ratio

Feed Conversion Ratio (FCR) was calculated as the weight of feed consumed divided by the unit egg weight.

Hen-day egg production

Hen-day egg production was calculated as the total number of eggs produced during the period divided by total number of hens alive during the same period.

Lipid measurement

For egg yolk cholesterol quantification, three eggs per replicate were prepared according to the procedure described by Elkin and Rogler (1990). Total cholesterol, High-Density Lipoprotein (HDL), and total triglyceride concentration in the egg yolk were determined using the respective cholesterol assay kit. Low-Density Lipoprotein (LDL) and Very Low-Density Lipoprotein (VLDL) were calculated as described by Friedewald et al. (1972).

Using two hens per replicate, the serum cholesterol level was determined using serum cholesterol kit (Cell Biolabs' HDL and LDL/VLDL Cholesterol Assay Kit) according to the procedure described by Siedel et al. (1981). At the end of the experiment, two hens per replicate were sacrificed and breast meat samples were collected for analysis of cholesterol level in meat. Approximately 2 g of sample was saponified according to a modified version of the method described by Stewart et al. (1992).

Intestinal microbial load

Two birds per replicate were sacrificed and ileal digesta was collected for intestinal microbial load evaluation. The microbial count was done using the method described by Barrow and Feltharn (1993).

Statistical analysis

Data were analyzed using descriptive statistics and GLM procedure in SAS software (SAS Institute Inc., USA) and among treatments, means differences were separated using Duncan's multiple range test at P=0.05.

Table 1. Proximate chemical composition of control and treatment diets supplemented with L-Dopa

Item	Control diet	0.1% L-Dopa	0.2% L-Dopa	0.3% L-Dopa	0.4% L-Dopa
Metabolisable energy (kcal/kg)	2755	2761	2756	2755	2745
Dry matter (%)	93.14	93.11	93.05	93.20	93.21
Crude protein (%)	16.94	15.12	16.40	16.49	17.83
Crude fiber (%)	3.20	3.30	3.10	3.20	3.10
Ether extract (%)	7.30	8.20	7.50	7.50	7.50
Ash (%)	15.00	11.00	14.00	17.00	18.00
Nitrogen free extract (%)	50.70	55.49	52.05	49.01	46.78

RESULTS AND DISCUSSION

Production performance

The effects of L-Dopa supplemented diets on the performance of layers are shown in Table 2. There were no significant ($p>0.05$) differences in terms of FCR, feed intake, and egg weight among the treatments. This finding is in agreement with a study conducted by Omidiwura et al. (2017) who found that the feed intake, weight gain, and FCR were not significantly influenced by L-Dopa inclusion in broiler diets, but contradicts the findings of Vadivel and Pugalenthi (2010) and Omidiwura (2019). Also, this study revealed that the supplementation of L-Dopa in tested levels had no significant effect on the hen-day egg production of layers. This was not in line with the results obtained by Wilson and Meier (1989), who reported that feeding considerable levels of L-Dopa resulted in changes in body growth and reproductive performance. Bhatt and Chaturvedi (1993) also revealed that dopaminergic activity may affect the neuroendocrine-gonadal axis which could induce and maintain reproductive conditions.

Lipid profile

The effect of L-Dopa supplemented diets on blood cholesterol of laying birds is shown in Table 3. The L-Dopa inclusion had no effect on HDL and LDL of birds on the treatment diets. There were significant differences in triglycerides and total serum cholesterol ($p<0.05$). As the level of L-Dopa increased in the diet the level of triglycerides and cholesterol tended to decrease. Although birds fed 0.2%, 0.3% and 0.4% L-Dopa supplemented diets had similar concentrations of triglycerides and VLDL. Cholesterol levels of birds fed the control diet and 0.1% L-Dopa supplemented diets were similar but significantly higher than those fed 0.3% and 0.4% L-Dopa supplemented diets. This finding showed that the L-Dopa lowered the triglycerides and total serum cholesterol values. This may be a result of an increase in availability of norepinephrine and epinephrine, supplied by L-Dopa supplementation, which is involved in the hydrolysis of triglyceride. Carew et al. (2003) reported a reduction in serum cholesterol may be due to the adsorption of intestinal cholesterol by dietary fiber and rapid excretion of some other components in *Mucuna*. Similar reports by Jayaweera et al. (2010) suggested that *Mucuna pruriens* had a strong cholesterol-lowering effect in broilers.

The significant effect of L-Dopa on VLDL was in line with the findings of Iauk et al. (1993) who observed a reduction in blood cholesterol when birds were fed raw *Mucuna* based diets.

Egg cholesterol concentration

The effect of L-Dopa diet supplementation on egg cholesterol of laying birds is shown in Table 4. No significant differences in terms of the cholesterol, triglycerides, HDL, LDL, and VLDL were observed among the treatment diets. Contrarily, Prasad et al. (2007) reported that the incorporation of L-Dopa in the feed of poultry reduced the cholesterol content and also the ratio of saturated to unsaturated fatty acids in eggs.

Lipid profile of chicken meat

The lipid profile of meat produced by laying hens fed L-Dopa supplemented diets is shown in Table 5. There were no significant differences in terms of triglyceride concentration in meat. The HDL level in the meat of birds fed 0.1% L-Dopa supplemented diets was significantly higher than that those fed other diets. The Cholesterol and LDL concentration in birds fed the control diet was significantly higher than 0.1% and 0.3% L-Dopa supplemented diets but similar to other diets. The inclusion of L-Dopa at 0.3% dietary level reduced the level of cholesterol and LDL in the meat.

Intestinal microbial population

The result of L-Dopa supplemented diets on the intestinal microbial population of layers is shown in Table 6. Significant differences were observed in the microbial population of *E. coli* and *Lactobacillus.* Population of *E. coli* ($p<0.05$) was highest (7.25×10^4 cfu/mL) in birds on the control diet and was least (1.35×10^4 cfu/mL) in birds fed 0.3% L-Dopa diet. Treatment diets did not influence the total coliform count. *Lactobacillus* count was significantly ($p<0.05$) higher in birds on L-Dopa supplemented diets compared to the control diet. According to the results, the population of *E. coli* in the control diet was higher than that in the diet supplemented with 0.1%, 0.2%, 0.3% and 0.4% L-Dopa. This simply means that L-Dopa greatly reduced the population of *E-coli* in the laying birds. Moreover, L-Dopa inclusion in diet had a significant effect on the population of *Lactobacillus* in the layers. Intestinal *Lactobacillus*

population of birds on L-Dopa supplemented diets in 0.1% and 0.2% inclusion levels were found to be higher than that of chickens on other diets. The population of *Lactobacillus* was found to be the lowest in layers fed the control diet. This finding indicates that for an effective increase in the population of *Lactobacillus*, a diet needs little quantity of L-Dopa supplementation. The information on the influence of L-Dopa on animal's gut microbiota is very scanty. However, the study by Rekdal et al. (2019) on the treatment efficacy of L-Dopa in Parkinson's disease suggested possible interplay between L-Dopa and human gut microbiota. The current study showed the efficacy of L-Dopa in improving the gut integrity of laying hens.

Table 2. The effects of diets supplemented with graded levels of L-Dopa on the performance of laying hens

Characteristics	L-Dopa inclusion level (%)					SEM	*P*-value
	0.0	0.1	0.2	0.3	0.4		
Hen-day egg production (%)	83.93	82.44	79.76	84.62	81.15	3.26	0.83
Daily feed intake (g/hen)	101.87	98.65	103.18	102.52	100.48	1.75	0.39
FCR (g/feed/g/egg)	0.91	0.92	1.18	0.85	1.16	0.15	0.35
Egg weight (g)	58.26	57.49	60.65	60.73	61.76	1.92	0.48

SEM: Standard Error of Mean. FCR: Feed Conversion Ratio

Table 3. The effects of diets supplemented with graded levels of L-Dopa on lipid profile of laying hens

Parameter (mg/dL)	L-Dopa inclusion level (%)					SEM	*P*-value
	0.0	0.1	0.2	0.3	0.4		
Triglyceride	337.06^{a}	331.63^{a}	286.30^{b}	255.21^{b}	254.02^{b}	15.30	0.0003
HDL	10.23	7.67	12.97	8.031	6.15	2.52	0.3687
Total serum cholesterol	107.51^{a}	102.32^{a}	83.74^{ab}	48.09^{c}	64.65^{bc}	10.09	0.0007
LDL	20.24	26.33	20.01	9.31	15.08	4.85	0.1647
VLDL	67.41^{a}	66.33^{a}	57.26^{b}	51.04^{b}	50.80^{b}	3.06	0.0003

Means in the same row with different superscript letters are significantly ($p< 0.05$) different; SEM= Standard Error of Mean; HDL: High-Density Lipoprotein; LDL: Low-Density Lipoprotein; VLDL: Very Low-Density Lipoprotein

Table 4. Lipid profile of eggs from laying hens fed on diets supplemented with graded levels of L-Dopa

Parameter (mg/dL)	L-Dopa inclusion level (%)					SEM	*P*-value
	0	0.1	0.2	0.3	0.4		
Cholesterol	88.77	63.00	100.30	94.37	72.23	10.33	0.1084
Triglycerides	429.06	378.67	417.58	435.05	400.34	39.66	0.8509
HDL	5.58	5.79	11.38	9.37	6.92	2.68	0.5038
LDL	4.64	0.00	11.05	15.22	3.89	6.38	0.4829
VLDL	85.81	75.73	82.77	87.01	80.07	8.11	0.8638

SEM= Standard Error of Mean. HDL: High-Density Lipoprotein; LDL: Low-Density Lipoprotein; VLDL: Very Low-Density Lipoprotein

Table 5. The effects of diets supplemented with graded levels of L-Dopa on lipid profile of chicken meat

Parameter (mg/dL)	L-Dopa inclusion level (%)					SEM	*P*-value
	0	0.1	0.2	0.3	0.4		
Triglyceride	31.38	33.89	43.05	32.22	41.56	5.58	0.4569
Cholesterol	177.33^{a}	87.12^{b}	205.52^{a}	60.31^{b}	171.81^{a}	17.1	<0.0001
HDL	5.35^{b}	17.50^{a}	6.18^{b}	5.28^{b}	5.29^{b}	1.07	<0.0001
LDL	165.71^{a}	57.84^{b}	190.74^{a}	48.58^{b}	158.21^{a}	16.53	<0.0001

Means in the same row with different superscript letters are significantly different ($p < 0.05$). SEM: Standard Error of Mean; HDL: High-Density Lipoprotein; LDL: Low-Density Lipoprotein.

Table 6. The effect of L-Dopa supplementation on intestinal microbial population of 40 weeks-old layers

Bacterial count ($\times 10^4$ cfu/ml)	L-Dopa inclusion level (%)					SEM	*P*-value
	0	0.1	0.2	0.3	0.4		
E-coli	7.25[a]	3.38[b]	3.08[b]	1.35[c]	2.75[b]	1.97	<0.0001
Total Coliform	4.75	3.00	2.60	3.18	5.25	5.09	0.039
Lactobacillus	2.05[b]	6.98[a]	7.00[a]	5.50[a]	4.88[a]	3.74	<0.0001

Means in the same row with different superscript letters are significantly different (p<0.05). SEM: Standard Error of Mean.

CONCLUSION

The present study demonstrated that the dietary supplementation of L-Dopa, especially at 0.3% level, improved the lipid profile in the blood and meat. Also, this study showed the efficacy of L-Dopa in improving the gut integrity of laying hens. Dietary L-Dopa supplementation at 0.1 to 0.4% did not have any deleterious effect on the performance of layers. It is recommended that further studies should be conducted to investigate the influence of L-Dopa in other species of poultry.

DECLARATIONS

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Authors' contributions

All authors were involved in planning and execution of the study. Omidiwura B. R. O prepared the manuscript and others reviewed it.

Competing interests

No competing interest exists.

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Comparison of Overfeeding Influence on Slaughter Performance, Small Intestinal Physiology and Microbiota between Gang Goose and Tianfu Meat Goose

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ABSTRACT

The objective of this research was to compare the influence of overfeeding on slaughter performance and small intestinal physiology and microbiota between Gang goose and Tianfu Meat Goose. Fifty Gang geese were randomly divided into the control group and overfed group, as were fifty Tianfu Meat geese. All geese were slaughtered after 3 weeks of overfeeding. After overeating, the results indicated that the liver weight, villus height to crypt depth ratio in duodenum and ileum, and the activity of invertase and maltase enzymes of Tianfu Meat goose was higher than those of Gang goose. However, the subcutaneous adipose tissue weight of Gang goose was higher than that of Tianfu Meat goose. Moreover, the Enterobacterial Repetitive Intergenic Consensus-Polymerase Chain Reaction (ERIC-PCR) fingerprint indicated that the band number of intestinal bacteria in each small intestinal segment decreased in Gang goose and increased in Tianfu Meat goose. The *Enterobacter* had a higher gene expression in the jejunum of Gang goose than that of Tianfu Meat goose and *Escherichia coli* the gene expression decreased in the ileum of Tianfu Meat goose after overfeeding. In conclusion, Tianfu Meat goose's liver weight, small intestinal digestion and absorption capacity and microbiota homeostasis were much better than those of Gang goose. Therefore, Tianfu Meat goose is more suitable for *foie gras* production. The results of the present study will provide a reference for the use of goose breeding for overfeeding and the relationship between intestinal physiology and the mechanism of goose fatty liver formation.

Key words: Digestive enzyme, Goose, Intestinal flora, Intestinal morphology, Overfeeding

INTRODUCTION

China has the largest number of geese in the world and has a number of indigenous and developed goose breeds. Goose breeding is the primary determinant for *foie gras* production. Researching the production performance of these breeds under overfeeding is essential for the use of these breeds for *foie gras* production. The small intestinal is the most important place where nutrients have been digested and absorbed. The growth of the animal relies on the digestion and absorption of the nutrients. Therefore, it is important to investigate the influence on small intestinal structure morphology, digestive enzymes activity and flora to improve the yield of *foie gras* through overfeeding.

Different goose breeds have a very different performance from *foie gras.* Landes Goose is the special breed for *foie gras* production and has the best foie gras performance (Geng et al., 2016). Some researchers reported that there is a negative effect on the digestion, absorption, and utilization of nutrients when the waterfowls were overfed (Zhang et al., 2007). Different duck genotypes have different intestinal microbiota compositions (Vasai et al., 2014). A study of Langdes Goose indicated that the prosperity and diversity of the bacterial communities in the ileum and cecum decreased after overfeeding (Tang et al., 2018). Liu et al. (2016) reported that *Firmicutes* in the duodenal, jejunum and ileum were more densely distributed than in caeca, and their abundance was affected by overfeeding, in overfed geese.

At the present experiment, an influence of overfeeding on the morphology of the intestine structure,

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and the activity of digestive enzyme has rarely been reported in geese. Flora researches mainly focused on Landes goose and there have been few reports of other goose breeds. Gang goose is an excellent indigenous goose breed distributing in southwest China. It is characterized by large body size, fast growth rate and strong fat storage capacity. Tianfu Meat goose is a developed goose breed from the Sichuan Agricultural University with many outstanding achievements, for example, excellent egg-laying, fast growth rate and strong adaptability. In the present study, a comparison was performed of the influence of overfeeding on slaughter performance, the morphology of the small intestine structure, digestive enzymes activity and the flora between Gang goose and Tianfu Meat goose. The present study will provide a reference for the development and use of breed resources for *foie gras* production and provide a foundation for further investigation of intestinal health and the mechanism of goose fatty liver formation.

MATERIALS AND METHODS

Ethical Approval

All authors in the present study had to be approved by the Institutional Animal Care and Use Committee (IACUC) of Sichuan Agricultural University (Permit No. DKY-B20141401), and carried out in accordance with the approved guidelines. All efforts were made to minimize the animals suffering. The movement of birds was not restricted until the age of 90 days.

Birds, Experiment design and sampling

Fifty newborn male Tianfu Meat geese and fifty newborn male Gang geese were raised. At the age of 13 weeks, the geese of each breed were randomly divided into two groups, each containing a control group from Gang goose and an overfed group from Gang goose, the control group from Tianfu Meat goose and an overfed group from Tianfu Meat goose, each group comprising 25 geese. The control group geese were fed as before; the geese of the overfed group were overfed with boiled maize (5 minutes boiled maize, supplemented with 1% vegetable oil and 1% salt), the daily feed intake reached 600-750 gram (g) (4 meals per day) and given free access to water. The temperature and the relative humidity of the room were maintained at 24° centigrade (°C.), and 65 to 70%, respectively, until the end of the experiment. Geese and overfeeding places were provided by Xichang Huanong Poultry Compony, Sichuan, China. All geese were slaughtered of age if overfeeding. After 12 hours of fasting, the geese were killed. The geese were weighed before slaughter; the geese slaughter weight was weighed after slaughter. The samples of abdominal fat, subcutaneous adipose tissue and liver were collected and weighed immediately. The weight and length of each section of the intestine were measured and weighted after slaughtering and dividing. Five geese in each group were anesthetized with an intraperitoneal injection of sodium pentobarbital (60 milligram per kilogram (mg/kg)), and then immediately sacrificed for small intestinal tissue and content. Small intestinal tissue was washed in ice-cold saline (0.9% NaCl; 4 °C) and fixed in 4% formaldehyde-phosphate buffer to determine intestinal morphological, and the small intestinal contents were frozen in liquid nitrogen and then kept at −80 °C for digestive enzyme activity, Enterobacterial Repetitive Intergenic Consensus-Polymerase Chain Reaction (ERIC-PCR) and quantitative fluorescence Polymerase Chain Reaction (PCR) of the intestinal flora assay.

Small intestine morphological examinations

According to the methods of Cao et al. (2015), the cross-sections from the middle of the duodenum, jejunum, and ileum were preserved in 4% formaldehyde-phosphate buffer were prepared using standard paraffin embedding techniques, sectioned (5 μm) and stained with Hematoxylin and Eosin (HE), and then sealed by neutral resin size, and then examined by microscope photography system (Olympus, Tokyo, Japan). Each slice was observed and 5 visual fields were randomly scanned. Visual measurements of villus height, crypt depth, and intestinal wall thickness were measured 10 times and measured in each field of view with 40× magnification using imaging software (Image Pro Plus 6.0, Media Cybernetics, Bethesda, MD, USA)

Small intestine digestive enzymes activity examinations

According to the previous research (Cao et al., 2018), approximately 0.1 g of frozen small intestinal contents were accurately weighed, and placed in sterile Eppendorf tubes containing 9 volumes (weight / volume) of ice-cold normal saline (0.7 gram / milliliter). The mixture of small intestinal contents and normal saline was centrifuged at 4000 revolution / minute for 15 minutes at 4°C. The supernatant was then obtained and kept at −20°C, which was used for the study of the enzyme activity. The protein concentration of the samples was employed to calculate digestive activities, and assayed using a protein quantification kit (Bicinchoninic Acid

Assay, Beyotime Biotechnology, Beijing, China); the kits, which assayed the activity of amylase, maltase, invertase, chymotrypsin were provided by the Nanjing Jiancheng Bioengineering Institute (Nanjing, China). All operations were carried out according to the instructons.

Extraction of total DNA of small intestinal bacteria

The fecal DNA extraction kit (DP328, Tiangen, Beijing, China) was used to extract bacterial genomic DNA from small intestinal contents; then the concentration of the extracted DNA was determined with an ultraviolet spectrophotometer (Nano Drop 2000, Thermo, USA). The purity and quality of the entire DNA was assessed by spectrophotometric absorption at 260/280 nanometer (nm) and the integrity of DNA was detected by agarose gel electrophoresis and the results were observed and photographed in the gel imaging system (Bio-Rad, USA).

Detection and analysis of intestinal flora by ERIC-PCR

The ERIC-PCR assay was performed according to the method described by Staji et al. (2018). The total DNA extracted from small intestinal bacteria was used to perform ERIC-PCR amplification using primers (ERIC-1: 5'-ATGTAAGCTCCTGGGGATTCAA-3'; ERIC-2: 5'-AAGTAAGTGACTGGGGTGAGCG-3') (BGI, Beijing, China) (Wang et al., 2014). Amplification reactions were performed in a volume of 25 microliter (μL) containing 1.5 μL of each primer (25 picomole), 2 μL of 2.5 millimole (mM) dNTPs, 2.5 μL of 10 × Buffer, 2 μL of 25 mM $MgCl_2$, 0.5 μL of 5 U/μL Taq Polymerase (Sigma Aldrich, Shanghai, China), and 10 nano-grams (ng) of template DNA. The procedure was initially denatured at 94 °C for 7 minutes, followed by 30 denaturation cycles at 94 °C for 60 seconds, annealing at 52 °C for 60 seconds, and elongation at 65 °C 8 minutes, followed by a 10 minutes extension at 65 °C. The ERIC-PCR products were isolated by electrophoresis (100 voltage, 30 minutes) in agarose gel (1.5%), with the DNA marker DL5000 (Takara, Japan), and the electrophoresis results were observed and photographed in the gel imaging system (Bio-Rad, USA).

Detection of bacterial relative gene expression levels

Detection of small intestinal bacterial relative gene expression levels was performed according to the method described by Dewar et al. (2017). The quantitative fluorescence PCR was performed with the CFX 96 instrument (Bio-Rad, USA), using a Takara ExTaq RT-PCR kit and SYBR Green as the detection dye (Takara, Japan). The reaction system and procedure of the quantitative fluorescence PCR were operated according to the Prime Script ^{TM} RT reagent Kit manual operation (TaKaRa, Japan). The relative gene expression levels of genes were normalized to *β-actin* and *18S* using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Table 1 listed the quantitative fluorescence PCR Primers (BGI, Beijing, China).

Table 1. Primers for quantitative fluorescence quantitative PCR

Gene name	Primers	Product size (bp)	Reference
Escherichia coli	F: TACCCGCAGAAGAAGCACC R: CGCATTTCACCGCTACACC	215	Ren et al. 2012
Enterobacter	F: CATTGACGTTACCCGCAGAAGAAGC R: CTCTACGAGACTCAAGCTTGC	195	Lu, et al., 2019
Enterococcus	F: CCCTTATTGTTAGTTGCCATATT R: CTCTACGAGACTCAAGCTTGC	144	Bartosch, et al., 2004
18S	F: TTGGTGGAGCGATTTGTC R: ATCTCGGGTGGCTGAACG	129	Benjamino and Graf, 2016
β-actin	F: CAACGAGCGGTTCAGGTGT R: TGGAGTTGAAGGTGGTCTCG	92	Feng et al. 2012

F = forward primer, R = reverse primer, bp = base pair.

Data analysis

The gel imaging system was used to take pictures of the stained gel and Quantity One image analysis software (Bio-Rad, USA) was used for ERIC-PCR image analysis. Using SAS 9.2 (SAS Institute, USA), the comparisons of several groups were analyzed by General Liner Model

(GLM), and the means were assessed for significant differences using the SNK-q test. All results were expressed as means ± Standard Deviation and p-value below 0.05 was considered statistically significant.

RESULTS

Influence of overfeeding on slaughter performance

As shown in table 2, overfeeding could significantly increase body weight, slaughter weight, abdominal fat pad weight, subcutaneous adipose tissue weight, and the liver weight of Tianfu Meat goose and Gang goose ($p < 0.05$). After overfeeding, there was no significant difference between the body weight, slaughter weight, and abdominal fat weight of the two goose breeds ($p > 0.05$). However, the liver weight of Tianfu Meat goose was higher than that of Gang goose, and the subcutaneous adipose tissue weight of Gang goose was higher than that of Tianfu Meat goose after overfeeding ($p < 0.05$).

Influence of overfeeding on intestinal morphology

As shown in table 3, duodenal weight, duodenal length, jejunum weight, ileum weight, and ileum length obviously increased in both Tianfu Meat goose and Gang goose after overfeeding ($p < 0.05$). The villi height, the crypt depth, and the wall thickness of different small intestinal segments were measured, the results are shown in table 4. The wall thickness became thin except for the jejunum of the Gang goose after overfeeding ($p < 0.05$). Overfeeding increased the villus height of all small intestinal segments of two breeds ($p < 0.05$). Compared to the control group, the crypt depth of all small intestine parts in the overfed group of Tianfu Meat goose decreased ($p < 0.05$). The ratio of villus height to crypt depth of Tianfu Meat goose after overfeeding was higher than that of Gang goose in duodenum and ileum ($p < 0.05$).

Influence of overfeeding on digestive enzyme activity in small intestine

As shown in table 5, the activity of amylase, and chymotrypsin significantly in the duodenum of two breeds decreased after overfeeding ($p < 0.05$). The activity of maltase, invertase, and chymotrypsin increased significantly in the jejunum of Gang goose after overfeeding ($p < 0.05$). The activity of amylase, chymotrypsin, and maltase in the ileum of the Gang goose decreased significantly after overfeeding ($p < 0.05$). The activity of amylase and chymotrypsin decreased and the activity of invertase and maltase increased in the ileum of Tianfu Meat goose after overfeeding ($p < 0.05$). Compared to the overfed group of Gang goose, the activity of maltase and invertase in the duodenum and ileum of Tianfu Meat goose overfed group was higher ($p < 0.05$).

ERIC-PCR results of the small intestinal microbiota after overfeeding

Figure 1 indicates that the ERIC-PCR amplified bands, which were bigger than 500 base pair (bp), had the greatest difference between the control group and the overfed group in each intestinal segment of the overfed group. The number of amplified bands of intestinal microbiota in each intestinal segment of the Gang goose overfeeding group was less than that of the control group. In contrast, the number of amplified bands of intestinal microbiota was increased in each intestinal segment in Tianfu Meat goose after overfeeding.

The Quantity One Analysis Software was used to analyze the similarity of the ERIC-PCR map (Figure 2). In comparison, it was intuitive to see that the difference in the composition of the intestinal microbiota is between different intestinal segments. The similar index between the microbiota in different intestinal segments varied from 0.13 to 1.00. The cluster analysis indicated that force-feeding had an obvious influence on the intestinal microbiota in both goose breeds.

Overfeeding influence on the bacterial distribution in each intestinal segments

The Relative gene expression level of bacteria in the duodenum is shown in figure 3A. The gene expression of *Enterobacter* was higher in the Tianfu Meat goose than in the Gang goose ($p < 0.05$). There was no significant difference in *Escherichia coli* and *Enterococcus* gene expression between two breeds after overfeeding ($p > 0.05$). The relative gene expression level of bacteria in the jejunum is shown in figure 3B. The gene expression of *E. coli* and *Enterococcus* in the jejunum had no significant difference between the Tianfu Meat goose and the Gang goose after overfeeding ($p > 0.05$). *Enterobacter* gene expression of the Gang goose was higher than Tianfu Meat goose ($p < 0.05$). In figure 3C, the relative gene expression level of bacteria in the ileum was shown. There was no significant difference in *Escherichia coli* and *Enterococcus* gene expression in the ileum between two breeds after overfeeding ($p > 0.05$). However, *Escherichia coli* gene expression in Tianfu Meat goose decreased after overfeeding ($p < 0.05$).

Table 2. Comparison of overfeeding influence on the slaughter performance between Gang goose and Tianfu Meat goose

Characteristics	Control group of Gang goose	Overfed group of Gang goose	Control group of Tianfu Meat goose	Overfed group of Tianfu Meat goose
Alive body weight	4189.66 ± 436.31^{b}	6099.51 ± 805.15^{a}	4027.94 ± 468.44^{b}	6282.11 ± 566.29^{a}
Slaughter weight	3496.15 ± 1007.10^{b}	5533.63 ± 688.32^{a}	3385.19 ± 367.43^{b}	5679.26 ± 519.89^{a}
Abdominal fat weight	96.44 ± 29.73^{b}	366.51 ± 81.93^{a}	97.61 ± 35.97^{b}	428.13 ± 120.18^{a}
Subcutaneous fat weight	632.13 ± 46.38^{c}	1440.46 ± 184.84^{a}	440.52 ± 63.37^{d}	1240.96 ± 104.16^{b}
Liver weight	60.6 ± 6.04^{c}	266.54 ± 37.52^{b}	54.35 ± 8.07^{c}	397.94 ± 80.02^{a}

Values are means ± Standard Deviation (n = 25). $^{a, b, c, d}$ = values within the same row with different superscripts mean significant difference ($p < 0.05$). g = gram.

Table 3. Comparison of overfeeding influence on the weight and length of the small intestine between Gang goose and Tianfu Meat goose

Characteristics	Control group of Gang goose	Overfed group of Gang goose	Control group of Tianfu Meat goose	Overfed group of Tianfu Meat goose
Duodenal weight (g)	8.85 ± 1.22^{c}	24.74 ± 7.51^{a}	8.85 ± 0.93^{c}	15.32 ± 2.41^{b}
Duodenal length (cm)	37.75 ± 11.06^{b}	53.10 ± 10.96^{a}	32.38 ± 1.49^{b}	47.60 ± 5.14^{a}
Jejunal weight (g)	16.38 ± 3.16^{b}	24.98 ± 3.50^{a}	16.55 ± 0.94^{b}	23.00 ± 1.88^{a}
Jejunal length (cm)	59.75 ± 6.30^{b}	73.90 ± 5.55^{a}	74.00 ± 2.94^{a}	75.90 ± 4.39^{a}
Ileal weight (g)	16.20 ± 2.53^{b}	23.86 ± 3.45^{a}	15.15 ± 4.52^{b}	21.48 ± 1.89^{a}
Ileal length (cm)	59.25 ± 11.64^{b}	85.40 ± 9.04^{a}	70.75 ± 4.99^{b}	88.2 ± 18.72^{a}

Values are means ± Standard Deviation (n = 25). $^{a, b, c}$ = values within the same row with different superscripts mean significant difference ($p < 0.05$). g = gram, cm = centimeter.

Table 4. Comparison of overfeeding influence on small intestinal histology between Gang goose and Tianfu Meat goose

Intestinal section		Control group of Gang goose	Overfed group of Gang goose	Control group of Tianfu Meat goose	Overfed group of Tianfu Meat goose
Duodenum	Villus height (μm)	379.74 ± 57.58^{c}	426.67 ± 133.85^{b}	393.28 ± 102.49^{c}	526.25 ± 77.82^{a}
	Crypt depth (μm)	129.06 ± 28.95^{a}	123.44 ± 19.07^{a}	142.07 ± 42.84^{a}	98.05 ± 21.96^{b}
	Villus/crypt ratio	3.12 ± 0.87^{b}	3.64 ± 1.58^{b}	3.29 ± 1.96^{b}	5.56 ± 1.25^{a}
	Gut wall thickness (μm)	227.38 ± 125.70^{b}	189.54 ± 46.57^{c}	466.48 ± 169.32^{a}	237.54 ± 108.36^{b}
Jejunum	Villus height (μm)	301.54 ± 46.45^{d}	509.27 ± 136^{a}	395.15 ± 150.42^{c}	444.01 ± 215.55^{b}
	Crypt depth (μm)	129.96 ± 30.64^{b}	123.08 ± 23.28^{b}	188.47 ± 63.76^{a}	98.17 ± 214.23^{c}
	Villus/crypt ratio	2.49 ± 0.85^{b}	4.38 ± 1.66^{a}	2.35 ± 1.25^{b}	4.73 ± 1.00^{a}
	Gut wall thickness(μm)	235.14 ± 54.93^{b}	233.92 ± 53.29^{b}	408.99 ± 197.51^{a}	287.35 ± 131.71^{b}
Ileum	Villus height (μm)	332.35 ± 117.04^{b}	423.11 ± 89.58^{a}	303.07 ± 48.03^{b}	379.46 ± 46.2^{a}
	Crypt depth (μm)	106.01 ± 26.57^{b}	121.69 ± 27.61^{a}	99.69 ± 26.33^{b}	85.51 ± 18.93^{c}
	Villus/crypt ratio	3.44 ± 1.77^{b}	3.68 ± 1.22^{b}	3.20 ± 0.78^{b}	4.63 ± 1.07^{a}
	Gut wall thickness (μm)	442.86 ± 279.94^{a}	177.9 ± 41.13^{d}	338.15 ± 157.64^{b}	258.93 ± 139.6^{c}

Values are means ± Standard Deviation (n = 5). $^{a, b, c, d}$ = values within the same row with different superscripts mean significant difference ($p < 0.05$). μm = micrometer.

Table 5. Comparison of overfeeding influence on small intestinal digestive enzyme activity between Gang goose and Tianfu Meat goose

Intestinal section		Control group of Gang goose	Overfed group of Gang goose	Control group of Tianfu Meat goose	Overfed group of Tianfu Meat goose
Duodenum	Chymotrypsin (U / mgprot)$^{\Delta}$	371.57 ± 76.01^{a}	283.36 ± 120.69^{b}	374.09 ± 90.05^{a}	264.48 ± 59.91^{b}
	Amylase (U / mgprot)	6407.23 ± 317.92^{a}	4624.37 ± 241.80^{b}	6545.65 ± 291.29^{a}	5046.17 ± 524.72^{b}
	Invertase (U / mgprot)	1480.11 ± 239.26^{a}	782.59 ± 287.07^{b}	1536.89 ± 186.00^{a}	1394.96 ± 126.74^{a}
	Maltase (U / mgprot)	4700.95 ± 345.56^{b}	3088.29 ± 364.67^{b}	6508.92 ± 415.00^{a}	6038.36 ± 336.35^{a}
Jejunum	Chymotrypsin (U / mgprot)	157.72 ± 20.00^{b}	200.42 ± 56.57^{a}	137.36 ± 17.32^{b}	148.44 ± 16.27^{b}
	Amylase (U / mgprot)	5278.36 ± 261.53	5950.41 ± 532.03	5259.59 ± 189.00	5387.69 ± 195.89
	Invertase (U / mgprot)	1210.43 ± 386.95^{b}	2272.92 ± 128.92^{a}	738.97 ± 102.12^{c}	645.05 ± 228.88^{c}
	Maltase (U / mgprot)	5781.68 ± 750.08^{b}	7928.71 ± 918.54^{a}	3529.73 ± 560.71^{c}	3282.79 ± 544.71^{c}
Ileum	Chymotrypsin (U / mgprot)	140.84 ± 20.15^{a}	87.73 ± 1.94^{b}	219.74 ± 12.34^{a}	83.73 ± 26.62^{b}
	Amylase (U / mgprot)	5658.06 ± 183.10^{b}	4495.21 ± 196.86^{c}	7118.45 ± 168.24^{a}	5328.67 ± 165.74^{b}
	Invertase (U / mgprot)	869.08 ± 129.69^{c}	766.17 ± 147.12^{c}	1138.15 ± 156.47^{b}	1314.76 ± 136.02^{a}
	Maltase (U / mgprot)	4645.29 ± 323.60^{a}	3927.61 ± 195.76^{b}	2785.75 ± 167.45^{c}	4645.29 ± 223.61^{a}

Values are means ± Standard Deviation (n = 5). $^{a, b, c}$ = values within the same row with different superscripts mean significant difference ($p < 0.05$). $^{\Delta}$= The protein concentration of the samples was employed to calculate digestive activities, U / mgprot = the enzyme activity of samples (U) / the protein concentration of the samples (mg); mgprot = milligram protein.

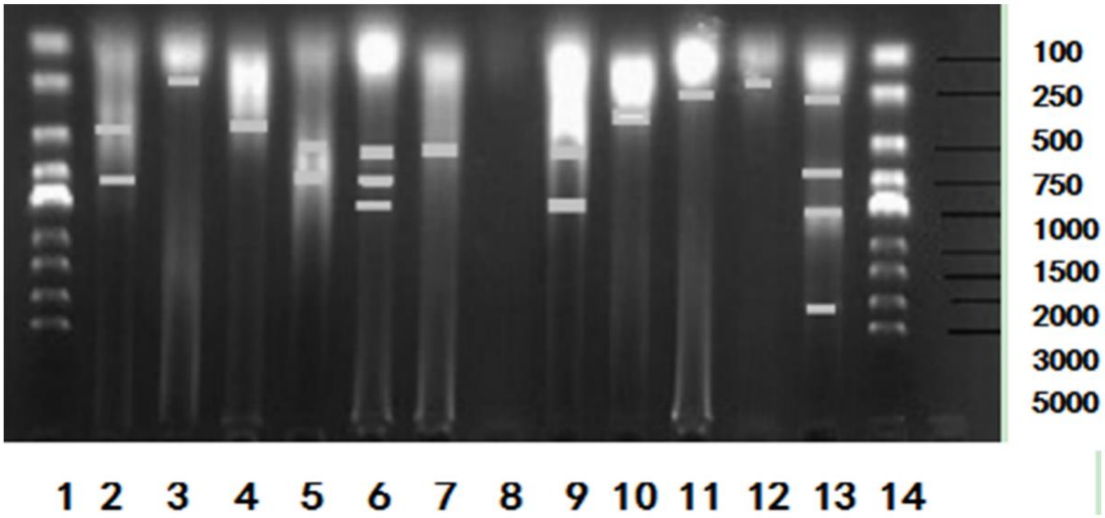

Figure 1. ERIC-PCR results of the intestinal microbiota in Gang goose and Tianfu Meat goose. 1 and 14: DNA marker DL5000; 2: duodenal microbiota of the control group of Gang goose; 3: duodenal microbiota of the overfeed group of Gang goose; 4: duodenal microbiota of the control group of Tianfu Meat goose; 5: duodenal microbiota of the overfeed group of Tianfu Meat goose; 6: jejunal microbiota of the control group of Gang goose; 7: jejunal microbiota of the overfeed group of Gang goose; 8: jejunal microbiota of the control group of Tianfu Meat goose; 9: jejunal microbiota of overfeed group of Tianfu Meat goose; 10: ileum microbiota of the control group of Gang goose; 11: ileum microbiota of the overfeed group of Gang goose; 12: ileum microbiota of the control group of Tianfu Meat goose; 13: ileum microbiota of the overfeed group of Tianfu Meat goose.

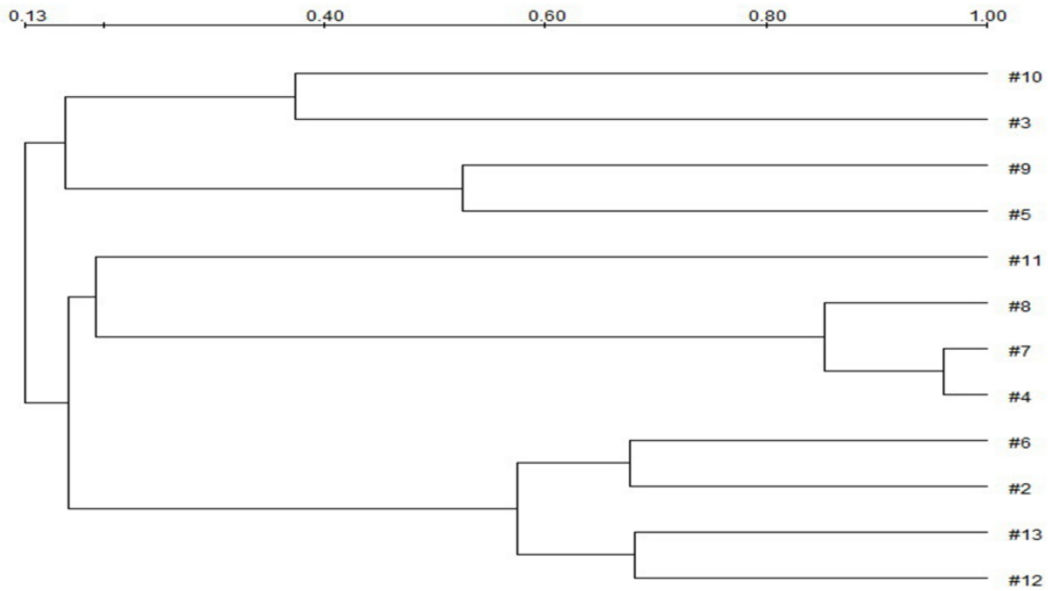

Figure 2. The dendrogram of ERIC-PCR in the intestinal microbiota of Gang goose and Tianfu Meat goose. #2: duodenum microbiota of the control group of Gang goose; #3: duodenum microbiota of the overfeed group of Gang goose; #4: duodenum microbiota of the control group of Tianfu Meat goose; #5: duodenum microbiota of the overfeed group of Tianfu Meat goose; #6: jejunum microbiota of the control group of Gang goose; #7: jejunum microbiota of the overfeed group of Gang goose; #8: jejunum microbiota of the control group of Tianfu Meat goose; #9: jejunum microbiota of the overfeed group of Tianfu Meat goose; #10: ileum microbiota of the control group of Gang goose; #11: ileum microbiota of the overfeed group of Gang goose; #12: ileum microbiota of the control group of Tianfu Meat goose; #13: ileum microbiota of the overfeed group of Tianfu Meat goose.

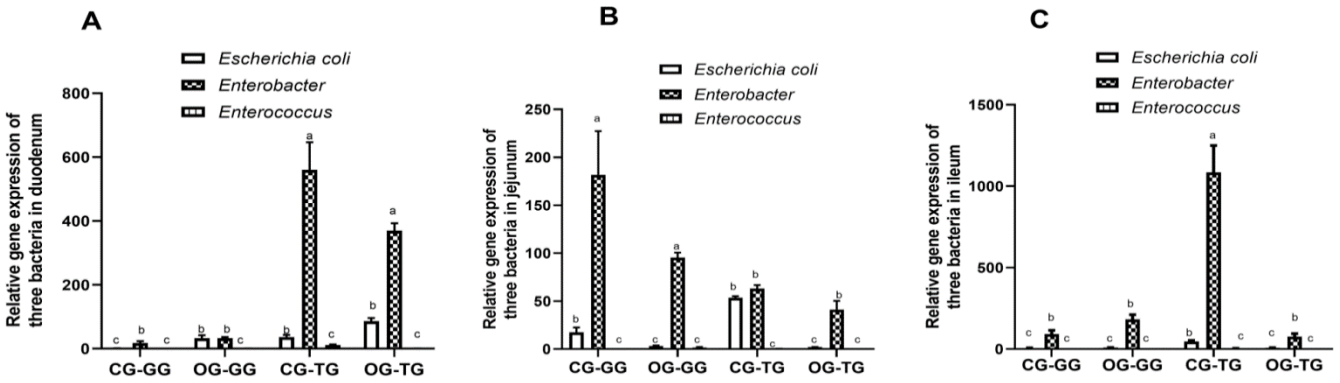

Figure 3. Relative gene expression level of bacteria in small intestine of Gang goose and Tianfu Meat goose. CG-GG: control group of Gang goose; OG-GG: overfed group of Gang goose; CG-TG: control group of Tianfu Meat goose; OG-TG: overfed group of Tianfu Meat goose. The experimental values are the means ± Standard Deviation (n = 5). [a, b, c] =The different lowercase above the bars represent significant differences ($p < 0.05$).

DISCUSSION

The main purpose of overfeeding was to increase body fat deposition and produce *foie gras* in ducks and geese. The impact of overfeeding on production performance has been well discussed in waterfowl (Wen et al., 2016). The present study found similar results that body weight and liver weight increased, and fat was deposited in the subcutaneous and abdominal areas. Overfeeding-induced synthesis of triglycerides (TG) in the liver and peripheral adipose tissue could lead to a significant accumulation of TG in the liver and in the peripheral adipose tissue (Davail et al., 2000; Liu et al., 2020). Overfeeding induced a significant increase in liver weight (Arroyo et al., 2017; Liu et al., 2019). In the present trial, liver weight and subcutaneous fat weight increased significantly in the overfed group. Tianfu Meat goose's liver weight was higher than that of Gang goose after overfeeding, indicating that Tianfu Meat goose performed better for *foie gras* production. The subcutaneous adipose tissue weight of Gang goose was higher than that of Tianfu Meat goose after overfeeding, indicating that Gang goose had better fat storage capacity in subcutaneous adipose tissue.

The small intestine is the main place where the nutrient is digested and absorbed, which plays an important role in the digestion, absorption, and transportation of nutrients (El Aidy et al., 2015; Lamot, et al., 2019). Mitchell and Smith studied three broiler strains with different growth rate. The result indicated that the fastest growing strain had the highest absolute intestinal weight and length (Mitchell and Smith, 1991). Abdelfattah-Hassan and El-Ghazaly (2019) reported that the small intestine length of the duck increased, the villi length increased after the addition of organic acids to duckdiet, and the digestion and absorption surface area increased. Significant differences in the duodenum weight and length, the jejunum weight, the ileum weight and length were observed in the present research, indicating that the intestine accelerated its own growth and development to adapt to the high-intensity digestion and absorption.

The integrity of the intestinal mucosa morphology is the basis for maintaining normal intestinal physiological activity in animals (Houshmand et al., 2012). The villus height, crypt depth, the ratio of the villus to crypt and wall thickness of the small intestine are important indicators to measure the function of small intestine digestion and absorption (Simon et al., 2019). The higher the height of villi, the greater the number of intestinal epithelial cells. The larger the contact area in which the small intestinal mucosa is in contact with nutrients, the greater the nutrients absorption (Caspary, 1992; Abdelfattah-Hassan and El-Ghazaly, 2019). The depth of the crypt reflects the rate of proliferation and maturity of the crypt cells. The crypt becomes shallow, indicating that maturation rate of crypt cell increased, the secretion function enhanced (Al-Fataftah and Abdelqader, 2014), and the crypt cells migrate and differentiate from the base of the crypt to the end of a villus from which the absorptive chorionic villi and supplement normal exfoliated chorionic epithelium (Yang et al., 2016). The higher ratio of villus height to crypt depth reflects higher nutrient absorption capacity (Wu et al., 2004; Li et al., 2019). The villus height, the ratio of villus height to crypt depth increased and the crypt depth became shallow in intestine of Tianfu Meat goose and some segments of Gang goose intestine after

overfeeding, which explained that the intestinal absorption capacity increased. Compared to the Gang goose, the ratio of villus height to crypt depth of the Tianfu Meat goose after overfeeding was higher in the duodenum and in the ileum, which indicated that the intestinal absorption capacity of the Tianfu Meat goose was higher than that of Gang goose. The thickness of the small intestinal mucous membrane and myometrium is closely related to the rhythmic contraction of the small intestine and the efficiency of the mechanical digestion of the chyme. However, in the present experiment, the intestinal wall thickness decreased after overfeeding. This was possibly due to the physical expansion caused by overfeeding, on the other hands the thickness became thin, which was beneficial for the diffusion absorption dependent on the nutrients concentration.

Animals have to digest all kinds of nutrients under the action of digestive enzymes. In the present experiment, amylase activity decreased, but some other digestive enzyme activities increased after overfeeding. These changes ensured that nutrients were fully utilized. When overfeed, a large number of carbohydrates obtained from geese were converted into blood sugar, which continuously supplied the liver with the synthesize TG, so that the rate of using blood glucose to synthesize TG in the liver of geese would increase dramatically. When the synthesis of TG in the liver exceeded the secretion of apolipoprotein and the rate of fatty acids β-oxidation, fat began to accumulate in the liver (Fournier et al., 1997). Invertase and maltase are disaccharidase and catalyze disaccharide that was created by cracking starch in glucose. The activity of invertase and maltase from Tianfu Meat goose was higher in the duodenum and ileum and lower in the jejunum than that of Gang goose after overfeeding. In combination with the present trail the total length of duodenum and ileum was longer than the length of jejunum, so that the activity of invertase and maltase from Tianfu Meat goose after overfeeding in the small intestine was higher than that of Gang goose. As previously mentioned, the ratio of villus height to crypt depth of the Tianfu Meat goose after overfeeding, was higher than that of the Gang goose. So, the digestion-absorption capacity of Tianfu Meat goose was higher than that of Gang goose. Thereby, more glucose was absorbed into blood and TG was synthesized in the liver of Tianfu Meat goose, and the liver weight of Tianfu Meat goose was higher than that of Gang goose after overfeeding.

In the present study, the ERIC-PCR fingerprint technique was applied to analyze the community structure of the small intestinal microbiota. The experimental results indicated that overfeeding decreased the band number of intestinal bacteria in each intestinal segment of Gang goose and increased the band number of intestinal bacteria in each intestinal segment of Tianfu Meat goose, indicating that the small intestinal microbial diversity was significantly influenced by overfeeding, which was similar to the finding reported by Vasai et al. (2014). The reduction in intestinal microbial diversity reduces intestinal homeostasis and resistance to pathogens (Mountzouris et al., 2015; Das et al., 2019). The overfeeding and intestinal homeostasis of Tianfu Meat goose was, therefore, higher than that of Gang goose. However, the bacterial species represented by the specific bands of each segment, still need to be investigated.

It has been found in the livestock, the *Escherichia coli* are pathogenic bacteria (Kittler et al., 2020; Xu et al., 2020; Yuan et al., 2020). *Enterococcus faecium* belongs to *Lactobacillus*, and is a normal beneficial bacterium in the gastrointestinal tract of mammals (Zhao et al., 2013; Shanmugasundaram et al., 2019). It was reported that when *Enterobacter cloacae B-29* is given orally to germ-free mice and providing the high-fat diet could induce obesity and insulin resistance in mice (Fei and Zhao, 2013). Therefore, *Enterococcus*, *Escherichia coli*, and *Enterobacteria* were selected for analysis in the present study, and the overfeeding effect on the gene expression level of the three bacteria in different intestinal segments of Gang goose and Tianfu Meat goose was measured. The general distribution ratio of *Enterococcus*, *Enterobacter*, and *Escherichia coli* was similar in each part of the intestine (*Enterobacter*>*Escherichia coli*>*Enterococcus*) in the groups of Gang goose and Tianfu Meat goose. Pathogenic bacteria entering the intestinal tract reduce the species and number of intestinal microorganisms by producing toxins and harmful substances and reducing the diversity of intestinal flora (Barman et al., 2008; Ayiku et al., 2020). In the present study, there was no significant difference in the gene expression of *Enterococcus*. The gene expression of *Escherichia coli* decreased after overfeeding in Tianfu Meat goose. Therefore, intestinal microbiota had better stability in Tianfu Meat goose. The *Enterobacter* gene expression of Gang goose was higher in the jejunum and ileum and lower in the duodenum than that of Tianfu Meat goose before overfeeding. The *Enterobacter* gene expression of Gang goose was higher in the jejunum and lower in the duodenum than that of Tianfu Meat goose after overfeeding, furthermore, the total length of the jejunum and ileum was longer than the length of the duodenum, so that the number of *Enterobacter* in the small intestine of the Gang goose

overfeeding group was greater compared to the Tianfu Meat goose overfeeding group. The subcutaneous adipose tissue weight of Gang goose was higher than that of Tianfu Meat goose after overfeeding. This was consistent with new research that *Enterobacter cloacae* administration induced subcutaneous fat accumulation in mice fed high-fat diet (Keskitalo et al., 2018).

CONCLUSION

In conclusion, the liver weight, lipid deposition in the liver, the digestion-absorption capacity, gut microbiota diversity, and gut microbiota stability of the Tianfu Meat goose after overfeeding were higher than those of the Gang goose. Thereby, Tianfu Meat goose is the better breed for *foie gras* production for prolonged overfeeding. The subcutaneous adipose tissue weight and small intestine *Enterobacter* gene expression of Gang goose were higher than those of Tianfu Meat goose after overfeeding, so Gang goose is suited to overfeeding in a short time for gain body weight and subcutaneous fat as an overfed duck for roast duck.

DECLARATION

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Competing interest

We declare that all authors have no conflict of interest in this manuscript.

Author's contributions

The experiments were conceived and designed by Chunchun Han, and Rongxue Wei and were performed by Fengjiang Ye, Fang He, Qi Song, and Xiangping Xiong. Wenlan Yang and Xianyin Zeng carried out the data analysis. Reagents, materials, and analysis tools were contributed by Ling Chen, Hongyong Xu, and Liang Li. Hehe Liu was administered the project. The draft manuscript was written by Chunchun Han and Rongxue Wei, and Rongxue Wei was also reviewed and edited the text of article. All authors were involved in the general discussions of the data and implications.

Consent to publish

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

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The acute toxicity assesment of Mospilan RP and Actara 25 WG for White Mice

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ABSTRACT

In this article an attempt was made to present the results of toxicity assessment of insecticides from the group of neonicotinoids, Mospilan RP (active substance acetamiprid) and Actara 25 WG (active substance thiamethoxam) in white mice. The aim was to investigate the acute toxicity of Mospilan RP and Actara 25 WG in white mice. The half-lethal dose (LD50) of Mospilan RP was found to be131.25 ± 34.12 mg/kg Body Weight (BW) per active substance and 656.25 ± 170.6 mg/kg BW per drug, and the Lethal Dose (LD100) was 200 mg/kg BW by the active ingredient (1000 mg/kg BW by drug). The half-life dose (DL50) of Actara 25 WG for white mice was 907.81 ± 24.03 mg/kg BW for the active substance, and 3631.24 ± 96.12 mg/kg BW for the drug; The lethal dose (LD100) was also 1200 mg/kg BW per active ingredient (4800 mg/kg BW per drug). The acute course of poisoning by Mospilan RP and Actara 25 WG in mice was characterized mainly by nervous disorders (lesions of the central and peripheral nervous systems), which is evidenced by the clinical features of poisoning including depression, convulsions, ataxia (impaired movement coordination), tremor and impaired breathing. It has been established that Mospilan RP belongs to the third toxicity class according to the Hygienic classification of substances by skin-resorptive toxicity (DL50> 2000 mg/kg).

Key words: Actara 25 WG insecticides, Acute toxicity, Insecticides toxicity, Mospilan PP, Neonicotinoids, White mice

INTRODUCTION

In Ukraine, Imidacloprid (included in the drug Confidor, 20% bp), Thiacloprid (Calypso drug, 48% bp), Acetamiprid (Mospilan drug, 20% pp), Thiamethoxam (Actor 25 WG preparation for the cultivation of vegetative crops), and neo-nicotinic insecticides are used as seed dressing agents for protecting of orchards (apple, plum), vineyards and field crops (corn, potatoes, tomatoes, cucumbers, hops, sugar beets, cereals, rapeseed, sunflower) (Bazaka et al., 2018).

In recent years, there has been an increasing amount of using anti-ectopatasite agents for dogs and cats in the veterinary practice which are based on two active substances; neonicotinoids -Nitenpyram (tablets Capstar, by Novartis Switzerland) and Imidacloprid, manufactured by the German company, Bayer Krop Sayens AG. The agents containing active substances, Imidacloprid or Thiamethoxam have been registered for home usage, public health or veterinary practice for the purposes of disinfestation. Granular baits such as Kvik Byte VG 10 (10% imidacloprid, Kvizda Agro GmbH, Austria), Agita (10% thiamethoxam, Novartis, Switzerland), and Adamant bait for flies (1% thiamethoxam, Russia) are allowed to be used for fly control in the livestocks (Smith et al., 2016).

It is believed that Neonicotinoids act selectively on the target sites, and its toxicity to animals and humans is not much significant (Ford and Casida, 2008). According to the chemical structures, Neonicotinoids are charecterized to the class of nitromethylene heterocyclic compounds. They are suggested to be highly efficient at low cost rates, and relatively harmless to non-target organisms and the environment (Abrieux et al., 2016). Despite its safty and efficacy, there is evidence of Neonicotinoid toxicity to bees and other pollinator insects.consenquently, there are some restrictions imposed on the use of Imidacloprid, Thiamethoxam and Clothianidin in several European countries. What is not yet clear is the untraceable side effects of Neonicotinoids

on the animals' body, due to their vast usage in the agriculture. Previous studies in Japan have demonstrated a high sensitivity of rats' brain receptors to the effects of Imidacloprid and Acetamiprid in low doses (Kimura-Kuroda et al., 2012).

In addition, a group of Turkish scientists have found the effects of Imidacloprid at doses (0.5; 2 and 8 mg/kg Body Weight (BW) for three months) on the reproduction system of male rats, indicating impaired mobility and morphological structure of sperms, as well as a significant decrease in the level of blood testosterone, activation of germ cell apoptosis, DNA fragmentation, and changes in the composition of fatty acids (Bal et al., 2012a).

Clotianidin at a dose of 32 mg/kg/day has been also found to have negative effects on the reproductive functions of male rats, as a result of a significant decrease in the absolute weight of the right testicle appendage and the seminal vesicles, a decrease in the concentration of sperms, testosterone and glutathione, as well as a decrease in the number of abnormal germ cell forms, and DNA fragmentation (Bal et al., 2012b). However, the same authors indicated that Clothianidin has a slight effect on the reproductive capacity of male rats in later publications (Bal et al., 2013).

Imidacloprid and Thiacloprid have been discussed to have embryotoxic effects. While the authors do not describe the type of changes in the reproductive organs of males and females, it is only mentioned that pesticides provoke abortions in the pregnant females (Basaka et al., 2018). Neonicotinoids are not believed to be absorbed by the skin due to their poor solubility in lipids, so acute toxicity is more evident when they are taken orally. Inacute cases of toxicity, neonicotinoids are considered mild and hazardous compounds. The prolonged exposure of the active substances in this group to laboratory animals is characterized by a general effect on the body with a predominant hepatotoxic effect (Felsot, 2001).

Acute oral toxicity of Acetamiprid (DL50) (active ingredient in the commercial drug Mopilan) for rats is 146–217 mg/kg BW, and for mice is 184–198 mg/kg BW. Acute dermal toxicity of > 2000 mg/kg BW to rats does not irritate rabbits' skin and their mucous membranes. Acute oral toxicity for quails is 180 mg/kg BW, and Lethal Concentration 50% (CL50) (24–96 years)is more than 100 mg/l, CL50 (3–6 h) for craps, and more than 1000 mg/l for Daphnias (Basaka et al., 2018).

The clinical features of intoxication are characterized by decreased activity, salivation, tremor, convulsions, ataxia, lateral recumbency, and in severe cases, hemorrhages in the lungs may cause death.

Acetamiprid is low toxic on the skin of rats for 24 hours. No clinical signs of intoxication and no irritant effects were found in DL50 in males and females at the doses greater than 2000 mg/kg BW.additionally, no macroscopic changes have been observed in the internal organs (Basaka et al., 2018). Oral toxic dose of Thiamethoxam (DL50) (active ingredient of the commercial preparation of Actara) is 1563 mg/kg BW in rats, and 871 mg/kg BW in mice. Its acute dermal toxicity (DL50 24 hours) is more than 2000 mg/kg parts per million (ppm) in rats. However, recent studies about to determine the toxicity of Neonicotinoids to bees by A.I. Illarionov (Illarionov, 2012) indicates a relationship betweenthe death of bees and contacting to the plants treated with Thiamethoxam or Imidacloprid on the day of drugs use. Moreover, several studies have been made to show the potential danger of neonicotinoids for bees (Decourtye et al., 2003; Decourtye and Devillers, 2010).

Overall, these studies provide important insights into the toxicological characteristics of Neonicotinoids. They will allow to expand and supplement the information on the potential danger of Neonicotinoids and will promote their safe usage in theagriculture and veterinary medicine.

MATHERIELS AND METHODS

Experimental studies on white mice were prepared according to the procedure of Toxicological Control of New Animal Protection guidelines (Denisenko, 2013), existing documents organizing the work with laboratory animals, and the principles of the European Convention on the Protection of Vertebrate Animals for experimental and scientific purposes ”(Strasbourg, 1986) and Art. 26 of the Law of Ukraine No. 5456-VI of 16.10.2012. “On the Protection of Animals from Cruelty”. Afterward, 104 nonlinear white mice weighing 18–20 gram were examined for acute toxicity of Mospilan RP and Actara 25 WG.

Previous study based its criteria on the method established by G. Kerber to measure the half-life dose. The direct results of the study were used to calculate the LD50. The mice were divided into equivalent groups (at least 6 mice in each one). Regarding the doses, they included LD50 and LD100 taken in different intervals for 4-5 times which were believed to be sufficient. The study was conducted in the vivarium of the Veterinary Medicine Faculty of the National University of Life and Environmental Sciences of Ukraine. Before starting the study, the mice were kept for 7 days during the adaptation period, in which a daily close observation of their clinical

condition was done. They have been hungry for 3–4 hours before the experiment started. The drug solutions were administered orally using a calculated probe, so that the solution volume did not exceed 0.4 mililiter (ml). The dose was calculated in milligram (mg) of active substance (AS) per 1 kg body weight (BW).

Twelve groups (6 groups of mice to determine the acute toxicity of Mospilan RP and 6 groups of mice to determine the acute toxicity of Actara 25 WG), and one control group (in each group n = 8) were set to conduct the studies on the determination of acute toxicity parameters.The mice of the experimental groups for Mospilan RP acute toxicity took 400; 200; 150; 100; 50 and 25 mg/kg body weight of the drug orally including the active substance.

the mice grouped for parameter determination of Actara 25 WG acute toxicity also took the drug orally at a rate of 1200.0; 1000.0; 875.0; 600.0; 437.5 and 218.8 mg/kg ppm in terms of AS. Mice in the control group were administered distilled water in a volume of 0.4 ml.

During the study, the clinical condition of the animals were carefully monitored for 14 days. The animals were constantly monitored on the first day. Theappearance, reaction to stimuli, changes in body position, behavior, food and water intakes, the intensity and type of locomotor activity, the condition of the skin and mucous membranes were taken into account, and the development of intoxication and their death were recorded.

As a result of the studies in mice, lethal (DL100) and semi-lethal (DL50) doses of the studied drugs were used.

RESULTS AND DISCUSSION

One of the first and most important steps for determining the toxicological characteristics of a substance is to study its acute toxicity. Thepurpose of studying acute toxicity is to find out the toxic effects of the drug with a single injection, and to determine lethal, toxic and non-toxic doses. Acute toxicity studies include the recording of specific and nonspecific symptoms of intoxication, the general pattern of poisoning, its onset, course and consequences. The main parameter of acute toxicity is the DL50 (which leads to the death of 50% of animals after single or multiple administration over a short period of time). The average lethal (semi-lethal) dose (DL50) of the toxic substance is determined in mg/kg BW, and administered orally (or subcutaneously, intravenously, intraperitoneally, subcutaneously) in weeks.

The available literature indicated that Acetamiprid and Thiamethoxam are low-toxic substances. The half-life of Acetamiprid (DL50) is 213 mg/kg BW for white rats, and 98 mg/kg BW for poultry. The DL50 of Thiamethoxam is 1563 mg/kg BW for white rats, and 576 mg/kg BW for poultry.

In order to find the lethal dose of Mospilan RP, the mice were separated to six experimental groups and a control one (n=8). Themice in the experimental groups were orally administered an aqueous solution of 0.4 ml of Mospilan RP at doses of 2000, 1000, 750, 500, 250, 125 mg/kg BW (which for DR was 400; 200; 150; 100; 50 and 25 mg/kg BW), and the control group took distilled water in a volume of 0.4 ml.

The response of the experimental groups to the drug was as same as the control one's, while the mice hada a reaction to the stress caused by manipulation, There was a slight increase in locomotor activity in the first few seconds, followed by sedation of the animals. Thirst was not noticed. Changes in the general condition of micewere found in 1–5 min in animals in groups 1, 2 and 3 after they took the drug (Mospilan RP at the dose of 2000, 1000, 750 mg/kg BW). the development of signs such as depression, rapid breathing, clonic-tonic convulsions, tremor, and bouncing were exactly observed. The mice moved individually in the cage, 5 to 8 minutes after drug administration.clinical signs of inhibition increased, and the animals were supine. The average time of death for animals in the 1st and 2nd experimental groups (all were killed) was 6.5 minutes. In the group 3, 5 out of 8 animals were killed within 5 hours. No further death was observed. Relating to the mice in the group 4, the signs described were less pronounced and longer, and 2 animals were killed within 12 hours. The animals in the experimental group 5 showed mild signs of impaired locomotor activity during the first 1.5 to 2 hours, which then disappeared. The condition of animals in the experimental group 6 did not differ from the animals in the group 7 (control group).

The results of the determination of the acute toxicity of Mospilan RP are given in table 1. The data in the table show a strong evidence of Mospilan RP toxicity to white mice on oral administration at the doses of 100 mg/ kg or more. From the data, it can be seen that the deaths of mice in the experimental groups range from two to eight mice.

$$DL_{50} = DL_{100} - \frac{\sum zd}{m}$$

DL_{100}: the dose of the substance investigated that caused the death of all animals in the group.

d: the interval between two adjacent doses; *z:* = arithmetic mean of animals killed by two adjacent doses; *m:* the number of animals in each group.

The results of these calculations are shown in table 2.

Table 1. Protocol and the results of the acute experiment with oral administration of Mospilan RP to white mice in 2016, based on the Department of Pharmacology and Toxicology of the National University of Life and Environmental Sciences of Ukraine.

Dose, mg/kg		Number of animals in a group	The number of animals dead					The average time of death (h)
			In days			all	in%	
By acceptable level (active substance)	By the за drug		1	2	15			
400	2000	8	8	-	-	8	100	0.1
200	1000	8	8	-	-	8	100	0.1
150	750	8	5	-	-	5	62.5	3
100	500	8	2	-	-	2	25	12
50	250	8	0	-	-	0	0	-
25	125	8	0	-	-	0	0	-

This data is sufficient to calculate the DL50.

Table 2. Toxicity assessment of Mospilan RP according to G. Kerber method on white mice weighing 18–20 grams in 2016, based on the Department of Pharmacology and Toxicology of the National University of Life and Environmental Sciences of Ukraine.

Dose, mg/kg (by active substance)	25	50	100	150	200
Survived	8	8	6	3	0
Dead	0	0	2	5	8
Z	0	1	3.5	6.5	
d	25	50	50	50	
Zd	0	50	175	325	
Σzd	550				

d: = the interval between two adjacent doses; z: arithmetic mean of animals killed by two adjacent doses

In the present study, m = 8; DL_{100} = 200 mg/kg of body weigh by Acceptable Level (AL);

DL_{50} = 200 – (550/8)=200 – 68.75 = 131.25 mg/kg of BW by AL; DL84 and DL16 were calculated using the two-point method, which examined two doses of substances selected in such a way that the frequency of the alternative effect was less than 50% in one case and higher in the other. The equation of a line passing through two points was used:

$$\frac{Y-Y_1}{Y_2-Y_1}=\frac{X-X_1}{X_2-X_1}$$

X1 and X2 are the values of the two doses tested;

Y1 and Y2 are the respective mortality rates.

$\frac{84-25}{62.5-25}=\frac{X-100}{150-100}$, thus we get X = DL_{84} = 178,67 mg/kg BW by active substance.

Similarly, DL16 is calculated.

$\frac{16-25}{62.5-25}=\frac{X-100}{150-100}$, thus we get X= DL_{16}=88,0 mg/kg of boy weight by active substance .

The confidence limits of the DL50 are found by the method of K. Miller and M. Tainter by the formula DL50 ± mt. In this case, 2σ = DL84 - DL16, and the mean error (m) of the half-lethal dose is

$$m=\frac{2\sigma}{\sqrt{N'\times 2}},$$

N 'is the total number of animals in groups in which at least one animal died or survived.

According to our data, 2σ = 90.7;

$$m=\frac{90,7}{\sqrt{16\times 2}}=\frac{90,7}{5,66}=16,02$$

The value of t is found from the Student's table, guided by a given value of P = 0.05, for the number of degrees of freedom f = N '- 1.

t = 2.13

Confidence limits DL_{50}

mt = 16.02×2.13 = 34.12

DL_{50} ± mt = 131.25 ± 34.12 mg/kg BW by active substance.

As a result of the experiment, it was found that the half-lethal dose of Mospilan RP insecticide preparation is DL50 ± mt = 131.25 ± 34.12 mg/kg BW for active substance (656.25 ± 170.6 mg/kg for the preparation).

Lethal dose, DL100 = 200 mg/kg ppm for active substance (1000 mg/kg for drug). Due to the certain acute toxicity indicators according to the toxicity classification of substances, the investigated insecticidal drug Mospilan RP belongs to class IV – low toxic (DL50 = 501–5000 mg/kg BW), according to the classification of chemicals in

accordance with the degree of danger (GOST 12.1.007-76) it belongs up to the third class (DL50 = 151–5000 mg/kg BW).

Six experimental groups of mice and one control group (n = 8) were formed to determine the acute toxicity parameters of Actara 25 WG preparation. The experimental drug was administered orally to the animals at a rate of 4800.0 mg/kg BW in the first group, 4000.0 mg/kg BW in the second, 3500.0 mg/kg BW in the third, 2400.0 mg/kg BW in the fourth; 1750 mg/kg BW in the fifth, and 875.2 mg/kg BW in the sixth experimental group, which for DR was 1200.0, 1000.0, 875.0, 600.0, 437.5 and 218.8 mg/kg BW, respectively. For the control group, distilled water in a volume of 0.4 ml was administered to the mice. The animals were monitored for 14 days, and their dynamics of changes in their clinical conditions were observed. On the first day, the animals were under constant surveillance.

According to the results of the experiment, a lethal (DL100) and a half-lethal (DL50) dose of the study drug was set up. The animals' response in the experimental groups to the drug introduction was as same as that of the animals of the control group when the placebo was taken, while the mice had a stressful reacrion to manipulation.. Although there was a slight arousal for the first few seconds, the mice settled down after a while. After the grug was administered, changing in their general conditions were observed for 5 to 10 minutes in the mice of groups 1 and 2 (at doses of 4800, 4000 mg/kg BW). Frequent breathing, convulsions, and muscle tremors were noticed, and the micebounced and moved individually in the cage. Over time, in 10–20 minutes after the drug used, signs of depression increased and the mice took a supine position.

The average death time of the animals in group 1 (all killed) was 15 minutes. In group 2, 7 out of 8 mice were killed within an hour. In group 3, 2 out of 8 mice were killed in 2 hours. No further death was observed. In the group 4, the signs described were less pronounced and prolonged, and no death was observed. In the mice of group 5, there was a mild inhibition during the first 1.5–2 hours. There were no significant differences in the condition of the mice in group5 and group 7.

Table 3 indicates the toxic effects of Actaras 25 WG when administered orally to white mice in doses of 875 mg/kg BW in DR. As a result, the deaths of mice in the experimental groups ranges from two to eight mice. This data is sufficient to enable the DL50 to be calculated.

The results of calculations of the half-life dose of Actara 25 WG in accordance with the results obtained from the experiment (Table 3) using the Kerber method are shown in table 4.

Table 3. A protocol of the results of the oral administration of Actara 25 WG to white mice in 2016, basis on the Department of Pharmacology and Toxicology of the National University of Life and Environmental Sciences of Ukraine.

Dose, mg/kg		Number of animals in a group	The number of animals dead					The average term of death (h)
			In days			all	in%	
By active substance	By the drug		1	2	15			
1200	4800	8	8	-	-	8	100	0.25
1000	4000	8	7	-	-	7	87.5	1
875	3500	8	2	-	-	2	25	2
600	2400	8	0	-	-	0	0	-
437.5	1750	8	0	-	-	0	0	-
218.8	875.2	8	0	-	-	0	0	-

This data is sufficient to calculate the DL50.

Table 4. Toxicity determination of Actara 25 WG by G. Kerber method

Dose, mg/kg (by AL)	600	875	1000	1200
Survived	8	6	1	0
Dead	0	2	7	8
Z	1	4.5	7.5	
d	275	125	200	
Zd	275562 1500			
Σzd	2337.5			

d = the interval between two adjacent doses; *z* = arithmetic mean of animals killed by two adjacent doses; AL: Acceptable Level

m = 8; DL_{100} = 1200 mg/kg BW by active substance;

DL_{50} = 1200 – (2337,5/8) = 1200 – 292.19 = 907.81 мг/кг мт.

The average error (m) of the half-lethal dose is equal:

$$m = \frac{2\sigma}{\sqrt{N' \times 2}},$$

N' is the total number of animals in the groups in which at least one animal has died or survived.

In the present case (by the method of K. Miller and M. Tainter) $2\sigma = DL_{84} - DL_{16}$. The indicators DL84 and DL16 are calculated using the two-point method, in which substances were studied in two doses selected in such a way that the frequency of the alternative effect was less than 50%in one case and greater in the other. the equation of a line passing through two points is used:

$$\frac{Y - Y_1}{Y_2 - Y_1} = \frac{X - X_1}{X_2 - X_1},$$

X1 and X2 are the values of the two doses tested;

Y1 and Y2 are the respective mortality rates.

After calculations on the respective parameters of the mice in the experimental groups 2 and 3, it was found that DL84 = 993 mg/kg for active substance, and DL16 = 857.0 mg/kg of metric tons for AS. So if 2σ = 993 - 857 = 136, then: $m = \frac{136}{\sqrt{16 \times 2}} = \frac{136}{5.66} = 24.03$

DL_{50} = 907.81 ± 24.03 mg/kg BW by active substance.

DL_{50} = 3631.24 ± 96.12 mg/kg of BW.

The half-lethal dose of the Actara 25 WG insecticide drug for white mice was DL_{50}±mt = 907.81±24.03 mg/kg BW (3631.24 ± 96.12 mg / kg BW); lethal dose DL_{100} = 1200 mg/kg BW for active substance and 4800 mg/kg BW for drug.

On the basis of certain indicators of acute toxicity according to the classification of toxic substances , the investigated insecticidal drug Aktara 25 WG belongs to the class IV – low toxic (DL50 = 501 - 5000 mg/kg m.); according to the classification of chemicals about the degree of its danger (GOST 12.1.007-76), it belongs to up to the third class (DL_{50} = 151 - 5000 mg/kg BW).

According to the toxicity classification of substances,the results of half-lethal dose determination of Mospilan RP and Actara 25 WG when they are administered orally once to white mice indicate that the investigated insecticides are low-toxic. The DL50 values of the investigated preparations for white mice were 656.25 ± 170.6 mg/kg BW for Mospilan RP (131.25 ± 34.12 mg/kg BW for DR), and 3631.24 ± 96.12 mg/kg BW for Actara 25 WG (907.81 ± 24.03 mg/kg BW for DR), which was somewhat dissimilar from the DL50 of active substances for white mice determined by another author (Tomlin, 2009).

The physicochemical properties of the auxiliaries and formants included in the preparations could be the reason of differences in the results.

The acute course of poisoning by Mospilan RP and Actara 25 WG in mice was characterized mainly by nervous disorders (lesions of the central and peripheral nervous systems), as evidenced by the clinical features of poisoning, including depression, convulsions, ataxia (impaired movement coordination), tremor and impaired breathing. Ataxia could be a consequence of impulse transmission inhibition in the neuromuscular synapses (curative action), and interneuronal impulse transmission in the central nervous system. It is found that, at high concentrations of Acetylcholine in the blood and the effector organs, the inhibitory reaction developing as a result of the mediator's action at the central synapses is determined by the nervous system. Then, the inhibition extends to every part of the central nervous system, which is evidenced by the disorder of movement coordination in animals. The onset of tremor, and also convulsive syndrome in more sever cases result from central nervous system over-excitation. The leading role in this case was the developement of tissue hypoxia to which brain cells are most sensitive (Lavryshyn et al., 2016).

Respiratory disorders associated with the development of toxic encephalopathy and disorders of the regulatory function of the central nervous system are a pathogenetic basis for the development of hypoxia, which leads to a dysfunction of all vital organs and systems (Livanov, 2008).

Considering the mechanism of action of Neonicotinoids on insects, specifically their interaction with H-cholinoreceptors (Matsuda et al., 2001; Jones and Sattelle, 2010), it was assumed that the consequence of the excessive intake of nicotinic receptor agonists in animals was a violation of the efferent impulse transmissions in vegetative ganglia, cerebral ganglia, cerebral ganglion of synapses, in chemoreceptors and generation of afferent impulses in the carotid glomerulus, as well as in the interneuron transmission of excitation in the central nervous system. In this case, there was a two-phase effect on H-cholinoreceptors. the excitation stage was changed by the inhibition effect.

Overally, the pattern of acute toxicity of Mospilan RP and Actaraa 25 WG in this study was shown to have the

same results as the other reaserch (Mohamed et al., 2009; See et al., 2009; Kammon et al., 2010; Iyadurai et al., 2010; Mossa et al., 2018).

CONCLUSION

According to the classification of substances by toxicity, the studied drugs Mospilan RP and Actara 25 WG are found to be in class IV – low toxic. Furthermore, according to the classification of chemicals in accordance with the degree of danger, they belong to up to class III, because the half-lethal dose of Mospilan RP for white mice was DL_{50} ± mt = 656.25 ± 170.6 mg/kg BW, and for Actors 25 WG was DL_{50} ± mt = 3631.24 ± 96.12 mg/kg BW. It has been established that Mospilan RP belongs to the third toxicity class according to the Hygienic classification of substances by skin-resorptive toxicity (DL50 > 2000 mg/kg).

DECLARATIONS

Author's contribution

Volodymyr Dukhnytskyi conducted the research, collected data and performed the statistical analysis. Vasily Sokolyuk, Petro Boiko, Irina Ligomina and Vladimir Goncharenko wrote the manuscript. All authors have read and approved the final manuscript.

Competing interests

The authors have declared that no competing interest exists.

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Detection of *Campylobacter jejuni* among Commercial Broiler Chickens in East-Coast Malaysia

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ABSTRACT

Human campylobacteriosis is associated with contaminated foods and drinks or direct contact with the source of organisms. Broilers are the main reservoir of *Campylobacter* as well as the primary source of meat in many countries. The microorganism load in broilers is converted into the rate of food-related campylobacteriosis. The present study aimed to determine the *Campylobacter jejuni* colonization load in commercial broiler chickens in east coast Malaysia. Cloaca swabs were taken after consent from the owners of selected broiler farms in Kelantan, Malaysia. Swabs were kept in Amie's transport media before being placed in enrichment broth. The DNA was extracted directly from the broth and specific Polymerase Chain Reaction (PCR) was performed, which targeted hippurate hydrolase (hipO) gene of *C. jejuni*. Three broiler farms from different districts in Kelantan were identified. A total of 120 cloacal swabs were analyzed, 20% (24/120) confirmed by PCR-positive for hipO gene when the amplified product with the band size of approximately 344 bases per was visualized on agarose gel. It is concluded that the prevalence of *C. jejuni* colonization among living broiler flocks in Kelantan was at a low level. However, further studies with bigger sample size and involvement of more farms are needed.

Key words: Broiler chicken, *Campylobacter jejuni*, Cloaca swab, Polymerase Chain Reaction, Hippurate hydrolase

INTRODUCTION

More than 30 *Campylobacter* species has been identified to date (Pitkanen and Hanninen, 2017). However, *Campylobacter jejuni* and *Campylobacter coli* are the two predominant species that cause gastrointestinal infections, based on analysis of 1020 inflammatory stool samples (Ranjbar et al., 2017). The person acquired the infection after consuming contaminated food or water or after having direct contact with the source. Since the 1970s, the broiler has been the main cause of campylobacteriosis, which has been identified in connection with human nutrition. The presence of high *Campylobacter* rate in broiler chickens and their carcasses is one of the major risk factors for human acquisition (Marotta et al., 2015; Skarp et al., 2016).

Broiler chickens, specifically *Gallus gallus domesticus*, have a significant contribution to meat production worldwide (Skarp et al., 2016). It was estimated that around 20 to 30% of campylobacteriosis in the European Union was related to chicken meat consumption, as reported by European Food Safety Authority. Another report indicated 50–80% infections might be attributed to the entire chicken reservoir. It is also emphasized that consumption of broiler meat production contributed to campylobacteriosis in different regions (Skarp et al., 2016; Ranjbar and Babazadeh, 2017).

There are limited reports on *Campylobacter* species in living broiler chickens. Furthermore, no data are available for the Kelantan District, where the study is being conducted. To reduce the incidence of campylobacteriosis in human, understanding the colonization status or contamination of poultry by *Campylobacter* species is very necessary for intervention strategies and monitoring programs in the primary broiler production chain. Thus, the aim of this study was to

determine the colonization load of *C. jejuni*, which is the predominant species in local broiler farms.

MATERIALS AND METHODS

Ethical approval

The study was approved by the Institutional Animal Care and Use Committee of the Universiti Sains Malaysia (USM) (USM/IACUC/2018/ 113, 928).

Sample collection

Three commercial broiler chicken farms were identified in the three locations in Kelantan state on the east-coast Malaysia. The farms had about 2000 to 6000 broiler each. One hundred cloacal samples were taken from the first two farms (50 samples from each farm) and another 20 samples were taken from the last farm. The samples were taken according to the availability of the broiler during sampling and with consent of owners. The samples were taken using a moistened sterile cotton swabs and immediately kept in Amie's transport medium. Within 1-2 hours, the swabs were placed in 3 mL of Blood Free *Campylobacter* broth (Oxoid, France) and incubated at 37^{o}C for 48 hours to enhance *Campylobacter* growth.

PCR

The DNA extraction was performed using a boiling lysis method directly from the broth according to the published protocols with modifications (Singh et al., 2011). The boiling method is a classic alternative procedure of DNA extraction using TE buffer to lyse the cells, followed by rapid boiling at 95^{o}C to obtain the DNA. About 100 µL of broth was mixed with 100 µL of sterile distilled water and centrifuged at 10,000 rounds per minute (rpm) for three minutes. The supernatant was removed, and the remained pellets were suspended again with 100 µL TE buffer. The samples were vortexed to mix them up and boiled at 95^{o}C for 10 minutes to lyse the bacteria cells. The samples were centrifuged again at 10,000 rpm for three minutes. The supernatant was used directly as a template in a PCR reaction. A set of specific forward (5'-GAC TTC GTG CAG ATA TGG ATG CTT-3') and reverse (5'-GCT ATA ACT ATC CGA AGA AGC CAT CA-3') primers were used, targeting hippurate hydrolase (hipO) gene characteristic of *C. jejuni*. The primers were proved to be specific for *C. jejuni* (Persson and Olsen, 2005). The conventional PCR was performed in a total reaction volume of 25 µL containing 12.5 µL of 2 x PCR buffers, 4.3 µL of PCR water, 1.6 µL of 5 µM of forward and reverse primers respectively and 5 µL of extracted DNA, which served as template DNA. The reactions prepared were run in the thermocycler with an initial denaturation of 6 minutes at 94^{o}C followed by 35 cycles of 94^{o}C (50 seconds), 57^{o}C (40 seconds) of annealing, and 72^{o}C (50 seconds) extension with a final extension at 72^{o}C for 3 minutes. The Polymerase Chain Reaction **(**PCR) products were analyzed on 1.5% agarose gel and a fragment with a size of 344 base per (bp) was identified as hipO gene.

RESULTS AND DISCUSSION

The PCR was considered positive for hipO gene when the amplified product with the band size of approximately 344 bp was visualized on agarose gel. A total of 120 cloaca swabs were taken and analyzed. Twenty-four (20%) samples were positive. A representative of the PCR analysis as shown in figure 1. *C. jejuni* was regarded as an intestinal flora of chickens but was recognized to cause campylobacteriosis in humans. Many researches were conducted to correlate the occurrence of the organism with the contamination and infection of human food (Skarp et al., 2016). The contamination was linked with the various stages of chicken meat production and processing chain until the consumption of chicken meat in the community. The production chain of chicken meat varied between regions, industrialized and developing countries. This would determine the contamination rate during the processes. The *Campylobacter* contamination in poultry farms subsequently reflected the contamination rate of carcasses and meat (Kaakoush et al., 2015; Skarp et al., 2016). In the recent study, the PCR was able to detect 24 (20%) positive samples among the living broilers compared to the previous data from Thailand, in which the prevalence of *Campylobacter* species in broilers ceca was reported 11.2% and 11 out of 98 ceca samples taken in the slaughterhouse were positive by culture method (Chokboonmongkol et al., 2013). Researchers postulated that the prevalence after avian influenza outbreaks declined drastically in 2004, with strict biosecurity measures in the broiler farms and the administration of antimicrobials were implemented to chickens less than 3 days old. Another study in 2017 showed a high prevalence and isolation rate around 40-60%, depend on the region and climate during the sampling (Prachantasena et al., 2017). A higher isolation rate was noted in Ecuador when the study was conducted from 2013 to 2014. About 64 to 70% *Campylobacter* species were isolated from ceca in the slaughterhouse. The chickens usually came from one identified breeding farm, but from different batches

(Vinueza-Burgos et al., 2017). Another high isolation rate was reported from Austria in 2015 and during the study of 15 broiler flocks, 80% (n = 12/15) were positive for *Campylobacter* species by some detection methods. At least samples were positive by one of the detection methods (lateral flow, direct plating, Bolton broth enrichment and real-time PCR). *C. jejuni* was detected as predominant species in poultry flocks (Schallegger et al., 2015). A study in the United Kingdom noted the *Campylobacter* species being colonized at the slaughterhouse and the prevalence was almost 95.9%. However, the methods of detection influenced the outcome of the study (Rodgers et al., 2017).

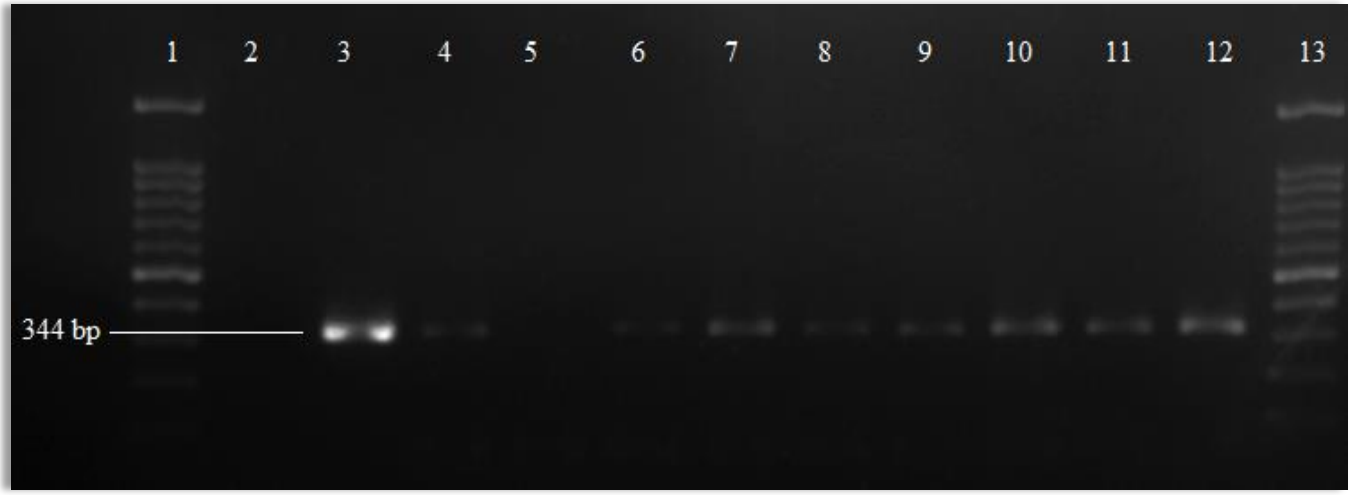

Figure 1. Conventional PCR analysis of hipO gene from broilers in east-coast region of Malaysia. Lanes 1 and 13: DNA Ladder (100 bp); Lane 2: Negative control; Lane 3: Positive control (344bp); Lanes 4–12: Cloaca samples (Lane 4 and Lanes 6-12: Positive; Lane 5: Negative)

CONCLUSION

It is concluded that the colonization load of *C. jejuni* in local broiler farms is still low in study locations compared to other reports. However, further studies with larger sample sizes and involvement of more farms in different locations are very necessary. There is a lot of routs for improvement in order to prevent human campylobacteriosis. The control and prevention program requires a multidisciplinary approach that includes both food-chain production and the consumer as the end-users. Regular monitoring of *Campylobacter* species load in commercial broiler chickens is necessary for successful control of human campylobacteriosis in connection with meat production.

DECLARATIONS

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Competing interests

The authors have declared that no competing interest exists.

Authors' contributions

Nur-Aziera-Aina CMN had performed the field-work, laboratory analysis and drafting the manuscript. Nur-Syafiqah MN had also performed the field-work and laboratory analysis, and Zaidah AR supervised the field-work, monitored the laboratory analysis, drafted the manuscript. All authors approved the final revised manuscript.

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Effect of Dietary Supplementation of Palm Oil Waste Fermented with *Phanerochaete chrysosporium* and *Neurospora crassa* on Performance and Some Egg Characterizes of Laying Japanese Quails

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ABSTRACT

The aims of present study was to determine the effect of dietary supplementation of palm oil waste fermented with *Phanerochaete chrysosporium* and *Neurospora crassa* (POWF) with ratio 1:1 on performance and some egg characterizes of laying Japanese quails. This experiment used 200 quails (*Coturnix-coturnix japonica*) aged 20 weeks, with 70% egg production. This study used an experimental method with a completely randomized design (CRD) with four treatments (0%, 8%, 16%, and 24% POWF and five replications). The results of this research showed that the use of POWF in the diet was not affected to feed consumption, daily egg production, egg mass, egg weight, and feed conversion. But, a significant difference in blood total cholesterol, LDL, HDL, egg cholesterol, and index egg yolk color was obsereved, and no significant effect on triglycerides and egg yolk fat. In conclusion, feeding the mixture of palm oil waste fermented with *Phanerochaete chrysosporium*, and *Neurospora crassa* with ratio1:1 can be used up to 24% in laying quail rations, without any adverse effect on egg production. It may decrease the blood cholesterol and egg yolk cholesterol.

Key words: Eqq quality, *Neurospora crassa,* Palm oil waste, Performance, *Phanerochaete chrysosporium,* Japanese quail

INTRODUCTION

The composition of the feed positively influences high egg production, especially in quails (Suwarta and Suryani, 2019; Bejar, 2017). The provision of quality and continuous feeding, especially for poultry, still has problems, because of difficulty in obtaining feed ingredients that do not compete with human needs and high feed prices. About 60-70% of production costs is attributed to the cost of feeding (Thirunalaisamy et al., 2019). The main reason of this expensiveness, especially in developing countries, is due to a great amount of imported ingredients. High feeding costs can be overcome by the use of alternative feeds such as palm oil waste (Sugiharto et al., 2018). According to the TCESI (2017), the area of oil palm planting in Indonesia (2017) is 12.037.677 hectares, producing 7.071.877 tons in 2017. Each hectare of palm oil can produce 4 tons of oil per year, which is obtained around 16 tons of fresh fruit bunches. Based on these data, the palm oil waste can be used as a potential alternative food ingredients because of its abundant availability.

Based on the results of Nuraini et al. (2017), palm oil sludge has nutritional contents; namely crude protein (11.30%), crude fiber (25.80%), lignin (19.19%), cellulose (16.15%), Cuprum (28,169 ppm), and metabolic energy (1550 kcal/kg). According to Nuraini et al. (2019), a palm kernel cake has nutritional contents; crude protein (16.30%), crude fiber (20.42%), lignin (14.19%), cellulose (13.26%), Cuprum (44,62 ppm), and metabolic energy (2017.87 Kcal/kg). According to Nuraini et al. (2017), palm kernel cake can be used in 10% of the ration , and palm oil sludge can only be given as much as 5% in broiler rations (Djulardi et al., 2018). Palm oil sludge and palm kernel cake have a limiting factor in the form of crude fiber content; especially that lignin, and cellulose are high consentrated, so it is difficult for the livestock to digest them (Nuraini et al.,2019).

Fermentation using *Phanerochaete chrysosporium* fungi can produce enzymes of ligninase and cellulase (Wang, 2016). Nuraini et al. (2015) reported that *Neurospora crassa* fungi are orange molds, which provide high β-carotene compared to other carotenogenic fungi

that have been isolated from corn cobs. β-carotene can reduce cholesterol by inhibiting the work of the enzyme Hydroxymethyl Glutaryl-COA reductase (HMG-COA reductase), which plays a role in the formation of mevalonate in the process of cholesterol synthesis. *Neurospora crassa* can produce amylase, cellulase, and protease enzymes. Fermentation is influenced by several factors including the composition of the substrate, inoculum dose, and length of fermentation (Nuraini et al., 2017). Fermentation using fungi requires a media/substrate containing carbon, nitrogen, and mineral sources to support the growth and development of mycelium to the fullest.

According to Nuraini et al. (2019), the optimum condition is 7% inoculum dose and duration of seven days fermentation time with *Phanerochaete chrysospurium* and *Neurospora crassa* with ratio1:1 on a mixture of palm oil sludge and palm kernel cake. This condition may be provided with 13.25% of crude fiber content and 52.87% of crude fiber digestibility, 27.88% of crude protein content, 58.01% of nitrogen retention, 25.42 ppm of Cuprum, and 145.50 mg/kg of β-carotene. Increased crude protein, decreased crude fiber of palm oil waste fermented with *Phanerochaete chrysosporium*, and *Neurospora crassa* were expected to increase the use of palm oil waste in quail rations. The use of a mixture of palm oil waste fermented with *Phanerochaete chrysospurium* and *Neurospora crassa* for laying quails has not been studied yet.

So, the present study aimed to determine the effect of dietary palm oil waste fermentation fermented with *Phanerochaete chrysosporium* and *Neurospora crassa* on performance and some egg characterizes of laying Japanese quails.

MATERIALS AND METHODS

Materials

The material used in this study was a mixture of palm oil waste fermented with *Phanerochaete chrysosporium* and *Neurospora crassa*, yellow corn, bran, 126 concentrates (PT. Charoen Phokphand), bone meal, soybean meal, coconut oil, top mix, and CaCo3 meal. The livestock used in this study were quail (*Coturnix-coturnix japonica*) layer phase of 200 animals, aged 20 weeks with 70% egg production. Food ingredients, food content (%), and metabolic energy (kcal/kg) of components composing rations (as feed) can see in table 1.

Table 1. Feed ingredients, food content and metabolic energy of the diet in laying Japanese quails [a]

Feed ingredients (as feed)	Crude Protein (%)	Fat (%)	Crude Fiber (%)	Calsium (%)	Available Phospor (%)	Energy metabolism[c] (Kcal/kg)	Methionine (%)[c]	Lysine (%)[c]	B-carotene
Concentrate126[b]	38.00	4.00	3.50	5.50	1.00	2910.00[a]	1.00[a]	1.76[a]	-
Milled yellow corn	8.58	2.66	3.90	0.38	0.19	3300.00	-	0.30	33.00
Soybean meal	43.35	2.49	3.50	0.23	0.36	2240.00	0.50	0.60	-
Rice Bran	9.50	5.09	12.84	0.69	0.26	1640.00	0.27	0.67	-
Coconut oil	-	100[b]	-	-	-	8600.00[b]	-	-	-
POWF	20.54	6.97	11.84	0.52	0.26	2200.00[a]	0.42[a]	0.98[a]	95.50
Bone meal	-	-	-	24.00	12.00	-	-	-	-
CaCo3	-	-	-	40.00	-	-	-	-	-
Top mix[d]	-	-	-	0.06	-	-	0.003[c]	0.003[c]	-

Note : [a] Nuraini et al. (2019); [b] PT. Charoen Pokphan (consist of fish meal, soybean meal, dicalcium phosphate, NaCl, niacin, trace mineral and antioxidant) Nutrinet content at this label: crude protein 38-40%, crude fat min 3%, crude fiber max 8%, ash max 20%.). [c]Scott et al. (1982); POWF: Palm Oil Waste fermented with *Phanerochaete chrysospurium* and *Neurospora crassa* . [d]Top mix from PT Medion (Composition /10kg : vitamine A=12.000.000 IU, vitamine D3=2.000.000 IU, vitamine E= 8.000.000 IU, vitamine B1= 2.000mg, vitamine B2= 5.000mg, niacine= 40.000,methionine= 30.000 mg, lysine = 30.000, manganese = 120.000mg, iron= 20.000mg, iodine =200mg, zinc= 100.000mg, cobalt=200mg,copper= 4.000mg)

Fermentation of palm oil waste with *Phanerochaete chrysospurium* and *Neurospora crassa*

Five hundred gram of substrate consisted of 80% palm oil waste (200 g palm oil sludge and 200-gram palm kernel cake), and 20% (100-gram rice bran) with 35 ml of mineral solution added. The mineral composition consisted of $MgSO_47H_2O$ (2.5 g), $FeSO4_7H_2O$ (1 g), KH_2PO_4 (0.01 g), $ZnSO_44H_2O$ (1 g), $MnSO_44H_2O$ (0.01 g), thiamine hydrochloride (0.1225 g) and urea (50 g) and sterile water 1000 ml. The substrate was sterilized in an autoclave (121^0C for 15 minutes). The substrate was inoculated with 8% inoculum of *Phanerochaete chrysosporium* and *Neurospora crassa* with a ratio of 1:1. The substrate was stirred until become homogeneous and flattened to a thickness of 2 cm, and incubated for nine days (Nuraini et al., 2019).

Methods

Experimental design

This research was conducted using the Completely Randomized Design (CRD) method with four treatments and five replications, in the following procedures: group A (control) contained 0% Palm Oil Waste Fermented with *Phanerochaete chrysospurium* and *Neurospora crassa* (POWF), group B contained 8% POWF, group C contained 16% POWF, and group D contained 24% POWF in diets. The composition of the treatment diet was indicated in table 2, and the content of the treatment diet in table 3.

Measurement of variables

The variables in laying quail have included: feed consumption (g/head/day), quail egg production per day (%), egg weight (g/head/day), quail egg mass (g/head/day), feed conversion, blood cholesterol (mg/dl), HDL (mg/dl), Triglyceride (mg/dl), LDL (mg/dl), egg yolk cholesterol (mg/100g), egg yolk fat (%), and egg yolk color index. The egg yolk color index was evaluated visually by the usual La Roche scale (Bovšková et al., 2014).

Data analysis

The data was statistically analyzed by a one-way analysis of variance in CRD. Significant differences among treatments were determined using Duncan's Multiple Range Test (DMRT), P values<0.05 was considered to be substantial.

Table 2. Composition of experimental rations

Feed ingredients	Treatment (%)			
	A	B	C	D
Yellow corn	46.25	43.25	41.25	39.00
Concentrate126	28.50	28.50	28.50	28.50
Soybean meal	10.50	6.75	3.25	0.00
Rice Bran	8.50	5.25	2.75	0.25
Coconut oil	1.25	1.25	1.25	1.25
POWF	0.00	8.00	16.00	24.00
Bone meal	3.00	3.00	3.00	3.00
Top Mix	0.50	0.50	0.50	0.50
CaCO3	1.50	1.50	1.50	1.50
Total	100.00	100.00	100.00	100.00

Note: POWF: Palm Oil Waste fermented with *Phanerochaete chrysospurium* and *Neurospora crassa*

Table 3. Food content and metabolic energy of the experimental ration

Substance content	Ration			
Food and Energy Metabolism	A	B	C	D
Crude protein	20.16	20.14	20.20	20.35
Crude Fiber	3.80	4.22	4.61	5.06
Fat	4.31	4.60	4.88	5.17
Ca	3.19	3.18	3.17	3.17
P available	0.73	0.73	0.74	0.74
ME (kkal/kg)	2837.70	2835.10	2834.00	2821.95
Methionine	0.36	0.37	0.38	0.39
Lysine	0.76	0.79	0.83	0.86
B-carotene	15.26	22.41	29.55	36.45

*Note: The data of this table is calculated based on tables 1 and 2

RESULTS

The effects of inclusion POWF on the production performance of laying quails was indicated in table 4.

Food consumption

The food consumption of laying quail was not affected by the levels of POWF presenting in the diet. Utilization of POWF until 24% POWF (24.22 g/head/day) was similar to food consumption in control (24.09 g/head/day).

Hen-day egg production

The levels of POWF in the diet were not significantly affected the hen-day egg production of laying quails ($p > 0.05$). Increasing POWF levels to 24% (79.93 g/head/day) was similar to 0% POWF (79.80 g/head/day).

Egg mass production

The egg mass production of laying quail was not affected by the levels of CPF in the diet (P>0.05). Increasing fermented product levels to 24% POWF (7.97 g/head/day) in the diet was similar to that in the 0% POWF (7.84 g/head/day) control group on egg mass production.

Egg weight

The egg weight of laying quail was not significantly affected (*ns*) by utilization of POWF in the diet. The egg weight in the control treatment (9.81 g/egg) was similar to that in the 24% POWF (9.96 g/egg) group.

Food conversion

The food conversion ratio of laying quail was not affected (*ns*) by the levels of POWF in the diet. Increasing POWF levels to 24% (3.04) was similar to the control (3.08).

Plasma lipid profile

The total blood cholesterol significantly decreased (p<0.01) in POWF groups. The plasma lipid in the treated group with 24% POWF was decreased (152.26 mg/dl) compared to the group that had no POWF in the diet (298.86 mg/dl). The serum LDL content of laying quail was highly (P<0.01) decreased affected by POWF. The serum LDL content ranged in the treated group with 24% POWF was decreased (59.60 mg/dl) compared to the goup that received any POWF treatment (102.18 mg/dl). The serum HDL content of laying quail was significantly (P<0.01) increased affected by POWF. The serum HDL in treated group with 24% POWF was increased (113.18 mg/dl) compared the group with no POWF (82.04 mg/dl). Triglycerides in the group received any POWF (442.64 mg/dl) was not significantly different from the group with 24% POWF (422.86 mg/dl).

Egg Yolk Cholesterol

The inclusion of POWF in the diet of laying quails highly affected (P<0.01) on the egg yolk cholesterol. Increasing the amount of POWF has decreased the egg yolk cholesterol content. The egg yolk cholesterol in the 24% POWF treated group was reduced (681.51 mg/100g) compared to that in the group consumed no POWF treatment (854.94 mg/100g).

Egg yolk fat

The egg yolk fat of laying quail was not affected by the levels of POWF in the diet. The egg yolk fat in the control group was 28.40%, and 28.42% in the 24% POWF treatedgroup.

Egg yolk color index

The inclusion of POWF in the diet of quails was highly increased (P<0.01) on the egg yolk color index. The index of egg yolk color in the group treated with 24% POWF was increased (6.63) compared to that one with 24% POWF (7.91).

Table 4. The effects of inclusion Palm Oil Waste fermented with *Phanerochaete chrysospurium* on the production performance of Japanese laying quails aged 20-25 weeks

Treatment	Feed consumption (g/head/day)	Egg production (%)	Egg weight (g/egg)	Egg mass (g/head/day)	Feed conversion
A (0% POWF)	24.09	79.80	9.81	7.84	3.08
B (8% POWF)	24.07	79.20	9.89	7.88	3.05
C (16% POWF)	24.18	79.27	9.93	7.91	3.06
D (24% POWF)	24.22	79.93	9.96	7.97	3.04
SE	0.09	0.69	0.08	0.12	0.05

*Note: ns = not significantly different effect (P> 0.05); SE = Default error; POWF: Palm Oil Waste fermented with *Phanerochaete chrysospurium* and *Neurospora crassa*

Table 5. Plasma lipid profile for each treatment at the end of the experiment in laying Japanese quails aged 20-25 weeks influenced by palm oil waste fermented in the diet

Treatment	Total cholesterol (mg/dl)	Triglycerides (mg/dl) ^{ns}	HDL (mg/ dl)	LDL (mg/ dl)
A (0%POWF)	298.86^{a}	442.64	82.04^{c}	102.18^{a}
B (8%POWF)	274.68^{a}	426.94	96.26^{b}	99.07^{a}
C (16%POWF)	197.24^{c}	426.50	99.48^{b}	72.80^{b}
D (24%POWF)	152.26^{d}	422.86	113.18^{a}	59.60^{c}
SE	7.73	6.80	3.65	3.14

Note: ^{a,b} = Superscript difference in the same column affected highly significant (P<0.05). POWF: Palm Oil Waste fermented with *Phanerochaete chrysospurium* and *Neurospora crassa*

Tabel 6. Effect of utilization of palm oil waste fermented in the diet of Japanese quails aged 20-25 weeks on the quality of egg

Treatment	Egg Cholesterol (mg/100g) **	Egg Yolk Fat (%) ns	Egg Yolk Color **
A (0%POWF)	854.94[a]	28.40	6.63[b]
B (8%POWF)	742.70[b]	28.21	6.56[b]
C (16%POWF)	732.56[b]	28.67	7.48[a]
D (24%POWF)	681.51[b]	28.42	7.91[a]
SE	9.25	0.68	0.13

Note: [a,b =] Superscript difference in the same column affected highly significant ($p < 0.05$). POWF: Palm Oil Waste Fermented with *Phanerochaete chrysospurium* and *Neurospora crassa*

DISCUSSION

Effects of dietary supplementation POWF on the Japanese laying quail performance

The effects of the utilization of a mixture of POWF with *Phanerochaete chrysosporium* and *Neurospora crassa* with ratio 1:1 on the food consumption, quail day egg production, egg weight, egg mass, and food conversion are shown in table 4. There was no signficant differences in feed consumption in treatment groups A, B, C, and D which showed that the use of POWF in the diet preferred by quail. Althoughtreatments B, C, and D contain POWF, and provide a small amount of corn, concentrate, and bran, they have similar palatability to treatment A (without a mixture of POWF). however they contain more corn, soybean meal, and bran. According to Djulardi et al. (2018), the factors influenced food consumption in poultry are palatability, food quality, crude fiber in the food, and taste of food.

Palatability was similar among treatments A and treatments B, C, and D showing that the quality of the rations were as same as reach other. The fermentation process can break down the complex food substances into simple ones to improve foods' quality and digestibility. Nuraini et al. (2017), fermented products can produce a desired flavor, having several preferred vitamins to the livestock compared to the original products. food consumption of laying quail at the age of 20 weeks in treatment D (24% POWF) was 24.22 g/head/day. Castro (2018) reported food consumption of laying quail from 23.5 up to 25.5 g/head/day.

According to Indreswari (2016), food consumption, especially protein intake, affects egg production. Daily egg production was not different in treatment A, in comparison with treatments B, C, and D, due to the food consumption; especially protein intake was also similar. similar protein intake may result in equal daily egg production.

Reducing the use of corn, bean, and soybean meal in treatments B, C, and D has resulted in a decrease in amino acid content of lysine and methionine. However, a mixture of POWF and fermented bran with *Phanerochaete chrysospurium* and *Neurospora crassa* can cover the deficiency of amino acid lysine and methionine. The amino acid content of lysine and methionine in POWF with *Phanerochaete chrysospurium* and *Neurospora crassa* includes 0.98% lysin and 0.42% methionine. Daily egg production of laying quail at the age of 20 weeks in treatment D (24% POWF) is 79.93 g/head/day.

Because of same food intake, food conversion in treatment A, it was similar to treatment B, C, and D , hence egg mass was also identical. Food conversion can indicate the production coefficiency; a smaller value indicates more efficient use of food to produce eggs. Khairani (2016) suggested that the food's conversion values will remain balanced even if there is an increase in food conversion affected by food intake and egg mass

The food conversion of laying quail at the age of 20 weeks in treatment D (24% POWF) was determined to be in the ratio of 3.04. Indreswari (2016) reported that food conversion of laying quail was 3.06-3.80.

Effects of POWF utilization on the blood cholesterol

The blood cholesterol of quail (mg/dl) treated with POWF was indicated in table 5. The use of a mixture of POWF with *Phanerochaete chrysospurium* and *Neurospora crassa* with ratio 1:1 was found to reduce cholesterol levels in the serum of laying quail. Utilization of POWF with *Phanerochaete chrysospurium* and *Neurospora crassa* with rate 1:1 up to the level of 24% can reduce blood cholesterol by as much as 50.5%. The blood cholesterol reduction in treatments C and D compared to treatments A and B is related to the use of POWF with *Phanerochaete chrysospurium* and *Neurospora crassa* containing β-carotene. The higher the use of POWF

with *Phanerochaete chrysospurium* and *Neurospora crassa* in the ration, the higher the content of β-carotene in the diet; so that it can reduce cholesterol levels in the serum of laying quail. According to Nuraini et al. (2015), β-carotene can lower the cholesterol by inhibiting Hydroxymethyl Glutaryl reductase enzyme (HMG-KOA reductase). This enzyme is needed in the formation of mevalonate in the process of cholesterol synthesis.

The table 5, shows that the blood cholesterol of quail given POWF with *Phanerochaete chrysospurium* and *Neurospora crassa* range 152.26 - 298.86 mg/dl. This research has shown differnt results in comparison with findings of Aetin et al. (2017) suggested that blood cholesterol content of quails ranged 222-322.2 mg/dl. The average serum LDL of quails fed a mixture of POWF with *Phanerochaete chrysospurium* and *Neurospora crassa* ranged 59.60-102.18 mg/dl. The results of this research on POWF supplementation with *Phanerochaete chrysospurium* and *Neurospora crassa* showed a decrease in serum LDL levels in quails' blood. The LDL content in the study was higher than that of Aetin et al. (2017), ranged 37.48 - 51.60 mg/dl. The decrease in LDL levels is related to the β-carotene content, which can prevent lipid oxidation, and be able to inhibit the activity of the HMG CoA reductase enzyme resulting in mevalonates formation, which is needed for cholesterol synthesis. The inhibition of HMG-CoA reductase will reduce the cholesterol synthesis in liver, thereby reducing the combination of APO B, and increasing LDL receptors on the surface of the liver (Rahastuti et al., 2011).

The average serum HDL of quails' blood with the use of a mixture of POWF and fermented bran with *Phanerochaete chrysospurium* and *Neurospora crassa* ranged 82.04 - 113.18 mg/dl. The HDL content in this study showed a high value compared to the research of Khabib Arrosichin et al. (2016), where the HDL content of quails' blood at ranges of 86.6 - 99.8 mg/dl. The average of serum triglycerides of quails (80 days) with the use of a mixture of POWF with *Phanerochaete chrysospurium* and *Neurospora crassa* ranged 422.86-442.64 mg/dl. The triglycerides content in this study was higher than Parizadian (2011) reported that the triglycerides blood of quail (70 days) ranged 149.80 - 154.62 mg/dl

Effect of POWF on egg quality of the Japanese quails

The use of POWF in the diet is found to reduce egg yolk cholesterol in quails, due to an increase in β-carotene content in the diet. The β-carotene content in treatment A (no POWF) was 15.26 mg/kg, while in treatment D (24% POWF) was 36.45 mg/kg. Ramakrishnan et al. (2017), also recommended that β-carotene is a compound that can reduce cholesterol by inhibiting the action of the HMG-CoA reductase enzyme. According to USDA (2017), the egg yolk cholesterol content of quails was 844 mg/100 g. Table 5 indicate that the egg yolk fat content in treatment A (no POWF) is 28.40%, and in treatment D (24% POWF) is 28.42%. Because crude fat content in both treatment diets were not much different, egg yolk fat content in both treatments A and D was similar (Tatiana, 2018).

Increasing utilization of POWF also caused an increase in the β-carotene content, so that the intensity color of egg yolk was also higher. The treatment diet with 24% POWF containing high β carotene content increased the score of egg yolk (orange-yellow) compared to the control treatment. β carotene is an unstable carotenoid group compound because it quickly oxidizes to xanthophyll. Xanthophyll serves to stain the yolk. The quails' body cannot synthesize xanthophyll; therefore, xanthophyll is obtained from diets consisting of xanthophylls in the ingredients. The egg yolk color index obtained by using 24% POWF in the diet of 7.91. El-Tarabany (2016) indicated the range of egg yolk color index between 7 - 9 with the treatment of cage stocking density on egg quality traits in Japanese quail.

CONCLUSION

Based on the results of the present study, it is concluded that the use of a mixture of palm oil waste fermented with *Phanerochaete chrysospurium* and *Neurospora crassa* with ratio 1:1 up to 24% level in diet of laying quail can be adequate. In other words, with this kind of dietary supplementation, food consumption was 24.22 g/head/day, egg production was 79.93%, egg weight was 9.96 g/head/day, egg mass was 7.97 g/head/day, food conversion was 3.04, total cholesterol was 152.26 (mg/dl), triglycerides was 422.86 (mg/dl), LDL was 59.60 (mg/dl), HDL was 113.18 (mg/dl), egg cholesterol was 681.51 (mg/100g), egg yolk fat was 28.42%, and egg color index was 7.91.

DECLARATIONS

Author's contribution

Nuraini contributed to created the idea, designed the experiment (fermentation and utilization POWF to quail), analyzed data, and wrote this article. Ade Djulardi committed to using POWF to quail. Dwi Yuzaria

contributed to analyzed data and checked the written report. All authors confirmed the final revised form of article for publishing in this journal.

Competing interests

The authors declared that they have no competing interests.

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A Review on the Use of *Azolla* Species in Poultry Production

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ABSTRACT

Aquatic plants are receiving a lot of attention in nutrition research, which is due to their broad range of uses in animal and human food. *Azolla* is one of the commonly used these floating plants. Currently, different *Azolla* species are used as maintainable feed alternatives for cattle, pigs, poultry, and fish as dried flakes or fresh form. In addition to high protein and essential amino acid content of *Azolla*, the fern is rich in other nutrients such as minerals, vitamins, and pigments. There is a discrepancy in the use of *Azolla* to improve the productivity of poultry. However, most studies have indicated that the incorporation of broiler ration with certain levels of *Azolla* promotes feed intake, body weight gain, feed conversion rate, and general health conditions as *Azolla* comprises growth promoters. In layers, the use of *Azolla* improves productivity in terms of egg quantity and quality as *Azolla* contains pigments, minerals, and essential amino acids. Moreover, *Azolla*s improves carcass traits at processing. Therefore, this review article provides information on *Azolla* plant, its composition, and the significance of its supplementation for different poultry species.

Key words: *Azolla*, Broilers, Immunity, Layers, Performance

INTRODUCTION

As a result of declining food resources around the world and increasing their costs, the search for unconventional resources for poultry production has become increasingly important. In addition, there is a serious effort to replace the conventional feed ingredients of poultry ration with cheap items to reduce the costs. Aquatic plant species do not appear to accumulate secondary plant compounds due to their growth habits and therefore offer greater potential than tree leaves as a source of protein for monogastric animals (Bacerra et al., 1995; Wagner, 1997). Several studies had been done on the nutritive value of aquatic plant *Azolla*. *Azolla* is a small aquatic and floating fern of the family Azollaceae and the order Pteridophyta. The name is referred to azo (to dry) and allyo (to kill) as the fern can be killed after exposure to drought conditions. In the world, at least eight species of *Azolla* are known, namely *Azolla pinnata*, *Azolla nilotica*, *Azolla caroliniana*, *Azolla japonica*, *Azolla circinata*, *Azolla microphylla*, *Azolla rubra*, and *Azolla Mexicana*, of which most common is *Azolla pinnata* (Mathur et al., 2013). *Azolla* is an abundantly available aquatic fern in the stagnant water of ponds, drains, rivers, canals, marshy fields and wetland paddy in tropical and subtropical countries of the world. This fern can be cultivated under natural and controlled environmental conditions (Senthilkumar and Manivannam, 2016). The blue-green symbiotic cyanobacterial partner alga (*Anabaena azollae*) grows in *Azolla* leaves cavities (Becking, 1979). *Azolla*, in turn, provides carbon source, nutrients, and a protective cavity to *Anabaena* colonies in exchange for fixed high amount of atmospheric dinitrogen as well as growth promoters (Pillai et al., 2005). This special symbiotic relationship makes *Azolla* a great and protein-rich plant (Mooventhan et al., 2019).

Azolla was originally used as green manure but is now used as mosquito inhibitor, herbicide, water saver and purifier, fertilizer saver (Van Hove and Lejeune, 1996), cough medicine (Raja et al., 2012), saline soils reclaimer (Raja et al., 2012), biogas producer (Van Hove, 1989; Das et al., 1994) and bioremediator (Sood et al., 2012; Yadav et al., 2014). *Azolla* fern is promising in terms of the ease of cultivation, the minimal water for propagation, the rapid biomass production, the growth in unexploited niches, productivity and nutritive value (Singh and Subudhi, 1978; Lumpkin and Plucknett, 1982; Van Hove and Lejeune, 1996; Pillai et al., 2002; Alalade and Iyayi, 2006; Prabina and Kumar, 2010). It is reported that the addition of *Azolla*

in rations significantly reduces the feed cost (Escobin, 1987; Bacerra et al., 1995; Lawas et al., 1998; Sujatha et al., 2013). The bio-composition of *Azolla* makes it one of the cheapest, economic, potential, efficient, and maintainable feed alternatives for livestock and poultry (Pannaerker, 1988; Kathirvelan et al., 2015).

Azolla pinnata meal has long been used successfully for broiler and layer chickens (Castillo et al., 1981; Querubin et al., 1986; Bhuyan et al., 1988; Sreemannarayana et al., 1993; Basak et al., 2002; Alalade and Iyayi, 2006; Balaji et al., 2009; Dhumal et al., 2009; Naghshi et al., 2014), ducks (Escobin, 1987; Bacerra et al., 1995; Lawas et al., 1998; Sujatha et al., 2013; Acharya et al., 2015), quails (Rathod et al., 2013; Shamna et al., 2013; Varadharajan et al., 2019), fish (Nwanna and Falaye, 1997) and rabbits (Wittouck et al., 1992; Sreemannarayana et al., 1993; Sadek et al., 2010; Anitha et al., 2016a). Moreover, dietary *Azolla pinnata* has been applied for feeding of shrimps, goats, cattle and buffalo calves (Sudaryono, 2006; Hossiny et al., 2008; Indira et al., 2009; Mandal et al., 2012; Rawat et al., 2015), while *Azolla filiculoides* has been added to diets of sows as protein source replacer (Leterme et al., 2010) and for growing fattening pigs (Bacerra et al., 1995).

Therefore, this review article provides an overview of *Azolla* plant composition and the significance of its supplementation for different poultry species.

Composition of *Azolla*

Azolla is a rich and potential source of protein (25-35%), nitrogen (Lumpkin, 1984), almost all essential amino acids (7-10%) especially lysine (Van Hove, 1989), essential minerals such as iron, calcium, phosphorous, magnesium, manganese, potassium, iron and copper (10-15%), vitamins like vitamin A and vitamin B12 (Bacerra et al., 1995; Lejeunea et al., 1999), carotenoids, chlorophyll a and b, bio-polymers, probiotics, and growth-promoting intermediates (Ivan and Thuget, 1989; Tamany et al., 1992; Lejeune et al., 2000; Pillai et al., 2005; Alalade and Lyayi, 2006; Lakshmi and Sailaja, 2012; Mathur et al., 2013; Parashuramulu et al., 2013; Cherryl et al., 2014; Henry et al., 2017). Thus, *Azolla* is considered a significant source of nutrients. Furthermore, cyanobiont of *Anabaena azollae* contains chlorophyll a, phycobiliproteins, and carotenoids (Tyagi et al., 1980). However, its contents of carbohydrate and oil are low. *Azolla* is highly digestible because of lignin and high protein contents (Anitha et al., 2016b). A study by Namra et al. (2010) revealed the presence of high levels of energy in *Azolla* which is important for both digestion and availability of nutrients. *Azolla* appears to be a possible biofertilizer due to nitrogen contribution to rice crops (Kannaiyan, 1992).

Effects of *Azolla* supplementation on production performance

Broiler chickens

The addition of *Azolla* to the poultry diet economizes production. *Azolla* is a cheap and plentiful alternative plant protein source that improves FCR, energy efficiency, and performance with no adverse effects on livestock, poultry, and humans (Lejeunea et al., 1999; Alalade and Lyayi, 2006; Namra et al., 2010). Although there are inconsistent results, the majority of the data show enhancement of production and reproduction of livestock and poultry fed with diet containing *Azolla*.

Many studies have demonstrated the efficiency of using *Azolla* at different replacement concentrations in the ration of broiler chickens. However, Ali and Leeson (1995) found that feeding of broilers on *Azolla* resulted in similar body weight and growth like those kept on a maize-soybean meal. Also, Sarria and Preston (1995) found an increase in the growth of broilers when soybean protein was replaced by *Azolla* up to 15% level. Sundararaju et al. (1995) investigated the performance modulating effects of dietary *Azolla*, *Sesbania*, and *Leucaena* proteins on broilers and found that the addition of them to broiler chicken diet at a level of 6% significantly improved the body weight at 4, 6 and 8 weeks of age. Improvement in the FCR as a result of *Azolla* feeding was also reported by Ardakani et al. (1996). Seth et al. (2013) found an increase in efficiency at 5-15% *Azolla* level in the ration of broilers, while Balaji et al. (2009, 2010); Namra et al. (2010); Ara and Adil (2012); Sujatha et al. (2013); and Naghshi et al. (2014) found this improvement at 5% level. Incorporation of *Azolla* up to 5% as a feed ingredient to replace sesame meal in the ration of 2 to 6-week-old broilers improved growth rate, FCR, and energy efficiency without deleterious effect on palatability and mortalities (Basak et al., 2002), along with promising economic returns (Parthasarathy et al., 2002). The use of 20% fresh *Azolla* was suggested as a substitute for commercial feed in chicken diets as it could increase the body weight (Subudhi and Singh, 1978). A significant difference in the feed intake was observed when *Azolla* increased up to 15% in the diet (Querubin et al., 1986). The study by Dhumal et al. (2009) demonstrated a significant increase in feed intake with an increase in *Azolla* levels up to 30%. However, Bacerra et al. (1995) indicated a benefit from *Azolla* supplementation at a low

level of inclusion. Similarly, Ara et al. (2015) found a linear reduction in feed intake with increasing *Azolla* levels in the diets of broiler chickens. It was found that *Azolla pinnata* meal can be safely included up to 15% in growing pullet ration with no health issues (Alalade et al., 2007) but 10% inclusion level in pullet chick diet has given the best performance. Incorporation of the concentrate feed with *Azolla* at 7.5% level resulted in an increase in the body weight up to 2.6% (1.99 kg) compared to control diet (1.93 kg). In addition, the consumed feed was lower in broilers fed on 7.5% *Azolla* (Prabina and Kumar, 2010). Seth et al. (2013) also detected increased weight gain in Vanaraja chicken fed on 5% or 10% *Azolla* over control. Saikia et al. (2014) demonstrated that the highest body weight gain was in the group supplemented with 5% *Azolla* while the lower one was in the group fed with 15% level and they explained that increasing the fiber content in high concentrations of *Azolla* negatively affects the appetite of the birds and consequently reduces the growth rate. They also concluded that *Azolla* can be added to the broiler diet by 10% level without adverse effect in the performance. Improvement in body weight gain as well as FCR was detected after the inclusion of *Azolla* at 5% or 10% level in the feed (Acharya et al., 2015) and up to 7.5% level (Kumar et al., 2018). Moreover, the feed cost of production was considerably reduced by the inclusion of *Azolla* at either level. The inclusion of *Azolla* at 10% level showed the maximum economic benefit. A study carried out by Henry et al. (2017) indicated that fresh *Azolla* supplementation (30 g/bird/day) reduced feed consumption with no effects on growth performance parameters in 7-week-old turkeys, which might be due to high protein level and mineral content of *Azolla*. The addition of dried *Azolla* to Vencobb broilers ration can be used safely up to 5% without any adverse effects, however, 2.5% addition level of *Azolla* is effective in improving both growth and biochemical parameters (Rana et al., 2017). It was revealed that inoculation of *Azolla* at levels of 5% or 7% is appropriate for safe and profitable production of broilers due to reduced FCR, mortalities, and production costs, as well as improved net profit (Islam and Nishibor, 2017). The addition of *Azolla* to the basal ration could improve FCR with no adverse effects on blood biochemistry and immune parameters (Shukla et al., 2018). Recently, Samad et al. (2020) suggested that the addition of *Azolla* up to 15% level enhances the growth performance traits without negative effects on the nutrient digestibility of broiler chickens.

On the other side, some researchers found no or low effects of *Azolla* supplementation on poultry production performance. Higher levels compared to low levels of aquatic plants result in lower body weight, which may be related to the high level of neutral detergent fiber (Buckingham et al., 1978) and tannin contents (Tamany et al., 1992) as limiting factors for FCR as well as efficient nutrient utilization (Muzlar et al., 1978). The decrease in the consumed feed may be due to reduced palatability (Bested and Morento, 1985) and increased bulkiness of *Azolla* (Bacerra et al., 1995) which reduces its utilization. Castillo et al. (1981), Bhuyan et al. (1988), and Sreemannarayana et al. (1993) found that inclusion of *Azolla* by 15% in broilers ration did not affect feed consumption. The discrepancy in the trend of feed intake could be attributed to the differences in the nutrient composition of the diets and *Azolla* species used. Moreover, another study on *Azolla* revealed a proportional decrease in the body weights of broilers (Parthasarathy et al., 2002). The results of Biplob et al. (2002) and Balaji et al. (2009) indicated no changes in the production performance of broiler chickens after feeding on *Azolla* compared to control. In addition, Alalade and Iyayi (2006) attributed the lower growth rate of broilers fed on high levels of *Azolla* to the lower feed intake and subsequently the reduction of metabolizable energy intake. Alalade et al. (2007) demonstrated that addition *Azolla* to the ration of pullets up to 10% level induced non-significant differences in growth parameters.

Limited researches have been conducted on evaluating the effects of *Azolla* supplementation on the carcass traits. Higher dressing percentage of broiler chicken with giblet percentage at 15% was reported in the treatment group fed on 5% *Azolla* (Basak et al., 2002) and this improvement was attributed to the higher body weight gains. Feeding on 5% *Azolla* powder significantly increased the carcass yield percentages of broiler chicks, while lowest percentage was observed in 15% *Azolla* supplemented group (Naghshi et al., 2014) However, Ara et al. (2015) found no adverse effect on carcass traits on feeding *Azolla* up to 20% level.

Ducks

Some studies also showed the effects of *Azolla* as a supplement in the ration of ducks. *Azolla* as an unconventional nutrient source could be added by 10% to the basal diet of white Pekin broiler ducks (Acharya et al., 2015). In the study of Escobin (1987), the author found no differences in the production efficacy among different groups after partial replacement of growing Muscovy ducks ration with *Azolla* at levels of 20, 30, and 40%. In

addition, 15% replacement of soybean meal with *Azolla* increased the daily weight gains of ducks (Bacerra et al., 1995), however, at levels 20, 45, or 60% of *Azolla*, the growth rate was reduced. Similar findings on the production performance of ducks were reported (Lawas et al., 1998; Sujatha et al., 2013). In the same line, Lawas et al. (1998) observed that feeding of Mallard ducks with *Azolla* at levels of 75 or 250 g/bird/day lowered the FCR. In a trial with ducks, Bacerra et al. (1995) demonstrated that by addition of 15 to 60% *Azolla* in diet supplying 15.2-30.3% of the total protein, the FCR decreased with increasing *Azolla* consumption. However, studies with long periods are needed to determine the effect of *Azolla* supplementation on performance at older ages.

Quails

Shamna et al. (2013) reported that feeding of quails on *Azolla* at 5% replacement level of the basal ration enhanced the growth and FCR, also it was more economic than feeding on basal diet alone. Varadharajan et al. (2019) concluded that *Azolla* meal up to 6% can include in quails diet without affecting feed consumption and carcass traits. Rathod et al. (2013) reported non-significant effects of *Azolla* supplementation on feed consumption of Japanese quails.

Layer chickens

The pigmenting ability of group fed with *Azolla* was demonstrated by its higher Roche fan color score and this could be attributed to the ability of *Azolla* to pigment the yolk as it is rich in β carotene pigment. A similar effect on yolk color due to the inclusion of *Azolla* in layer ration was reported by Bastian (1987). Ali and Leeson (1995) found that the addition of *Azolla* powder to chicken feed significantly improved the carotene status of the chicken and increased egg production. Moreover, Khatun et al. (1999) investigated the replacement of sesame oil in the ration of layers by *Azolla* 200 g/kg and reported good egg mass output and FCR. A similar better egg production performance was detected by Kannaiyan and Kumar (2005) in terms of higher egg yield after *Azolla* inclusion at the level of 100 g/bird/day. Lakshmanan et al. (2017) stated supplementation of *Azolla* in layers diets resulted in increasing egg production, improving the nutrient value as well as saving the concentrated feed. The positive effect of *Azolla* on eggshell strength was referred to the high calcium content of *Azolla*. It has been found that the eggshell consists of minerals especially calcium carbonate that deposits in the organic matrix (Austic and Nesheim, 1990). It was reported that inoculation of *Azolla* in the ration of layer chickens at levels up to 20% had no effect on alanine aminotransferase and aspartate aminotransferase levels, which indicates no toxic effect of *Azolla cristata* supplementation (Ara et al., 2018).

Effects of *Azolla* supplementation on immunity

Prabina and Kumar (2010) demonstrated higher values of antibody titer against the Ranikhet virus in broilers fed on 10% dried *Azolla* in comparison with those received 7.5% dried *Azolla*. The authors explained increased immunity in *Azolla* treated birds by the ability of the fern to assimilate atmospheric dinitrogen through algal symbionts (*Anabaena*) in its leaves. The immune-modulatory effect of *Azolla* meal without any toxicity was detected by Mishra et al. (2016) as birds treated with 5%, 7.5%, and 10% *Azolla* showed higher cell-mediated immune responses. Contrary results were reported by Sujatha et al. (2013) who noted a non-significant difference between the mean hemagglutination-inhibition antibody titer and anti-mercaptoethanol resistant antibodies titer between the control group and the group fed with 200 g fresh *Azolla*/chick/day from 45 to 60 weeks in Nicobari fowls. Moreover, the haemagglutinating and immunoglobulin M responses to 1% chicken red blood cells were comparatively better in the choice-feeding group compared to the 5% *Azolla*-fed group. In turkeys, Bhattacharyya et al. (2016) also reported that antibody response to sheep red blood cells was positively affected by 5.5% *Azolla pinnata* replacement in the conventional ration.

CONCLUSION

It can be concluded that *Azolla* species can be used as a source of protein and other essential nutrient elements for poultry species. Further research is required to determine the effects of using *Azolla* as a feed supplement in poultry ration.

DECLARATIONS

Competing interests

The author has no conflict of interest.

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Gallibacterium anatis: Moleculer Detection of Tetracycline Resistance and Virulence Gene

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ABSTRACT

Gallibacterium anatis causes infections in the reproductive tract of egg-laying hens and it is associated with increased mortality and decreased egg production. For this study we used singeleplex and multiplex PCR with specific primers to assess the presence of tetracycline resistance (Tcr) (*tet* A, B, C, D, E, G, H, K, L, M, O, S, P, Q and X), virulence [cytotoxic (RTX-like toxin, *gtxA*) and fimbrial (*flfA*)] genes and antibiotic resistance in *G. anatis* isolates. Among the 20 isolates tested, the highest antimicrobial resistance patterns were observed in erythromycin, streptomycin, tilmicosin (100%) followed by colistin sulphate (65%), cephalexin and tulathromycin (50%). Among 20 isolates examined, 10 (50%) carried tetracycline resistance genes, 7 (35%) had *tet*(B), 2 (10%) had *tet*(G), and 1 (5%) had *tet(*A), (D), (M) or (L). Of these *G .anatis* isolates were carried out 6 (30%) *gtxA* but none of *flfA* gene. Based on present results, it is concluded that virulence and Tcr genes could contribute to pathogencity of *G. anatis*, which is a major risk to poultry health.

Key words: Antibiotic resistance, *G. anatis*, Poultry, Virulence genes, Tetracycline resistance genes

INTRODUCTION

Major health problems in the poultry industry can affect egg production. In particular, infectious diseases can reduce egg production and egg quality by directly affecting the reproductive system of hens. Such diseases also can indirectly diminish the overall health status of poultry (Clauer, 2009). *Gallibacterium anatis* (*G. anatis*) is a resident of normal microflora of the lower genital and upper respiratory tract in chickens and many other avian species (Bojesen et al., 2004; Rzewuska et al., 2007; Jones et al., 2013; Paudel et al., 2013; Persson and Bojesen 2015; Lawal et al., 2018). Decreased egg production associated with salpingitis, respiratory system problems and mortality in commercial laying hens therefore, *G. anatis* infections have been the topic of researchers' works in recent years (Bojesen et al., 2011a; Sing, 2016; Chaveza et al., 2017). The knowledge of bacteria-host interactions and antimicrobial susceptibility to *G. anatis* in laying hens remains limited (Bisgaard et al., 2009; Johnson et al., 2013). Among the most important *G. anatis* virulence factors involved in colonization and invasion of the epithelium in the trachea, oropharyngeal tissues and oviduct are the IgG destructive protease, RTX-like toxin, *gtxA* and hemagglutinin, which suppress the host immune response (Vaca et al., 2011; Lucio et al., 2012). Bacterial fimbria are also important not only as a virulence factor, but as a target for preventative vaccines (Kudirkiene et al., 2014; Sorour et al., 2015). Tetracycline resistance determinants (Tcrs) are widespread among both Gram negative organisms and *Pasteurellaceae* family and are often found in multi-drug resistant bacterial species (Levy et al., 1989; Roberts, 1996). To better understand *G. anatis* pathogenicity in poultry, this study amied to determine the prevalence of Tcr genes and virulence-specific factor genes in *G. anatis* isolates from laying hens.

MATERIAL AND METHODS

Bacterial Isolates

In the present study, 20 *Gallibacterium anatis* isolates from laying hens obtained from the previous study at the Department of Microbiology, Faculty of Veterinary Medicine, Mehmet Akif Ersoy University, Burdur, Turkey were analysed (Yaman and Sahan Yapicier, 2019).

Gallibacterium anatis Strains

G. anatis F149T (non-hemolytic strain, ATCC 43329) and 12656-12 strain (hemolytic strain) was used for analysis in this study.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility test was carried out by the agar disk diffusion method on Mueller-Hinton agar (Oxoid Ltd, Hampshire, UK) supplemented with 5% sheep blood according to the guidelines from Clinical and Laboratory Standards Institute (CLSI, 2017). The following antibiotics (spiramycin and tulatromycin, Bioanalyse, Turkey), 18 out of 20 (Oxoid, UK) commonly used in veterinary medicine were selected: ampicillin (10µg; AMP), amoxicillin (10µg; AX), amoxicillin clavulanic acid (30µg; AMC), cephalexin (30µg; CL), ceftiofur (30µg; FUR), ciprofloxacin (5µg; CIP), colistin sulphate (10µg; CT), doxycilin (30µg; DO), enrofloxacin (5µg; ENR), erythromycin (15µg; E), florfenicol(30µg; FFC), gentamicin (10µg; CN), tetracycline (30 µg; T), penicillin (10units; P), spiramycin (100 µg; S), streptomisin (10 µg; S), tilmicosin (15µg; TIL), trimethoprim sulphamethoxazole (25µg; TS), tulathromycin (30µg; TUL), tylosin (30µg; TY). The results were obtained by measuring the diameter of the growth inhibition zone around the antibiotic disc for each isolated bacteria and recorded as sensitive, intermediate and resistant according to the interpretive standards of CLSI and antimicrobials manufacturers' instructions. Isolates displaying resistance to ≥3 antimicrobial agents tested were defined as exhibiting multi-drug resistance (MDR) (Tenover et al., 1987; Schwarz et al., 2010). *E. coli* ATCC 10536 was used as a quality control strain.

Primers

A primer pair specific for 14 tetracycline resistance genes and *G. anatis* virulence genes were listed in Tables 1 and 2 (Ng et al. 2001; Bager et al. 2013; Paudel et al. 2013).

DNA Extraction

DNA extraction from *G. anatis* isolates were performed according to the instructions of the GeneJET Genomic DNA Purification Kit (Thermo Scientific, USA). DNAs were stored for use as template DNA at -20°C until amplification.

PCR Conditions

Singeleplex PCR assay was carried out for virulence genes. 25 µl reaction volumes containing 3 µl MgCl (25 mM), 0.5 µl dNTP (10 mM), 10 pmols of primers and 0.2 µl Taq polymerase (5U/µl). The following cycling conditions were used: 3 min at 94°C, followed by 30 cycles of 1 min at 94°C (denaturation) and 1 min at 54°C (primer annealing), 1 min at 72°C (extension), and 7 min at 72°C (final extension). Multiplex PCR was performed for tetracycline resistant genes and these genes grouped (Group I: *tet*(B), *tet*(C) and tet(D); Group II: *tet*(A), *tet*(E) and tet(G); Group III: *tet*(K), *tet*(L), *tet*(M), *tet*(O) and *tet*(S); Group IV: *tet*A(P), *tet*(Q) and *tet*(X)) described by Ng et al. (2001). Each multiplexed group's PCR reaction mix concentration and amplification conditions were carried out following the previous research (Zhao and Aoki, 1992).

Table 1. Tetracyline resistance specific primers

Tcrs	primer sequence (5'-3')	Amplicon size (bp)
tet(A)	GCT ACA TCC TGC TTG CCT TC CAT AGA TCG CCG TGA AGA GG	210
tet(B)	TTG GTT AGG GGC AAG TTT TG GTA ATG GGC CAA TAA CAC CG	659
tet(C)	CTT GAG AGC CTT CAA CCC AG ATG GTC GTC ATC TAC CTG CC	418
tet(D)	AAA CCA TTA CGG CAT TCT GC GAC CGG ATA CAC CAT CCA TC	787
tet(E)	AAA CCA CAT CCT CCA TAC GC AAA TAG GCC ACA ACC GTC AG	278
tet(G)	GCT CGG TGG TAT CTC TGC TC AGC AAC AGA ATC GGG AAC AC	468
tet(G)	CAG CTT TCG GAT TCT TAC GG GAT TGG TGA GGC TCG TTA GC	844
tet(K)	TCG ATA GGA ACA GCA GTA CAG CAG ATC CTA CTC CTT	169
tet(L)	TCG TTA GCG TGC TGT CAT TC GTA TCC CAC CAA TGT AGC CG	267
tet(M)	GTG GAC AAA GGT ACA ACG AG CGG TAA AGT TCG TCA CAC AC	406
tet(O)	AAC TTA GGC ATT CTG GCT CAC TCC CAC TGT TCC ATA TCG TCA	515
tet(S)	CAT AGA CAA GCC GTT GAC C ATG TTT TTG GAA CGC CAG AG	667
tet(P)	CTT GGA TTG CGG AAG AAG AG ATA TGC CCA TTT AAC CAC GC	676
tet(Q)	TTA TAC TTC CTC CGG CAT CG ATC GGT TCG AGA ATG TCC AC	904
tet(X)	CAA TAA TTG GTG GTG GAC CC TTC TTA CCT TGG ACA TCC CG	468

Table 2. Spesific primers for virulence genes of *G. anatis*

Virulence genes	Primer sequence (5'-3')	Amplicon size (bp)
GalNtx	TGCGCAAGTGCTAAATGAAG GGATAATCGTTGCGCTTTG	925
flfA	CACCATGGGTGCATTTGCGGATGATC C TATTCGTATGCGATAGTATAGTTC	538

Ethical Approval

This study was approved by Animal Research Ethics Committee of Burdur Mehmet Akif Ersoy University, Burdur, Turkey (Protocol No. MAKU-HADYEK/ 2017-314).

RESULTS

Antimicrobial Susceptibility Test

The highest antimicrobial resistance patterns in 20 isolates tested were observed for erythromycin, streptomycin, tilmicosin (100%) followed by colistin sulphate (65%), cephalexin and tulathromycin (50%) which are shown in table 3. 100% of the *G. anatis* isolates exhibited sensitivity to doxycilline while 15% and 85%, respectively, showed intermediate resistance to tetracycline.

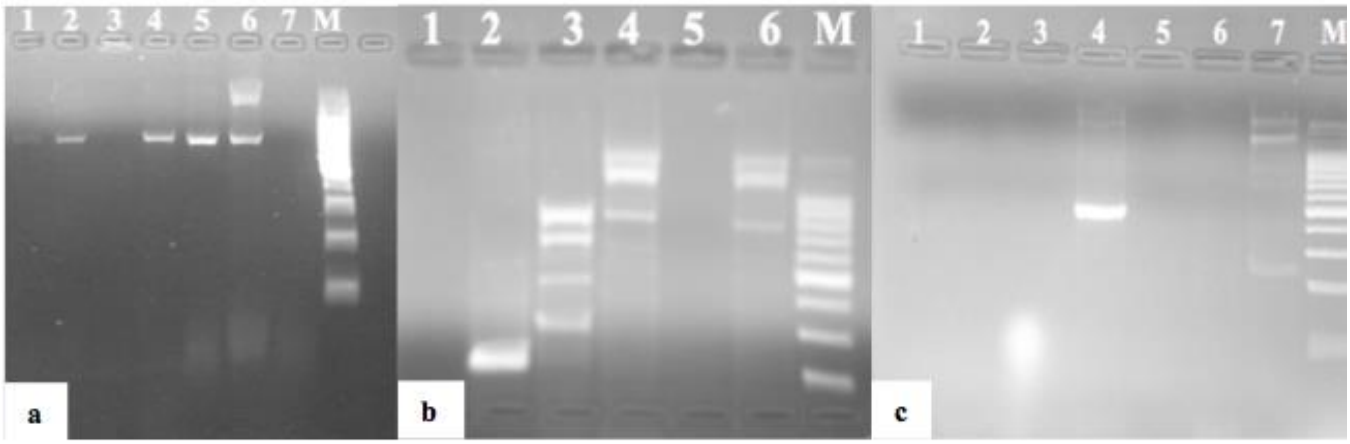
Molecular Detection of *tet* Genes and Virulence Genes

Twenty isolates of *G. anatis* contained 10 (50%) tetracycline resistance, 7 (35%) *tet*(B), 2(10%) *tet*(G), 1(5%) *tet*(A), (D), (M) and (L). 2(%10) were found to carry both *tet*(B) and *tet*(G); 1(5%) were carried both *tet*(B), (D) and (A) genes (Figure 1). The amplification of genes by PCR showed that 6 (30%) strains contains *gtxA* and no *flfA* genes encoded. Based on the correlation of isolates; one of the two isolates carried both *tet*(B), (D) and *gtx* gene and the other carried *tet* (B) and *gtx* genes.

Table 3. Antimicrobial resistance of 20 *G. anatis* isolates

Items	S (n%)	I (n%)	R (n%)
AMP	20(100)	0	0
AMC	20(100)	0	0
AX	20(100)	0	0
CIP	15(75)	0	5(25)
CL	12(50)	0	12(50)
CN	16(80)	0	4(20)
CT	7(35)	0	13(65)
DO	20(100)	0	0
E	0	0	20(100)
ENR	20(100)	0	0
FFC	20(100)	0	0
FUR	20(100)	0	0
P	20(100)	0	0
SP	15(75)	0	5(25)
S	0	0	20(100)
TE	0	17(85)	3(15)
TIL	0	0	20(100)
TS	19(95)	0	1(5)
TUL	10(50)	0	10(50)
TY	15(75)	0	5(25)

S: Sensitive, I: Intermediate, R: Resistant, AMP: Ampicillin, AMC: Amoxicillin clavulanic acid, AX: Amoxicillin, CIP: Ciprofloxacin, CL: Cephalexin, CN: Gentamicin, CT: Colistin sulphate, DO: Doxycillin, E: Erythromycin, ENR: Enrofloxacin, FFC: Florfenicol, FUR: Ceftiofur, P: Penicillin: SP: Spiramycin, S: Streptomisin, TE: Tetracycline, TIL: Tilmicosin, TS: Trimethoprim sulphamethoxazole, TUL: Tulathromycin, TY: Tylosin



Figures 1. Multiplex PCR assay was performed using Group I-II-III Tcr primers respectively. M: 100bp marker; 1, 2, 4, 5: *tet* (B), 6: *tet* (B) and (D); 2: *tet* (A), 3, 4, 6: *tet* (G); 4: *tet*(M), 7: *tet* (L)

DISCUSSION

G. anatis is commonly found among normal flora of both the upper respiratory tract and lower genital tract of chickens and other avian species, and can therefore be regarded as an opportunistic pathogen. The pathogenesis of *G. anatis* is not well-characterized, particularly at the molecular level, and little is known about which antibiotic resistace, genes and mechanisms are associated with the ability of *G. anatis* to cause disease.The current investigation is the first study of the antimicrobial resistance, *tet* and virulence genes of *G. anatis* in Turkey. Among the 20 isolates tested, the highest antimicrobial resistance patterns were observed for erythromycin, streptomycin, tilmicosin (100%) followed by colistin sulphate (65%), cephalexin and tulathromycin (50%) which are shown in table 3 The majority of the isolates were exhibited susceptibility against to amoxicillin clavulanic acid, ceftiofur, enrofloxacin, florfenicol, gentamicin, trimethoprim sulphamethoxazole which is in agreement with the other studies (Jones et al., 2013; El-Bastawy, 2014; El-Adawy et al., 2018; Lawal et al., 2018). About 100% of the *G. anatis* isolates exhibited sensitivity to doxycilline while 15% and 85%, respectively, showed intermediate resistance to tetracycline. Especially high level of tetracycline resistance was similar with the previous researches (Bojesen et al., 2011b; Jones et al., 2013; Abd El-Hamid et al., 2016; Lawal et al., 2018). In contrast to these findings, Lin et al. (2001) also reported moderate sensitivity to tetracycline. Multi-drug resistance reveals that 13 isolates representing large percentage (65%) resistance against three or more antibiotics. Especially, MDR patterns in this study were similar to those observed in previous study (Bojesen et al., 2011b). In this study, singleplex and multiplex PCR were used to detect Tcr and virulence genes in *G. anatis* isolates from laying hens. This study can be one of the first tries to examine the prevalence of these genes in *G. anatis* isolates in Turkey and also to test for the presence of *tet* (P), (Q), (S), and (X) in addition to the previously studied *tet* (A), (B), (C), (D), (E), (G), (H), (K), (L), (M) and (O) genes (Hansen et al., 1993; Bojesen et al., 2011b). Four multiplex PCR groups were used in this study to detect 14 tetracycline resistance genes and singleplex PCR to target virulence-associated *gtx*A and *flf*A genes. Twenty isolates of *G. anatis* contained 10 (50%) carried genes for tetracycline resistance, 7 (35%) had *tet*(B), 2 (10%) had *tet*(G), and 1(5%) had *tet*(A), (D), (M) or (L). Another 2 (10%) carried both *tet*(B) and *tet*(G) while 1 (5%) had *tet*(B), (D) and (A) genes. None of the other resistance genes were detected. Together, *tet*(A), (B), (D), (G), (M) and (L) genes, which are associated with efflux and/or ribosomal protection mechanisms of *G. anatis* were detected (Ng et al., 2001; Michalova et al., 2004). Unsuprisingly, presence of these genes was explained according to the previous studies (Kehrenberg et al., 2001; Kehrenberg et al., 2006; Bojesen et al., 2011b). It is indicated that group I *tet*(B) genes had the most numbers among the 20 isolates, which is consistent with a report by Bojesen et al. (2011b). The *tet*(B) gene compared to the others, represented especially among *Enterobacteriaceae* (Roberts, 1996; Levy, 1998; Kehrenberg et al., 2006) and reported to be widely distributed among *Pasteurellacea* (Vaca et al., 2011; Lucio et al., 2012; Bager et al., 2013; Kudirkiene et al., 2014; Persson and Bojesen 2015; Zhang et al., 2017). The pathogenicity of *G. anatis* is influenced by various factors encoded by different virulence genes that play important roles in different pathogenic activities such as adhesion, invasion, intracellular survival, systemic infection, and toxin production (Kristensen et al., 2011; Persson and Bojesen, 2015; Sorour et al., 2015; Sing et al., 2016). In particular, the *gtx* toxin is responsible for the hemolytic and leukotoxic affects of *G. anatis* (Bager et al., 2013; Kudirkiene et al., 2014; Persson and Bojesen, 2015). The *flfA* gene is also implicated in *G. anatis* virulence and is a target for prevention of diseases caused by *G. anatis* in laying hens (Bager et al., 2013; Kudirkiene et al., 2014; Persson and Bojesen 2015). PCR amplification of these genes (*gtxA* and *flfA*) in this study showed that 6 (30%) of the tested strains carried *gtxA*, but none had *flfA*. All of the isolates in this study displayed hemolytic characteristics, which is consistent with the expectations about the value of detecting *gtx* for determination of pathogenic activity. A previous study that focused on hemolytic strains of *G. anatis* found that *gtx* was present in 7/12 (58%) and 5/13 (38.4%) samples from chickens and ducks, respectively (Sorour et al., 2015). Meanwhile, a study by Kristensen et al. (2011) revealed that *gtx* is associated with non-hemolytic *G. anatis* strains. The other studies found high incidences (50-75%) of *flf*A gene (Kudirkiene et al., 2014; Sorour et al., 2015), whereas none of the isolates in present study had *flf*A. Moreover, the absence of fimbria in the isolates that examined could have contributed to the lower pathogenicity of these *G. anatis* strains. The findings of this study indicated no correlation between the presence of Tcr genes and genes associated with virulence in the isolates tested. The virulence mechanisms

associated with the ability of *G. anatis*, which is typically a non-pathogenic component of the normal respiratory microflora of animals, to induce opportunistic respiratory tract infections under conditions that compromise immune responses or those that cause stress, such as inadequate nutritional intake (Bojesen et al., 2003), require further investigation.

CONCLUSION

The present study detected the genes associated with virulence and tetracycline resistance of *Gallibacterium anatis* that isolated from laying hens in Turkey for the first time and presented the first evidence to support the use of specific primers for *tet* P, Q, S and X genes in this breed. The findings of this study can increase the knowledge of *Gallibacterium anatis* pathogenicity in poultry.

DECLARATIONS

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

OSY planned and designed the study. OSY performed the experiments, SY and OSY contributed to the analysis and interpretation of data. OSY drafted the manuscript. All authors read and approved the final manuscript.

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Effect of Water Acidification and Sanitation on Performance, Gut Microbial Population and Carcass Characteristics of Broiler Chicken

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ABSTRACT

Management of water is of utmost importance in order to ensure better performance of poultry birds. A study was thus conducted to evaluate the effect of water acidification and sanitation on performance, gut microbial population and carcass characteristics of broiler chicken. A total of 144 broiler chickens aged one-week were utilized for the study up to 6 weeks of age. The birds were distributed into 4 treatment groups of three replicates of 12 chickens. Untreated drinking water was routinely used in the control group (T1). In T2 and T3, acidifier and sanitizer were used in an amount of 1ml and 5ml/20 liters of water, respectively, while in T4 a combination of acidifier and sanitizer was used in similar doses. There was a significant effect of acidification and sanitization of water on body weight gain and feed conversion ratio of broiler chicken. The combination of acidifier and sanitizer (T4) was found to be highly effective in improving chickens' performance, followed by sanitizer alone (T3) and acidifier alone (T2), compared to the control group. There was no significant effect on various carcass characteristics of broiler chicken except for the gut pH and intestinal length. There was a significant effect on the pH value of various intestinal segments in broiler chickens using acidifier treated water (T2) compared to T1 and T3, but no statistical effect was noticed between T2and T4. A similar trend was noticed in the length of intestines of broiler chickens in various treatment groups. There was a significant reduction in Caecal Coliform Count (in all the treatment groups that was used acidifier and sanitizer compared to the control group.

Key words: Acidifier, Broiler chicken, Gut microbiology, Performance, Sanitizer

INTRODUCTION

It has been estimated that there will be a lack of clean water in almost half of the world by 2025 (Micciche et al., 2018). Water is the most important nutrient and is physiologically required in all animals, including poultry. Therefore, the quantity and quality of water should be supplied daily according to the age and breed of the birds to maintain all physiological functions. Moreover, from a health perspective, the amount of water consumed on a daily basis by commercial poultry birds is considered as a prime indicator (Manning et al., 2007). In addition to the production perspective, providing adequate and good quality water is listed as a basic animal welfare criterion (NCC, 2010).

The use of acidifiers in poultry is relatively new. Almost all acidifiers were considered safe for animal use (Center for Food Safety Applied Nutrition, 2018). A wide range of acidifiers with variable physical and chemical properties are available for poultry, many of which are used in the drinking water or mixed with the feed (Huyghebaertet al., 2011; Menconi et al., 2014). It has been documented that the use of acidifiers benefit the young chickens by their role in competitive exclusion, improving intestinal health and nutrient utilization, and the performance of birds (Adil et al., 2010; Saki et al., 2012). The acidifiers penetrate the cell wall of certain bacteria in non-dissociated form and disrupt their physiology (Dhawale, 2005). Besides antimicrobial activity, acidifiers decrease gut pH, increase secretions from pancreas and exert trophic impact on mucosa of gastro-intestinal tract (Dibner and Buttin, 2002).

Sanitizers such as quaternary ammonium compounds are also used in poultry operation to disinfect the water and water system (Schwartz, 1994). Biofilm formation exposes birds to various pathogens (Maharjan et al., 2017). The oxidability of these chemicals in water destroyed most bacteria and viruses within a few seconds (Yang, 2006)

and maintained the biologically safe and stable environment in the water, which prevented the regrowth of microbes, algal blooms and biofilm formation in the water distribution systems (Sparks, 2009). Daily water sanitation at poultry farms had improved the performance and profitability of poultry birds (Tablante et al., 2002).

In view of the beneficial effects of acidifiers and sanitizers and the scares literature available on the use of a combination of acidifiers and sanitizers in poultry production, the present study was undertaken.

MATERIALS AND METHODS

Ethical approval

The present study was approved by the Institutional Animal Ethics Committee after approval in Research Council Meeting of SKUAST-Kashmir, India.

Methodology and Experimental design

The study was carried out using144 commercial Cobb straight broiler chickens, purchased from local supplier. On arrival of day old birds, they were offered sugar solution (8%) and ground maize for initial 12 hours. In order to avoid stress, the water-soluble vitamins and electrolytes were added to the drinking water in the first 3 days. At the age of 7 days, the chickens were divided into 4 treatment groups with 3 replicates of 12 chickens. The 24 hours lighting schedule was maintained and exhaust fans were used for proper ventilation. The birds were vaccinated against New castle and Gumboro diseases. Fresh food and water were provided *ad libitum* daily. The feeding program consisted of a starter diet up to 21 days and a grower diet up to the age of 42 days for all broiler chickens. Untreated drinking water was used routinely in the control group (T1). In T2 and T3 groups, acidifier and sanitizer were used in the amount of1ml and 5ml/20 liters of water respectively, while in T4 group a combination of acidifier and sanitizer at similar doses was used.

Parameters recorded

The body weight and feed intake per replicate were recorded weekly and subsequently the feed conversion ratio per replicate was determined. After the completion of the trail, 6 days were randomly selected and slaughtered from each treatment. The length of gastrointestinal tract was measured with a tape measure. The intestine was exposed on both sides. The carcass characteristics were evaluated. The contents of caeca were collected, weighed (1 gram), and then homogenized in sterile tubes in the ration 1:1 with 0.9% normal saline solution. Then the solutions were mixed on vortex. Serial dilutions of the samples were made up to the sixth dilution. 0.1 ml was withdrawn from each dilution, and distributed evenlyon Brain Heart Infusion (BHI) agar and the caecal coliforms count was calculated. The plates were incubated at 37˚C for 48 hours. Bacterial colonies were counted by the pour plate method (Quinn et al., 1992). The average number of colonies was multiplied by reciprocal of the dilution factor and expressed as cfu/gram of contents.

Statistical analysis

The data obtained were statistically assessed by the analysis of variance (ANOVA) through the General Linear Model procedure of SPSS (20.0) software, considering replicates as experimental units. The values were expressed as means ± Standard Error (SE). Duncan's multiple range test was used to test the significance of the difference between the means by considering the significant differences at $p<0.05$.

RESULTS AND DISCUSSION

The feed consumption showed no statistical difference ($p>0.05$) between the treatment groups compared to the control group (Table 1). These results were in agreement with the results of Banday et al. (2015), who found no difference in the cumulative feed consumption between the groups in which acidifiers were used and the control group. There was a significant ($p<0.05$) improvement in the chickens FCR using acidifier and sanitizer in water alone or in combination compared to the control group (Table 2). The combination of acidifier and sanitizer (T4) was found to be highly effective in improving the chickens FCR, followed by sanitizer alone (T3) and acidifier alone (T2) compared to the control group (T1). The improvement in the FCR could possibly be due to better use of nutrients, which leads to in increased body weight gain in the birds (as can also be seen in the present study), since the water was used based on acidifying and sanitizer agents. These results were consistent with other researchers (Adil et al., 2011; Sultan et al. 2014; Banday et al., 2015), who reported that the addition of acidifiers and sanitizers improved the feed conversion ratio in poultry birds.

The body weight gains were significantly ($p<0.05$) improved by addition of acidifier and sanitizer alone or in combination in water of broiler chicken compared to the control group (Table 3). The combination of acidifier and sanitizer (T4) was found highly effective in improving the body weight gains of broiler chicken, followed by sanitizer

alone (T3) and acidifier alone (T2) compared to control group (T1). The results of present study regarding acidifier coincides with the results of other researchers (Aoet al., 2009; Adil et al., 2011; Banday et al., 2015) who reported that the supplementation of acidifiers improves the body weight gain in poultry birds compared to control group. Likewise, Tablante et al. (2002) and Jacobs et al. (2019) reported that water sanitation improved the performance of poultry.

Table 1. Feed consumption of cobb broiler chickens in different treatments wherein acidifiers and sanitizers used in water in 2019 at the farm of Faculty of Veterinary Sciences SKUAST-K in Kashmir region, India

Age	Treatment Groups			
	T1 (control group)	T2 (Acidifier)	T3 (Sanitizer)	T4 (Acidifier + Sanitizer)
1-2 weeks	276.13±0.9*	273.67±1.6	272.26±1.3	273.11±4.9
2-3 weeks	508.05±6	537.27±18.6	532.56 ±12.1	548.01±13.1
1-4 weeks	1190.39±31.2	1209.58±37.7	1213.44±25.4	1221.14±41.8
1-5 weeks	2054.41±55.7	2065.50± 13.8	2082.25± 51.2	2071.78±61.4
1-6 weeks	3183.16±95.	3149.83±80.7	3172.06± 52.7	3238.80±90.1

*: means ±standard error

Table 2. Feed conversion ratio of Cobb broiler chickens in different treatments wherein acidifiers and sanitizers were used in water in 2019 at the farm of Faculty of Veterinary Sciences SKUAST-K in Kashmir region, India

Age	Treatment Groups			
	T1 (control group)	T2 (Acidifier)	T3 (Sanitizer)	T4 (Acidifier+ Sanitizer)
1-2 weeks	1.39^{a}± 0.06	1.38^{a}± 0.05	1.38^{a}± 0.01	1.37^{a}± 0.01
2-3 weeks	1.54^{b}±0.11	1.45^{a}±0.17	1.43^{a}±0.12	1.41^{a}±0.17
1-4 weeks	1.68^{b}±0.17	1.56^{a}± 0.23	1.52^{a}±0.13	1.50^{a}±0.16
1-5 weeks	1.83^{b}±0.01	1.73^{a}±0.02	1.71^{a}±0.02	1.68^{a}±0.03
1- 6 weeks	1.96^{b}± 0.34	1.84^{a}±0.03	1.82^{a}±0.01	1.80^{a}±0.03

$^{a, b}$ = Means within the same row with different superscripts are significantly different ($p<0.05$); *: means ±standard error

Table 3. Body weight gain of Cobb broiler chickens in different treatments wherein acidifiers and sanitizers were used in water in 2019 at the farm of Faculty of Veterinary Sciences SKUAST-K in Kashmir region, India

Age	Treatment Groups			
	T1 (control group)	T2 (Acidifier)	T3 (Sanitizer)	T4 (Acidifier+ Sanitizer)
1-2 weeks	198.65^{a}±1.4	198.32^{a}±2	197.47^{a}±2.4	199.33^{a}±2.848
2-3 weeks	330.0^{b}±6.3	370.21^{a}±8.3	372.28^{a}±5.5	388.39^{a}±8.2
1-4 weeks	708.50^{b}±11.2	775.17^{a}±12.7	798.85^{a}±22.8	813.31^{a}±18.5
1-5 weeks	1122.74^{b}±23.3	1194.29^{a}±19.9	1217.41^{a}±17.6	1232.28^{a}±19.7
1- 6 weeks	1623.89^{c}±30	1711.0^{b}±17	1742.81^{ab}±17.9	1798.56^{a}± 21.4

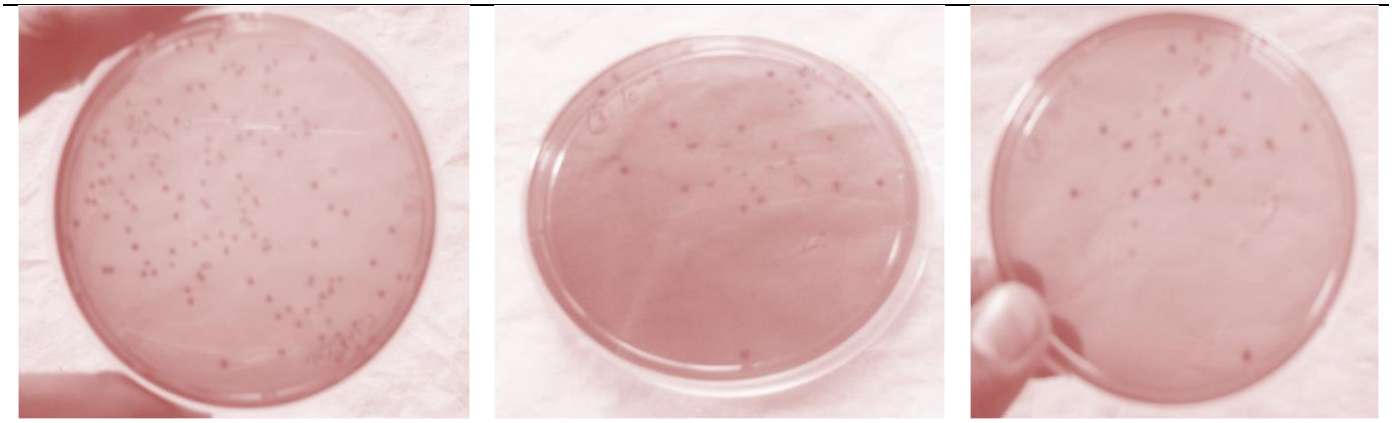
$^{a, b}$ = Means within the same row with different superscripts are significantly different ($p<0.05$); *: means ±standard error

Table4. Carcass characteristics, gut pH and gut microbiology of Cobb broiler chickens offered water treated with acidifier and sanitizer in 2019 at the farm of Faculty of Veterinary Sciences SKUAST-K in Kashmir region, India

Parameters	T1 (Control)	T2 (Acidifier)	T3 (Sanitizer)	T4 (Acidifier +Sanitizer)
Dressing percentage	71.23^{a}±0.51	70.44^{a}±0.79	72.67^{a}±1.31	71.34^{a}±1.17
Ready to cook yield (%)	75.28^{a}±1.05	75.45^{a}±0.82	76.19^{a}±0.69	76.32^{a}±0.58
Length of small intestine (cm)	168.43^{a}±1.57	179.30^{b}±2.04	169.52^{a}±2.13	179.95^{b}±1.76
Weight of small intestine (g)	41.62^{a}±0.93	47.23^{b}±1.12	40.66^{a}±1.01	46.85^{b}±1.27
Crop pH	4.83^{a}±0.02	4.57^{b}±0.03	4.99^{c}±0.01	4.54^{b}±0.02
Duodenum pH	5.71^{a}±0.01	5.45^{b}±0.02	5.79^{a}±0.03	5.46^{b}±0.01
Caecal coliform count	5.64^{a}±0.04	5.03^{b}±0.01	5.11^{b}±0.01	5.08^{b}±0.02

(cfu/gram)

[a, b =]Means within the same row with different superscripts are significantly different ($p<0.05$); *: means ±standard error



Control (T1) Acidifier (T2) Sanitizer (T3)

Figure 1. The effect of acidifiers and sanitizers in water on caecal coliform count of broiler chickens at the farm of Faculty of Veterinary Sciences SKUAST-K in Kashmir region, India

The improved body weight gain due to addition of acidifiers and sanitizers in water is probably due to the beneficial effect on gut flora as observed in the present study because of reduced caecal coliform count, reduced gut pH and beneficial effect on intestinal morphology (Table 4 and figure 1). A significant reduction in caecal coliform count in all the treatment groups was observed wherein acidifier and sanitizer treated was used when compared to control. The combination of acidifier and sanitizer (T4) was found highly effective in reducing the caecal coliform count. Similar results were observed by Adil et al. 2011 and Owens et al. (2008) who reported significant ($p<0.05$) reduction in total viable caecal coliforms in broiler chicken as a result of acidifier supplementation. A significant decrease in the number of total and gram-negative bacteria has been reported when using acidifiers (Gunal et al., 2006). Samanta et al. (2010) also reported that acidifiers reduce E. coli and other harmful bacteria which mayenhance poultry growth. Antibacterial effect of acidifiers has been associated with the fact that undissociated organic acids pass through the cell membrane of the bacteria and afterward dissociate forming H^+ions and this, in turn, decreases the pH value ofthe bacterial cell. In order to restore the normal balance, bacteria use its energy. Whereas RCOO- anions produced from the acid can disrupt DNA, hampering protein synthesis and putting the organism in stress. As a result the organism cannot multiply rapidly and decrease in number Nursey (1997). Similarly, sanitizers have been reported to control microbes or inhibit biofilm formation because of antimicrobial activity (Maharjan et al., 2016). Because of antibacterial activity of acidifiers and sanitizers, there would have been a decrease in the competition for the host nutrients, thereby improving the protein and energy digestibility and subsequent overall performance of the broiler chickens.

Further, this antibacterial activity gets augmented by pH reducing property of acidifiers as was seen in the present study (Table 4). The use of acidifiers resulted in decreased ($p<0.05$) pH in crop and duodenum of broiler chicken. The reduced pH is helpful for the growth of favorable bacteria and at the same time prevented the growth of harmful bacteria which require a relatively higher pH for growth (Adil et al., 2011). Moreover, no effect ($p>0.05$) on carcass characteristics was observed as a result of addition of acidifiers and sanitizers in drinking water of broiler chicken except for the weight and length of small intestine (Table 4). There was a significant ($p<0.05$) effect on pH value of various segments of gut in broiler chicken wherein acidifier treated water (T_2) was used when compared to T_1 and T_3, however no statistical effect was noticed between T_2 and T_4. Similar trend was noticed in the length of intestines of broiler chicken among various treatment groups. Adil et al. (2011) also reported that acidifiers resulted in remarkable increase in the intestinal weight and length of broiler chicken. These results could be attributed to the fact that acidifiers have direct stimulatory effect on the gastro-intestinal cell proliferation as was reported by other workers with short chain fatty acids. The short chain fatty acids are believed to increase plasma glucagon-like peptide 2 (GLP-2) and ileal pro-glucagon mRNA, glucose transporter (GLUT2)

expression and protein expression, which are all signals which can potentially mediate gut epithelial cell proliferation Tappenden and McBurney (1998). Le Blay et al. (2000) and Fukunaga et al. (2003) also reported that short chain fatty acids can accelerate gut epithelial cell proliferation, thereby increase improve intestinal morphology. Besides antibacterial activity, this improved intestinal morphology effect augments performance of birds by improved digestion of nutrients.

CONCLUSION

In conclusion, addition of acidifiers and sanitizers @ 1ml and 5ml/20 liters of water of broiler chickens improve their performance and subsequent profitability out of a poultry enterprise. The beneficial effects were achieved by antibacterial and improved gut health properties of acidifiers and sanitizers, it is thus recommended to improve the quality of water at poultry farms by adding acidifier and sanitizer products.

DECLARATIONS

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Competing interests

The authors declare that they have no competing interests.

Author's contributions

MT Banday and IU Sheikh designed the research; S Adil performed the research and wrote the manuscript; IA Baba and B Zafferassisted in collection of data and AA Khan analysed the data. All authors read and approved the final version of the manuscript.

Consent to publish

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

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Immune Profile of Broilers between Hatch and 9 Days of Age Fed Diets with Different Betaine and Fibre Concentrations

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ABSTRACT

An experiment was designed to determine the immune profile of broiler chickens between hatch and 9 days of age when fed diets with different fibre and betaine concentrations. A total of 240 day-old Cobb 500 male chickens were allocated to 16 cages with 15 chickens each. Treatments were arranged in a 2 x 4 factorial design, with 2 replicate cages per treatment. Treatments consisted of two feed formulations (low and high fibre diets) and four levels of betaine (0, 1, 3 or 5 kg/tonne). Before the start of trial (hatch), 10 broilers were weighed and blood samples were collected by cardiac puncture, then euthanised by cervical dislocation and jejunal samples collected for the determination of gene expression of claudin 1, claudin 5, occludin and interleukin 2 by PCR. Mononuclear cells populations in the blood samples were determined by flow cytometry. On days 4 and 9, five birds/cage (10 birds/treatment) were selected, euthanised and samples taken as per the start of hatch. Gene expression of claudin 1, claudin 5 and occludin was reduced between 4 and 9 days, independent of the group tested, while interleukin tended to increase between hatch and 4 days and decreased thereafter. Betaine inclusion reduced claudin 5 and occludin gene expression and increased CD8- CD28+ and CD8+ CD28+, suggesting it may aid in accelerating maturation of both the gastrointestinal tract and immune system for broilers in the early days post-hatch.

Key words: Betaine; Fibre; Gene expression; Maturation; Immune response

INTRODUCTION

The genetic selection imposed on broilers for improving weight gain and feed conversion ratio has reduced its immunocompetence and as a result, increased the disease susceptibility (Bridle et al., 2006). The generation of an unnecessary immune response represents a significant expense of energy and nutrients that could be targeted for other processes such as body weight gain. On other hand, failure to generate an immune response when required, is even more detrimental due to the morbidity and mortality it may cause. The immune system not only react to pathogenic challenges but also support the nervous system maintaining animal homeostasis (Tada, 1997).

At hatch, broilers do not have a fully developed immune system (Kogut, 2017). Both cellular and humoral immune systems develop during the first few days post hatch, including the development of the bursa of Fabricius and the thymus (Dibner et al., 1998) that increase proportionally more than other organs at early ages, receding as the animal gets older (Cazaban et al., 2015).

The gastrointestinal tract is not fully developed at hatch (Uni et al., 2003). The association in development of these two systems is linked because the development of the immune system depends on the availability of nutrients and energy absorbed via the gastrointestinal tract. Moreover, the gut functions as a sensory organ for the development of the immune system (Furness et al., 2013). Impaired nutrient absorption can influence the initial development of the immune system, compromising immunocompetence of the animal (Dibner et al., 1998). At the same time, maintenance of the immune status in the gastrointestinal tract has a high cost for the animal (Kogut et al., 2017).

At hatch, gastrointestinal enterocytes are not tightly linked to each other, allowing the passage of large molecules via paracelullar routes directly into the interstitial spaces (Karcher and Applegate, 2008). Opening of paracellular passages is a route used by pathogenic bacteria and viruses while other bacteria actually increase rigidity of these junctions thereby reducing permeability (Sommer and Backhed, 2013). Gut-associated lymphoid tissues are agglomeration of immune cells which protect the body against the passage of microbiota from the gastrointestinal tract to other organs that are more concentrated in the hindgut and especially the ceca where the microbiota population is highest (Yegani and Korver, 2008).

The gut microbiota changes as soon as the animal starts to eat (Ballou et al., 2016). The composition of the feed influences the immune status of the animal and the species of bacteria that colonize the tract (Oakley and Kogut, 2016). The diet also influences the osmolarity of the intestines, with hyperosmolarity often stimulating inflammatory responses (Schwartz et al., 2009). Thus, the composition of the feed can influence the immune status of broilers. High fibre diets have been shown to increase the digesta transit time and stimulate bacteria growth due to a reduction in nutrient digestibility (Adeola et al., 2016) and also increase gut fermentation and possibly increase the inflammatory status of the digestive tract (Celi et al., 2017). On the other hand, fibre fermentation in the lower gut stimulates volatile fat acid such as butyrate, a trophic substance that stimulates villus growth (Rahmatnejad and Saki, 2016), increases gap junction protein expression, reduces inflammatory responses and endogenous losses during a coccidial challenge (Adedokun et al., 2012), lowers the presence of pathogenic bacteria in internal organs and improves animal performance (Rezaei et al., 2011).

Betaine is an osmoprotectant and is recognised as a chemical chaperone preventing protein denaturation and thus maintaining cell activity in hyperosmotic situations (Schwahn et al., 2003). Birds fed betaine have been shown to have reduced inflammatory responses when subsequently exposed to a coccidial challenge and maintain higher water flow and villus size (Kettunen et al., 2001), but similar effects were not observed when the betaine was offered at the same time as the coccidial challenge (Matthews and Southern, 2000).

The objective of the present study was thus to evaluate the proportion of circulating immune cells, and the tight protein and interleukin 2 gene expression in broilers at hatch, 4 and 9 days of age when fed diets with different levels of fibre and inclusion rates of betaine.

MATERIALS AND METHODS

Ethical approval

The study design was reviewed and approved by the Animal Use Ethics Committee of the Agricultural Science Campus of the Universidade Federal do Paraná (Protocol number 002/2015).

Animals, diets and experimental design

Two hundred and forty day-old male Cobb 500 broilers were sourced specifically for the experiment and housed in 16 stainless steel cages with 15 birds/cage. A further 10 birds were included for sampling at hatch, for measurement of the parameters of interest at the outset of the experiment. Room temperature was controlled with electric heaters to meet the breeder recommendations for the age of the bird (Cobb-Vantress Inc., 2015). Cage dimensions were 0.90 m × 0.40 m, and 0.30 m height. Birds had *ad libitum* access to water and feed. Treatments were designed in a 2 × 4 factorial arrangement with two levels of fibre concentration (low and high) and four inclusion rates of betaine (0, 1, 3 and 5 kg betaine/tonne of feed). Experimental diets were based on corn, soybean and rice bran as previously described (Santos et al., 2019) with Betaine (Vistabet® 96, AB Vista – Marlborough, United Kingdom) included at the targeted rate for each treatment by replacing the relevant weight of washed sand (Table 1).

Sample collection

At hatch, 10 birds that were not assigned to any treatment were weighed and separated for initial sampling. Blood was collected by cardiac puncture. Birds were then euthanized by cervical dislocation. The intestine was collected from the cardiac junction to the cloaca. One jejunal sample, defined as the section between the duodenal loop and 1cm distal of the Meckel`s diverticulum (Teirlynck et al., 2009), was taken from each bird. At 4 and 9 days of age, 10 birds per treatment were separated and the same collection process was followed for blood and jejunum collection.

Flow cytometry analysis

Approximately 2 mL of blood was collected into a heparin treated vacutainer tube. Initially, the mononuclear cells in the blood were isolated using Histopaque-1077 (Sigma Aldrich, St. Louis, USA), as described by Fair et al. (2008). Briefly, blood samples were centrifuged at 400 x g for 30 min after dilution with Phosphate-Buffered Saline (PBS) and Histopaque-1077, and white cells then collected as the layer above the erythrocytes. The white cells were then washed with PBS and centrifuged at 400 x g for 7 min. Samples were fixed with 1% paraformaldehyde and counted using a Neubauer counting chamber.

Once the cell concentration in the blood was determined, flow cytometry was performed as reported by Stabel et al. (2000). Briefly, 50µl of cell suspension (equivalent to 10^6 mononuclear cells) was incubated for 30 min at 37 °C with specific primary antibodies and fixed in paraformaldehyde for 30 min at 4 °C. This process was repeated for the secondary antibody. Monoclonal antibodies to the specific subset of cells were supplied by Southern Biotechnology. "CD4" specificity for T helper lymphocytes; "CD8β" specificity for T cytotoxic lymphocytes; "CD28" specificity for memory T lymphocytes; "Mo" specificity for monocytes; "MHCII" specificity for antigen-presenting cells and "TCR αβ Vβ1" specificity for T lymphocytes were prepared.

Reading was performed within 2 h of staining on a FACSCalibur flow cytometer (Becton Dickinson, United Kingdom), as reported by Beirão et al. (2012). Green fluorescence was detected on the FL1 channel (530/30 nm), and orange fluorescence was detected on the FL2 channel (585/42 nm). Cells were analysed at up to 10,000 events in the lymphocyte gate and data were analysed with FlowJo software (TreeStar Inc., United States).

Gene expression

Jejunal samples were immediately put in dry ice after sampling and stored at -80 ^oC. A 5 mm^2 section of each sample was collected and processed as described by Adedokun et al. (2012). RNA extraction was proceed using RNAzol® RT kit as per the manufacturer's protocol (Molecular Research Center Inc., Cincinnati, OH, USA). The RNA pellet was dissolved in nuclease-free water (Omnipure, EMD Chemicals Inc., Gibbstown, NJ) and its concentration determined using a 260/280 nm nanodrop spectrophotometer (Fisher Scientific, St. Louis, MO) and extracted RNA stored at -80^oC (Beirão et al., 2012).

Primers were designed for real-time PCR with the Oligo Analyzer 3.1 tool from Integrated DNA Technologies for the (complementary DNA) cDNA sequence obtained from the NCBI database. Genes evaluated in this study were glyceraldehyde-3-phosphate dehydrogenase (GAPDH) which served as the housekeeping gene, claudin 1 and 5, occludin and interleukin 2. A list of the primers used in the present study is reported in table 2.

PCR analysis was performed as described by Bustin et al. (2009). Complementary DNA was synthesized from 1µg of total RNA using iScript Select cDNA Synthesis kit (BioRad) with Oligo $(dT)_{20}$ primer mix and GSP enhancer solution. The synthesis reaction was follows: 25 ^oC for 5 min, 42 ^oC for 1 h, and 70 ^oC for 15 min. The reaction for PCR were carried out on a RotorGene Real-Time PCR System (Quiagen Inc., Thousand Oaks, CA) in an EvaGreen fluorescent system. The following PCR conditions were used: 95 ^oC for 10 min, followed by 40 cycles of 60 ^oC for 10 sec and 95 ^oC for 10 sec. All samples were processed in triplicate and expressed relative to the house-keeping gene. For the analysis of gene expression at different ages, the expression was calculated based on the average expression of the target gene and housekeeping gene as reported by Pfaffi (2001) using the results from the low fibre diet without betaine inclusion as the reference.

Statistical analysis

Data was log-transformed prior to analysis as described by Beirão et al. (2012). Data were subjected to ANOVA using the GLM model for completely randomised design procedure of JMP Pro (SAS Institute Inc., Cary, NC, USA). Treatment means at hatch, 4 and 9 days of age were separated by Tukey`s Significant Difference test. Results at 4 and 9 days of age were also analysed using a two-way interaction considering fibre and betaine inclusion levels for the gene expression data and inclusion of a third factor, age, to generate the three-way interaction for the leukocyte population. A three-way interaction for gene expression was not considered as the samples were not collected from the same animals. Each animal served as the experimental unit. Statistical significance was accepted at $p < 0.05$.

Table 1. Ingredients and chemical composition of experimental diets (g/kg, as-fed basis).[1]

Ingredients	Low fibre diet	High fibre diet
Corn (8% CP[2])	533.60	463.80
Rice bran (12.5% CP[2])	-	70.00
Soybean oil	35.00	43.50
Soybean meal (46% CP[2])	385.00	376.50
Limestone	10.60	10.90
Dicalcium phosphate	18.00	17.50
Salt	4.60	4.60
Vitamin/Mineral premix[3]	2.00	2.00
Lysine HCl	2.05	2.05
DL Methionine	3.25	3.25
L- Threonine	0.65	0.70
Washed sand	5.00	5.00
Choline Cloride	0.25	0.20
Chemical composition		
Crude protein	220	220
Metabolizable energy, kcal/kg	3000	3000
Crude fibre	29.50	43.50
Sol. Arabinoxylane	0.53	0.60
Insol. Arabinoxylanse	19.21	21.81
Sol NSP[4]	19.88	19.30
Insol NSP[4]	89.48	93.74
Ether extract	60.00	77.00
Ash	34.00	39.00
Calcium	9.50	9.50
Phosphorous	6.80	7.60
Available Phosphorous	4.50	4.50
Sodium	2.00	2.00
Digestible Lysine	12.50	12.50
Digestible Methionine+Cysteine	9.10	9.10
Digestible Threonine	8.10	8.10

[1]Betaine included at the expense of inert. [2]Crude Protein. [3]Supplied per kilogram diet: iron (Ferrous Sulphate), 60 mg; manganese (Manganese Sulphate and Manganese Oxide), 120 mg; zinc (Zinc Oxide), 100 mg; iodine (Calcium Iodate), 1 mg; copper (Copper Sulphate), 8mg; selenium (Sodium Selenite), 0.3mg, vitamin A, 9,600 IU; vitamin D_3 3,600 IU; vitamin E, 18mg; vitamin B_{12}, 15 mcg; riboflavin, 10 mg; niacin, 48 mg; d-pantothenic acid, 18 mg; vitamin K, 2 mg; folic acid, 1.2 mg; vitamin B_6, 4 mg; thiamine, 3 mg; d-biotin, 72 mcg. [4]Non-starch polysaccharides.

RESULTS

Average body weights were 47, 121 and 260 g at hatch, 4 and 9 days of age, respectively, which is close to the expected weight of the genetic line (Cobb-Vantress Inc., 2015) and no differences were observed between treatments. A detailed description of results has previously been published (Santos et al., 2019).

Claudin, occludin and interleukin 2 gene expression

Gene expression for claudin 1 and claudin 5, occludin and interleukin 2 was reduced ($p < 0.001$) at 9 days compared to hatch and 4 days of age (Table 3). At 4 days of age, inclusion of 1 and 3 kg/tonne of betaine reduced ($p < 0.05$) gene expression of claudin 5 and occludin while inclusion of 5 kg/tonne had intermediate results (Table 4). At day 9, reduction was dependent on the fibre content in the diet as betaine inclusion reduced ($p < 0.0001$) claudin 1 and 5, occludin and interleukin 2 gene expression in the low fibre diets but no effect was observed in high fibre diets. When betaine was not included in the diet, broilers fed the high fibre diets also presented lower ($p < 0.0001$) gene expression for claudin 1 and 5, occludin and interleukin 2 (Table 4).

Peripheral blood mononuclear cells

Peripheral blood mononuclear cells population varied depending on the age and inclusion of betaine, while fibre concentration had no effect. The population of CD8-CD28+ and CD8+CD28+ cells increased ($p < 0.0001$) from hatch to 4 days of age and fell thereafter, while CD8+CD28- cells further increased ($p < 0.0001$) at day 9 (Table 5). Betaine inclusion increased ($p < 0.0001$) the proportion of both CD8-CD28+ and CD8+CD28+ cells independent of the dose and age, but had no effect on the population of CD8+CD28- cells (Table 5). On the other hand, the proportion of CD4+TCRvβ1+ and CD4+TCRvβ1- cells increased ($p < 0.0001$) from hatch to 4 days of age and fell afterwards, whilst CD4-TCRvβ1+ increased ($p < 0.0001$) from hatch to 4 days and 4 to 9 days of age (Table 5). The impact of betaine on the proportion of CD4+TCRvβ1+; CD4+TCRvβ1- and CD4-TCRvβ1+ populations depended upon age (Table 6). At 4 days of age, inclusion of 3 kg/tonne of betaine increased ($p < 0.0001$) the proportion of CD4-TCRvβ1+; and inclusion of 5 kg/tonne increased ($p < 0.0001$) the proportion of both CD4+TCRvβ1+ and CD4+TCRvβ1- cells, but no effect of betaine inclusion was observed at 9 days of age. The proportion of both Mo+MHCII+ and Mo-MHCII+ cells compared with total cells increased ($p < 0.0001$) in birds at 4 days of age while that of Mo+MHCII- fell ($p < 0.0001$) between 4 and 9 days of age (Table 5). Age affected the CD4:CD8 ratio with the CD4+:CD8+ ratio falling ($p < 0.001$) between days 4 and 9, but no difference was observed between these ages and birds at hatch.

Table 2. Primers used for real-time PCR

Genes	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	Fragment size (base pair)
GAPDH[1]	TCTGGAGAAACCAGCCAAGT	GAGACAACCTGGTCCTCTGTG	104
CLDN1[2]	CCAATGAAGAGGGCTGAT	GTGCATGGAGGATGACCA	185
IL2[3]	TCCCAGGTAACACTGCAGAGTTT	TTGGAAAATATCAAGAACAAGATTCATC	92
CLDN5[4]	ATCTGTGCGCCTTTGAGACT	GCGACCTGCAATGAGTTCG	149
OCLN[5]	GTGGGTTCCTCATCGTCATC	GTTCTTCACCCACTCCTCC	156

[1]Glyceraldehyde 3-phosphate dehydrogenase; [2]Claudin 1; [3]Interleucine 2; [4]Claudin 5; [5]Occludin

Table 3. Claudin 1, Claudin 5, Occludin and Interleukin 2 gene expression (log of the proportion of GADPH expression) on jejunal samples of broilers at hatch, 4 and 9 days of age.[1]

Age (days)	Claudin 1	Claudin 5	Occludin	Interleukin 2
hatch	-0.055[a]	-0.588[a]	-0.176[a]	-0.798[a]
4	-0.374[a]	-0.687[a]	-0.469[a]	-0.108[a]
9	-2.828[b]	-2.521[b]	-2.143[b]	-2.184[b]
SEM	0.109	0.211	0.137	0.202
p-value	<0.001	<0.001	<0.001	<0.001

[1]Means and standard error of the mean (SEM) represent 10 birds at hatch, 4 and 9 days of age. [a,b]Mean within columns with different superscripts are statistically different ($p < 0.05$).

Table 4. Claudin 1, Claudin 5, Occludin and Interleukin 2 gene expression (log of the proportion of GADPH expression) on jejunal samples of broilers at 4 and 9 days of age and fed diets with different fibre concentration and betaine inclusion.[1]

Treatment		Claudin 1		Claudin 5		Occludin		Interleukin 2	
		Age (days)							
		4	9	4	9	4	9	4	9
Fibre									
Low[2]		-0.145	-1.820	-1.008	-2.081	-0.918	-1.591	-0.482	-3.087
High[3]		-0.136	-2.526	-0.734	-2.768	-0.605	-2.143	-0.472	-3.769
SEM		0.095	0.125	0.147	0.145	0.143	0.117	0.098	0.142
Betaine (kg/tonne)									
0		-0.001	-1.277	-0.359[a]	-1.680	-0.237[a]	-1.354	-0.356	-2.255
1		-0.228	-2.279	-1.161[b]	-2.780	-1.095[b]	-2.021	-0.500	-3.672
3		-0.182	-2.678	-1.183[b]	-2.632	-1.050[b]	-1.918	-0.504	-4.005
5		-0.153	-2.432	-0.774[ab]	-2.660	-0.660[ab]	-2.071	-0.549	-3.781
SEM		0.135	0.178	0.208	0.204	0.203	0.166	0.139	0.203
Fibre × Betaine(kg/tonne)									
Low	0		-0.134[a]		-0.426[a]		-0.304[a]		-0.874[a]
	1		-1.886[b]		-2.455[b]		-1.723[bc]		-3.218[b]
	3		-2.854[b]		-2.618[b]		-1.990[bc]		-4.160[b]
	5		-2.611[b]		-2.932[b]		-2.426[bc]		-4.097[b]
High	0		-2.548[b]		-2.934[b]		-2.666[c]		-3.635[b]
	1		-2.934[b]		-3.105[b]		-2.518[bc]		-4.126[b]
	3		-2.502[b]		-2.646[b]		-1.846[bc]		-3.850[b]
	5		-2.176[b]		-2.388[b]		-1.564[bc]		-3.466[b]
SEM			0.229		0.293		0.238		0.291
p-value									
Fibre		0.928	0.001	0.167	0.002	0.111	0.002	0.940	0.001
Betaine		0.658	<0.001	0.021	0.001	0.013	0.046	0.776	<0.001
Fibre × Betaine		0.388	<0.001	0.945	<0.001	0.866	<0.001	0.604	<0.001

[1]Means and standard error of the mean represent 10 birds/treatment at 4 and 9 days of age. [2]Corn-soybean meal diet. [3]Corn-rice bran-soybean meal diet. [a-c]Mean within columns with different superscripts are different ($p < 0.05$).

Table 5. Leukocyte population determined by flow cytometry (log of the proportion of the population) on blood samples of broilers fed diets with different fibre concentration and betaine inclusion at hatch, 4 and 9 days of age.[1]

Leukocytes	CD8- CD28+	CD8+ CD28+	CD8+ CD28-	Mo+ MHCII-	Mo+ MHCII+	Mo- MHCII+	CD4- TCRvβ1+	CD4+ TCRvβ1+	CD4+ TCRvβ1-	CD4:CD8 Ratio
Age (days)										
hatch	0.553[b]	0.012[c]	-1.425[c]	-0.496[a]	0.044[b]	-0.266[b]	-0.375[c]	0.360[c]	-0.128[c]	2.91[ab]
4	1.141[a]	0.603[a]	-0.399[b]	-0.457[a]	0.476[a]	0.028[a]	0.286[b]	0.959[a]	0.568[a]	3.15[a]
9	0.726[b]	0.276[b]	0.106[a]	-0.839[b]	0.430[a]	0.007[a]	0.382[a]	0.563[b]	0.307[b]	2.01[b]
SEM	0.028	0.029	0.031	0.053	0.032	0.034	0.028	0.028	0.031	0.15
Fibre										
Low[2]	0.930	0.445	-0.139	-0.647	0.469	0.045	0.366	0.774	0.415	2.60
High[3]	0.902	0.437	-0.111	-0.682	0.432	-0.015	0.308	0.756	0.443	2.49
SEM	0.026	0.027	0.031	0.052	0.031	0.033	0.026	0.027	0.029	0.14
Betaine (kg/tonne)										
0	0.824[b]	0.286[b]	-0.224	-0.801	0.431	-0.043	0.259	0.690	0.339	2.84
1	0.971[a]	0.495[a]	-0.164	-0.660	0.497	0.028	0.370	0.773	0.436	2.35
3	0.977[a]	0.466[a]	-0.079	-0.602	0.475	0.042	0.318	0.774	0.465	2.48
5	0.964[a]	0.516[a]	-0.112	-0.537	0.404	0.039	0.384	0.823	0.515	2.50
SEM	0.037	0.037	0.044	0.073	0.043	0.046	0.036	0.038	0.041	0.21
p-value										
Age	<0.001	<0.001	<0.001	<0.001	<0.001	<0.010	<0.001	<0.001	<0.001	<0.001
Fibre	0.719	0.822	0.727	0.560	0.356	0.172	0.120	0.629	0.457	0.489
Betaine	0.010	<0.001	0.107	0.082	0.435	0.536	0.074	0.107	0.027	0.423
Fibre × Betaine	0.949	0.497	0.561	0.162	0.058	0.224	0.318	0.852	0.985	0.385
Fibre × Age	0.402	0.849	0.428	0.188	0.519	0.903	0.632	0.413	0.478	0.437
Age × Betaine	0.118	0.377	0.545	0.393	0.125	0.191	0.011	0.050	0.036	0.216
Fibre×Age×Betaine	0.671	0.684	0.962	0.974	0.712	0.962	0.611	0.688	0.554	0.082

[1]Means and standard error of the mean represent 10 birds at hatch day of age and 10 birds/treatment at 4 and 9 days of age. [2]Corn-soybean meal diet. [3]Corn-rice bran-soybean meal diet. [a-c]Mean within columns with different superscripts are different ($p < 0.05$).

DISCUSSION

At hatch, the paracellular junctions between enterocytes in the intestine are not well developed, allowing the paracellular transport of large molecules from the yolk sac. This route of absorption reduces rapidly as these paracellular junctions tighten. The results from the present experiment support this hypothesis as the gene expression for claudin 1 and claudin 5 and occludin was greater between hatch and 4 days compared with birds at day 9. This suggests a greater rate of production of these proteins to increase the strength of these junctions during the first few days post hatch. The percentage of enterocyte membrane involved in tight junctions increases in the 3 days after hatch (Karcher and Applegate, 2008), supporting that during these early days post hatch, the tight junctions become more rigid.

Gene expression of interleukin 2 tended to increase gene expression between hatch and 4 days and reducing afterwards. Interleukin 2 is a proinflammatory interleukin involved in the proliferation of lymphocytes by stimulating the transition of activated T cells from the G_1 to the S phase (Han et al., 2010). An increase in interleukin gene expression between hatch and 4 days of age may be related to a transitory proinflammatory status as birds may be reacting to the presence of digesta in the gastrointestinal tract. Most of the materials in the gastrointestinal tract, although not pathogenic, are capable of stimulating an immune response (Huges, 2005).

At hatch the gastrointestinal tract of chickens is empty, with absorption of fat and readily available proteins in the yolk sac taking place majorly from the yolk sac membrane and the intestine directly into the blood (Noy and Sklan, 2001). Once birds hatch and start to consume feed with more complex nutrients (more complex proteins, carbohydrates, fibre, etc), the intestine may react immunologically due to the presence of these nutrients in the lumen. Given at hatch the digestive tract of chicken is not completely developed (Geyra et al., 2001), the low digestibility and absorption rates can result in a hyperosmotic solution that further contributes to a proinflammatory response (Hubert et al., 2004; Schwartz et al., 2009). It is estimated that 3% of the metabolised energy of the chicken could be directed towards the

maintenance of a feed-induced immune response (Kogut, 2017).

At 4 days of age, the inclusion of 1 and 3 kg/tonne of betaine in the diet reduced gene expression for claudin 5 and occludin, which suggests there has been a quicker development of the gastrointestinal tract and the demand for tight junction formation has reduced. Tight junction formation is affected by enterocyte differentiation and replication (Karcher and Applegate, 2008). Betaine inclusion increases villus height, modulates immune response and improves gastrointestinal tract recovery after a coccidial challenge (Kettunen et al., 2001; Klasing et al., 2002). As an osmoprotectant, betaine may help the gastrointestinal tract maintain its physiology when facing a transitory hyperosmotic period. At 9 days of age, gene expression of claudin 1, claudin 5, occludin and interleukin 2 were higher in broilers fed the low fibre diet and these were reduced by both betaine inclusion or fibre addition. Fibre interferes with the inflammatory responses in broilers but it is dependent upon the type of fibre used (Teirlynck et al., 2009). Inclusion of soluble fibre increases inflammatory responses while insoluble fibre reduce these inflammatory responses and stimulate villus growth (Rezaei et al., 2011; Adedokun et al., 2012). Fibre composition in rice bran is mainly represented by insoluble fibre.

Cytotoxic T cells are classified dependent on the presence of CD8 and CD28 in the superficia of their membrane. T cells that express both CD8 and CD28 are recognised as T cells in initial stages of activation or memory cells (CD8+CD28+) and T cells that express CD28 but not CD8 are recognised as non-activated T cells (CD8-CD28+) (Nabeshima et al., 2002). When T cells are activated for a long period of time, they lose the expression of CD28 while maintaining CD8 expression and are classified as activated cytotoxic T cells (CD8+CD28-). These cells are specific to target antigens and cannot replicate (Nabeshima et al., 2002; Pawelec et al., 2004).

The proportion of cytotoxic T cells in the leukocyte population increased between hatch and 4 days, and a reduction in this percentage was observed for CD8-CD28+ and CD8+CD28+ between 4 and 9 days, while CD8+CD28- increased further. The initial increase in the population of cytotoxic T cells may be related to the development of the immune system. Beirão et al. (2012) observed that under normal commercial conditions the levels of cytotoxic T cells increase in proportion to other T cells from day of age to 5 weeks This suggests that the changes noted in the present study is related to an early development of the immune system. Betaine inclusion increases the proportion of CD8+CD28+ and CD8-CD28+ suggesting it aids in the development of the immune status but no effect was observed in the population of CD8+CD28- as the effect of betaine is likely not related to specific antigens.

Helper T cells are classified by the presence of CD4 and TCRvβ1 phenotypes. Helper T cells with CD4+ and TCRvβ1- (CD4+TCRvβ1-) reaction are recognised as classical helper T cells and are responsible for activation of lymphocytes and adaptative immune response (Chan et al., 1988). When TCRvβ1 is expressed (CD4+ TCRvβ1+) cells tends to migrate to the mucosa where they are responsible for IgA production (Cihak et al., 1991). Other helper T cell that express TCRvβ1, but not CD4, are classified as cytotoxic mucosal T cells (CD4- TCRvβ1+) and have an important role in cellular immunity (Lillehoj, 1994). Birds challenged by *Salmonella* spp. have reduced concentrations of CD4 and CD8 in the peripheral blood (Flores et al., 2012) which actually increases their susceptibility to *Salmonella* infection (Kamalavenkatesh et al., 2005). Inclusion of betaine increased the percentage of helper T cells at 4 days but not at 9 days of age. As the objective of the present study was not to measure the response to a specific pathogen, but to evaluate the immune system development in young birds, it could be concluded that betaine may play a role as an immune modulator during the transition period following hatch and improve immune competence.

Monocytes (Mo+ MHCII+) are precursors of macrophages in different tissues and express Mo marker in the membrane and represents a connection between the innate and adaptative immune responses. There are other cells that are not monocytes (Mo-) that may also be antigen-presenting cells when expressing the MHCII marker in its membrane (Mo-MHCII+). Monocytes that do not present the MHCII marker reduce lymphocyte activity, classified as suppressor monocytes (Mo+MHCII-) (Masternak and Reith, 2002). An increase in the proportion of both Mo+MHCII+ and MO-MHCII+ between hatch and 4 days of age and the reduction of suppressor monocytes (Mo+MHCII-) shows how the chick prepares for any immune response during these early stages. The betaine and fibre concentration in the diet did not affect the proportion of these populations.

The ratio between CD4 and CD8 cells may be used as an indicator of cell-mediated immune response (Cheng et al., 2001). Higher ratios of CD4:CD8 are usually an indication of immunocompetence (Beirão et al., 2012), whilst reduced values are usually associated with

challenging environmental factors, immunization or challenges such as bronchitis (Beirão et al., 2012). Genetic selection for higher growth and egg production is also associated with a reduction in CD4:CD8 ratio (Cheng et al., 2001). In the present evaluation, the CD4:CD8 ratio was reduced ($p < 0.001$) at day 9 compared with day 4 but the results were still greater than 1.5 which is considered as a normal ratio (Cheng et al., 2001) when cellular immune mechanisms are not impaired and survivability is not compromised (Reid and Tervit, 1995; Levinson and Jawetz, 1996). A reduction of the CD4:CD8 ratio can be associated with a natural exposure to immune estimulators in the environment as the ratio is reduced in birds raised commercially compared with birds raised specifically to be pathogen-free (Flores et al., 2012). Some degree of immune challenge may be required to develop the immune system by increasing the relationship between helper and cytotoxic cells (Beirão et al., 2012). Neither fibre concentration nor betaine inclusion affected the CD4:CD8 ratio in the current study. A lack of response may be related to the development of the immune system being a result of exposure to a moderate bacterial challenge, but not a challenge by specific pathogens.

CONCLUSION

Betaine inclusion reduced expression of claudin 5 and occludin, and increased the proportion of cytotoxic T cells, non-activated helper T cells, and helper T cells. These results suggest that betaine can be used as a modulator of immune maturation during the early days post hatch, probably not by acting directly on these immune cells but helping the birds to support and adapt to this initial period and then reducing the inflammatory response associated with this adaptation process. Fibre content had no effect on the gene expression and leukocyte population in broilers until 9 days of age. As fibre will affect the microbiota population in the gut, it is possible that the effect of fibre on the immune status of broilers takes longer to develop. In both cases, the fluctuation of the local gene expression for tight junction proteins and leukocyte populations shows that gut development is closely linked with development of the systemic immune system due to the role of the gut as a sensory organ.

DECLARATIONS

Authors' contributions

Tiago Tedeschi dos Santos, Suelen Baal and Ana Vitória Fisher da Silve designed the trial and developed it. Celso Favero worked on Laboratory analysis. Mike Bedford, Sophie Lee and Tiago Tedeschi dos Santos analysed the statistical data and wrote the article. All authors confirmed the final version of the original article for publishing in this journal.

Competing interests

All authors declare no conflicts of interest.

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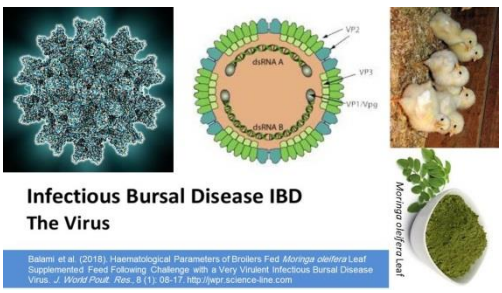

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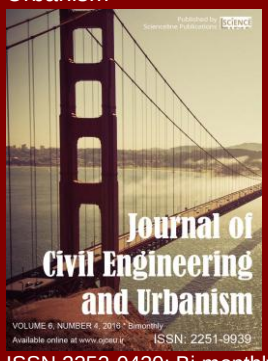

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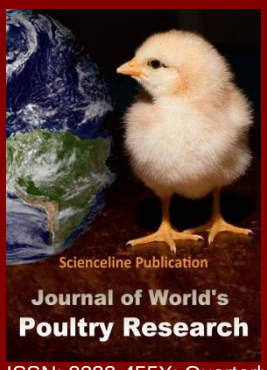

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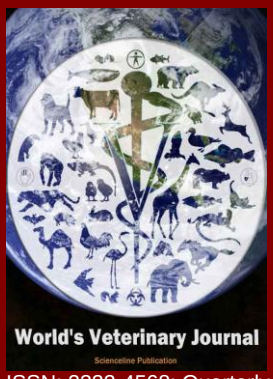

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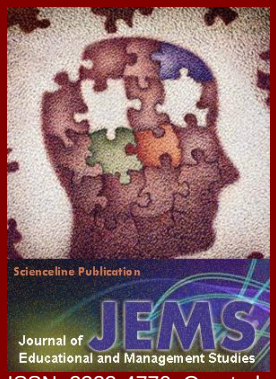

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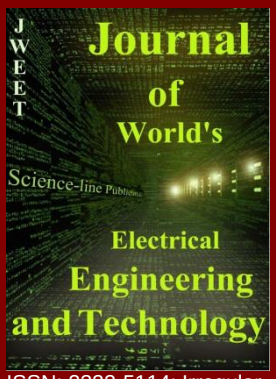

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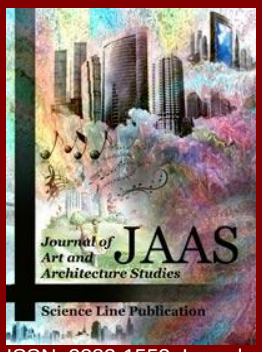

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