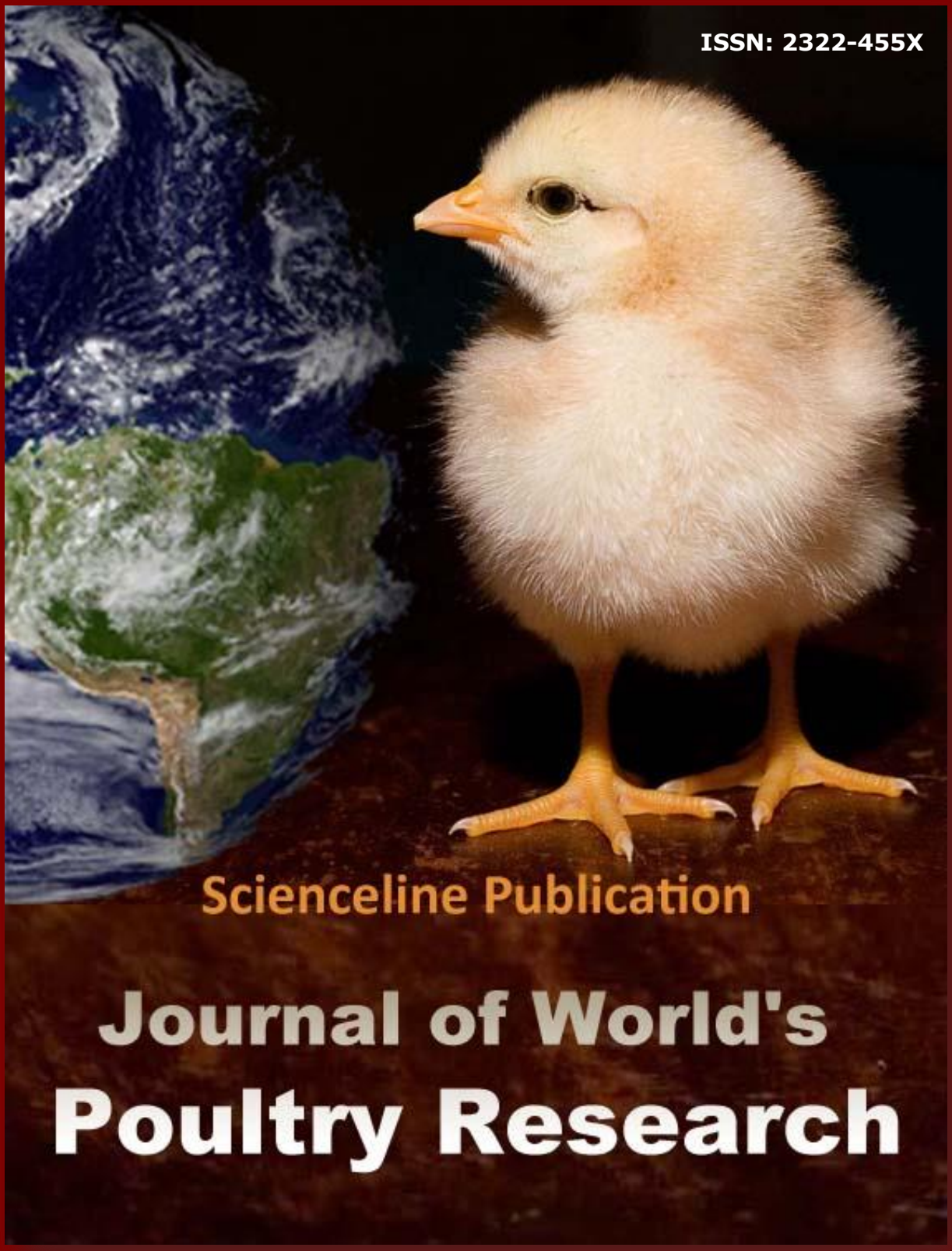
In the Name of God



An international peer-reviewed journal which publishes in electronic format

Volume 9, Issue 2, June 2019

Journal of World's Poultry Research

J. World Poult. Res. 9 (2): 32-116; June 25, 2019

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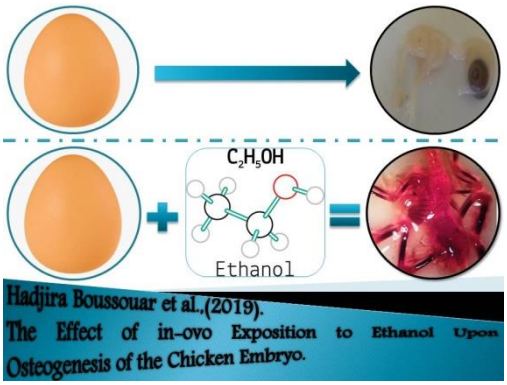
Volume 9 (2); June 25, 2019

Research Paper

The Effect of *In Ovo* Exposition to Ethanol Upon Osteogenesis of the Chicken Embryo.

Boussouar H, Khenenou T, Bennoune O and Berghiche A.
J. World Poult. Res. 9(2):32-37; pii:S2322455X1900005-9



ABSTRACT
Excessive alcohol consumption by a pregnant woman may delay foetal development and may cause malformations. In this study, the model of the chicken embryo to demonstrate the teratogenic effect of ethanol (33%) on the chicken osteogenesis on the 10th day of embryonic development have been used. 49 fertilized eggs were used in present investigation. Hence, different doses of ethanol were injected into the chicken embryos at 33% (20, 40, 80µl) in the air space at gastrulation and, on the other hand, an equivalent amount of the mentioned doses of distilled water were injected into the control-group eggs which was done once in every two days in order to maintain a high concentration in the blood. Experiments were repeatedly and independently carried out for three times. The eggs were incubated in a humid incubator at the temperature of 37.7 °C and at 60-65% of humidity. On the 10th day of incubation, the embryos were taken out and fixed in formalin at 10%. After that, the eggs were sectioned at 5µm of thickness with a Leica micrtome and, then, stained with the Hematoxylin and eosin. Histological examination has revealed that the exposition of chicken embryos to ethanol (33%) delays the skeletal development in a dose-dependent manner by reducing the length of the cartilaginous proliferation zone and hypertrophic zone during the bone formation period. Furthermore, under the effect of ethanol, the cell proliferation activities were repressed. In conclusion, present results indicated that using ethanol to treat chicken embryos at early stages caused considerable malformations and a decreased in the embryo survival rate. The exposition to alcohol affects the chicken osteogenesis in a dose-dependent manner.

Keywords: Chicken embryo, Ethanol, Malformations, Osteogenesis, Teratogenic effect

[Full text-PDF]

Research Paper

Effects of Feeding Different Levels of Baker's Yeast on Performance and Hematological Parameters in Broiler Chickens.

Mulatu K, Ameha N and Girma M.
J. World Poult. Res. 9(2):38-49; pii:S2322455X1900006-9

ABSTRACT
The effects of feeding baker's yeast performance of Cobb 500 broilers were studied. Four nearly isocaloric and isonitrogenous starter and finisher rations were prepared. 240 chicks with an average initial body weight of 42g were randomly divided into 12 groups contained 4 treatments with 3 replications for each treatment. Treatment rations were containing 0, 0.5, 1.5 and 2.5% of baker's yeast as T1, T2, T3 and T4 respectively. At the end of the trial, 3 males and 3 female chickens from each replication were slaughtered for carcass evaluation. The Crude Protein (CP) and Metabolizable Energy (ME) contents of baker's yeast were 48% and 3615 kcal/kg DM, respectively. The CP content of the rations during the starter and finisher phases were 22% and 20%, respectively. The ME content of the rations during the starter and finisher phases were 3100 kcal/kg and 3200 kcal/kg respectively. Feed intake during the starter phase and entire trial period was lower for T4, whereas during the finisher phase in control diet group showed the highest feed intake than the other supplemental groups. The highest daily body weight gain was recorded in broilers fed T4 rations during starter phase, finisher phase and entire experimental period. Feed conversion ratio of T4 and T3 groups was better than T2 and T1. T3 and T4 groups had higher eviscerated percentages. Blood parameters results showed that fed broilers yeast containing ration had higher WBC, PCV and Hb. Partial budget analysis indicated that the highest net income, marginal rate of return and chicks' sale to feed cost were obtained for T3 followed by T4. Baker's yeast can be an important feed additive, which can be included up to 2.5% of the total ration and improve the overall performance of broilers without compromising the hematological indices of broiler chickens.

Keywords: Baker's yeast, Blood constituents, Broiler, Carcass and Growth

[Full text-PDF]

Research Paper

Effect of Different Bedding Materials on the Hematological and Serum Biochemical Parameters of Broiler Chickens.

James G, Garba DJ, Adeolu AS, Adamu Z and Mamma Z.
J. World Poult. Res. 9(2):50-58; pii:S2322455X1900007-9

ABSTRACT
The aim of the present study was to determine the effect of different bedding materials on the, hematological and serum biochemical parameters of the broiler chickens. A completely randomized design was employed for the research in which the treatment were five bedding materials (rice hulls, groundnut hulls, wood shaving, sharp sand and control). The treatments were replicated three times each with ten birds in each experimental unit. The birds were brooded for two weeks before the experiment begin. The blood samples were collected at day 56 of experiment for hematological and serum biochemical analysis. There was no significant difference in the glucose, serum total protein, globulin, calcium, sodium, total bilirubin, conjugated bilirubin, and serum alanine transferase, but there was a significant difference in serum cholesterol, albumin, bicarbonate and serum aspartate transferase within the groups. The findings also indicated in the hematological parameters that bedding materials caused significant difference in the hemoglobin content, white blood cell count and mean corpuscular hemoglobin, but no significant differences in the other parameters (Heterophils, monocytes, basophils, lymphocytes, mean cell volume and packed cell volume) were seen. The results suggested that rice, hulls, groundnut hulls and sharp sand can serve appropriately as bedding materials for broiler production, with compatible effects on serum biochemical and hematological parameters.

Keywords: Bedding materials, Broilers, Hematology, Serum biochemistry

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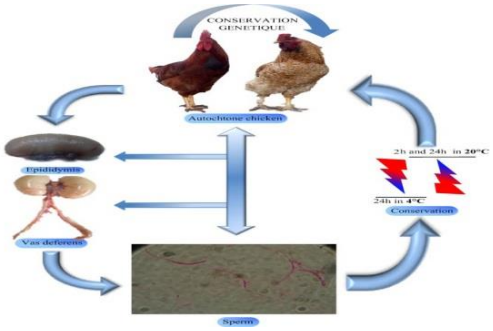
Research Paper

The characterization of Post -Mortem Sperm of Local Chicken Cocks inEastern Algeria.



Ouennes H, Afri Bouzebda F, Bouzebda Z, Majdoub S, Djaout A and Adnane Smadi M.
J. World Poult. Res. 9(2):59-67; pii:S2322455X1900008-9

ABSTRACT
The present aimed to investigate for the first time the characteristics and conservation of post-mortem sperm, obtained from epididymis and the vas deferens of 18 pairs of adult local chicken cocks from the east of Algeria (age, 12-24 months, body weight 1.50-2.53 kg). And compare the sperm quality at the epididymis and the vas deferens levels, the efficacy of two post-mortem sperm retrieval techniques, the flushing and float-out methods in the collection of local chicken cock's sperm, and the effects of conservation in situ at different temperatures (2h and 24h at 20°C and after refrigeration at 4°C for 24h). The quality was significantly higher (0.13±0.05ml vs 0.72±0.12ml) in the vas deferens compared to the epididymis, whereas spermatozoa abnormalities and chromatin quality did not differ in both the epididymis and vas deferens. No significant difference was observed between the flushing and float-out methods. Concentration (3.33±1.63 million sperm vs 1.75±0.76 million sperm), initial motility (77.50±6.89% vs 75.83±8.61%), viability (75±10.39% vs 74.67±10.15%) and abnormality (30.33±4.68% vs 30.33±4.68%), only the volume was significantly higher (0.72±0.12ml vs 0.17±0.08ml). However, the effects of conservation in situ, at 25°C and 4°C for 24h, showed a significant difference for viability and motility of the spermatozoa recovered from vas deferens, and no significant difference for abnormality and acrosome integrity. Therefore, it can be concluded that good quality semen samples can be collected from the vas deferens with the flushing method, and semen of Algerian local cocks can be preserved at 20°C for 24h.

Keywords: Conservation, Eastern Algeria, Epididymis, Local cocks, Post-mortem, Vas deferens

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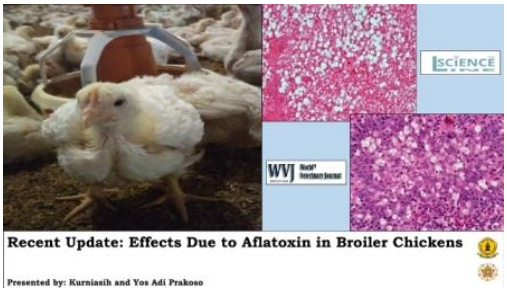
Research Paper

Recent Update: Effects of Aflatoxin in Broiler Chickens.



Kurniasih and Prakoso YA.
J. World Poult. Res. 9(2):68-77;pii: S2322455X1900009-9

ABSTRACT
Aflatoxin is a worldwide problem in poultry industries as it is known to

contaminate poultry feed. Aflatoxin induces stress and increases mortality rate during infection in poultry, especially broiler chickens. The objectives of this study was to observe the pathological effects due to aflatoxicosis in broiler chickens. A total of 120 chickens were divided into four groups, group A fed with a basal diet without aflatoxin contamination, group B with aflatoxin (> 1 ppb 51 ppb 101 ppb < 150 ppb). The data were collected and analysed on day 7, 14, and 21. The results showed that diet with high aflatoxin contamination in group D impaired the physical and laboratory performances of the chickens, increased the risk of residue in the poultry's final products. The varying doses of aflatoxin contamination in the chicken feed causes the problems on the broiler chickens with different level of severity.

Keywords: Aflatoxin, Antibody, Broiler, Haematology, Immunohistochemistry, Residue

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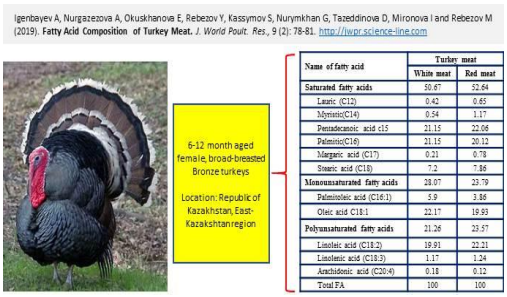
Research Paper

Fatty Acid Composition of Turkey Meat.

Igenbayev A, Nurgazezova A, Okuskhanova E, Rebezov Y, Kassymov S, Nurymkhan G, Tazeddinova D, Mironova I and Rebezov M.
J. World Poult. Res. 9(2):78-81;pii: S2322455X1900010-9



ABSTRACT
This paper aimed to study the fatty acid composition of turkey meat. Red and white turkey meat were sampled from the local markets of Semey city, republic of Kazakhstan. The proximate composition showed a significant difference in the fat content of red and white meat. The fatty acid composition of turkey meat was as follows: saturated fatty acids 50.67% in white and 52.64% in red meat; monounsaturated fatty acids 28.07% in white and 23.79% in red meat; polyunsaturated fatty acids 21.26% in white and 23.57% in red meat. Palmitic and pentadecanoic are the major saturated fatty acids, where the oleic and linoleic acids are in a large amount in monounsaturated and polyunsaturated fatty acids, respectively.

Keywords: Fatty acid, Polyunsaturated fatty acid, Red meat, Turkey meat, White meat

[Full text-PDF] [XML]

Research Paper

Evaluation of Dietary Medicinal Plants and algae in Laying Japanese Quails.

Habibi H, Ghahtan N and kohanmoo MA.
J. World Poult. Res. 9(2):82-88; pii:S2322455X1900011-9

ABSTRACT
The current study was conducted to examine the influences of *Sargassam cristafolium*, *Gracilaria pulvinata*, *Rhus coriaria*, and *Punica granatum* peel dried powder in two levels (10 and 20gr/kg) of dietary on productive performance and some egg quality characteristics of laying Japanese quail. A total of 675 (49 days old) Japanese quail were randomly distributed into nine groups with three replicates of 25 birds in each. Results showed that egg weight, shell weight, albumen weight and shell thickness were not influenced by treatments. The effects of medicinal plants on weekly egg production differ depending on the dietary medicinal plant type and dosage. Moreover, the birds fed diet supplemented with *Sargassum cristaefolium* at 10g/kg had greater weekly egg production compared to others. Haugh unit was reduced by diet supplemented with *R. coriaria*. Diet supplemented with *S. cristaefolium* at 20 g/kg increased yolk weight. Furthermore, greater albumen protein and thiobarbituric acid content of the tenth day's eggs ($TBAd_{10}$) were perceived in birds fed diet supplemented with *G. pulvinata*. In present study, the lowest levels of cholesterol (9.66 mg/g) was determined in the egg yolk of groups *R. coriaria* and *G. pulvinata,* and the control group showed to have the most amounts (12.00 mg/g). Based on obtained data, addition of 0.5% and 2% of *S. cristafolium*, *G. pulvinata*, *R. coriaria*, and *P. granatum* peel to the diet lead to be progress in egg production rate, enhanced the egg quality and egg biochemical properties in Japanese quail.

Keywords: Egg production, Egg quality, Laying quails, Medicinal plants, Thiobarbituric acid

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Review

A Meta-Analysis on Antibiotic Residues in Meat of Broiler Chickens in Developing Countries.

Berghiche A, Khenenou T and Labiad I.
J. World Poult. Res. 9(2):89-97;pii: S2322455X1900012-9

ABSTRACT
Present study consisted of performing a meta-analysis on data about the detection of antibiotic residues in chicken meat achieved from all over the researches with a wide collection and very strict selection criteria of data. The databases were searched quantitative inputs from the available scientific publications using important keywords, in order to evaluate all studies about antibiotic residue and detection methods and the reliability of the results obtained by the international researchers. Then an advanced statistical analysis on collected data was done, the first phase was a descriptive study of positive and negative cases followed by a modeling of two cases with a prediction of the values obtained and ended with an analysis of the main compounds (population size, residue detection methods and positive case rates). All performed steps are reported in detail. The results indicated that the accuracy of the detection technique is a factor that influenced on reports of residues, and caused differences in reports, there are still antibiotic residues in meat of intensively broiler chicken farms (45. 26% of the samples analysed are positive), It is concluded that residue detection requires a high-precision qualitative analysis protocol.

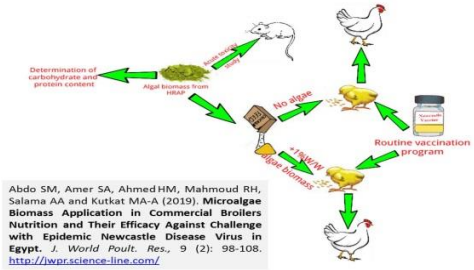
Keywords: Antibiotic residues, Broiler chicken, Databases, Meta-analysis.

[Full text-PDF] [XML]

Research Paper

Microalgae Biomass Application in Commercial Broilers Nutrition and Their Efficacy Against Challenge with Epidemic Newcastle Disease Virus in Egypt.

Abdo SM, Amer SA, Ahmed HM, Mahmoud RH, Salama AA and Kutkat MA-A.
J. World Poult. Res. 9(2):98-108; pii:S2322455X1900013-9



ABSTRACT
Using microalgal biomass in animal diets has been studied recently. Many species of cultivated algae were found effective in maintaining animal growth performance, and in improving body weight. Using of microalgae collected from high rate algal ponds (HRAP) as a feed additive to broilers ration was studied. One hundred and twenty broiler chicks were divided into 6 groups of 20 birds, three of them have fed on balanced broiler ration supplied with 1% weight per weight (W/W) of microalgae biomass and have variable vaccination schemes of live attenuated and inactivated Newcastle disease virus (NDV) vaccines genotype II or either non-vaccinated control. In addition, the other 3 groups have fed on free microalgae biomass balanced ration with the same vaccination treatment. Furthermore, weight gain, antibody response, mortalities, viral shedding and normal viability of chickens were estimated in order to assess the efficiency of microalgae as a feed additive. The results showed that the microalgae have no hazard effect on feed and water intake as well as enhanced viability of chickens. And in regards to immune function and body weight, they have similar effect with the free microalgae groups in normal serological response and viral shedding post vaccination with NDV vaccines as well as similar protection rate and body weight gain. In conclusion, microalgae can be used in broiler ration with no deleterious effect on growth rate, weight gain, poultry viability and immune response. In conclusion dried microalgal biomass harvested from HRAP can be used in broiler ration with no deleterious effect on growth rate, weight gain, poultry viability and immune response. Furthermore, future studies should be applied with increasing microalgae percent in poultry feed up to 5, 10 or 20% (W/W) in order to assess better performance on poultry production.

Keywords: Algae biomass, Immune response, Newcastle disease virus, Poultry feed

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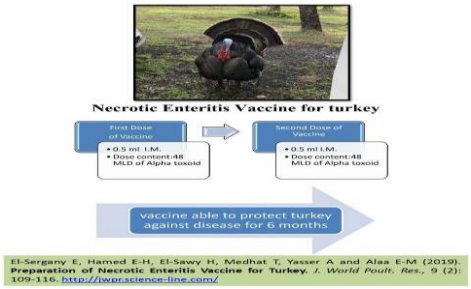
Research Paper

Preparation of Necrotic Enteritis Vaccine for Turkey.

El-Sergany E, Hamed E-H, El-Sawy H, Medhat T, Yasser A and Alaa E-M.
J. World Poult. Res. 9(2):109-116; pii:S2322455X1900014-9



ABSTRACT
Clostridium perfringens is the most important cause of enteritis in domestic animals, in chicken and turkey it well known as pathogen responsible for necrotic enteritis; hepatitis, and cholecystitis. The disease in turkey characterize by either severe form with high rate of mortalities or subclinical form of reduce growth rate and increase condemnation rate. The major factor responsible for pathogenicity of *Clostridium perfringens* was alpha toxin. The aim of present study was to prepare of *Clostridium perfringens* alpha Toxoid vaccine for controlling the necrotic enteritis disease. The vaccine was prepared at different doses depend on lethality of toxin (24, 48 and 96 Minimum Lethal Dose) for controlling necrotic enteritis disease. Antibody titer elicited by vaccination was measured by toxin neutralization test, ELISA, and challenge test. It revealed that antibody titer expressed by international antitoxin unit per ml was 7.4, 4.1 and 1.26 respectively according to the mentioned dose, and also the protection percent against challenge was 100%

when vaccinated with either 48 or 96 Minimum Lethal Dose, while it gave 80% when vaccinated with 24 Minimum Lethal Dose.It concluded that use of *Clostridium perfringens* alpha Toxoid with recommended dose of 48 MLD able to protect turkey for 6 months.

Keywords: Alpha toxin, *Clostridium perfringens,* Turkey, Type A, Vaccine

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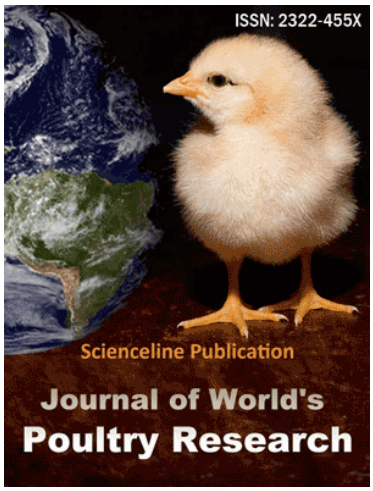
Journal of World's Poultry Research



ISSN: 2322-455X

Frequency: Quarterly

Current Issue: 2019, Vol: 9, Issue: 2 (June 25)

Publisher: SCIENCELINE

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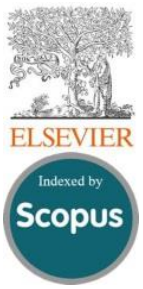

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J. World Poult. Res. 9(2): 32-37, June 25, 2019

Research Paper, PII: S2322455X1900005-9

The Effect of *In Ovo* Exposition to Ethanol Upon Osteogenesis of the Chicken Embryo

Hadjira Boussouar[1]*, Tarek Khenenou[2] , Omar Bennoune[3] and Amine Berghiche[4]

[1] *Department of Veterinary Sciences, Institute of Veterinary Sciences and Agronomic Sciences, Batna University 1, Batna 05000, Algeria*
[2] *Laboratory of Animal Productions, Biotechnology and Health, Institute of Agronomic and Veterinary Sciences, Mohamed Cherif Messaadia University, Souk Ahras, Algeria*
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Received: 17 Apr. 2019
Accepted: 17 May 2019

ABSTRACT

Excessive alcohol consumption by a pregnant woman may delay foetal development and may cause malformations. In this study, the model of the chicken embryo to demonstrate the teratogenic effect of ethanol (33%) on the chicken osteogenesis on the 10th day of embryonic development have been used. 49 fertilized eggs were used in present investigation. Hence, different doses of ethanol were injected into the chicken embryos at 33% (20, 40, 80 µl) in the air space at gastrulation and, on the other hand, an equivalent amount of the mentioned doses of distilled water were injected into the control-group eggs which was done once in every two days in order to maintain a high concentration in the blood. Experiments were repeatedly and independently carried out for three times. The eggs were incubated in a humid incubator at the temperature of 37.7 °C and at 60-65% of humidity. On the 10th day of incubation, the embryos were taken out and fixed in formalin at 10%. After that, the eggs were sectioned at 5µm of thickness with a Leica micrtome and, then, stained with the Hematoxylin and eosin. Histological examination has revealed that the exposition of chicken embryos to ethanol (33%) delays the skeletal development in a dose-dependent manner by reducing the length of the cartilaginous proliferation zone and hypertrophic zone during the bone formation period. Furthermore, under the effect of ethanol, the cell proliferation activities were repressed. In conclusion, present results indicated that using ethanol to treat chicken embryos at early stages caused considerable malformations and a decreased in the embryo survival rate. The exposition to alcohol affects the chicken osteogenesis in a dose-dependent manner.

Key words: Chicken embryo, Ethanol, Malformations, Osteogenesis, Teratogenic effect

INTRODUCTION

Alcohol is considered as the most common teratogen in the occidental world. Its consumption during pregnancy may pave the way to a disease called Foetal Alcohol Syndrome or, under its softest form, Foetal Alcohol Effects (Chaudhuri, 2004). This is very worrying, if anyone consider the increase of alcohol consumption by women in the world (Chaudhuri, 2004). Despite the fact that the rate of alcohol consumption has been relatively steady through the last 30 years, the rate of women who consumed alcohol increased by 12.4% in 1991 and by 16.3% in 1995. Meanwhile, the number of newborns suffering from the Foetal Alcohol Syndrome (FAS) quadrupled between 1979 and 1993 with an incidence rate of 6.7 per 10 000 births in 1993 (Miller et al., 2001). The negative clinical results of alcohol consumption during pregnancy may include an increased neonatal mortality, head and facial anomalies, a microcephaly, body organs disorder (Bupp Becker and Shibley, 1998). Including growth delay, muscle and skeletal anomalies and intellectual and behavioural disability (McGill et al., 2009). Various animal models have been used by researchers in order to study the factors and effects of various alcohol-related diseases. Such models have helped researchers to explore the mechanisms by which short-term and long-term consumption may interfere with the normal embryo-development processes (Kamran et al., 2011).

To cite this paper: Boussouar H, Khenenou T, Bennoune O and Berghiche A (2019). The Effect of *In Ovo* Exposition to Ethanol Upon Osteogenesis of the Chicken Embryo. *J. World Poult. Res.*, 9 (2): 32-37.

The chicken embryo (*Gallus gallus*) is an extremely useful model for the development biology, experimental embryology and teratology (Drake et al., 2006). Accurate and full descriptions of the development of chicks are widely available (Drake et al., 2006). Using chicken models is very beneficial for the developmental researches. Fertile eggs are cheap, easy to find at stores and only an incubator is required for their development (Smith and Nagy, 2008). The shell can easily be fenestrated to view or manipulate the embryo directly. It can also be easily obliterated so that to continue the development process (Smith and Nagy, 2008). Maternal metabolism lacks an *in ovo* system that allows the direct isolation of teratogenic effects. All in a nutshell, the embryo model offers a cheap and practical system in which it is possible to apply modern experimental tools to know how a teratogen interferes with specific mechanisms underlying the organogenesis and the morphogenesis (Drake et al., 2006).

Hence, the aim of this work is to study the teratogenic effect of the *in ovo* exposition to ethanol on the osteogenesis in a system of a chicken-embryo model considering the injected ethanol dose and the embryonic development stage.

MATERIALS AND METHODS

Ethical approval

The experiment was carried out according to the National Regulations on Animal Welfare and Institutional Animal Ethical Committee.

Experimental design

Present investigation was carried out on a group of 49 embryonated of the broiler chickens (Hubbard F15) eggs, brought from the private hatchery "Coopérative agricole Errakhaà Wa Izddihar", located in Theniyetsaida (Ain Yagout – Batna) through the period from April 2017 to March 2018, Algeria. The eggs were divided into three groups: a non-treated negative control group (n=7), a positive control group, treated with distilled water, and which, in turn, was subdivided into three subgroups of six eggs each. These subgroups received respectively (20, 40 and 80 µl) of distilled water. A last group, exposed to ethanol 33%, was also subdivided into three subgroups of eight eggs each. These subgroups received respectively (20, 40 and 80 µl) of ethanol 33% (Table 1).

Substance injection

This particular step is crucial and necessitates a maximum sterility. Hence, a Bunsen burner was used for the substance injection in the air sacs (Cevik et al., 2007).

Incubation

The eggs were incubated during 240 hours (10 days) in a humid incubator at 37°C and 60-65% of humidity, and were automatically turned every four hours (Cevik and Lale Satiroglu, 2003; Aguilera et al., 2005).

Visualisation of the skeleton

The skeletal staining was done by the technique described by Sadeghi (2014): the embryos were eviscerated and the skeletons were cleared without being damaged. After that, they were fixed in ethanol absolute. The skeletal staining was done with Alizarin red at 0.001 %. The samples were then rinsed by KOH at 1%, 3 times during a week. The last step was the treatment of the samples with ascending concentration sets of glycerol in KOH at 1% during 24 hours in every step. Finally, the samples were placed in glycerol.

Histological examination

The samples were fixed with 10% buffered formalin, passed through to a series of ethanol from 80% to 100%, then cleared with xylene during one hour in each shower and then incorporated in paraffin. 5 µm bone sections were taken by a microtome (Leica), the slides were then H&E stained and analysed under an optic microscope Carl Zeiss Axioskop 20 connected to a digital video camera DOM 300 (Laanani et al, 2015; Khenenou et al, 2017; Khenenou et al, 2019).

Table 1. Treatments, subgroups and number of eggs in each gruoup of study

Group	Treatment	Subgroup	Number of eggs
A : Negative control	No traited		7
B : Positive control	Distilled water	B1: 20 µl	6
		B2: 40µl	6
		B3: 80 µl	6
C : Treated; ethanol	EtOH (33%)	C1: 20 µl	8
		C2: 40 µl	8
		C3: 80 µl	8

Statistical analysis

The statistical analysis of the experimental data was carried out using statistical software Graph Pad Prisme 5.01. The data concerning the length of the proliferation and hypertrophic zones were identified using a T test for 2 independent samples or an analysis of the one-way variance (One-way ANOVA); for both tests. $P<0.05$ was considered as statistically significant between the control groups and those exposed to ethanol.

RESULTS AND DISCUSSION

Ethanol effects on the embryonic viability

Present findings indicated that the *in ovo* injection of the distilled water at low quantities (20µl) had a negligible effect on the embryonic viability whose average embryo survival rate was 83.33%; only a slight difference was noticed in comparison to the control group (85.72). In contrast, it was noticed a remarkable diminution of the embryonic (58.33%) viability at higher water quantities (40 and 80µl). When the groups exposed to ethanol 33%, it was noticed a diminution of the embryo viability rate depending on the volume (the bigger the volume gets; the more the embryo mortality is), the average of the survival rate of embryos treated with 20µl was 56.28%, but with an increase of the injected amount reaching 40µl and 80µl, the average of the survival rate was reduced to 37.5% and 12.5% respectively (Table 2). The data illustrated in table 1 and figure 1 indicated that ethanol affects the embryonic survival rate at an early stage. Present results are in complete concordance with the study done by Zulifqar et al. (2015) which demonstrated that the treatment of the chicken embryos at early stages causes a decrease in the survival rate of chicken embryos.

Table 2. Average of survival rates in broiler chicken embryos treated with ethanol

Group \ Quantity (µl)	20	40	80
Negative control ; no traited	85.72%	85.72%	85.72%
Positive control; distilled water	83.33%	58.33%	58.33%
Treated; ethanol	56.28%	37.50%	12.50%

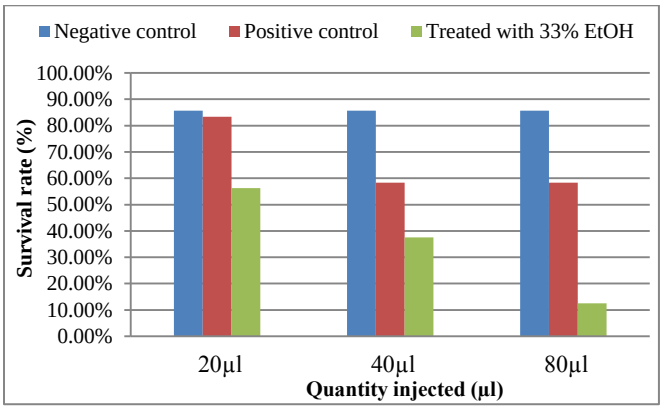

Figure 1. The effect of ethanol on broiler chicken (Hubbard F15) embryo survival rate, Algeria (April 2017 to March 2018).

Special staining

The stereomicroscopic observation of the embryos stained with Alizarin red has shown a dark red stain noticed on the long bones (Figure 2); Alizarin red staining has an affinity for calcium ions. This explains that there is an ossification process at the level of these bones. Hence, it was noticed that the ossification of long bones, such us the humerus bone, starts on the 10th day of incubation for chicken embryos. This confirms the study done by Sawad et al. (2009).

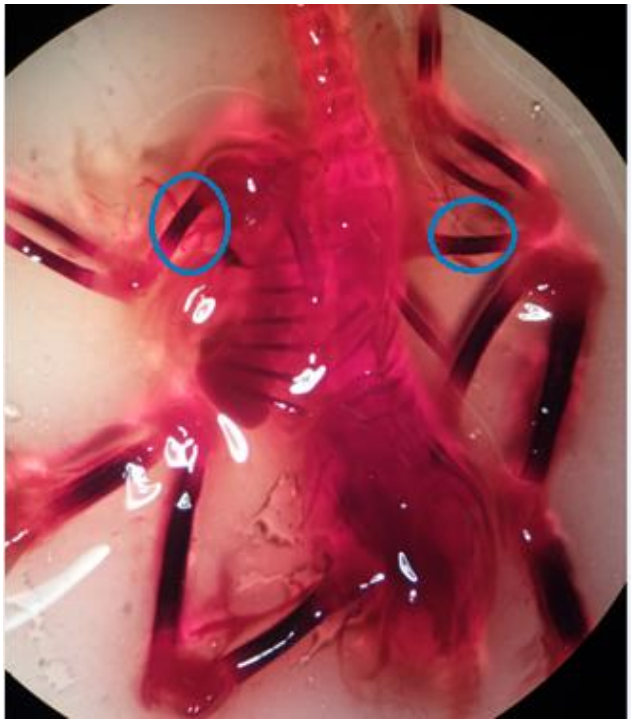

Figure 2. A broiler chicken (Hubbard F15) embryo after 10 days of staining with Alizarin red, Algeria (April 2017 to March 2018).

The effect of ethanol on the length of the proliferation and cartilaginous-hypertrophy zones

Long bones like the humerus bone develop by endochondral ossification, length measurements of the proliferation and cartilaginous-hypertrophy zones, particularly of the developing humerus bone, show the results below:

The length of the humerus-bone's proliferation zone of chicken embryos of the negative control group (515.7±1.28µm, n = 4) was considerably (P<0.005) larger than that of the three groups exposed to ethanol (20µl, 40µl and 80µl) whose lengths are (505.9±1.14µm, 498.1±1.93µm and 489.9±0.83µm) respectively (Figure 3). Also, the cartilaginous-hypertrophy zoneof the humerus bone of the control group (543.2±0.98 µm, n=4) was

considerably (P<0.005) superior to that of the three groups exposed to ethanol (20 µl, 40µl and 80µl) whose length is (523.4±1.15 µm, 515.5±0.88 µm, and 493.9±2.84 µm) respectively (Figure 4).

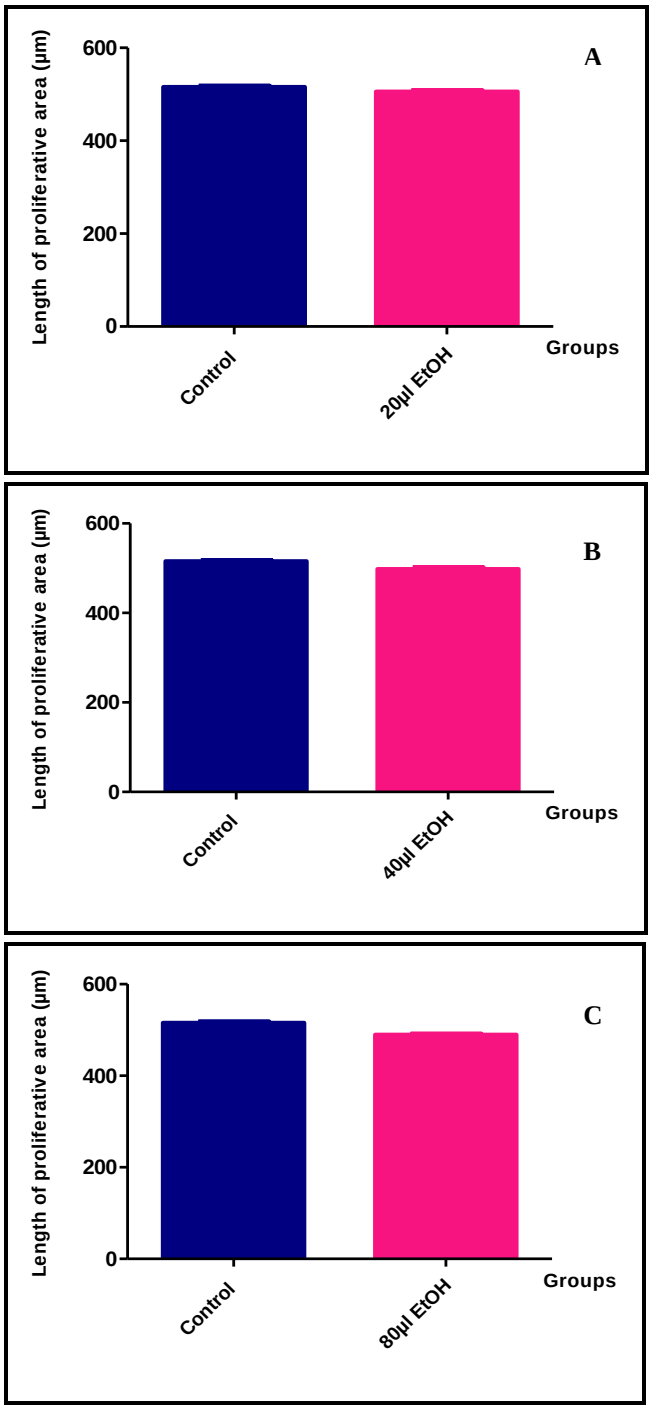

Figure 3. The ethanol effect on the length of the proliferative zone of the humerus of the broiler chicken (Hubbard F15) embryo, Algeria (April 2017 to March 2018) **A.** Exposition at 20µl of EtOH, **B.** Exposition at 40µl of EtOH, **C.** Exposition at 80µl of EtOH.

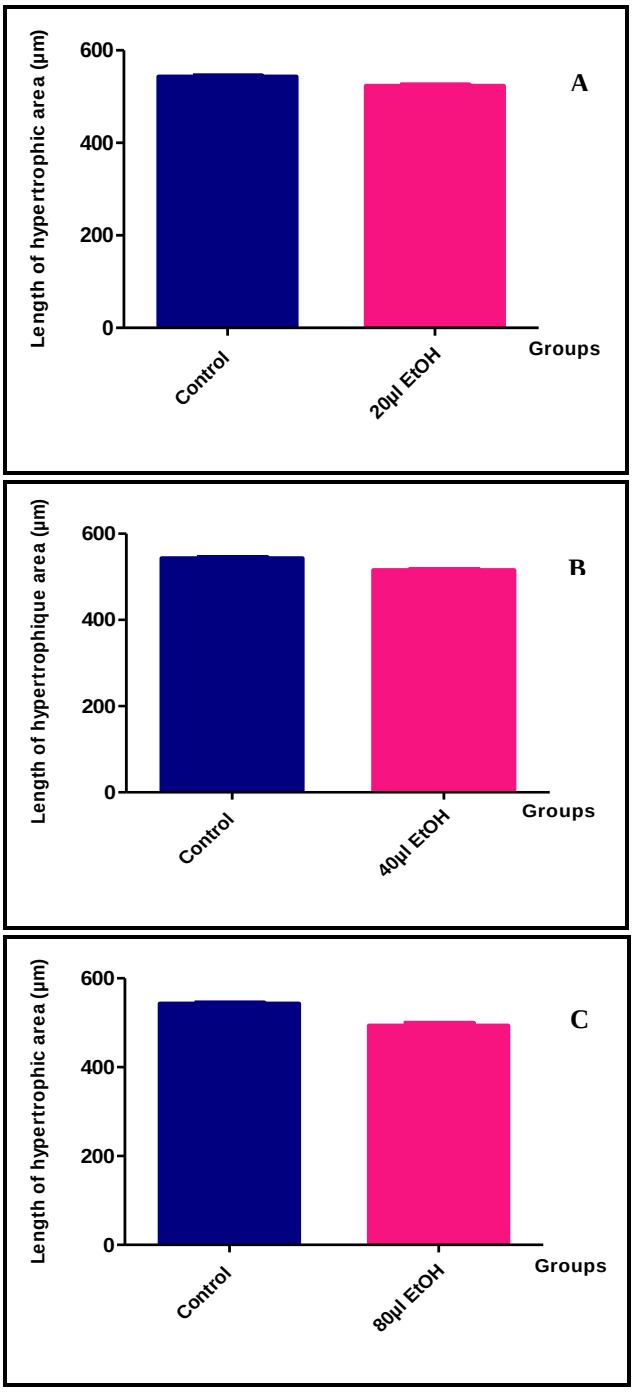

Figure 4. The ethanol effect on the length of the hypertrophic zone of the humerus of the broiler chicken (Hubbard F15) embryo, Algeria (April 2017 to March 2018); **A.** Exposition at 20µl of EtOH; **B.** Exposition at 40µl of EtOH; **C.** Exposition at 80µl of EtOH.

Present results demonstrated that exposure to ethanol, causing skeletal disturbances, affects on long bones by delaying ossification and total bone length which already reported by Clissmann and Brennan (2017).

Present results revealed that ethanol inhibited the cell proliferation and the cartilaginous-hypertrophy process. This may have shortening effects on the proliferation and the cartilaginous-hypertrophy zones, because it was noticed that, at high ethanol concentrations, the length of the two zones of proliferation and cartilaginous hypertrophy of the humerus bone was considerably shortened.

Many studies were performed to determine the toxic effects of naturel and synthetic products on the embryos of chickens, including the studies undertaken by Afzal et al. (2019); Chaudhuri (2019) and Patel et al. (2019). Although the present research was planed base on chemical products that used in pharmaceutical production and considered as a less expensive and good antiseptic (Presterl et al., 2019).

The exposition to ethanol indicated the ossification process of chicken embryos in a dose-dependent manner. Therefore, it can be understood that the exposition to ethanol delays the skeletal development of chicken embryos at gastrula stage by repressing osteogenesis. Furthermore, ethanol effects on osteogenesis seem to be dose dependent, which has already been demonstrated by Zhong-yang et al. (2016).

CONCLUSION

Present findings indicated that ethanol is harmful and directly affects the embryonic viability. Also, it is noticed that exposition to ethanol at early stages causes a decrease in the survival rate of embryos in a dose-dependent manner. The embryos exposed to ethanol have indicated a delay in the skeletal development by a shortening of the length of the proliferation and cartilaginous-hypertrophy zones. This effect is considerable at higher doses. In conclusion, ethanol has negative effects on the embryonic development; these include a delay in the growth and skeletal anomalies.

DECLARATIONS

Competing interests

The authors have no competing interests to declare.

Consent to publish

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

Author`s contributions

Boussouar , Khenenou and Bennoune were involved in the collection of data, statistical analysis and drafting of the manuscript. Khenenou and Bennoune read and approved the final manuscript.

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JWPR
Journal of World's Poultry Research

2019, Scienceline Publication
J. World Poult. Res. 9(2): 38-49, June 25, 2019

Research Paper, PII: S2322455X1900006-9

Effects of Feeding Different Levels of Baker's Yeast on Performance and Hematological Parameters in Broiler Chickens

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Received: 21 Apr. 2019
Accepted: 18 May. 2019

ABSTRACT

The effects of feeding baker's yeast performance of Cobb 500 broilers were studied. Four nearly isocaloric and isonitrogenous starter and finisher rations were prepared. 240 chicks with an average initial body weight of 42g were randomly divided into 12 groups contained 4 treatments with 3 replications for each treatment. Treatment rations were containing 0, 0.5, 1.5 and 2.5% of baker's yeast as T1, T2, T3 and T4 respectively. At the end of the trial, 3 males and 3 female chickens from each replication were slaughtered for carcass evaluation. The Crude Protein (CP) and Metabolizable Energy (ME) contents of baker's yeast were 48% and 3615 kcal/kg DM, respectively. The CP content of the rations during the starter and finisher phases were 22% and 20%, respectively. The ME content of the rations during the starter and finisher phases were 3100 kcal/kg and 3200 kcal/kg respectively. Feed intake during the starter phase and entire trial period was lower for T4, whereas during the finisher phase in control diet group showed the highest feed intake than the other supplemental groups. The highest daily body weight gain was recorded in broilers fed T4 rations during starter phase, finisher phase and entire experimental period. Feed conversion ratio of T4 and T3 groups was better than T2 and T1. T3 and T4 groups had higher eviscerated percentages. Blood parameters results showed that fed broilers yeast containing ration had higher WBC, PCV and Hb. Partial budget analysis indicated that the highest net income, marginal rate of return and chicks' sale to feed cost were obtained for T3 followed by T4. Baker's yeast can be an important feed additive, which can be included up to 2.5% of the total ration and improve the overall performance of broilers without compromising the hematological indices of broiler chickens.

Key words: Baker's yeast, Blood constituents, Broiler, Carcass and Growth

INTRODUCTION

Broiler production represents nearly 33% of global meat production and is a source of protein that plays an important role in human nutrition (FAO, 2010). Modern intensive poultry production produces market ready broiler chickens within six weeks of their age. This achievement arises from improved productivity via genetic selection, improved feeding and health management practices involving usage of antibiotics as therapeutic agents to treat bacterial diseases and as feed additives for growth promotion (Apata, 2009). One of the major challenges faced by the poultry industry in the developing world is improving efficiency of production. To meet this challenge and maintain the efficiency of feed utilization, series of attempts have been made by different researchers and organizations. These include incorporation of genetics selection, antimicrobials and other natural products, such as antibiotics as therapeutic agents to treat bacterial diseases and as feed additives for growth promotion, probiotics, vitamin supplements and antibodies to animal feeds and pelleting of feed, all decrease the time that an animal requires to reach market weight, reducing feed and overall cost (Kanwal et al., 2017). Two main groups of feed additives are the nutrient feed additives and non-nutrient feed additives. The nutrient feed additives are added in the feed to correct quantity of the deficient nutrients in the rations, such as vitamin mix, mineral mix and single or the mixture of amino acids. While the non-nutrient feed additives such as color and taste enhancers, appetizers, enzymes, yeast, growth promoters and probiotics are added in the feed to improve or to accelerate the rate of feed or nutrient utilization (Kemal et al., 2001).

Addition of Live yeast to animal feed has been known for improving the animals health symptoms

To cite this paper: Mulatu K, Ameha N and Girma M (2019). Effects of Feeding Different Levels of Baker's Yeast on Performance and Hematological Parameters in Broiler Chickens. *J. World Poult. Res.*, 9 (2): 38-49.

(Kanwal et al., 2017). For a long time, yeast products have been successfully included in feed as natural growth promoters for livestock and poultry production. Many types of yeast have been fed to animals either in the form of yeast fermented mash produced on farm, yeast by-products from breweries or distilleries and commercial yeast products (Kemal et al., 2001; Saied et al., 2011). Currently, in many parts of the world, food additives, such as probiotics and prebiotics are being experimented to alleviate the problems associated with the withdrawal of antibiotics from feed. Probiotic is defined as a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance (Fuller, 2000). It is a biological product, which stimulates the immunity system (Toms and Powrie, 2001), produces the digestive enzyme and increases its defensive activity against pathogenic bacteria and stops the implementation of those bacteria over the mucosa of the intestine (Rolfe, 2000).

Baker's yeast (*Saccharomyces cerevisiae)* is considered as one of the live microorganism probiotic that is used as feed additive. It can improve body weight gaining, feed efficiency, stimulate the immunity system and increase its defensive activity against pathogenic bacteria and also reduces feed cost by shortening the length of feeding (Fietto et al., 2004; Graff et al., 2008; Patane et al., 2017; Mohamed et al., 2015). Even though in Ethiopia incorporating yeast in ration specially baker's yeast is not widely used, it is one of the most widely available in Ethiopia. Therefore, the current experiment was conducted with the following objectives:

To evaluate the effects of feeding different levels of baker's yeast *(S. cerevisiae)* on feed intake, growth performance and carcass characteristics of broiler chicks; To assess the effects of baker's yeast on hematological indices of broiler chicks and to determine the economic profitability of using baker's yeast in the ration of broilers.

MATERIALS AND METHODS

Experimental site

The experiment was conducted at Haramaya University poultry farm, Ethiopia, which is located at an altitude of 1980 meters above sea level and 515 km east of Addis Ababa. The area is located at latitude and longitude of 9^0 26' N and 42^0 3' E, respectively. The average annual rainfall of the area is 780 mm with an average minimum and maximum temperatures are 8^0C and 24^0C, respectively (Samuel, 2008).

Experimental feeds

Feed ingredients of the study were formulated using the common broiler feeds. The ingredients used for ration formulation were soybean meal, noug seed cake, corn grain, wheat short, vitamin premix, di-calcium phosphate, limestone, baker's yeast, salt, lysine and methionine. The food ingredients that are corn grain and noug seed cake were hammer milled at 5mm sieve size and mixed based on dry matter basis. Lysine, methionine, di-calcium phosphate and vitamin premix were added to the feed during mixing without hammer milling. Representative samples of food ingredients soybean meal, noug seedcake, corn grain and wheat short as well as additive which are baker's yeast were analyzed for chemical composition before ration formulation.

For the period of both starter and finisher phases, treatment rations containing baker's yeast at levels of 0%, 0.5%, 1.5%, and 2.5 % of the total ration were formulated for T1, T2, T3 and T4, respectively by using feed win software. The four treatment rations used in the study were formulated to contain approximately 22% and 20% CP and 3100 and 3200 kcal/kg ME for starter's and finisher's diet respectively. The starter phase was until the age of 3 weeks. The finisher phase was offered from fourth up to sixth weeks. The starter and finisher diets were formulated separately as indicated in tables 1 and 2.

Experimental design and treatments

Completely Randomized Design (CRD) with four treatments and three replicates was used in the study. Twelve pens were used for the two hundred and forty day old chicks and 20 chicks were randomly assigned to each pen (Table 3).

Table 1. Percentage of ingredients used in formulating starter ration of broiler chicken

Ingredients (%)	T1	T2	T3	T4
Corn grain	44.0	44.0	44.0	44.0
Wheat short	11.0	11.0	10.5	10.0
SBM	25.0	25.0	25.0	25.0
NSC	17.5	17	16.5	16.0
BY	0.0	0.5	1.5	2.5
Lime stone	1	1	1	1
Common salt	0.5	0.5	0.5	0.5
VP	0.4	0.4	0.4	0.4
DCP	0.3	0.3	0.3	0.3
Lysine	0.1	0.1	0.1	0.1
Methionine	0.2	0.2	0.2	0.2
Total	100.00	100.00	100.00	100.00
ME(kcal)	3038.23	3043.71	3054.7	3062.37
CP (%)	21.7	21.82	21.98	22.06

SBM= soybean meal; NSC= noug seed cake; BY= baker's yeast; VP= vitamin premix; DCP= di-calcium phosphate; ME= metabolizable energy; CP= crude protein.

Table 2. Percentage of ingredients used in formulating finisher ration of broiler chicken

Ingredients (%)	T1	T2	T3	T4
Corn grain	55.0	55.0	55.0	55.0
Wheat short	8.0	7.5	7.5	7.0
SBM	22.0	22.0	22.0	22.0
NSC	12.5	12.5	11.5	11.0
BY	0.0	0.5	1.5	2.5
Limestone	1	1	1	1
Salt	0.5	0.5	0.5	0.5
VP	0.4	0.4	0.4	0.4
DCP	0.3	0.3	0.3	0.3
Lysine	0.1	0.1	0.1	0.1
Methionine	0.2	0.2	0.2	0.2
Total	100	100	100	100
ME(kcal)	3100.51	3104.34	3112.01	3123
CP %	19.47	19.93	20.00	20.03

SBM= soybean meal; NSC= noug seed cake; BY= baker's yeast; VP= vitamin premix; DCP= di-calcium phosphate; ME= metabolizable energy; CP= crude protein.

Table 3. Experimental design

Treatment	No of Replication	Starter Phase			Finisher Phase		
		Chicks/ Replication			Chicks/ Replication		
		R1	R2	R3	R1	R2	R3
T1	3	20	20	20	18	19	17
T2	3	20	20	20	18	19	19
T3	3	20	20	20	20	18	20
T4	3	20	20	20	19	19	18

Management of experimental chicks

The experimental house was cleaned and disinfected 3 weeks before the chicks' arrive. The pens were washed and sprayed with commercial disinfectant labeled for use in the poultry farm. The feeding and drinking troughs were properly cleaned, dried and disinfected before chicks' arrival. 240 day old chicks with 42 g average weigh (Cobb 500, commercial broiler strains) were purchased from Debre Zeit Elere Farm, Ethiopia. For these chicks, 12 pens were used and their floors were covered with disinfected wood shaving. Each pen was also equipped with a 250-watt infrared heat bulb. Feeding was twice a day at 08:00 and 16:00 hours *ad libitum*. Watering was also given *ad libitum* by washing the watering troughs properly. The chicks were vaccinated against common diseases in the area. Other health precautions and disease control measures were taken throughout the study period.

Chemical analysis of food ingredients. The chemical analyses of experimental feeds were done at Haramaya University Nutrition Laboratory by taking representative samples. Samples were taken from each food ingredients. Each ingredient was analyzed for their nutrients composition of DM, CP, EE, CF and total ash using the Weende or Proximate analysis method of the AOAC (1995). Metabolizable energy (ME) content of the feed ingredients as well as experimental diets was determined by using indirect method of Wiseman (1987) as follows: ME (kcal/kg DM) = 3951 + 54.4 EE - 88.7 CF - 40.80 Ash

Feed intake. Daily feed consumption was determined as the difference between the feed offered and refused. Feed offered and refusals were weighed and recorded every day in the morning. Mean daily Feed intake per bird was computed as;

$$\text{Mean daily feed intake} = \frac{\textbf{Mean total feed intake}}{\textbf{No of experimental days}}$$

Body weight gain. Body weight gain was assessed every week by weighing the chicks with sensitive balance. The body weight gain of birds was computed by subtracting mean initial weight from the mean final weight. Daily body weight gain (ADG) was determined as a difference between mean final and mean initial body weights divide by the number of experimental days.

Feed conversion ratio. The mean feed conversion ratio was determined by dividing the average daily feed intake (DFI) with a mean daily body weight gain (DBWG).

Carcass measurements. At the end of the experiment, six broilers were randomly picked from each replication for carcass evaluation. The birds were slaughtered after being starved for about 12 hours and weighed. After slaughtering, bleeding and de-feathering dressing percentage (DP%) was calculated as

$$\textbf{DP\%} = \frac{\textbf{Carcass weight}}{\textbf{Live weight}} \times \textbf{100}$$

Eviscerated percentage was calculated by removing the viscera, head, shank, trachea and lungs but with giblets (heart, liver, and gizzard) and skin and expressed as percent of live weight. Abdominal fat was determined by weighing the fat trimmed from proventriculus up to cloacae. Its percentage was calculated as the proportion of slaughter weight and multiplied by hundred. Breast meat weight was measured individually and equated with percent live weight. Drumstick and thigh together were measured and expressed as percent of the live weight. The edible offal (heart, liver and gizzard), kidney and spleen were weighed after separated from the visceral and their percentages were determined in relation to slaughter weight. The length and weight of small intestine, ceaca, proventriculus and crop were measured using a centimeter tape and sensitive balance.

Hematological parameter analysis. At the end of experiment period, six broilers were randomly selected from each replicate of each treatment groups and blood samples were taken from the bronchial vein with a syringe on a tube containing anticoagulant (heparin solution) for analysis of hematology parameters (Hemoglobin, Packed Cell Volume, Total white blood cell and red blood cell). Hemoglobin (Hb) was determined from samples before spinning in centrifuge by the method of Acid hematin. Packed cell volume (PCV) was determined by spinning blood filled capillary tubes in a centrifuge at 1200 revolutions per minute (rpm) for 5 minutes and reading on hematocrit reader. Total white blood cell (WBC) and red blood cell (RBC) counts were determined by using hemocytometer. The hematological parameters were determined as described by Dacie and Lewis (1991). At the time of slaughter, gastrointestinal tract and organs were examined for any pathological symptoms and gross lesions were recorded when observed.

Mortality. Mortality was recorded as it occurred and was determined for each treatment as a percentage of the total mortality at the end of the whole experiment.

Partial budget analysis

The net profits from broiler were calculated based on the cost of feed that each bird consumed from the respective treatments and the other costs. To estimate net benefit of baker yeast feeding, the partial budget was analyzed by consideration of the whole feed expense according to the principles developed by put (Upton, 1979). The partial budget analysis involves calculation of the variable cost and benefits. Partial budget measures the chicken cost, feed and others if any and the profit after the experiment, or differences between gains and losses for the proposed change.

Total variable cost includes the cost of feeds and other costs. The selling prices of broilers were determined by using the average current market price of broiler carcass per kilogram. Total return (TR) was considered as the difference between sale and purchase price in the partial budget analysis. The net income (NI) was expressed by subtracting total variable cost (TVC) from total return (TR). NI =TR - TVC The change in net income (ΔNI) was expressed as the difference between the changes in total return (ΔTR) and total variable cost (ΔTVC). The marginal rate of return (MRR) measures the increase in net income (ΔNI) related with each additional unit of expenditure (ΔTVC) as follows;

$$MRR = \frac{\Delta NI}{\Delta TVC}$$

The sale of chicks to cost of feed ratio was calculated as additional parameter to evaluate profitability and use efficiency of the rations as;

$$\frac{\text{Sales of chick's (Birr)}}{\text{Cost of feed (Birr)}}$$

Ethical approval

This research was carried out as a part of Master of Science in Agriculture (Animal production) research after the approval of competent authority of the director of research and post graduate study, Haramaya University, Ethiopia.

Statistical analysis

Data of the experiment was subjected to ANOVA using the General Linear Model (GLM) procedure (SAS, 2008). When the analysis of variance revealed significant differences, treatment means were compared using Least Significant Difference (LSD) test ($P<0.05$). The model used for data analysis was;

$Yij = \mu + Ti + eij$

Where,

Yij = is an observation (experimental unit)

μ = Overall means

Ti = Treatment effect

eij = Random error term

RESULTS AND DISCUSSION

Chemical composition of experimental diets

The results of laboratory analysis for the different feed ingredients are shown in table 4. Among all ingredients, yeast contains the highest protein value and with respect to the total protein content, it seems to be good protein feed additive for poultry. Even though the total protein composition of a given feed is important, the quality of the essential amino acids and their composition determine to a considerable extent its nutritive value in poultry ration (Sukanya et al., 2017).

The rations CP and ME contents were almost in line with in the recommended levels of 22% and 20% and ME value of 3100 and 3200 kcal/kg for broilers during the starter and finisher phases, respectively on tables 1 and 2 (Leeson and Summers, 2005).

Feed consumption

The average daily feed intake of the four groups of chicks during the starter, finisher phases as well as the whole growth period are shown in table 5. Average daily and total intake of feed during the starter phase was not affected ($P>0.05$) by dietary treatment. But in finisher phase and entire experimental period average and total

feed intake had significant difference (P<0.05). During finisher phase, total feed intake was higher for T1 than T3 and T4 but similar with T2 and average feed intake also high in T1 and T2 than T3 and T4. For the whole experimental period, total feed intake for all treatment groups was significantly different (P<0.05) and at the level of supplemental yeast increases, the birds consume less feed. In terms of total feed intake, the present results showed that chicks fed a diet containing 0% baker's yeast (T1) consumed more quantity of feed than the yeast containing treatment groups. The present study is in agreement with those of Adebiyi et al. (2012) and Nihar et al. (2016), who reported the lowest fed intake in all chickens given probiotic and highest in the control group. In addition, Shoeib and Madian (2002) also described low feed intake in the chickens fed on probiotic as compared to the control group.

Table 4. Chemical composition of feed ingredients

Ingredients	Chemical composition							
	DM (%)	CP (%DM)	EE (%DM)	CF (%DM)	ASH (%DM)	ME (kcal/kg DM)	Ca (%DM)	P (%DM)
Corn grain	89.07	10.28	3.1	5.72	6.08	3364.22	0.04	0.25
WS	90.02	15.36	3.3	12.34	4.60	2848.28	0.15	0.71
SBM	94.22	38.84	2.7	7.42	5.45	3217.37	0.37	0.32
NSC	90.89	30.76	7.2	15.74	10.55	2516.10	0.35	0.83
BY	94.46	48.0	2.94	3.29	5.01	3614.71	0.12	1.40

DM= Dry Matter; CP= Crude Protien; EE= Ether Extract; CF= Crude Fiber; P= Phosphorus; Ca= Calcium; ME= Metabolizable Energy, WS= Wheat short; SBM= Soybean meal; NSC= Noug seed cake; BY= Baker's yeast.

Table 5. Feed and nutrient intake of broilers fed diets with different levels of supplemental baker's yeast during the starter phase (1-21 days), finisher phase (22-45 days) and the whole growth period (1-45 days)

Parameters	Treatments				SEM	SL
	T1	T2	T3	T4		
Starter phase						
Feed intake (g/bird)	978.55	962.22	960.47	947.35	9.85	NS
Feed intake (g/bird/day)	46.6	45.82	45.73	45.11	0.47	NS
Finisher phase						
Feed intake (g/bird)	3377.09^{a}	3355.16^{ab}	3314.69^{bc}	3292.65^{c}	9.35	*
Feed intake (g/bird/day)	140.71^{a}	140.03^{a}	138.11^{b}	137.19^{b}	0.36	*
Whole period						
Feed intake (g/bird)	4355.64^{a}	4317.38^{b}	4275.16^{c}	4240^{d}	4.38	*
Feed intake (g/bird/day)	96.41^{a}	95.04^{ab}	95.9^{a}	94.22^{b}	0.35	*

^{abcd} Means within a row with different superscript letters are significantly different (P<0.05); NS = non-significant; SEM = Standard error of the mean; SL = Significance level; FI= Feed Intake; T1 = diet containing 0% of baker's yeast; T2 = diet containing 0.5% of baker's yeast; T3 = diet containing 1.5 of baker's yeast; T4 = diet containing 2.5% of baker's yeast.

Body weight gain

The growth rate of the experimental chicks during the starter, finisher and the whole growth period are presented in table 6. In this experiment chicks fed 2.5% baker's yeast (*Saccharomyces cerevisiae*) supplementation in ration were significantly (P<0.05) increase the body weight gaining compared with other groups. Meanwhile, chicks fed 2.5% baker's yeast has higher feed conversion efficiency compared with the other dietary treatments (control and 0.5% baker's yeast). No negative effects were exerting on the addition of at all inclusion levels on internal body organs as compared with control.

At the end of starter phase, final body weight of bird was greater for T4 as compared to T1 and T2 but, similar with T3 groups. Results showed that the final body weight of birds during finisher phase and at the end of whole experimental period had higher body weight for T4 than T1 but, similar to other yeast containing treatment groups. Average daily body weight gain during the starter phase and the whole experimental period was significantly affected by treatment (P<0.05). The obtained results confirmed the previous findings of several researchers (Zhang et al., 2005; Paryad and Mahmoudi, 2008) that

yeast supplementation in broiler ration had a significant effect on body weight gain and feed conversion ratio.

Gudev et al. (2008) and Patane et al. (2017) also reported that *Saccharomyces cerevisiae* improved feed/gain ratio and body weight gain. The present study revealed that baker's yeast supplementation had a positive effect on the body weight of broiler chickens in T4 for the whole trail period. This may be explained as one of the critical roles of probiotic yeast in the metabolic function is promoting a healthy or pathogen free gastrointestinal tract environment for the proper functioning of endogenous enzymes to break down the energy nutrients of the experimental rations and the competition between probiotic microorganism and pathogenic microorganisms for energy and nutrients are reduced. Then energy and nutrients are efficiently absorbed in Gastrointestinal tract of broilers. This in turn may have improved body weight and feed conversion efficiency of the chicks fed yeast added rations as compared to the chicks fed control or non-yeast added diets.

Table 6. Body weight change of broilers fed diets with different levels of supplemental baker's yeast during the starter phase (1-21 days), finisher phase (22-45 days) and the whole growth period (1-45 days)

Items	Treatments				SEM	SL
	T1	T2	T3	T4		
Starter Phase						
Initial wt (g/bird)	43.28	42.76	42.58	42.37	0.71	NS
Final wt (g/bird)	478.63^{b}	494.36^{b}	495.56^{ab}	527.67^{a}	7.1	*
Total weight gain (g/bird)	435.35^{b}	451.77^{b}	452.46^{b}	485.3^{a}	7.02	*
ADG (g/bird/day)	20.73^{b}	21.51^{b}	21.54^{b}	23.11^{a}	0.33	*
FCR (g feed/g gain	2.25^{a}	2.13^{ab}	2.12^{ab}	1.95^{b}	0.04	*
Finisher Phase						
Initial wt (g/bird)	478.63^{b}	494.36^{b}	495.56^{ab}	527.67^{a}	7.1	*
Final wt (g/bird)	1784.72^{b}	1802^{ab}	1849.09^{ab}	1863.85^{a}	16.68	*
Total weight gain (g/bird)	1306.09	1307.64	1353.53	1336.17	16.68	NS
ADG (g/bird/day)	54.42	54.48	56.4	55.67	0.69	NS
FCR (g feed/g gain	2.59	2.57	2.45	2.46	0.03	NS
Whole Period						
Initial wt (g/bird)	43.28	42.76	42.58	42.37	0.71	NS
Final wt (g/bird)	1784.72^{b}	1802^{ab}	1849.09^{ab}	1863.85^{a}	16.68	*
Total weight gain (g/bird)	1741.44^{b}	1759.41^{ab}	1806^{ab}	1821.48^{a}	16.61	*
ADG (g/bird/day)	38.7^{b}	39.1^{ab}	40.13^{ab}	40.47^{a}	0.37	*
FCR (g feed/g gain)	2.5^{a}	2.45^{ab}	2.37^{bc}	2.33^{c}	0.02	*

^{abc} Means within a row with different superscript letters are significantly different (P<0.05); NS = non-significant; SEM = standard error of the mean; SL = Significance level; ADG = Average daily body weight gain; g = gram; T1= diet containing 0% of baker's yeast; T2 = diet containing 0.5% baker's yeast; T3 = diet containing 1.5% baker's yeast; T4 = diet containing 2.5% of baker's yeast; wt = weight.

Feed conversion ratio

Feed conversion ratio of broilers during the starter, finisher phases and entire growth period of the experiment are presented in Table 6. Results of the experiment indicated that there was significant difference (P<0.05) in feed conversion ratio in broilers fed the starter rations. During finisher phase feed conversion ratio showed insignificant difference (P>0.05) among treatments. Feed conversion ratio (FCR) expressed as feed to gain and during whole growth period feed conversion ratio was showed significant difference (P<0.05) and the value was being greater for T1 and T2 than T3 and T4. A significant improvement in FCR was recorded in the supplemental yeast containing treatment groups. Yeast acts by reducing the feed conversion ratio, resulting in an increase in daily life weight gain. Birds that have a low FCR are considered as efficient users of feed. So attributable to this FCR of birds in T4 (2.5% baker's yeast) and T3 (1.5% baker's yeast) were low and efficiently feed utilizers than other groups.

The result is in agreement with Leeson and Summers (2006) who written 2-4 feed conversion ratios for poultry.

Zhang et al. (2005) had also reported significant improvement in feed/gain ratio. In addition, Bansal et al. (2011) and Hana et al. (2015) reported significant and better feed conversion efficiency on probiotic supplementation in the diet of commercial broiler chicks. In the present study, this improvement of feed conversion ratio in yeast supplemented groups might be due to the one beneficial effects of yeast in improvement of the intestinal lumen health and thereby increasing the absorption and utilization of the dietary nutrients.

Carcass parameters

Results of the present study indicated that the average slaughter weight was not significantly differ (P>0.05) among the treatment groups (Table 7). However, significant difference (P<0.05) was observed in dressed and eviscerated carcass weight and percentage. In breast meat percentage, drumstick-thigh, abdominal fat, thigh, wing and drumstick weight and percentage there was no significant difference (P>0.05) among treatments. But, there was significant difference in breast meat weight for T4 compared to other treatments. Breast meat often has a higher economic value than meat from other parts of the poultry carcass (Sasidhar, 2006 and Eltazi et al., 2014). The author also reported that the main concern of people producing broilers is unnecessary accumulation of carcass fat, particularly in the abdominal area, as this fat is not accepted by consumers it becomes a waste to the processor. Even if the statistical results showed insignificant difference in abdominal fat weight and percentage among treatments, it was a bit higher for the groups kept on the control group than T2, T3 and T4.

Table 7. Carcass yield characteristics of broilers fed different level of supplemental baker's yeast from 1-45 days of the period.

Parameters	Treatments				SEM	SL
	T1	T2	T3	T4		
Slaughter wt (g)	1881.42	1877.08	1944.17	1966.25	46.4	NS
Dressed carcass wt (g)	1599.14^{b}	1616.77^{b}	1750^{a}	1747.95^{a}	36.08	*
Dressing percentage (%)	85.02^{c}	86.36^{c}	90.02^{a}	88.85^{ab}	0.65	*
Eviscerated wt(g)	1278.14^{ab}	1269.88^{b}	1378.54^{a}	1388.08^{ab}	33.42	*
Eviscerated percentage (%)	67.93^{ab}	67.66^{b}	71.43^{a}	70.13^{ab}	0.8	*
Drumstick -thigh wt (g)	348.42	350.77	362.71	367.33	9.1	NS
Drumstick -thigh (%)	18.53	18.68	18.89	18.45	0.28	NS
Breast meat wt (g)	457.46^{b}	452.15^{b}	497.08^{ab}	505.33^{a}	14.0	*
Breast meat (%)	24.29	24.1	25.56	25.71	0.49	NS
Abdominal fat wt (g)	36.69	32.59	31.53	31.08	2.7	NS
Abdominal fat (%)	1.95	1.74	1.62	1.57	0.11	NS
Thigh wt (g)	188.87	189.99	195.63	198.33	5.9	NS
Thigh (%)	10.05	10.12	10.19	9.96	0.23	NS
Wing wt (g)	71.31	71.84	74.75	77.75	1.48	NS
Wing (%)	3.79	3.82	3.84	3.96	0.07	NS
Drumstick wt(g)	159.55	160.77	167.08	169	4.72	NS
Drumstick (%)	5.82	8.56	8.7	8.49	1.24	NS
Liver wt (g)	35.33^{b}	30.3^{b}	43.93^{a}	46.87^{a}	1.89	*
Liver (%)	1.87^{bc}	1.61^{c}	2.26^{ab}	2.38^{a}	0.1	*
Heart wt (g)	9.87^{b}	9.08^{b}	10.77^{ab}	13.2^{a}	0.64	*
Heart (%)	0.52^{b}	0.49^{b}	0.55^{ab}	0.67^{a}	0.03	*
Gizzard wt (g)	28.0	30.33	32.0	33.33	1.25	NS
Gizzard (%)	1.47	1.62	1.64	1.67	0.05	NS
Kidney wt (g)	10.68^{c}	11.1^{bc}	13.13^{ab}	13.87^{a}	0.51	*
Kidney (%)	0.57^{b}	0.59^{ab}	0.67^{ab}	0.7^{a}	0.03	*
Spleen wt (g)	2.0^{b}	1.83^{b}	2.83^{ab}	3.33^{a}	0.34	*
Spleen (%)	0.11	0.09	0.15	0.17	0.02	NS

^{abc} Means within a row with different superscript letters are significantly different (P<0.05); NS = non-significant; SEM = standard error of the mean; SL= Significance level; g = gram; wt= weight; T1 = diet containing 0% of baker's yeast; T2 = diet containing 0.5% of baker's yeast; T3 = diet containing 1.5% of baker's yeast; T4 = diet containing 2.5% of baker's yeast; wt = weight.

In study, Kalavathy et al. (2003) found that supplementation of *S. cerevisiae* reduces (P < 0.05) abdominal fat pad. Similarly, Anjum et al. (2005) and Safalaoh (2006) also reported that supplementation of yeast had produced low level (P<0.05) of abdominal fat pad. This result is similar with several studies that reported lowering of abdominal fat by yeast supplementation than non-supplementation (control group), indicating the fact that baker' yeast enhance efficient energy usage.

Baker's yeast (*S. cerevisiae*) affect significantly spleen weight, liver, heart and kidney weight and percentage, crop length, caeca and small intestine weight. The results agreed with that of Ivanov (2004); Penkov et al. (2004); Dimcho et al. (2005) and Onwurah and Okejim (2014) reported more improvements in liver, and heart of broilers, mules and ducklings by supplementing diets with probiotics. However, it is in contrast with the findings by Hussein and Selim (2018) who reported that dietary probiotic supplementation did not increase the liver weights of broiler chickens. As indicated on Table 8 entire mass of the small intestine in T3 and T4 groups were heavier than the weights of intestine from other experimental groups. Gao et al. (2008) also noted that yeast culture inclusion at a level of 0.25% increased (P<0.05) small intestine weight in broilers. This result is in contrast with the findings by Alcicek et al. (2004), who reported that dietary supplementation of probiotics lowered the weight of the small intestine. Finally, non-significant differences were seen in spleen percentage, gizzard, crop, proventriculus weight and percentage and caeca and small intestine length. In addition, there was no significant effect of the probiotic on the weights of organs like crop and gizzard (Çınar et al., 2009).

Table 8. Non-edible offal components of broilers fed different level of supplemental baker's yeast from 1-45 days of the period.

Parameters	Treatments				SEM	SL
	T1	T2	T3	T4		
Crop weight (g)	8.58	8.3	11.27	11.43	1.13	NS
Crop length (cm)	13.79^{b}	16.34^{a}	15.83^{ab}	15.79^{ab}	0.45	*
Proventriculus weight (g)	9.66	11.88	10.16	10.66	1.48	NS
Proventriculus length (cm)	9.87	12.15	9.14	7.58	1.21	NS
Caeca weight (g)	12.66^{b}	14.38^{ab}	18.75^{a}	19.5^{a}	1.3	*
Caeca length (cm)	27.45	25.21	29.42	29.12	1.15	NS
Small intestine weight (g)	60.67^{c}	66.33^{bc}	101.75^{a}	85.75^{ab}	5.32	*
Small intestine length (cm)	177	168	196.67	180.22	7.15	NS

^{abc} Means within a row with different superscript letters are significantly different (P<0.05); NS = non-significant; SEM = Standard error of the mean; SL = Significance level; cm = centimeter; g = gram; T1 = diet containing 0% of baker's yeast; T2 = diet containing 0.5% of baker's yeast; T3 = diet containing 1.5 of baker's yeast; T4 = diet containing 2.5% of baker's yeast.

Hematology evaluation

The values obtained for all hematological parameters of broilers fed graded levels of baker's yeast in ration (Table 9) showed that Hb (10.96 - 12.5 g/dl) and PCV (32.42 - 35.63%) were within normal range of 6.0-13.0 g/dl and 29 - 38% for Hb and PCV, respectively (Nworgu, 2007). Hematological constituents reflect the physiological state of the animals to its internal and external environment (Chowdhury et al., 2005). RBC was also within the range of 1.0-3.0 ($x10^6/mm^3$) and no reduction in total WBC were recorded in chicks of all treatment groups with or without yeast at its four graded levels within the normal range of 1.099.06 x $10^3/mm^3$ reported by Douglas et al. (2010). These indices could have contributed to the better performance of the broilers at both phases. The use of baker's yeast had no significant effects on RBC for all treatment groups, but differences between treatments were significant for Hb and PCV (P < 0.05). All yeast fed chicks in compare to control diet had more WBC. The yeast can stimulate immune system of chick's body so, it affects WBC. Mohamed et al. (2015) reported a positive correlation between dietary levels of *S.cerevisiae* with the haematological indices like, RBC, WBC and PCV in rabbit and broiler chickens. The results agreed with Shareef and Al-Dabbagh (2009) that reported there was no reduction in total white blood cells and hemoglobin with supplemental yeast fed to broilers.

Normal hematological values reveal the nutritional status of animal. Thus, the normal values observed in the present study indicate the adequacy of nutrients for the birds. Oladele et al. (2001) reported that linked lower values of these parameters to inadequate nutrition. It also

implies that the immune systems of the chicks are adequate. Even though Hb, PCV and RBC values are within the normal range, the higher values observed in broilers consumed ration containing yeast as compared to the control diet suggest that yeast improved nutrient utilization and assimilation in to the blood stream for use by the birds and enhanced blood formation due to availability of essential nutrients.

Mortality

Rate of mortality recorded from the experimental chicks are shown in table 9. During the trial period there was no observable sign of morbidity recorded but mortaliti es occurred fortuitously within the first 2 weeks of the study. May be due to stress and mechanical injury during transportation. Mortality percent of broilers during experimental period was 10.00, 6.67, 6.67 and 3.33 (SEM = 2.89) for T1, T2, T3 and T4, respectively and there was no significant difference (P>0.05) in mortality percentage among the treatments. But numerically the highest mortality rate was seen in T1 and the lowest mortality rate was observed in T4. All mortalities occurred during the first phase of feeding trail and it was not reported in all groups of this experiment during the second phase. This observation could be in accordance with that mention yeast is used to stimulate the animal's immune system, thereby further reducing the risk of disease (Laegreid and Bauer, 2004). Also several workers (Shashidhara and Devegourda, 2003; Goa et al., 2008) reported that (*Saccharomyces cerevisiae*) improved the efficiency of immune system of broilers. Similar findings were obtained by Świątkiewicz et al. (2014) who found positive effect of dietary (*Saccharomyces cerevisiae*) on mortality rate of broiler. In addition to Karaoglu and Durdag (2005) reported that, the use of probiotic (*Saccharomyces cerevisiae*) in the broiler diet reduced or prevented the mortality.

Table 9. Effect of graded levels of baker's yeast fed in ration of broiler on haematological indices and mortality rate during 1-45 days of age.

Parameters	Treatments				SEM	SL
	T1	T2	T3	T4		
Hemoglobin (g/dl)	10.96^{b}	12.06^{ab}	12.17^{a}	12.5^{a}	0.24	*
Packed cell volume (%)	32.42^{b}	34.63^{ab}	34.94^{ab}	35.63^{a}	0.7	*
RBC(10^6/mm^3)	1.76	2.09	2.07	2.17	0.89	NS
WBC (10^4/mm^3)	2.23^{b}	2.44^{a}	2.40^{ab}	2.46^{a}	0.56	*
Mortality %	10	6.67	6.67	3.33	2.89	NS

^{abc} Means within a row with different superscript letters are significantly different (P<0.05); NS = non-significant; SEM = Standard Error of the Mean; SL= Significance level; RBC= red blood cell; WBC = white blood cell; g/dl= gram per deciliter; T1= diet containing 0% of baker's yeast; T2 = diet containing 0.5% of baker's yeast; T3 = diet containing 1.5% of baker's yeast; T4 = diet containing 2.5% of baker's yeast.

Table 10. Partial budget analysis for broilers fed different levels of baker's yeast during 1-45 days of age.

Variables	Treatment			
	T1	T2	T3	T4
Purchase price/bird (birr)	20	20	20	20
Price/kg of carcass (supermarket)	140	140	140	140
Selling price/bird (birr)	178.94	177.78	193.00	194.33
Feed cost/bird (birr)	26.96	27.07	28.6	30.93
Other cost/bird (birr)	1.15	1.15	1.15	1.15
TVC/bird (birr)	28.11	28.22	29.75	32.08
TR (birr)	158.94	157.78	173	174.33
NR (birr)	130.83	129.56	143.25	142.25
ΔNR	0.00	-1.27	13.69	-1
ΔTVC	0.00	0.11	1.53	2.33
MRR	0.00	-11.54	8.95	-0.43
Chicks sale/feed cost	6.64	6.57	6.75	6.28

ETB = Ethiopian Birr, TR= total return, NR= net return, ΔTVC = change in total variable cost, ΔNR = change in net return; MRR = marginal rate of return; T1= diet containing 0% of baker's yeast; T2 = diet containing 0.5% of baker's yeast; T3 = diet containing 1.5% of baker's yeast; T4 = diet containing 2.5% of baker's yeast.

Partial budget analysis

Differences in feed cost, chick sale and chick's sale to feed cost ratio were noticed among the treatment groups (Table 10). The highest net income and marginal rate of return were obtained for T3 ration followed by T4. Therefore, T3 appeared to be economical in economic parameters used in the study. The chicks' sale to feed cost ratio was estimated as additional parameter to see the importance of inclusion baker's yeast on ration of broilers during both starter and finisher phases. The birds in T3 score highest chicks' sale to feed cost than T1, T2 and T4. The lower ratio of chick's sale to feed cost was resulted from higher price of baker's yeast and low body weight gain. Therefore, the results of this study indicated that ration containing 1.5% addition of baker's yeast is potentially profitable than the other levels of inclusion in the ration under the condition of the present experiment.

CONCLUSION

In conclusion, baker's yeast can be an important feed additive, which can be included up to 2.5% of the total ration and improve the overall performance of broilers without compromising the hematological indices of broiler chickens. Generally, the present study can be a gate-way for further researches on how to use baker's yeast as an efficient protein feed additive in poultry ration.

DECLARATIONS

Competing interests

The authors declare that they have no conflict of interest.

Author's contributions

This manuscript is part of Kassech Mulatu MSc Thesis, where Negassi Ameha and Meseret Girma were advisors of her. Meseret Girma prepared of the manuscript. All authors read and approved the manuscript.

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JWPR
Journal of World's Poultry Research

2019, Scienceline Publication
J. World Poult. Res. 9(2): 50-58, June 25, 2019

Research Paper, PII: S2322455X1900007-9

Effect of Different Bedding Materials on the Hematological and Serum Biochemical Parameters of Broiler Chickens

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Received: 03 Apr. 2019
Accepted: 03 May. 2019

ABSTRACT

The aim of the present study was to determine the effect of different bedding materials on the, hematological and serum biochemical parameters of the broiler chickens. A completely randomized design was employed for the research in which the treatment were five bedding materials (rice hulls, groundnut hulls, wood shaving, sharp sand and control). The treatments were replicated three times each with ten birds in each experimental unit. The birds were brooded for two weeks before the experiment begin. The blood samples were collected at day 56 of experiment for hematological and serum biochemical analysis. There was no significant difference in the glucose, serum total protein, globulin, calcium, sodium, total bilirubin, conjugated bilirubin, and serum alanine transferase, but there was a significant difference in serum cholesterol, albumin, bicarbonate and serum aspartate transferase within the groups. The findings also indicated in the hematological parameters that bedding materials caused significant difference in the hemoglobin content, white blood cell count and mean corpuscular hemoglobin, but no significant differences in the other parameters (Heterophils, monocytes, basophils, lymphocytes, mean cell volume and packed cell volume) were seen. The results suggested that rice, hulls, groundnut hulls and sharp sand can serve appropriately as bedding materials for broiler production, with compatible effects on serum biochemical and hematological parameters.

Keywords: Bedding materials, Broilers, Hematology, Serum biochemistry

INTRODUCTION

Poultry litter is used in confined buildings met for raising broilers, turkeys and other birds. The bedding materials such as wood shavings sawdust, peanut hulls, shredded sugarcane, straw, sand and other dry, absorbent, low-cost organic materials. The main function of bedding materials is to help in the absorption's moisture, preventing the excess production of ammonia and harmful pathogens (Monira et al., 2003).

The materials used as bedding can also have a significant impact on carcass quality and bird's performance as well as on their biochemical characteristics. Some specific procedures must be adhered, to properly maintain the litter and maximize the health and productivity of the birds raised on it (Monira et al., 2003).

The poultry usually grown on dirty floors with some type of bedding material. Concrete floors and some specialized raised flooring are used at some facilities. Most of poultry flocks are raised on the floor with a layer of bedding called, "litter," spread over the top of the floor. Unlike livestock where bedding material is typically clean and unused, in poultry flocks the term bedding material can also mean used bedding material that includes manure, spilled feed, water and feathers. Good litter should be nontoxic to the birds, be free of contaminants, such as pesticides and metals, be very absorbent with a short drying time and be readily available and relatively inexpensive (Monira et al., 2003). The purpose of using litter on floor is to absorb moisture from birds' droppings in order to keep the floor reasonably dry and ensure comfortable conditions for birds. It also gives birds a suitable medium on which feeding, watering and other management practices are carried out. Sawdust, rice husk, sugarcane bagasse, chopped straw, paper and sand amongst others are the common types of litter used in poultry houses throughout Nigeria (Monira et al., 2003).

The considerable individual differences in biochemical indices in birds are the main reason for the wide physiological ranges specified for chickens (Pedersen et al., 2000). The major factors for successful poultry production are high genetic potential, balanced nutrition and health maintenance (Horsted and Hermansen, 2007). Their values are influenced by species, age, sex,

season, geographic region, nutrition, and physiological condition. They are also indicators of the health of birds housed in cage systems. Variables such as total protein, albumin, cholesterol, glucose, calcium, phosphorus, uric acid, aspartate aminotransferase, lactate dehydrogenase, and lactate are commonly measured biochemical characteristics for poultry (Hrabčáková et al., 2014).

It has been noted that significant changes in the serum biochemical parameters can be done to assess the both pathological and nutritional status of animals (Akpodiete and Ologbodo, 1998). Serum concentrations of glucose, cholesterol, triglyceride and uric acid level have been reported to be significantly affected by different feeding treatments, whereas total protein, albumin and creatinine level were not significantly affected by the treatments (Ibrahim and Albokhadaim, 2016). Serum total protein and albumin have been reported to be directly related to protein intake and quality (Onifade, 1998).

Biochemical valuables can be used to confirm the maturity and to monitor any changes in the quality of water and related soils (Paria, 2014). The evaluation of hematology and serum biochemistry in birds allows for the determinations of metabolic changes due to endo- and exogenous factors including the genetic type, husbandry conditions, season, sex and age (Ross el at., 1978; Bowes et a1., 1989; Melluziet al., 1992; Krasnodêbska et al., 2000; Harr, 2002; Juraniet al., 2004; Rajmanet al., 2006).

Therefore, the objectives of the present study were to evaluate the hematological and serum biochemical parameters of broiler's chickens reared in different bedding materials.

MATERIALS AND METHODS

The study was conducted at the poultry house in the research and teaching farm of the Department of Agricultural Education of Federal College of Education, Kontagora, Niger State Nigeria. Geographically, Kontagora is located at latitude $10^0$24'1" North and longitude $5^0$28'1" East and at an elevation of 335m above sea level (Niger State Bureau of statistics, 2011). The birds (broilers) Arbor acre breedwas purchased at day old from Top Link, an accredited poultry hatchery based in Ibadan, Nigeria. They were fed with top feed at day old to the eight weeks, vaccines, antibiotics and anticoccidial were the main routine drugs used for the preventives measures

A Completely Randomized Design (CRD) was used for present study. Five bedding materials (rice hulls, groundnut hulls, Wood Shavings (WS), sharp sand and control, concrete floor) were utilized. The treatments (bedding materials) were replicated three times each, that is, there were fifteen experimental units. Ten birds were placed in each experimental unit, to get a total number of 150birds that have been brooded from day old to two weeks of age.

The blood was collected at day 56 (eighth week) of the experiment. Blood sample was collected from the wing (brachial vein) and neck slitting of bird per replicate. 5ml of blood was collected from one bird per replicate into a tube containing Ethylene Diamine Tetra Acetate (EDTA) to determine hematological parameters (packed cell volume, white blood cell, Haemoglobin, Red blood cell, Neutrophil, lymphocyte, Eosinophil, Basophile, monocytes and conjugated billirubin) while another 5ml was collected to determine some serum biochemical parameters (Ca^{++}, Na^{++}, Bicarbonate, Glucose, cholesterol, Total protein, Albumin, Globulin, Total billirubin, Conjugated billirubin, Serum glutamic pyruvic transaminase, serum glutamic oxaloacetic transaminase, and Alkaline phosphate.

Packed cell volume

The percentage of Packed Cell Volume (PCV) in the blood was determined using the haematocrit centrifuge method as described by Dacie and Lewis (1984; 1995). A capillary tube was dipped into sample to fill it to about three-quarter length. Excess blood on the side of the capillary tube was wiped off in other to keep accurate reading. One end of the tube was sealed over a Bunsen burner. The capillary tube was put into a micro-haematocrit reader and the level of the packed cell was regarded as the PCV.

Red blood cell

Blood was diluted with 0.9% NaCl. The diluted blood was mounted on a haemocytometer and the number of erythrocytes in the cream scribed volume ($0.02mm^{3)}$ was counted microscopically. Calculated erythrocytes were expressed in million per cubic meter (Jain, 1986). MCHC = Hb / PCV (grams/litre).

White blood cell

The estimate of the total blood cells was carried out immediately after collection of blood sample from experimental animals using Neubauer haemocytometer counting chamber (Jain, 1986). From blood sample of test animals 2ml of blood sample was pipette and mixed with 4ml of White Blood Cell (WBC) diluting fluid (WBC fluid made up of 3% aqueous solution of acetic acid and 1% gentian violet) (Jain,1986). The sample was then put into the haemocytometer and cell counted and expressed as 10^9 WBC per liter of blood.

Haemoglobin

The Haemoglobin (Hb) concentration of each blood sample was determined by cyanmethaemoglobin method as described by Jain (1986). From each blood sample of experimental animal, 20µl of blood was mixed with 4ml of modified Drabkin's solution, Drabkin's solution was prepared by mixing 200 mg potassium ferricynide, 50 mg potassium cyanide and 140 mg potassium dihydrogen phosphate; volume was made up to 1 liter with distilled water and pH adjusted to 7.0). The mixture of blood sample of experimental animal and Drabkin's solution was allowed to stand for 3 minutes before reading the Hb concentration using a spectrophotometer at wavelength of 540nm. The actual value of Hb was extrapolated from a standard curve (Jain, 1986).

Serum cholesterol

The cholesterol of the serum was determined by using enzymatic endpoint method as described by Ahur et al. (2012). The absorbance of the sample was measured against the blank reagent within 60 minutes and the reading was taken at wave length of 520nm (Ahur et al., 2012).

Total protein

This was determined using Biuret method as described by Ahur et al. (2012). The reagent and serum was mixed and incubated for 30 minutes at 20-25^{o}C and absorbance of the sample and that of the standard was measured against the blank at a wave length of 540 nm. The total protein was calculated as follows:

$$g/d = \frac{\text{Absorbance of sample} \times \text{Concentration}}{\text{Absorbance of standard}}$$

$$g/d = \frac{1}{\text{grams per decimeter.}}$$

Albumin

The Bromo Cresol Green (BCG) method as described by Donmas (1971) were employed. The absorbance of the sample and the standard were taken against the reagent blank at a wave length of 620nm.

$$\text{Albumin (g/d) concentration} = \frac{\text{Absorbance of sample} \times \text{concentration}}{\text{Absorbance of standard} \quad 1}$$

Aspartate transferase

Concentration of the oxaloacetate hydrazoneformed with 2,4– dinitrophenylhydrazine was measured by absorbance it produces, 0.1ml of test samples was added to 0.5ml phosphate buffer containing q–oxoglutarate and L – aspartate. It was mixed and allowed to stand for 30 minutes at 37oc 0.5ml solution containing 2, 4–dinitrophenylhydrazine were added and allowed to stand for 20 minutes at 25^{o}C. Later 5ml solution hydroxide was added, mixed and the absorbance of the color produced was read using spectrophotometer and concentration was determined from Aspartate transferase (AST) activity table (Ahur et al., 2012)

Alanine transferase

Concentration of the oxaloacetate hydrazone formed with 2, 4 – dinitrophenylhydrazine was measured by absorbance it produces, 0.1ml of test sample was added to 0.5ml phosphate buffer containing q – oxoglutarate and L – aspartate. It was mixed and allowed to stand for 30 minutes at37oc' 0.5ml solution containing 2, 4 – dinitrophenylhydrazine was added and allowed to stand for 20 minutes at 25oc. Later 5ml sodium hydroxide was added, mixed and the absorbance of using spectrophotometer and concentration was determined from Alanine transferase (ALT) activity table (Ahur et al., 2012).

Calcium

Lx20 system was used to dilute Ion Selective Electrode (ISE) methodology to measure calcium concentration in serum. The system will determine calcium concentration by measuring calcium ion activity in solution. The sample of buffer mixture contact the electrode, calcium ions complex with the ionophore at the electrode surface. The reference signal was used to calculate the analyzed concentration based on the Nernst equation (Ahur et al., 2012).

Bicarbonate

Bicarbonate reacts with Phosphoenolpyruvate (PEP) in the presence of Phosphoenolpyruvate Carboxylase (PEPC) to produce oxaloacetate and phosphate. This reaction occurs in conjunction with the transfer of a hydrogen ion from Nicotinamide Adenine Dinucleotide Hydrogenase (NADH) to oxaloacetate using MDH. The resultant of Nicotinamide Adenine Dinucleotide (NAD) causes a decrease in absorbance in the uv range (320 – 400 nm). The change in absorbance was directly proportional to the concentration of bicarbonate in the sample being assayed (Ahur et al., 2012).

Glucose

Hexokinase catalyses the phosphorylation of glucose by adenosine triphosphate. G-6-PD was oxidized to 6-phosphogluconate in the presence of NAD by the enzymes glucose -6- phosphate dehydrogenase (Ahur et al., 2012).

Sodium

An ISE made use of unique properties of certain membrane materials to develop an electrical potential Electromotive Forces (EMF) for the measurement of ions in solution. The electrode has a selective membrane in contact with the both test solution and an internal filling solution (Ahur et al., 2012).

Statistical analysis

Analysis Of Variance (ANOVA) using the general linear model SAS (2000) was employed. Duncan's Multiply Range Test (DMRT) was used to indicate the significant diferences at level of $P < 0.05$.

Ethical approval

Federal College of Education, Kontagora has adopted the University of Ibadan institutional animal care and use committee which works on animal care and research ethics. The aim was to provide a platform geared towards educating the scientific world on global best practices as it concerns humane handling and use of laboratory/experimental animals for research purposes.

RESULTS

The serum glucose levels were 8.10, 8.27, 8.60, 9.07 and 7.57 mmol/l for the control, rice hull, groundnut hull, WS and sharp sand respectively. The mean values of this parameter indicated that WS and sharp sand had the highest and lowest serum glucose content of 9.07 and 7.57mmol/l respectively. There was no significant ($p>0.05$) difference in the serum glucose content for the broilers raised on the different bedding materials.

There was not a significant different ($p\leq0.05$) between treatment groups for values of serum cholesterol. However broilers raised on the rice hull and control grroups had insignificantly higher values than the other groups ($p>0.05$) (Table 1).

The albumin level of the birds raised on the sharp sand (4.27 g/dl) was significantly ($p<0.05$) higher than the birds raised on WV (3.20 g/dl). There was no significant difference ($p>0.05$) between the mean values of the globulin levels of the birds reared on different bedding materials (Table 1).

There was significant difference ($p<0.05$) in the serum bicarbonate content of the broilers raised on the rice hull (28.67 mmol/l) having significantly higher ($p<0.05$) serum bicarbonate content than the birds reared on the WS (24.33 mmol/l). Calcium contents in the serum showed no significant difference ($p>0.05$) between the treatments (Table 1).

The serum sodium contents in control group (154.63 mmol/l) was significanly higher than the WS group (112.90 mmol/l) ($p<0.05$). However no significant different with other treatments. No significant differences ($p>0.05$) were recorded between the treatments about bilirubin and conjugated bilirubin contents (Table 1).

The serum levels of ALT in broilers were 3.30, 5.47, 4.50, 2.57 and 3.17iu/l whereas the levels of AST were 2.60, 2.53, 1.84, 1.87 and 2.27iu/l for the birds raised on the control, rice hull, groundnut hull, WS and sharp sand respectively. There was no significant difference ($p>0.05$) in the levels of AST between the broilers reared in different treatments (bedding materials) but the level of ALT in the birds reared on the rice hull was significantly ($p<0.05$) higher than the birds raised on the WS and sharp sand (Table 1). Statistically, there were no significant differences ($p>0.05$) in the heterophils and lymphocytes for the broilers raised on different bedding materials (Table 1).

There were significant differences ($p<0.05$) in the monocytes between birds raised on rice hull when compared with control and sharp sand; also, WBC was significantly higher ($p<0.05$) in broilers of control group when compared with other groups (Table 1). The RBC content were 2.05, 1.95, 1.88, 1.93 and 1.95iu/l for the broilers that were reared on the control, rice hull, groundnut hull, WS and sharp sand respectively. The recorded the significantly highest RBC content was established in birds reared on sharp sand ($p<0.05$) when compared other bedding materials (control, rice hull, groundnut hull) and insiginificantly higher than WS group (Table 1).

The Hb content of the birds raised on the control (12.20 iu/l) was significantly higher ($p<0.05$) than those for the other treatments (6.02, 5.62, 7.46 and 7.66 iu/l).

There was no significant difference in PCV level ($p>0.05$) between the broilers reared on different bedding materials.

Table 1. The effect of different bedding materials on hematological and serum biochemical parameters of broiler chicken (Arbor acre) between February and April 2018 at research and teaching farm of federal college of education, Kontagora Niger state, Nigeria

Parameters	Control	Rice Hull	Groundnut Hull	Wood Shaving	Sharp Sand
Heterophils (iu/l)	53.67±7.26^{a}	50.33±11.35^{a}	55.00±2.65^{a}	48.00±7.81^{a}	44.67±6.17^{a}
Monocytes (iu/l)	1.67±0.88^{a}	6.00±1.53^{b}	3.00±1.15^{ab}	4.33±1.45^{ab}	1.33±0.67^{a}
Lymphocytes (iu/l)	44.67±6.39^{a}	43.67±10.48^{a}	42.00±2.08^{a}	47.67±9.24^{a}	54.00±5.51^{a}
Haemoglobin (iu/l)	12.20±0.85^{b}	6.02±1.62^{a}	5.62±0.42^{a}	7.46±1.39^{a}	7.66±1.03^{a}
White blood cell (iu/l)	66.71±3.41^{b}	22.0$^{1\pm}$13.68^{a}	13.16±8.10^{a}	24.95±15.60^{a}	29.73±14.62^{a}
Red blood cell (iu/l)	2.05+0.22^{a}	1.95+0.32^{a}	1.88+0.42^{a}	2.12+0.50^{ab}	2.92+0.23^{b}
Packed cell volume (%)	19.78±1.56^{a}	12.71±4.00^{a}	16.18±3.73^{a}	17.57±5.44^{a}	17.28±4.78^{a}
Mean cell volume % (MCV)	107.67±6.81^{a}	112.33±2.73^{a}	112.00±4.00^{a}	113.00±8.89^{a}	120.67±2.03^{a}
Glucose level (mm02/I)	8.10±0.62^{a}	8.27±0.62^{a}	8.60±0.09^{a}	9.07+0.45^{a}	7.57±0.67^{a}
Total protection level (g/dl)	6.80±0.20^{a}	6.73±0.29^{a}	6.87±0.09^{a}	6.47±0.33^{a}	6.63±0.33^{a}
Albumin level (g/dl)	3.50±0.31^{a}	3.53±0.17^{a}	3.77±0.35^{ab}	3.20±0.06^{a}	4.27±0.90^{b}
Globulin level (g/dl)	30.97±11.40^{a}	35.20±1.89^{a}	30.33±6.67^{a}	39.20±5.77^{a}	43.67±6.17^{a}
Aspartate transferase (iu/l)	2.60±0.57^{a}	2.53±0.38^{a}	1.84±0.06^{a}	1.87±0.08^{a}	2.27±0.42^{a}
Alanine transferase level (iu/l)	3.30±0.25^{a}	5.47±1.16^{b}	4.50±0.20^{ab}	2.57±0.33^{a}	3.17±0.18^{a}
Total bilirubin level (mg/dl)	0.77±0.010^{a}	0.60±0.10^{a}	0.68±0.08^{a}	0.62±0.05^{a}	0.06±0.09^{a}
Conjugated bilirubin level (mg/dl)	0.09±0.33^{a}	0.11±0.07^{a}	0.10±0.05^{a}	0.14±0.01^{a}	0.17±0.06^{a}
Calcium level (mg/dl)	8.57±0.87^{a}	7.73±0.12^{a}	8.13±0.59^{a}	8.13±0.33^{a}	9.43±0.72^{a}
Bicarbonate level (mmol/l)	27.33±0.88^{ab}	28.67±0.88^{b}	26.33±1.20^{ab}	24.33±1.55^{a}	26.00±1.00^{ab}
Sodium level (mmdl/l	154.63±1.90^{b}	124.93±12.95^{ab}	127.833±18.41^{ab}	112.90±1.55^{a}	140.20±5.23^{ab}
Cholesterol level (mmol/dl)	4.37±0.53^{ab}	4.47±0.38^{ab}	3.67±0.023^{a}	3.73±0.19^{a}	3.57±0.09^{a}

Data are presented as (mean ± SEM). $^{a, ab, b}$ within the same row with different superscripts declare significant differences ($P \leq 0.05$).

DISCUSSION

Similar haematological values obtained from broiler chickens reared on different bedding materials with those in the control group is indicative of the bedding materials used. According to Rath et al. (2006), RBC, WBC and PCV values in broilers were not affected, which is an agreement with present findings.

Similarly, Cetin et al. (2006) reported that supplementation of humic acid to laying hens had no effects on WBC and PCV but affected on RBC and Hb levels. However Banaszkiewicz and Drobnik (1994) reported that Hb, PCV and RBC were observed to increase in rats treated with humic acid. The inconsistency of results in the various studies might be attributed to the composition and nature of the bedding materials.

The increase of Ca and Na levels in blood serum caused by effect of different bedding materials in this studied agreed with findings of Boling et al (2001), Abdo and Zeinb (2004) which is attributed to the lowering of gastrointestinal tract pH, which increases the absorption of the minerals from the gut into the blood stream. Improvement in the utilization of calcium and sodium by organic acids supplementation which was revealed by Boling et al. (2001). Increase in blood calcium of broiler chicks fed on dietary acidifier was observed by Abdo and Zeinb (2004) . Furthermore Kishi et al. (1999) reported that dietary acetic acid prevented osteoporosis, through the reduction in bone turnover, as it enhanced intestinal Ca absorption by improving Ca solubility in ovariectomized rats.

The levels of some essential minerals (such as Ca, Al and Fe) in serum, liver and muscles increased after humate feeding (Stepchenko et al., 1991).

The result of aspartate aminotransferase and alanine aminotransferase means that broiler chickens could tolerate the effect of different bedding materials without any deleterious effects on liver functions. Abdel-Azeem et al. (2000) reported that level of aspartate aminotransferase was reduced, although alanine aminotransferase was significantly affected by bedding materials which is not in agreement with Abdel-Azeem et al.(2000) in this studied.

These findings corroborates the conclusion that when birds are reared on alternative bedding materials, their performance is equal to or worse than that of birds reared on WS (Benabde and Ayach, 1996; Grimes et al., 2002). Contradictory results have been reported that broilers performance and welfare found to be improved when the birds are reared on WS over other materials used as bedding, (Neme et al., 2000; Tasistro et al., 2007) reported significantly lower weight gained when birds were reared on wheat straw relative to WS.

Also, performance of flocks raised on rice hulls was reported to be lower when compared with WS (Garcia et al., 2010). The enhanced performance of the birds on the sharp sand over other bedding materials could be attributed to the possession of good physical properties such as fines and high absorbance capacity due to its pervious nature which allowed for easy movement of liquid and air in addition to easy drying. Sharp sand provides a suitable condition that can encourage social behavior such as sand bathing which has been reported to help reducing aggressive behavior and improves general leg health conditions, leading to better mobility and better flock welfare (Shields et al., 2004). Sharp sand also offers a high option for use as bedding material in poultry production, particularly in the area of study as it meets the most selection criteria listed by Garcia et al. (2010) such as availability, accessibility, ease of handling, cheapness, low fermentation, environmental impact, ability of avoiding compacting, amongst others. Gracia et al. (2010) had reported that sand litter was four times heavier than WS posing potential problems in handling and transportation. However, sand allows producers to rear multiple flocks while only removing small portions of litter, which could compensate for these aspects and make it a convenient bedding source (Bowes et al., 2003).

The levels of glucose for the broilers raised on the different bedding materials of this studied were below the normal levels of glucose concentration in the majority of birds which is usually in range of 11.1–27.8 mmol/L which is not in agreement of Coles et al. (1970) and Hrabčáková et al. (2014) findings may be due to the poor absorption of the feeds by broilers. They reported that glucose values that were in the normal range for pheasants. It is also documented by Lloyd and Gibs (2006) in range of 19.5-20.08 mmol/L and Nazifi et al. (2012) reported in range of 2.6 mmol/L in pheasants females.

The values of cholesterol for the broilers were within the standard range of values in domestic fowl which is 3.55 to 10.25 mmol/L reported by Sugiharto (2014). The finding of this present studies is in accordance with the findings of Sugiharto (2014). As already been published, the wide variation in cholesterol levels between species of birds may be due to the circadian rhythms, effects of diet (Harr, 2002; Villagrá et al., 2011) gender and age (Meluzzi et al., 1992). However, (Hrabčáková et al., 2014) reported that the increases in the cholesterol levels could be due to increased biosynthesis and accumulation of egg yolk in the layers, which is not peculiar with broilers.

The values for total protein content in the majority of birds fall was in range of 30–50 g/L, (Lloyd and Gibson, 2006; Nazifi et al., 2012). Significant differences in albumin and serum total protein among females and males during breeding season might be due to the egg production that can influence on concentration of the blood proteins (Schmidt et al., 2007). The serum protein levels in the birds of present studies reared on the different bedding materials were under the reported normal range which agreed with findings of Lloyd and Gibson (2006); Nazifi et al (2012). Serum total protein and albumin are directly related to protein intake and feed quality. Serum proteins are involved in the formation of immunoglobins responsible for the development of antibodies. High or low serum globulin content may implicate a better disease resistance and immune response of chickens reported by Abdel-Fattah et al. (2008) which was observed in this present studied when compared with control.

Generally, Biochemical parameters can be used as physiological indicators in birds. Their values are influenced by species, age, sex, season, geographic region, nutrition, and physiological condition (Paria, 2014). They are also indicators of the health of birds housed in cage deep liters systems. Parameters such as total protein, albumin, cholesterol, glucose, calcium, and aspartate aminotransferase are commonly measured as biochemical characteristics of poultry (Hrabčáková et al., 2014). It has been noted that significant changes in the serum biochemical parameters can be related to the both pathological and nutritional status of individual animals (Aldu-hafeez et al., 2009)

The values of examined hematological parameters were according to the reported range of broilers (Talebi et al., 2005). The measured parameters of present experiment were age dependent which are disagrees with findings of Talebi et al. (2005) in broiler chickens. The changes in Hb concentration, erythrocyte count, haematocrit level and differential leukocytes may indicate stress as reported by Hrabčáková et al. (2014), while the changes in erythrocyte, Hb and pack cell volume may reflect an alteration of energy status in chickens as reported by Karamanlis et al. (2008) and Alabi et al. (2015). Aside from the physiological and nutritional aspects, haematological variables can also be used as an indicator of health in birds, (Hrabčáková et al., 2014). Indeed, several factors have been obtained that influence on haematological variables including species, age, sex, environment, nutrition, infection and physiological conditions, (Hrabčáková et al., 2014). Leukocytes are cells of the immune system that protect the body against infections. The findings of WBC in this studied agreed with Sugiharto et al. (2014) findings due to the high numbers of leukocytes in control when compared with bedding materials. This may due to the lack of comfort leading to stress in the control. High number of leukocytes seems therefore to imply in a superior ability of chickens to respond to infections. However, this assumption may be ambiguous as high number of leukocytes may also indicate stress (Sugiharto et al., 2014).

CONCLUSION

Since, serum biochemical parameters can indicate and influence the behavior and health conditions of birds, it is important that alternative bedding materials be investigated for their influences on these parameters in order to determine if they can replace the conventionally known bedding materials such as WS that are becoming scarce in recent times. The compatibility of the values of the haematological parameters evaluated for the other bedding materials to those of WS suggest that they can successfully be utilized in the rearing of broilers without detrimental effects on the health and physiology of the birds.

From the findings of the research, rice hull, groundnut hull and sharp sand can be effectively used as alternative bedding materials to WS without detrimental effects on the physiology of broilers, particularly in the locality of Kontagora and its environs where rice and groundnut are intensively produced, and sharp sand can be sourced with ease and at cheaper cost. Rice hull, groundnut hull and sharp sand can function effectively in this regard and performance of broilers would be maintained and even improve as associated with WS. Sharp sand presents the best alternative as it is not and may not be in shortage, and does not have unnecessary competition for other uses that limit its availability as rice and groundnut hulls, which face serious competition as livestock feeds during the dry season.

DECLARATIONS

Authors contributions

Dr. Gana James. Carried out the main work, which were the blood analysis for hematological and serum biochemical parameters, compilation and discussion of the results. He has gone through the work and approved for publication. Dr. Adekojo S.A: Collected the blood samples for hematological and serum biochemical analyses. He has read through the research work and recommended the final manuscript for publication. Mr. Danwake J.G: Carried out statistical analysis of obtained results. He has read through and recommended the final manuscript for publication. Mr. Zarma Adamu: Restrained the birds for the collections of blood samples and assisted the corresponding author in the compilation and discussion of the results. He has read through and approved the final manuscript for publication. Mr. Zakari Mamman: Prepared the brooding house, feeding of the birds, daily supplied of water, administration of vaccines/medications and changed the bedding materials. He has read through and approved the final manuscript for publication.

Consent to publish

The authors have given permission for the manuscript should be published.

Competing interests

There was no conflict of interest reasons being that research results were not influenced by external factors or any scientific misconduct and was free of bias. Thus, professional judgments and objectives of the research were not harmed.

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JWPR
Journal of World's Poultry Research

2019, Scienceline Publication
J. World Poult. Res. 9(2): 59-67, June 25, 2019

Research Paper, PII: S2322455X1900008-9

The characterization of Post-Mortem Sperm of Local Chicken Cocks in Eastern Algeria

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Received: 24Apr. 2019
Accepted: 23May. 2019

ABSTRACT

The present aimed to investigate for the first time the characteristics and conservation of post-mortem sperm, obtained from epididymis and the vas deferens of 18 pairs of adult local chicken cocks from the east of Algeria (age, 12-24 months, body weight 1.50-2.53 kg). And compare the sperm quality at the epididymis and the vas deferens levels, the efficacy of two post-mortem sperm retrieval techniques, the flushing and float-out methods in the collection of local chicken cock's sperm, and the effects of conservation in situ at different temperatures (2h and 24h at 20°C and after refrigeration at 4°C for 24h). The quality was significantly higher (0.13±0.05ml vs 0.72±0.12ml) in the vas deferens compared to the epididymis, whereas spermatozoa abnormalities and chromatin quality did not differ in both the epididymis and vas deferens. No significant difference was observed between the flushing and float-out methods. Concentration (3.33±1.63 million sperm vs 1.75±0.76 million sperm), initial motility (77.50±6.89% vs 75.83±8.61%), viability (75±10.39% vs 74.67±10.15%) and abnormality (30.33±4.68% vs 30.33±4.68%), only the volume was significantly higher (0.72±0.12ml vs 0.17±0.08ml). However, the effects of conservation in situ, at 25°C and 4°C for 24h, showed a significant difference for viability and motility of the spermatozoa recovered from vas deferens, and no significant difference for abnormality and acrosome integrity. Therefore, it can be concluded that good quality semen samples can be collected from the vas deferens with the flushing method, and semen of Algerian local cocks can be preserved at 20°C for 24h.

Key words: Conservation, Eastern Algeria, Epididymis, Local cocks, Post-mortem, Vas deferens

INTRODUCTION

Actually, a plethora of research focused on the conservation of genetic resources and biodiversity (Blesbois, 2007). The importance of granting poultry genetic resources from unexpected accidents or disease outbreaks cannot be overemphasized (Sasaki et al., 2010). Local poultry breeds may cause an interesting different option to commercial lines, supplying high-quality products of great interest for local and regional markets (De Marchi et al., 2005). Poultry products, meat and eggs, obtained from native breeds show specific features (De Marchi et al., 2005; Castellini et al., 2006; Zanetti et al., 2011) which distinguish them from standard ones, moreover, breeds can be reared in outdoor free-range systems and even used to reintroduce agricultural activity in marginal rural areas. Traditionally, the creation of banks of semen doses from endangered breeds is performed using the protocols developed for commercial breeds or lines (Blesbois, 2007).

In addition to experimental lines and commercial stocks contributing to the range of chicken genetic diversity, traditional chicken breeds are still highly connected to cultural values, geographical origins and adapted to local environments; however, many of them have undergone a major decrease in their population size, thus increasing erosion of their genetic diversity (Woelders et al., 2006). The loss of valuable genes and rapid decrease in biodiversity as a result of a smaller number of selected breeds used for breeding has resulted in an urgent need to create gene banks and databanks

(Tisdell, 2003). In poultry species, semen cryopreservation is currently the most practical method for long-term storage of genetic material (Blesbois, 2007).

The most frequent method of semen collection in birds is Dorso-abdominal massage (Chelmonska et al., 2008). But it has been proven that this method of harvesting is harmful to sperm due to sperm contamination during urinary retrieval (Blanco et al., 2002). The use of the post-mortem harvest of spermatozoa by the various methods (like Swim-up and mincing), was a way of overcoming the inconveniences of the abdominal massage, which has already been found in Philippine roosters (Salting et al., 2016). Germplasm banks for endangered avian species are still being set up. Gunn et al. (2008) described and compared different techniques for post-mortem sperm collection in wild pukekos (*Porphyrio porphyrio melanotus*), although none of the cells obtained was motile. Some authors, because they did not evaluate the viability of the sperm nor the effectiveness of its extension suggested that the pukeko sperm is not mobile in the vas deferens and becoming active only after ejaculation, or that those recovered are simply dead (Gunn et al., 2008).

Sadly, the avian spermatology is not simple, which is necessary to improve sperm cryopreservation (Villaverde-Morcillo, 2016). However, sperm may also be useful to create germplasm banks of sperm from dead birds by feeding information on the physiological status of the donor used for reproduction (Kumar and Holt, 2014). Semen evaluation prior to its further processing is essential and an important prerequisite. In order to propose the devising methods for the storage of fowl spermatozoa, it is necessary to evaluate the basic quality of sperm (Łukaszewicz et al., 2008). To ensure the better cryopreservation, it is essential to appreciate the mobility, morphology and the metabolism of fresh semen (Blesbois, 2007). This study was conducted to characterize post-mortem sperm of local Algeriancocks collected from the epididymis and the vas deferens, find the suitable methods of extract the sperm and the effects of conservation in situ at different temperatures (2 hours and 24 hours at 20°C and after refrigeration at 4°C for 24 h).

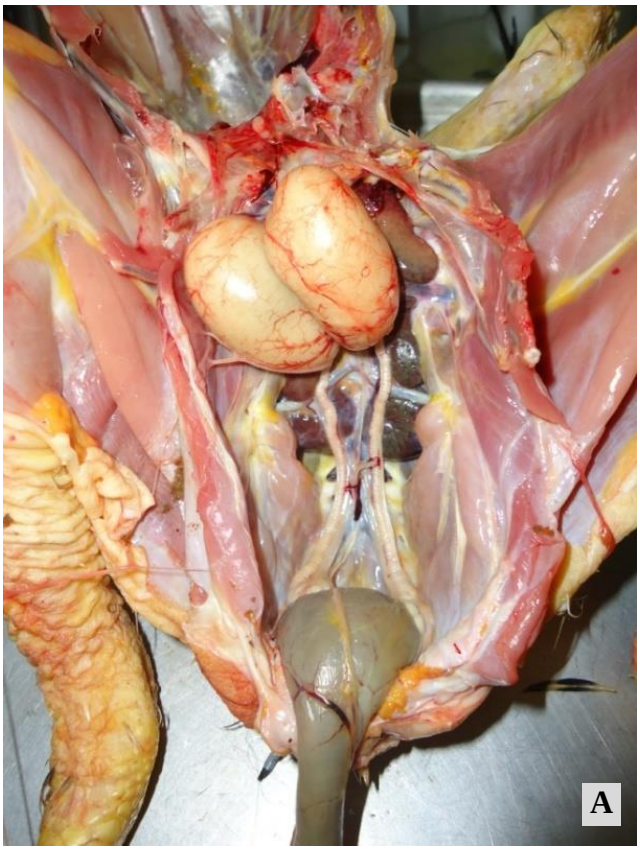
MATERIALS AND METHODS

Ethical approval

The experiment was carried out according to the national regulations on animal welfare and institutional animal ethical committee of national center for biotechnology research, Constantine, Algeria.

Semen collection and evaluation

The birds used in present investigation were local cocks from the east of Algeria (El Tarf and Constantine). 12-24 months of age and 1.50- 2.53kg live body weight were used in this experiment. Procedures were performed using sperm obtained post-mortem from the epididymis and the vas deferens of 18 pairs adult cockshumanely euthanized by cervical dislocation. Testes with their corresponding vas deferens were collected within one hour after slaughtering by opening the carcass along the midline. All the viscera were removed and the testes extracted together with the ureter and renal vasculature, thus damages to the vas deferens was avoided. The ureter and renal vasculature were carefully dissected out to minimize blood and urine contamination of the sperm sample (Figure 1). Once the epididymis and vas deferens were isolated from the testes and adjacent tissues, sperm collection was performed either by the flushing or float-out method from the vas deferens (one testis via the flushing method, and the other by the float-out method), and the epididymis employed simply by the mincing method. Following sperm collection, sperm variables were assessed before refrigeration and 24 hours at environment temperature, then after cooling in situ at 4°C for 24 hours.



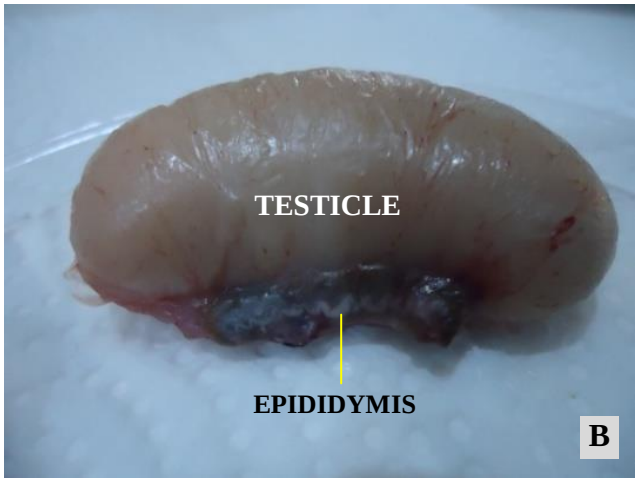


Figure 1. Anatomy of reproductive tract of adult local cocks' in Eastern Algeria(A and B)

Mincing method

The Mincing technique involves isolating the epididymis from the testis, placed in microcentrifuge tubes containing 400µl of Phosphate Buffered Saline (PBS) and were minced by using sterile surgical scissors, and then left to stand for 10-15 minutes. The upper liquid portion was transferred to another microcentrifuge tube (Salting et al., 2016).

Flushing method

Samples were obtained by injecting 1.5 ml of saline solution at 38°C into the proximal extreme of the vas deferens using a 27G needle attached to a 5 ml syringe (Figure 2). The entire volume of the saline solution and the collected sperm were placed in a sterile plastic Petri, from which they were transferred to a polystyrene tube (2 ml). The samples were then incubated at 38°C for 15 min (Villaverde-Morcillo, 2016).

Figure 2. Extraction the sperm from Vas deferens 2hours after slaughtering of local cocks' in Eastern Algeria by flushing method

Float-out method

The vas deferens was cut into 0.5 cm-long pieces which were submerged in 1.5 ml of saline solution at 38°C in a 2 ml tube. These samples were then incubated at 38°C for 15 min (Villaverde-Morcillo, 2016).

Sperm assessment

Semen volume (ml) was measured visually using a graduated collection tube. The total volume retrieved after flushing or float-out was considered as the final sperm volume to calculate sperm concentration. The concentration of spermatozoa (million per millilitre) was determined by using a calibrated spectrophotometer. The wavelength is set at 650 nm. The addition of 3 ml of a 2.9% sodium citrate solution (PH 7.0). A drop of semen (10 µl) was placed on a clean, pre-warmed (41°C) microscope slide using a micropipette. A clean cover slip was placed gently to avoid air bubble formation. Sperm motility was demonstrated by the progressive wavy movement of sperm cells. Briefly, 20 µl aliquot from sperm suspension was stained by 20 µl Eosin–Nigrosine dye. Then, smears were prepared on a warm slide and the stain spread with a second slide. Twenty hundred sperm were counted under phase-contrast at 1000× magnification. Acrosomal integrity was studied using Giemsa staining (Watson, 1975).

Acidic aniline blue staining

Sperm chromatin condensation was disturbed (NCI) when lysine-rich somatic histones were not sufficiently substituted by arginine-rich protamines during spermiogenesis. Histone-containing spermatozoa can be visualized using acidic aniline blue staining solution, which reacts with lysine residues of persisting histones (Shanmugam et al., 2016). The percentage of sperm heads partially or entirely stained dark blue was examined according to the procedures described by Boitrelle (2004).

Statistical analysis

Data were analyzed using SPSS software (version 19). The effect of the level of epididymis or the vas deferens, two sampling techniques (Float-out and flushing methods), and the time of conservation of the gonads (2h, 24h at 25°C and 24h at 4°C) on the sperm quality were compared by the one-way ANOVA test followed by the Student Newman-Keuls multiple comparison test. Mean values were considered significantly ($P<0.05$), data are expressed as values ± Standard Deviation (SD).

RESULTS AND DISCUSSION

Various parameters of spermatozoa collected from the epididymis and the vas deferens

Mean values of volume, initial motility, concentration, viability, abnormality and aniline blue negative spermatozoa (chromatin quality) were presented in table 1. The volume, concentration, motility and viability of spermatozoa were significantly (P<0.05) influenced by the level of sampling. They were significantly higher (P<0.001) in the vas deferens compared to the epididymis. While spermatozoa abnormalities and chromatin quality do not differ in the epididymis and the vas deferens (P>0.5).

Table 1. Comparison of sperm quality at the level of the epididymis and the vas deferens from local cocksin Eastern Algeria

Items	Epididymis	Vas deferens	P
N	12	12	value
Volume (ml)	0,13±0,05	0,72±0,12	***
Concentration (10^6)	250±137,84	5333,33±1861,90	***
Initial motility (%)	65,83±4,92	80,83±7,36	**
Viability (%)	53,17±9,13	86,50±2,59	***
Abnormality (%)	21,17±3,37	20,67±13,43	ns
Aniline blue negative (%)	78,17±4,75	76,17±6,62	ns

Nombre (n), no significant difference (ns)** P<0.01 and *** P<0.001

The results confirmed the findings of Munro (1935 and 1938), which reported that sperm motility increased somewhat in the rudimentary epididymis of birds, but the maximum motility was seen in the vas deferens. Avian spermatozoa are not motile in the testes, although they have been found to be capable of fertilizing eggs when taken from the testes. Because spermatozoa are mature at this stage (Jones and Lin,1993). The role of epididymal region of the rooster reproductive tract is the production of fertile sperm. But testicular effluent is concentrated and surface of sperm cells altered by passing through the excurrent ducts of the epididymis (Clulow and Jones, 1988). These changes presumably revealed base on differences in the function of sperm recovered from the testis and deferent duct (Howarth, 1983). Clulow et al. (1988) reported that before the sperm stored for an extended period in the ductus deferens, these ducts resorb nearly 90% of the testicular plasma outputwhen the sperm traverse this region of the male reproductive tract rapidly. The assessment of the basic quality of sperm is indispensable for the purpose of devising methods for the storage of fowl spermatozoa *in vitro* for long periods of time (Łukaszewicz et al., 2008). Salting et al. (2016) expressed for choosing semen at good quality level, colour must be free of any contamination caused by cloacal products, the volume greater than 0.3 ml, sperm mobility greater than 65%, and sperm concentration greater than 1×10^9 sperm cells /ml. This study addressed the contribution of the epididymis to the attainment of functional maturity of local cocks' spermatozoa. In contrast to their mammalian counterparts, where it is well established that spermatozoa undergo a number of important changes in the epididymis, present study obtained few changes in the functional profile of indigenous cocks spermatozoa at epididymis. These results are in accordance with the fundamental differences in the anatomy and biology of the avian and mammalian reproductive systems (Jones et al., 2003). Accordingly, the vas deferens (leading from the testes to the cloaca) should be the ideal site to harvest mature sperm (Gunn et al., 2008). The results of the study proved that good quality semen samples can be collected from the vas deferens of local cocks with parameters comparable to ejaculated sperm of some strains. Samples collected from the vas deferens can be considered as a better source for future rooster semen studies regarding the development of methods of chicken sperm cryopreservation and, eventually, in cryopreservation per of valuable genetic resources. Because, the basis of sperm can be a suitable indicator in AI practices (Mavi et al., 2017).

Spermatozoa retrieved of the vas deferens by float-out and flushing methods

This study was the first comparing two post-mortem sperm retrieval techniques of cocks' sperm from the vas deferens of Algerian local cocks, retrieved by flushing and float-out methods. Table 2 presents the comparison of motility, viability, concentration and percent abnormality observed in the samples retrieved using flushing method and float-out method. It indicates that the volume of sperm (ml) retrieved by flushing was 0.72±0.12, but with the float–out method, it was 0.17±0.08. The difference was significantly higher (P<0.01), the volume harvested by float-out method was lower than Aseel (0.36 ± 0.08) and Kadaknath (0.30 ± 0.06 ml) breed (Mavi et al., 2017), and of Venda cockerels (0.3ml) (Mphaphathi et al., 2016), obtained by abdominal massage technique.

The concentrations of sperm (billion cells per millilitre) retrieved by flushing and float-out methods found were 3.33±1.63 and 1.75±0.76, respectively. The t-test did not obtain significant (P>0.05) differences between the concentrations of samples retrieved using

flushing and float-out methods. In the present study, the methods of collecting caused low concentration than harvested by abdominal massage technique (6.8×10^9/ml) from Venda chicken (Mphaphathi et al., 2016). Siudzinska and Lukaszewicz (2008) reported an average sperm concentration of 4.7×10^9/ml in White Crested Black Polish cocks and 4.2×10^9/ml in the Black Minorcas breeds. Tuncer et al. (2008) and Obidi et al. (2008) reported sperm concentrations at levels of 2.4×10^9/ml in Gerze cocks and 3.6×10^9/ml in Shikabrown cocks similar to present findings.

The study of Villaverde-Morcillo et al. (2016) demonstrated the interaction between recovery methods and sperm diluents did not exert significant influence on sperm variables. Irrespective of the extender used, significantly more sperm was retrieved by the flushing method than by the float-out method (5965754 million sperm per vas deferens 3410876 million sperm per vas deferens, P< 0.05), indeed, the number was similar to that recorded for the ejaculated sperm (6303782 million sperm). The float-out was among the easiest techniques to perform, and although a low amount of sperm was obtained, there was a much lower incidence of extraneous cells in the sample, making it easier to find sperm. This technique was also the least likely to damage sperm and has the greatest likelihood for obtaining mature sperm, as only those sperm that was free in the lumen was included in the solution (Gunn et al., 2008). According to Salting et al. (2016), the concentrations of samples retrieved from the excurrent ducts, regardless of the retrieval method used, were found at the normal range in ejaculated samples.

Table 2. Comparison of two post-mortem sperm retrieval techniques of local cocks' in Eastern Algeria

Items	Float-out method	Flushing method	P value
N	6	6	
Volume (ml)	0.17±0.08	0.72±0.12	***
Concentration (10^6)	1.75±0.76	3.33±1.63	ns
Initial motility (%)	75,83±8.61	77.50±6.89	ns
Viability (%)	74.67±10.15	75.00±10.39	ns
Abnormality (%)	30.33±4.68	30.33±4.68	ns

Number (n),*** p<0.001 and no significant difference (ns)

Results impliedthat concentrations of spermatozoa obtained from the excurrent ducts were comparable with the concentrations of spermatozoa in samples obtained via dorso-abdominal massage method. The normal concentration of spermatozoa varies between individuals of the same species (Glover, 2012), it has been estimated between 1.7 and 3.5 billion spermatozoa per millilitre of chicken (Hicks, 1992), these numbers are consistent with the results found in this study.

As shown on the table 2, the motility of samples retrieved using the float-out was 75.83±8.61% and 77.50±6.89% for flushing method. T-test revealedno significant difference (P>0.05) between the motility of the float-out and the flushing retrieved spermatozoa indicating that the retrieval method did not significantly affect or improved the resulting percentage of motile and progressive spermatozoa in the sample collected. Which was higher (65.5±4.97%) than findings obtained by Salting et al. (2016) (swim-up method) and 65±4.71% (mincing method). Higher sperm motility has been observed in freshly ejaculated semen of cocks (66.67±6.67% to 86.5±0.78%) as reported in earlier studies (Almahdi and Ondho, 2014; Churchil et al., 2014). The flushing method affected more negatively than float out method on a percentage of sperm showing progressive motility (Villaverde-Morcillo et al., 2016). The float-out method, which has been used with the pukeko (Gunn et al., 2008) and mice (Mohammadzadeh et al., 2011), that was easier to perform and faster than the flushing method, but the sperm samples were usually contaminated with blood cells and urates, adversely affecting the long-term storage of the sperm, because the presence of white blood cells may increase the production of reactive oxygen species, according to Aitken and Bennetts (2006). Unlike the flushing method reduced contamination by blood cells and urates, according to Villaverde-Morcillo et al., (2016), and have shown that the flushing method was more effective. However, recovering roughly the same number of sperm is in a single vas deferens as in ejaculates.

Percentage of live spermatozoa in the sample retrieved by the float-out was found at 74.67±10.15% whereas, for flushing methods retrieved spermatozoa, viability was 75.00±10.39%. Statistical analysis shows no significant (P>0.05) difference between the viability of the float-out and flushing methods retrieved spermatozoa (Figure 3). Results indicated that the retrieval method had no effect on the resulting percentage of viable sperm in the sample. The viability of samples obtained in this study was lower than that obtained of the excurrent ducts by Salting et al. (2016), 85.65 ±7.88% (float-out method) and 84.20±8.79 % (mincing method), and to the results of these earlier studies (Tabatabaei et al., 2009; Tarif et al., 2013; Churchil et al., 2014). The percentage of live spermatozoa in the excretory ducts would be similar to that of ejaculated samples in domestic poultry because the spermatozoa mature and become fully fertile in the vas

deferens. It was also in the vas deferens where sperm produced by the testicles were stored until the rooster's companion (Salting et al., 2016). These differences may be explained by breed, location, nutrition, age and climate differences because the sperm parameters can vary depending on these factors (Das et al., 2016; Kuzlu and Taskin, 2017; Mavi et al., 2018).

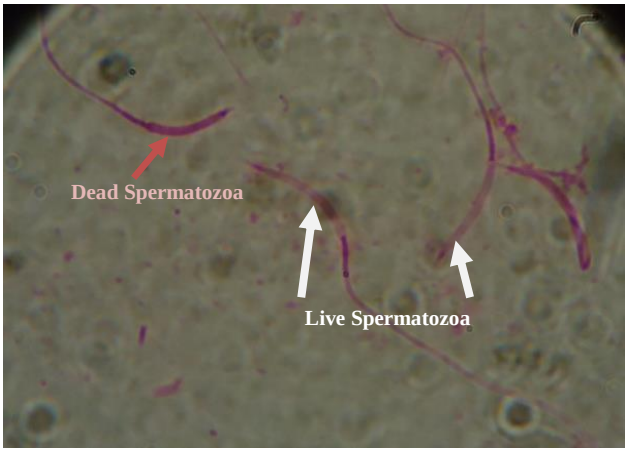


Figure 3. Assessment of sperm viability at local cocks' in Eastern Algeriawith eosin nigrosin (white: live spermatozoa, pink: dead spermatozoa)

As presented in table 2, the percentage of morphologically abnormal spermatozoa in samples retrieved by float-out was 30.33±4.68 % while 30.33±4.68 % abnormality was observed in flushing retrieved samples too. No difference was found between the percentages of abnormality of spermatozoa retrieved using float-out and flushing method. The percentages of abnormal spermatozoa observed in this study from vas deferens collected by both techniques (float-out and flushing method) were higher than those observed from ejaculated spermatozoa of various strains in studies conducted by Tarif et al. (2013) and Churchil et al. (2014), which ranged from 4.52±10% to 23.33±6.67% and lower than reported by Tabatabaei et al. (2009) in exotic Ross-308 and indigenous chicken in Iran ranged from 41.04±10.19% to 44.1±0.26%, respectively. Although this may be attributed to the genetic or intrinsic factors of Algerian local cocks, unfortunately, pieces of literature regarding the characterization of semen ejaculates of local breeds or strains were lacking. In this study, the isolation of spermatozoa from vas deferens of Algerian indigenous cocks was made possible and the difference between the quality of spermatozoa retrieved by float-out and flushing method was also studied. The flushing method allows for obtaining higher numbers of sperm. This technique can be easily performed on wild and domestic birds found dead or critically injured, which can be interesting in breeding and conservation programs. Many studies have shown that different factors include species, age, weight and others play significant roles in the differences in semen parameters (Salting et al., 2016)

A similar study of the harvest and storage specific to the specific strain for Algerian local chickens was recommended. In addition, other in vitro tests and subsequent correlation with cryo survivability have been suggested.

The effects of conservation the vas deferens at different temperatures

The effect of temperature on sperm quality during storage in situ, 2h after slaughter (20°C), 24h at 20°C and 24h at 4°C is shown in table 3. In this study, the quality of sperm samples obtained from vas deferens of local cocks, stored 24 h at 4ºC and at 20°C, after slaughtering was also determined. Holding spermatozoa at temperatures below body temperature prior cryopreservation was commonly done in many species with acceptable results. Since refrigeration devices were widely available, testes of animals could be kept at 4°C during its storage.

Table 3. Effects of conservation the vas deferens at different temperatures of localcocks' in Eastern Algeria

	2h	24h 4°C	24h 20°C	P
N	**6**	**3**	**3**	**value**
Initial motility (%)	81,00±2,19	55,00±5,00	83,00±7,21	***
Viability (%)	75,50±11,11	48,33±3,51	81,00±7,21	**
Abnormality (%)	32,00±4,65	32,00±4,36	29,67±2,52	ns
Acrosome integrity (%)	75,67±10,61	75,33±2,52	81,67±7,51	ns

Number (n), hours (h)** p<0.01 and *** p<0.001, no significant difference (ns)

In the present study, the motility of spermatozoa collected from the vas deferens, and conserved at 24 h in 4°C (55±5%), while, at 20°C (83±7.21%), were significantly higher (P<0.01),in comparison to that of control spermatozoa from non-refrigerated vas deferens (immediatelyafter slaughtering) (81±2.19%) and after conservation at 4°C. Unlike in present study, the motility decreases significantly (P<0.01) during *in-vitro* storage and after 24 h at 20°C, was seen 30.6 %. Semen samples stored at 5°C revealed an overall motility more than 50 % after 24 h (Mphaphathi et al., 2016), semen samples of the Venda breed record the motility of 55 % after 24h *in vitro*storage at 5°C while, semen samples stored at 25°C, caused a drastic reduction to 30 % after 24 h (Mphaphathi

et al., 2016). According to Clarke et al. (1982), cock semen stored at 41°C drastically decreased the sperm motility compared to that stored at 5, 15 and 25 °C. There were also significant differences concerning, the viability of the spermatozoa recovered from vas deferens stored at 4°C and at 20°C (48.33±3.51% and 81±7.21%). In contrast, abnormal spermatozoa (32±4.36%, 29.67±2.52%) and acrosome integrity (75.33±2.52%, 81.67±7.51%) did not obtain significant differences.

CONCLUSION

It was concluded that good quality of spermatozoa could be collected from vas deferens, and it is possible to preserve semen of Algerian local cocks at 4°C and 20°C for 24 hours. It is obtained that high quality semen from vas deferens of local cocks can be purposed for a further study on cryopreservation in order to create a gene bank of the local Algerian chicken cocks

DECLARATIONS

Acknowledgments

The authors are thankful to Mr Seidira Chamssedine, from biotechnology animal lab (national center for biotechnology research -CRBt), for sample collection.

Author's contribution

Bouzebda Afri Farida and Bouzebda Zoubir were mainly responsible for the tabulation of experimental data and article writing under the supervision of Majdoub Sara, Djaout Amel and Smadi Moustapha Adnane.

Consent to publish

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

Competing interests

The authors declare that they have no conflict of interest with respect to the research, authorship, and/or publications of this article.

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JWPR
Journal of World's Poultry Research

2019, Scienceline Publication
J. World's Poult. Res. 9(2): 68-77, June 25, 2019

Research Paper, PII: S2322455X1900009-9

Recent Update: Effects of Aflatoxin in Broiler Chickens

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Received: 25 Apr. 2019
Accepted: 23 May. 2019

ABSTRACT

Aflatoxin is a worldwide problem in poultry industries as it is known to contaminate poultry feed. Aflatoxin induces stress and increases mortality rate during infection in poultry, especially broiler chickens. The objectives of this study was to observe the pathological effects due to aflatoxicosis in broiler chickens. A total of 120 chickens were divided into four groups, group A fed with a basal diet without aflatoxin contamination, group B with aflatoxin (> 1 ppb < 50 ppb), group C with aflatoxin (> 51 ppb < 100 ppb), group D with aflatoxin (> 101 ppb < 150 ppb). The data were collected and analysed on day 7, 14, and 21. The results showed that diet with high aflatoxin contamination in group D impaired the physical and laboratory performances of the chickens, increased the risk of residue in the poultry's final products. The varying doses of aflatoxin contamination in the chicken feed causes the problems on the broiler chickens with different level of severity.

Key words: Aflatoxin, Antibody, Broiler, Haematology, Immunohistochemistry, Residue

INTRODUCTION

Aflatoxin is the most prevalent mycotoxin synthesised by *Aspergillus flavus*, *Aspergillus parasiticus*, and *Aspergillus nomius* in the ideal temperature and humidity (Morrison et al., 2017). Prolonged storage of the chicken's feed creates a high synthesis of the aflatoxin (Sarma et al., 2017). Aflatoxin induces severe cellular defects and carcinogenesis (Kim et al., 2016). The disease is caused by aflatoxin known as aflatoxicosis (Wogan et al., 2012). The prevalence of aflatoxicosis is not limited to some limited geographical area. The clinical manifestations of aflatoxicosis are lethargy, anorexia, lower growth rate, microbial stress, economic losses and toxicity (Sarma et al., 2017). The aflatoxin found as a residue on the poultry's final product (Denli et al., 2009), and it represents the port of entry of aflatoxin exposure in human (Manafi et al., 2014).

Mostly, the previous study has shown the effects of a single type of aflatoxin such as Aflatoxin B1 (AFB1) (Bintvihok and Kositcharoenkul, 2006). AFB1 is commonly reported because it has the highest toxicity (Mughal et al., 2017). However, poultry aflatoxicosis naturally is caused by several types of toxins, unlimited to AFB1 and it may be with varying doses. Both of doses (low and high) and types of aflatoxin exposes to human and animal can causes several impacts (Qureshi et al., 2015). The differing types and amount of synthesised aflatoxin in one sample have defined as the total aflatoxin (Kamala et al., 2018). It is necessary to explore profoundly toward the impacts of the aflatoxin contamination in the poultry. This study aimed to explore the impacts of natural contamination of aflatoxin in the chicken's feed.

MATERIALS AND METHODS

Ethical approval

This study was approved by the ethical clearance committee of the faculty of veterinary medicine, university of Gadjah Mada, with license number 0010 / EC – FKH / Int / 2017.

Experimental animals and design

The experiment was performed in the integrated laboratory, faculty of health, the university of Muhammadiyah Sidoarjo from November 2017 until April 2018. All the experimental procedure followed the federal guidelines for the care and use of laboratory animal. 120 of one-day-old chickens of broiler strain Cobbwere randomly divided into four groups. Group A was control

group that fed a diet with aflatoxin undetectable, group B was fed a diet naturally contaminated with aflatoxin (> 1 ppb < 50 ppb), group C with aflatoxin (> 51 ppb < 100 ppb) and group D with aflatoxin (> 101 ppb < 150 ppb). The chickens were treated in 24 hours light schedule with decreasing of its intensity after 16 hours per day, 30 ^{o} C temperature, and 65 % humidity for 21 days. The water and feed were given *ad libitum*. The chickens were vaccinated by Newcastle Disease (ND) and Infectious Bursal Disease (IBD) vaccine on day three, and Avian Influenza (AI) vaccine on day five.

Diet

The chickens were fed with a broiler starter diet contained 23% of crude protein and 3200 kcal metabolizable energy. The total aflatoxin of chicken feed was routinely tested by Enzyme–Linked Immunosorbent Assays (ELISA). The chicken feed was incubated based on the following method that was indicated by the previous study (Prakoso et al., 2018). The total aflatoxin was measured each day during the study with a triple of replication. The observation results of aflatoxin levels are reported in table 1.

Table 1. Total aflatoxin level (ppb) of feed of broiler chickens from day 1 until day 21

Groups / Day	A	B	C	D
1	0	22.57	74.85	120.56
2	0	49.96	79.21	137.84
3	0	28.15	94.56	141.25
4	0	36.22	78.55	149.03
5	0	36.36	90.04	115.94
6	0	29.45	88.48	140.18
7	0	46.81	94.23	139.44
8	0	40.52	80.00	135.21
9	0	28.19	68.29	128.68
10	0	25.44	78.86	130.65
11	0	28.91	95.52	140.78
12	0	30.15	89.01	145.24
13	0	33.47	96.66	136.22
14	0	38.32	77.18	144.68
15	0	29.01	86.25	133.00
16	0	40.11	79.18	148.07
17	0	43.78	90.09	136.88
18	0	39.18	88.87	140.15
19	0	44.83	89.21	129.38
20	0	44.05	90.03	148.55
21	0	47.18	93.05	140.00
Mean ± SD	0	36.32± 8.02	85.81±7.83	137.22±8.64

SD = standard of deviation.

Body weight, feed intake and feed conversion rate

The chickens were weighed using the body weight scale on day 7, 14 and 21 during the treatments. Following the weight recording, the chicken's FI and FCR were measured using the formulae, FCR = feed intake (g)/ body weight (g).

Sample collection

10 chickens from each group were euthanised by cervical replacement on day 7, 14 and 21. The chicken's body weight was measured, and the blood and serum samples were collected before the euthanasia. The liver, kidney, spleen, Bursa of Fabricius (BF) and thymus were weighed using scale and expressed as the Relative Organ Weight (ROW). It was measured using the formulae, ROW = organ weight (g)/ body weight (100 g). After the measurement, those organs were divided into two part. The first parts were stored in the sterile plastic and saved in the refrigerator for ELISA test against total aflatoxin. The second parts were stored in 10% neutral buffer formalin for immuno-histopathological examination.

Histopathology and immunohistochemistry

The broiler chicken's organs (liver, kidney, spleen, BF and thymus) were dehydrated using the graded ethanol and xylene, embedded and blocked in the liquid paraffin. The tissue samples were prepared into 5µm diameter with duplication. The first sections were mounted on the glass slide for histopathology. The second section of spleen, BF and thymus were mounted on the slide coated with poly-lysine for immunohistochemistry. The histopathology was performed using the Hematoxylin and Eosin (H&E) staining and immunohistochemistry was done using antibody anti-CD3+, CD4+, and CD8+. The morphometry of H&E staining was performed for the organs of broiler chickens on day 7, 14, and 21 by a single pathologist. All the observed histopathological changes were reported. The immunohistochemical slides of the broiler chicken's organs were analysed on day 21 using the scoring system from 0 to 4 as follow: absence = 0, minimal = 1, mild = 2, moderate = 3 and severe = 4.

Haematological, heterophil phagocytic activity, average phagocytic bacteria, and phagocytic index

The blood samples were analysed by the routine haematological test following the standard laboratory procedure. The blood parameters contained erythrocytes total/ Red Blood Cells (RBC), Packed Cells Volume (PCV), Haemoglobin (Hb), Mean Corpuscular Volume (MCV), Mean Corpuscular Haemoglobin (MCH), Mean

Corpuscular Haemoglobin Concentration (MCHC), leucocytes total/ White Blood Cells (WBC) and differential count of leucocytes. The Heterophil Phagocytic Activity (HPA) against *Staphylococcus aureus* (ATCC® BAA-1690) was measured and analysed following the previous study (Sornplang et al., 2015). The Phagocytic Index (PI) was measured using the formula, PI = [percentage of heterophils containing bacteria) × (the Average number of Phagocytic Bacteria (APB) per ingesting heterophils] × 100.

Titre antibody measurement

The serum samples are tested by Haemagglutination Inhibition (HI) test against titre antibody of ND, IB, and AI following the standard procedure. The HI test results reported as the Geometric Mean Titre (GMT).

Enzyme-linked immunosorbent assays

The ELISA test was performed to analyse the residue of aflatoxin level in the broiler chicken's organ (liver, kidney, spleen, BF, thymus, skin and muscle). The organ samples were weighed and about 2gr, extracted, centrifuged then the aliquots reacted with peroxidase conjugate and antibody solution against aflatoxin. The samples were incubated for 30 minutes, washed, and added the stop reagent to end the further reaction. The absorbance was measured with ELISA reader using 450 nm wavelength using the standard curve.

Data analysis

The data of the chicken body weight, FI, FCR, the relative weight of the organ, haematology, HPA, APB, PI, GMT, immunohistochemistry and aflatoxin residue were analysed by SPSS (version 16) using two-way ANOVA and post hoc tests in significance level of 0.05. However, the histopathological data were analysed by using Kruskal – Wallis and Mann Whitney U tests.

RESULTS AND DISCUSSION

The results showed that contamination of the aflatoxin in the chicken's feed affected the muscle mass formation. It is approved by the significantly different regarding chicken's body weight ($P<0.05$). Those effects are caused by decreasing of FI in chickens of the group D that treated with high aflatoxins ($P<0.05$). FI is an indicator of the broiler industry used to determine the chicken's productivity. Commonly, the FI of the broiler chicken is increasing when the broiler has treated in suitable milieu including the humidity, temperature and feeds. In this case, the decrease of FI was promoted by stress and immune-depression of broiler during aflatoxin exposure. Moreover, it can be elucidated using the FCR as a further indicator. The highest FCR was indicated in group D ($P<0.05$) compared to the other groups. This finding approved that aflatoxin has impaired the chicken's metabolism through the disturbance of gut and liver activity (Zuidhof et al., 2014). It is supported by the significant gross histopathological changes of liver, kidney, spleen, BF, and thymus in group C and D with high exposure doses of aflatoxin ($P< 0.05$) (Table 2).

Gross pathological change on the visceral organ decreases cellular absorption and metabolism. Aflatoxin increases the relative weight of the liver and kidney due to the cellular defect. The liver has a role in detoxification and kidney in filtration and excretion (Matur et al., 2010). Every secondary metabolite of toxins was metabolised in the liver and stored in its cytoplasm. The aflatoxin impairs the equilibrium of the endogenous antioxidant in the cellular biological system and causes the increasing level of the oxidative stress (Yaman et al., 2016).

The oxidative stress accumulates in the hepatocytes,induce inflammation and cellular necrosis (Baptista et al., 2008). Those cellular effects are shown by liver enlargement macroscopically. The increase of liver relative weight in present study is supported by the histopathological finding which had been shown the severe inflammation in both perilobular and interlobular area, degeneration and bile duct proliferation ($P<0.05$) (Table 3). The liver hepatocytes play an essential role in the detoxification of the toxin and xenobiotics (Colakoglu and Donmez, 2012). High accumulation of the toxin promotes the degeneration (Figure 1a) that is induced by the oxidative stress and impairment of cellular metabolism (Guo et al., 2013). Chronic oxidative stress is aggravated by prolonged inflammation in the liver (Figure 1b), and it caused severe necrosis (Figure 1c). The bile duct is proliferating as the compensatory effects during toxin excretion from the liver (Figure 1d). Those histopathological changes are observed massively in group D (Figure 1a-1d).

The similar consequences due to aflatoxicosis are illustrated by the histopathological finding in the kidney ($P<0.05$) (Table 3). As the main excretory organ, the kidney plays an essential role in filtering blood and removing waste products. In this study, the imbalance of kidney function is demonstrated in both macroscopic and microscopical examination. Further, the tubular degeneration is observed on the group treated with high aflatoxin contamination (Figure 2a).

Table 2. The effects of aflatoxin exposure on the broiler chicken's body weight, feed intake, feed conversion rate and relative weight of the organs at day 7, 14 and 21

Parameters		Day	Groups			
			A	B	C	D
Body weight (g)		7	120.24 ± 2.11[a]	120.00 ± 1.21[a]	119.30 ± 1.55[a]	116.32 ± 2.36[b]
		14	338.09 ± 10.89[a]	333.85 ± 5.03[a]	328.74 ± 7.46[a]	317.50 ± 8.33[b]
		21	686.21 ± 11.36[a]	684.62 ± 11.82[a]	674.56 ± 12.08[a]	641.09 ± 27.75[b]
Feed intake (gram)		7	175.90 ± 2.96[a]	171.50 ± 1.64[a]	171.10 ± 1.52[a]	165.20 ± 3.48[b]
		14	567.90 ± 5.19[a]	560.50 ± 7.21[a]	555.60 ± 5.77[a]	548.10 ± 9.19[b]
		21	1228.37 ± 10.28[a]	1219.70 ± 16.64[a]	1221.23 ± 18.07[a]	1204.50 ± 20.56[b]
Feed conversion rate		7	1.46 ± 0.03[a]	1.42 ± 0.01[a]	1.43 ± 0.02[a]	1.42 ± 0.04[b]
		14	1.68 ± 0.05[a]	1.67 ± 0.03[a]	1.69 ± 0.03[a]	1.72 ± 0.03[b]
		21	1.79 ± 0.03[a]	1.78 ± 0.03[a]	1.81 ± 0.04[a]	1.88 ± 0.09[b]
Relative Weight of Organ (Organ Weight/ 100g Live Body Weight)	Liver	7	2.28 ± 0.15[a]	2.29 ± 0.14[a]	2.34 ± 0.11[a]	2.60 ± 0.34[b]
		14	2.26 ± 0.12[a]	2.25 ± 0.15[a]	2.30 ± 0.13[a]	2.45 ± 0.17[b]
		21	2.34 ± 0.15[a]	2.37 ± 0.17[a]	2.42 ± 0.10[a]	2.53 ± 0.10[b]
	Kidney	7	0.62 ± 0.03[a]	0.64 ± 0.05[a]	0.73 ± 0.04[b]	0.78 ± 0.03[c]
		14	0.60 ± 0.03[a]	0.60 ± 0.03[a]	0.70 ± 0.03[b]	0.79 ± 0.05[c]
		21	0.61 ± 0.04[a]	0.62 ± 0.04[a]	0.79 ± 0.03[b]	0.81 ± 0.02[c]
	Spleen	7	0.13 ± 0.02[a]	0.13 ± 0.02[a]	0.11 ± 0.01[b]	0.10 ± 0.01[c]
		14	0.14 ± 0.02[a]	0.15 ± 0.02[a]	0.14 ± 0.01[b]	0.12 ± 0.01[c]
		21	0.13 ± 0.01[a]	0.14 ± 0.01[a]	0.11 ± 0.01[b]	0.10 ± 0.01[c]
	Bursa of Fabricius	7	0.23 ± 0.02[a]	0.22 ± 0.01[a]	0.22 ± 0[a]	0.20 ± 0.01[b]
		14	0.23 ± 0.01[a]	0.23 ± 0.01[a]	0.22 ± 0.01[a]	0.22 ± 0.02[b]
		21	0.25 ± 0.02[a]	0.23 ± 0.01[a]	0.23 ± 0.01[a]	0.21 ± 0.01[b]
	Thymus	7	0.27 ± 0.01[a]	0.26 ± 0[b]	0.25 ± 0.01[b]	0.23 ± 0.01[c]
		14	0.27 ± 0.02[a]	0.26 ± 0.01[b]	0.24 ± 0.01[b]	0.23 ± 0.03[c]
		21	0.26 ± 0.02[a]	0.26 ± 0.02[b]	0.25 ± 0.01[b]	0.22 ± 0.01[c]

[a, b, c] the different superscript on the same row showed significantly different values ($P < 0.05$)

Table 3. The effects of aflatoxin exposure on the histopathological change of broiler chicken's organs

Organs	Histopathological Change	Day	Groups			
			A	B	C	D
Liver	Necrosis	7	0/10[a]	0/10[a]	2/10[b]	3/10[c]
		14	0/10[a]	1/10[a]	4/10[b]	6/10[c]
		21	2/10[a]	0/10[a]	6/10[b]	10/10[c]
	Fatty degeneration	7	2/10[a]	2/10[a]	3/10[b]	3/10[c]
		14	0/10[a]	1/10[a]	6/10[b]	10/10[c]
		21	2/10[a]	1/10[a]	8/10[b]	10/10[c]
	Hydropic degeneration	7	0/10[a]	0/10[a]	0/10[a]	1/10[a]
		14	0/10[a]	1/10[a]	1/10[a]	0/10[a]
		21	0/10[a]	0/10[a]	1/10[a]	2/10[a]
	Perilobular inflammation	7	0/10[a]	0/10[a]	1/10[b]	2/10[c]
		14	0/10[a]	1/10[a]	3/10[b]	4/10[c]
		21	2/10[a]	1/10[a]	3/10[b]	8/10[c]
	Interlobular inflammation	7	1/10[a]	1/10[a]	2/10[b]	3/10[c]
		14	0/10[a]	1/10[a]	2/10[b]	7/10[c]
		21	2/10[a]	1/10[a]	3/10[b]	6/10[c]
	Bile duct proliferation	7	0/10[a]	1/10[a]	1/10[b]	5/10[c]
		14	2/10[a]	2/10[a]	6/10[b]	7/10[c]
		21	0/10[a]	3/10[a]	6/10[b]	10/10[c]
Kidney	Tubular degeneration	7	0/10[a]	1/10[a]	1/10[a]	4/10[b]
		14	0/10[a]	2/10[a]	2/10[a]	2/10[b]
		21	1/10[a]	2/10[a]	1/10[a]	6/10[b]
	Inflammation	7	0/10[a]	0/10[a]	0/10[a]	3/10[b]
		14	0/10[a]	2/10[a]	2/10[a]	3/10[b]
		21	0/10[a]	0/20[a]	4/10[a]	5/10[b]
Spleen	Lymphoid depletion	7	0/10[a]	0/10[a]	2/10[b]	3/10[c]
		14	0/10[a]	2/10[a]	3/10[b]	4/10[c]
		21	2/10[a]	1/10[a]	3/10[b]	8/10[c]
Bursa of Fabricius	Lymphoid depletion	7	1/10[a]	2/10[a]	2/10[a]	5/10[b]
		14	2/10[a]	1/10[a]	4/10[a]	9/10[b]
		21	2/10[a]	3/10[a]	3/10[a]	10/10[b]
Thymus	Cortical depletion	7	0/10[a]	0/10[a]	2/10[b]	2/10[c]
		14	0/10[a]	2/10[a]	4/10[b]	4/10[c]
		21	1/10[a]	0/10[a]	3/10[b]	6/10[c]

[a, b, c] the different superscript on the same row showed significantly different values ($P < 0.05$).

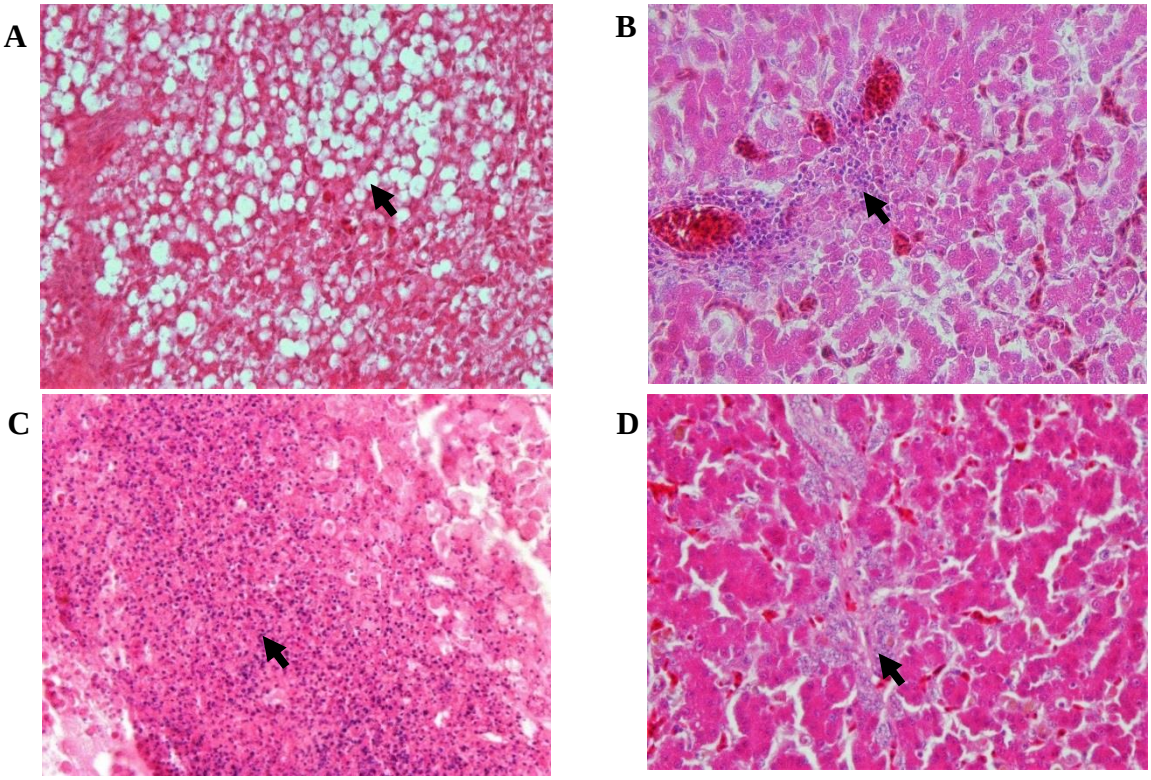

Figure 1. Effects of the aflatoxin on liver histopathology of the broiler chickens. Fatty degeneration (**A**), lymphocytic inflammation (**B**), severe necrosis (**C**), and bile duct proliferation (**D**) of the liver due to the high exposure of aflatoxin (> 101 ppb < 150 ppb) in on day 21. All histopathological changes are marked by the arrow. H&E, 100× (A, C), 1000× (B, D).

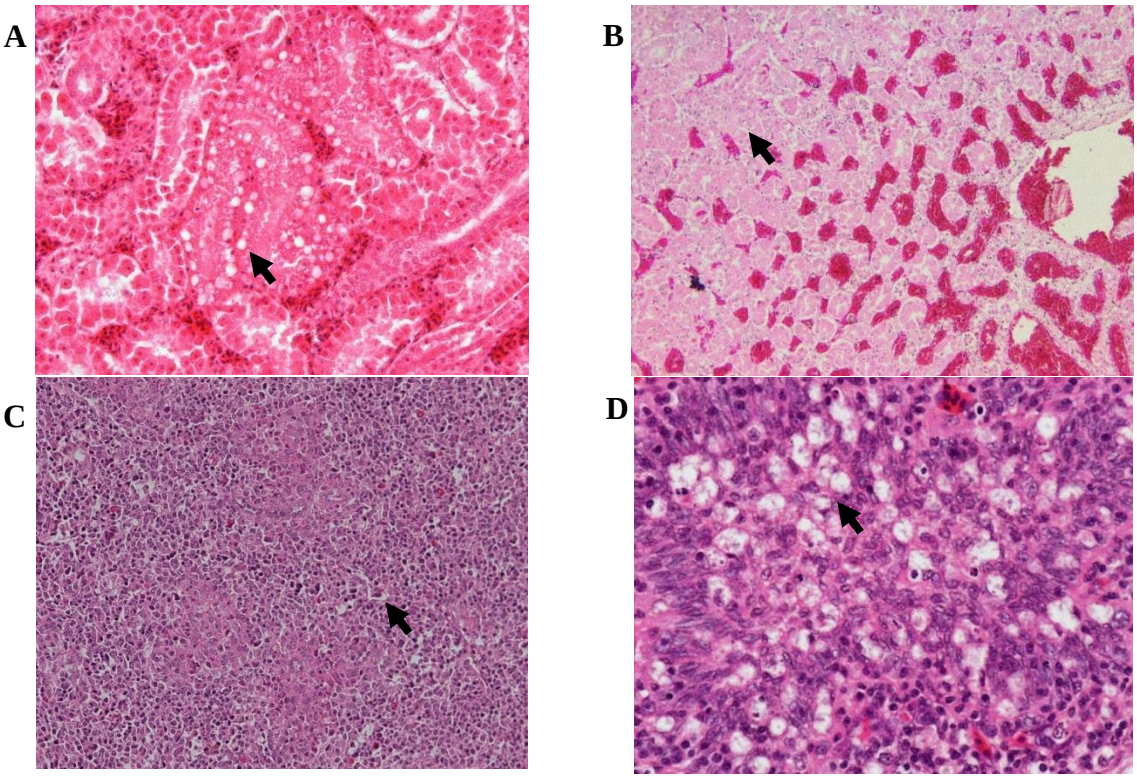

Figure 2. The effects of the aflatoxin on the histopathology of kidney, spleen and lymphoid organ due to the high exposure of aflatoxin (> 101 ppb < 150 ppb) in broiler chickens on day 21 of experiment. Tubular degeneration with congestion (**A**), congestion and tubular necrosis (**B**) of the kidney; white pulp depletion (**C**) and lymphoid depletion (**D**) of spleen and bursa of Fabricius. All histopathological changes are marked by the arrow. H&E, 100× (A), 10× (B), 4× (C) and 1000× (D).

Those effects were caused by the chaos of exchange of sodium pump in the renal tubule that generates water and sodium retention. Renal cells swelling induces tubular occlusion and decrease the excretory function of the kidney (Liang et al., 2015). Further exploration in the kidney revealed that severe degeneration was followed by tubular necrosis and congestion (Figure 2b). These phenomena are causing the accumulation of aflatoxin in the plasma that gradually aggravates the lesion to another organ (Tokonami et al., 2013).

Aflatoxin significantly affected the relative weight of the lymphoid organ of broiler chickens, especially in group D ($P<0.05$) (Table 2). It is supported by the depletion of lymphoid tissue in spleen and BF (Figure 2c and 2d). Moreover, the vacuolisation observed in the cortical area of the thymus ($P<0.05$) (Table 3). The secondary metabolite of aflatoxin accumulates inside the splenocytes, bursal cells and thymocytes that induces the cellular defects (Peng et al., 2015). This accumulation causes the apoptosis of the immune cells, and impair the antibody synthesis. As the base of immune cells proliferation, the lymphoid organ is used as the indicator of immunity in the vertebrates. The immunohistochemistry showed that the decreasing immune expression of CD3+, CD4+, and CD8+ occur synergistically with aflatoxin exposure level ($P<0.05$) (Table 4). CD3+, CD4+, and CD8+ are the molecular surface of the T cells subset. CD3+ is the prominent T cell produces the antibody clone with high reactivity (Nagel et al., 2014). CD4+ and CD8+ potentially play as cell-mediated immunity during the antibody synthesis and support to destruct the infected cells (Kempashi et al., 2017). The shrinkage of T cells subset in the lymphoid tissue dramatically changes the circulatory phenomenon not only in the haematological aspects but also the antibody synthesis (Yang et al., 2018).

The haematology results obtained that high exposure of aflatoxin causes the severe non-regenerative anaemia indicated by the decreasing number of RBC, PCV, and Hb ($P<0.05$), without increasing of MCV. No statistical differences were observed regarding the MCV, MCH and MCHC in all groups ($P>0.05$) (Table 5). Further, the lymphocytes are significantly decreased in both group C and D that indicated a low synthesis of antibody ($P>0.05$). This result is supported by the previous study that aflatoxin was able to change haematology and biochemistry parameters before the developing of the clinical signs (Tessari et al., 2010; Mahfouz and Sherif, 2015). In this study, the aflatoxin induced normocytic normochromic anaemia due to the inhibition of protein synthesis. However, lymphocytopenia was caused by dramatical increasing of apoptosis in the lymphoid organs that inhibit the proliferation and maturation of the circulatory lymphocytes (Donmez et al., 2012). Chronic inflammation and severe cellular necrosis play as the chemo-attractant in monocyte infiltration. Further, it induces monocytosis. Monocyte is the phagocytic mononuclear cells to destroy and recycle tissue debris. The monocytes infiltration in the aflatoxicosis induces external trap formation of macrophages (An et al., 2017).

Present study proved that the aflatoxin inhibited the numbers of circulatory heterophil and its phagocytic activity ($P< 0.05$) (Table 6). The phagocytosis is the first lineage of a defensive strategy against the pathogenic agents in the animal including broiler chickens. In this study, the phagocytic activity of the heterophil was investigated against the *Staphylococcus aureus* that commonly infects the chickens. The heterophil is activated during the first lineage of infection (Guriec et al., 2018). It`s actively engulfment the bacteria in the circulatory system by forming the cytoplasmic pseudopodia (Mohd et al., 2016). The decrease of the heterophils phagocytic index is suspected due to the shrinkage of mature circulatory heterophil. The systemic inflammation depresses on bone marrow to release the immature heterophil on the circulation. Moreover, it may be caused by the decreasing level of arginine-specific adenosine diphosphate ribosyltransferase (ADP-ribosyltransferase) in the serum that down-regulates the migration, attachment, and phagocytosis of heterophil. Further, the cellular damage of the heterophil impairs the dectin-1-receptor on its surfaces to identify the pathogenic bacteria. The phagocytic activity of the heterophil and monocytes influence the antigen presenting cells that mediated the synthesis of antibody. It is approved by the present findings that indicatedlow antibody against ND, IBD, and AI after vaccination. The highest GMT level only indicated on day 21 in the group treated with high level of aflatoxin (> 101 ppb < 150 ppb). It was the compensatory impacts of lymphoid organs depletion during the aflatoxicosis compared to the control. This result was different from the previous study that reported chicken was the most resistant species against the aflatoxin in the poultry industry (Lazona and Diaz, 2006). It is caused by consumption of the aflatoxin during growth that leads the lymphoid tissues atrophy in the broiler chickens. Moreover, vacuolisation in the bursa of fabricius, spleen, and thymus aggravates the synthesis of antibody after vaccination via depression of the lymphocytes in tissue (Chen et al., 2014) and circulatory system (Pandey et al., 2007).

Table 4. The effects of aflatoxin exposure on the immunohistochemistry of CD3+, CD4+, and CD8+ on day 21 in the lymphoid organ of broiler chickens

Parameters	Organ	Groups			
		A	B	C	D
CD3+	Spleen	2.40 ± 0.51[a]	1.70 ± 0.48[b]	1.40 ± 0.69[b]	0.80 ± 0.42[c]
	Bursa of Fabricius	2.10 ± 0.73[a]	1.20 ± 0.91[b]	1.00 ± 0.81[b]	0.60 ± 0.51[c]
	Thymus	2.40 ± 0.69[a]	1.60 ± 0.96[b]	1.40 ± 0.96[b]	1.00 ± 0.81[c]
CD4+	Spleen	1.90 ± 0.56[a]	1.50 ± 0.97[b]	1.40 ± 0.84[b]	1.00 ± 0.66[c]
	Bursa of Fabricius	2.00 ± 0.81[a]	1.40 ± 0.84[b]	1.20 ± 0.78[b]	0.90 ± 0.31[c]
	Thymus	2.00 ± 0.66[a]	1.70 ± 0.94[b]	1.40 ± 0.69[b]	0.80 ± 0.63[c]
CD8+	Spleen	1.50 ± 0.52[a]	1.00 ± 0.81[b]	1.20 ± 0.63[b]	0.80 ± 0.78[c]
	Bursa of Fabricius	1.60 ± 0.51[a]	1.30 ± 0.67[b]	1.00 ± 0.81[b]	0.70 ± 0.67[c]
	Thymus	1.70 ± 0.67[a]	1.20 ± 0.78[b]	1.20 ± 0.63[b]	0.60 ± 0.51[c]

[a, b, c] the different superscript on the same row showed significantly different values (P< 0.05).

Table 5. The effects of aflatoxin exposure on the haematological profile of broiler chickens

Parameters		Day	Groups			
			A	B	C	D
Red blood cells (× 10^6/ µL)		7	2.25 ± 0.02[a]	2.26 ± 0.05[a]	2.26 ± 0.08[a]	2.24 ± 0.04[b]
		14	2.39 ± 0.16[a]	2.44 ± 0.15[a]	2.34 ± 0.08[a]	2.31 ± 0.13[b]
		21	2.46 ± 0.03[a]	2.41 ± 0.04[a]	2.44 ± 0.11[a]	2.31 ± 0.10[b]
Packed cells volume (%)		7	27.30 ± 1.14[b]	27.29 ± 1.05[b]	26.81 ± 1.24[b,c]	26.15 ± 1.08[c]
		14	27.54 ± 0.64[b]	27.39 ± 0.52[b]	26.70 ± 1.21[b,c]	26.46 ± 1.04[c]
		21	27.53 ± 0.89[b]	27.37 ± 0.83[b]	27.44 ± 0.48[b,c]	27.08 ± 0.47[c]
Haemoglobin (g/ dL)		7	11.10 ± 0.20[b]	10.73 ± 0.48[b]	10.48 ± 0.23[b,c]	10.37 ± 0.48[c]
		14	12.04 ± 2.58[b]	11.28 ± 0.85[b]	10.88 ± 0.59[b,c]	11.04 ± 1.04[c]
		21	11.24 ± 0.71[b]	11.28 ± 0.40[b]	11.12 ± 0.84[b,c]	10.24 ± 0.17[c]
Mean corpuscular volume (fL)		7	121.29 ± 4.15[a]	120.46 ± 5.37[a]	118.28 ± 6.42[a]	116.62 ± 6.28[a]
		14	115.40 ± 9.36[a]	112.33 ± 7.64[a]	114.25 ± 7.88[a]	115.03 ± 10.46[a]
		21	111.55 ± 4.16[a]	113.21 ± 4.71[a]	112.53 ± 4.87[a]	117.36 ± 7.24[a]
Mean corpuscular haemoglobin (Pg)		7	49.33 ± 0.95[a]	47.37 ± 2.69[a]	46.25 ± 1.76[a]	46.25 ± 2.12[a]
		14	50.50 ± 12.05[a]	46.12 ± 3.04[a]	46.50 ± 2.41[a]	47.86 ± 4.24[a]
		21	45.53 ± 2.76[a]	46.68 ± 3.21[a]	45.56 ± 3.36[a]	44.37 ± 2.17[a]
Mean corpuscular haemoglobin concentration (%)		7	40.72 ± 1.95[a]	39.32 ± 1.23[a]	39.15 ± 1.50[a]	39.75 ± 2.53[a]
		14	43.72 ± 9.49[a]	41.17 ± 3.10[a]	40.90 ± 3.98[a]	41.82 ± 4.52[a]
		21	40.86 ± 2.86[a]	41.23 ± 2.29[a]	40.55 ± 3.38[a]	37.84 ± 0.78[a]
White blood cells (× 10^3/ µL)		7	21.22 ± 1.92[a]	21.42 ± 1.63[a]	21.41 ± 2.43[a]	20.92 ± 1.01[b]
		14	21.71 ± 0.98[a]	21.75 ± 1.28[a]	20.53 ± 0.72[a]	19.61 ± 0.40[b]
		21	20.82 ± 0.99[a]	21.02 ± 0.74[a]	18.98 ± 1.32[a]	18.29 ± 1.24[b]
Differential count of leucocytes (× 10^3)	Heterophils	7	5.62 ± 0.58[a]	5.44 ± 1.01[a]	5.86 ± 0.72[a]	6.06 ± 0.31[a]
		14	5.69 ± 0.50[a]	6.00 ± 0.42[a]	5.62 ± 0.74[a]	5.60 ± 0.40[a]
		21	5.45 ± 0.55[a]	5.56 ± 0.28[a]	5.12 ± 0.52[a]	4.90 ± 0.73[a]
	Lymphocytes	7	13.21 ± 0.12[b]	13.63 ± 0.11[b]	12.08 ± 0.21[c]	11.52 ± 0.16[c]
		14	13.35 ± 0.12[b]	13.08 ± 0.07[b]	12.50 ± 0.10[c]	11.06 ± 0.12[c]
		21	12.42 ± 0.06[b]	12.94 ± 0.08[b]	11.13 ± 0.09[c]	0.97 ± 0.06[c]
	Monocytes	7	1.77 ± 0.71[a]	1.94 ± 1.08[a]	2.66 ± 0.41[a]	2.45 ± 0.83[b]
		14	1.75 ± 0.48[a]	2.28 ± 0.42[a]	1.85 ± 0.63[a]	2.33 ± 0.64[b]
		21	1.75 ± 0.24[a]	1.79 ± 0.60[a]	1.79 ± 0.93[a]	2.42 ± 0.78[b]
	Eosinophils	7	0.54 ± 0.49[a]	0.31 ± 0.22[b]	0.57 ± 0.56[a]	0.70 ± 0.44[b]
		14	0.64 ± 0.57[a]	0.28 ± 0.25[b]	0.45 ± 0.38[a]	0.47 ± 0.59[b]
		21	0.86 ± 0.40[a]	0.54 ± 0.29[b]	0.78 ± 0.5[a]	0.92 ± 0.38[b]
	Basophils	7	0.06 ± 0.13[a]	0.08 ± 0.25[a]	0.22 ± 0.27[a]	0.17 ± 0.28[a]
		14	0.26 ± 0.27[a]	0.08 ± 0.15[a]	0.10 ± 0.14[a]	0.13 ± 0.25[a]
		21	0.32 ± 0.35[a]	0.16 ± 0.27[a]	0.14 ± 0.32[a]	0.29 ± 0.51[a]

[a, b, c] the different superscript on the same row showed significantly different values (P< 0.05).

Table 6. The effects of aflatoxin exposure on the heterophil phagocytic activity, average phagocytic bacteria, and phagocytic index against *Staphylococcus aureus* bacteria in broiler chicken

Parameters	Day	Groups			
		A	B	C	D
Heterophil phagocytic activity (%)	7	50.10 ± 0.05[a]	0.48 ± 0.05[b]	0.46 ± 0.03[b]	0.40 ± 0.04[b]
	14	48.50 ± 0.05[a]	0.48 ± 0.06[b]	0.46 ± 0.06[b]	0.39 ± 0.05[b]
	21	51.00 ± 0.04[a]	0.47 ± 0.04[b]	0.41 ± 0.03[b]	0.39 ± 0.04[b]
Average phagocytic bacteria (cells)	7	1.77 ± 0.33[a]	1.90 ± 0.38[b]	1.57 ± 0.22[b]	1.50 ± 0.29[b]
	14	1.86 ± 0.23[a]	1.89 ± 0.25[b]	1.71 ± 0.16[b]	1.59 ± 0.19[b]
	21	1.81 ± 0.22[a]	1.81 ± 0.31[b]	1.43 ± 0.23[b]	1.17 ± 0.25[b]
Phagocytic index (%)	7	88.48 ± 18.23[a]	92.62 ± 25.74[a]	72.33 ± 13.19[b]	60.72 ± 15.58[c]
	14	90.59 ± 17.10[a]	91.92 ± 17.82[a]	79.93 ± 14.28[b]	62.11 ± 9.83[c]
	21	92.77 ± 16.36[a]	85.65 ± 20.25[a]	59.83 ± 9.86[b]	46.34 ± 8.16[c]

[a, b, c] the different superscript on the same row showed significantly different values (P< 0.05)

Aflatoxin was undetected as the residue on the broiler chicken organs in group A, B, and C during the observation periods. It is detected on the liver (0.10 to 0.12 ppb), skin (0.02 to 0.09 ppb), and muscle (0.03 to 0.10 ppb) only in birds treated with high level of aflatoxin (> 101 ppb < 150 ppb) on day 21. The liver becomes the organ with the highest aflatoxin residue because of its activity to detoxify and metabolise the toxins. The hypodermis consists of the adipose tissue arranged by adipocytes that potential as the storage of the energy and metabolite products such as toxin and antibiotics and also it is similar to muscle tissues (Sinique et al., 2017). The chicken's breast muscle is the prominent part of the poultry's final product with a high economic value and the highest of human consumption rate. The monitoring of aflatoxin contamination on the chicken stuff is necessary to be conducted routinely in the poultry industry. It is because of the aflatoxin residue in the poultry's final products also found in the edible tissue such as muscle and liver that is commonly consumed by a human (Diaz-Zaragosa et al., 2014). In the long period of consumption of aflatoxin promotes the carcinogenesis and mutagenesis even at a low level (Adam et al., 2017).

CONCLUSION

The varying doses of aflatoxin in the chicken feeds caused the problems with different severity and impacts. High level of aflatoxin (> 101 < 150 ppb) was significantly adverse to the physical performance (body weight, FI, FCR, the relative weight of the organ) and laboratory indicators (histopathological changing, immune-expression of CD3+ and CD4+, promote the non-regenerative anaemia, decrease the role of leucocytes, heterophil phagocytic activity, phagocytic index, depress the synthesis of antibody), and also increasd risk of residue in the poultry's final products.

DECLARATIONS

Acknowledgements

This study is partially funded by M. J. Ahmed Farm with grant number [07.07.Ah.2017]. All the laboratory technicians form the integrated laboratory, university of Muhammadiyah Sidoarjo, faculty of veterinary medicine, university of Wijaya Kusuma Surabaya, and disease investigation Centre Yogyakarta, were acknowledged for their assistant during this study.

Author's contribution

Kurniasih and Yos Adi Prakoso participated in design, experimental procedure, writing, revised, and reviewing the manuscript.

Competing interests

The authors have declared that no competing interest exists.

Consent to publish

The authors guarantee that this work has not been published elsewhere and that any person named as a co-author of this work is aware of the fact and has agreed to be so named.

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JWPR
Journal of World's Poultry Research

2019, Scienceline Publication
J. World Poult. Res. 9(2): 78-81, June 25, 2019

Research Paper, PII: S2322455X1900010-9

Fatty Acid Composition of Female Turkey Muscles in Kazakhstan

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Received: 29 Apr. 2019
Accepted: 28 May 2019

ABSTRACT

This paper aimed to study the fatty acid composition of turkey meat. Red and white turkey meat were sampled from the local markets of Semey city, republic of Kazakhstan. The proximate composition showed a significant difference in the fat content of red and white meat. The fatty acid composition of turkey meat was as follows: saturated fatty acids 50.67% in white and 52.64% in red meat; monounsaturated fatty acids 28.07% in white and 23.79% in red meat; polyunsaturated fatty acids 21.26% in white and 23.57% in red meat. Palmitic and pentadecanoic are the major saturated fatty acids, where the oleic and linoleic acids are in a large amount in monounsaturated and polyunsaturated fatty acids, respectively.

Key words: Fatty acid, Polyunsaturated fatty acid, Red meat, Turkey meat, White meat

INTRODUCTION

Adequate nutrition is fundamental to human life and activities. In the condition of the unfavorable environmental situation, the nutrition should also have the long term pharmaceutical and health effects (Aslanova et al., 2018). Turkey meat is an essential part of healthy eating due to its dietary, hypoallergenic, safe and nutritive properties (Lisitsyn et al., 2018). Turkey meat is rich in B group vitamins, which help to prevent anemia maintain the normal functioning of the cardiovascular and nervous system (Amirkhanov et al., 2017).

Chickens, turkeys, ducks, goose, guinea fowls and quails are domesticated birds. Among these birds, turkey is one of the biggest. Turkey meat compares favourably with high protein content (up to 28%) and low-fat content (2-5%), more B group vitamins and lowest cholesterol content rather than in other poultry meat (Okuskhanova et al., 2017a).

Turkey growing and turkey meat processing is a fast growing branch of poultry production. Nowadays, USA, France, Italy, Russia, Germany, Poland, Morocco and Portugal are the main producers of turkey meat in the world (Aksenova et al., 2015).

Turkey meat is widely used in the production of meat products. For example, Tscvetkova (2012) developed the sausages replacing beef with turkey meat. The nutritive value of turkey meat sausages was not lower than beef sausages (Tscvetkova, 2012). Igenbayev (2017) used turkey meat in the formulation of the protein additive comprised turkey meat, lentil, wheat germ and broth. Further, the protein additive was used in the formulation of meat pate (Igenbayev, 2017).

Fatty acid composition of turkey meat represented with high content of polyunsaturated fatty acids. The lipids of turkey meat have practically zero content of β-carotene (Gargaeva and Gurinovich, 2017). Polyunsaturated fatty

acids (PUFAs) are responsible for significantly low levels of blood lipids and a low incidence of hypertension (De Almeida et al., 2006). Also it is indicated that the significant part of fat in human body is consumed as an energetic material. However, fats are a plastic material, as they are part of cellular components and especially in membranes (Staykov et al., 2015).

The purpose of this work was to evaluate the fatty acid composition of turkey meat from meat markets of Semey city, republic of Kazakhstan.

MATERIALS AND METHODS

Turkey meat (predominately, 6-12 month of age, female, broad-breasted Bronze turkeys) was sampled from the three local meat markets of Semey city, Kazakhstan. Totally, 15 samples weighing around 200g each were grinded with 3mm plate and stored at -2 to -4 °C before analysis.

The proximate composition (moisture, fat, ash and protein) was determined according to the standard methods, fully described in (Okuskhanova et al., 2017b). The fatty acid composition was determined at the certified laboratory of limited liability partnership "Nutritest" according to the standard method of GOST 55483-2013 which determine the fatty acids composition by gas chromatography in meat and meat products" (Antipova et al., 2001). The method is based on the liquid extraction of animal lipids by organic solvents, allowing to isolate 90% -95% of all cellular lipids and triglycerides by hydrolysis.

Statistical analyses

Statistical analysis was performed using Statistica 12.0 (STATISTICA, 2014; StatSoft Inc., Tulsa, OK, USA). The differences between samples were evaluated using ANOVA method. The differences were considered to be statistically significant at $P \leq 0.05$.

RESULTS AND DISCUSSION

The proximate composition of turkey meat was 18.8% protein, 21.7% fat, 0.9% ash for red meat and 21.7% protein, 12.0% fat and 1.1% ash for white meat (Amirkhanov et al., 2017).

White and red turkey meat has small differences in fatty acid composition. The total amount of saturated and polyunsaturated fatty acids is higher in red meat, while monounsaturated fatty acids predominate in white meat. The higher content of pentadecanoic (21.15% in white and 22.06% in red meat), palmitic (21.15% in white and 20.12% in red meat), oleic (22.17% in white and 19.93% in red meat), and linoleic (19.91% in white and 22.21% in red meat) acids were observed in turkey meat.

Table 1. Proximate composition of broad-breasted Bronze female turkey meat, g/100 g

Parameter	6-12 month old turkeys	
	red meat (g/100 g)	white meat (g/100 g)
Water	56.9±0.01	63.8±0.01
Protein	18.8±0.04	21.7±0.04
Fat	21.7±0.01	12.0±0.01
Ash	0.9±0.01	1.1±0.01

Table 2. Fatty acid composition of the white and red broad-breasted Bronze female turkey meat

Name of fatty acid	Turkey meat	
	white meat	red meat
Saturated fatty acids	50.67	52.64
Lauric (C12)	0.42	0.65
Myristic(C14)	0.54	1.17
Pentadecanoic acid c15	21.15	22.06
Palmitic(C16)	21.15	20.12
Margaric acid (C17)	0.21	0.78
Stearic acid (C18)	7.2	7.86
Monounsaturated fatty acids	28.07	23.79
Palmitoleic acid (C16:1)	5.9	3.86
Oleic acid C18:1	22.17	19.93
Polyunsaturated fatty acids	21.26	23.57
Linoleic acid (C18:2)	19.91	22.21
Linolenic acid (C18:3)	1.17	1.24
Arachidonic acid (C20:4)	0.18	0.12
Total FA	100	100

The daily nutrients ratio of protein to fat should be 1.0 (0.8-.2) in food rations of a healthy human. Such kind of ratio is more favourably for the satisfaction of energy and nutrient sources of the human body. Fat quality is defined by the content and balance of fatty acids (saturated and unsaturated fatty acids).

The best proportion of saturated and unsaturated fatty acids in the human diet is 30% to 70%. The physiological requirement of fat for a man is 70-154 g/day, for woman 60-102 g/day (Ustinova et al., 2005). The content of monounsaturated and polyunsaturated fatty acids does not significantly ($P \geq 0.02$) differ from the study of Gassilina et al. (2010) (29.56% in white and 25.89 in red meat of Monounsaturated Fatty Acids (MUFA) and 22.33% in white and 25.43% in red meat of Poly-unsaturated fatty acids (PUFA).

Thus, by the content of saturated fatty acids white turkey meat has higher content of palmitic acid, while others are lower than in red meat. Among the MUFA the content of palmitoleic and oleic acids was higher in white meat rather than in red meat. However, PUFA content in red meat, namely linoleic and linolenic fatty acids were more in red meat, while the concentration of arachidonic acid was higher in white meat.

The study conducted by Mauric et al. (2016) indicated that in breast meat of Dalmatian turkeys the content of Saturated Fatty Acids (SFA), MUFA and PUFA were 38.04, 34.62 and 26.54% of total fatty acids. A slight difference was observed in thigh meat: SFA 39.98%, MUFA 33.68% and PUFA 25.77%.

Coskuntuna et al. (2015) reported that the collected samples of turkey meat from the regional farm of Istanbul, Turkey contained 36.6% of SFA, 39.70% of MUFA and 23.60% of PUFA.

Higher content of PUFA (31.40%) and MUFA (34.40) in turkey meat from Belarus was reported by Meleschenya et al. (2013). Among the PUFA the content of ω-6 was 28.10% and ω-3 was 1.40% (Meleschenya, 2013). Marudova et al. (2018) observed that in turkey breasts from Bulgaria the fat content was 2.7%. Fatty acids predominately contain SFA (47.9%), following by MUFA 42.0% and PUFA 10.1% (Marudova et al., 2018). Polish scientists conducted experiments on feeding the turkeys with soybean or linseed oil containing feed mixtures and studied fatty acid compositions of turkey breast. It was observed the high content of PUFA (31.42-34.22%) (Stadnik et al., 2018).

Palmitic acid promotes synthesis of collagen, elastin, glycosaminoglycans and hyaluronic acid. However, the high concentration of palmitic acid leads to an increased level of cholesterol (Agostoni and Bruzzese, 1992). Oleic acid reduces the level of bad cholesterol (low-density lipoprotein) and increases the level of high-density lipoproteins. It can also slow the progression of the heart diseases and promotes antioxidant activity (Hur et al., 2005).

CONCLUSION

White and red turkey meat is the most healthy and nutritive parts of turkey. Base on chemical composition, white turkey meat contains more protein and water, while red turkey meat has more fat. The fatty acid composition is characterized by high content of PUFA with an abundance of linoleic acid. Among the MUFA mainly represented by omega-9 (oleic acid), while SFA most includes palmitic and pentadecanoic acid. Given data on the fatty acid composition of turkey meat can evaluate the qualitative profile of turkey meat and furthermore precisely modelling the formulation of food products with turkey meat.

DECLARATIONS

Competing interests

The authors have no competing interests to declare.

Consent to publish

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

Author`s contribution

All authors participated in making the design, performing the experiment, analyses of the data, and writing paper.

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JWPR
Journal of World's Poultry Research

2019, Scienceline Publication
J. World Poult. Res. 9(2): 82-88, June 25, 2019

Research Paper, PII: S2322455X1900011-9

Evaluation of Dietary Medicinal Plants and algae in Laying Japanese Quails

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Received: 01 May 2019
Accepted: 28 May 2019

ABSTRACT

The current study was conducted to examine the influences of *Sargassam cristafolium*, *Gracilaria pulvinata*, *Rhus coriaria*, and *Punica granatum* peel dried powder in two levels (10 and 20gr/kg) of dietary on productive performance and some egg quality characteristics of laying Japanese quail. A total of 675 (49 days old) Japanese quail were randomly distributed into nine groups with three replicates of 25 birds in each. Results showed that egg weight, shell weight, albumen weight and shell thickness were not influenced by treatments. The effects of medicinal plants on weekly egg production differ depending on the dietary medicinal plant type and dosage. Moreover, the birds fed diet supplemented with *Sargassum cristaefolium* at 10g/kg had greater weekly egg production compared to others. Haugh unit was reduced by diet supplemented with *R. coriaria*. Diet supplemented with *S. cristaefolium* at 20 g/kg increased yolk weight. Furthermore, greater albumen protein and thiobarbituric acid content of the tenth day's eggs ($TBAd_{10}$) were perceived in birds fed diet supplemented with *G. pulvinata*. In present study, the lowest levels of cholesterol (9.66 mg/g) was determined in the egg yolk of groups *R. coriaria* and *G. pulvinata*, and the control group showed to have the most amounts (12.00 mg/g). Based on obtained data, addition of 0.5% and 2% of *S. cristafolium*, *G. pulvinata*, *R. coriaria*, and *P. granatum* peel to the diet lead to be progress in egg production rate, enhanced the egg quality and egg biochemical properties in Japanese quail.

Key words: Egg production, Egg quality, Laying quails, Medicinal plants, Thiobarbituric acid

INTRODUCTION

Protein with animal source such as eggs are of a major significance for balanced and adequate human nutrition and health (Al-Daraji et al., 2010). Recently, antibiotics have been used as growth promoters for higher yields in poultry production (Sinar et al., 2013; Mehdi et al., 2018). However, the use of antibiotics in animal nutrition has a negative consequence because animals will become resistant to antibiotics and residues of antibiotics in animal products will be carried away over eggs and meat (Abd El-Galil and Henda, 2015). Thus, search for effective alternatives to antibiotics in intensive animal production is a very important topic, considering not only the impact in animal welfare but also in human health (Abd El-Galil and Henda, 2015). The use of herbs and algae as poultry feed ingredients is continuously increasing after the ban of nutritive antibiotics in the European union (Gerzilov et al., 2015; Abouelezz, 2017). During the last ten years, the beneficial nutritional aspects and pharmaceutical of seaweeds have been advertised extensively worldwide, and therefore the algae enterprises started to gain a clear interest among producers (De Almeida et al., 2011; Abouelezz, 2017). There is a wide variety of active compounds such as carotenoids, vitamin B_{12}, vitamin C, thiamin, riboflavin, pyridoxine in algae, and it could be applied in the diets of layers and chicken, to improve the quality of yolk and flesh (Abouelezz, 2017; Aljumaily and Taha, 2019). Fucosan or sulfated fucans is a polysaccharide mainly produced by brown algae. Fucoidan has been reported with high and various bioactivities such as immunostimulating activity, anticancer, anti-inflammatory, antiviral, anticoagulant, anti-thrombosis, anti-liver fibrosis and antioxidant (Isnansetyo et al., 2016).

Herbal products are harmless to humans and animals with no undesired side effects. In addition, there is no complication of drug resistance due to their consumption (Behnamifar et al., 2015). Aminzade et al. (2012)

recommended the use of dietary antioxidants to reduce lipid peroxidation in the feeding animal and to preserve product quality. *Punica granatum* and *R. coriaria* are medicinal plants with antioxidant properties (Sharbati et al., 2015; Yassein et al., 2015). In one study, it was found that diet supplemented with 15 g PPP/kg (pomegranate peel powder) led to improve in production, physiological parameters as well as meat shelf-life in Japanese quails (Yassein et al., 2015). Also, seed pulp of pomegranate (<15% in poultry diet) improved egg production, however, higher amounts showed detrimental effects on laying performance (Saki et al., 2014a). In another study, *R. coriaria* L. and pomegranate peel were reported to contain high levels of tannins, flavonoids, and phenolics with antioxidant properties and improvement digestion and metabolism (Abbas et al., 2017; Gurbuz and Salih, 2017).

The present experiment was designed to study the possibility of using *S. cristafolium*, *G. pulvinata*, *P. granatum* and *R. coriaria* dried powder as supplements to Japanese quail diets to evaluate its effect on productive performance, egg quality and egg biochemical compounds.

MATERIALS AND METHODS

Preparation of herbal materials

The plants *Sargassam cristafolium*, *Gracilaria pulvinata*, *Punica granatum* and *Rhus coriaria* were harvested from their natural habitat in Bushehr province, south-west of Iran, and then were dried in a dry and dark environment. Then, obtained dried seeds were powdered.

Experimental birds

All procedures used during this study were approved by animal care committee of Persian Gulf University, Bushehr, Iran. At the beginning of the experiment, 1200 day-old quail were grown simultaneously and with the same diet until 49 days old. Then among them a total of 675 (49 days old) Japanese quail were randomly distributed into nine groups with three replicates of 25 birds each. The birds' average body weight (212.00±4.00) did not differ between the experimental groups when placed in the cages at the beginning of trial period. All birds were allowed to adapt for a period of seven days, consuming a commercial laying quail's diet ad libitum (Table 1). The same diet was fed to the control group during the experimental period. Control group (C) was fed a commercial laying diet. The control group was fed with a diet containing no supplements, while other groups received diets containing 10 gr/kg or 20 gr/kg of *S. cristafolium*, *G. pulvinata* dried powder, *R. coriaria* dried powder and *P. granatum* peel dried powder. Under commercial conditions, the birds were offered feed and water ad libitum during a period of 28 days.

Table 1. Composition of basal diet

Ingredient	g/kg
Corn	518.00
Soybean meal	355.00
Soybean oil	31.40
Dicalcium phosphate	7.00
Limestone	75.00
Sodium chloride	2.80
Sodium bicarbonate	1.00
L-Lys-HCl	1.30
DL-Met	3.40
Vitamin and mineral premix[1]	5.00
Phytase 10000	0.10
Total	1000.00
Analysis	
Metabolizable energy, Kcal/kg	2800.00
Crude protein	19.84
Calcium	3.10
Available phosphorous	0.32
Sodium	0.15
Chloride	0.23
Lysine	1.08
Methionine	0.48
Methionine + Cysteine	0.88
Threonine	0.65
Tryptophan	0.22
Arginine	1.26
Isoleucine	0.77
Valine	0.83

Basal diet was provided (per kg): vitamin A, 10000 IU; vitamin D3, 4500 IU; vitamin E, 65 IU; vitamin K3, 3 mg; vitamin B1, 2.5 mg; vitamin B2, 6.5 mg; vitamin B3, 60 mg; vitamin B5, 18 mg; vitamin B6, 3.2 mg; vitamin biotin, 0.22 mg; folic acid, 1.9 mg; vitamin B12, 0.017 mg; choline chloride, 1400 mg, Mn, 120 mg; Zn, 110 mg; Fe, 20 mg; Cu, 16 mg; I, 1.25 mg; Se, 0.3 mg

Production performance

Egg collection was done daily from eight weeks of age, and eggs' weight were measured by a digital balance (Olawumi and Ogunlade, 2014). The yolk was separated from the albumen and then weighed while the albumen weight was found by subtracting the weights of yolk and shell from egg weight. Haugh unit (HU) values were calculated using the following formula (Aboonajmi et al., 2010):

$$HU = 100 \text{ Log } (H + 7.57 - 1.7 \times W^{0.37})$$

Where H is the albumen height (mm) and W is the egg weight (g).

Biochemical factors

Yolk and albumin of three eggs obtained from each replicate were separated and their chemical composition was determined according to the methods of Association of Official Agricultural Chemists (AOAC, 1990). In order to determine the egg yolk cholesterol concentration, 1g of pooled yolks of each replication was added to 9ml of 2% NaCl solution. Samples were shaken for 2h. Then, 1 ml of the diluted yolk was re-diluted 10 times. In this study, 10µl of this sample was mixed with 100µl of salt solution and 1ml of the enzymatic reagent. The same procedure was also implemented for the standard of cholesterol. As the blank sample, 10µl of deionized water was used instead of sample or standard of cholesterol. Samples were incubated in a water bath at 37°C for 15 min and then the light absorbance of 500 nm was measured (Behnamifar et al., 2015).

Statistical analysis

All data were subjected to ANOVA using the general linear models procedure of SAS software (SAS, 2004). Treatment means were tested using the Duncan's (1995) multiple range test, and statistical differences declared at $P<0.05$ (Duncan, 1955).

Ethical approval

The present study was approved by the Ethics Committee of the Agriculture College of Persian Gulf University. The experiments were performed in the poultry research ward, Agricultural and Natural Resources College Persian Gulf University, Boushehr, Iran.

RESULTS AND DISCUSSION

Egg production and egg quality traits

Herbal plant powder could encourage the digestive system in poultry, to improve the function of digestive enzymes as well as liver efficacy and increase the pancreatic. Expansion of the metabolism of herbal plant powder, generally carbohydrates and proteins in the major nutrient would increase growth rates (Pooryousef and Hosseini, 2012). Many medicinal plants and their extracts are used widely in poultry diets because the herbs have biological activities and stimulate the digestive system (Hamodi and Al-Khilani, 2014). Results of the effects of different levels of *S. cristafolium*, *G. pulvinata*, *P. granatum* peel dried powder and *R. coriaria* on Egg production and egg quality traits have been reported in tables 2, 3 and 4.

Most egg weight and week eggs production were due to the treatment with *S. cristafolium* powder content. As macro-algae contain high levels of vitamin and trace element, as well as properties of lipid mobilization, and suitable absorption rates can lead to improvement in performance (Morshedi et al., 2018). Abd El-Galil and Henda, (2015) mentioned that anti-inflammatory, restoratives, antibacterial and anti-parasitic properties of medicinal plants can enhance the productive performance in poultry.

Table 2. Effect of different dietary herbal plant food supplement ratios on egg weight and egg production of Japanese quail from 8 to 10 weeks of age.

Treatment	Egg weight (g)	Weekly egg production (%)			
		Week 8	Week 9	Week 10	Week 11
C	11.33	3.33^{c}	24.66^{b}	63.33^{b}	72.00^{b}
S-1.0	11.56	12.33^{a}	38.66^{a}	75.33^{a}	85.33^{a}
S-2.0	11.39	7.33^{ab}	29.33^{b}	63.66^{b}	79.00^{b}
G-1.0	10.43	10.66^{a}	37.66^{a}	66.00^{b}	80.33^{ab}
G-2.0	11.10	10.00^{a}	29.66^{b}	61.33^{b}	77.00^{bc}
R-1.0	10.96	1.33^{c}	17.00^{c}	64.66^{b}	71.33^{cd}
R-2.0	11.12	2.66^{bc}	15.33^{c}	61.33^{b}	70.00^{d}
P-1.0	10.56	0.66^{c}	24.33^{b}	61.00^{b}	72.66^{dc}
P-2.0	10.95	2.66^{bc}	14.66^{c}	53.66^{c}	67.33^{d}
SEM	0.86	0.93	1.73	1.16	1.18
P value	0.47	0.001	0.001	0.001	0.001

Egg weight was calculated as the mean of whole experimental period. $^{A-d}$ Means within a column sharing a common superscript are not different ($P < 0.05$). C: control. S-1.0: *Sargassum cristafolium* 1.0%. S-2.0: *Sargassum cristafolium* 2.0%. G-1.0: *Gracilaria corticata* 1.0%. G-2.0: *Gracilaria corticata* 2.0%. R-1.0: *Rhus coriaria* 1.0%. R-2.0: *Rhus coriaria* 2.0%. P-1.0: *Punica granatum* 1.0%. P-2.0: *Punica granatum* 2.0%. S.E.M: standard error of the means.

Table 3. Effect of different dietary herbal plant food supplement ratios on egg quality parameters of Japanese quail on 70 days of age.

Treatment	HU	SW	YW	AW	ST
C	70.33[a]	1.80	4.13[b]	6.03	0.17
S-1.0	69.66[a]	1.83	4.40[ab]	6.03	0.17
S-2.0	71.33[a]	1.76	4.73[a]	6.20	0.15
G-1.0	71.33[a]	1.63	4.16[b]	6.06	0.18
G-2.0	70.66[a]	1.60	4.16[b]	6.06	0.17
R-1.0	66.00[b]	1.66	4.40[ab]	6.00	0.18
R-2.0	65.33[b]	1.80	4.60[ab]	5.23	0.17
P-1.0	70.00[a]	1.70	4.13[b]	5.96	0.17
P-2.0	69.66[a]	1.63	4.13[b]	5.96	0.14
SEM	0.88	0.04	0.05	0.08	0.00
P value	0.00	0.93	0.03	0.38	0.18

HU; Haugh unit, SW; Shell weight(g), YW; Yolk weight (g), AW; Albumen weight (g), ST; Shell thickness (mm), [A-d] Means within a column sharing a common superscript are not different (P<0.05). C: control. S-1.0: *Sargassum cristafolium* 1.0%. S-2.0: *Sargassum cristafolium* 2.0%. G-1.0: *Gracilaria corticata* 1.0%. G-2.0: *Gracilaria corticata* 2.0%. R-1.0: *Rhus coriaria* 1.0%. R-2.0: *Rhus coriaria* 2.0%. P-1.0: *Punica granatum* 1.0%. P-2.0: *Punica granatum* 2.0%. S.E.M: standard error of the means.

Table 4. Effect of different dietary herbal plant food supplement ratios on egg biochemical factors of Japanese quail on 70 days of age.

Treatment	Yolk cholesterol	Albumen protein	Egg protein	$TBAd_1$	$TBAd_{10}$
C	12.00	82.66[abc]	46.33	2.05	2.59[a]
S-1.0	10.00	83.66[abc]	46.66	1.99	2.50[a]
S-2.0	11.33	84.33[ab]	47.66	1.95	2.48[ab]
G-1.0	9.66	85.00[a]	46.66	1.94	2.52[a]
G-2.0	11.33	85.00[a]	48.00	1.95	2.51[a]
R-1.0	10.33	84.00[ab]	46.33	1.96	2.28[bc]
R-2.0	9.66	83.33[abc]	46.66	1.93	2.24[c]
P-1.0	10.66	83.66[abc]	46.33	1.86	2.24[c]
P-2.0	11.66	82.00[c]	45.33	1.87	2.19[c]
SEM	0.23	0.23	0.24	0.01	0.03
P value	0.07	0.01	0.34	0.27	0.00

Yolk cholesterol (mg/g), Albumen protein (%) and egg protein (%) were measured at the end of experiment. $TBAd_1$ (µmol/g); Thiobarbituric acid content of the first day's eggs, $TBAd_{10}$ (µmol/g); Thiobarbituric acid content of the 10[th] day's eggs. [A-c] Means within a column sharing a common superscript are not different (P<0.05). C: control. S-1.0: *Sargassum cristafolium* 1.0%. S-2.0: *Sargassum cristafolium* 2.0%. G-1.0: *Gracilaria corticata* 1.0%. G-2.0: *Gracilaria corticata* 2.0%. R-1.0: *Rhus coriaria* 1.0%. R-2.0: *Rhus coriaria* 2.0%. P-1.0: *Punica granatum* 1.0%. P-2.0: *Punica granatum* 2.0%. S.E.M: standard error of the means.

Therefore, they are considered as a promising dietary supplement, but data concerning their use in quail diets are limited (Abd El-Galil and Henda, 2015). Saleh et al. (2017) showed that feeding pomegranate peel diet significantly improved the daily weight gain and decreased the feed to gain ratio of broiler chickens. The effects of medicinal plants on weekly egg production differ depending on the dietary medicinal plant type and dosage. Moreover, the birds fed diet supplemented with *S. cristafolium* at 10 g/kg had greater weekly egg production compared to others. HU was reduced by diet supplemented with *R. coriaria* (sumac). Diet supplemented with *S. cristafolium* at 20 g/kg increased yolk weight. As noticed in table 4, shell thickness was increased (P<0.05) in *G. pulvinata* and *R. coriaria* supplemented quail compared with that in those fed on control diet during 70d period. In a research conducted by Saki et al. (2014a) the pomegranate seed pulp can be a potential feed supplement when added to the diets of laying hens up to 5% without negative effects on performance and egg production and quality. Higher levels will have a negative effect on egg production and quality in layers as shown by increased

cholesterol level (Saki et al., 2014a). *P. granatum* and *R. coriaria* are medicinal plants with antioxidant properties (Sharbati et al., 2015; Yassein et al., 2015). It seems that an increase in yolk quality by *S. cristafolium* is due to antioxidative properties of *S. cristafolium*. This antioxidant potency can protect yolk membrane against oxidation and damages, and increase standing up quality of yolk (Jafarzadeh et al., 2014; Jahanian et al., 2017).

Egg biochemical factors

The nutritive value and functional properties of eggs make them as important animal protein source. However, consumption of eggs is often considered as a reason for some health problems due to high cholesterol content leading to coronary heart disease (Miranda et al., 2015). The medicinal plants are expected to decrease the level of cholesterol. On the other hand, herbal essential oils including fenchone, geraniol, menthone, citral, and borneol fenchyl alcohol may diminish the activity of liver HMG-CoA reductase (Behnamifar et al., 2015). Results of present study shows that dietary inclusion of *G. pulvinata* and *R. coriaria* decreased the concentration of egg yolk cholesterol in Japanese quail (Table 4). Medicinal active products of sumac plants are tannins, phenolic acids, anthocyanins, malic, citric and tartaric acids. Sumac's antioxidant properties are reported because of Phenolic compounds. This is probably due to the reduction of quail egg cholesterol by sumac due to the compounds present in this plant (Sharbati et al., 2015). Similar results were found by Habibi and Ghahtan (2019) who reported that the dietary addition of 20 g/kg *R. coriaria* powder reduced cholesterol levels on quail. Englmaierova et al. (2013) showed that supplementing layers with Algae not only increased the concentration of lutein and zeaxanthin, but also improved feed conversion ratio, shell quality, and the oxidative stability of yolk lipids of fresh and stored eggs (Englmaierová et al., 2013). Saki et al. (2014b) have shown that serum triglyceride and cholesterol did not significantly affected by supplementation of a phytogenic feed in layer diets.

The average of egg protein and albumen protein values of Japanese quail under different dietary treatments at ten week of age are presented in table 4. Egg protein (45%) and albumen contents (85%) are higher in 20g/Kg plant of *G. pulvinata* groups when compared to control and other experimental groups, this difference is statistically significant ($P \geq 0.05$).

Results of present study shows that supplementation of Japanese quail diet with *S. cristaefolium*, *G. pulvinata*, *P. granatum* peel and *R. Coriaria* at the rate of 10 or 20g/Kg did positively influence TBA status of Japanese quail eggs (Table 4). The least TBA content of the tenth day's eggs (TBAd10) were perceived in birds fed diet supplemented with *P. granatum* peel. It seems that a decrease in thiobarbituric acid by *P. granatum* peel is due to antioxidative properties of *P. granatum* peel. Pomegranate peel extract with an abundance of flavonoids and tannins has been shown to have a high antioxidant activity (Yassein et al., 2015).

CONCLUSION

The results of this study demonstrated that using mentioned *R. coriaria*, *G. pulvinata*, *S. cristafolium*, and dried powder of *P. granatum* peel could affect egg production rate, egg quality and egg biochemical compounds of laying Japanese quails depending on the dietary medicinal plant type and dosage. Addition of 20 g/kg *S. cristafolium* to the diet improved yolk weight. Furthermore, diet supplemented with *G. pulvinata* led to higher levels of albumen protein and thiobarbituric acid content of the tenth day's eggs ($TBAd_{10}$). Based on the findings of this study, the lowest value egg yolk cholesterol was found in the quails received *G. pulvinata* and *R. coriaria* while the highest amounts were detected in the control group.

DECLARATIONS

Competing interests

The authors have no competing interests to declare.

Consent to publish

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

Acknowledgements

The authors would like to thank Ms. Afsane Esmaeili for her help with the project during experimental period. Also, we appreciate the initial improvements in English usage made by Peter Lowther through the Association of Field Ornithologists' program of editorial assistance.

Author`s contributions

Habibi and Ghahtan were involved in the data collecting, statistical analysis and drafting of the manuscript. Kohanmoo read and approved the final manuscript

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JWPR
Journal of World's Poultry Research

2019, Scienceline Publication

J. World Poult. Res. 9(2): 89-97, June 25, 2019

Review Paper, PII: S2322455X1900012-9

A Meta-Analysis on Antibiotic Residues in Meat of Broiler Chickens in Developing Countries

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Received: 02 May 2019
Accepted: 01 June 2019

ABSTRACT

Present study consisted of performing a meta-analysis on data about the detection of antibiotic residues in chicken meat achieved from all over the researches with a wide collection and very strict selection criteria of data. The databases were searched quantitative inputs from the available scientific publications using important keywords, in order to evaluate all studies about antibiotic residue and detection methods and the reliability of the results obtained by the international researchers. Then an advanced statistical analysis on collected data was done, the first phase was a descriptive study of positive and negative cases followed by a modeling of two cases with a prediction of the values obtained and ended with an analysis of the main compounds (population size, residue detection methods and positive case rates). All performed steps are reported in detail. The results indicated that the accuracy of the detection technique is a factor that influenced on reports of residues, and caused differences in reports, there are still antibiotic residues in meat of intensively broiler chicken farms (45. 26% of the samples analysed are positive), It is concluded that residue detection requires a high-precision qualitative analysis protocol.

Key words: Antibiotic residues, Broiler chicken, Databases, Meta-analysis.

INTRODUCTION

Antibiotic residues in food of animal origin remain a topical issue throughout the world, because the massive use of these molecules, for preventive and curative therapeutic purposes and also as food additives or growth promoters. (Preziosi et al., 2012; Berghiche et al., 2018). The lack of international regulations to control the use of antibiotics in poultry farming, especially in developing countries (possession, distribution and use are delayed), where many farmers treat their animals without knowing the conditions and quantities to administer or the withdrawal times (Mills et al., 2015).

Recent literature suggested that the term meta-analysis refers precisely to Glass (1976), who refers to the fact that results are evaluated at a higher level and from a more general perspective than the original studies. Glass thus focuses on the analysis of analyses. This method of analysis appeared in the 1970s in the fields of classical experimental sciences and psychotherapy (Webster, 2019).

From the 1990s onwards, the first meta-analyses appeared in the field of economics, particularly in environmental economics (Boyle et al., 1994; Schwartz, 1994; Smith and Huang, 1995) and also in labour economics (Jarrell and Stanley, 1990; Hedges et al., 1994; Card and Krueger, 1995).

Meta-analysis is a statistical approach, which aims to combine the results of a series of available and independent studies on the subject and then make a reproducible synthesis (Bouras et al., 2019).

The principles of meta-analysis are as follows: It should include all available trials for the treatment studied, positive and negative, published and unpublished (exhaustive research) (Marshall et al., 2019). It must be based on a precise protocol, to avoid any bias in the choice of studies included. Finally, this statistical method is applied to contextualize the probability of the outcome of all studies (Cervesato et al., 2019).

In total there are 23 studies that match the criteria for inclusion in this meta-analysis. In detail there were 17 studies in eight African countries (Congo, Sudan,

Morocco, Nigeria, Senegal, Tunisia, Egypt and Algeria), four studies of them in three Asiatic countries (Turkey, Iraq and Palestine) and one in one European country (Bulgaria).

The uncontrolled use of antibiotics to form high-risk residues for the consumer, this work is focused on these residues through studies performed for its detection by the different methods based on a statistical analysis of the meta data available on this subject, the objective of present research was to evaluate the relationship between antibiotic therapy in broiler chickens and residues of antibiotic compounds in the white meat, a statistical analysis was based of the meta-data available on the subject.

The meta-analysis, which makes it possible to estimate this correlation by synthesizing similar studies that have common characteristics.

MATERIALS AND METHODS

Experimental design

The protocol was developed prior to the meta-analysis. It is defined in three steps described below: Identification, obtaining and selection of articles that were related to present research criteria, data extraction and statistical analysis.

Defining the variables

Different factors are likely to influence the presence of antibiotic residues in each study that included:

- The organic materials used broiler chicken meat or offal (liver, gizzard).
- Type of food (feed additive).
- Antibiotic treatments performed.
- The route and dose of administration.
- Breeding method (traditional or modern).
- Breeding condition, include the type of soil used, relative humidity, the total number of animals per farm, the surrounding temperature and the level of hygiene are all environmental factors that can vary from one farm to another.
- The methods used for the study are sources of variability.
- The age and weight of the chickens studied may also be different.
- Judgement criteria, included the endpoint was the variable measuring at the end of the experiment to define the effectiveness of the test product.

In order to understand all the factors of variation in order to define a protocol that avoids sources of bias as much as possible.

Searching for articles related to the issue

After defining the main characteristics of the studies, it is necessary to search them exhaustively.

Pubmed. Pubmed being a free, easy-to-use and very complete search database, firstly to perform present search using this site.

The search performed is as follows:

Reading of bibliographies. After sorting the articles obtained through pubmed, and reading the bibliography of each article, in order to retrieve other articles.

Google scholar. Google scholar, allowed the searching of academic work. It was used last to ensure that no studies dealing had been forgotten.

Selection of usable articles. Initially, articles were selected based on their title and abstract, when available. The rest of the selection was made after obtaining the entire items; the selected items should have certain characteristics, which are sample size, type of antibiotic sought and method used for detection.

Selection on title and summary. The first selection was made simply from the title of the articles, also keywords related to antibiotic residues in broiler chicken meat. The selected articles were further developed by reading their abstracts. A decision was taken to include only articles in English or French languages. This first phase excluded non-native language articles and studies on the detection of antibiotic residues in foodstuffs apart on white meat. Unfortunately, the reviewing of titles and abstracts sometimes leaves some doubt as to whether or not a study can be included in the meta-analysis. In this case the selection was made base on the entire article.

Selection on the entire article. The quality of some articles was not always adequate and some articles just did not meet the requirements of the meta-analysis.

The authors of this study decided that evaluate the quality of the article for including in present study base on following condition:

- Animal species has been specified, is the broiler chicken.
- The antibiotics used in poultry farming that anybody looking for them (like Tetracycline, Sulfonamides, Aminosides, Macrolide, Beta lactamine, Tylosin, Colistin etc.).
- The research method used (four boxes reference method)

Characteristics of the articles. The details of the articles, those were not essential, but interesting and allowing to explain some calculations. The exclusion criteria for each item were clearly stated, in this study

only those concerning the detection of chloramphenicol was excluded, because this molecule is currently not commercialized (Fowler, 1992).

Studies included in the meta-analysis

The contamination of animal food origin by antibiotic residues has been reported by many authors, the selected research data are listed in the following summary table (Table 1).

Table 1. Summary table of studies concerned by the meta-analyses on antibiotic residues in meat of broiler chickens (Data included)

Study	Authors	Years	Counrty
01	Okombe et al.	2016	Congo
02	Hind et al.	2014	Sudan
03	Titouche et al.	2016	Algeria
04	Karmi	2014	Egypt
05	Mansouri	2007	Algeria
06	Omotoso	2015	Nigeria
07	Benghalem et al.	2016	Algeria
08	Alambedji et al.	2004	Senegal
09	Rezgui	2009	Tunisia
10	Abiola	2005	Senegal
11	N'kaya	2004	Senegal
12	Ramdane	2015	Algeria
13	Ben Mohand	2008	Algeria
14	Chaiba et al.	2017	Morocco
15	Ezenduka, et al.	2014	Nigeria
16	Onurdağ et al.	2013	Turkey
17	Shareef et al.	2009	Iraq
18	Elmanama and Albayoumi.	2016	Palestine
19	Shamsa.	2013	Iraq
20	Al Pavlov et al.	2008	Bulgaria
21	Berghiche et al.	2017	Algeria
22	Berghiche et al.	2018	Algeria

Studies not included in the meta-analysis

The objective of this study was to detect antibiotic (chloramphenicol) residues in chicken liver, kidney and muscle by three methods: four box methods, high performance liquid chromatography and enzyme-linked immunosorbent assay test (Tajik et al., 2010), this study was excluded because the use of the antibiotic concerned is currently banned so the determination of its minimum residual limit is irrelevant. Chloramphenicol is a broad-spectrum antibiotic active against Gram-positive and Gram-negative bacteria. It is an effective therapeutic agent for the treatment of many animal infections. However, historical epidemiological data have shown that its use in humans may be associated with hematological disorders, including aplastic anemias. During the evaluation, it was not possible to set a threshold value base on the available data. This inability to set the threshold value and the shortcomings of the documents have led to a classification as a prohibited substance for use in food-producing animals in the European community since 1994 (Mensah, 2014).

Statistical analysis

The collected data are logged and processed using the program (Microsoft Office Excel, 2007) to perform the description and evaluation, for the advanced statistical part a various statistical software was used (XLSTAT, Past 3 and Pro Origin).

The first phase was a descriptive study of positive and negative cases followed by a modelling of two cases with a prediction of the values obtained and ended with an analysis of the main compounds (population size, residue detection methods and positive case rates), a value of (P=0.05) which is considered significant throughout all the results obtained

RESULTS

Summary and description of the studies concerned by the meta-analysis

In a summary table an exhaustive description of the studies already carried out in the field of detection of antibiotic residues in the chicken meat in an exhaustive way (Table 2).

Prevalence of antibiotic residues across the different studies

As shown in figure 1, the presence of antibiotic residues with a rate of 45.30% was detected in 1144 cases out of a total of 2525. There is a significant difference ($P<0.05$) between the study areas with the highest rate of antibiotic residues reported in Africa with 649 positive cases followed by Europe and Asia with 462.460 positive cases respectively (Nisha, 2008; Berghiche, 2019). A boxplot is a graph that gives a good indication of how the values of the data are distributed, the arrangement of the data on its variability or dispersion.

The comparison between the two distributions shows that the different results that are negative are compact (Figure 2A) and for the positive ones are dispersed (Figure 2B).

Table 2. Recuperative table of results obtained across the different studies

No.	Country	Total No. of analysed samples	Cas positives	Methods used	Antibiotics researched
01	Congo	67	7 (10.44%)	Four boxes	Tetracycline (100%) Tylosine (100%) Colistine (75%)
02	Soudan	221	60 (27%)	Three plates	Quantitative detection
03	Algeria	145	124 (85.51%)	Four boxes	Betalactamine /tetracycline (75.81%) Macrolide /betalactamine (44.35%) Sulfamide (36.29%) Aminoside (13.71%)
04	Egypt	50	42 (83.33%)	Four boxes	Beta lactamine (0%) Tetracycline (51%) Sulfamide (53%) Macrolide (0%) Aminoside (15%) Quinolone (39%)
05	Algeria	120	79 (65.7%)	Four boxes	Sulfamide (87.65%) Macrolide /tetracycline (43.20%) Betalactamine /Tétracycline (12.34%) Aminosides (11.11%)
06	Nigeria	80	44 (55%)	one box	Ciprofloxacin (51%) Norfloxacin (55%) Ofloxacin (40%)
07	Algeria	50	32 (64%)	Four boxes	Betalactamine /Tétracycline Bétalactamine /macrolide Sulfamide Aminoglucoside
08	Senegal	37	21 (50.5%)	Four boxes	Tetacycline (14%) Sulfamide (8%) Nitrofuraines (41%) Chloramphenicol (5%)
09	Tunisia	12	2 (16.66%)	Recepteur assay	Tétracycline Sulfamide Quinolone
10	Senegal	100	20 (20%)	Four boxes	Quantitative detection
11	Senegal	91	9 (10%)	Four boxes -thin-layer chromatography- Recepteur assay	Aminosides (8.33%%) Sulfamide (14.28%) Beta lactamine - Tetracyclines (17.86%) Beta lactamine – Macrolides (57.14%)
12	Algeria	90	54 (60%)	Four boxes	Betalactamine /tetracycline (37.5%) Sulfamide (18.75%) Aminoside (12.5%) Beta lactamine /macrolide(0%)
13	Algeria	30	16 (53.33%)	Four boxes/ high performance liquid chromatography	Beta lactamine (peni G) (100%) Macrolide (6.66%)
14	Morocco	50	18 (36.15 %)	High-performance liquid chromatography coupled to diode array UV detection and mass spectrometry / Four boxes	Tetracycline (26%) Sulfonamides (6%) Aminosides (6%) Macrolide / Beta lactamine (12%)
15	Nigeria	70	42 (60%)	Thin-layer chromatography	Quantitative detection
16	Turkey	127	58 (45.7%)	Enzyme-linked immunosorbent assay	Quinolone (45.7%)
17	Iraq	75	39 (52%)	Thin-layer chromatography	Oxytetracycline (24%) Sulfamide (24%) Neomycin (0%) Gentamycin (0%)
18	Palestine	365	88 (24.1%)	Four boxes	Tetracycline (43.15%) Aminoside (27.36%) Betalactamine (21%) Macrolide (8.42%)
19	Iraq	40	17 (42.5%)	Thin-layer chromatography	Sulfamide (27.5%) Oxytetracycline (20%) Neomycin (10%)
20	Bulgaria	462	35 (15.8%)	Four boxes	Quantitative detection
21	Algeria	50	18 (34%)	Four boxes	Tetracycline Erythromycin
22	Algeria	120	58 (69.6%)	Four boxes / Spectrophotometry	Quantitative detection
Total		2695	1220 (45.26%)		/

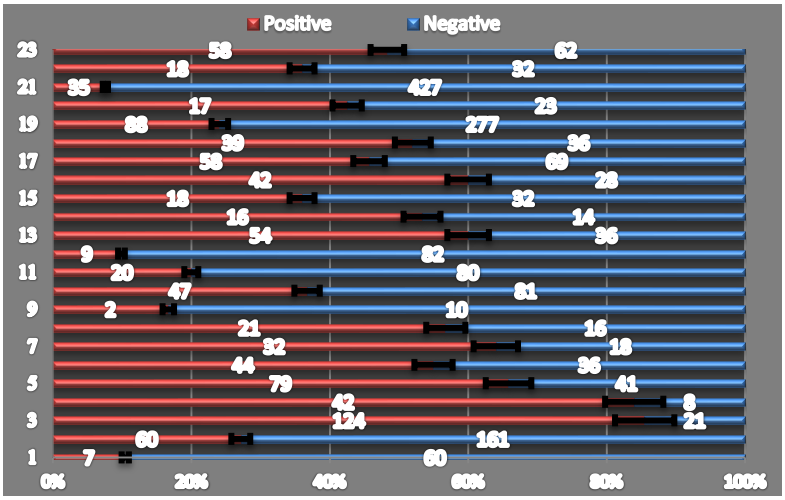

Figure 1. Recuperative results obtained of the detection of antibiotic residues used in poultry farming across the different studies in developing countries.

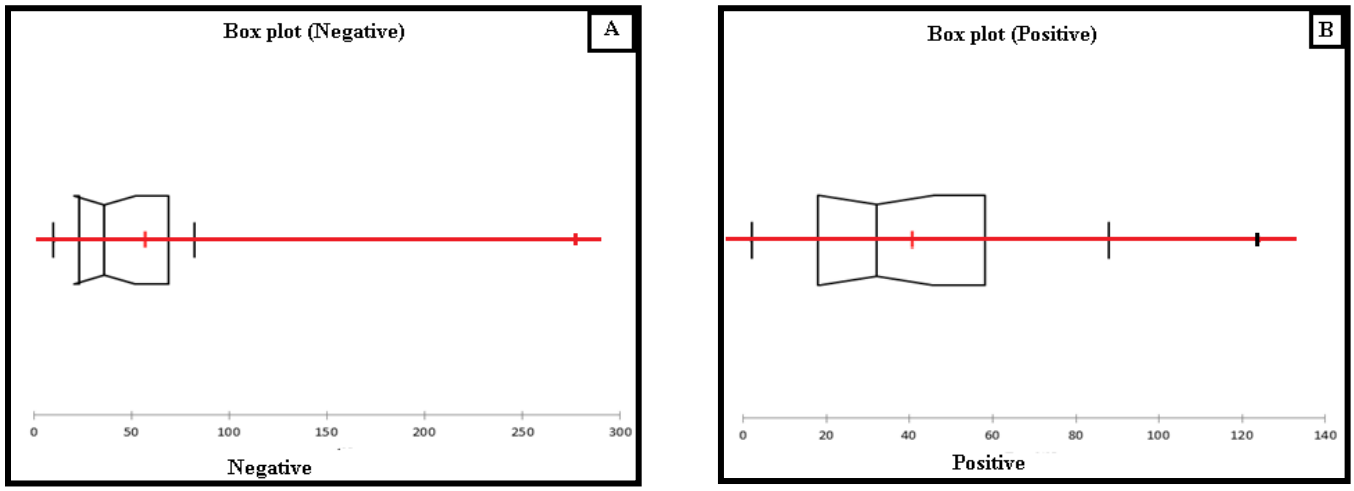

Figure 2. Results obtained from the detection of antibiotics used in poultry farming presented in Box Plot: (A) Positive cases, (B) Negative cases

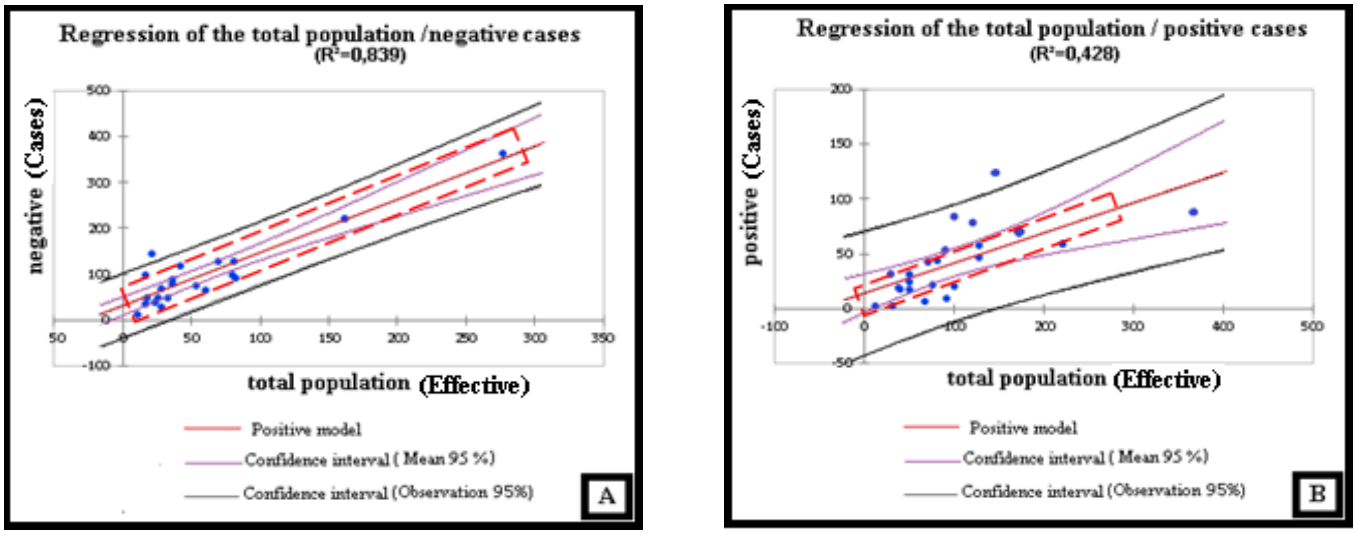

Figure 3. Modelisation of the results obtained from the detection of antibiotics used in poultry farming by linear regression in developing countries: (A) Negative cases**, (**B) Positive cases (R^2: correlation coefficient)

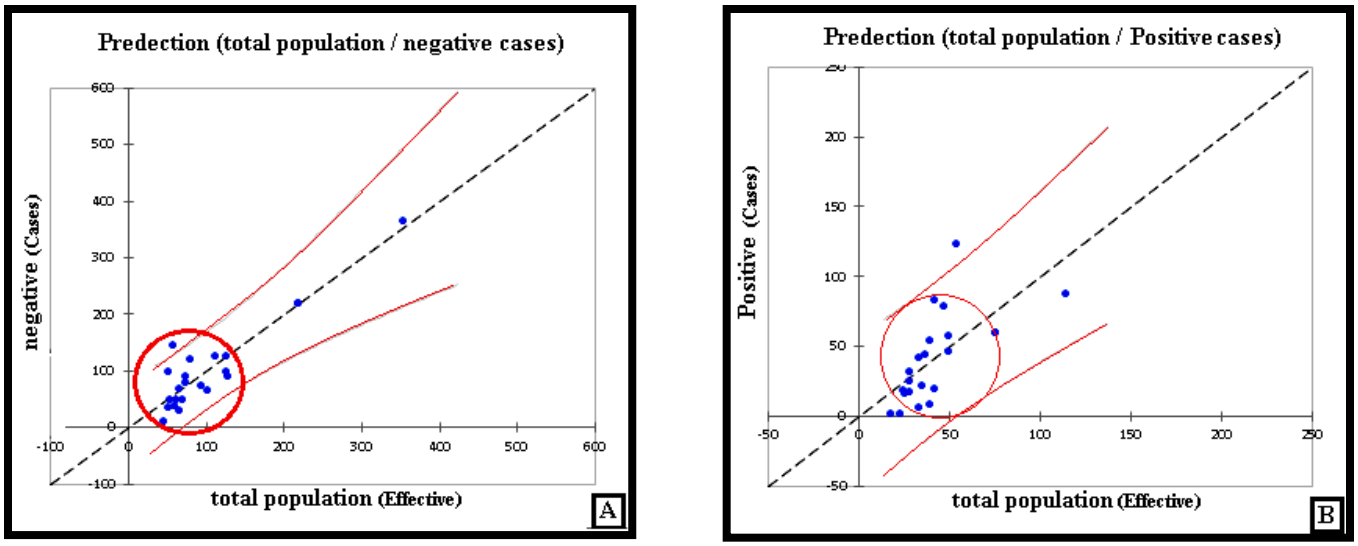

Figure 4. Results obtained from the detection of antibiotics used in poultry farming in developing countries (predetermination of the density of the values): (A) Positive cases**, (**B) Negative cases (Specified section: aggregation of prediction values)

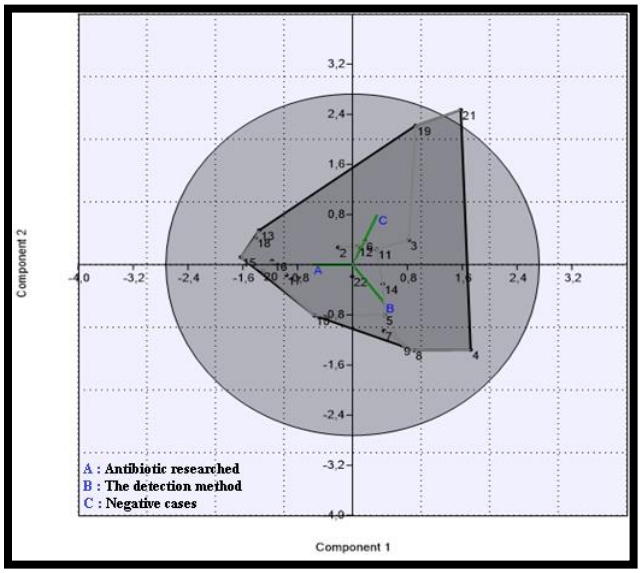

Figure 5. Factors affecting the precision of antibiotic residue detection used in poultry farming in different studies in developing countries (Analysis of the main compounds)

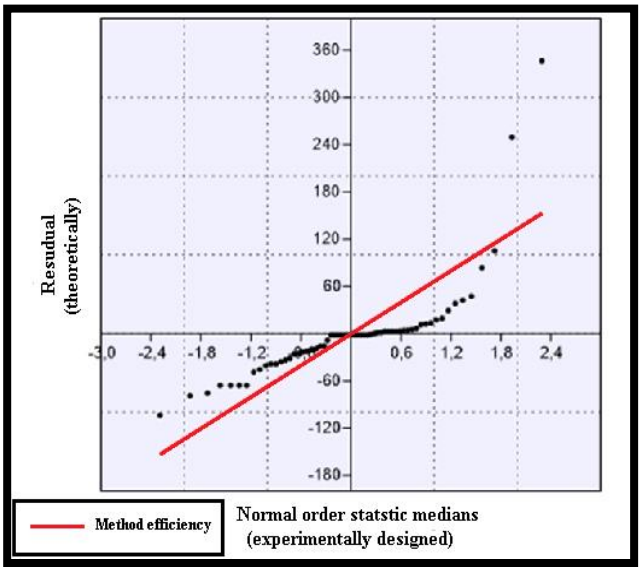

Figure 6. Effect of the Efficiency of the method in the detection of antibiotics used in poultry farming and the accuracy rate (Analysis of residual and normal order)

To properly compare the results for the two classes (positive and negative), The first step consists in developing a regression model for only 2 classes on the associated variable (Figure 3). In the second step, regression by Correlated Components is used to predict values based on detection methods to determine which ones are most reliable in the studies performed (Figure 4). Separate models were developed and then the models for the two classes were compared for the ideal explanation.

The use of this two-step method over two classes improves prediction compared to the traditional correlation coefficient method. The comparison between positive correlation coefficient (R^2 = 43%) and negative correlation coefficient (R^2 = 84%) since the variability of actual dependent detection (poultry samples) is explained by the variable 'detection methods' although both cases are statistically significant (R^2 = 0.84 and p=0.05**)**, for the confirmation of this hypothesis it is necessary to analyze

the main compounds statistically (sample size, type of antibiotic sought and method used for detection).

The interpretation of the statistical matrices (Figures 5 and 6) shows that the detection method is the impact factor on the results, for this purpose it can be said that the methods used by the authors are not generally reliable and that the sensitivity of the techniques influenced. Through the results of these previous studies, it must be taken into consideration that the correct determination of residues in white meat involves high-precision techniques.

DISCUSSION

Among African countries, the highest percentage of residues was observed in Algeria "Souk Ahras" (100%), while the lowest in Congo (10.7%). (Okombe et al., 2016; Berghiche et al., 2018). In Asian countries, a high residue rate was detected in Gaza (88%), while the lowest in Iraq (42.5%), while in Europe, the presence of residues was reported lowest level in Bulgaria (15.8%). (Al Pavlov et al., 2008; Shamsa, 2013; Elmanama and Albayoumi, 2016).

The high percentage of positive cases is due to non-compliance with waiting times, as well as self-medication of animals by farmers, who do not know the conditions and doses administered. This may be due to the misuse of antimicrobials probably related to animal treatment followed by insufficient withdrawal time (Corpet and Brugere, 1995; Bonfoh, 2003). Contamination of food of animal origin has been reported by many authors. Indeed, in Algeria, numerous studies have reported the presence of residues, in the Tizi Ouzou region according to Hakem et al. in 2015, sample analyses revealed the presence of 124 positive samples out of 145 collected, representing a percentage of 86.2%. In the same region, Ramdane (2015) reports a 62.5% positive sample rate. In the El Taref region, Mansouri (2007) found that 65.7% of his samples containing antibiotic residues. In the Algiers region, according to Ben Mohand (2007), 3.33% of the samples analysed contained residues of white meat.

A study conducted in northwest Algeria (Benghalem et al. (2016) found that 32 samples of chicken meat from three different regions (Tlemcen, Ain Témouchent and Sidi Bel Abbes) were positive, representing 64% of the total. At the international level, the problem of residues of veterinary drugs in foodstuffs of animal origin, particularly chicken meat, is a real problem. In Senegal, Bada-Alambedji et al. (2004) found 4 positive samples out of a total of 41 samples surveyed, representing a percentage of 9.8%. Two other studies conducted in Senegal (Dakar) by Abiola (2005) and N'kaya (2013) revealed positivity rates of 50.5% and 20% respectively. In Ankara, according to Er B1et al. (2013) 45 ,7% of the samples analysed were contaminated by residues. In Egypt Karmi (2014) revealed 42 positive samples out of a total of 50 samples analysed, i.e. a percentage of 83.33%. In Tunisia Rezguie (2009) found 2 positive samples, i.e. a rate of 16.66%. Two studies conducted in Nigeria by Omotoso (2015) and Ekene (2014) found the presence of antibiotic residues with positivity percentages of 55% and 60% respectively. In Khartoum State (Sudan) (Hind et al., 2014) revealed that 27% of samples were positive for the presence of antibiotic residues. A study conducted in Morocco (Chaiba et al., 2017) on a total of 50 poultry meat samples revealed that 18 samples were positive for the presence of antibiotic residues, representing a rate of 36.15%. Another study conducted in Palestine by Elmanama (2016) revealed 88 positive samples out of a total of 365 samples analyzed, a percentage of (24.1%).

In Bulgaria (Al Pavlov et al., 2008) found that 15.8% of samples are contaminated with antibiotic residues. Two other studies conducted in Iraq by Shareef et al. (2009) and Shamsa (2013) reported positive rates of 52% and 42.5% respectively. In Congo (Okombe, 2016) revealed that 7 positive samples out of a total of 67 samples of chicken meat tested, i.e. a rate of 10.44%. Depending on the method used, antibiotic residues were detected in 944 (37.38%) of the cases by a microbiological inhibition method, including 660 (69.91%) cases using the standard four-boxes method as the experimental protocol.

Four-boxes method has the advantage of being sensitive, with inexpensive reagents, and easy to carry out, because it allows the detection of the following four families of antibiotics, betalactamines and macrolides, betalactamines and tetracyclines; sulfamides and aminosides.

The other advantage of four boxes method is that it is less restrictive and less expensive than other microbiological methods. From our results the most important points that we have noticed, the detection of antibiotic residues in chicken meat requires firstly a high precision technique, a good understanding of the antibiotics marketed in the region that will be studied and a representative sample of white meat for a correct estimation.

CONCLUSION

The application of meta-analysis in biological domains becomes a qualitative leap, where this statistical technique

can give reliability and meaning in life science. Despite the fact that the meta-analysis requires a lot of time and that this technique is very complicated compared to reviews. The results obtained from our meta-analysis showed that the precision of the technique for detecting antibiotic residues in chicken meat is the key factor in determining the validity of the results and the strength of its authenticity, even though the variations in the data analyzed, the application of meta-analysis allowed to summarize the results and identify defects in the detection techniques, which encourages scientists in this field to apply it to other topics.

DECLARATIONS

Competing interests

The authors have no competing interests to declare.

Consent to publish

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

Author`s contributions

Berghiche wrote the paper, created the idea and the design the study, Labiad and Berghiche collected data, Berghiche achieves all statistical analysis and drafting of the manuscript. Khenenou and Berghiche read and approved the final manuscript.

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JWPR
Journal of World's Poultry Research

2019, Scienceline Publication
J. World Poult. Res. 9(2): 98-108, June 25, 2019

Research Paper, PII: S2322455X1900013-9

Microalgae Biomass Application in Commercial Broilers Nutrition and Their Efficacy Against Challenge with Epidemic Newcastle Disease Virus in Egypt

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Received: 07 May 2019
Accepted: 04 June 2019

ABSTRACT

Using microalgal biomass in animal diets has been studied recently. Many species of cultivated algae were found effective in maintaining animal growth performance, and in improving body weight. Using of microalgae collected from high rate algal ponds (HRAP) as a feed additive to broilers ration was studied. One hundred and twenty broiler chicks were divided into 6 groups of 20 birds, three of them have fed on balanced broiler ration supplied with 1% weight per weight (W/W) of microalgae biomass and have variable vaccination schemes of live attenuated and inactivated Newcastle disease virus (NDV) vaccines genotype II or either non-vaccinated control. In addition, the other 3 groups have fed on free microalgae biomass balanced ration with the same vaccination treatment. Furthermore, weight gain, antibody response, mortalities, viral shedding and normal viability of chickens were estimated in order to assess the efficiency of microalgae as a feed additive. The results showed that the microalgae have no hazard effect on feed and water intake as well as enhanced viability of chickens. And in regards to immune function and body weight, they have similar effect with the free microalgae groups in normal serological response and viral shedding post vaccination with NDV vaccines as well as similar protection rate and body weight gain. In conclusion, microalgae can be used in broiler ration with no deleterious effect on growth rate, weight gain, poultry viability and immune response. In conclusion dried microalgal biomass harvested from HRAP can be used in broiler ration with no deleterious effect on growth rate, weight gain, poultry viability and immune response. Furthermore, future studies should be applied with increasing microalgae percent in poultry feed up to 5, 10 or 20% (W/W) in order to assess better performance on poultry production.

Key words: Algae biomass**,** Immune response, Newcastle disease virus, Poultry feed

INTRODUCTION

Microalgae considered as a good source of a wide range of metabolites that are suitable for animal feed. These metabolites include protein, carbohydrate, fat, vitamins, minerals, and other organic compounds (Abdelnour et al., 2019; Andrade et al., 2018). Microalgae are identified as microscopic, unicellular and photosynthetic organisms and can grow in saline and fresh water which are rich source of nutrients and biologically active compounds, including proteins, amino acids, polyunsaturated fatty acids (LCPUFA n-3), microelements, vitamins, antioxidants, as well as carotenoids which have a long history of application as a food for human (Belay et al., 1996). Microalgae of different species can be successfully included into poultry diets, for example as a defatted biomass byproduct from biofuel production, which can have a beneficial influence on birds' health, performance, and the quality of meat and egg (Abdelnour et al., 2019). Especially important for the poultry industry are recent studies where microalgal biomass was efficiently used in the production of eggs containing health-promoting lipids, i.e. eggs enriched with health promoting long-chain n-3 polyunsaturated fatty acids (LCPUFAs n-3) (Wu et al., 2012). Microalgae has many advantages including high growth rates, growth potential during the year and minimal land and water requirements compared to crop plants (Rawat et al., 2011). Furthermore, the high capital costs for large scale production of microalgal biofuel currently

precludes this when coupled with wastewater treatment, it may become viable in the future (Rawat et al., 2011; Benemann, 2008).

High Rate Algal Ponds (HRAP) is an advanced open pond system that was found to offer more effective wastewater treatment technologies, since it performs high removal rates for nutrients, in addition to lower capital and operation costs, in comparison with traditional treatment ponds (Sutherland et al., 2014; Craggs et al., 2014). HRAP systems have the added advantage for resource recovery from the wastewater, via algal biomass, using as fertilizer, feed or as a feedstock for biofuel production, with the later use receiving considerable attention in recent years (Craggs et al., 2014; Park and Craggs, 2011).

Newcastle Disease Virus (NDV) causes one of the most important infectious diseases of poultry, Newcastle Disease (ND) found worldwide and causes losses from mortality and condemnation of carcasses (OIE, 2012). There are many countries that have endemic NDV, with increasing occurring year after year. Also known as avian paramyxovirus serotype-1 virus, NDV is a member of the genus Avulavirus in the Paramyxoviridae family (Mayo, 2002). Feeding of β-glucan derived from micro algae resulted asignificant increase in the antibody titer against NDV and Infectious Bronchitis Virus (IBV). It was suggested that dietary β-glucan and BA-pro might be useful in treating against viral diseases because of immunostimulating activity as reported by An et al. (2008).

The objective of the present study was to investigate the dietary effects of micro-algae biomass on growth performance, humoral immunity and viral shedding against challenge with velogenic NDV (VDNV) genotype VII in broiler chicks.

MATERIALS AND METHODS

Ethical approval

The animal experiment was conducted in strict accordance with and adherence to the relevant policies regarding animal handling as mandated under international national, and /or institutional guidelines for the care of animals and was approved by the research ethical committee at the national research centre, Cairo, Egypt.

Algal biomass dominated by *Microcystis* sp.

Race way-type pond (HRAP) made of glass-fiber reinforced plastics material, with 6.5 m^3 capacity without connection or separation. Its dimensions are 7m length × 3m width × 40cm depth and the effective wastewater depth is 0.3 m. The HRAP was constructed and installed in Zenin wastewater treatment plant- Giza Company for water and wastewater (Figure 1). Algal biomass collected from HRAP (1 m^3) is harvested biweekly and precipitated by cationic starch. Algal biomass was dried (using sun drier) and grinded to fine particles (0.1 mm).

Figure 1. High rate algal pond system used for water treatment an algal biomass production

Identification of algal community contained in the algal biomass

Along one year, samples were collected twice a week and subsamples were dispensed into glass Sedgewick-Rafter cells and examined using OLYMPUS CX41® microscope (20×power). Species composition and dominance in the samples were determined semi-quantitatively. Algal identification has been done according to the main references used in phytoplankton identification (Streble and Krauter, 2006).

Toxicity study for coagulants used in the precipitation of algae

Animals

Thirty Swiss mice with 25g average body weight were housed in standard cages (six rats), under specific pathogen-free conditions in facilities maintained at controlled room temperature (21-24°C) with a 40-60% relative humidity and under normal environmental dark–light cycles (from November to March, 7-9 hours of light and from April to October 9-12 hours of Light in Cairo, Egypt). All animals had free access to rat chow diet and water *ad libitum* and were acclimated for two weeks prior to initiation of the experiment in the laboratory of in the national research centre.

Acute toxicity study. Selected thirty mice of uniform weight are taken and divided into five groups each of six. The coagulants (cationic starches 1, 2, 3 and 4) and aluminum sulfate were dissolved in distilled water then given orally to four groups of mice in graded doses (1gm, 2gm, 3gm 4gm) up to 5 g/kg (El Naggar et al., 2018). In

addition, the Non-Polar Fraction of dried Algal biomass (NPFA) was suspended in distilled water then given orally to rats in graded doses up to 5 g/kg. The control group received the same volumes of distilled water. The percentage mortality for extracts was recorded 24 hours later (Desoukey et al., 2016). Observation of rats for 14 days, for any changes in the skin and fur, respiratory, circulatory, autonomic, central nervous systems, somatomotor activity and behavior pattern. Particular observation for tremors, convulsions, salivation, diarrhea, lethargy, sleep, and coma were done (El Naggar et al., 2018).

Table 1. Composition of the experimental diets applied in broiler feed

Item	Starter (1 to 21 day)	Grower (22 to 40 day)
Ingredient (%)		
Corn	59.2	64.65
Soybean meal	30.0	24.0
Corn gluten meal	2.5	3.0
Soybean oil	2.8	3.0
Limestone	1.2	1.2
Dicalcium phosphate	1.7	1.6
Vitamin-mineral premix*	1.0	1.0
Salt	0.3	0.3
l-Lysine	0.15	0.15
dl-Methionine	0.15	0.1
Algae	1.0	1.0
Analyzed chemical composition		
ME (kcal/kg)	3,122	3,188
CP (%)	20.72	18.93
Ether extract (%)	4.14	5.79
DM (%)	89.1	88.4
Lysine (%)	1.17	1.0
Methionine (%)	0.5	0.41
Methionine + cysteine (%)	0.65	0.6
Threonine (%)	0.7	0.6
Tryptophan (%)	0.17	0.16
Calcium (%)	0.9	0.84
Phosphorus (%)	0.6	0.5

* Vitamin-mineral mix (per kilogram of diet): vitamin A: (10000 IU/g) 8000 IU, vitamin D3: (200000 IU/g) 1600 IU, vitamin E: (20000I U/g) 11 mg, rboflavin: (53 mg/g) 9 mg, α calcium pantothenate: (80 g/lb), vitamin B12: (60 mg/g) 13 mg, niacin: 26 mg; choline chloride: (50%) (74% choline) 900 mg, vitamin K: (Hetrazeen 35.27 mg/g) 1.5 mg, folic acid: (13.23 mg/g) 1.5 mg, biotin: (2%) 0.2 mg, santoquin: (25%) 125 mg, manganous oxide: (56% Mn) 55 mg, selenium premix: (200 mg/kg) 0.1 mg, zinc oxide: (80% Zn) 50 mg, copper sulfate: (25% Cu) 5 mg, ferrous oxide: (69.94% Fe)30 mg, ME: (kcal/kg) metabolizable energy (kilocalorie per kilogram), CP: crude protein, DM: Dry matter

Determination of carbohydrates content

Total carbohydrate content of algal biomass was determined where 0.1g of sample was treated with 25ml of 1 normal sulfuric acid (1N H_2SO_4) was added and the mixture was hydrolyzed for two hours on a boiling water bath. At the end of hydrolysis, a flocculent precipitate was noticed. This was freed of sulphate by precipitation with barium carbonate. The mixture was filtrated and completed to 100ml. one ml of the filtrate mixed with one ml 5% phenol and 5 ml concentrated H_2SO_4 (sulfuric acid). The absorbance was measured spectrophotometrically at wave length at 485nm (Albalasmeh et al., 2013).

Determination of total protein content

Total protein content was determined by kjeldahl method and then multiplied with a factor of 6.25 to convert total measured organic nitrogen to total Protein to give the total protein (Stephen et al., 2013).

Viruses and vaccines

Challenge virus. Very Virulent (vv) NDV used in the challenge was kindly supplied by poultry diseases department, veterinary research division, National Research Centre, Dokki, Egypt, characterized by sequencing as vvNDV genotype VIId designated as NDV/Chicken/EG-MN/NRC/2015 under accession number (MF418020.1) on gene bank. The virus challenge dose equal 6-Log-10 EID_{50} given 0.5 ml / bird via Intramuscular route (IM) (OIE, 2012).

Live NDV vaccine. Freeze-dried vaccine containing live NDV LaSota strain (Jovac ND LaSota ® 1000 doses, Jordan) supplied by local agency. The vaccinal dose equal 6-Log^{-10} EID_{50}/ bird was given via occulonasal route as recommended by manufacturer.

Inactivated NDV genotype II vaccine. Inactivated NDV genotype II vaccine (Boehringer Ingelheim® AI ND 1000 doses, Germany) supplied by local agency. The vaccinal dose equal 8.2-Log-10 EID_{50} given 0.5ml / bird via subcutaneous route (SC) as recommended by manufacturer.

Serology

Blood was collected pre and post challenge from all birds at 14, 21, 28 and 35 days of age, serum was extracted then tested by Hemagglutination Inhibition (HI) assay. The HI assay was performed using LaSota NDV antigen

according to standard procedures with four haemagglutination units virus/antigen in 0.025 ml (OIE, 2012).

Virus shedding

Virus Isolation (VI) was performed to identify virus shedding conducted on oral and cloacal swabs as All VI-positive swabs were titrated in 9–11 days old specific pathogen free embryonated chicken eggs. Virus titers were calculated reported as mean embryo infectious dose (EID_{50}/ 0.1 ml) on a Log 10 scale (OIE, 2012).

Experimental design

One hundred and twenty broiler chicks (Cobb 500®) one day-old supplied from commercial hatchery, were divided into six groups of 20 birds in separate units with strict biosecurity level. Conventional animal welfare regulations and food standards were taken into account (Table 2).

Table 2. Experimental design for usages of microalgae biomass in Broilers diet treated with different vaccines of Newcastle disease against velogenic Newcastle disease virus challenge

Group no.	Birds no.	Vaccination regime		Microalgae Addition with 1% In feed	Challenge at age / day [c]
		Type	Age / days		
1	20	Live GII [a]	5 and 18	+	28
2	20	Live. GII Inact.NDV.GII [b]	5 and 18 7	+	28
3	20	None	None	+	28
4	20	Live GII	5 and 18	None	28
5	20	Live. GII Inact.NDV GII	5 and 18 7	None	28
6	20	None	None	None	28

[a] Live NDV vaccine genotype II (LaSota strain) . The vaccinal dose equal 6-log-$_{10}$ EID_{50} / bird given via occulonasal route, [b] Inactivated oil emulsion NDV vaccine genotype II (LaSota strain). The vaccinal dose equal 8.2-Log-$_{10}$ EID_{50} given 0.5 ml / bird via SC route, [c] Challenge with velogenic Newcastle disease virus (genotype VII). The virus challenge dose equal 6-Log-$_{10}$ EID_{50} given 0.5 ml/bird via IM route. no: number

RESULTS

Algal dynamics and predominance in the high rate algal pond

The high rate algal pond operation started on 30 June 2017 (summer months) which lead to the predominance of *Microcystis flos aquae*, *Microcystis aeruginosa* and other species of *Microcystis*. Always, different *Microcystis* sp. represent the upper most layer, while near the bottom layer of the pond different green and diatoms group are present. Many species include *Scenedesmus obliquus* and *Scenedesmus quadricauda*, *Ankistrodesmus*, *Coelastrum microporum*, *Selenastrum* and *Micractinium pusillum* (green algae group), *Oscillatoria limnetica* (blue green algae group) and *Nitzschia linearis* (diatoms group) was the most pre dominant species. Since the beginning of November 2017, the community structure of HRAP completely changed where the *Microcystis* sp. disappeared and the dominance of different algal species took place. In addition, all algal species mixed and floated in pond water column. The predominant algal species are *Scenedesmus obliquus*, *Scenedesmus quadricauda*, *Ankistrodesmus*, *Coelastrum microporum*, *Selenastrum*, *Oocystis parva*, *Dictyosphaerium pulchellum*, *Coelastrum reticulatum*, *Pediastrum gracillimum*, *Siderocells elegans*, *Eudorina elegans*, *Clamydomonas reinhardi* and *Micractinium pusillum* (green algae group), *Euglena sp.* (Euglenophyta), *Oscillatoria limnetica* (blue-green algae group) and *Nitzschia linearis* (diatoms group) by microscopical examination.

Along one year, algal biomass (from inside the algal pond) were harvested biweekly and precipitated using cationic starch and then dried. The dried algal biomass grinded to fine particles (0.1mm) and mixed thoroughly.

Acute toxicity study

The results obtained no mortality after 24h of oral administration of NPFA at increasing doses up to a 5 g/kg. Also, after 15 days of single oral administration of cationic starch as well as NPFA, the results revealed that no

obvious changes were detected in skin and fur, respiratory, circulatory, autonomic, central nervous systems, as well as somatomotor activity and behavioral pattern were apparently normal.

Proteins and carbohydrates content were detected in order to evaluate the possibility of using the biomass for feeding properties. Total protein content detected was ranging from 232.1 to 371.3 mg/gm. Referring to carbohydrates content, total carbohydrates content was ranged from 100-138 mg/gm. Microalgae was reported to have high percentage of proteins, lipids and carbohydrates and could be used in the manufacture of different products. Microalgae can be grown in open ponds or closed photobioreactors (Kit et al., 2017; Koopmans, 2013).

Serological response and hemagglutination inhibition assay

Similar immunological response was detected between treated and non-treated algae groups. HI geometric means titers were ranged from 2.81 to 5.43 versus 2.83 to 5.23 HI titers in G1 and G4, respectively. While, ranged from 3.93 to 6.64 versus 3.86 to 6.61 HI titers in G2 and G5, respectively. Whereas, non-vaccinated infected controls G3 and G6 were, 1.73 to 1.91 HI titers against 1.36 to 1.86 HI titers, respectively before and after challenge (Table 3).

Table 3. Serological response of different vaccines against velogenic Newcastle disease virus challenge after algal biomass addition in broilers diet

Group no.	Birds no.	Vaccination regime		Microalgae Addition with 1% In feed	Challenge at age/day [c]	HI [d] titre means Log-2 at age/days (N = 5)			
		Type	age/days			14	21	28	35
1	20	Live GII [a]	5 and 18	+	28	2.81	3.56	4.42	5.43
2	20	Live GII Inact.NDV GII [b]	5 and 18 7	+	28	3.93	4.86	5.21	6.64
3	20	None	None	+	28	1.73	1.61	1.91	NT
4	20	Live GII	5 and 18	None	28	2.83	3.61	4.33	5.23
5	20	Live GII Inact.NDV GII	5 & 18 7	None	28	3.86	4.52	5.36	6.61
6	20	None	None	None	28	1.36	1.65	1.86	NT

[a] Live NDV vaccine LaSota strain. The vaccinal dose equal 6-log$_{10}$ EID$_{50}$/bird given via occulonasal route. [b] Inactivated oil emulsion NDV vaccine genotype II. The vaccinal dose equal 8.2-Log$_{10}$ EID$_{50}$ given 0.5 ml/bird via SC route. [c] Challenge with velogenic Newcastle disease virus (genotype VII) and The virus challenge dose equal 6-Log$_{10}$ EID$_{50}$ given 0.5 ml/bird via IM route. [d] HI titre ≤ 2 Log- 2 considered negative (OIE, 2012). N: Number of tested samples. no: Number, NT: None tested.

Protection from clinical disease and post-mortem gross lesions

The objective of this study was to estimate the effect of microalgae in poultry feed on viability, weight gain and enhancement of immune response and lesser shedding of viral infection that already circulated in field. Accordingly, chickens in all groups were monitored for one-week Post-Challenge (PCH). Vaccinated-challenged groups (G1, G2, G4 and G5 either with or without algae in feed, respectively) indicated similar degree of protection against clinical disease in the form of mild respiratory signs, dull appearance and decreased in feed and water intakes for three days of PCH. While non-vaccinated-challenged groups (G3 and G6 with or without algae in feed, respectively) showed more severe signs of foamy conjunctivitis, swollen eye lids, respiratory sounds, slight depression and marked decrease in feed and water intake. Obvious greenish diarrhea, marked depression with sleepy appearance at three days PCH. Severe respiratory sounds, conjunctivitis, nasal discharge four days PCH, and paresis ended by nervous signs (torticollis) seven days PCH.

On the other side, all dead birds were necropsied for Post-Mortem (pm) gross lesions assays revealed that, vaccinated-challenged birds showed mottled and enlarged spleens with either hemorrhagic spots and/or petechial hemorrhages on the proventricular glands (Figure 2). While non-vaccinated infected controls had severe pm gross lesions including severe congestion in the trachea, necrosis and congestion of liver, mottled and enlarged spleen, either hemorrhagic spots and/or petechial hemorrhages on the proventricular glands and enteritis with greenish intestinal contents (Figure 2).

Body weight value of microalgae

Since the nutritional value of microalgae varies considerably with the species used, 1% treated algae ration was not enough to add value in compare with non-treated groups (Table 4). The G1 average Body Weight **(**BW) in

compared with G4 was 1.43 kg versus 1.46 kg, respectively. Whereas, G2 in compared with G5 was 1.41 versus 1.42 kg, respectively. Furthermore, non-vaccinated controls G3 1.3 versus G6 1.25 kg, respectively all at 28 days of age. In addition to, viability of chickens and rate of feed and water consumption was nearly equal and in normal if comparing algae and non-algae treated groups.

Protection against mortalities

One of the major purposes of this study was to determine the role of micro algae treated ration in enhancement of better viability and fair protection against mortalities when challenge birds with vvNDV post vaccination regimes. Table 5 indicates 95% of non-vaccinated infected controls were dead at one week PCH in both G3 and G6. While, mortalities in G1 compared with G4 was 60% versus 55%, respectively. Furthermore, was 25% in both G2 and G5 one week PCH.

Virus shedding

Viral shedding is one of the most relevant parameter in judgment of vaccine efficacy in correlation with the differences in immunological response obtained from microalgae supplement. Accordingly, No obvious differences were detected in bio-algae feeded groups in compared with non-feeded ones. Viral titers (Log 10) expressed as mean embryo infectious doses per 0.1 ml in G1 against G4 were 4.1 and 5.7 versus 4.2 and 5.6, respectively at three and seven days PCH. While, in G2 and G5 were 2.9 and 4.1 versus 2.8 and 4.2, respectively at three and seven days PCH. In addition, viral titer was 6.5 in both non-vaccinated challenged controls (G3 and G6) three days PCH (Table 6 and Figure 4).

Table 4. Weight gain after algal biomass addition in broilers diet treated with different vaccines against velogenic Newcastle disease virus challenge

Group no.	Birds no.	Vaccination regime		Microalgae Addition with 1% In feed	Challenge at age / day [c]	Average BW in kilograms at 28 days of age
		Type	Age / days			
1	20	Live GII [a]	5 and 18	+	28	1.43
2	20	Live GII Inactivated NDV GII [b]	5 and 18 7	+	28	1.41
3	20	None	None	+	28	1.3
4	20	Live GII	5 and 18	None	28	1.46
5	20	Live GII Inactivated .NDV GII	5 and 18 7	None	28	1.42
6	20	None	None	None	28	1.25

[a] Live NDV vaccine LaSota strain. The vaccinal dose equal 6-log-$_{10}$ EID_{50} /bird given via occulonasal route. [b] Inactivated oil emulsion NDV vaccine genotype II. The vaccinal dose equal 8.2-Log-$_{10}$ EID_{50} given 0.5 ml / bird via SC route. [c] Challenge with velogenic Newcastle disease virus (genotype VII), The virus challenge dose equal 6-Log-$_{10}$ EID_{50} given 0.5 ml / bird via intramuscular route. BW: body weight; no: Number.

Table 5. Mortality percent after algal biomass addition in broilers diet treated with different vaccines against velogenic Newcastle disease virus challenge

Group no.	Birds no.	Vaccination regime		Microalgae Addition with 1% In feed	Challenge at age/day [c]	Mortalities one week PCH	
		Type	age/days			no.	%
1	20	Live GII [a]	5 and 18	+	28	12	60
2	20	Live GII Inactivated NDV GII [b]	5 and 18 7	+	28	5	25
3	20	None	None	+	28	19	95
4	20	Live GII	5 and 18	None	28	11	55
5	20	Live GII Inact.NDV GII	5 & 18 7	None	28	5	25

6	20	None	None	None	28	19	95

[a]Live NDV vaccine LaSota strain. The vaccinal dose equal 6-log-$_{10}$ EID_{50}/bird given via occulonasal route. [b]Inactivated oil emulsion NDV vaccine genotype II. The vaccinal dose equal 8.2-Log-$_{10}$ EID_{50} given 0.5 ml / bird via subcutaneous route (S/C). [c]Challenge with velogenic Newcastle disease virus (genotype VII). The virus challenge dose equal 6-Log-10 EID50 given 0.5 ml / bird via intramuscular route. PCH: post challenge, no: Number.

Table 6. Viral shedding after algal biomass addition in broilers diet treated with different vaccines against velogenic Newcastle disease virus challenge

Group no.	Birds no.	Vaccination regime		Microalgae Addition with 1% In feed	Challenge at age/day [c]	[d] Shedding at days post-challenge	
		Type	age/days			3 days (OP swabs)	7 days (CL swabs)
1	20	Live GII [a]	5 and 18	+	28	4.1	5.7
2	20	Live GII Inactivated NDV GII [b]	5 and 18 7	+	28	2.9	4.1
3	20	None	None	+	28	6.5	NT
4	20	Live GII	5 and 18	None	28	4.2	5.6
5	20	Live GII Inact.NDV GII	5 & 18 7	None	28	4.8	4.2
6	20	None	None	None	28	6.5	NT

[a] Live NDV vaccine LaSota strain. The vaccinal dose equal 6-log-$_{10}$ EID_{50} / bird given via occulonasal route, [b] Inactivated oil emulsion NDV vaccine genotype II. The vaccinal dose equal 8.2-Log-$_{10}$ EID_{50} given 0.5 ml / bird via subcutaneous route, [c] Challenge with velogenic Newcastle disease virus (genotype VII). The virus challenge dose equal 6-Log-$_{10}$ EID_{50} given 0.5 ml/bird via intramuscular route, [d] Viral titers (log10) expressed as mean embryo infectious doses per 0.1 ml from oral and cloacal swabs taken at day 3 and 7 post-challenge from the challenged birds. OP: oropharyngeal, CL: cloacal, NT: None tested, no: number of birds in each group.

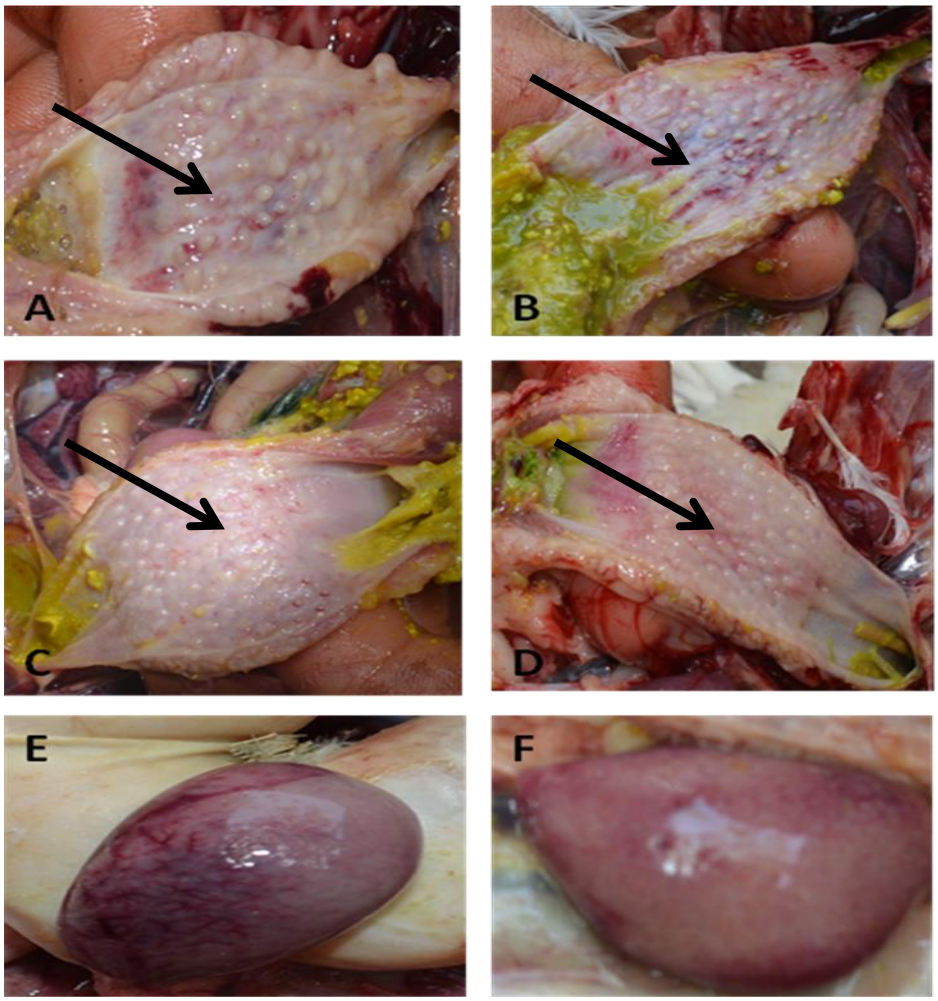

Figure 2. The post mortem lesions in broiler chickens treated with different vaccines of Newcastle disease against velogenic Newcastle disease virus challenge. **A** and **B:** Petechial hemorrhage on proventricular glands of infected controls Groups 3 and 6, respectively at 4 days after challenge. **C** and **D**: petechial hemorrhage on proventricular glands of infected vaccinates Groups 2 and 5, respectively at 5 days after challenge. **E** and **F**: mottled and enlarged spleen of infected vaccinates groups 1 and 4, respectively at 5 days after challenge.

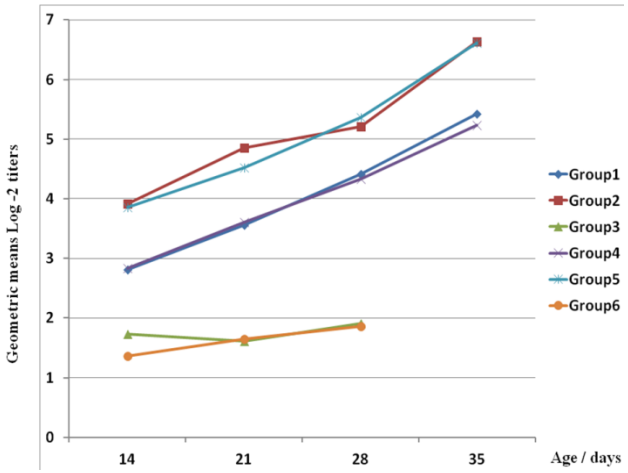

Figure 3. Serological response evaluation after microalgae biomass application in commercial broilers feed and received different Newcastle disease virus vaccination schemes against velogenic Newcastle disease virus challenge at 14, 21, 28 and 35 days post challenge with haemagglutination titers of the same rang of both algae-treated groups or no-algae feeded ones either challenged or non-infected controls.

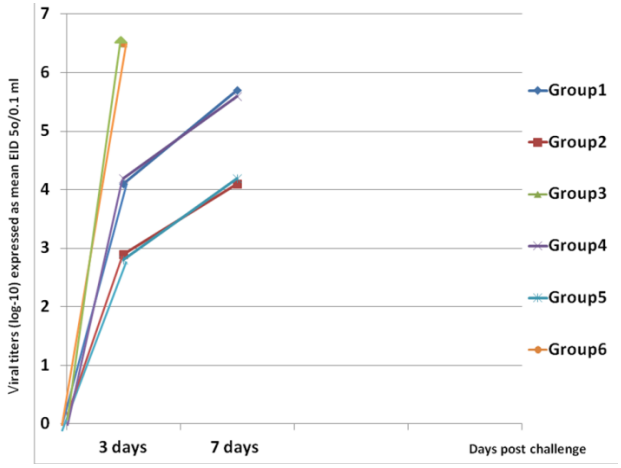

Figure 4. Viral shedding after velogenic Newcastle disease virus challenge at 3 and 7 days from oropharyngeal and cloacal swabs for evaluation of microalgae biomass application effect in commercial broilers feed treated with different Newcastle disease virus vaccination schemes. Showing viral titers of the same rang of both algae-treated groups or no-algae feeded ones either challenged or non-infected controls.

DISCUSSION

Using microalgal biomass in feeding animals has recently applied in poultry as Microalgae improve skin color, shanks and egg yolks. Numerous nutritional and toxicological evaluations revealed the appropriateness of algal biomass as a valuable feed supplement or used instead of conventional protein sources (soybean meal, fish meal, rice bran, etc.) (Becker, 2007). In addition to, using algae as a protein source for livestock, many were used for health benefits (i.e. improved immune response, improved fertility, better weight control, healthier skin and a lustrous coat) (Pulz and Gross, 2004).

This study aims to explore the effects of dietary micro algae supplementation in broiler chicks as enhancer of BW and immune status in broilers in a challenged setting. No considerable differences were found in BW among the treatment groups with 1% bioalgae supplement (Andrade, 2018; Abdelnour et al., 2019). Nonetheless earlier studies have reported changeable results debating the effects of micro algae on performance in poultry. Zhang et al. (2008) found that β-glucans extracted from the algae at the percentage of 50 and 75 mg/kg in the diet increased BW. In a similar work, (Rathgeber et al., 2008) observed higher BW in broilers that fed on β-glucan during the growth phase. On the other hand, other researchers reported low performance after B-glucan supplementation in chickens (Huff et al., 2006). In harmony with our findings, several cases reported no significant effects of micro algae on growth performance where algae feeding do not negatively affect performance in either non-challenged reports (Cheng et al., 2004; Chae et al., 2006; Morales-Lopez et al., 2009) or challenged settings (Chen et al., 2006; Chen et al., 2008). The conflicting results found in those studies could be due to differences in the source of the algae or the presence and type of challenge used, or both.

The use of live and inactivated vaccines has been used in the prevention and control of NDV (Zhao et al., 2014). Vaccines induce antibody production of IgA, IgG, and IgM to high levels of vaccinated chicks (Russell and Ezeifka, 1995). Because micro algae can play a role in stimulating these immune responses, the use of algae products in poultry vaccinated with NDV has been studied. In a study by An et al. (2008), algal B-glucan was fed to NDV vaccinated broilers at levels of 0, 0.025, and 0.1% for 35 days. The inclusion of algal B -glucan

significantly increased (P<0.05) at day 35 BW of NDV vaccinated broilers than control. Additionally, NDV specific antibody titers were elevated through the supplementation of algal B-glucan at 0.1%. The cell mediated immune response was suggested by (Cheng et al., 2004) to be improved by algal β-glucan through modulating macrophage activity. These observations are supported by (An et al., 2008) who also observed an increase in day 35 Newcastle virus antibody titers when feeding algal B-glucan at 0.05 and 0.1%, suggesting dosage level of β-glucan impacted the increase in antibody titers. These data confirm that an algal supplement can improve early performance parameters in a non-challenge setting, increase Newcastle virus specific antibody titers, and eliminate growth performance reductions in Eimeria challenged broilers. These data also confirm that dosage level of algae plays a significant role in performance effects in both non-challenged and challenged settings. These three experiments demonstrate the effectiveness of micro algae in poultry production although additional research must be conducted to further confirm proper dosage level. The results of Morales-Lopez et al. (2009) supporting our results, revealing that no benefits to performance or NDV specific antibody titers of a purified algae when fed to NDV vaccinated broilers.

In recent work and in agreement with our study Evans et al. (2015) showed that the incorporation of 16% of dried algae into a broiler diet has no negative effects on the performance of chicks. In addition, (Ross and Dominy, 1990) showed similar results in his work where found that no significant differences in performance of broilers fed a diet containing 1.5, 3, 6 or 12% dehydrated algae in feed. As well as, (Raach-Moujahed et al., 2011) also reported that fed with or without 4 or 8% of algal biomass in the diet has no difference in growth performance of broilers. In contrast, (Shanmugapriya et al., 2015) recently observed improvement in body weight gain and food conversion rate in broilers when fed a diet with algal biomass. Furthermore, Mariey et al. (2012) reported that including algal biomass in low concentration (0.02 or 0.03%) not only improved performance in broilers, but also increased dressing percentage, meat color score, blood morphology was improved and relative abdominal fat weight was decreased.

A lot of projects in many countries are going on since the algal biomass production has concerned, currently there are lot of projects going on in many countries. We concluded that, the present study has established the safety and acceptability of micro algae biomass as poultry feed up to 1% replacement and may be incorporated into practical broiler diets that utilized by broiler chicks without causing detriment to performance, immunity or either viability and body weight gain. So as to, further research is required to identify the optimal dosage of microalgal biomass supplementation for consistent favorable results in poultry.

DECLARATIONS

Acknowledgments

The authors sincerely thank the academy of scientific research and technology, ministry of higher education, Egypt, for the kind support and providing infrastructure facilities to carry out this work. This work was developed within the scope of the financed project entitled “novel approach to maximize the use of stabilization pond in Egypt: A model for water and energy food nexuses” ID: 1343.

Author’s contribution

Sayeda, Sameh, Hagar, Rehab, Abeer and Mohamed Kutkat participated in design, experimental procedure, writing, revised, and reviewing the manuscript.

Competing interests

The authors have declared that no competing interest exists

Consent to publish

The authors guarantee that this work has not been published elsewhere and that any person named as a coauthor of this work is aware of the fact and has agreed to be so named.

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JWPR
Journal of World's Poultry Research

2019, Scienceline Publication

J. World Poult. Res. 9(2): 109-116, June 25, 2019

Research Paper, PII: S2322455X1900014-9

Preparation of Necrotic Enteritis Vaccine for Turkey

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Received: 14 May 2019
Accepted: 12 June 2019

ABSTRACT

Clostridium perfringens is the most important cause of enteritis in domestic animals, in chicken and turkey it well known as pathogen responsible for necrotic enteritis; hepatitis, and cholecystitis. The disease in turkey characterize by either severe form with high rate of mortalities or subclinical form of reduce growth rate and increase condemnation rate. The major factor responsible for pathogenicity of *Clostridium perfringens* was alpha toxin. The aim of present study was to prepare of *Clostridium perfringens* alpha Toxoid vaccine for controlling the necrotic enteritis disease. The vaccine was prepared at different doses depend on lethality of toxin (24, 48 and 96 Minimum Lethal Dose) for controlling necrotic enteritis disease. Antibody titer elicited by vaccination was measured by toxin neutralization test, ELISA, and challenge test. It revealed that antibody titer expressed by international antitoxin unit per ml was 7.4, 4.1 and 1.26 respectively according to the mentioned dose, and also the protection percent against challenge was 100% when vaccinated with either 48 or 96 Minimum Lethal Dose, while it gave 80% when vaccinated with 24 Minimum Lethal Dose.It concluded that use of *Clostridium perfringens* alpha Toxoid with recommended dose of 48 MLD able to protect turkey for 6 months.

Key words: Alpha toxin, *Clostridium perfringens*, Turkey, Type A, Vaccine

INTRODUCTION

Enteric disorders were one of the most important groups of diseases that affect poultry and continuing to cause high economic losses in many areas worldwide due to increased mortality rates, decreased weight gain increased medication costs and increased feed conversion rate. The causal of enteric disorders either pathogens (viruses, bacteria, parasites) as a monocausal due to specific pathogen or synergy with different other microorganisms (multicausal) or non-infectious causes such as feed and /or management related factors. One of the most important pathogens cause severe enteric disorders either mono or multicausal infection is *Clostridium perfringens* (*C. perfringens*) type A that causes severe clinical manifestation and lesions include necrotic enteritis; necrotic dermatitis; cholangiohepatitis as well as gizzard erosion in addition it had been indicated to cause food poisoning in human (Lovland and Kaldhusdal**,** 2001; McClane et al., 2006; Novoa-Garrido et al., 2006). The ban and voluntary withdrawal antibiotics have resulted in resurgence of necrotic enteritis. Moreover, the demands of consumers for antibiotic free poultry products has continued to grow (Mwangi et al., 2018)

The necrotic enteritis can be caused by one or number of predisposing factors such as the high levels of non-starch polysaccharides and fishmeal, pathogenic *Clostridium perfringens,* and the factors that damage the epithelial cells like fusarium mycotoxin. Previous studies have revealed that different predisposing factors caused similar shifts in intestinal microbiota composition and reduced the abundance when the poultry chickens fed a fishmeal based diet; *Eimeria* infection, and mycotoxin contaminated diet (Antonissen et al., 2016)

Alpha toxin was one of the most important lethal and dermonecrotic toxins produced by *C. perfringens,* known as phospholipase C (PLC). It was produced in different amounts by all types (A, B, C, D and E) of *C. perfringens* and was considered as a primary virulence factor involved in clostridial myonecrosis (Williamson and Titball, 1993; Awadet al., 1995). In *C. perfringens* type A, the alpha-toxin was the unique lethal protein produced during vegetative growth. Owing to its role in gas gangrene disease, food poisoning and animal enterotoxemia, *C. perfringens* type A strains, had been the subject of intense investigations over the past 60 years (McDonel, 1980). Alpha-toxin, produced by *C. perfringens*, was a

metalloenzyme with molecular weight of 43 kDa (Takahashi et al., 1974; Hale and Stiles, 1999) and LD50 of 40 ng/mL-1 (Naylor et al., 1997) and catalyzed the hydrolysis of lecithin and phospholipids (Saint-Joanisetal., 1989; Hale and Stiles, 1999).

A common commensally inhabitant of the healthy broiler chicken gut microflora *C. perfringens* was frequently found in the feces of livestock and poultry at high levels (Tschirdewahn et al., 1991). However, its overgrowth in fowl can be considered an imbalance of the gut ecosystem at the microbial level resulting in gastrointestinal dysbacteriosis and necrotic enteritis (Wages and Opengart, 2003). Poultry necrotic enteritis was associated with predisposing factors (Elwinger et al., 1992) such as *Eimeria* spp. infections (coccidiosis); the incorporation of dietary fish meal, rye, barley and wheat as major feed components. The withdrawal of dietary subtherapeutic Antibiotic Growth Promotants (AGPs). While the impact of AGP withdrawal on chicken health, welfare and production efficiency had been studied to a limited degree (Casewell et al., 2003), it was known that the exclusion of ionophore coccidiostat antibiotics, which were generally anticlostridial, from the broiler chicken diet had resulted in higher rates of necrotic enteritis in broiler chickens raised in AGP free settings (Elwinger et al., 1992; Elwingeret al., 1998).

Necrotic enteritis is caused by *C. perfringens* type A total has been positively limited by administered antibiotics to the diet of poultry, however recent concerns regarding the influences of this practice on ehancing antibiotic resistance in pathogens of human have led to consider alternative schedules such as vaccination, immune responses against alpha toxin can provide partial protection against Necrotic enteritis (Joseph et al., 2018). So the aim of present study was to prepare of *C. perfringens* alpha toxoid vaccine for controlling the necrotic enteritis disease.

MATERIALS AND METHODS

Ethical approval

All procedures performed according to Egyptian ethical standards of the national research committee. Approved from Institutional Animal Care and Use Committee (IACUC) Cairo University under number 1221 /2013 review and approve all activities involving the use of vertebrate animals prior to their initiation.

Strain

Clostridium perfringens type A was previously isolated and identified in Anaerobic Bacterial Vaccine Research Department of Veterinary Serum and Vaccine Research Institute (VSVRI) from turkey farms in Egypt from different localities (Giza, Qalyobia, Ismalia governorates) suffered from necrotic enteritis and high mortalities, the identification of the isolates was depended on culture characters; biochemical tests and serological tests (Nagler Test, Toxin Neutralization test and Dermonecrotic reaction) beside was PCR and DNA sequence (El-Helw et al., 2014).

Vaccine preparation

The lyophilized strain was rehydrated into cooked meat medium and then inoculated into the primary toxin production medium incubated at 37°C for four hours and then transferred into main toxin production medium according to El-Helw et al. (2017) and incubated at 37°C for four hours. Then about 10 ml of this culture was taken for determination of Minimum Lethal Dose (MLD) according to Fu et al. (2004) and the rest of culture was inactivated by adding 0.5% (Formaldehyde 37%) (v/v) until complete inactivation for 7 days. Microfiltration and ultra-filtration by using millipore® filter was applied for separation of unwanted cells from toxoid and concentration of toxoid. Aluminum hydroxide gel adjuvant was added at 20% (v/v) to alpha toxoid and homogenized by using magnetic stirring at room temperature. Sterility and Safety test of the above vaccine was done according to OIE (2016).

Evaluation of the prepared vaccine in turkey poults

Vaccinal dose. It was adjusted according to MLD to contain different three MLD (96, 48 and 24) per 0.5 ml dose.

Vaccination schedule. Fifty Orlopp Bronze turkey poults aged 4-6 weeks was divided into four groups, the first three groups containing 15 birds in each, and fourth group containing five birds was left as control. Vaccination schedule of these poults was indicated in table 1.

Table 1. Vaccination schedule of Orlopp Bronze turkey at 5 weeks of age

Groups	No. of turkey/ group	Dose /Rout of Injection	Booster dose
1	15	96 MLD/0.5 ml , I/M	It was done in all birds with the same dose and route after 21 days from first vaccine in each group
2	15	48 MLD /0.5 ml , I/M	
3	15	24 MLD/0.5 ml , I/M	
U* (Control)	5	0.5 ml saline for each bird	

No: Number, MLD: Minimum Lethal Dose, U*:Unvaccinated

Blood samples were taken after two weeks from second dose, and then every month for six months, serum were collected and stored at -20°C until evaluation of antibody titers.

Determination of antibody titer

Toxin neutralization test. It was done according to Barile et al. (1970) firstly L+/2 dose of alpha toxin of *C. perfringens* type A determined and after that serum samples were serially diluted and equal volume of alpha toxin dose (L+/2) was added for each dilution then the mixture was incubated at 37°C for one hour two mice were injected from each dilution with 0.2 ml I/V and observed for 24 hours. The reciprocal of the highest dilution of serum that cause death of all mice multiplied by two was regarding as the antibody titer which expressed by International Unit (IU).

ELISA. It was done according to Lee et al. (2012) and antibody titer was calculated against standard curve by weighted parallel linear regression according to Grabowska et al. (2002) according to equation:

$U_s=U_R\ Df^D$

Were

U_R: number of units in reference serum and Us: number of units of serum samples; D: horizontal distance between the sample and reference line; Df: dilution factor (equal to 2 were used twofold dilution).

The estimated slope in the new weighted Parallel Linear Line slop for both sample and reference

$b_{wPLL}=(S_{XYs}+S_{XYR})/(S_{XXs}+S_{XXR})$ and the horizontal distance , D was then

$D= X_S-X_R-\frac{(Ys-YR)}{bwPLL}$

1- Hemolysin Inhibition Assay it was done according to Jayappa et al. (2006) serial twofold dilution from serum samples were made in v bottom microtiter plate, 100µl of four hemolysin unit of alpha toxin was added to each well. The plates were incubated at 36°C for one hour , then 100µl of 0.5% of sheep Red Blood cells added to each well and incubated at 36°C for three hours

The reciprocal of the highest dilution of tested sample that indicated no hemolysis was considered the endpoint.

2- Challenge test was done according to Kaldhusdal and Hofshagen (1992). In brief the lyophilized strain of *C. perfringens* was reconstituted into cooked meat medium and serially subculture into fluid Thioglycollate broth medium increasing the volume at each step to reach volume (1 L), this culture was mixed with feed in a ratio of three parts cultures, four parts of high protein feed. The mixture which had a paste like consistency was placed in feed tray and to birds.

Five birds from each vaccinated groups (one, two and three) and three from control unvaccinated group were fed on the previous mentioned culture with feed for four successive days, two weeks after second vaccination. Birds were observed for presence of any clinical signs of necrotic enteritis. These birds were slaughtered and score of lesions were determined (0-4) where 0: No lesion; 1+: friable small intestine; 2+: Focal necrosis; 3+: large patches of necrosis, and 4+: severe necrosis.

The challenge test was repeated after six months of second dose of vaccination as the same first one.

Statistical analysis

All statistical analyses were performed with Easy R in Saitama Medical Center, Jichi Medical University, Japan .21) One-way ANOVA followed by the Tukey's test was used to evaluate differences among three or more groups. Differences were considered to be significant for values of $p<0.05$.

RESULTS

Clostridium perfringens type A, the alpha-toxin is the unique lethal protein produced during vegetative growth at logarithmic phase, at this study the toxin production medium that used produce alpha toxin at 120 MLD/ml and then submitted to microfiltration by using Millipore® filter (0.22µm) for clarification of alpha Toxoid and then using Millipore® ultrafiltration (10000 n mol. w.) was one of the first author to employ high-pressure ultrafiltration to obtain alpha-toxin in high concentration, the efficiency of high-pressure ultrafiltration for purification of alpha-toxin was evaluated the ultrafiltration process was less time-consuming, easier to perform and less laborious than ammonium sulfate and acetone precipitations. So after ultrafiltration of alpha Toxoid made double fold concentration to achieved 240 MLD/ml, so the group one vaccinated with dose one after addition of aluminum hydroxide gel as adjuvant containing 96 MLD/0.5 ml; group two vaccinated with dose two the origin was double fold dilution so it containing 48 MLD/0.5ml, and group three vaccinated with dose three dilution of origin ¼ so it containing 24 MLD/ml.

Antibody titers in sera of vaccinated turkey in groups one and two as indicated in table 2 revealed that the mean antibody titer was 7.4 IU/ml and 4.13 IU/ml respectively. While in group three received dose 24 MLD the antibody titer was 1.26 IU/ml. Antibody titer in sera of vaccinated

bird measured by ELISA which calculated according to linear regression fit where the standard serum of known titer measured by TNT used against unknown serum, the coefficient of variance was 0.9983 and the intercept (0.3165), slop (0.584).

Mean Antibody titer measured by ELISA in group one was 8.04 U/ml which came in parallel with Toxin Neutralization Test (TNT) 7.4 IU/ml, there was no significantly difference (P>0.05), but in groups two and three the mean antibody titer measured by ELISA were (4.83 and 1.78 u/ml) respectively had a significant difference (P<0.05) with results obtained by TNT. These results were due to high sensitivity of ELISA for measurement of antibody titer especially in a low level.

Geometric mean titer of antibodies measured by hemolysin Inhibition assay revealed that in Group one vaccinated with 96 MLD/dose was 1176.26 HU, Group two vaccinated with 48MLD/dose was 466.8, and in Group three vaccinated with 24 MLD/dose was 25.39. Challenge test for vaccinated turkey and control after two weeks of second dose revealed that in group one and two the vaccine gave 100% protection for all birds against challenge and the average score lesions was 0. But in group three vaccinated with 24 MLD dose gave 80 % protection and score lesions was 0.4. These results were parallel with antibody titers which cover the permissible level required for vaccine release, but in group three after six months post vaccination repeat challenge test on present birds revealed that protection became only 10% and score lesion where three, but in groups one and two antibody titer was one and 0.5 IU/ml and protection % (60%), score lesions (1, 1.4 respectively), so from these results revealed that group two vaccinated with 48 MLD/ dose able to cover the permissible level of antibody titer required for release the vaccine and able to maintained the antibody titer and protection of birds for six months so it could be recommended for revaccination of birds after six months when used this birds as breeder but for broiler farms only vaccination on two weeks with two doses 21 days apart.

Duration of immunity in vaccinated turkey

As indicated in group one there was a gradual rise in antibody titer after two weeks of post second dose of vaccination were reach to 9IU, 10.2 EU and 2048 HU measured by TNT; ELISA, and Hemolysin Unit respectively (Table 4). After two months, then gradual decrease in antibody titer from three months to six months after vaccination where reach to 1IU, 1.56 EU and 50.5 HU and in group two the antibody titer reach to 0.5IU, 0.8 EU and 32 HU, while in group three antibody titer was 0.2 IU, 0.28 EU and 22 HU. Also as indicated in table five the challenge test was done in vaccinated turkey after six months revealed that in group one and two the protection rate was 60%, while in group three it was 10%.

Table 2. Antibody titer in sera of vaccinated turkey after 2 weeks past of second doses

Bird No.	Vaccinated with 96 MLD/0.5 ml (Group 1)			Vaccinated with 48 MLD/0.5 ml (Group 2)			Vaccinated with 24 MLD/0.5 ml (Group 3)		
	TNT (IU)*	ELISA	Hemolysin Unit**	TNT (IU)	ELISA	HU**	TNT (IU)	ELISA	HU**
1	7	7.22	1024	4	5.12	128	2	2.09	64
2	8	9.45	2048	4	4.78	512	1	1.77	32
3	8	10.02	1024	4	4.55	64	1	1.89	16
4	6	5.88	512	4	3.87	128	1	1.94	16
5	9	9.78	2048	4	4.77	512	1	0.89	32
6	8	8.95	1024	5	5.89	512	1	1.67	32
7	8	8.12	1024	4	4.12	1024	2	2.45	16
8	8	7.99	1024	4	5.09	1024	1	1.67	16
9	9	9.47	2048	4	4.89	512	1	1.83	16
10	6	6.94	1024	4	5.3	1024	2	2.09	64
11	8	8.23	2048	5	5.99	1024	2	2.33	32
12	8	8.44	2048	4	4.55	512	1	1.49	16
13	6	6.87	1024	4	4.89	512	1	1.74	32
14	6	6.39	512	4	3.99	1024	1	1.67	16
15	6	6.9	1024	4	4.76	512	1	1.22	32
Mean	7.4±1.08	8.04±1.2	1178.2	4.13±0.33	4.83±0.5	466.8	1.26±0.44	1.78±0.3	25.3
Control group	0	0	0	0					

TNT*: Toxin Neutralization Test, HU**: Hemolysin Unit, ELISA: Enzyme Linked Immunosorbant Assay, MLD: Minimum Lethal Dose, No.: Number, IU: International Units

Table 3. Response of vaccinated Orlopp Bronze turkey aged 5 weeks to challenge with *C. perfringens* two weeks after second dose of vaccination

Treatment	No. of birds with gross lesion (%)	Average lesion score	Protection (%)
Vaccinated with 96 MLD (Group1)	0/5 (0%)	0	100
Vaccinated with 48 MLD (Group2)	0/5 (0%)	0	100
Vaccinated with 24 MLD (Group3)	1/5 (20%)	0.4	80
Non vaccinated, challenged	3/3 (100%)	3.33	-

No: Number, MLD: Minimum Lethal Dose

Table 4. Duration of antibody titer measured by TNT and ELISA, and hemolysin inhibition assay expressed by hemolysin unit in sera of vaccinated Orlopp Bronze turkey aged 5 weeks against necrotic enteritis

After second dose of vaccination	GROUP (1)			GROUP (2)			GROUP (3)		
	TNT (IU)	ELISA	Hemolysin Unit	TNT (IU)	ELISA	Hemolysin Unit	TNT(IU)	ELISA	Hemolysin Unit
1 month	8.3	9.23	2048	5	5.89	560	2.5	3.09	288
2 month	9	10.2	2048	6	6.48	670	3.5	4.22	388
3 month	6	6.95	1020	4	4.78	465	2	2.66	64
4 month	4	5.22	461.2	2	2.77	130	1	1.44	40
5 month	3	3.96	382	2	2.49	125	0.5	0.89	30
6 month	1	1.56	50.5	0.5	0.8	32	0.2	0.28	22
7 month	0.3	0.366	24	0.2	0.19	15.5	0.1	0.16	10

TNT: Toxin Neutralization Test, ELISA: Enzyme Linked Immunosorbant Assay, IU: International Unit

Table 5. Response of vaccinated Orlopp Bronze turkey aged 5 weeks to challenge with *C. perfringens* after six months of post vaccination

Treatment	No.* of birds with gross lesion (%)	Average lesion score	Protection (%)
Group (1) vaccinated with 96 MLD	2/5 (40%)	1	60
Group (2) vaccinated with 48 MLD	2/5 (40%)	1.4	60
Group (3) vaccinated with 24 MLD**	4/5 (90%)	3	10
Non vaccinated, challenged	3/3 (100%)	3.33	0

*No: number, **MLD: minimum lethal dose

DISCUSSION

*Clostridium perfringens*is the most important cause of clostridial enteritis in domesticanimals. In broilerchickens, C. *perfringens* has been known for decades as apathogen responsible for necrotic enteritis (NE), hepatitis,and cholecystitis. NE exists in a clinical form with severeoutbreaks and mortality and a subclinical form, which wasmainly characterized by a reduced growth rate and anincreased condemnation rate. *C. perfringens* was member of the normal intestinal microflora in poultry and consequently, the mere presence of this bacterium in the intestine was not sufficient to provoke disease; this happens only when high numbers were present. A number of predisposing factors had been identified, among them high wheat, barley or fish meal contents in the feed, and an underlying coccidiosis problem.

Necrotic enteritis via of proliferations of *Clostridium perfringens* type A and associated toxins in the small intestine of birds can be a harmful enteric disease which cause unexpected mortality, sudden diarrhea and mucosal necrosis. It also has contributed to high economic losses in global poultry industry (Yang et al., 2019)

An important defense system that constantly utilized and trigger was the adaptive immune system through application of vaccines. Vaccines had proven to be effective tools to prevent or lessen the impact of diseases which biosecurity alone cannot prevent. However, it needed to realized that many of vaccines caused do cause a transient immunosuppression and that all label claims clearly state that "only healthy animals should be vaccinated. So problems could occur when a flock was going through an undetected subclinical challenge and a vaccine was applied. The vaccine may either precipitate

clinical disease or the vaccine is unable to work leaving the flock vulnerable to future challenge by that pathogen. The other essential consideration is the timing between vaccinations or between vaccination and a major stressor such as moving the flock from brooding to the finishing barns. For example, flocks that were vaccinated for Hemorrhagic Enteritis Virus (HEV) and then moved one week later may become ill, because major stressors (maximum vaccine replication and moving to a new environment, especially during the winter months) had coincided.

So this result pass in parallel with previous result of (Elham et al., 2014) in which they using a fixed dose (60 MLD/ml) for vaccination of rabbits against rabbit clostridial enterotoxaemia and the antibody titer was 5 IU/ml, and this titer was over the permissible limits (4 IU/ml) according to United States Department of Agriculture (USDA) (2002), which surpass requirements to receive a conditional license pass standardized test by the development of a serum antibody concentration of at least four international antitoxin units per ml in at least 80 % of vaccinated animals that were seronegative prior to vaccination. These findings agree with Garcia-Moreira et al. (2016) that prepared recombinant trivalent vaccine containing epsilon, alpha and beta using $Al(OH)_3$ adding 200 µg of each of the three antigens of the vaccine can induce 5.19 IU/ml for alpha toxin, thus there was a similarity for determination a fixed dose of antigen to be added to the vaccine either minimum lethal dose of toxin or protein concentration. From the above result it could be concluded that used of 48 MLD /Dose for turkey in vaccine preparation was enough for coverage the permissible limit required for approved vaccine.

There was parallel between antibody titer measured by TNT; ELISA, and hemolysin assay approved by several authors report the efficiency of the ELISA technique in the quantification of vaccine antigens. However, as this study demonstrated, and calculated by weighted parallel linear regression for accurate calculation of antibody titer. For instance, the sandwich ELISA (El-Idrissi and Ward, 1992a, 1992b), was capable of detecting, in a linear form, concentrations between 7.8 and 125ng/mL and the minimum detectable 2ng/mL. Nagahama et al. (1991) reported that the sensitivity of ELISA with specific antibodies for the detection of beta, epsilon and iota toxins of *C. perfringens* may reach up to 1.0mg/mL for the purified iota and beta toxins and 0.1ng/mL of purified epsilon toxin. (Uzal et al., 2003) reported the detection of 0.075 DL50/mL of epsilon toxin in the intestinal content. By comparing the sandwich ELISA and the serum neutralization for the detection of beta and epsilon toxin in intestine content, a study found a sensibility and specificity of 90.5% and 89.2% for the beta toxin and 97.4 and 95.6 for the epsilon toxin (El-Idrissi and Ward, 1992a and 1992b). On the other hand, this study indicated promising results when utilizing the indirect ELISA measured by linear regression.

The results of challenge test revealed that 40% of turkey in groups one and two developed necrotic enteritis lesion when challenged with virulent strain of *Clostridium perfringens* type A after 6 months post vaccination with 0.5 IU/ml antibody titer, while in group three 90 % of vaccinated turkey had lesions of necrotic enteritis associated with antibody titer below 0.5 IU/ml so the protection of intestine and resist to challenge and developed of lesions depend on immunity of gut by vaccination and production of antibodies and also by presence of gut miroflora as mentioned by Keerqin et al. (2017) that found miroflora play an important role in protecting poultry from necrotic enteritis infection where both ileal and cecal inoculants containing *Lactobacillus spp*. are widely regarded as beneficial bacteria in poultry.

The duration of immunity in vaccinated turkey revealed that there was a relation between antibody titer in sera of vaccinated turkey and protection against challenge test were the antibody titer of 0.5 IU/ml sufficient to made protection up to 60%, this relation achieved by lovland et al. (2004**)** use toxoids vaccines based on *C. perfringens* type A and C toxoids to vaccinate breederflocks. The IgG responses in vaccinated parent hens weredistinct and the levels of antibodies to *C. perfringens* alpha - toxin in progeny of the vaccinated hens was highenough to protect the progeny against subclinical *C. perfringens* associated necrotic enteritis. Also, Heier et al. (2001) provided that active and passive immunity using vaccination against *C. perfringens* and its toxins appears to offer protection. Found out that broiler flocks with high titers of maternal antibodies against *C. perfringens* alpha-toxin had lower mortality during the production period than flocks with low tiers.

CONCLUSION

It concluded that a vaccine comprising of *Clostridium perfringens* alpha Toxoid wherein containing 48 MLD/Dose was sufficient to elicit at least 4 IU/ml antialpha toxin antibody in vaccinated turkey which was sufficient for protect turkey for six months. There was linear parallel between antibody titer measured by Toxin Neutralization Test; ELISA, and Hemolysin Inhibition

Test so for evaluation of further recent batches of vaccine can be used either ELISA or Hemolysin Inhibition Test as test replacement of TNT for limitation used of mice. And also from economic point of view it better for control of necrotic enteritis disease in turkey flock used the vaccine to subside used of antibiotic as food additive which cause risk of antibiotic resistance for human.

DECLARATIONS

Competing interests

All authors have no conflict of interest.

Consent to publish

The author(s) grant(s) the publisher the sole and exclusive license of the full copyright in the contribution. Consequently, the publisher shall have the exclusive right throughout the world to publish and sell the contribution in all languages and all other forms of electronic publication.

Author`s contribution

Elham El-sergany and El-Helw Hamed prepared and evaluated Necrotic enteritis vaccine; Hala El-Sawy vaccinated turkey and taken blood samples; Taha Medhat and Abdalla Yasser writing the paper and made statistical analysis, and El-Meneisy Alaa reviewed the article.

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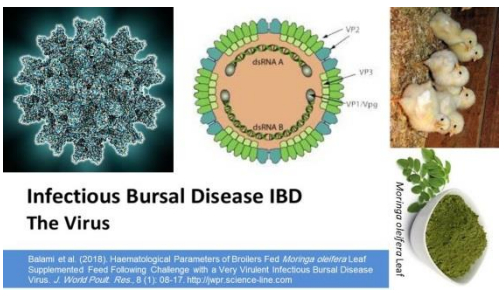

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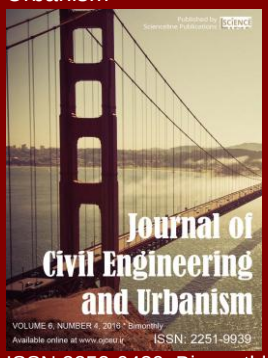

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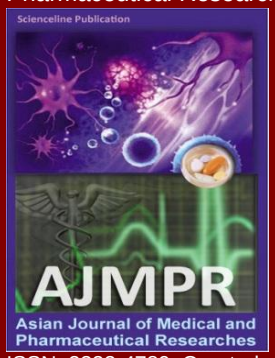

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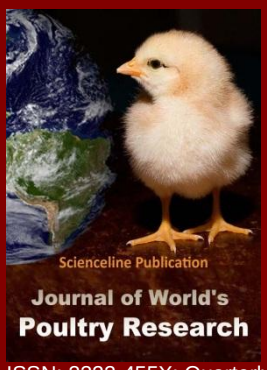

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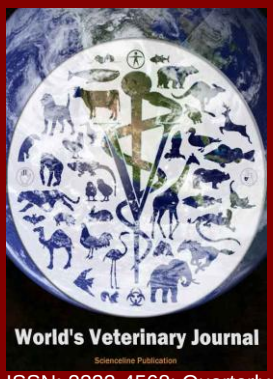

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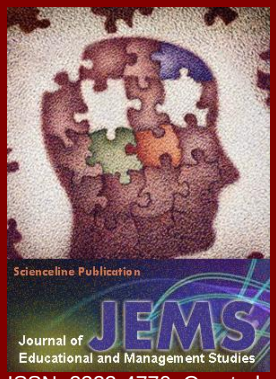

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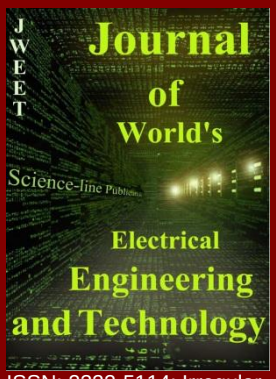

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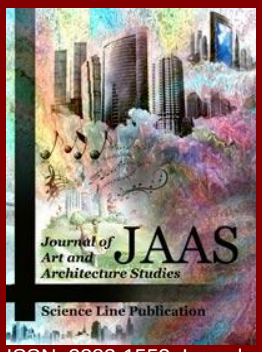

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