Immunomodulatory potentials and serum biochemical indices of Ross 308 broiler chickens exposed to different post hatch feeding time and methionine levels

***Oshifade E. O, Ajayi F. O and George, O. S**
Department of Animal Science, University of Port Harcourt, Port Harcourt
Corresponding author -ddoyin012@gmail.com; 08060183641

Abstract

The effect of post hatch feeding time and inclusion levels of methionine on biochemical parameters and cytokine levels were studied in a 56-day experiment in Ross 308 broiler chickens. A total of 270-day old broiler chickens in a completely randomized design using factorial arrangement: were randomly assigned to 9 groups of 30 chickens each 24hrs, 0.4% methionine; 30 hours 0.4% methionine;36 hours, 0.8% methionine; 24hrs, 0.48 methionine; 30 hours 0.8% methionine;36 hours, 0.8% methionine; 24hrs, 1.2methionine; 30 hours 1.2%methionine;36 hours, 1.2% methionine. The chickens were fed maize-soyabean based basal diet. Feed and water were provided ad libitum. The research lasted for 56 days. The values from serum biochemical indices showed that total cholesterol, triglyceride, high density lipoprotein, glucose, phosphorus, creatinine, uric acid were significantly influenced by the dietary treatments. The birds on the 0.8% methionine diet had significantly more total cholesterol and glucose. The birds on 1.2%dietary methionine had significantly higher level of phosphorus than the other treatments. the effect of post hatch feeding time had a significant effect on plasma cholesterol, triglyceride, high density lipoprotein, total protein, glucose, calcium, phosphorus, creatinine and uric acid were all significantly ($p<0.05$) different. In the caecal tonsil, IFN-γ was significantly down regulated. However, no significant difference was observed in the IFN-γ expression in the spleen. The IL2 gene expression increased with increased methionine in the diet. In this study, it was observed that negative relationship exist between IL2 and IFN-γ and serum albumin and Total Protein. These serum components are the traditional markers of nutritional status. This suggests that the negative effect of delayed access to feed can be reduced in broiler production by supplementing the feed with about 0.8% dietary methionine.

keywords. Methionine, Post hatch Feeding Time, Biochemical parameters, Cytokine, Ross308

Potentiels immunomodulateurs et indices biochimiques sériques des poulets de chair Ross308 exposés à différents temps d'alimentation après l'éclosion et niveaux de méthionine

Résumé

L'effet du temps d'alimentation après l'éclosion et des niveaux d'inclusion de méthionine sur les paramètres biochimiques et les niveaux de cytokines a été étudié dans le cadre d'une expérience de 56 jours menée sur des poulets de chair Ross 308. Au total, 270 poulets de chair âgés de 270 jours ont été répartis de manière aléatoire en 9 groupes de 30 poulets chacun, selon un plan factoriel complètement randomisé : 24 heures, 0,4 % de méthionine ; 30 heures, 0,4 % de méthionine ; 36 heures, 0,8 % de méthionine ; 24 heures, 0,48 % de méthionine ; 30 heures, 0,8 % de méthionine ; 36 heures, 0,8 % de méthionine ; 24 heures, 1,2 % de méthionine ; 30 heures, 1,2 % de méthionine ; 36 heures, 1,2 % de méthionine. Les poulets ont été nourris avec un régime de base à base de maïs et de soja. Ils ont été nourris à volonté et abreuvés à volonté. La recherche a duré 56 jours. Les valeurs des indices biochimiques sériques ont montré que le cholestérol total, les triglycérides, les lipoprotéines de haute densité, le glucose, le phosphore, la créatinine et l'acide urique étaient significativement influencés par les traitements alimentaires. Les oiseaux nourris avec un régime à 0,8 % de méthionine présentaient un taux de cholestérol total et de glucose significativement plus élevé. Les oiseaux nourris avec un régime contenant 1,2 % de méthionine présentaient un taux de phosphore significativement plus élevé que les autres groupes. La durée d'alimentation après l'éclosion a eu un effet

significatif sur le cholestérol plasmatique, les triglycérides, les lipoprotéines de haute densité, les protéines totales, le glucose, le calcium, le phosphore, la créatinine et l'acide urique, qui présentaient tous des différences significatives (p < 0,05). Dans l'amygdale cœcale, l'IFN-γ était significativement régulé à la baisse. Cependant, aucune différence significative n'a été observée dans l'expression de l'IFN-γ dans la rate. L'expression du gène IL2 augmentait avec l'augmentation de la méthionine dans l'alimentation. Dans cette étude, il a été observé qu'il existe une relation négative entre l'IL2 et l'IFN-γ et l'albumine sérique et les protéines totales. Ces composants sériques sont les marqueurs traditionnels de l'état nutritionnel. Cela suggère que l'effet négatif d'un accès retardé à la nourriture peut être réduit dans la production de poulets de chair en ajoutant environ 0,8 % de méthionine alimentaire à la nourriture.

Mots-clés: méthionine, temps d'alimentation après l'éclosion, paramètres biochimiques, cytokine, Ross308

Introduction

The global broiler industry has emerged over time as a result of the economic traits of broiler chickens (Castro,2023). Global broiler production has increased faster than any other livestock sector, especially in third world countries that are key producers of broiler meat (FAO,2010). This trend can be traced to the inherent feed conversion efficiency and improved management practices carried out in intensive poultry production (Taha, 2003).

According to Schmidt *et al*. (2009), genetic selection has made it possible to develop broilers that can eat less and grow more. Present day broilers can double their starting body weight in the first 72 hours (Burt, 2004). However, selection for performance traits alone has adversely affected the immune competence of modern-day birds (Cheema *et al*., 2007). Kleyn *et al.* (2021) reported the benefit of chicks receiving feed early post hatch and that chicks benefit more from feed with a balanced nutrient content especially the protein and amino content of the feed. For a continuous broiler production, attention has started to shift from diets just supplying nutrients for production and body maintenance to specialized areas such as immuno nutrition (Field *et al*., 2000; Okamoto *et al*., 2009). Protein and energy content of the feed has immuno- modulating effect, i.e. they affect rate of cytokine production in birds (Perween *et al*., 2016). It is therefore imperative to choose ingredients that will ensure nutrient availability during broiler feed formulation. (Ravindran, 2005).

In addition to the benefit of timely access to feed and water, the nutritional composition of diets is also important for overall growth and muscle development. Birds require access to solid, semi-solid, or liquid nutrients grow. Also, growth rates and breast meat yield have been reported to climax at higher dietary lysine and methionine concentrations when the dietary crude protein content was more than 23% (Sklan and Noy, 2003; Vieira *et al*., 2004). Methionine is an essential and first limiting amino acid for livestock especially in poultry because it is limited in plant protein-based diet (Baker, 2006) and it is also highly required for feather growth. (Bucnhaak, 2009). Availability of methionine in poultry diet is of utmost importance as methionine is of high nutritional value, its content in poultry diet determines the nutritional value of the feed. In poultry, methionine is needed for growth promotion (Mirzaaghatabar *et al*., 2011), detoxification (Kim *et al*., 2013), anti-cancer and anti-tumor (Yen *et al.,* 2002; Li *et al*., 2009), Coccidium infection resistance (Rama Rao *et al*., 2003), Methionine has been noted to have significant effect on specific and non-specific immune functions. (Swain and Johri, 2000; Konnashi *et al*., 2000; Guerrero and Reuter 2002; Zhang and Li 2008). Methionine plays an important function in cellular and humoral immunities of broilers. It is involved in the production of antibodies as well as in cell mediated immune responses (Liu *et al*., 2007).

Avila *et al*. (2000) observed that the deficiency of dietary methionine caused a pathological and ultra-structural changes of broiler thymus, decreased the serum Interlukin-2 (IL2) level and the population of T- cell , stimulate less proliferation of T cells and high percentage of dead cells in the spleen. Additionally, GSH, a metabolite from methionine was noted to regulate nuclear transcription factor $_{K}$B pathway, the cell function of T cell helper, antibody and interferon-γ (IFN- γ) production in broilers immune response to infectious challenge (Li *et al*., 2007). Cytokines are the small molecular proteins which are produced by cells of the immune system to transmit information between the cells and a means of information between the immune systems and the body tissues. Cytokines can be classified on the basis of their activities into pro inflammatory, anti-inflammatory, Th1, Th2, Th3/Tr 1. There are over a hundred cytokines in nature, however, only three- Interleukin 1B, TNF- , IL6 have been extensively researched because of their cross regulation and effect on whole body metabolism, nutritional status and body composition (Wigley and Kaiser, 2003; Zheng, 2013; Duque and Descoteaux, 2014). A negative association exists between cytokines and nutritional status of farm animals (Humphrey, 2010).. Immune system activation has been reported to reduce the growth performances of birds (Humphrey, 2010). Though, it is beneficial for birds to be diseases resistant, the performance of the birds is lowered in order to compensate for this. The reduced performance observed is the consequence of immune system activation (Humphrey, 2010). One way of improving the efficiency of animal production systems and the welfare of birds is by increasing the resistance of birds to diseases and at the same time maintaining a high level of growth performance (Humphrey, 2010). These can be ensured by evaluating the nutritional and health status of farm animals regularly. These two important factors can easily be monitored using cytokines and the serum biochemistry. Hence, the need for this study to monitor cytokines as a measure of nutritional status in broiler chickens and determine the relationship that exists between cytokine and serum biochemistry.

Materials and Methods

A total of 270 1-day old Ross 308 broilers of mixed sex was purchased from a reliable hatchery in Ibadan. The birds were placed in the brooding house within the first 24 hours of life. The experiment lasted for 56 days. Completely Randomized Design using a factorial arrangement. Two factors each at 3 levels which resulted in 9 treatment combinations, each experimental unit received a treatment combination. There are 3 replicates per treatment combination and 10 birds per replicate. Three diets with 0.4 % (Control), 0.8 %and 1.2% methionine levels in the feed designated as Factor A, 3 feeding regimes of either immediate access to feed (FED) post arrival (24 hours post hatch), access to feed delayed by 30 hours (DELAYED) or access to feed delayed by 36 hours post hatch (HELD) designated as Factor B. Birds were allocated to treatment randomly to ensure that there is no bias. Birds in each group received a treatment combination of factors A and B.

Birds in the FED group were fed with water and feed *ad libitum* from arrival, those in the DELAYED were given only water on arrival and fed 6 hours afterwards (30 hours post hatch). The birds in the HELD group were fed after 12 hours and took only water at arrival (36 hours post hatch). The experiment was carried out at Doshfem Farms, located at New Rumukwurushi, Off Igbo Etche, road, Eleme Junction Port Harcourt, Rivers State. Port Harcourt is a coastal city located in the Niger Delta region of Nigeria. Port Harcourt lies within latitude $6^{0}58'$ - $7^{0}60'$E and longitudes $4^{0}40'$- $4^{0}55'$N. The monthly rainfall in Port Harcourt follows a sequence of increase from March to October before decreasing in the dry season months of November

to February (Uko and Tamunobereton-Ani 2013;
Lamidi and Ogunkunle,2016)

Table 1: Composition of experimental broiler starter diets

Ingredient (%)	**T1 (0.4 % Meth)**	**T2 (0.8 % Meth)**	**T3 (1.2 % Meth)**
Maize	45.60	45.60	45.60
Soybean meal	25.25	25.25	25.25
Palm oil	3.00	3.00	3.00
Bone meal	3.00	3.00	3.00
Wheat offal	6.80	6.60	6.40
Fish meal	5.00	5.00	5.00
Palm kernel cake	10.00	10.00	10.00
Lysine	0.30	0.30	0.30
*Vitamin premix	0.25	0.25	0.25
Methionine	0.40	0.80	1.20
Common salt	0.40	0.40	0.40
Total	**100**	**100**	**100**
Calculated Nutrient composition			
Crude Protein	20.68	20.62	20.55
Metabolisable Energy (Kcal/kg ME)	2773.00	2768.00	2763.00
Oil	5.60	5.58	5.56
Crude Fibre	4.79	4.83	4.75
Methionine	0.42	0.43	0.43
Lysine	1.302	1.302	1.302
Calcium	1.53	1.53	1.53
Available phosphorus	1.03	1.03	1.03

**Composition of vitamin premix per kg is as follows: Vitamin A,8000 IU; Vitamin D_3 1600 IU; Vitamin E_5 ; Vitamin K 0.200 mg ; Vitamins B, Thiamine $B_1$0.5 mg; Riboflavin B_2 4mg; Pyridoxine B_6 0.015 mg ; Niacin 0.015 mg; B_{12} 0.01mg ; Pantothenic acid 0.5 mg ; folic acid 0.5mg and Biotin 0.020mg ; Chlorine chloride 0.02mg ; Anti-oxidant 0.125 g and Minerals (Mn, Zn, Fe , Cu, Si, I, Co)0.0156*

Table 2: Composition of Experimental Broiler Finisher Diets

Ingredient (%)	T1(0.4 % Meth)	T2 (0.8 % Meth)	T3 (1.2 % Meth)
Maize	50.55	50.55	50.55
Soya bean meal	20.90	20.90	20.90
Palm oil	4.00	4.00	4.00
Bone meal	4.00	4.00	4.00
Wheat offal	6.90	6.70	6.50
Fish meal	5.00	5.00	5.00
Palm kernel cake	7.00	7.00	7.00
Lysine	0.30	0.30	0.30
*Vitamin premix	0.25	0.25	0.25
Methionine	0.40	0.80	1.20
Common salt	0.40	0.40	0.40
Total	**100**	**100**	**100**
Calculated Nutrient composition			
Crude Protein	18.40	18.34	18.28
Metabolisable Energy Kcal/kg	2289.00	2885.00	2879.00
Oil	7.11	7.09	7.07
Crude Fibre	4.30	4.26	4.22
Methionine	0.38	0.39	0.39
Lysine	1.30	1.30	1.30
Calcium	1.84	1.84	1.84
Available phosphorus	1.16	1.16	1.16

**Composition of vitamin premix per kg is as follows: Vitamin A,8000 IU; Vitamin D_3 1600 IU; Vitamin E_5 ; Vitamin K 0.200 mg ; Vitamins B, Thiamine $B_1$0.5 mg; Riboflavin B_2 4mg; Pyridoxine B_6 0.015 mg ; Niacin 0.015 mg; B_{12} 0.01mg ; Pantothenic acid 0.5 mg ; folic acid 0.5mg and Biotin 0.020mg ; Chlorine chloride 0.02mg ; Anti oxidant 0.125 g and Minerals (Mn, Zn, Fe , Cu, Si, I, Co)0.0156*

Data collection

Determination of serum biochemical characteristics

At 56 days old, two birds were chosen for blood collection from each replicate. About 5mls of llod was collected from each bird.The procedure involved puncturing the brachial vein with a 5ml scalp vein needle and syringe. Blood samples collected were centrifuged to obtain serum. Sera samples were analyzed for total protein, cholesterol and globulin using Spectrophotometry methods. Protein and albumin contents were determined using Randox Test Kits .

Tissue Collection and Total RNA Extraction and cDNA Synthesis

At 56 days old, 2 birds per treatment were selected randomly. Cecal tonsils and spleen samples were excised aseptically and stored inside RNA later, this was then stored at −4 °C till further analyses were carried out. Subsequently, spleen and cecal tonsils per broiler were homogenized (Separately) using mortar and pestle. Total RNA was extracted from the spleen and cecal tonsils using Jena Bioscience RNA Extraction Kit according to the manufacturer's protocol. The cDNA was synthesized using Jena Bioscience cDNA synthesis kit. The reaction consisted of 0.5 µg of total RNA, 1 µL of 50 µM

Oligo d(T)20 and nuclease-free water up to 13.5 µL, which was incubated at 80°C for 5 min and then cooled on ice. After cooling, 11.5 µL of the reaction mixture 5 µL of M-MLV RT 5x buffer (Promega), 1 µL of 10 mM deoxynucleoside triphosphate mix, 0.25 µL of RNasin (40 U/µL), 1 µL(200 U/ µL), and 4.25 µL of nuclease-free water was added. The total 25 µL reaction mixture was incubated at 55°C for 60 min and then heated at 90°C for 10 min to stop the reaction. After the cDNA synthesis, 25 µL of nuclease-free water was added to the cDNA. The cDNA was stored at -20ºC for ten days.

Real-time Quantitative PCR

Real-time PCR was done in 96 well microplates with an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA) and Jena Bioscience SYBR GREEN qPCR Kit. Each PCR reaction consisted of 10 µL of 2× master mix, 1 µL of 10 µM primer mixture (forward and reverse) of the target genes (Table 1), 2 µL of cDNA, and 7.0 µL of nuclease-free water for a 20-µL reaction volume. The reactions were incubated at 95 °C for 3 min, followed by 40 cycles of 95 °C for 5 s 60 or 62 °C for 20 s, 72 °C for 33 s. After which a melt curve analysis was performed to determine the reaction specificity. Each sample was measured in duplicates. The relative level of gene expression was estimated using the standard curve for each target gene as previously reported by Liu *et al*. (2006). Standard curves were constructed using serial dilutions of the purified PCR products of each gene in Table 3.2. The amount of sample cDNA for each gene was interpolated from the corresponding standard curve. All of the sample concentrations fell within the values of the standard curves. Glyceraldehyde-3-phosphate dehydrogenase was used as a normalizing gene at each sampling time because GAPDH expression was not affected by treatment. Each primer pair produced a unique dissociation curve, and randomly selected samples from all real-time qPCR reactions were resolved by agarose gel electrophoresis to ensure gene amplification specificity. A negative control, a well with no template, was included in each PCR reaction to detect possible contamination. Interferon gamma (*IFN-γ*), interlukin2, IL-2 and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) primers were designed with the PerlPrimer program v.1.1.19 (Marshall, 2004) using the GenBank sequences deposited on the National Center for Biotechnology Information and US National Library of Medicine (NCBI) shown in Table 3. The primers were selected so as to hybridize within the coding region of the transcripts. Furthermore 1 of the 2 primers was selected to hybridize at an intron/exon junction so as to exclude hybridization to even tiny residuals of genomic DNA. Afterwards primers were checked using the PRIMER BLAST algorithm against *Gallus gallus* genomic and mRNA databases to ensure that there was no genomic amplification and that there was a unique amplicon. Primers were checked for amplification efficiency which was found to range between 1.9 and 2.0 for all primers and the respective R squared (RSQ) values were presented in the Results section.

Table 3. Primer Sequence

Target	Primer sequence (5′ to 3′)[1]	Annealing temp, °C	PCR product size, bp	GenBank accession No.
GAPDH	F: GCTGAATGGGAAGCTTACTG R: AAGGTGGAGGAATGGCTG	60	216	NM_204305.1
IFN-γ	F:AGCTCCCGATGAACGAC R: CAGGAGGTCATAAGATGCCA	62	151	NM_205149.1
IL-2	F:AGTCTTACGGGTCTAAATCACAC R: GGACAGCAGATTAGTTAGCCA	62	219	AF000631.1

GAPDH = glyceraldehyde 3-phosphate dehydrogenase; *IFN-γ* = interferon-*γ*; *IL* = Interleukin;
F: Forward, R: Reverse.
Source: Parakeaus *et al*. (2017)

Statistical Analysis

The experimental data per treatment group were analyzed using the General Linear Model (GLM) – ANOVA procedure using the SPSS for Windows statistical package program, version 8.0.0 (SPSS Inc., Chicago, IL). Statistically significant effects were further analyzed and means were compared using Duncan's New Multiple Range Tests (Duncan1955). Statistical significance was determined at $P < 0.05$.

Statistical design

Data collected was subjected to analysis of variance in a Completely Randomized Design using a factorial arrangement with methionine levels and duration of access to feed post hatch as the sources of variation.

Statistical Model

$X_{ijk}=\mu + M_i + F_j + (MF)_{ij} + e_{ijk}$

Where μ= unknown constant

M_i= effect of varying levels of methionine (i= 0.4 %, 0.8 %, 1.2 %)

F_j= Feeding regime (j= 24 hours, 30 hours, 36 hours post hatch)

$(MF)_{ij}$ _= Interaction effect of Methionine and Feeding regime Post Hatch

e_{ijk}= Experimental error

Results and Discussion

Effect of Methionine on Biochemical Parameters of Broiler Chickens

Table 4 shows the effect of methionine on biochemical parameters. The values from serum biochemical indices showed that Total cholesterol, Triglyceride, High Density Lipoprotein, Glucose, Phosphorus, Creatinine, Uric Acid were significantly influenced by the dietary treatments while Low density Lipoprotein, Total Protein, Albumin and Calcium were not significantly influenced by the diet. Blood biochemical constituent shows the health and the nutritional status of the animal as well as the climatic and management conditions that the animals are subjected to (Minafra *et al*., 2010). Therefore, the percentage of blood biochemical parameters can serve as a bio indicator of metabolic disease and the productive performance of the animal. (Rotava *et al*., 2008). The Total Cholesterol was significantly influenced by the diets with birds on the 0.8% methionine level having significantly highest Cholesterol while the birds on the 0.4% methionine level had significantly low cholesterol values. The values are lower than the values reported by Duwa *et al*. (2012) but within the range of report of Akinola and Etuk (2015). Hernawan *et al*. (2002) reported that Triglyceride level in the blood is an indication of the rate of absorption of the hydrolysis of triglyceride fat from the digestive tract into the bloodstream and

the rate of its utilization by cells. Triglyceride level is highly regulated by the percentage of dietary carbohydrate and the response of the birds to lypolysis. According to Yang *et al*. (2009) triglyceride is the most important source of fatty acid for the accumulation of fat. The results of the analysis of variance shows that the level of Triglyceride in this study is significantly ($p<0.05$) influenced by the diet. The level of triglyceride reduces as the level of methionine increases, this is perhaps as a result of lipogenesis that occurs at high inclusion level of methionine in the diet of broilers. This result is consistent with the result of Ghasemi *et al*. (2014) who reported that increased inclusion of supplemental methionine can stimulate lipogenesis. Blood protein was not significantly influenced by dietary treatments. The values obtained in this study were within the range of serum protein reported by Udoyong *et al.* (2010) in their study. The blood protein value obtained in this study is lower than the values obtained in the experiment by Njidda *et al*. (2006). They explained that the blood pool is the major source of amino acids that is needed for protein synthesis; these values imply that the protein level in the diet was sufficient to sustain the normal protein levels in the blood. Njidda *et al*., (2006) suggest that higher value of blood protein shows that there is enzymatic hydrolysis of dietary proteins. Blood calcium is the major factor in the formation and maintenance of the bones (Duwa *et al*., 2012). The blood calcium was not significantly influenced by the dietary treatments. Minerals have been identified to be part of hormones and as enzymes activators of (NRC, 1977). The level of blood phosphorus was significantly influenced. The level of phosphorus increases with the level of dietary methionine. The birds on 1.2%dietary methionine had significantly higher level of phosphorus than the other treatments. This implies there is better absorption of nutrients at 1.2% methionine level as the pH of the Gastro Intestinal Tract is lowered. Blood Glucose was significantly influenced by the level of methionine in the diet. The highest was obtained for diet with 0.80% methionine while the lowest was recorded for those on 0.40% methionine. The values obtained in this study where not in agreement with the normal range stated by Banerjee, (2009) and the values reported by Akinola and Etuk, (2015). According to Oni *et al*. (2015), higher serum glucose level may reflect enhanced energy metabolism due to stimulation of endogenous digestive enzymes by methionine and due to the release of adequate substrate (glucose) needed for mechanical work and body maintenance. The HDL percentage was significantly influenced by the methionine. The HDL level increased significantly with increased level of methionine. In their study, Mohammadzad *et al. (*2011) observed that increased Methionine levels leads to high level of serum HDL and reduced level of VLDL and LDL. According to Mohammadzad *et al.* (2011) and Shin *et al*. (2009), HDL influences the elimination of free cholesterol in the blood circulation due to the influence of HDL on liver metabolism. The level of Uric Acid was significantly influenced. The birds on 0.8% methionine had the significantly highest Uric Acid followed by the birds on 1.2% dietary methionine. Uric acid is the product of the degradation of amino acids. In the liver, nitrogen is converted to urea in mammals and uric acid in birds. Kohn *et al*. (2005) and Donsbough *et al*. (2010) reported that serum uric acid level indicates amino acid utilization. Uric acid elimination leads to a lower rate of muscle protein deposition. From the result, creatinine level increases significantly with increased level of methionine.

Table 4. Effect of Methioninelevels on Biochemical Parameters of Broiler Chickens

Parameters	**(T1) 0.4%**	**(T2) 0.8%**	**(T3) 1.2%**	**SEM**
TC (mg/dL)	2.63[c]	3.1[a]	2.86[b]	0.6
TCG (mg/dL)	0.85[b]	0.99 [a]	0.89[b]	0.2
HDL(g/dL)	0.60 [c]	0.83[b]	0.92 [a]	0.13
LDL(g/dL)	1.72	1.82	1.65	0.70
TP(g/dL)	60.16	58.33	60	0.75
Albumin(g/dL)	26.83	26.83	28.33	0.54
Glu(mg/dL)	4.5 [b]	4.97 [a]	4.65 [b]	0.01
Cal(mg/dL)	2.33	2.34	2.34	0.01
Phos (g/dL)	6.533 [b]	6.6 [b]	8.45 [a]	0.01
Cr(mg/dL)	1.22 [c]	1.26 [b]	1.31[a]	0.12
Uric Acid (mg/dL)	441.17b	483.83 [a]	459.83 [ab]	7.95

[abc] Means within the same column with different superscript are significantly different

HDL- High Density Lipoprotein, LDL- Low Density Lipoprotein, GLU-Glucose, Cal-Calcium,Phos-Phosphorus,Cr-Creatinine. TC-Total Cholesterol, TCG- Triglyceride,TP- Total Protein

T1- 0.4g/kg Methioine, T2- 0.8g/kg Methioine, T3- 1.2 g/kg Methioinine

Effect of PHFT on the Biochemical Parameters of Broilers.

The effect of Post Hatch feeding time is shown in table 5. Plasma Cholesterol, triglyceride, High Density Lipoprotein, Total Protein, Glucose, Calcium, phosphorus, creatinine and uric acid were all significantly ($p<0.05$) different in the study while Low density Lipoprotein and Albumin were not significantly influenced by Post hatch feeding. The values observed for plasma cholesterol, triglycerides and High-Density Lipoprotein shows significant effect ($p<0.05$) of Post Hatch Feeding Time on these parameters.TC, TG and HDL values were higher in birds that were subjected to 24 hours and was lower in birds submitted to 30 hours and 36 hours Post Hatch Feeding Time. The values obtained in this study supports the previous work of Balog *et al*. (2004), they observed a lower level of Total cholesterol in Fasted chickens.

Demir *et al*. (2004) observed that delayed feeding leads to an increase in plasma total cholesterol content compared with broilers fed *ad libtum* while others have found no effects (Mohebodini *et al*., 2009; Onbasılar *et al*., 2009). The discrepancies in the results reported by different authors may be as a result of variations in lipoprotein lipase activity (Zhan *et al*., 2007). Plasma Protein values in this study show that Post Hatch Feeding Time significantly influence the Total Protein. Birds that are fed 36 hours Post Hatch have significant higher Total Protein than those fed 24 hours and 30 hours. This result corroborates the findings of Pires *et al*. (2017) that chicks that are fed 48hrs post hatch had higher Total Protein than birds that were given feed and water immediately they arrived. High level of TP may be as a result of increased protein catabolism by the body in order to maintain the physiological functions during fasting. According to Pires *et al*. (2017), high values of TP in fasted chickens reveals that fasted chickens can use depletion of protein to obtain energy and metabolic water.

Post hatch Feeding Time had a significant influence on Plasma Glucose. Birds that were fed immediately had significantly higher plasma glucose than birds that were fed later. This shows that Post Hatch fasting do influence Plasma Glucose depletion. The high values of glucose in the 24 hours fed chicken is probably related to the utilization of liver glycogen as glucose is the major end product of energy in chickens. Whereas, the low values of Glucose indicate the depletion of liver glycogen. This study is consistent with the findings of Pires *et al*. (2017), they reported an increase in Total Protein and a low level of Plasma Glucose in birds that were fasted Post Hatch. Corduk *et al*. (2013) reported that delayed access to feed and water for 24 or 48 h post hatch resulted in a significantly low serum glucose levels at 21days compared with birds that have immediate access. They explained that newly hatched chicks have low liver and muscle glycogen and need to be fed as soon as possible. On the other hand, chicks that subjected to delay in access to carbohydrates feed in the initial 48 hours to 72 hours after hatching lose BW. The BW losses consequently reduce the energy requirement of chickens. As a result, the liver and muscle glycogen resources are initially reduced followed by blood glucose level (Poljičak-Milas *et al*., 2003). Calcium and Phosphorus levels show significant effect of Delayed Post Hatch Feeding Time. The birds fed 36hours Post Hatch had significant higher serum calcium than birds fed 24 hours and 30hours Post hatch while the birds fed 24 hours Post Hatch had significant higher Serum phosphorus than birds fed 30 hours and 36hours. Serum creatinine values were significantly different between treatments as the values increase as the access to feed was delayed. According to Umit *et al.* (2011) creatinine is a chemical waste molecule produced as a result of muscle metabolism and the kidney is reported to help in regulating its normalcy. The values of uric acid increased significantly as the length of feeding increases. According to Adeleye *et al*.

(2017) the significant higher value of serum uric acid as time of feeding was delayed indicated protein wastage. Fafiolu (2007) reported that plasma uric acid is a function of the quality of protein quality offered to the animal and that high levels indicate low protein efficiency utilization.

Table 5 Effect of PHFT on biochemical parameters of broiler chickens

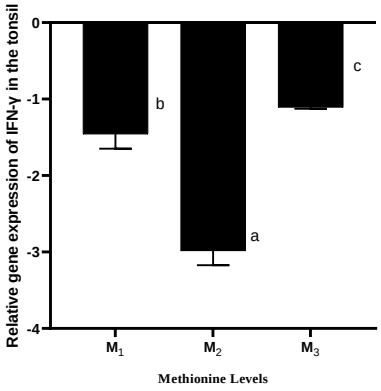
Parameters	24 hours	30 hours	36 hours	SEM
TC(mg/dL)	3.22[a]	2.63[b]	2.75[b]	0.06
TCG (mg/dL)	1.08 [a]	0.80 [b]	0.85 [b]	0.02
HDL(g/dL)	0.94 [a]	0.75 [b]	0.66 [c]	0.01
LDL(g/dL)	1.71	1.66	1.81	0.07
TP(g/dL)	60.17[b]	55.67[c]	62.67[a]	0.75
Albumin(g/dL)	27.50	26.80	27.67	0.54
Glu(mg/dL)	4.92 [a]	4.57[b]	4.62 [b]	0.09
Cal(mg/dL)	2.31[b]	2.30[b]	2.41[a]	0.01
Phos(g/dL)	8.20 [a]	6.85 [b]	6.55 [b]	0.09
Cr(mg/dL)	1.38 [a]	1.22 [b]	1.19[b]	0.01
Uric acid (mg/dL)	528.00[a]	408.50 [c]	448.33[b]	7.95

[abc] Means within the same column with different superscript are significantly different
HDL-High Density Lipoprotein, LDL- Low Density Lipoprotein, GLU-Glucose, Cal-Calcium,Phos-Phosphorus,Cr-Creatinine. TC-Total Cholesterol, TCG- Triglyceride,TP- Total Protein, PHFT- Post Hatch Feeding Time.

Effect of Methionine on Cytokine Expression.

Figure 1 and 2 show the effect of methionine in cytokine expression in broilers. In the spleen, the production of IL-2 was not sufficient enough to be detected by RT-PCR. In cecal tonsils, dietary methionine had a significant effect on IL-2 expression, the expression of IL-2 was significantly down-regulated in birds that are on the 0.4 % methionine and significantly up regulated in birds on 0.8% and 1.2% treatment. IL-2 is a pro inflammatory cytokine, which stimulate the growth, proliferation and differentiation of T cells (Yang *et al*., 2017). Interleukins are a part of a class of cytokines that influence the immune system through their important physiological and pathological role in the inflammatory response process. An imbalance in cytokine secretion or cytokine process disorders may result in a variety of pathological disorders (Tayal & Kalra, 2008), this shows that the broilers in the 0.4% methionine group had less of an inflammatory reaction during growth. Significant difference was also observed in the expression of IFN-γ in the ceacal tonsil. In the caecal tonsil, IFN-γ was significantly down regulated. However, no significant difference was observed in the IFN-γ expression in the spleen. Splenic T lymphocyte concentration is an essential factor in maintaining immune homeostasis of the spleen and the fluctuations can indicate cellular immunologic status. Interleukin-2 and IFN-γ are examples of cytokines with multiple functions in the regulation of immune reaction and inflammation. IFN-γ is a proinflammatory cytokine and is also secreted by activated T lymphocytes. IFN-γ functions in both innate and acquired immunity. It is also an important cytokine that is involved in maximizing the potential and proliferation of Cytotoxic T Cells (Schoder *et al*., 2004). Our experiment indicated that Methionine supplementation decreased IFN-γ level as the quantity of methionine included increased. Lai (2018),

however, showed that Methionine has a stimulative effect on the production of IFN-γ in birds vaccinated against Eimeria spp. This contradiction may be due to the immune response of IFN-γ in models of inflammation, as opposed to this study where inflammation was not a pathological condition. It has been reported that IFN-γ have the ability to \modulate chemokine secretion in response to the other cytokines and affect cellular adhesion and transmigration. Thus, our result suggests that the reduced level of IFN-γ after methionine supplementation can reduce the risk of inflammation. Also, IFN-γ is able to down regulate the expression of surface adhesion factors in lymphocytes. This present study is in line with the report of Bhanja *et al*. (2014), they reported increased IL2 gene expression with increased methionine in the diet. Sirimongkolkasem (2007) also suggested that Methionine plays an important role in growth and humoral immunity (antibody production), and Cysteine is in greater demand than lysine for humoral immunity. Cysteine a product from methionine also added to the glutathione and accessory protein produced by liver during the acute phase response (Grimble, 2006). In another study, Wang (2017) also reported severity of Newcastle Disease infections in chicks fed methionine deficient diet. He also reported a lower serum IL2 in birds fed methionine deficient diet.



[abc]Means within the column with different superscript are significantly different.

Figure 1: The Effect of Methionine Levels on the Relative Gene Expression of IFN-γ inthe tonsil of broiler Chickens

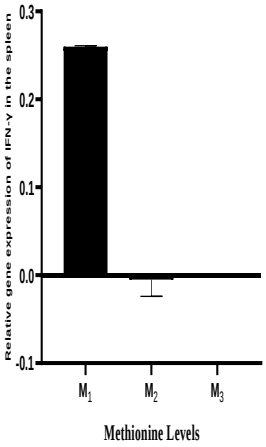
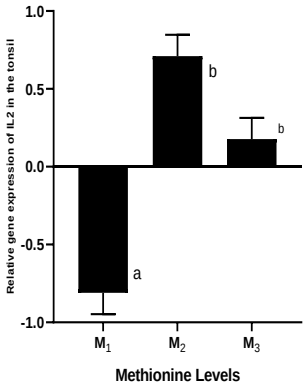
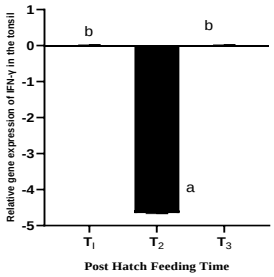


Figure 2: The Effect of Methionine Levels on the Relative Gene Expression of IFN-γ in the Spleen of Broiler Chickens



[ab] Means within the column with different superscript are significantly different.

Figure 3: The Effect of Methionine Levels on the Relative Gene Expression of IL2 in the tonsil



[ab] Means within the column with different superscript are significantly different.

Figure 4: The Effect of Post Hatch Feeding Time on the Relative Gene Expression of IFN-γ inthe tonsil of broiler Chickens

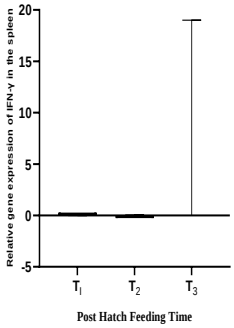
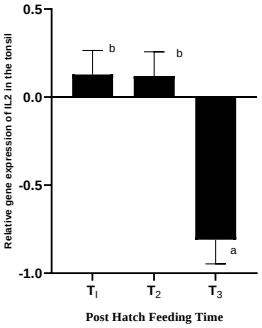


Figure 5: The Effect of Post Hatch Feeding Time on the Relative Gene Expression of IFN-γ in the Spleen of Broiler Chickens



[ab] Means within the column with different superscript are significantly different.

Figure 6: The Effect of Post Hatch Feeding Time on the Relative Gene Expression of IL2 in the Tonsil of Broiler Chickens

Effect of Post Hatch Feeding Time on Cytokine Expression

Figure 4-6 presents the result of the effect of post hatch feeding time on the relative expression of IL-2 and *IFN-γ* in the spleen and in the tonsil of broilers. The expression of IL-2 in the spleen was not enough to be detected by QT-PCR. In the spleen, PHFT caused a significant effect on the expression of *IFN-γ*. The expression of *IFN-γ* in the spleen was up regulated in the birds fed 30 hours and 36 hours respectively, while it was down regulated in the birds fed 24 hours post hatch. In the tonsil, IL2 expression was down regulated in birds fed at 36 hours post hatch and was upregulated in birds fed at 24 hours and 3o hours post hatch. A significant effect of *IFN-γ* was also observed, however, *IFN-γ* was upregulated in the birds fed 24 hours and 36 hours while it was downregulated in the birds fed 30 hours post hatch.

Correlation Between Cytokine Expression and Some Biochemical Parameters

The correlations between cytokine expressions in the spleen and tonsils and biochemical parameters of broilers are presented in table 4:14. Of the biochemical parameters tested, there exist a negative significant relationship between IL-2 and Albumin, IL2 and Total Protein, IL-2 and HDL.Uric Acid had a significant correlation with IFN-γ both in the spleen and in the tonsil. A low significant negative correlation exists between Spleenic IFN-γ and HDL. Inflammation initiates an acute-phase response, resulting in the activation of monocytes and/or macrophages, which in turn release more pro inflammatory cytokines.

These cytokines stimulate acute-phase response by stimulating the release of IL-6, which amplifies this acute-phase response, this leads to the inhibition of various protein synthesis in the liver, such as visceral proteins, albumin, pre-albumin, and transferrin. These proteins are traditionally considered markers of the nutritional status.

Serum albumin is produced by the liver. Pro-inflammatory cytokines stimulate acute-phase response in the liver, thereby increasing serum concentration of C Reactive Protein, fibrinogen and amyloid A protein. Acute phase response leads to a reduction in the synthesis of serum albumin and an increase in degradation of albumin that result in hypoalbuminaemia. Even though serum albumin is an acute-phase protein, it has been reported that its concentration may be independently reduced by inflammation caused by pro inflammatory cytokines. Tang *et al*. (2009) and Cockerill *et al*. (2001) has described situations where HDL acts as an anti-inflammatory particle in human subjects. The negative significant relationship that exists between the HDL and the cytokines under research may be due to the anti-inflammatory properties exhibited by HDL. Correlation analysis between IFN-γ and Uric acid show a strong positive correlation, this is probably due to the antioxidant effect of Uric acid in Broilers. Several authors have reported that UA is a potent antioxidant and major antioxidant defense mechanism used by birds (Klandorf *et al*., 2001; Simoyi *et al*., 2002; Machin *et al*., 2004; Seaman *et al*., 2008). Settle *et al*., (2015) reported that a reduced level of Uric Acid in the liver will result in inflammation, oxidative damage and a decline in the health of birds.

Table 6. Correlation Between Cytokine Expression and Some Biochemical Parameters

Parameters	IL-2	IFN-γ Tonsil	IFN-γ SPLEEN
Glucose(mg/dL)	0.257	0.205	-0.368
Albumin(mg/dL)	-0.511*	0.241	-0.449
HDL(mg/dL)	0.536	0.069	-0.062*
Total Protein(mg/dL)	-0.672**	0.536	-0.363
Creatinine(mg/dL)	0.210	0.175	0.489
Uric Acid(mg/dL)	0.489	0.0501	0.750**
Triglyceride(mg/dL)	0.269	0.414	0.459

*- $p<0.05$ **- $p<0.01$

HDL- High Density Lipoprotein, IL-2- Interlukin 2, IFN-γ - Interferon gamma

Conclusion

This research shows that methionine and Post Hatch Feeding Time influences the immune system. It was also discovered that the percentage of dietary methionine needed by broilers to maintain optimum nutritional and health status may be higher than the requirement for growth, Furthermore, it can be concluded that the expression of IL-2 and IFN-γ in broilers can be used to monitor the nutritional status of the birds. In this study, it was observed that negative relationship exists between IL-2 and IFN-γ and serum albumin and Total Protein. These serum components are the traditional markers of nutritional status

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