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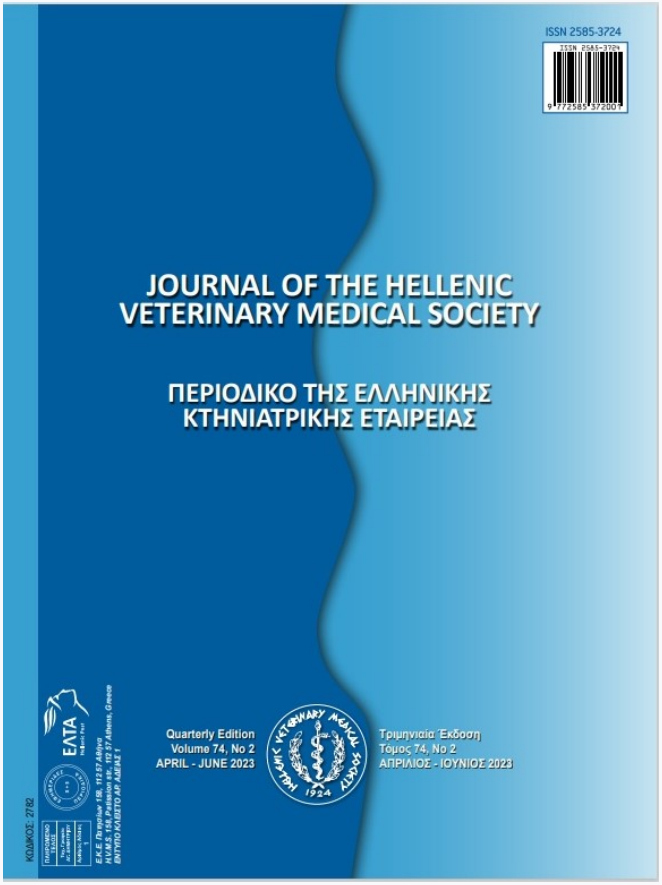

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Research article
Ερευνητικό άρθρο

Effects of different levels of methionine in pre-starter diets on immunity function and DNA methylation in broilers

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ABSTRACT: The study was carried out to determine the effects of different levels of methionine in Pre-starter diets on PPAR gene expression and immune function of broiler chickens. Methionine (Met) is necessary to achieve a fast growth rate in chickens. A total of 240 Ross 308 broilers were equally assigned to 8 treatments with 3 replicates. The treatments included T1: diet with 20% methionine less than Ross catalog recommendation. T2: Standard diet in accordance with the recommendations of the Ross catalog. T3, T4, T5, T6, T7 and T8: diets with 20, 40, 60, 80, 100 and 120 % methionine more than Ross catalog recommendation. The results showed that there was a significant difference in the expression of PPAR gene with the difference in diet Met levels in the 8 groups ($P<0.05$). PPAR controls the expression of several genes involved in the proliferation and differentiation of adipose tissue cells. Gene expression in broiler chickens with Met deficiency and excess may have compensated for this deficiency and excess in the birds. Also, the results indicated that increasing in the levels of Met in pre-starter diets of chickens the antibody production against ND increased significantly ($P<0.05$). Besides, the antibody production against IBD increased significantly ($P<0.05$). Furthermore, the antibody production against H9N1 not affected ($P>0.05$). Studies suggest that dietary protein deficiency reduces the concentration of most amino acids in plasma and compromises the immune system. Totally it is suggested that the high levels of Met in the diet maybe beneficial and it needs more studies.

***Keywords*:** Broiler chickens; DNA methylation; immune function; PPAR gene expression.

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INTRODUCTION

High dietary concentrations of essential amino acids, including methionine (Met) is necessary to achieve a fast growth rate in chickens. The lower Met content of raw materials of birds feed is measured and mentioned (NRC, 1994). Therefore, in the usual cereal/soybean meal-based diets, Met is the first essential amino acid that limits the biological values of protein (Meirelles et al., 2003; Lemme et al., 2005; Kim et al., 2006; Matsushita et al., 2007). Methionine is important because participates in protein synthesis, also is a glutathione precursor, a tripeptide that reduces reactive oxygen species (ROS) and thus protects cells from oxidative stress furthermore, Met is required for the synthesis of polyamines (spermine and spermidine), which take part in nucleus and cell division events. Besides as a non-proteinogenic function in the body, Met serves as a methyl group giver. The addition of methyl group to DNA in DNA methylation procedure, which regulates cell differentiation and development, is the product of the conversion of Met to S-adenosyl methionine (Niculescu& Zeisel, 2002). Methionine content of diet in poultry could affect the methylation of skeletal muscle tissues and the meat quality of broiler chickens (Liu et al., 2010). Some substances complicated in sulphur containing antioxidant system and Met as a sulphur-contained amino acid is a precursor of other sulphuric amino acids (taurine and L-cysteine) and other substances (Mukwevho et al., 2014). It is verified that Met positively affects the immune system, with improving both cellular and humoral immune responses. The reports showed that Met requirements for optimal growth are lower than for optimal immunity (Swain &Johri, 2000; Shini&Bryden, 2005), and so deficiency of sulfur amino acids (SAA) resulting in acute lymphocyte reduction in intestinal tissue (Peyer's patches) and lamina propria (Swain &Johri, 2000). Proliferation of T cells is one of the mechanisms suggested to clarify Met interference in the immune system, which are sensitive to intracellular glutathione and cysteine amounts, compounds also take a part in Met metabolism (Kinscherf et al., 1994).

In mammals, peroxisome proliferator-activated receptor (PPAR) controls the expression of several genes involved in the proliferation and differentiation of adipose tissue cells. It is reported in rats, starvation and levels of Dietary fatty acids affect the expression of PPAR gene (Vidal-Puig et al., 1996). Fasting in pigs also reduces the expression of PPAR gene in adipose tissue. It has been reported that the profile of dietary fatty acids, especially unsaturated acids, regulates the expression of PPAR gene, thereby affecting cell differentiation. The adipose tissue of the ventricular area is affected (Sato et al., 2004). Affect the expression of some genes involved in energy metabolism. The receptor gene affected by gamma-type proxisome amplifiers (PPAR) one of the genes studied by Rosen & Spiegelman (2001) regulates the metabolism of glucose and lipid toxins. Research's has shown that this gene is expressed in broiler chickens in different tissues, but its highest expression has been reported in adipose tissue of chicken ventricular area (Meng et al., 2005). High expression of this gene in adipose tissue affects the differentiation of this tissue (Wang et al., 2008). Reports suggest that sex related hormones can somehow directly and indirectly affect the expression of PPAR gene. Studies on Mammals show that mammalian adipose tissue contains sex hormone receptors such as estrogen and progesterone (Mizutani et al., 1994; Dieudonne et al., 1995). Sex related hormones can affect adipose tissue and fat metabolism through these receptors. An important purpose in the broiler industry is to reduce carcass fat, which is an attempt to achieve this goal through genetics and genetic manipulation as well as nutrition management. Due to the limited resources that can be used both as a fat supplement and is economically optimal, it led to the use of some by-products of industries such as fats. The aim of this study was to investigate the effect of dietary fatty acid profile and bird sex on the function and expression of PPAR gene in adipose tissue of broilers. With these aspects the study was carried out to determine the effects of different levels of methionine in Pre-starter diets on DNA methylation and immune function of broiler chickens.

MATERIALS AND METHODS

Animal experiments were approved by the Animal Care Committee of the Islamic Azad University and all experiments were performed in accordance with the regulations and guidelines established by this committee(no. 1361-IAU. 06.09.2020).

Animals and Experimental Diets

A total of 240 Ross 308 broilers one-day old (both sex) were equally assigned to 8 treatments with 3 replicates (each replicate contain 10 birds). The treatments were as follows: 8 different groups of broilers, including group fed with diet 1 (diet with 20% methionine less than Ross catalog recommendation) and group fed with diet 2 (Standard diet in accordance with

the recommendations of the Ross catalog), group fed with diet 3 (diet with 20% methionine more than Ross catalog recommendation), group fed with diet 4 (diet with 40% methionine more than Ross catalog recommendation), group fed with diet 5 (diet with 60% methionine more than Ross catalog recommendation), group fed with diet 6 (diet with 80% methionine more than Ross catalog recommendation), group fed with diet 7 (diet with 100% methionine more than Ross catalog recommendation) and group fed with diet 1 (diet with 120% methionine more than Ross catalog recommendation). The experiment started from day first and lasted for a week then the diets of all treatments was equal and formulated with regards to Ross catalog recommendation (table 1).

RNA Extraction, and Real-time PCR Analysis

To examine the effects of different levels of methionine in pre-starter diets on DNA methylation of broiler chickens, the total RNA was extracted from liver tissue using TRIZOL method (Hamidi et al. 2022). The amount of RNA was determined spectrophotometrically (260 nm and A260/280 = 1.8-2.0), with the samples stored at − 70 °C. Reverse transcription was performed with 7 μg of total RNA extracted from liver tissue of broiler chickens. For RT-PCR process, cDNAwas synthesized in a 20 μL reaction mixture containing 1 μg of RNA, the oligo (dT) primer (1 μL), a 5 × reaction buffer (4 μL), an RNAse inhibitor (1 μL), a 10 mM of dNTP mix (2 μL), and M-MuLV Reverse Transcriptase (1 ml), according to the manufacturer's protocol(Fermentas, GmbH, Germany). The cycling protocol for 20 μL reaction mixes included 5 min at 65 °C, followed by 60 min at 42 °C, and 5 min at 70 °C to terminate the reaction. PCR conditions were as follows:

One cycle at 97°C for 5 minutes, 25 cycles at 30°C for the incubation phase, 30°C at the connection stage for 67°C and 72°C for the expansion phase at 72°C. Final cycle at 72°C for 8 minutes. At first, band normalization was performed based on GAPDH. At this stage, after staining the PCR products, the bands ob-

Table 1. Ingredient and nutrient composition (%) of basal diets.

	Pre-starter								Starter	Grower	Finisher
Age, d	0-7								8-14	15-24	25-35
	T1	T2	T3	T4	T5	T6	T7	T8			
Ingredients											
Yellow corn	46.83	50.18	46.78	46.64	46.58	46.52	46.46	46.4	50.18	55.19	58.87
Soybean meal	34.13	33.87	34.13	34.13	34.13	34.13	34.13	34.13	33.87	30.25	27.5
Gluten	7	5	7	7	7	7	7	7	5	5.64	3.5
Vegetable oil	7.1	5.87	7.1	7.1	7.1	7.1	7.1	7.1	5.87	4.4	5.5
Common salt	0.29	0.29	0.29	0.29	0.29	0.29	0.29	0.29	0.29	0.26	0.27
Sodium bi-carbonate	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.23	0.24
Dicalcium phosphate	2.06	2.06	2.06	2.06	2.06	2.06	2.06	2.06	2.06	1.84	1.85
Calcium carbonate	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99	1.4	1.5
DL-methionine	0.25	0.35	0.372	0.44	0.5	0.56	0.62	0.68	0.35	0.2	0.18
L-lysine	0.53	0.52	0.53	0.53	0.53	0.53	0.53	0.53	0.52	0.09	0.09
L-Threonine	0.1	0.14	0.1	0.1	0.1	0.1	0.1	0.1	0.14	-	-
Choline chloride (60%)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	-	-
Premix[1]	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Nutrients[2]											
Metabolizable energy, kcal/kg	3000	3000	3000	3000	3000	3000	3000	3000	3000	3100	3200
Crude protein (%)	24	23	24	24	24	24	24	24	23	21.5	19.5
Ave. P (%)	0.48	0.48	0.48	0.48	0.48	0.48	0.48	0.48	0.48	0.51	0.51
Ca (%)	0.96	0.96	0.96	0.96	0.96	0.96	0.96	0.96	0.96	1.13	1.13
Na(%)	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.2	0.2
Met(%)	0.47	0.64	0.71	0.83	0.944	1.06	1.18	1.3	0.64	0.51	0.47
Lysine (%)	1.44	1.44	1.44	1.44	1.44	1.44	1.44	1.44	1.44	1.16	1.11
Threonine (%)	0.97	0.97	0.97	0.97	0.97	0.97	0.97	0.97	0.97	0.88	0.88

[1]Premix provided (mg/kg diet): thiamin 1; pyridoxine, 2; cyanocobalamine, 0.01; niacin, 15; pantothenic acid, 10; tocopherol, 10; riboflavin, 10; biotin, 0.08; menadione, 2; retinol acetate, 2.75; cholecalciferol, 0.03; choline, 650; copper, 8; iron, 45; manganese, 80; zinc, 60; selenium, 0.18; monensin sodium, 100; and hydrated sodium calcium alumino silicates, 800.
[2]Calculated based on analyzed values of individual feed ingredient except for energy which was based on published ME values.

served for the GAPDH gene must be completely identical in color intensity. To achieve this, it is sometimes necessary to take different amounts of cDNA samples for PCR. After normalization of the bands for the GAPDH gene (this gene is not affected by treatment), PCR is applied to the gene with the same amount of cDNA used for normalization. PCR products were loaded on 2% agarose gel and stained with ethidium bromide. Gene expression was analyzed using the relative PCR amplification analysis method (2−ΔΔCT). Changes in gene expression were referenced to the level of expression at ZT1.5 in the CON group. The specific primers for Gallus PPAR gene and GAPDH were designed and manufactured by CinnaGen (CinnaGen Co., Tehran, Iran). The primer pairs for RT-PCR are depicted in Table 2.

Serum antibody titres

Serum antibody titres against Newcastle disease (ND) and Avian Influenza (H9N1) were determined by means of hemagglutination inhibition (HI) test using standard methods described in OIE (2009). Antibody titre to infectious bursal disease virus was determined by commercial ELISA kits (Pars Azmon, Iran), according to manufacturer's instructions.

Statistical Analysis

The obtained data of immune function were analyzed with SPSS software (SPSS pack. 21). A one-way analysis of variance (ANOVA) was carried out to determine significant differences between groups.

RESULTS

Effects of Feeding Low- and High-Met Diets on the Expression of PPARA Gene in Liver of broilers

The expression level of PPARA gene, which was measured in 8 different groups of broilers, including group fed with diet 1 (diet with 20% methionine less than Ross catalog recommendation) and group fed with diet 2 (Standard diet in accordance with the recommendations of the Ross catalog) and groups fed with diet 3, 4, 5, 6, 7 and 8 (diets with 20, 40, 60, 80, 100 and 120 % methionine more than Ross catalog recommendation), was upregulated in treatments 1 and 2, then downregulated in treatments 3, 4, 5 and

Table 2. Primer used for quantitative real-time PCR analysis.

Gene	Forward primer (from 5_ to 3_) reverse primer (from 5_ to 3_)	Product size (bp)	NCBI gene bank accession number
PPARA	AGGCCAAGTTGAAAGCAGAA GTCTTCTCTGCCATGCACAA	217	NM 001001464.1

(Made by TakapoZist co. Iran)

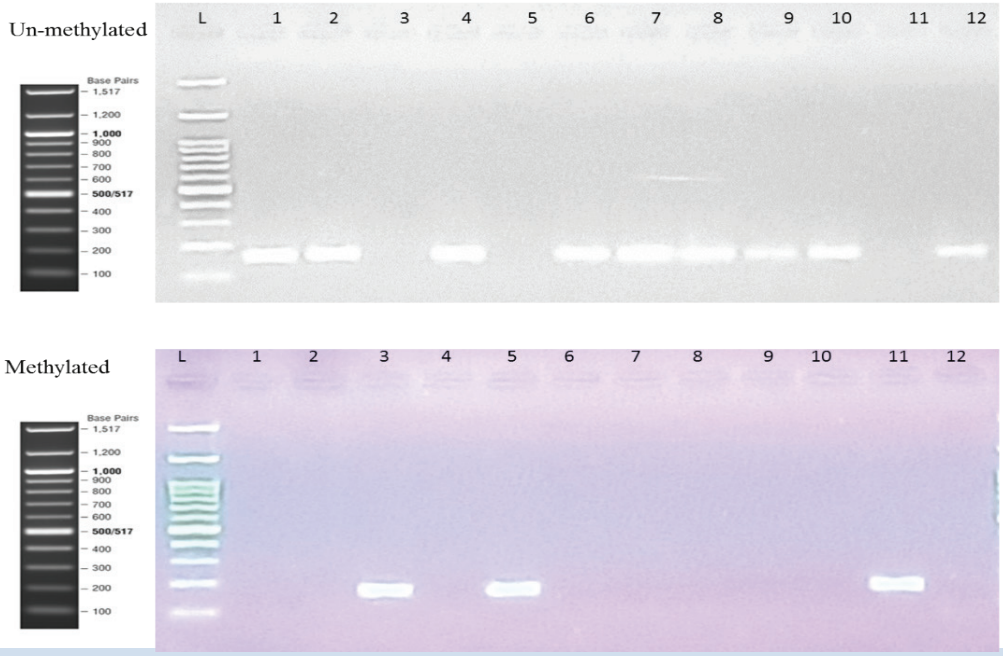


Figure 1. Electrophoresis of PCR product with methylated and non-methylated primers for 12 samples (the replicates of treatments 1 to 4) in 2% agarose gel. 100 bp DNA Ladder

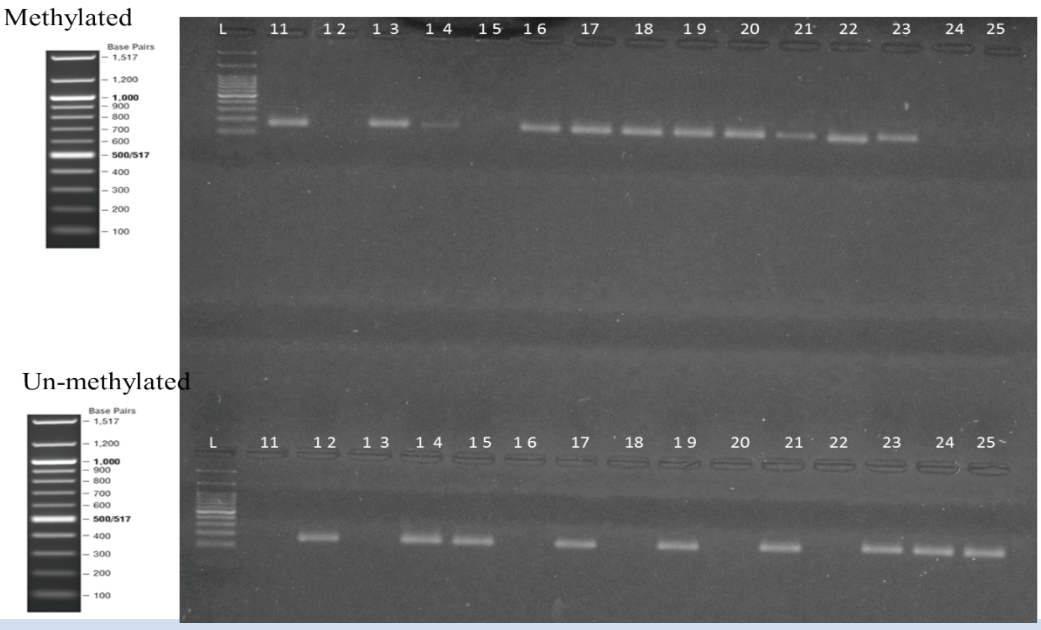
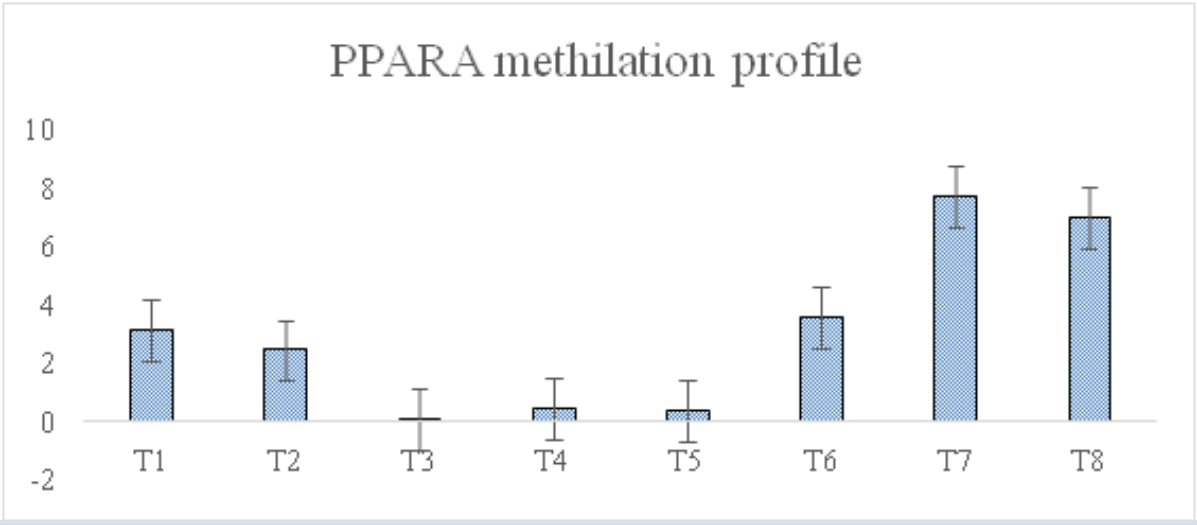


Figure 2. Electrophoresis of PCR product with methylated and non-methylated primers for 12 samples (the replicates of treatments 5 to 8) in 2% agarose gel. 100 bp DNA Ladder



Graph 1. PPARA gene expression

6 at that point upragulated again in treatments 7 and 8 (graph 1). In this regard, no report from previous research was found for comparison.

The results showed that there was a significant difference in the expression of their PPAR gene with the difference in diet methionine levels in the 8 groups. Gene expression in broiler chickens with methionine deficiency and excess may have compensated for this deficiency and excess in these birds.

Methionine is one of the most expensive components of broiler diets. Therefore, the level of methionine in the diet should be minimized in such a way that it can provide maximum efficiency so that proper growth of the body is also provided. This issue needs to be considered in future research.

Immune function

Furthermore, the effects of different levels of methionine in Pre-starter diets on antibody titre against Newcastle disease (ND), Infectious Bursal Disease (IBD) and Avian Influenza (H9N1) virus in broiler chickens was measured. The results showed that with increasing the levels of methionine in Pre-start-

Table 3. Effect of different levels of methionine on ND, IBD and H9N9 antibody titer of broilers

	T1	T2	T3	T4	T5	T6	T7	T8	SEM	P value
ND	1.9^{c}	2.0^{bc}	2.0^{bc}	2.5^{abc}	2.37^{abc}	2.6^{ab}	2.7^{a}	2.6^{ab}	0.0829	< 0.05
IBD	2.88^{b}	2.98^{b}	3.38^{ab}	3.46^{ab}	3.48^{ab}	3.66^{a}	3.50^{ab}	3.65^{a}	0.0818	< 0.05
N9H1	1.28	1.30	1.27	1.26	1.23	1.22	1.24	1.25	0.0166	ns

T1: diet with 20% methionine less than Ross catalog recommendation. T2: Standard diet in accordance with the recommendations of the Ross catalog. T3, T4, T5, T6, T7 and T8: diets with 20, 40, 60, 80, 100 and 120 % methionine more than Ross catalog recommendation; a-c represents means within the same row with different superscripts differ (p < .05).

er diets of chickens the antibody production against ND increased significantly (P<0.05). Also, the antibody production against IBD increased significantly (P<0.05) with increasing the levels of methionine in Pre-starter diets of broilers. Besides, the antibody production against H9N1 not affected (P>0.05) by different treatments (Table 3).

DISCUSSION

Effects of Feeding Low- and High-Met Diets on the Expression of PPARA Gene in Liver of broilers

The expression level of PPARA gene, which was measured in 8 different groups of broilers, including group fed with diet 1 (diet with 20% methionine less than Ross catalog recommendation) and group fed with diet 2 (Standard diet in accordance with the recommendations of the Ross catalog) and groups fed with diet 3, 4, 5, 6, 7 and 8 (diets with 20, 40, 60, 80, 100 and 120 % methionine more than Ross catalog recommendation), was upregulated in treatments 1 and 2, then downregulated in treatments 3, 4, 5 and 6 at that point upragulated again in treatments 7 and 8 (graph 1). In this regard, no report from previous research was found for comparison. PPAR controls and regulates the expression of various genes that play a role in lipid homeostasis, especially cholesterol (Lee et al., 2018). On the other hand, fatty acids (especially trans-unsaturated fatty acids, or in fact 15 cis) act as ligands for PARs; following binding of the ligand to PPAR, a complex is formed which activates the target genes by binding to 16 promoters (Koronowicz et al., 2017). Methionine is an essential amino acid and a precursor to poultry protein (Lee et al., 2020). When this raw material is not available for protein synthesis, the endocrine system is likely to increase the secretion of insulin-like growth hormone from breast muscle tissue to compensate for this deficiency in broilers that have been reduced by methionine-free diets. To further stimulate protein production and the body can use methionine, which is another component of the diet, to build muscle protein (Yang et al., 2018).

It is proved that chickens fed diets containing balanced protein had less abdominal fat than low-protein diets and the reduction of the protein of the diet increasing abdominal fat content (Van Milgen, 2021). In this regard it is reported that increasing dietary protein reduces abdominal fat in broilers. Rezaei et al. (2004) also observed that reducing crude dietary protein increases the percentage of abdominal fat. There seems to be a close relationship between the percentage of abdominal fat and the ratio of energy to protein (Rezaei et al., 2004). The smaller this ratio, the less fat will be stored in the body. By reducing crude protein, the energy to protein ratio changes and more energy is available, thus increasing carcass fat. One of the effective factors in reducing carcass fat when using high protein diets is increasing energy costs to convert excess nitrogen to uric acid because the excretion of excess nitrogen in the form of uric acid requires 6 moles of ATP per gram of nitrogen (Van Milgen, 2021). Also, in low-protein diets, in addition to lower energy loss related to uric acid excretion, the activity of the enzyme acetyl COA carboxylase is also increased and consequently the intensity of fat production in the liver is increased.

Immune function

Further studies show that amino acid supplements in the diet of animals and humans, in contrast to malnutrition and infectious diseases, improve the immune status and thus reduce the prevalence of disease and mortality. Besides, metabolism of amino acids in leukocytes is critical to prevent immune-related diseases (Tan et al., 2013). Recent studies suggest that dietary protein deficiency reduces the concentration of most amino acids in plasma and compromises the immune system (Adedokun&Olojede, 2019). In recent years, fundamental cellular and molecular mechanisms have begun to detect these effects (Adedokun&Olojede, 2019). In determining the needs of poultry, not only should their performance be considered, but their safety should be considered too. In 1994, the NRC recommended poultry nutrient requirements based on

the lack of clinical signs of deficiency. On the other hand, early feeding appears to affect poultry performance and safety. Different effects have been reported on the effect of methionine supplementation in broiler diets on cellular or humoral immunity (Lee et al., 2020). The need for sulfur-containing amino acids for optimal function is different from the need for optimal immunity: Adequate intake of methionine and cysteine from the diet is essential for the synthesis of immune proteins (Jankowski et al., 2014.). Methionine and cysteine supplements are beneficial for the immune system under different catabolic conditions. For example, increasing the level of methionine from 0.35 to 1.2% in the diet of broilers with Newcastle disease, obviously immune reactions, proliferation of T cells in response to mitogenic stimuli, plasma levels of immunoglobulin G (Tsiagbe et al., 1987) increases leukocyte migration and antibody titer (Swain et al., 2000). Also, the addition of methionine to soybean meal-based diets increases antibody titer and increases metogenic stimulation (Tsiagbe et al., 1987). Some researchers have reported that supplementation of the diet with Zn-methionine at 60 mg/kg diet increases antibody response and antibody titer and improves immune system function (Hamidi&Pourreza, 2009). In addition to protein production, methionine has been shown to have other roles in cell division and hypoxia, which can be effective in reducing immunity (Kalbande et al., 2009).

CONCLUSIONS

Totally the writers suggest that the high levels of Met in the diet maybe beneficial to fat metabolism therefore it could improve the carcass traits of broilers and it may improve immune function but it needs more studies.

CONFLICT OF INTEREST DECLARATION

The authors declare that they have no conflict of interest.

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COMPLIANCE WITH ETHICAL STANDARDS

Animal experiments were approved by the Animal Care Committee of the Islamic Azad University and all experiments were performed in accordance with the regulations and guidelines established by this committee.

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