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Effect of Threonine Supplementation on Growth Performance, Metabolizable Energy, Morphological Changes and Immune Response in Broiler Chickens Challenged with Coccidia

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Abstract

This study was performed to investigate the effects of dietary threonine (Thr). level on performance, metabolizable energy, intestinal morphology, and immune system in coccidian–infected broiler chickens. The diets contained: 88%, 100% (Non challenged (NC) and challenged control (PC)), 112%, 124%, and 136% of Thr requirement according to Cobb 500 recommendation and fed during grower (pre challenged) and finisher (post challenged) periods. On d 23 (end of grower period), each bird received 0.5 mL of distilled water or received around 24000 sporulated oocysts. On d 23 and 31, one bird per replicate was slaughtered to measure the performance criteria. Mean dietary apparent metabolizable energy corrected for nitrogen (AMEn) and digestible energy were greater in NC birds than the challenged birds fed on 88% or 100% Thr diets. Feed intake and blood parameters were not significantly influenced by increasing levels of Thr in the diet. Compared with unchallenged birds with coccidia (NC), the growth performance, morphological parameters (not crypt depth), and immune responses decreased ($P < 0.05$) in the birds (PC) that were challenged with coccidia, and oocyte numbers were enhanced. Growth performance, jejunal morphology, and immune responses improved and oocyte count decreased in coccidian- challenged birds fed on the diets with greater levels of Thr ($P < 0.05$). Feeding the challenged birds with the diet containing greater levels of Thr improved ($P < 0.05$) their growth performance, morphology, and immune responses and decreased oocyte number. The birds fed on the diet with 124% Thr demonstrated a similar response as the NC birds. Increased diet Thr level linearly increased average daily gain and decreased feed conversion ratio in the grower and the whole experimentation periods. The AMEn and digestibility of crude protein were enhanced linearly Thr level increased in coccidian-challenged birds. It is concluded that diets containing 124% of Thr recommendation led to the greatest efficacy on the intestinal immune response and normal growth maintenance of the birds contaminated with coccidia.

Introduction

Beyond its principle function of breaking down and metabolizing nutrients, the gut also plays a key role in barrier defense. Given that more than 70% of immune system cells are present in the gastrointestinal, it is not unexpected that the gut is the largest organ in the immune system (Vighi *et al.,* 2008). The intestine is also one of the largest body organs, comprising immune cells spread throughout the entire of the gut-associated lymphoid tissue (GALT) (Celi *et al.,* 2019). It is becoming increasingly important to describe the relationship between the immune system and the gastrointestinal, not only in the function of the gastrointestinal but also in comprehensive studies of health and well-being (Celi *et al.,* 2017). A major physiological role of the gastrointestinal tract is to act as a barrier against antigens and pathogens because gastrointestinal is the most extensive area of contact between the host and their environment.

Diet composition (ingredients and additives) can influence the development and functionality of the gastrointestinal tract. It is demonstrated that diet composition containing ingredients, nutrients, and

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additives can regulate the function and development of the gastrointestinal. Additionally, diet composition also plays a key role in immune-nutrition. Moreover, the components of diet play an important role in the immune-nutrition system (Klasing, 2007). Food and additive digestive response studies enable nutritionists to arrange the best nutrients and additives for maximum immune efficiency (Azzam *et al.,* 2011; Celi *et al.,* 2019). Findings from both animal and human model studies indicate an important function of dietary amino acids in preserving intestinal health and prohibiting intestinal diseases has been recognized.

Among the essential amino acids, threonine (Thr) is used in the synthesis of mucin that covers the surface area of the mucosa, and subsequently has a high impact on intestinal functionality (Ahmad *et al.,* 2019). It is proven that Thr has a vital function in maintaining intestinal immune function through the mucus component (Zaghari *et al.,* 2011). Threonine is known as one of the most necessary mucosal components, containing 40% of the mucoglycoproteins of the gastrointestinal tract (Corzo *et al.,* 2007). Mucins are not highly digestible, and the associated Thr cannot be recovered. Mucin is poorly digested and the related Thr is not able to be recovered. The dietary requirements of Thr for broilers have been precisely examined in terms of growth performance and carcass yields (Ahmadi and Golian, 2010; Xie *et al.,* 2014). Nevertheless, little is known about the effect of dietary Thr on the intestinal maintenance, immune system functionality, and intestinal morphology in broiler chickens, especially in stressful conditions, such as inflammation created by diseases (Chee *et al.,* 2010). Although it should be considered that during inflammation, Thr availability may be restricted by mucins synthesis, which can result in a defect in gut barrier function. Increased dietary Thr can enhance mucins synthesis and stabilize the intestinal flora, protecting the intestine, and improving the mucosal membrane (Faure *et al.,* 2006). It has been found that diets deficient in Thr result in a decrease in mucin protein synthesis in rats. Moreover, a healthy diet that supplies the recommended Thr level is not sufficient when the animal experiences a gut challenge, because the requirement for gastrointestinal health is often greater than the Thr requirement for growth (Faure *et al.,* 2006).

On the other hand, intestinal parasites are the main stressors that cause malnutrition and reduce the performance and productivity of poultry. Coccidian protozoa of the genus Eimeria spp are known to cause Coccidiosis which is an intestinal infection. Coccidia parasites pose a serious threat to the growth and feed consumption of poultry (Mohiti *et al.,* 2015). The proliferation of parasite in epithelial cells can destroy tissues and increase the chance of diarrhea and intestinal bleeding which impedes digestion and consequently leads to weight gain, reduced feed intake, and eventually declining feed conversion ratio (Hafez, 2008).

In this study, coccidiosis has been used as the infection model to determine the Thr requirement of birds suffering from intestinal disease. Therefore, the present study aimed to evaluate the effects of increasing dietary levels of Thr on intestinal morphometry, immune responses, metabolizable energy, and growth performance in broiler chickens infected with coccidiosis.

Material and Methods

Animals and experimental diets

A total of 500 male day-old broilers (Cobb 500) were obtained from a commercial hatchery and raised collectively for 10 days in a controlled house. On day 11, 396 of them were randomly divided into 36 groups of 11 birds each and assigned to six treatments of six replicate groups. The facility was measured to be 32°C when the chicks were placed, as the experiment went on this temperature was gradually reduced. All birds were given the freedom to feed and drink water freely throughout the course of the study. The lighting program consisted of 23L:1D throughout the study. The experiment was conducted at the growing (11 to 22 days) and finishing (23-37 day) periods. The basal diet was prepared with the same ingredients for all periods (starter, grower, and finisher). The diets were formulated to meet the necessary nutrient requirements according to the Cobb 500 rearing guidelines (Cobb, 2012). There were five dietary treatments at the grower period (11-22 days of age) for evaluating the effect of different levels of threonine. The dietary treatments contained: 88, 100 (two control treatment groups with a similar diet in this period), 112, 124, and 136 percent of Cobb-500 rearing guideline recommendations in a completely randomized design. All the birds were challenged with coccidiosis at 23 days of age, except one of two control treatment groups. So, there were two control treatments fed the diet with 100% Thr requirement in that time (finisher period). One of them was negative control (NC), received no *Eimeria*, oocysts and the other control group was positive control (PC), challenged with mixed *Eimeria* oocysts. In this way, there were six treatments that the groups were arranged into five challenged and one unchallenged. The L- threonine was later added to each individual part at a rate of 0.61, 0.69, 0.77, 0.85 and 0.93 g/kg for the grower diet and 0.57, 0.65, 0.73, 0.81 and 0.89 g/kg for the finisher diet. Five diets were subsequently produced with 88, 100, 112, 124, and 136% digestible threonine levels for either grower or finisher periods. The ingredients and chemical composition of diets are shown in Table 1.

Table 1. Ingredient composition and calculated values of the basal diet (as-fed basis) [a]

Ingredients (%)	Starter (0-10 days)	Grower (11-22 days)	Finisher (23-36 days)
Corn	59.80	63.06	65.58
Soybean meal	33.83	29.85	26.89
Soybean oil	2.23	3.06	3.85
Salt	0.37	0.36	0.36
Dicalcium phosphate	1.78	1.79	1.59
Limestone	0.80	0.91	0.84
DL-Methionine	0.33	0.27	0.23
L-Lysine	0.28	0.20	0.16
Threonine	0.08	0.00	0.00
Vitamin premix [b]	0.25	0.25	0.25
Mineral premix [c]	0.25	0.25	0.25
Calculated nutrient values			
Metabolizable energy (kcal/kg)	3008	3086	3167
Crude protein	21.06	19.24	18
Calcium	0.90	0.84	0.76
Available phosphorous	0.45	0.42	0.38
Sodium	0.16	0.16	0.16
Digestible Arginine	1.26	1.15	1.06
Digestible Lysine	1.21	1.05	0.95
Digestible Methionine	0.61	0.53	0.48
Digestible Methionine + Cystine	0.89	0.80	0.74
Digestible Threonine	0.74	0.607	0.57
Digestible Tryptophan	0.22	0.19	0.18

[a] The diets were provided in a way that a batch basal diet (lowest digestible threonine concentration) was made and then divided into 5 equal portions, the L- threonine was added at the rate of 0.61, 0.69, 0.77, 0.85 and 0.93 g/kg for grower diet and 0.57, 0.65, 0.73, 0.81 and 0.89 g/kg for finisher diet on the top of each portion at the expense of filler (corn starch) and mixed to provide five diets with 88, 100, 112, 124, and 136% digestible threonine levels for either grower or finisher periods.
[b] Provided per kilogram of diet: 15,000 IU of vitamin A (retinol); 3,750 IU of vitamin D3 (Cholecalciferol); 37.5 mg of vitamin E (tocopheryl acetate); 2.55 mg of vitamin K3; 3 mg of thiamin; 7.5 mg of riboflavin; 4.5 mg of vitamin B6 (pyridoxine); 24 µg of vitamin B12 (cyanocobalamin); 51 mg of niacin; 1.5 mg of folic acid; 0.2 mg of biotin; 13.5 mg of pantothenic acid; 250 mg of choline chloride; 100 mg of antioxidant.
[c] Provided per kilogram of diet: 37.5 mg of Zn (ZnO, 80.35% Zn); 37.5 mg of Mn (MnSO4.H2O, 32.49% Mn); 37.5 mg of Fe (FeSO4·7H2O, 20.09% Fe); 3.75 mg of Cu (CuSO4·5H2O); 0.83 mg of I (KI, 58% I); 62.5 mg of Sulphur; 0.23 mg of Se (NaSeO3, 45.56% Se).

Growth performance and immune organs

Birds and feeds were weighed at day 11, 22, and 38 on a pen basis and then average daily weight gain (ADG), and daily feed intake (ADFI) were calculated. Mortality was checked daily and birds were weighed to determine FCR adjustments. At day 23 and 31, one bird per replicate (6 birds per treatment) were slaughtered. Immune organs (Bursa, and spleen) were all weighed and measured to calculate body weight percentages.

Experimental Challenge

At 23 days of age, all experimental groups except the negative control group (NC) were challenged with 20 doses of the Livacox T® (Biopharm Co., Prague, Czech Republic) orally, to produce a mild coccidiosis infection in birds. Livacox T® is a live attenuated coccidiosis vaccine for broilers that contains three lines of the important species *(E. acervulina, E. maxima,* and *E. tenella*). Each dose of vaccine was determined to contain 1200 sporulated oocysts of these three species. So, each bird received around 24000 sporulated oocysts at 23 days of age but the birds of the NC group were inoculated with a placebo dose of 1 mL of distilled water (Mansoori *et al.,* 2012).

Oocyst counts

The fecal samples were collected on days 5, 7, 9, and 11 post-inoculation. Samples were refrigerated before oocyst per gram of feces (OPG) determination. The oocyst counting was performed using McMaster. Fecal samples (2 g) were mixed in 10% (w/v) NaCl solution and transferred to a McMaster chamber using a micropipette (Chand *et al.,* 2016). Oocysts were enumerated using a brightfield upright microscope (Chand *et al.,* 2016).

Lesion scoring

One bird per replicate was randomly selected and slaughtered to score the duodenum, jejunum, and ileum lesions on d 31. The lesions were scored as described by Tanweer *et al.* (2014).

Histological Analysis

At 23 and 31 days of age, one bird per replicate (6 per treatment) were randomly selected for slaughtering. The intestines were washed with distilled water and jejunum

segments (from Meckel's diverticulum to ileocecal junction) were separated for morphological analysis. Fixing and staining of the samples were carried out by 10% formalin and hematoxylin-eosin, respectively. Each jejunal sample from each bird was cross-sectioned four times and 5 measurements per cross-section were carried out. Numerating the goblet cells was done under a light microscope based on the acid-Schiff staining method, as proposed by Wils-Plotz and Dilger, (2013). Hereby, some parameters containing villus height (VH), villus width (VW), crypt depth (CD), midway up the villi, villus height to crypt depth ratio (VH: CD), villus surface area (VSA; [2π × (VW/2) × VH]), and lamina propria thickness (LPT) were investigated (Wils-Plotz and Dilger, 2013).

Determination of apparent metabolizable energy corrected for nitrogen (AMEn) and total tract apparent digestibility of nutrients

The total tract apparent digestibility of nutrients and AMEn of finisher diets was determined during d 26 to 32 by the inclusion of chromic oxide (Cr2O3) as an analytical marker. Chromium oxide has been carefully added to the diet at a concentration of 5 g Cr2O3/kg diet. To prevent fermentation and loss of nutrients, excreta samples were collected twice a day at 12-hour intervals in plastic-lined trays and immediately placed in a freezer at -20°C. According to Williams *et al*., (1962) the concentration of chromium was analyzed in all samples and diets using atomic absorption spectrophotometry (model 2380, PerkinElmer, Norwalk, CT) (Paraskeuas *et al*., 2017). Dry matter (DM), crude protein (CP), ash, and organic dry matter (ODM in diet and excreta were evaluated according to the procedures proposed in AOAC International (2006). An adiabatic bomb calorimeter (model 6200, Parr Instrument Co., Moline, IL) was used to determine the gross energy.

The apparent digestibility coefficients of nutrients in the experimental diets were calculated as based on the method used by Dilger *et al.* (2004):

$$AD\% = 100 - [(Cr_{diet}/Cr_{excretal}) \times (Nutrient_{excretal}/Nutrient_{diet}) \times 100]$$

Cr_{diet} is the chromium concentration of dietary intake; $Cr_{excretal}$ is the chromium concentration of excreted digesta; $Nutrient_{excretal}$ is the nutrient concentration of excreted digesta, and $Nutrient_{diet}$ is the nutrient concentration of dietary intake.

Nitrogen retention and AMEn were calculated as in Dilger *et al.* (2004):

$$N_{retained} = N_{diet} - [(N_{excreta} \times Cr_{diet})/Cr_{excreta}],$$

where N_{diet} and $N_{excreta}$ (%) represent the analyzed N concentration of diet and excreta samples, respectively. The following equation was adapted from Lammers *et al.* (2008):

$$AMEn = \{GE_{diet} - [(GE_{diet} \times Cr_{diet})/Cr\ excreta] - 8.22 \times N_{retained}\}$$

where AMEn (kcal/kg) is the N-corrected AME content of the diet; GE_{diet} and $GE_{excreta}$ (kcal/kg) are the GE concentrations of the diet and excreta, respectively; Cr_{diet} and $Cr_{excreta}$ (%) are the chromic oxide concentrations in the diet and excreta, respectively; 8.22 is the energy value attributed to uric acid; and $N_{retained}$ (g/kg) is the N retained by the chickens per kilogram of feed intake (Wils-Plotz and Dilger, 2013).

Immune responses

For evaluating the antibody response, sheep red blood cells (SRBC) test was used as T-dependent antigens. Six broiler chickens from each treatment were injected in duplicate with SRBC (5% suspension in PBS, 0.5 mL/bird) intramuscularly at days 23 and 30. Collecting the blood samples was carried out 7 d after the first and the second injection. The serum samples were collected, heat-inactivated at 56 ˚C for 30 min, and then analyzed for total immunoglobulins (Ig), IgG as proposed by Hajati *et al.* (2018). All antibody titers were reported as $\log_2$ of the reciprocal of the last dilution in which agglutination was detected. IgG was subtracted from total Ig to calculate the amount of IgM (mercaptoethanol [ME]–sensitive antibody against SRBC).

Hematological parameters

On day 31 of age, one bird from each pen was used for blood collection. Blood samples were collected from the bird's wing vein in EDTA tubes and analyzed for parameters of cell blood count (CBC) like white blood cells (WBC), monocyte (M), eosinophil (E), heterophil (H), lymphocyte (L), heterophil to lymphocyte ratio (H/L) (Saadatmand *et al.,* 2019).

Statistical analysis

The GLM procedure of SAS (SAS Inst. Inc., Cary, NC) was used to carry out the statistical analyzing the data. The selected experimental design was a completely randomized design with the pen being defined as the experimental unit. Differences among the means were compared using Tukey's test. Significant differences were considered to be less than $P < 0.05$. Linear and quadratic effects of dietary threonine levels were investigated using polynomial contrasts. The linear and quadratic effects of the threonine levels were evaluated between the challenged groups.

Results

Growth Performance

Overall mortality was low during the study (0.5%) and the recorded deaths were not found to be associated with any specific treatment. In both growth period and the whole study period, the performance of birds received non-challenged (NC) and 124% level of threonine (Thr) was greater than both the birds received challenged (PC) and a diet containing 88% levels of Thr ($P < 0.05$) (Table 2).

Table 2. Effects of dietary threonine levels on growth performance in male broiler chickens during pre (grower period) and post challenged (finisher period) with Eimeria spp.[1]

Diet	Grower (d 11-22) Pre-challenge			Finisher (d 23-36) Post challenge			Whole (d 11-36)		
Threonine levels[2]	ADG	ADFI	FCR	ADG	ADFI	FCR	ADG	ADFI	FCR
88%	43.37^{c}	68.63	1.591^{a}	83.76^{b}	178.79	2.159^{a}	54.93^{b}	120.24	2.212^{a}
100%	52.12^{ab}	75.91	1.451^{ab}						
100% NC				108.56^{a}	180.33	1.661^{b}	71.14^{a}	123.75	1.739^{b}
100% PC				90.97^{ab}	189.26	2.159^{a}	62.52^{ab}	128.85	2.088^{a}
112%	55.93^{a}	72.94	1.309^{b}	101.41^{ab}	175.02	1.729^{ab}	69.35^{a}	120.43	1.743^{b}
124%	56.08^{a}	73.43	1.308^{b}	103.93^{ab}	175.39	1.691^{b}	70.59^{a}	121.14	1.719^{b}
136%	48.36a^{bc}	73.66	1.526^{ab}	95.92^{ab}	175.53	1.859^{ab}	63.04 ^{ab}	121.33	1.932^{ab}
SEM[4]	1.761	3.493.	0.057	5.380	6.378	0.107	2.671	3.335	0.073
P-value	<0.0001	0.682	0.004	0.032	0.608	0.003	0.001	0.447	<0.0001
Polynomial contrasts[5]									
Linear	0.0192	0.4992	0.1421	0.0387	0.2517	0.0079	0.0082	0.5473	0.0011
Quadratic	<0.0001	0.4235	0.0005	0.0694	0.7707	0.1097	0.0014	0.4797	0.0026

[a-c] In each column, means with the uncommon superscript letter are significantly different ($P < 0.05$).

[1] Data represent the mean value of 6 replicate pens of 11 birds each.

[2] Percentage of the Cobb 500 recommended threonine levels for broiler chickens. Negative control (NC) birds received a corn-soybean meal basal diet with 100% of threonine and were not challenged with coccidia; positive control (PC) birds received the basal diet with 100% of threonine and were orally challenged with one mL of coccidia.

[3] ADFI: Average daily feed intake, ADG: average daily gain, FCR: feed conversion ratio.

[4] *SEM*: Standard error of means.

[5] The linear and quadratic effects of the threonine levels were evaluated between the challenged groups fed diets contained 88%, 100% (PC), 112%, 124% and 136% Thr recommendation and the unchallenged treated birds (NC) were omitted.

In general, the ADFI was affected by no experimental treatments in all ages. Birds fed on the diets containing 112% and 124% level of Thr supplementation had greater ADG in the grower period (before the coccidia challenge) compared to the birds received a diet containing 88% or 136% level of Thr. Also, in this period birds fed on the diet with 112% and 124% levels of Thr improved FCR when compared to the birds received a diet containing 88% level of Thr ($P < 0.05$). In the finisher period, ADG increased in the NC group compared to the group fed diet containing 88% level of Thr ($P < 0.05$). In this period (finisher period), the FCR of birds fed diets with 124% level of Thr and NC groups were greater than the birds fed the diet with PC and diet containing 88% level of Thr, and there was no significant difference between the other treatments. From 11 to 36 days of age (whole period), the data of the experiment showed that ADG of the NC treatment was greater than those fed diet with 88% level Thr ($P < 0.05$) and proved to be similar to the other treatments. Furthermore, in the whole period, the groups of NC, 112% level of Thr, and 124% level of Thr supplementation improved FCR and diet containing 136% level of Thr performed to a comparable extent.

Increasing threonine levels enhanced ADG in a linear ($P < 0.05$) and quadratic ($P < 0.05$) trend in the grower and the whole periods, and FCR decreased quadratically only in the grower period. However, the FCR decreased linearly in the finisher period and both linearly and quadratically in the whole period.

Intestinal Morphology

Mean VH of the birds fed the diet with 124% Thr and 136% Thr diet treatments were greater than those fed 88%, 100% Thr ($P < 0.05$) (Table 3). No statistical difference was observed in CD, VH: CD ratio, VW, LPT, and GCN among the birds fed the diet with different Thr supplementation ($P > 0.05$). Birds fed 124% Thr diet had greater ($P < 0.05$) VSA than those 88% Thr diet, however, the results were still close to birds fed 100% Thr, 112% Thr, and 136% Thr diets (Table 3)

Table 3. Effects of dietary threonine levels on jejunal morphology of male broilers chickens before challenge with Eimeria spp at d 23.[1]

Threonine levels[2]	Parameters [3]						
	VH (µm)	CD (µm)	VH/CD	VW (µm)	VSA(µm)^2	LPT(µm)	GCN/1 mm length
88%	647.82^{b}	130.88	5.00	101.72	1505^{b}	78.68	104.09
100%	635.57^{b}	129.73	4.89	98.16	1596^{ab}	83.96	103.21
112%	650.40^{ab}	126.76	5.18	105.37	1704^{ab}	77.32	111.66
124%	676.43^{a}	126.03	5.38	110.26	1866^{a}	72.80	109.86
136%	674.27^{a}	124.73	5.42	104.66	1785^{ab}	73.63	111.31
SEM[4]	9.286	4.033	0.138	3.989	76.364	3.449	6.459
P-value	0.018	0.822	0.042	0.308	0.019	0.751	0.267
Polynomial contrasts[5]							
Linear	0.0013	0.2404	0.0025	0.1003	0.0020	0.2059	0.0569
Quadratic	0.8785	0.9341	0.7883	0.2627	0.3207	0.7109	0.7481

[a-c] In each column, means with the uncommon superscript letter are significantly different ($P < 0.05$).
[1] Data represent the mean value of 6 birds. There was one sample per chick, 3 cross-sections per sample (18 cross-sections per treatment), and 10 measurements per cross-section for a total of 180.
[2] Percentage of the Cobb 500 recommended threonine levels for broiler chickens.
[3] Parameters include: Villus height (VH), Crypt depth (CD), villus height/crypt depth (VH/CD), villus width (VW), villus surface area (VSA), lamina propria thickness (LPT), and goblet cell number (GCN).
[4] *SEM*: Standard error of means.
[5]The linear and quadratic effects of the threonine levels were evaluated between the challenged groups fed diets contained 88%, 100% (PC), 112%, 124%, and 136% Thr recommendation and the unchallenged treated birds (NC) were omitted.

Mean VH and VSA increased, in the groups fed the diet with NC and diet containing 124% level of Thr compared with those received PC and diet containing 88% level of Thr, but they were similar to the birds 112% Thr and 136% Thr groups (Table 4). The VH: CD ratio was less in the PC and diet containing 88% level of Thr, and greater in the NC and diet containing 88% level of Thr ($P < 0.05$), the diets containing 112% and 136% level of Thr being intermediate (136% level of Thr, which was less than a diet containing 112% level of Thr and similar to the diet containing 124% level of Thr). The GCN increased in the birds maintained on NC, diets containing 112% level of Thr. Also, diet containing 124% level of Thr increased ($P < 0.05$) compared to the PC, and the diet containing 88% level of Thr, but no statistically significant differences were observed when compared to birds fed the diet with 136% level of Thr. In the pre-challenge (d 23) the VH, VH: CD ratio, VSA, and GCN linearly ($P < 0.05$) increased as dietary Thr increased. However, the VH, VH: CD ratio, VSA, and GCN responses increased linearly and quadratically in the post-challenge (d 31).

Table 4. Effects of dietary threonine levels on jejunal morphology of male broiler chickens on d 31 after 8 d post challenged with Eimeria spp. at d 31.[1]

Threonine levels[2]	Parameters[3]						
	VH (µm)	CD (µm)	VH/CD	VW (µm)	VSA (mm)[2]	LPT(µm)	GCN/1 mm length
88%	751.51[b]	201.48	3.76[c]	138.32	0.325[b]	104.28	118.17[b]
100% NC	900.64[a]	187.15	4.82[a]	155.26	0.439[a]	99.97	136.42[a]
100% PC	770.17[b]	203.86	3.78[c]	136.31	0.328[b]	108.63	117.45[b]
112%	857.68[ab]	200.53	4.28[abc]	143.70	0.388[ab]	101.55	138.05[a]
124%	883.04[a]	195.54	4.52[ab]	157.29	0.435[a]	97.72	142.50[a]
136%	794.39[ab]	203.33	3.92[bc]	142.56	0.354[ab]	101.55	131.08[ab]
SEM[4]	24.876	4.598	0.154	7.866	0.023	3.338	4.741
P-value	0.0004	0.121	<0.0001	0.317	0.003	0.666	<0.0001
Polynomial contrasts[5]							
Linear	0.0238	0.7649	0.0452	0.2410	0.0293	0.8264	<0.0001
Quadratic	0.0090	0.6165	0.0171	0.5131	0.0440	0.3789	0.0125

[a-c] In each column, means with the uncommon superscript letter are significantly different (P < 0.05).
[1] Data represent the mean value of 6 birds. There was one sample per bird, 3 cross-sections per sample (18 cross-sections per treatment), and 10 measurements per cross-section for a total of 180.
[2] Percentage of the Cobb 500 recommended threonine levels for broiler chickens. Negative control (NC) birds received a corn-soybean meal basal diet with 100% of threonine and were not challenged with coccidia; positive control (PC) birds received the basal diet with 100% of threonine and were orally challenged with one mL of coccidia;
[3] Parameters include: Villus height (VH), Crypt depth (CD), villus height/crypt depth (VH/CD), villus width (VW), villus surface area (VSA), lamina propria thickness (LPT), and goblet cell number (GCN).
[4] *SEM*: Standard error of means.
[5]The linear and quadratic effects of the threonine levels were evaluated between the challenged groups fed diets contained 88%, 100% (PC), 112%, 124%, and 136% Thr recommendation and the unchallenged treated birds (NC) were omitted.

Determination of apparent metabolizable energy corrected for nitrogen (AMEn) and total tract apparent digestibility of nutrients

The non-challenged birds had greater AMEn and digestible CP than the challenged birds fed on the diet with 88% or 100% Thr recommendations (Table 5). The digestibility of DM, DOM, and ash in non-challenged and challenged birds fed different levels of Thr were similar. Overall, increasing Thr supplementation enhanced the digestibility of CP and AMEn of diet linearly (Table 5).

Table 5. Effects of dietary threonine levels on apparent metabolizable energy and apparent digestibility of nutrients of male broilers chickens at 9 d post challenged with Eimeria spp.[1]

Threonine levels[2]	Parameters [3]				
	AMEn (kcal/kg)	DCP (%)	DDM (%)	DODM (%)	Ash (%)
88%	2049[b]	66.67[b]	83.74	71.95	28.55
100% NC	2356[a]	76.17[a]	88.34	75.12	30.25
100% PC	2036[b]	66.67[b]	82.02	71.48	26.54
112%	2243[ab]	73.33[ab]	84.16	77.30	29.42
124%	2308[ab]	74.83[ab]	86.22	74.79	31.49
136%	2207[a]	74.00[ab]	85.96	73.97	29.20
SEM[4]	65.878	2.075	2.728	2.871	1.599
P-value		0.006	0.653	0.726	0.387
Polynomial contrasts[5]					
Linear	0.0076	0.0006	0.2216	0.4112	0.1912
Quadratic	0.1931	0.3328	0.7314	0.3925	0.8087

[a-c] In each column, means with the uncommon superscript letter are significantly different (P < 0.05).
[1] Apparent nutrient digestibility and AMEn values are means of 6 replications each.
[2] Percentage of the Cobb 500 recommended threonine levels for broiler chickens.
[3] Apparent digestibility of crude protein (CP), dry matter (DM), organic dry matter (ODM), Apparent metabolizable energy corrected for nitrogen (AMEn)
[4] *SEM*: Standard error of means.
[5]The linear and quadratic effects of the threonine levels were evaluated between the challenged groups fed diets contained 88%, 100% (PC), 112%, 124%, and 136% Thr recommendation and the unchallenged treated birds (NC) were omitted.

Oocyst Counts

The oocysts present per gram of feces (OPG) in the challenged birds fed different levels of Thr supplementation was greater in comparison with the NC on days 5, 7, 9, and 11 post coccidia inoculation (Table 6). In general, diets containing 124% and 136%

levels of Thr showed a decreased OPG count in the challenged birds, but they did still possess less OPG count than the challenged birds which were fed 88% Thr and 100% Thr diets. There was no significant difference for OPG between the birds received a diet containing 124% and 136% levels of Thr. On days 5 and 7 post coccidia inoculation, the OPG count was highest in birds fed diet with 88% level of Thr when compared to the other groups. Also, the OPG count of the diet containing 124% level of Thr was less than PC and similar to the diets containing 112% and 136% levels of Thr. However, on the day 9 post-challenge, OPG in the birds fed on the diet with 112%, 124%, and 136% levels of Thr were less than those fed the diet containing 88% level of Thr and PC groups. On day 11 post-challenge, the highest OPG count was related to birds fed the diet with 88% level of Thr and PC, with similar results found for the diet containing 112% level of Thr. However, OPG of the same groups were greater than those birds fed the diet with 124% and 136% levels of Thr. Overall, on days 5, 7, 9, and 11 post-challenge, the number of oocytes decreased linearly and quadratically with the lowest of OPG observed in birds fed 124% and 136% of the Thr recommendation diet (Table 6).

Table 6. Effects of dietary threonine levels on the mean oocyst per gram of **feces** of male broiler chickens challenged with Eimeria spp**.**

	Parameters (log10(X + 1))			
Threonine levels[1]	5 d post-challenge	7 d post-challenge	9 d post-challenge	11 d post-challenge
88%	58.15[a]	5825[a]	33.58[a]	29.07[a]
100% PC	45.860[b]	4072[b]	31.76[a]	26.84[a]
112%	37.24[bc]	2789[bc]	18.25[b]	21.76[ab]
124%	31.33[c]	2163[c]	20.13[b]	16.54[b]
136%	27.62[c]	2635[bc]	17.99[b]	17.63[b]
SEM[2]	2.896	387.454	1.636	1.979
P-value	<0.0001	<0.0001	<0.0001	0.0003
Polynomial contrasts[3]				
Linear	<0.0001	<0.0001	<0.0001	<0.0001
Quadratic	<0.0001	<0.0001	<0.0001	<0.0001

[a-c] In each column, means with the uncommon superscript letter are significantly different (P < 0.05).
[1] Percentage of the Cobb 500 recommended threonine levels for broiler chickens. Negative control (NC) birds received a corn-soybean meal basal diet with 100% of threonine and were not challenged with coccidia; positive control (PC) birds received the basal diet with 100% of threonine and were orally challenged with one mL of coccidian. The non-challenged group (NC) has not included in the statistical analysis of OPG counts.
[2] *SEM*: Standard error of means.
[3]The linear and quadratic effects of the threonine levels were evaluated between the challenged groups fed diets contained 88%, 100% (PC), 112%, 124%, and 136% Thr recommendation and the unchallenged treated birds (NC) were omitted.

Immune Responses

Dietary Thr did not affect cell blood count in broiler chickens ($P > 0.05$) (The data has not been shown). The NC treatment increased primary and secondary total SRBC antibodies compared to all other groups, excluding the diet containing 124% level of Thr, which demonstrated the same primary and secondary total SRBC antibodies compared to NC, the diets containing 112%, and 136% level of Thr ($P < 0.05$) (Table 7). Also, birds fed diets with NC, 112%, and 124% level of Thr had greater levels of IgG titers as a primary and secondary response to SRBC when compared to PC and the diet containing 88% level of Thr. Primary IgM response was similar ($P > 0.05$) in all the treatments, but secondary IgM response was significantly greater ($P < 0.05$) in NC, 112%, 124%, and 136% levels of Thr groups when compared to the responses of the PC and the diet containing 88% level of Thr. The primary and secondary total SRBC antibodies and IgG increased both linearly and quadratically ($P < 0.05$) with the increase diet Thr level. The highest primary and secondary total SRBC antibodies and IgG were obtained when fed diets contained a 124% level of Thr recommendation. The secondary IgM in birds increased linearly ($P < 0.05$) as dietary Thr increased from 88% to 136% of recommendation.

The percentage of bursa weight was not affected by experimental treatments ($P > 0.05$). Birds receiving NC and the diet containing 124% level of Thr supplementation increased the weight of spleen compared to PC and the diet containing 88% level of Thr supplementation ($P < 0.05$). The percentage of spleen weight in birds fed the diet containing 112% and 136% level of Thr were similar compared to the diet containing 124% level of Thr group ($P < 0.01$). The percentage of spleen weight increased linearly ($P < 0.05$) as dietary Thr increased from 88% to 136% of recommendation.

Table 7. Effects of dietary threonine levels on relative weights of immune organs and humoral immune response (SRBC) of male broiler chickens (d 23 and d 30) challenged with Eimeria spp**.**

	humoral immune response (log_2)						immune organs (g /% of BW)	
	Primary (23 d)			Secondary (30 d)				
Threonine levels[1]	Total Ig[2]	IgG	IgM	Total Ig	IgG	IgM	Spleen	Bursa of Fabricius
88%	3.31^{c}	2.18^{c}	1.13	3.90^{c}	2.72^{c}	1.18^{b}	0.091^{c}	0.183
100% NC	4.42^{a}	3.13^{a}	0.97	5.31^{a}	3.62^{a}	1.69^{a}	0.146^{a}	0.190
100% PC	3.61^{c}	2.38^{bc}	1.23	3.82^{c}	2.86^{bc}	0.96^{b}	0.073^{c}	0.178
112%	4.01^{b}	3.10^{a}	0.91	5.01^{b}	3.40^{a}	1.60^{a}	0.105^{bc}	0.201
124%	4.14^{ab}	3.13^{a}	1.00	5.07^{ab}	3.43^{a}	1.64^{a}	0.135^{ab}	0.193
136%	3.98^{b}	2.89^{ab}	1.10	4.99^{b}	3.24^{ab}	1.75^{a}	0.103^{bc}	0.187
SEM[3]	0.083	0.133	0.096	0.061	0.107	0.086	0.007	0.006
P-value	<0.0001	<0.0001	0.224	<0.0001	<0.0001	<0.0001	<0.0001	0.196
Polynomial contrasts[4]								
Linear	<0.0001	<0.0001	0.397	<0.0001	0.0002	<0.0001	0.0005	0.9375
Quadratic	0.0007	0.0054	0.302	<0.0001	0.0107	0.8418	0.2662	0.2656

[a-c] In each column, means with the uncommon superscript letter are significantly different (P < 0.05).

[1] Percentage of the Cobb 500 recommended threonine levels for broiler chickens. Negative control (NC) birds received a corn-soybean meal basal diet with 100% of threonine and were not challenged with coccidia; positive control (PC) birds received the basal diet with 100% of threonine and were orally challenged with one mL of coccidia;

[2] Ig: immunoglobulin, IgG: immunoglobulin G, Ig M: immunoglobulin M.

[3] *SEM*: Standard error of means.

[4]The linear and quadratic effects of the threonine levels were evaluated between the challenged groups fed diets contained 88%, 100% (PC), 112%, 124%, and 136% Thr recommendation and the unchallenged treated birds (NC) were omitted.

Discussion

Growth performance improved by the addition of grading Thr levels into the diets in both pre-challenged in the grower period and challenged birds in the finisher period. Overall, an increased diet Thr level linearly increased ($P < 0.05$) ADG in the grower and the whole periods. Also, an increase in diet Thr level led to a linear decrease ($P < 0.05$) in FCR in these periods. These results agree with Abbasi *et al.* (2014) report who showed that dietary Thr supplementation up to 110% of broiler chicken requirement improved BWG and FCR. Additionally, Ahmad *et al.* (2019) also reported that Thr supplementation above NRC recommended (10 and 20%) resulted in a greater growth performance than the control group. In contrast, Chen *et al.* (2016) reported no significant difference in performance for broiler chickens given three different levels of Thr was detected, the results were in agreement with Chee *et al.* (2010) findings which showed that dietary Thr in range of 8.0 to 10.5 g/kg, did not significantly affect the BWG, FI, and FCR in broiler chickens during the experiment. Similar results were also reported by Rezaeipour and Gazani (2014) and Gottardo *et al*. (2016), who observed no effects on weight gain when greater levels of Thr in diets of broiler chickens were utilized.

The present results showed that the greater levels of Thr than the recommended value in this experiment had positive effects on growth performance compared to those fed diets with 88% level of Thr and PC, but the highest level of Thr in the diet (136% Thr) resulted in a similar growth performance to PC and diet containing 88% level of Thr fed bird. Many amino acids, like Thr, can reduce growth performance with an excess dietary Thr level might be due to its toxic effects (Wils-Plotz and Dilger, 2013). An increase in the requirement of Thr during a parasitic infection could also be due to poor nutrient utilization (Wils-Plotz and Dilger, 2013). Additionally, *Eimeria spp*. challenge can damage the epithelium and reduce gut health which in turn, may lead to a suppressed whole growth performance. In the current study, the infected birds fed the diet with the low-Thr diet (88% Thr and PC) had shorter VH, VH: CD ratio, and VSA, while increased Thr levels led to a linear and a quadratic pattern of increase in jejunal VH, VH: CD ratio, and GCN.

Abbasi *et al.* (2014), Chen *et al.* (2016), and Najafi *et al.* (2017) reported extra Thr supplementation improved intestinal morphology in broiler chickens. They announced their results in accordance with Star *et al.* (2012), Wils-Plotz and Dilger (2013) which almost all support the hypothesis that extra nutritional Thr levels can positively affect small intestinal morphology. Greater VH to CD ratio may indicate a less tissue turnover, which would suggest decreased requirements to compensate for villus atrophy. Subsequently, less energy would be needed to support decreased tissue turnover. Greater VH indicates a greater volume of mature epithelial and improved absorptive efficiency due to the enhanced absorptive surface area (Abbasi *et al.,* 2014). Moreover, increased VH enhances the activity of the enzymes secreted from the tips of the villi, consequently leading to improved digestibility coefficients. The longer villi and shorter crypts are usually considered as markers of a healthy and well functional gut (Qaisrani *et al.,* 2015).

Among amino acids, the highest metabolism in the portal-drained viscera belongs to Thr (Schaart *et al.*, 2005). Based on the histological changes in our study, it seems that increasing dietary Thr levels improve intestinal absorptive surface area results. These results are in line with the findings of Abbasi *et al.* (2014) who demonstrated that VH in broiler chickens fed Thr-deficient diets declined in comparison with groups feeding with adequate Thr. Wu (1998) stated that small intestine directly absorbs 30% to 50% of essential Thr and other amino acids (Glu, Arg, Pro, Ile, Val, Leu, Met, Lys, Phe, Gly, Ser) and these amino acids are not available for extra-intestinal tissues. Therefore, enhancing the dietary level of the Thr diet can supply sufficient concentrations of the same major amino acids for the high circulation of mucosal tissue. Besides, due to the absorption of competitive receptors and the antagonism between amino acids, excess amino acids may have a negative impact on turnover (Gottardo *et al.*, 2016). Many factors inside the intestinal lumen and in the epithelium are responsible for regulating the intensity of the intestinal mucosa epithelial cells (Dignass, 2001). The positive effects of amino acid supplementation can be due to the presence of nutrients in the intestinal lumen. This means that the atrophic effect is not certainly caused by the absorption of nutrients (Schaart *et al.*, 2005).

Goblet cells can protect the intestinal mucosa and improve the overall protection of the intestinal absorptive area by secretion of mucins (Nichols and Bertolo, 2008). Mucins are particularly rich in threonine, proline, and serine, with Thr representing as much as 28% to 40% of the total amino acid profile of mucins (Najafi *et al*., 2017). In our study, goblet cell numbers found to be similar in all birds indicating greater levels of Thr help to lessen GC/mucin stimulation induced by sub-clinical coccidiosis challenge. Our findings are generally in agreement with the relevant beneficial effects of Thr levels on intestinal morphology and growth performance (Nichols and Bertolo, 2008).

In the current investigation, the jejunal goblet cell number reduced in those birds fed PC and diet containing 88% level of Thr. The number of these cells in the jejunal epithelium enhanced by increasing dietary Thr levels. As described previously by Schaart *et al.* (2005), one of the main applications of the absorbed Thr contributes to the synthesis of intestinal proteins, which are principally secreted into the lumen as mucus, where they protect the gut from pathogens and anti-nutritional factors. Mucin is a glycosylated protein that is excreted across the intestinal epithelium and plays a role in the release and absorption of nutrients in the digestive tract. In our study, the improved growth performance in broiler chickens was accompanied by enhanced gut health (high VH, VH: CD ratio, and VSA) that can affect AMEn and nutrient digestibility of the diet. The AMEn and digestibility of crude protein-enhanced linearly in groups challenged with *Eimeria spp*. The increased villus height led to a greater absorptive area. So, this might be a reason that birds who received the high-Thr diets had greater villus height as a mechanism to overcome the reduced nutrient absorbability and digestibility imposed by *Eimeria spp*. Finally, improved growth performance may reflect prioritizing nutrients during an immune challenge. A similar mechanism may describe the increase in surface area for challenged birds received a greater dietary Thr concentration. Ospina- Rojas *et al.* (2013) demonstrated that Thr can directly or indirectly affect the function of the intestinal mucosa and enhance dietary energy utilization. It was shown in a study that the synthesis of mucosal and mucin proteins was crucial to luminal Thr concentration, suggesting the importance of Thr absorption for gut metabolism (Nichols and Bertolo 2008). During an infection, the requirement for Thr may increase, because it is the main component of the intestinal mucins. Therefore, it is logical that increased demand for Thr to produce mucus and decrease of its supply due to a reduction in body weight would result in Thr deficiency (Star *et al.,* 2012).

On the other hand, Thr can increase mucin synthesis, which may increase the mucus layer and nutrient utilization, thus protecting the integrity of the intestinal epithelium (Ospina-Rojas *et al.,* 2013). As a result, during an infection, Thr requirement is enhanced, which may be due to the role which Thr plays in the immune system or barrier function. Moreover, an increase in the need for Thr in the body during a parasitic infection could also ameliorate the negative impact on nutrient utilization (Wils-Plotz and Dilger, 2013). Faure *et al.* (2007) pointed out that it is necessary to increase the amount of Thr absorbed in the diet to defeat acute increases in muscle in mobilizing muscle protein to stimulate the inflammatory response in rats. Therefore, supplemental Thr may exert protective effects in the maintenance of the mucus barrier during the inflammation of the intestine (Wang *et al.,* 2009). Chen *et al.* (2016) proposed that Thr participates in the synthesis of mucin and immunoglobulin, therefore, involves the maintenance of barrier integrity as an essential amino acid.

In our study, the oocyte count decreased quadratically as dietary Thr increased. The results of oocyst count in feces showed that appraisal of infection in this experiment was successful. Oocyst count was significantly reduced by supplementation of high levels of Thr. Greater levels of Thr was able to reduce the OPG. It is shown in various studies that reduced OPG can prevent Eimeria infection and may be more sensitive to cell-mediated interactions than antibodies (Yim *et al.,* 2011 and Mohiti *et al.,* 2015). In our study, elevated Thr levels had favorable effects on coccidiosis lesion scores numerically, and

improved gut health and immune response in coccidiosis challenged birds.

We also detected that high levels of Thr may lead to an enhancement of the humoral response (primary and secondary immunity condition) of *Eimeria* challenged birds after antigen stimulation. The immune system-related immune organs were greater in birds that received high levels of Thr than those fed PC and diet containing 88% level of Thr. In many cases, the immune parameters were similar in *Eimeria* challenged birds, which were fed diets with greater levels of Thr (112%, 124%, and 136%) to those of non-challenged birds. Overall, increasing dietary Thr level linearly and quadratically increased primary and secondary total SRBC antibodies and IgG. As Thr is a major component of immunoglobulins so, a greater level of Thr supplementation stimulates the synthesis of immunoglobulins (Chen *et al.*, 2016). Moreover, Thr can participate in the immune system establishment and has a very close relationship with organ development in the immune system. When birds receive sufficient Thr, antigens can stimulate different immune responses in the body. However, when sufficient Thr is not available, the body fails to produce adequate immunoglobulins, lymphocyte T and B, thus preventing the formation of antibodies and/or reducing the rate of antibody formation, consequently deteriorating the body's natural immune function. As a result, increasing dietary Thr level can advance the immunity of broilers and decline prevalence and mortality. Tarantino *et al.,* (2013) showed that spleen plays a role in the proliferation of immune cells and the production of antibodies. The thymus is the center of T cell proliferation and maturation in the immune system (Gordon and Manley, 2011). It is stated that broiler chickens raised in a built-up litter environment need a greater level of Thr to promote spleen and thymus development (Corzo *et al.* 2007). In consistence with our results, Corzo *et al.* (2007) and Ren *et al.* (2014) demonstrated that the addition of Thr to the diet increased the relative weight of the spleen, suggesting that a greater level of Thr, beyond the recommendation, could lead to valuable effects on the growth and development of immune organs in broiler chickens.

In conclusion, the current results indicate that increasing levels of dietary Thr can increase various intestinal health and immune functions, consequently lead to an improvement of the whole growth performance in the sub-clinically coccidiosis challenged broiler chickens. We confirm that Thr requirements for gut health and immune response in broiler chickens are greater than those for growth performance. Under commercial conditions, a greater level of Thr, a value exceeding the recommendation, may be required to have ideal immune function, growth performance, and overall, more ideal gut health status in poultry. Overall, high-Thr concentration (24% above recommendation) provided some measure of a protective effect against coccidiosis. The current findings suggest that the use of Thr, especially at 124% of recommendation, could be a useful management strategy to improve chicken health and welfare in a stressful condition such as *Eimeria* infection.

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