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Effect of L-threonine and NSP-degrading Enzyme on the Performance, Intestinal Morphometry and Immunocompetence of the Broiler Chickens Fed Wheat-based Diet during the Starter Period

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Abstract

This study was conducted to evaluate the effects of supplemental L-threonine and enzyme in a wheat-based starter diet (WBD) on the growth performance, intestinal morphology, and immune responses of male ROSS 308 broiler chickens. A total of 240 one-day-old chicks were allocated to six different dietary treatments in a completely randomized design with a 2×3 factorial arrangement. The treatments were two WBD (with or without enzyme) each supplemented with three levels of L-threonine (100%, 120%, and 140% of the NRC recommendation). To evaluate the humoral immune system, sheep red blood cells (SRBC) antigen was injected, and for evaluation of cutaneous basophil hypersensitivity (CBH) response, the phytohemagglutinin-P was injected subcutaneously. On day 21, two chicks were selected out of each replicate and were bled, slaughtered, and then their internal organs were dissected and weighed. Blood samples were used for malondialdehyde (MDA) measurement. Jejunal and ileal contents and tissues were sampled for the viscosity and morphometric measurements. The supplemental L-threonine at the level of 120% of the NRC recommendation improved body weight gain, feed conversion ratio, and anti-SRBC titer ($P < 0.05$). Also, the supplemental L-threonine at the level of 140% of the NRC recommendation increased the jejunal crypt depth and decreased the jejunal and ileal crypt width ($P < 0.05$). Nevertheless, the supplemental L-threonine did not affect serum MDA, the viscosity of intestinal contents, and the CBH response ($P > 0.05$). The enzyme supplementation decreased the viscosity of jejunal contents and increased the villus height to crypt depth ratio in the jejunum ($P < 0.05$). In conclusion, it seems that more dietary L-threonine is needed to support the performance and health of the broilers when fed the WBD. However, no synergism was observed when L-threonine and enzyme supplementation were simultaneously applied in this study.

Introduction

Threonine is usually considered as the third limiting amino acid after sulfur amino acids (methionine + cystine) and lysine within poultry diets (Kidd and Kerr, 1996). Besides, it has an important role in the synthesis and maintenance of body proteins and acts as a major constituent for intestinal development and its well-functioning (Stoll, 2006). For example, mucin which is a glycoprotein produced by goblet cells is mainly made from threonine (Schaart *et al*., 2005). Mucin plays a vital role in the intestinal wall protection against physical, chemical, bacterial, and enzymatic hazards throughout the intestinal lumen (Wils-Plotz and Dilger, 2013). Furthermore, mucin has a chief role in filtering, digestion, and absorption of nutrients (Smirnov *et al*., 2006). The effects of threonine on the improvement of intestinal morphological characteristics, the MUC2 gene

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expression, commensal microflora, and body antioxidative defense, have already been demonstrated by many researchers (Wang *et al*., 2010; Azzam *et al*., 2012).

Wheat is an important cereal that is largely cultivated all around the world. In many countries, wheat is one of the (or even the only) energy provision constituent of poultry diets (Pirgozliev *et al*., 2003). However, wheat contains several anti-nutritional factors, along with the majority of non-starch polysaccharides (NSPs). The main NSPs in wheat seeds are arabinoxylan polymers (Choct *et al*., 1995). Soluble compartments of the NSPs could insert several negative effects on the bird gastrointestinal tract and performance (Hetland *et al*., 2004). One of the sites which are negatively affected by the dietary NSPs is enteric mucosa (Rakowska *et al*., 1993). For example, research has revealed that the application of diets containing the NSPs in broilers nutrition has caused atrophic shortening and also thickening of jejunum villi and has increased the number of goblet cells per villus (Viveros *et al*., 1994). Similarly, Morel *et al*. (2003) reported an increase in intestinal mucin secretion and endogenous threonine losses, through the inclusion of arabinoxylan to the diet of weaner pigs.

Considering the importance of threonine in intestinal epithelium maintenance, it seems that the application of wheat-based diets (WBD) containing high amounts of NSPs would increase the requirements of threonine in broiler chickens. The purpose of the current study was therefore to investigate the effects of supplementing the WBD diet with L-Threonine (with and without exogenous enzyme) on the performance, immune system, and intestinal morphology of broiler chickens.

Materials and Methods

Experimental design, diets, and husbandry

The animal experimentation protocol used in this study was approved by Shahrekord University. A total of 240 one-day-old male broiler chicks (ROSS 308) were weighed and randomly assigned to 24 deep litter floor pens (1.8 × 1.5 m). A completely randomized design experiment with a 2 × 3 factorial arrangement was used. Six WBD were prepared using three levels of L-threonine (100%, 120%, and 140% of the NRC (1994) recommendations) and two levels of an enzyme (with and without enzyme). Four pens were then randomly allocated to each dietary treatment. The WBD was formulated for the starter period according to the NRC (1994) recommendations (Table 1). The NSP-degrading enzyme used in this study was a commercial preparation with 2,200 IU xylanase and 200 IU beta-glucanase activity per gram. The birds were reared in an environmentally controlled room while had free access to feed and water. The temperature was maintained at 30˚C for the first two days and then was gradually reduced to 22˚C (at the rate of 2.5˚C per week). The light was provided 23 h for the first week and then reduced to 18 h for the remaining period of the experiment. The feed intake (FI) and body weight gain (BWG) was recorded and the feed conversion ratio (FCR) was calculated weekly. The health status and mortalities were measured every day during the experimental period.

Sample collection

At the end of the experimental period (day 21), two birds were randomly selected from each replicate (eight birds per treatment), then weighed, bled via a brachial vein, and finally euthanized through cervical dislocation. The abdominal cavity was opened and internal organs including proventriculus, gizzard, liver, heart, pancreas, spleen and the bursa of Fabricius were dissected, rinsed with distilled water, wiped, and weighted. The small intestinal parts including the duodenum (from 1 cm after the gizzard's end up to the duodenal loop's end), jejunum (from the end of the duodenal loop towards the Meckel's diverticulum), and ileum (from the Meckel's diverticulum towards the ileocecal junction) were excised, weighed, and their lengths were measured. The weight of each organ was then expressed compared to the live body weight.

The viscosity measurement

To measure viscosity, jejunal and ileal contents were collected and placed into the polyethylene tubes and then were centrifuged (500 × g, 15 min). The supernatants were then used for the viscosity measurement using a viscometer apparatus (Brookfield, DV-II+ pro, Brookfield Inc., USA), according to the method used by Smits *et al*. (1997).

Morphological measurements

For the morphological measurements, two-cm sections from the middle parts of the jejunum and ileum were sampled, rinsed with normal saline, and fixed in a ten percent formalin solution (Chen *et al*., 2016). After the tissue processing, five-µm sections were prepared and stained by the hematoxylin and eosin (Gridley, 1960) and were imaged by a light microscope (×4 magnification). The prepared images were then analyzed using image analysis software (Image-Pro Plus, Media Cybernetics, USA). The villus height, the villus width, and the crypt depth were measured in 40 villi and crypts per treatment as described by Biloni *et al*. (2013).

Table 1. The ingredients and chemical compositions of the experimental diets (1-21 d).

Enzyme	Threonine level (% of NRC)					
	100	120	140	100	120	140
	-	-	-	+	+	+
Ingredient (g/kg)						
Wheat	480.5	477.9	474.3	478.8	476.0	472.5
Soybean meal (44% CP)	395.0	395.4	396.4	395.4	396.0	396.8
Soybean oil	84.7	85.6	86.6	85.2	86.1	87.2
Oyster shells	13.4	13.4	13.4	13.5	13.5	13.5
DCP	15.0	15.0	15.0	15.2	15.2	15.2
Sodium bicarbonate	1.8	1.8	1.8	1.8	1.8	1.8
Salt	2.4	2.4	2.4	2.4	2.4	2.4
DL-methionine	2.2	2.2	2.2	2.2	2.2	2.2
Vitamin premix[1]	2.5	2.5	2.5	2.5	2.5	2.5
Mineral premix[2]	2.5	2.5	2.5	2.5	2.5	2.5
L-threonine	0	1.3	2.9	0	1.3	2.9
Enzyme[3]	0	0	0	0.5	0.5	0.5
Chemical composition						
Metabolizable energy (kcal/kg diet)	3,100	3,100	3,100	3,100	3,100	3,100
Crude protein (%)	22.28	22.28	22.28	22.28	22.28	22.28
Calcium (%)	0.969	0.969	0.969	0.969	0.969	0.969
Available phosphorus (%)	0.436	0.436	0.436	0.436	0.436	0.436
Threonine (%)	0.83	0.96	1.12	0.83	0.96	1.12
Lysine (%)	1.17	1.17	1.17	1.17	1.17	1.17
Methionine + Cystine (%)	0.9	0.9	0.9	0.9	0.9	0.9

[1]Provides per kg of the diet: all-trans-retinyl acetate, 8144 IU; cholecalciferol, 2000 IU; all-rac-α-tocopheryl acetate, 4 IU; menadione (menadione sodium bisulfate), 2 mg; thiamine (thiamine mononitrate), 1.8 mg; riboflavin, 6.6 mg; Niacin, 9.8 mg; Ca-pantothenate, 29.7 mg; pyridoxine, 1.18 mg; folic acid, 1 mg; Cobalamin, 0.015 mg; D-biotin, 0.1 mg; choline chloride, 500 mg.
[2]Provides per kg of the diet: 76 mg Mn (as MnO_2); 66 mg Zn (as $ZnSO_4$); 40 mg Fe (as $FeSO_4.7H_2O$); 4 mg Cu (as $CuSO_4.5H_2O$); 0.64 mg I (as NaI); 0.2 mg Se (as $Na_2SeO_3 \cdot 5H_2O$).
[3]2200 IU xylanase and 200 IU beta-glucanase activity per gram.

The lipid peroxidation assay

Serum malondialdehyde (MDA) was measured as an indicator of lipid peroxidation using the method described by Placer *et al*. (1966) with minor modification. Briefly, 2.5 mL trichloroacetic acid (20%) and 1 mL thiobarbituric acid (6.7%) were mixed with 0.5 mL of serum. The mixture was then heated on a water bath (95˚C) for 30 min. After the cooling, the butanol (4 mL) was added to the mixture and centrifuged (2000 × g, 10 min). Finally, the optical density (OD) of the supernatants was measured spectrophotometrically at 532 and 572 nm. The difference between the two ODs was then applied for the calculation of the MDA concentration.

Antibody-mediated immunity

For the evaluation of the humoral immune response to dietary treatments, two chicks were immunized from each replicate (eight chicks per treatment) using the sheep red blood cells (SRBC) antigen. To this purpose, two percent (V/V) SRBC suspension was injected intramuscularly (2 mL/kg BW) into the breast muscle (pectoralis) seven days after they hatch (post-hatch). The blood samples were collected via the brachial vein 7 and 14 days after injection. Serum samples were then used to measure the antibody titer against the SRBC using a direct hemagglutination test (Haghighi *et al*., 2005). The highest serum dilution which was able to agglutinate an equal volume of the SRBC suspension was recorded as an anti-SRBC titer and expressed as $\log_2$ of the reciprocal dilution rate.

Cell-mediated immunity

To evaluate cell-mediated immunity reaction to dietary treatments, cutaneous basophil hypersensitivity (CBH) response was measured as described by Dibaiee-nia *et al*. (2017). In a brief, two chicks were randomly selected from each replicate, and phytohemagglutinin-P (PHA-P) solution in phosphate-buffered saline (PBS) (100 µg/0.1 mL) was subcutaneously injected into the toe web of the right leg. To correct the response to PBS alone, the PBS was simultaneously injected into the toe web of the left leg. The skin thickness was measured at the injection sites 12 h and 24 h after the injection, using a digital micrometer. Finally, the CBH response was calculated by subtracting the data from two legs' measurements in each recording time.

Statistical analysis

The collected data were analyzed using the general linear model (GLM) procedure of the SAS version 9.4 (2016). The main effects of the L-threonine and

xylanase together with their interaction were considered in the statistical model used for analysis. Where there was a significant interaction between L-threonine and xylanase for a trait, further analysis was performed using the slice option in the GLM procedure of the SAS version 9.4 (2016). The significance of means' differences was tested using Tukey's test and all differences were considered significant at $P < 0.05$.

Results

Growth performance

The Effects of dietary treatments on FI, BWG, and FCR are shown in Table 2. Neither L-threonine nor xylanase supplementation affected FI ($P > 0.05$). On the other hand, L-threonine at the level of 120% of the NRC (1994) recommendations, increased the BWG during all periods of the experiment, compared to the 100% level ($P < 0.05$). Moreover, an increase in dietary L-threonine to 120% of the NRC (1994) recommendations, improved FCR during the first and third week of age, compared to 100% dietary threonine level (*P<0.05*). In this study, supplementing the xylanase enzyme to WBD had no significant effect on performance ($P > 0.05$). Also, no L-threonine × enzyme interaction was seen regarding the FI, BWG and, FCR ($P > 0.05$).

Intestinal morphometry

The results of intestinal morphometry are exposed in Table 3. The supplementation of the WBD with different levels of L-threonine did not affect the villus height and villus height to crypt depth ratio in the jejunum and ileum, as well as the crypt depth of the ileum ($P > 0.05$). However, L-threonine at the level of 140% of the NRC (1994) recommendation was able to increase the jejunal crypt depth compared to the L-threonine at a 100% level ($P < 0.05$). In this study, the supplementation of the WBD with enzyme did not affect the villus height and crypt depth in the jejunum and ileum as well as the villus height to crypt depth ratio of the ileum ($P > 0.05$). However, enzyme supplementation increased the jejunal villus height to crypt depth ratio compared to the non-supplemented diet ($P < 0.05$). On the other hand, supplemental L-threonine decreased the jejunal and ileal villus width (120%) and the jejunal villus width (140%) in comparison with the L-threonine at 100% level ($P < 0.05$). Also, the supplementation of the WBD with enzyme decreased the jejunal and ileal villus width ($P < 0.05$). No L-threonine × enzyme interaction was perceived in intestinal morphometric measurements (Table 3, $P > 0.05$).

Intestinal length, relative weight, and digesta viscosity

The data obtained from the intestinal length, weight, and digesta viscosity are shown in Table 4. The effect of L-threonine supplementation on the jejunal and ileal length and relative weight was not significant ($P > 0.05$). However, the enzyme supplementation decreased the ileal and total small intestinal length compared to the non-supplemented diet ($P < 0.05$). A decrease in intestinal digesta viscosity was observed in response to the increase in dietary L-threonine level, either in the jejunum ($P = 0.15$) or ileum ($P = 0.06$). Also, enzyme supplementation decreased the jejunal digesta viscosity ($P < 0.05$). No L-threonine × enzyme interaction was detected concerning the intestinal relative weight and digesta viscosity ($P > 0.05$). Anyway, the L-threonine × enzyme interaction was significant as to the duodenum length ($P < 0.05$). The enzyme addition increased the duodenum length only when the dietary L-threonine was set at 100% of the NRC (1994) recommendation.

Relative organs weight

The effects of different dietary treatments on the organs' relative weight are demonstrated in Table 5. The supplementation of the WBD with graded levels of L-threonine had no significant effect on internal organ weights ($P > 0.05$). However, WBD supplementation with enzyme decreased the pancreas relative weight as well as the heart relative weight, compared to a non-supplemented diet ($P < 0.05$). There was a significant L-threonine × enzyme interaction regarding liver relative weight ($P < 0.05$). The enzyme addition decreased the liver relative weight only when the dietary L-threonine was set at 140% of the NRC (1994) recommendations.

Serum MDA concentration and immune system responses

The effects of supplemental L-threonine and enzyme in the WBD on MDA concentration, SRBC, and CBH responses, are illustrated in Table 6. The L-threonine and enzyme supplementation had no significant effect on the MDA serum concentration and CBH response ($P > 0.05$). However, the L-threonine inclusion at the level of 120% of the NRC (1994) recommendations increased the antibody titer against the SRBC, 14 days after the immunization ($P < 0.05$). Also, the enzyme supplementation increased the anti-SRBC titer seven days after the immunization ($P < 0.05$). A significant L-threonine × enzyme interaction was seen over the CBH response (12 h post-injection) and anti-SRBC titer (14 days post-immunization) ($P < 0.05$). Adding enzyme to the diet increased the CBH response only when the L-threonine was supplemented at 140% of the NRC (1994) recommendation. Also, the addition of enzyme increased anti-SRBC titer only when the dietary L-threonine was set at 100% of the NRC (1994) recommendation.

Table 2. Feed intake, body weight gain and feed conversion ratio of the broilers fed a wheat-based diet containing different levels of threonine with or without enzyme supplementation.

Treatments[1]		Feed intake (g/bird)				Body weight gain (g/bird)				Feed conversion ratio			
Threonine	Enzyme	1-7 d	8-14 d	15-21 d	1-21 d	1-7 d	8-14 d	15-21 d	1-21 d	1-7 d	8-14 d	15-21 d	1-21 d
100	-	111	283	463	857	100	223	316	644	1.12	1.27	1.47	1.33
120		113	280	463	856	106	237	338	675	1.05	1.18	1.37	1.27
140		114	289	456	859	106	226	321	653	1.08	1.28	1.42	1.32
100	+	114	267	451	833	115	208	305	629	1.00	1.29	1.48	1.32
120		110	286	469	865	101	235	335	671	1.08	1.22	1.39	1.29
140		111	284	451	846	101	220	318	639	1.10	1.29	1.41	1.32
SEM		3.6	12.4	13.5	25.2	2.8	5.6	7.3	12.8	0.029	0.052	0.028	0.023
Main effects													
Threonine	100	112	275	457	844	101[b]	216[b]	310[b]	637[b]	1.11[a]	1.28	1.47[a]	1.33
	120	111	283	466	857	110[a]	236[a]	337[a]	673[a]	1.02[b]	1.20	1.38[b]	1.28
	140	113	287	453	853	103[ab]	223[ab]	319[ab]	646[ab]	1.09[ab]	1.28	1.42[ab]	1.32
Enzyme	-	113	284	460	857	104	229	325	658	1.09	1.24	1.42	1.30
	+	112	279	457	848	106	221	320	646	1.06	1.26	1.43	1.31
***P*-value**													
Threonine		0.934	0.681	0.686	0.851	0.017	0.013	0.014	0.045	0.032	0.287	0.034	0.137
Enzyme		0.762	0.665	0.768	0.672	0.443	0.128	0.422	0.331	0.306	0.630	0.631	0.730
Threonine × Enzyme		0.665	0.729	0.832	0.823	0.079	0.612	0.849	0.907	0.443	0.970	0.880	0.840

[1]100, 120, and 140 are the percent of dietary L-threonine relative to the NRC (1994) requirements; (-), without enzyme; (+), with enzyme; SEM, Standard error of the means.

[a,b]The means placed at the columns with different superscript letters are significantly different ($P < 0.05$).

Table 3. Small intestinal morphologic measurements of the broilers fed a wheat-based diet containing different levels of threonine with or without enzyme supplementation measured on 21 day of age.

Treatments[1]		Villus height (µm)		Crypt depth (µm)		Villus height to crypt depth		Villus width (µm)	
Threonine	Enzyme	Jejunum	Ileum	Jejunum	Ileum	Jejunum	Ileum	Jejunum	Ileum
100	-	695	518	128	120	5.4	4.3	212	215
120		905	436	156	126	5.8	3.5	160	151
140		981	653	166	150	5.8	4.3	153	203
100	+	981	564	134	142	7.3	3.9	168	162
120		1032	516	132	148	7.6	3.8	112	122
140		973	465	158	125	6.2	3.7	116	102
SEM		78.8	54.7	7.8	15.4	0.57	0.38	8.1	16.0
Main effects									
Threonine	100	838	541	131[b]	131	6.3	4.1	190[a]	188[a]
	120	969	476	144[ab]	137	6.7	3.7	136[b]	136[b]
	140	977	559	162[a]	137	6.0	4.0	134[b]	152[ab]
Enzyme	-	860	536	150	132	5.7[a]	4.0	175[a]	189[a]
	+	996	515	141	138	7.0[b]	3.8	132[b]	128[b]
***P*-value**									
Threonine		0.261	0.384	0.012	0.915	0.560	0.527	<.001	0.033
Enzyme		0.086	0.692	0.254	0.676	0.023	0.552	<.001	0.001
Threonine × Enzyme		0.305	0.097	0.277	0.322	0.445	0.476	0.801	0.172

[1]100, 120, and 140 are the percent of dietary L-threonine relative to the NRC (1994) requirements; (-), without enzyme; (+), with enzyme; SEM, Standard error of the means.

[a,b]The means placed at the columns with different superscript letters are significantly different ($P < 0.05$).

Table 4. Small intestinal parts' relative weight, length, and viscosity of the broilers fed wheat-based diet containing different levels of threonine with or without enzyme supplementation measured on 21 day of age.

Treatments[1]		Length (cm)				Relative weight (g/100g BW)			Viscosity (centipoise)	
Threonine	Enzyme	Duodenum	Jejunum	Ileum	Total	Duodenum	Jejunum	Ileum	Jejunum	Ileum
100	-	24.2[b]	61.2	65.2	151.2	1.3	3.8	2.8	10.92	6.44
120		25.3[ab]	65.0	66.3	162.0	1.4	3.8	3.3	7.34	4.51
140		26.5[ab]	60.0	63.0	149.5	1.6	3.6	2.8	4.13	3.13
100	+	27.7[a]	61.0	62.2	151.0	1.4	3.8	3.0	2.81	4.02
120		25.7[ab]	59.5	58.0	143.2	1.5	3.4	3.0	3.66	3.13
140		24.7[ab]	58.7	56.7	139.7	1.9	3.0	2.9	3.28	2.74
SEM		0.95	2.25	3.68	5.73	0.24	0.30	0.20	1.548	0.919
Main effects										
Threonine	100	26.2	61.1	63.7	151.1	1.4	3.8	2.9	6.87	5.23
	120	25.7	63.7	63.1	152.6	1.4	3.6	3.1	5.50	3.82
	140	25.3	59.3	59.8	144.6	1.7	3.3	2.8	3.70	2.96
Enzyme	-	25.6	63.0	64.5[a]	154.2[a]	1.5	3.7	3.0	7.46[a]	4.71
	+	25.9	59.7	59.0[b]	144.6[b]	1.6	3.4	3.0	3.25[b]	3.30
***P*-value**										
Threonine		0.63	0.47	0.58	0.35	0.25	0.32	0.06	0.15	0.06
Enzyme		0.63	0.23	0.07	0.048	0.57	0.23	0.34	0.003	0.07
Threonine × Enzyme		0.04	0.51	0.78	0.29	0.92	0.63	0.88	0.09	0.57

[1]100, 120, and 140 are the percent of dietary L-threonine relative to the NRC (1994) requirements; (-), without enzyme; (+), with enzyme; SEM, Standard error of the means.

[a,b]The means placed at the columns with different superscript letters are significantly different ($P < 0.05$).

Table 5. Relative organs' weight of the broilers fed wheat-based diet containing different levels of threonine with or without enzyme supplementation measured on 21 day of age.

Treatments[1]		Relative organs' weight (g/100 g body weight)						
Threonine	Enzyme	Bursa	Spleen	Liver	Gizzard	Proventriculus	Pancreas	Heart
100	-	0.20	0.085	3.17^{ab}	2.94	0.715	0.67	0.64
120		0.24	0.102	3.10^{ab}	3.05	0.700	0.70	0.64
140		0.21	0.097	3.51^{a}	2.90	0.680	0.74	0.66
100	+	0.23	0.107	3.18^{ab}	3.12	0.667	0.40	0.41
120		0.21	0.075	3.36^{ab}	2.77	0.685	0.39	0.44
140		0.25	0.090	2.92^{b}	2.63	0.655	0.45	0.39
SEM		0.036	0.013	0.165	0.161	0.039	0.040	0.047
Main effects								
Threonine	100	0.22	0.096	3.18	3.03	0.691	0.53	0.52
	120	0.23	0.089	3.23	2.91	0.692	0.55	0.54
	140	0.23	0.094	3.22	2.77	0.667	0.60	0.53
Enzyme	-	0.22	0.095	3.26	2.96	0.698	0.70^{a}	0.65^{a}
	+	0.23	0.090	3.15	2.84	0.669	0.41^{b}	0.42^{b}
***P*-value**								
Threonine		0.92	0.80	0.94	0.21	0.77	0.27	0.90
Enzyme		0.66	0.67	0.43	0.19	0.37	<.001	<.001
Threonine × Enzyme		0.51	0.13	0.04	0.17	0.91	0.89	0.81

[1]100, 120, and 140 are the percent of dietary L-threonine relative to the NRC (1994) requirements; (-), without enzyme; (+), with enzyme; *SEM*, Standard error of the means.
[a,b]The means placed at the columns with different superscript letters are significantly different ($P < 0.05$).

Table 6. Malondialdehyde (MDA) concentration, cutaneous basophil hypersensitivity (CBH), and the sheep red blood cell (SRBC) responses of the broilers fed a wheat-based diet containing different levels of threonine with or without enzyme supplementation.

Treatments[1]		MDA	CBH (μm)		SRBC (log_2 RDF)	
Threonine	Enzyme	(μmol/L)	12 h PI	24 h PI	7 day PI	14 day PI
100	-	2.00	808^{a}	1366	1.00	2.00^{c}
120		2.14	774^{a}	1110	1.67	3.50^{a}
140		2.03	408^{b}	892	1.50	3.13^{ab}
100	+	2.06	563^{ab}	990	2.00	3.13^{ab}
120		1.80	519^{ab}	971	1.87	3.87^{a}
140		2.35	773^{a}	1006	2.13	2.62^{bc}
SEM		0.17	0.17	164.8	0.291	0.233
Main effects						
Threonine	100	2.03	685	1178	1.50	2.56^{b}
	120	1.97	646	1041	1.77	3.69^{a}
	140	2.19	590	949	1.81	2.87^{b}
Enzyme	-	2.06	663	1123	1.39^{b}	2.87
	+	2.07	618	989	2.00^{a}	3.21
***P*-value**						
Threonine		0.44	0.68	0.43	0.54	<.001
Enzyme		0.92	0.62	0.36	0.02	0.108
Threonine × Enzyme		0.19	0.01	0.38	0.45	0.010

[1]100, 120, and 140 are the percent of dietary L-threonine relative to the NRC (1994) requirements; (-), without enzyme; (+) with enzyme; PI, post-injection; RDF, reciprocal of dilution factor; *SEM,* Standard error of the means.
[a,b,c]The means placed at the columns with different superscript letters are significantly different ($P < 0.05$).

Discussion

In the current study, increasing the dietary L-threonine up to 120% of the NRC (1994) recommendations, improved the broiler performance (BWG and FCR). However, these positive effects were diminished by adding more L-threonine into the diet (140%). The higher level of the L-threonine application might impose a dietary amino acid imbalance which will eventually reduce the broiler performance. Min *et al.* (2016) similarly supplemented a corn-wheat based diet with graded levels of the L-threonine (from 85% up to 150% of the NRC recommendations) and reported a quadratically or cubically improvement in average daily weight gain (ADG) and the FCR of the broiler chickens in response to increasing dietary threonine

level. Also, Zaghari *et al*. (2011) reported an improvement in the BWG and FCR of the broiler chickens during the starter period by increasing the dietary threonine levels up to 0.7% of a corn-based diet. Sigolo *et al*. (2017) supplemented a low CP corn-based diet with L-threonine (up to 130% of ROSS recommendation) and reported the maximum ADG to be achieved by a 110% supplemental level. On the other hand, Azzam and El-Gogary (2015) provided a corn-based diet with graded levels of threonine (0.69% to 0.79% of the diet) to the broiler chickens from 18 to 42 days of age and reported no significant effect on performance by different levels of threonine inclusion. The enzyme used in this study was not able to significantly affect the growth performance of the broilers fed on the WBD. The xylanase inefficacy in the improvement of the BWG or FI has been indicated in some other reports as well (Gonzalez-Ortiz *et al*., 2017; Hosseini *et al*., 2017).

In the current study, the supplementation of WBD with L-threonine did not impact small intestinal villus' height and crypt depth. Nevertheless, the L-threonine supplementation decreased the jejunal and ileal villus width. Increasing the jejunal villus height and villus height to crypt depth ratio in response to dietary threonine supplementation has been underlined by some other researchers (de Barros Moreira Filho *et al*., 2015; Chen *et al*., 2016). Also, Min *et al*. (2016) in an experiment with broiler chickens pinpointed an increase in duodenal crypt depth when a corn-wheat-based diet was supplemented by the L-threonine at the level of 150% of the NRC (1994) recommendations. Threonine possesses an important role in the maintenance of small intestinal epithelium (Rhodes, 1989). In an experiment executed on piglets, it has been revealed that up to 50% of the dietary threonine is retained by the intestine (Stoll *et al*., 1998). Also, it has been exhibited that the dietary threonine imbalance could diminish the intestinal mucin synthesis over the young pigs (Wang *et al*., 2007).

The positive effects of NSP-degrading enzymes include the hydrolysis of non-starch polysaccharides, reduction in viscosity of the intestinal contents, and the improvement of the digestive system and nutrient utilization (Kalmendal and Tauson, 2012; Liu and Kim, 2016; Gonzalez-Ortiz *et al*., 2017). A significant increase was perceived in the jejunal villus height to crypt depth ratio and oppositely a decrease was detected in the jejunal and ileal villus width in response to enzyme supplementation (Table 3). In agreement with our results, Liu and Kim (2016) also reported that enzyme supplementation in the WBD improves the villus height and villus height to crypt depth ratio in different parts of the broilers' small intestine.

Although, Zaghari *et al*. (2011) reported a significant increase in the relative weight of duodenum and jejunum in response to threonine supplementation of a corn-based diet, consistent with our findings, Sigolo *et al*. (2017) supplemented a corn-based diet with threonine up to 130% of the ROSS recommendation and reported no significant effect of this diet on the relative weights of small intestinal parts (duodenum, jejunum, and ileum). Also, Shirzadegan *et al*. (2015) supplemented a corn-wheat-based diet with L-threonine (up to 1% of the diet) and represented no significant effect of the mentioned diet on relative weights and lengths of different parts of the small intestine. Although the effect of supplemental L-threonine on the jejunal and ileal digesta viscosity was not significant within the current study, a numerical linear reduction in this factor was found along with L-threonine level enhancement.

In our research, the enzyme supplementation decreased the ileal and total small intestinal length. Though, it has been proved in some studies that the xylanase supplementation does not affect the small intestinal relative weight and length (Kalmendal and Tauson, 2012; Gonzalez-Ortiz *et al*., 2017). A large part of the positive functions of NSP-degrading enzymes would be attributed to their diminishing impacts on intestinal viscosity. In the current study, a significant reduction in viscosity of jejunal contents was identified after supplementing the diet with xylanase.

Chen *et al*. (2016) supplemented a corn-based diet (containing 0.78% threonine) with 0.1% or 0.3% L-threonine and reported no significant effect of their suggested diet on the bursa of Fabricius relative weight; while the supplemental L-threonine increased the thymus and spleen relative weights. In another study, Corzo *et al*. (2007) investigated the effects of two dietary threonine levels (0.51% and 0.72% of the diet) in a corn-peanut meal-based diet under two different litter conditions (new and used) and reported no effect of their recommended diet on relative weights of the bursa of Fabricius and spleen. However, they believed that a higher dietary level of threonine in used litter conditions could increase the thymus relative weight. Insignificant effects of the dietary threonine on the liver, pancreas and gizzard weights have been reported in some other studies (Azzam and El-Gogary, 2015; Shirzadegan *et al*., 2015). In line with our results, Taheri and Shirzadegan (2017) discovered a significant decrease in relative pancreas and heart weight of the broilers fed on a WBD supplemented with multiple enzymes compared to the non-supplemented group. One of the major anti-nutritional effects of high-NSP cereals is assumed as increasing the digesta viscosity which could, in turn, decrease the efficacy of digestive enzymes (Bedford, 1993) which results in compensatory hypertrophy of the pancreas and more enzyme secretion. Through the use of exogenous

enzymes, the Physico-chemical properties of the digesta would improve and the need for excessive pancreatic secretion is eliminated.

Threonine has resulted in immune system improvement in some animals (Defa *et al*., 1999; Habte-Tsion *et al*., 2016). Abbasi *et al*. (2014), in favor of our findings, reported that increasing the dietary threonine up to the levels higher than the ROSS recommendation causes a significant improvement in anti-SRBC titer. In a similar study, Sigolo *et al*. (2017) indicated an improvement in antibody response to the SRBC by increasing dietary threonine level up to 120% of the ROSS recommendation. Tenenhouse and Deutsch (1966) accentuated that the chicken gamma globulin contains considerable amounts of threonine. The most significant gamma globulins are immunoglobulins (antibodies) (Tizard, 1992). Due to the threonine abundance in antibodies' structure, the deficiency of this amino acid could negatively affect immune system responses.

The enzyme supplementation of the WBD in this study brought about a significant improvement in humoral immune system response (anti-SRBC titer). Supporting our results, Gao *et al*. (2007) supplemented a WBD with a xylanase preparation and expressed an increase in serum antibody titers against the Newcastle disease virus. On the other hand, Basmacioğlu Malayoğlu *et al*. (2010) reported that the xylanase supplementation does not have any considerable effect on the immune responses of the broiler chickens fed on WBD.

Conclusion

In conclusion, this study demonstrated that the supplementation of a broiler starter WBD with dietary L-threonine at higher levels comparing the NRC (1994) recommendations, improved the growth performance, some morphological characteristics of the small intestine, and immune system responses of the broiler chickens. Hence, these results may indicate the greater importance of L-threonine supplementation when using the WBD in broiler nutrition. However, no synergistic effect was observed when dietary L-threonine and enzyme were simultaneously supplemented in WBD in this study.

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