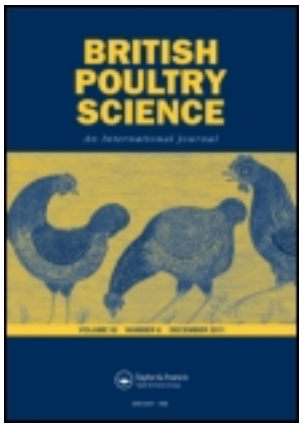

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Influence of L-threonine supplementation on goblet cell numbers, histological structure and antioxidant enzyme activities of laying hens reared in a hot and humid climate

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Influence of L–threonine supplementation on goblet cell numbers, histological structure and antioxidant enzyme activities of laying hens reared in a hot and humid climate

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Abstract 1. A previous experiment in our laboratory found that L-threonine supplementation at 0·2 and 0·3% increased egg production and the concentration of serum IgG, respectively. The objective of this current trial was to determine if both supplementation levels can positively influence histological structure, goblet cell numbers, or antioxidant enzyme activities.

2. Babcock Brown layers ($n = 576$), 40 weeks of age, were allocated to three treatment groups, each of which included 6 replicates of 32 hens. Each group received the same basal diet formulated with maize, peanut meal and crystalline amino acids. L-threonine was added to the basal diet at 0 (control), 0·2, and 0·3%, respectively for 8 weeks. Chemical analysis of the diets for threonine values were 0·47, 0·66 and 0·74 %, respectively.

3. The numbers of goblet cells did not change due to L-threonine supplementation. Also, L-threonine had no affect on the villus height and mucosal thickness. No differences were found due to treatments among groups in the activity of alkaline phosphatase (ALP) in jejunum or ileum. L-threonine supplementation at 0·2% maximised the concentration of superoxide dismutases (SOD) in both serum and liver.

4. In conclusion, L-threonine supplementation had no affect on gut morphology but may have an antioxidant function at 0·2%.

INTRODUCTION

High temperature not only reduces the laying performance of hens and egg quality (Njoya and Picard, 1994; Mashaly *et al.*, 2004) but has been reported also to impair the intestinal epithelial structure (Suzuki *et al.*, 1983; Burkholder *et al.*, 2008). Also, heat stress can lead to a reduction in the number of goblet cells (Sandikci *et al.*, 2004), which produce mucin glycoproteins (Forstner and Forstner, 1994). Moreover, studies *in vivo* and *in vitro* have reported that high temperatures can disturb the balance between the production of reactive oxygen species (ROS) and the antioxidant systems, and may further stimulate the formation of ROS (Mahmoud and Edens, 2003; Lin *et al.*, 2008; Feng *et al.*, 2008).

Threonine is an integral component of intestinal mucin glycoproteins (Van Klinken *et al.*, 1995; Burrin and Stoll, 2002), which are produced by goblet cells distributed along the villi (Uni *et al.*, 2003).

The goal of our study was to determine whether supplemental threonine can positively influence histological structure, goblet cell numbers, or antioxidant enzyme activities.

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MATERIALS AND METHODS

Birds and housing

A total of 576 commercial laying hens (Babcock Brown) were divided into three groups of 192 birds each, which consisted of 6 replications of 32 birds each. They were arranged using a completely randomised design. The hens were housed with 4 birds per cage under the same management conditions in a windowed poultry house. The experiment was carried out in accordance with the Chinese guidelines for animal welfare and approved by the animal welfare committee of Animal Science College, Zhejiang University. The photoperiod was 16L:8D throughout the experiment. The present study was carried out during June to August, considered as severe hot and humid months in the south of China. The temperature and humidity inside the barn were recorded twice daily (at 8 am and 2 pm). The average ambient relative humidity inside the barn was 85 ± 3% and the mean daily temperature was 30 ± 5°C. This study lasted 9 weeks, including a 1-week acclimation period and 8-week experimental period.

Diets

Hens were fed on the same basal diet based on maize, peanut meal, and crystalline amino acids. Feed in mash form and water were provided freely. Crystalline L-threonine (98·5% Thr, PT. Cheil Jedang, Indonesia) was added to the basal diet at 0 (control), 0·2, and 0·3%. Dietary treatments were achieved by the addition of crystalline L-threonine at the expense of inert filler. Chemical analysis of the diets for threonine values were 0·47, 0·66 and 0·74%, respectively. Ingredient composition and analysed nutrients are presented in Tables 1 and 2, respectively. To determine amino acids, samples were hydrolysed with 6 M HClat 110°C for 24 h, and the major amino acid composition of hydrolysates was analysed by HPLC (Hitachi L-8900 Amino Acid Analyser, Tokyo, Japan).

Table 1. *Composition and nutrient content of control diet*

Basal ingredients	g/kg
Yellow maize	602
Soybean meal 48%	40
Peanut meal 47·8%	196
Soybean oil	40
Dicalcium phosphate	18·5
Limestone (38% Ca)	80·0
Premix[1]	5
NaCl	3
Dl-Methionine	1·3
L- Lysine-HCl	1·2
L-Isoleucine	1·8
L-Tryptophan	0·3
L-valine	0·9
L- Threonine[2]	0·00
Filler[3]	10
Estimated concentration[4]	Analysed concentration
CP, g/kg 16·40	16·56
ME, MJ/kg 12·15	
Methionine, % 0·33	0·31
Lysine, % 0·73	0·70
Isoleucine, % 0·67	0·68
Tryptophan, % 0·19	
Valine, % 0·72	0·70
Threonine[5], % 0·47	0·48
Calcium, % 3·61	
Available phosphorus, % 0·47	

[1]Premix provided the following per kg of diet: retinyl palmitate, 3·96 mg; cholecalciferol, 0·06 mg; DL-α-tocopheryl acetate, 20 mg; menadione sodium bisulphite, 4 mg; thiamin mononitrate, 1·63 mg; riboflavin, 5 mg; niacin, 30 mg; pantothenic acid, 10 mg; folic acid, 0·5 mg; biotin, 0·22 mg; choline chloride, 250 mg; cyanocobalamin, 0·012 mg; Mn, 48 mg; Zn, 40 mg; Fe, 24 mg; Cu, 16 mg; I, 0·6 mg; Se, 0·12 mg; moisture ≤ 10%.
[2]The respective diet formulated to contain 0·47, 0·67, and 0·77% threonine.
[3]The dose titrations were achieved by addition of L-Threonine at the expense of washed builder's sand.
[4]Values were calculated from data provided by Feed Database in China (2009).

Sampling and sample processing procedure

At the end of the experiment, blood samples were collected from 6 hens (1 bird/replicate). After being separated naturally, the serum was centrifuged for 10 min (3000 rpm). Serum samples were aspirated by pipette and stored in 1·5-ml Eppendorf tubes at −70°C until analyses, and thawed at 4°C before analysis.

Immediately following slaughter, the jejunum (the small intestine from the distal end of the duodenal loop to Meckel's diverticulum) and ileum (the small intestine between Meckels diverticulum and the caeca) were obtained from 6 hens and about 1 cm of the jejunum and ileum was washed in physiological saline solution, and fixed in 10% buffered formalin for histology. The mucosa were scraped from the other parts of these tissues (jejunum and ileum) to measure alkaline phosphatase and snap-frozen in liquid N2, and stored at −80°C until determining ALP activity. The pancreas also was removed and snap-frozen in liquid N2. Thereafter, pancreas samples were transferred to a freezer at −80°C until required for enzyme activity measurements. Liver was snap-frozen with liquid nitrogen, and stored at −80°C until analysis. Serum glucose (GLU) was measured using a commercial kit (Shanghai Rongsheng Biotech Co. Ltd., Shanghai, China). Adrenocorticotropic hormone (ACTH) was measured by a microplate reader (SpectraMax M5, Molecular Devices, USA) using a commercial kit (R&D Systems Inc., Minneapolis, MN) according to the manufacturer's instructions.

Table 2. *Analysed concentrations of amino acids in the experimental diets*[1]

Amino acids	L-threonine[2] concentration (%)		
	0·0	0·2	0·3
Formulated dietary threonine	0·47	0·67	0·77
Analysed concentrations (%)			
Essential amino acids			
Arginine	1·29	1·36	1·30
Histidine	0·34	0·36	0·34
Isoleucine	0·68	0·66	0·68
Leucine	1·22	1·29	1·24
Lysine	0·70	0·71	0·70
Methionine	0·31	0·32	0·32
Phenylalanine	0·74	0·80	0·79
Threonine	0·48	0·66	0·74
Valine	0·70	0·71	0·71
Nonessential amino acids			
Alanine	0·77	0·75	0·78
Aspartic acid	1·53	1·64	1·57
Glutamic acid	3·20	3·43	3·25
Glycine	0·73	0·79	0·74
Proline	0·75	0·80	0·78
Serine	0·73	0·78	0·75
Tyrosine	0·49	0·50	0·50

[1]Chemical composition data are the results of a chemical analysis conducted in duplicate.
[2]Crystalline L-threonine added (98·5 % Thr, PT. Cheil Jedang, Indonesia).

Assay of antioxidant indices in liver and serum

Assay kits for superoxide dismutases (SOD), glutathione (GSH), glutathione peroxidase (GSH-Px), and malondialdehyde (MDA) were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). For biochemical assays, liver tissues were homogenised in ice-cold isotonic physiological saline to form homogenates at the concentration of 0·1 g liver/ml. The samples were centrifuged. Then, the supernatants and sera already prepared were subjected to the measurement of SOD, GSH-Px, and MDA concentrations by spectrophotometric methods using a spectrophotometer (UV-2000, Unico Instruments Co. Ltd., Shanghai, China). All of the assays followed the kit instructions. Activity of SOD was measured by the xanthine oxidase method, which monitors the inhibition of reduction of nitro blue tetrazolium by the sample (Winterbourn *et al.*, 1975). Total glutathione (GSH) was determined by measuring the rate of reduction of 5,5′-dithiobis-2-nitrobenzoate to 2-nitro-5-thiobenzoate (Zakowski and Tappel, 1978). Activity of GSH-Px was detected with 5,5′-dithiobis-p-nitrobenzoic acid, and the change of absorbance at 412 nm was monitored using a spectrophotometer (Hafeman *et al.*, 1974). The MDA concentration was analysed with 2-thiobarbituric acid, monitoring the change of absorbance at 532 nm with the spectrophotometer (Placer *et al.*, 1996). Enzyme activity was expressed as units per milligram of protein for tissues. GSH, SOD, GSH-Px, and MDA were determined by the same method in the serum.

Histological structure

Lengths (1 cm) from the medial portions of jejunum and ileum were washed in physiological saline solution; and fixed in 10% buffered formalin. Tissue samples were later embedded in paraffin, and a 2-μm section of each sample was placed on a glass slide and stained with Alcian Blue and periodic acid-Schiff reagent. Briefly, tissues were cleared and hydrated and then stained with Alcian Blue solution (1 g of Alcian Blue, 3 ml/l of acetic acid, 97 ml of distilled water, pH 2·5) for 30 min. After washing in water for 10 min, slides were oxidised in periodic acid (5 g/l) for 5 min, rinsed in lukewarm tap water for 10 min, and stained in Coleman's Schiff reagent as a counter stain for 10 min. Goblet cell number along the villi was determined visually by counting under a Nikon microscope (Nikon Corp., Japan). Villus height was measured from the top of the villus to the villus-crypt junction and total mucosal thickness was measured from the top of the villus to the border over the muscularis mucosa.

Alkaline phosphatase estimation

Previously frozen mucosa as well as the pancreas were homogenised in ice-cold 0·1 M sodium chloride solution. The homogenates were then centrifuged at 20 000 rpm for 30 min at 4°C and the supernatant was collected and stored at −70°C for enzyme assays. After thawing the homogenates and adjusting to room temperature, the activities of α-alkaline phosphatase (NC-IUBMB, EC 3·1·3·1) was analysed by a microplate reader (SpectraMax M5, Molecular Devices LLC, CA) using diagnostic kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the instructions of the manufacturer.

The protein concentrations were determined using the Coomassie Brilliant Blue G-250 reagent with BSA as a standard and enzyme activities were expressed as units/g protein.

Statistical analyses

Data were statistically analysed by one-way ANOVA of SPSS 16·0 for Windows (SPSS Inc., Chicago, IL). When significant differences were found ($P < 0·05$), Tukey post hoc tests were performed.

RESULTS AND DISCUSSION

A previous study in our laboratory showed that adding 0·2 and 0·3% L-threonine improved egg production and serum IgG, respectively (Azzam *et al.*, 2011*a*). Therefore, this study was conducted to investigate the effects of these two concentrations on histological structure, goblet cell numbers, and antioxidant enzyme activities of laying hens.

It has been reported that gut function is affected by many factors of stress such as heat stress (Suzuki *et al.*, 1983; Burkholder *et al.*, 2008), inflammatory bowel diseases such as ulcerative colitis and Crohn's disease (Corfield *et al.*, 2000). Compared with other essential amino acids, threonine content in proteins of the intestine represents up to 30% (Neutra and Forstner, 1987), suggesting that threonine affects intestinal functionality and maintenance. It was found that threonine supplementation affected villus height, epithelial thickness, goblet cell number, and the crypt depth (Zaefarian *et al.*, 2008). In the current study, L-threonine supplementation at 0·2 or 0·3% had no affect on the number of goblet cells, villus height, and mucosal thickness (Table 3). It has been reported that an increased supply of threonine, along with serine, proline, and cysteine, improved mucin synthesis during colitis, but this effect was weak in the small intestinal segments exempt from any sign of inflammation (Faure *et al.*, 2006). In our studies, hens were raised in cages and we did not see any enteric diseases and this explains why no significant changes in goblet cell, villus height, and mucosal thickness due to L-threonine supplementation were seen. Moreover, the lowest concentration of threonine in this experiment (0·47%; current NRC norm (NRC, 1994)) may be sufficient to protect the morphology of the gut from any atrophy. It has been reported that feeding piglets with 70% of threonine recommendations for 2 weeks induced villous atrophy (Hamard *et al.*, 2007). Recently, we found that expressions of jejunal and ileal MUC2 mRNA were increased linearly by increasing dietary threonine (Azzam *et al.*, 2011*b*). In general, proteins and specific amino acids have been found to change mucin secretion and may interact directly with goblet cells or with the enteric nervous system to elicit an alteration in mucin production (Montagne *et al.*, 2000; Claustre *et al.*, 2002; Faure *et al.*, 2005). Therefore, the absence of a difference in number of goblet cells per villus does not necessarily mean that the dietary treatments did not affect mucin production.

No differences were found due to treatments among groups in the activity of ALP in jejunum or ileum (Table 4). ALP is a marker of mucosal enterocyte maturation because it is expressed by the enterocytes on the top of the villi (Weiser, 1973; Traber *et al.*, 1992). So, the absence of significant differences between different groups in respect with ALP indicated that all sampled villi were at the same level of maturation.

Table 3. *Effect of L-threonine (Thr) supplementation on histological structure and goblet cell numbers (GCn), villus height (VH) and mucosal thickness (TMT), (mean,* n = *6 hens per group)*

Thr %	Jejunum			Ileum		
	GCn,/ villus	VH, µm	TMT, µm	GCn,/ villus	VH, µm	TMT, µm
0·0	101	1021·5	1201·9	133	835·4	1016·74
0·2	99	973·8	1190·7	121	807·5	997·19
0·3	119	1078·7	1277·7	125	834·1	983·43
SEM	15	87·69	99·79	7·60	39·23	38·97
***P*-Value**	0·35	0·49	0·64	0·28	0·72	0·69

Table 4. *Alkaline phosphatase (ALP, Units/g protein) in the mucosal homogenates of the jejunum and ileum of laying hens subjected to graded amounts of L-threonine (Thr), (mean, n = 6 hens per group)*

Thr %	Jejunum ALP	Ileum ALP
0·0	21·91	20·00
0·2	24·26	21·02
0·3	25·84	19·96
SEM	5·00	3·82
***P*-Value**	0·73	0·95

L-threonine supplementation at 0·2% maximised the concentration of superoxide dismutases (SOD) in both serum and liver ($P < 0·05$) (Table 5 and 6). Threonine is one of amino acids that can carry a small fraction of the copper in the blood (Shils *et al.*, 2006). It is known that copper-zinc superoxide dismutase acts as oxidation-retarding factor (Meyer *et al.*, 1994). Further studies are required to verify this result. SOD is one of the main antioxidant enzymes in scavenging the oxygen free radicals (McCord, 1979). One of the important strategies in poultry farms during the summer months is to maximise antioxidant ability and minimise lipid peroxidation. Therefore, adding threonine at 0·2% can maximise the concentration of SOD in the serum and may protect cells from oxidative injury by clearing superoxide anions.

L-threonine supplementation had no affect on serum MDA, GSH, and GSH-Px ($P > 0·05$) (Table 5). MDA can endogenously reflect lipid peroxidation, which is the consequence of diminished antioxidant protection as concentrations of ROS increase. Also, SOD, GSH and GSH-Px are

Table 5. *Effect of L-threonine supplementation on antioxidant enzyme activities and some serum parameters (mean, n = 6 hens per group)*

Variable	L-Threonine (%)				
	0	0·2	0·3	SEM	*P*-value
SOD, U/ml	149·16[b]	177·08[a]	168·47[ab]	7·44	0·006
GSH, μmol/l	15·03	15·46	16·7	1·80	0·63
GSH-PX, μmol/l	743·8	617·8	628·64	211·14	0·80
MDA, nmol/ml	7·95	7·13	5·13	2·40	0·51
ACTH, ng/l	75·08	65·83	65·03	14·82	0·75
GLU, mmol/l	12·33	10·41	12·86	1·23	0·33

[a,b]Within a row, values not sharing a common superscript letter are significantly different ($P < 0·01$).

Table 6. *Effect of dietary concentration of threonine on total protein, glutathione, glutathione peroxidase, and malondialdehyde concentrations of liver (mean, n = 6 hens per group)*

Items	L-Threonine (%)				
	0	0·2	0·3	SEM	*P*-value
TP, g/l	0·49	0·48	0·49	0·01	0·48
GSH, μmol/g protein	18·36	19·75	20·34	2·10	0·63
GSH-PX, U/mg protein	104·95	108·47	105·06	3·71	0·57
SOD, U/mg protein	262·62[ab]	281·84[a]	256·34[b]	9·60	0·04
MDA, nmol/mg protein	6·88	6·09	6·20	0·79	0·57

[a,b]Within a row, values not sharing a common superscript letter are significantly different ($P < 0·05$).

the main parameters used to assess oxidative status in the enzymatic system. Consistent with the increased activity of serum SOD, MDA concentration in the serum remained unchanged. In our study ACTH did not change due to L-threonine supplementation. The catabolic effect of ACTH in producing more glucose is well known. No difference between groups in the concentration of GLU suggests that high concentrations of threonine could not provoke activation of gluconeogenesis. According to Blatteis (2000) and Star *et al.* (2008) after combined exposure to two kind of stressors (heat stress; climatic stress and lipopolysaccharide; hygienic stress) increased the percentage of the mortality as compared to exposure to the single stressor. This means that animals are able to cope with one kind of single stressor. Therefore, high temperature (one kind of stressor) in our experiment may not have been enough to provoke MDA.

In conclusion, L- threonine supplementation had no affect on the gut morphology, but may have an antioxidant function under the summer weather. Further study is required to study the effect of supplemental L-threonine on these parameters under combined exposure to heat stress (climatic stress) and hygienic stress or malnutrition.

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