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Effect of *in ovo* injection of threonine on Mucin2 gene expression and digestive enzyme activity in Japanese quail (*Coturnix japonica*)

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ABSTRACT

A total of 540 Japanese quail eggs were assigned to 9 treatments of 4 replicates to investigate the effect of *in ovo* injection of threonine (THR) on mucin2 (MUC2) mRNA expression and digestive enzyme activity. Treatments were (non-injected) eggs and those *in ovo* injected with saline (0.05 or 0.1 ml) with or without THR (5 mg/ml) in two sites (in or under the air sac). On hatch day, 0.05 ml *in ovo* injected (under the air sac: TUAS) hatchlings were divided into three groups based on NRC recommendations for THR, while all 0.1 ml *in ovo* injected chicks were removed due to low hatchability. The remaining treatments received the NRC recommended diet until day 10 post-hatch. Treatments had no effect on protease and amylase activities, while TUAS increased MUC2 gene expression. In conclusion, the *in ovo* injection of THR increased MUC2 gene expression but had no effect on enzyme activity.

1. Introduction

It has been shown that there is an approximately 48–72 hr gap between hatch time and hatchlings' access to feed and water due to different hatch times, hatchery handling and transport times to the poultry farm (Bhuiyan et al., 2011; Willemsen et al., 2010). This delay results in a lowering of development and function of the gut (Potturi et al., 2005; Yang et al., 2009), thereby reducing final body weight (Noy and Sklan, 1999), decreasing immune response to pathogens (Dibner et al., 1998) retarding growth, and increasing mortality up to 5% (Willemsen et al., 2010). It has thus been suggested that the detrimental effects of this delay could possibly be overcome. Hatchery feeding (Kidd et al., 2007; Willemsen et al., 2010) and *in ovo* feeding (Uni and Ferket, 2004) are two most important clues suggested to overcome the detrimental effects of such delay. Hatchery feeding requires a high level of consistency in nutrients and diet formulation between hatchery management and the rearing farm, something that is practically difficult to achieve (Lilburn, 1998). Sharma and Burmester (1982) first used an *in ovo* method to vaccinate chicks against Marek's disease. In recent decades, many studies have been conducted to evaluate the effects of *in ovo* injection on poultry performance (Ohta and Kidd, 2001; Ohta et al., 2001). Finally, Uni and Ferket (2003) patented "*In ovo* Feeding" and stated that this method could beneficially affect hatchability (Uni et al., 2005), intestinal mucosa, and body weight at hatch and at 35 days of age (Uni and Ferket, 2004). Uni et al. (2003) reported that gastrointestinal functionality of *in ovo* treated chicks was the same as that of 2 day old birds fed immediately after hatching. To investigate the effect of *in ovo* feeding on the performance of chickens, various nutrients such as amino acids (Bhanja and Mandal, 2005; Bhanja et al., 2004, 2010), carbohydrates (Foye, 2005; Tako et al., 2004; Uni and Ferket, 2004), vitamins (Al-Daraji et al., 2012; Bhanja et al., 2007; Nowaczewski et al., 2012) and other nutrients (Moore, 2005; Tako et al., 2005; Zhai et al., 2008) have been administered to different poultry species. Among these nutrients, amino acids and, particularly, threonine (THR) have attracted more attention due to their effect on cellular (Tenenhouse and Deutsch, 1966) and humoral (Takahashi et al., 1994) immune responses, mucin structure (Gum, 1992; Lien et al., 1997), and digestive enzyme activity (Block et al., 1966; Yang et al., 1989). Kadam et al. (2008) reported that *in ovo* injection of THR caused better immunological responses in broilers, while having no effect on digestive enzyme activity. It was also demonstrated that digestive enzymes secreted by the intestinal lumen may degrade the thin mucosal layer of the intestine, enter the epithelial cell walls and cause ischemia (Godl et al., 2002). Similarly, THR can induce mucin secretion which results in an increase in the thickness of the mucosal layer and prevents enzymatic degradation of the intestinal mucous layer (Chang et al., 2012).

These studies were done only with poultry models (Kadam et al., 2008), so consequently there is a dearth of research on *in ovo* administration in quail. Thus, in the current study, we wanted to evaluate the

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Table 1
Description of different treatments used for *in ovo* injection of threonine in Japanese quail.

Treatments	Volume (ml)		Additive		Position		Period	
	0.05	0.1	Threonine	Saline	In the air sac (5 mm)	Under the air sac (8 mm)	Embryonic (E13)	Starter[b] (1–10 d)
Control (non-injected)	–	–	–	–	–	–	✓	✓
5SIAS[a]	✓	–	–	✓	✓	–	✓	✓
5SUAS	✓	–	–	✓	–	✓	✓	✓
1SIAS	–	✓	–	✓	✓	–	✓	–
1SUAS	–	✓	–	✓	–	✓	✓	–
5TIAS	✓	–	✓	✓	✓	–	✓	✓
5TUAS	✓	–	✓	✓	–	✓	✓	✓
1TIAS	–	✓	✓	✓	✓	–	✓	–
1TUAS	–	✓	✓	✓	–	✓	✓	–

[a] 5SIAS: injection of 0.05 ml saline (containing 0.9 g salt/l of distilled water) in the air sac; 5SUAS: injection of 0.05 ml saline under the air sac; 1SIAS: injection of 0.1 ml saline in the air sac; 1SUAS: injection of 0.1 ml saline under the air sac; 5TIAS: injection of 0.05 ml saline containing 5 mg/ml of threonine in the air sac; 5TUAS: injection of 0.05 ml saline containing 5 mg/ml of threonine under the air sac; 1TIAS: injection of 0.1 ml saline containing 5 mg/ml of threonine in the air sac; 1TUAS: injection of 0.1 ml saline containing 5 mg/ml of threonine.

[b] In rearing period (1–10 d), treatment injected 0.1 ml solution was removed due to low hatchability and other groups (except for 5TUAS) were received NRC recommendation (1994) diet. 5TUAS was divided to three groups: first group received all nutrients the same as NRC (1994) recommendations; second group received 10% higher threonine than NRC (1994) recommendations diet; third group received 20% higher threonine than NRC (1994) recommendations diet.

effect of *in ovo* injection of THR on MUC2 gene expression and digestive enzyme activity in quail hatchlings during the rearing period.

2. Materials and methods

2.1. Incubation, injection method and treatments

A total of 540 Japanese quail eggs were set in a single stage incubator. The relative humidity and temperature in the incubator for the hatchery (0–14 days) period were 68% and 37.8 °C, respectively, and for the setter (15–17 days) period were 78% and 36.8 °C, respectively. On day 11 of the embryonic period (E11), eggs were injected with different volumes of solutions containing physiological saline with or without threonine. Gauge 31 needles were used to inject all solutions into the air sac (IAS; depth of injection: 5 mm) or under the air sac (UAS; depth of injection: 8 mm) of quail eggs. The experiment was conducted as a completely randomized design with 9 treatments and 4 replicates of 15 eggs each. Treatments were consisted of (Table 1): (1) non injected (control) group; (2) IAS injection of 0.05 ml saline (containing 0.9 g NaCl/l of distilled water) (5SIAS); (3) UAS injection of 0.05 ml saline (5SUAS); (4) IAS injection of 0.1 ml saline (1SIAS); (5) UAS injection of 0.1 ml saline (1SUAS); (6) IAS injection of 0.05 ml saline containing 5 mg/ml of threonine (5TIAS); (7) UAS injection of 0.05 ml saline containing 5 mg/ml of threonine (5TUAS); (8) IAS injection of 0.1 ml saline containing 5 mg/ml of threonine (1TIAS); (9) UAS injection of 0.1 ml saline containing 5 mg/ml of threonine (1TUAS). Based on the lower hatchability of chicks receiving a 0.1 ml solution of (Kermanshahi et al., under revision) either IAS or UAS, quail hatched from these treatments were removed from the trial. Chicks from all other treatments (except those of 5TUAS group) received a corn-soybean meal basal diet formulated per all the nutrients based on NRC (1994) recommendations (Table 2) up to 10 days of age. In addition, quail from the 5TUAS group were divided into three groups consisting of 4 replicates with 3 quail per replicate and received the following diets: (1) control diet, a corn-soybean meal basal diet containing all nutrients based on NRC (1994) recommendations; (2) control diet except for threonine which was 10% higher threonine than NRC recommendations; (3) control diet except for threonine which was 20% higher threonine than NRC recommendations. Birds had free access to feed and water with a 23L/1D lighting program.

On hatch day and day 10 of the rearing period, 3 quail from each replicate (12 quail from each treatment) were euthanized by CO_2 asphyxiation, the adherent material and contents of the small intestine were carefully removed, and the duodenum, jejunum and ileum were carefully dissected and stored at –70 °C. By mild massaging of the intestine from the end of the duodenum to the ileocecal junction, homogenous digesta were collected and immediately frozen at –70 °C until used. Frozen jujenal samples were then divided into two groups in order to determine enzyme activity for analysis and MUC2 gene expression. The experimental protocols were reviewed and approved by the Animal Care Committee of the Ferdowsi University of Mashhad, Iran.

2.2. Muc2 mRNA expression assay

The assessment of MUC2 gene expression was performed on jejunal samples obtained on the day of hatch and 10 days after hatch. Total RNA was extracted from quail jejunum using the TRIzol

Table 2
Composition of the quail's experimental diet.[c]

Components (%)	1–10 days (starter)
Corn	55.58
Soybean meal (44%)	41.34
Methionine	0.12
Lysine	0.01
Threonine	0.11
Dicalcium phosphate	0.75
CaCO3	1.30
Common salt	0.15
Sodium bicarbonate	0.14
Vitamin premix[a]	0.25
Mineral premix[b]	0.25
Calculated nutrients analysis	
Metabolizable energy (kcal/kg)	2793.92
Crude protein (%)	23.072
Calcium (%)	0.784
Sodium	0.1468
Chlorine	0.1359
Available phosphorous (%)	0.291
Lysine (%)	1.264
Methionine (%)	0.475
Methionine + cysteine (%)	0.848
Arginine (%)	1.509
Threonine (%)	0.98

[a] Each kilogram of vitamin supplement contains: vitamin A, 3,600,000 IU; vitamin D3, 800,000 IU; vitamin E, 7200 IU; vitamin K3, 800 mg; vitamin B1, 720 mg; vitamin B2, 2640 mg; vitamin B3, 4000 mg; vitamin B5, 12,000 mg; vitamin B6, 1200 mg; vitamin B9, 400 mg; vitamin B12, 6 mg; biotin, 40 mg; choline chloride, 100,000 mg; antioxidant, 40,000 mg.

[b] Each kilogram of mineral supplement contains: Mn, 40,000 mg; Zn, 33,880 mg; Fe, 20,000 mg; Cu, 4000 mg; I, 400 mg; Se, 80 mg.

[c] T10 and T20 treatments were added 0.001% and 0.002% threonine to diets, respectively, in balance of other nutrients, especially amino acids.

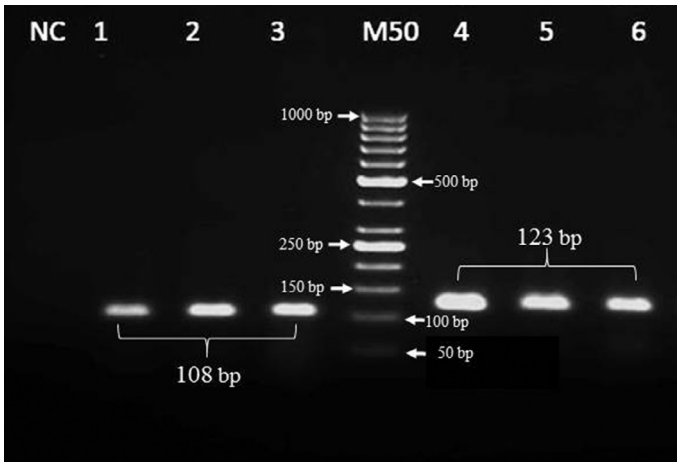

Fig. 1. Agarose gel electrophoresis (2.0% w/v) of mucin2 (lines 1, 2, 3) and beta-actin (lines 4, 5, 6) real-time PCR products in randomly selected tissue samples. M50, molecular weight marker (Thermo Scientific GeneRuler 50 bp DNA Ladder, contains 6 regularly spaced dsDNA bands ranging from 50 bp to 300 bp and 7 regularly spaced dsDNA bands ranging from 400 bp to 1000 bp. Each lane contains 50 ng of dsDNA); NC, negative control.

procedure (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The integrity of total RNA was checked by native RNA electrophoresis on 1.0% agarose gel in 1 × TAE buffer (Tris 2.0 M, acetic acid 1.0 M, and EDTA 0.1 M, pH 8.0). The quantity and purity of extracted RNA were assessed by the spectrophotometry method by measuring the absorbance in 260 nm and calculating the ratio of 260 to 280 nm, respectively (Fig. 1). Two micrograms of total RNA was used to synthesize the complementary DNA by MMLV (Moloney-murine leukemia virus reverse) transcriptase (Thermo Scientific, USA) according to the manufacturer's recommendation. The abundance of MUC2 mRNA was determined by a Real-Time PCR system (ABI 7300, Applied Biosystems, Foster City, CA) and chemicals were supplied from Thermo Scientific, USA (SYBR PrimeScript RT-PCR kit). Primer details are shown in Table 3. Each reaction was performed in a total volume of 20 μl in triplicate in addition to a negative control according to MIQE guidelines (Bustin et al., 2009). Product specificity was confirmed by plots of the melting curve derived by the 7300 software system and agarose gel electrophoresis of PCR products (Fig. 1). The relative mRNA abundance was calculated based on the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Gene expression data were normalized by the beta-actin as a housekeeper gene. Beta-actin was chosen as a housekeeper based on experiments demonstrating the stable expression of beta-actin mRNA in the intestine of Japanese quail (Singh et al., 2011; Ubuka et al., 2005). The values are represented as an n-fold difference relative to the calibrator. The fold changes in the experimental groups were compared to the non-injected group; thus, the control group was considered as one in all comparisons.

2.3. Digestive enzyme activity in small intestine contents and tissue

The amylase and protease activity were measured in intestinal and digesta samples obtained at d 10 of age. The small intestine samples containing the duodenum, jejunum and ileum were thawed at 4 °C and homogenized with 10-fold PBS (pH = 7), contents centrifuged at 18,000 × *g* for 20 min at 4 °C and supernatants separated for enzyme activity.

The digesta samples were homogenized in 10-fold cold PBS (pH = 7) based on the sample weight and centrifuged at 18,000 × *g* for 20 min at 4 °C. The supernatants were then collected in small microtubes and stored at –70 °C until analyzation for enzyme activity.

The methods described by Jin et al. (2000) were used to assess digestive enzyme activity. Amylase (EC 3.2.1.1) activity was determined according to the procedure described by Somogyi (1960). One unit of amylase activity was described as the amount of amylase that causes the formation of reducing power equivalent to 1 mg of glucose in 30 min at 38 °C per mg of intestinal tissue homogenates or digesta protein. A pure source of cornstarch was used as substrate. An analytical enzyme activity kit was purchased and all procedures were conducted in accordance with the manufacturer's instructions (SorenTech Co., Mashhad, Iran).

The modified procedure of Lynn and Clevette-Radford (1984) was used to determine the protease activity of intestinal tissue homogenates or digesta protein. The protease activity unit was declared as milligrams of casein degraded during 2 h incubation at 38 °C of samples with casein used as a substrate.

2.4. Statistical analysis

All data were analyzed in the general linear model (GLM) procedure with SAS statistical software (SAS, 2004), and significant differences between means ($P < 0.05$) were determined by Tukey's Honestly Significant Difference (HSD).

3. Results and discussion

To our knowledge, this is the first research on the *in ovo* injection of threonine in Japanese quail that has evaluated the effect of supplementation methods on MUC2 gene expression and digestive enzyme activities.

The results of the present study indicate that 5TUAS increased MUC2 gene expression on hatch day and at 10 days of age (Figs. 2 and 3, respectively). MUC2 is one of the subfamilies of protein secretory of mucin in the small and large intestines and is primarily responsible for the secretion of gel forming mucin (Jiang et al., 2013). MUC2 plays a fundamental role in stretching gel layers on the intestinal surface and protecting this surface from a variety of invading pathogens (Johansson et al., 2008). Any changes in the production of mucin in the lumen could thus affect the protection of epithelial cells from pathogens, nutritional absorption and subsequent performance (Forstner and Forstner, 1994). However, it was reported that the composition of digested proteins and amino acids might change the trend of mucin secretion in the small intestine

Table 3
Sequences of primer pairs used for amplification of target and reference genes.

Gene	Primer sequence	Annealing temperature (°C)	Amplication size	Accession number
Mucin2	Forward 5′ CCACAAGTCGTCCAGTACCTACA3′ Reverse 5′ AGGTTTCATAGTCACCACCATCTTC3′	60	108	XM_421035
Beta-actin	Forward 5′ CTGGCACCTAGCACAATGAA-3′ Reverse 5′ CTGCTTGCTGATCCACATCT3′	60	123	AF199488

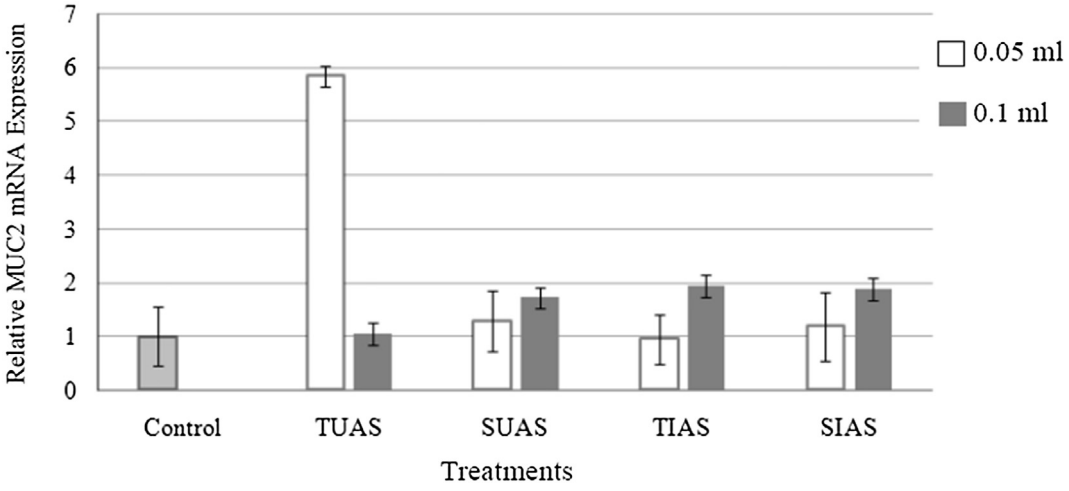

Fig. 2. Effect of threonine *in ovo* injection on jejunal mucin2 gene expression of Japanese quail on day of hatch. Treatment abbreviations: SIAS: saline in the air sac; SUAS: saline under air sac; TUAS: threonine under air sac; TIAS: threonine in the air sac. White and gray columns show 0.05 ml and 0.1 ml injection of supplementing solutions, respectively. Results are given as means (n = 12) for all treatments.

(Faure et al., 2005). Since THR serine build up the most molecular weight of mucin protein (about 50–80%) (Montagne et al., 2004), some researchers have supplemented this amino acid in poultry diets to evaluate the effect of THR levels on MUC2 gene expression as well as on following secretion (Azzam et al., 2011; Horn et al., 2009). However, others have reported that no change in MUC2 expression was observed following THR supplementation (Chee et al., 2010). The present results are consistent with those of Horn et al. (2009) who reported that increasing THR in duck diets up to 14 days of age resulted in significantly higher MUC2 gene expression. Azzam et al., 2011, in agreement with the present findings, also revealed that the addition of different levels of THR led to higher MUC2 gene expression in layers reared under thermal conditions. Present results also showed that 0.1 ml TUAS did not affect MUC2 gene expression. Although there is no well-known reason for such results, we hypothesized two possible reasons for the ineffectiveness of 0.1 ml TUAS. One reason might be the high volume of solution (0.1 ml) injected into the quail eggs. Another explanation might be the imbalance of amino acids following the injection of 0.1 ml threonine solution.

The results of THR *in ovo* injection on enzyme activity in the intestine showed that this supplementation had no significant effect on amylase activity in the small intestine segments and digesta samples in comparison with the control group (Table 4), while birds receiving 5TUAS and T20 had numerically higher amylase activity in jujenal and ileal samples as well as in digesta content. The same results were obtained with regard to protease activity in different samples, as THR injection did not significantly affect protease activity in the small intestine samples when compared to control birds, while 5TUAS and T20 tended to show higher protease activity in jujenal and ileal segments. Previous investigations showed that THR plays an important role in the basic structure of digestive enzymes (Yang et al., 1989), while Stoll et al. (1998) stated that THR forms the mucin backbone and subsequently supports intestinal mucosa. Kadam et al. (2008) reported that THR *in ovo* injection with different dilution rates in broilers had no significant effect on enzyme activity in the intestinal samples, which is consistent with the present results with quail. The same results were shown by Azzam et al. (2011) who added different levels of THR to laying hens' diet under different environmental conditions and reported that various levels of supplemented THR did not affect enzyme activity in jejunal and ileal segments.

It is well-documented that digestive enzymes such as trypsin involved in the epithelial cell disruption after ischemia disturb mucin

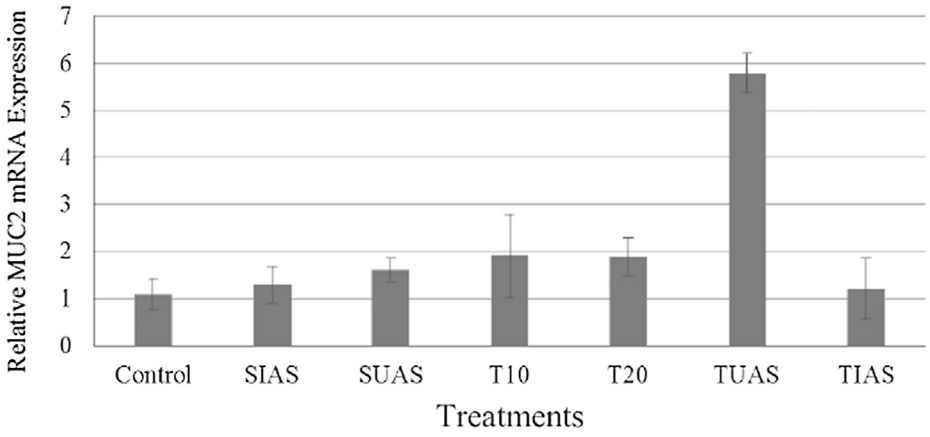

Fig. 3. Effect of threonine *in ovo* injection on mucin2 gene expression of Japanese quail on day 10 of rearing period. Treatment abbreviations: SIAS: saline in the air sac; SUAS: saline under air sac; TUAS: threonine under air sac; TIAS: threonine in the air sac; T10: 10% more threonine than NRC 1994 recommendations; T20: 20% more threonine than NRC 1994 recommendations. Results are given as means (n = 12) for all treatments.

Table 4
Effect of *in ovo* injection of threonine on enzyme activities (U/mg protein) in the small intestine and feces digesta in Japanese quails on d 10 of age*.

Treatments	Amylase				Protease			
	Duodenum	Jejunum	Ileum	Digesta	Duodenum	Jejunum	Ileum	Digesta
THR 0.05 UAS†	20.46 ± 0.38	41.79[a] ± 0.31	34.50[a] ± 0.24	14.12[a] ± 0.09	130.14 ± 1.10	327.20[a] ± 0.46	241.78[a] ± 0.35	25.32[a] ± 0.19
THR 0.05 IAS	20.23 ± 0.39	38.73[b] ± 0.36	31.51[b] ± 0.26	12.88[b] ± 0.18	130.12 ± 2.12	324.76[b] ± 0.56	239.22[b] ± 0.32	23.86[c] ± 0.26
Saline 0.05 UAS	20.34 ± 0.35	38.75[b] ± 0.41	31.55[b] ± 0.36	12.85[b] ± 0.19	130.72 ± 2.52	322.44[c] ± 0.49	239.11[b] ± 0.30	23.56[c] ± 0.22
Saline 0.05 IAS	20.32 ± 0.29	38.78[b] ± 0.45	31.55[b] ± 0.34	12.86[b] ± 0.22	130.33 ± 2.18	321.81[c] ± 0.52	239.00[b] ± 0.31	23.83[c] ± 0.28
T10	20.76 ± 0.24	40.07[ab] ± 0.32	33.72[ab] ± 0.28	13.59[ab] ± 0.17	130.37 ± 2.77	326.48[ab] ± 0.64	239.66[ab] ± 028	24.62[ab] ± 0.08
T20	20.74 ± 0.36	41.98[a] ± 0.39	34.42[a] ± 0.27	14.13[a] ± 0.14	130.6 ± 1.99	327.26[a] ± 0.58	241.57[a] ± 0.27	25.33[a] ± 0.16
Control	20.53 ± 0.40	39.93[ab] ± 0.31	33.83[ab] ± 0.38	13.63[ab] ± 0.08	130.63 ± 1.74	324.84[ab] ± 0.51	240.12[ab] ± 0.28	24.59[b] ± 0.13
P-value	0.9998	0.026	0.015	0.005	1.000	0.0004	0.047	0.0002

[a–c] Means sharing a common superscript in a column do not differ significantly ($P < 0.05$).
* Results are given as means ± SE ($n = 12$) for all treatments.
† THR: threonine; UAS: under air sac; IAS: in the air sac; T10: 10% more threonine rather than recommendation of NRC 1994; T20: 20% more threonine rather than recommendation of NRC 1994.

integrity resulting in the breakdown of the intestinal mucosal function with subsequent ischemia and hemorrhagic shock (Chang et al., 2012; Godl et al., 2002). The intestinal ischemia leads to the translocation of digestive enzymes from the lumen of the intestine into the intestinal wall, thereby initiating autodigestion (Chang et al., 2012). Although no significant increase in protease activity was observed in the present study, higher MUC2 mRNA expression obtained by 5TUAS might protect the mucosal layer of the intestine against degradation by digestive enzymes.

Overall, 5TUAS showed better performance with regard to a numerically higher enzyme activity and significantly higher MUC2 gene expression. The better performance of 5TUAS may be contributed to two factors: site of injection and solution volumes. Ohta and Kidd (2001) declared that the best sites for injection of amino acids are the yolk sac and extra amniotic fluid, the latter being the same under the air sac site as that considered in the present study. The second interfering factor is solution volume, as higher solution volumes resulted in lower hatchability and total performance of hatchlings (Zhai et al., 2011) and, as observed in this study, 0.05 ml showed better results than 0.1 ml.

According to the data obtained in the present study, it could be concluded that although supplemented THR via *in ovo* injection had no significant effect on amylase and protease activity in the intestine, 0.05 ml injection of THR solution under the air sac of quail eggs increased MUC2 gene expression. This might lead to higher mucin secretion in lumen and subsequently improve protection from pathogens and nutrients absorption in the small intestine, which are the most important factors for newly hatched chicks. Despite the beneficial results observed in this work and other similar ones, more research is necessary in order to support application of *in ovo* injection methods in the poultry industry.

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