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Effect of *In ovo* Injection of L-Threonine on Hatchability Followed by post-hatch Extra Level of Dietary Threonine on the Performance of Broilers

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Abstract

The aim of this study was to examine the effect of *in ovo* injection of L-threonine (L-Thr) on hatchability, growth performance, antioxidant capacity, carcass traits, immune organs weight, and intestinal histomorphology of Ross 308 broilers chicks. On day 18 of incubation, 258 fertile eggs were randomly allotted into 3 sets: non-injected group (NC group), saline injected group (sham control SC group), and (Thr group) L-Thr injected group. After hatch, each set was allocated into 2 groups (3 replicates of 10 birds/ replicate) and were fed on a basal diet (calculated Thr content, 0.87 %) supplemented with 0 (CON group), and 3 g/kg L-Thr, respectively. The groups were divided as follows: G1, G2, G3, G4, G5 and G6 where G1 is non injected group and fed basal diet, G2 saline injected and fed basal diet, G3 threonine injected and fed basal diet, G4 non injected and fed threonine supplemented diet, G5 saline injected and fed threonine supplemented diet and G6 threonine injected and fed threonine supplemented diet. Growth performance parameters were measured during different periods of the trial. At the end of rearing period serum antioxidative parameters (SOD and MDA) were measured, carcass traits and relative immune organs weight were calculated, in addition to intestinal histomorphology. Addition of Thr positively affected the FCR, and feed intake of broiler chicks compared to sham and control ($P < 0.05$) groups. Dietary Thr supplementation increased relative weight of spleen and thymus compared to the control non supplemented group and improved intestinal histomorphology. In conclusion, *in ovo* injection and dietary supplementation of L-Thr can improve growth performance, immunity of broiler chickens that may be mediated by the development of immune organs and improving the intestinal morphology of broilers as demonstrated by higher ratio of villus height to crypt depth.

KEYWORDS
Broiler, threonine, Growth performance, Immune organs, *in ovo* injection, Intestinal histomorphology

INTRODUCTION

Nutrient supply instantly after hatch is a critical factor for small intestinal development in chicks. It was found that feeding immediately post-hatch led to acceleration in intestinal morphological development, whereas late access to external feed resulted in delayed development of the small intestine's mucosal layer (Uni *et al.*, 1998; Uni and Ferket, 2003). A healthy 1-day-old chick is a crucial link between the hatchery and the broiler farm. The strategies of early feeding have been suggested and developed to diminish or possibly reverse the negative effects of delayed nutrition. These strategies range from *in ovo* feeding to specially designed post-hatch diets (Uni and Ferket, 2003; Uni and Ferket, 2004; Leeson, 2008). Delaying the onset of feeding and watering of newly hatched chicks could lead to a diminishing of their overall growth performance with adverse effects on breast meat. The most extreme consequence of delayed feeding is increased mortality (Willemsen *et al.*, 2010).

Optimal gastrointestinal functionality is important for sustainable animal production. Maintenance or improvement of gut health is critical for optimum growth, better efficiency of feed, and overall health of poultry (Yadav and Jha, 2019). Moreover, Effective functionality of the gastrointestinal tract (GIT) and its health are important factors in determining animal performance (growth, meat, and egg quality).

As incubation progresses, the embryo's body weight increases, as does the small intestine. However, the weight of small intestine increases at a much faster manner than the body does, the latter showing only a slight increase close to hatch. During the last days of incubation, the remaining part of yolk is internalized into the embryo's abdominal cavity facilitating its direct delivery into the small intestine through the yolk stalk, as well as transportation of the yolk lipids through the blood (Sklan, 2001). Following hatch, birds move from utilizing energy originated from endogenous nutrients in the yolk to utilizing the nutrients originated from an exogenous source (Sklan, 2001; Uni and Ferket, 2004).

A prominent early nutrition technique that could provide further opportunity to influence the development of a chick inside the egg and overcome the constraints of nutrient limitation during late incubation phase is *in ovo* feeding. This strategy offers the opportunity to provide the embryo with essential nu-

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trients, nutraceuticals, and functional foods to boost the status of growth and development of the embryo through injection of them also, that technique has been proven to provide adequate nutrients to the late-term embryo so protect it from the negative effect of starvation during the extended window of hatching (Uni *et al*., 2005). Several routes of *in ovo* injection including intra amniotic, yolk sac, and air sac have been used. Nutritional substances injected into the amniotic cavity are ingested and get deposited in the lungs and intestine due to the rhythmic respiratory movements of the late-term embryo (Jha *et al*., 2019). The whole chick's alimentary tract, especially the small intestine has the highest post-hatch relative growth throughout the first week of life (Dror *et al*., 1977; Katanbaf *et al*., 1988). Thus, an early feed deficiency can result in a lower growth rate of the intestine and reduced intestinal enterocyte length and villus surface area which adversely affects nutrient utilization and growth (Noy *et al*., 2001). Early nutrition is expected to induce the development of immune system through supplying nutrients for cell proliferation and differentiation or by providing substrates for immunomodulator activity leading to the production of several immunoglobulins (Jha *et al*., 2019).

Among the essential amino acids, Threonine (Thr) plays a vital role in the maintenance of integrity of the intestinal barrier and mucin synthesis (Law *et al*., 2007; Wang *et al*., 2010). Thr represents a higher concentration in the ileal endogenous protein losses by about 16% of the total amino acids, mostly in the mucins (Lien *et al*., 1997). Schaart *et al*. (2005) demonstrated that about 90% of dietary Thr is used by the intestine, and the majority of which is incorporated into mucosal proteins in piglets. Likewise, extra Thr supplementation can influence the animal's immunity through promoting the growth of the immune organs, enhance the synthesis of immunoglobulins and alleviate the immune stress induced by some viral and bacterial infection (Bhargava *et al*., 1971; Li *et al*., 1999; Wang *et al*., 2006; Corzo *et al*., 2007; Kadam *et al*., 2008; Azzam *et al*., 2012; Ren *et al*., 2014; Trevisi *et al*., 2015). In the present study, 3 g/kg was selected as the highest level for the extra L-Thr supplementation and hypothesized that extra Thr inclusion would exert a beneficial effect on broilers at the early age. Therefore, this study was performed to compare the effects of pre (*in ovo* injection) on hatchability and post hatch supplementation of L- Thr on the growth performance of broilers, antioxidant capacity, carcass traits, lymphoid organs relative weight, and intestinal histomorphology of broilers.

MATERIALS AND METHODS

Ethical approval

This feeding trial was carried out at the Poultry and Animal Research Center, Faculty of Veterinary Medicine, Cairo University, Egypt. The protocol was approved by the Animal Care Committee of the Faculty of Veterinary Medicine (Vet CU832022440).

Incubation and in ovo Supplementation

Two hundred and fifty-eight fertile eggs from Ross 308 breeders were obtained from a commercial farm. The eggs were randomly distributed in 3 sets (n = 86 per set). They were placed in egg incubator with standard temperature (37.7ºC) and humidity (60%) conditions and automatic turning at each hour. On the 11th day of incubation (DE11), non-fertile eggs were discarded after candling. Eggs with dead embryos were also separated and opened to determine the approximate age of the embryo. *In ovo* injection was performed on DE 18 according to Uni and Ferket (2003). All eggs were cleaned with 70% ethanol and punctured at the air chamber end. 0.5 mL nutritive solution (Thr enriched saline) was warmed to 30ºC and injected through the puncture in the amniotic fluid using 1-mL sterile syringes and 21G needles (Thr group). The second group (SC group) was injected with 0.5 ml sterile saline while the third group was not injected (NC group). The amino acid composition of the hen's egg, reported by Ohta and kidd (2001), was taken as a standard for the preparation of Thr solution. The Thr content of the eggs was calculated from egg weight. The required amount (30 mg) of crystalline L-threonine was dissolved in 0.5 ml of sterile saline. Inoculated eggs were placed in hatching trays and kept at 36.7ºC. After hatch, chicks were weighed individually, and incubation was assessed by chick weight at hatch, total hatchability, fertile hatchability, and embryo mortality.

Management

Hatchlings (n = 180) were weighed individually and distributed according to a completely randomized design with 6 treatments of 3 replicate with 10 bird per replicate and were fed on a basal diet supplemented with 0 (control group) and 3 g/kg L-Thr as presented in Table 1. The calculated value of Thr in the basal diet (0.87 %) is equivalent to 100% of the Ross 308 breed manual recommendation. Birds were housed on a deep litter system with a saw-dust floor and fed ad-libitum mash diet with free access to water in a temperature-controlled room with continuous lighting.

Table 1. Experimental design of groups.

Item	*In ovo* injection	Experimental diet
G1	Non-injected	Basal
G2	Saline (Sham control)	Basal
G3	Threonine	Basal
G4	Non-injected	Thr supplemented
G5	Saline	Thr supplemented
G6	Threonine	Thr supplemented

G1 (is non injected group fed basal diet), G2 (saline-injected and fed basal diet), G3 (threonine injected and fed basal diet), G4 (non-injected and fed threonine supplemented diet), G5 (saline injected and fed threonine supplemented diet) and G6 (threonine injected and fed threonine supplemented diet).

Diets

Two form of mash diets were formulated to each phase of broilers. The first diet was formulated with the minimum requirement of threonine according to the recommendation of Ross 308 breed manual (2019) recommendation about 0.87 % supplemented with 0 (control diet) while the second was supplemented with extra level of Thr (3 g/kg L-Thr) for 2nd treatment, the exra level of Thr was in accordance with Chen *et al*. (2017) as presented in Table 2.

Sampling and measurements

Growth performance

During rearing period, the chick's weights, feed intake and FCR were recorded weekly. At 35 d of age, birds were weighed individually after a 12-h feed withdrawal, and feed intake was recorded to calculate FCR. Three birds from each replicate were randomly selected for slaughter.

Table 2. Ingredients composition and chemical analysis of the control diet

Item	Diet		
	Starter	Grower	Finisher
Ingredients			
Yellow corn	55.1	58.35	62.68
Corn gluten meal (60% CP)	2.4	2.2	2.3
Soybean meal (46% CP)	37.54	34	28.8
Soybean oil	0.67	1.55	2.57
Dicalcium phosphate	1.94	1.7	1.5
Limestone	1.14	1.08	1
Common salt	0.35	0.35	0.35
DL-Methionine	0.14	0.12	0.1
L-Lysine	0.1	0.05	0.1
Threonine	0.02	--	--
Broiler premix[1]	0.3	0.3	0.3
Toxin binder	0.15	0.15	0.15
Sod bi carbonate	0.15	0.15	0.15
Total	100	100	100
Calculated analysis			
Metabolizable energy (Mcal/kg)	3009.01	3101.13	3212.24
Crude protein, %	23	21.5	19.5
Crude fat, %	2.54	2.6	2.8
Crude fiber, %	2.33	2.29	2.22
Calcium, %	0.96	0.87	0.79
Available phosphorus, %	0.48	0.43	0.39
Threonine, %	0.97	0.89	0.79

[1]per Kg premix: 12000000 IU vit. A, 2200000 IU vit. D3, 10000 mg vit. E, 1000mg vit. B1, 5000 mg vit. B2, 1500 mg vit. B6, 10 mg vit. B12, 3000 mg niacin, 50 mg biotin, 10000 mg pantothenic acid, 1000 mg folic acid, 50000mg Zn, 60000 mg Mn, 30000 mg Fe, 4000 mg Cu, 1000 mg I, 100 mg Co, 100 mg Se. (Multimix®; Multi Vita).

Antioxidant capacity

Five mL of whole blood sample were collected to separate serum and stored at −20ºC until analysis. Lipid peroxidation (MDA marker) was assayed according to Ohkawa *et al*. (1979) using lipid peroxide (MDA) colorimetric kit (Biodiagnostic, Egypt). The method is based on the reaction between Thiobarbituric acid (TBA) and malondialdehyde (MDA) in acidic medium at temperature of 95ºC for 30 min to form thiobarbituric acid reactive product. The absorbance of the resultant pink product can be measured at 534 nm. SOD was assayed according to Nishikimi *et al*. (1972) using SOD colorimetric kit (Biodiagnostic, Egypt). This assay relies on the ability of the enzyme to inhibit the phenazine methosulphate-mediated reduction of nitroblue tetrazolium dye. The absorbance was measured at 560 nm.

Carcass traits, and Immune organs

After slaughter and measuring the carcass weight, relative breast and thigh weights were recorded, also lymphoid immune organs weights (spleen, thymus, and bursa of fabricius) were recorded.

Intestinal histology and histomorphometry

Three cm segments from the jejunum were dissected out and kept in 10% neutral buffered formalin. After fixation, the tissue samples were processed, embedded in paraffin and stained by Hematoxylin and Eosin (H and E) (Bancroft and Gamble, 2008). The villus height (µm) was measured from the villus tip to the villus crypt junction, while crypt depth (µm) was measured as the distance between the basement membrane and the mouth of crypt (Shokryazdan *et al*., 2017) using Image J software (NIH, USA) and villi to crypt ratio (V:C) was calculated. Twenty measurements were recorded from each sample.

Statistical analysis

Statistical analysis was performed using Minitab software version 17 Minitab-17 Statistical Software (Minitab Inc., 2013). Tukey's multiple range test was used to determine the differences between individual treatment means when interactions were observed. Significant differences between mean values were declared at $P \leq 0.05$.

Data of intestinal histomorphology were expressed as means± SE, data were analyzed using one-way ANOVA procedure of SPSS 25 program, Post Hock test (Tukey test) used for multi-comparison between raw means. Significance was considered if $P \leq 0.05$. Graphs were made using Graph prism.

RESULTS

Hatchability

The effects of control, sham and *in ovo* threonine injection on hatchability of broiler chicks are presented in Table 3. Hatchability was decreased with *in ovo* injection of Thr compared to control group, whereas no such reduction was detected compared with sham.

Table 3. Fertile hatchability and hatching weight of fertilized eggs injected with Thr, Saline (sham SC) and (NC) non-injected group.

Item	Fertilized eggs		
	Threonine injected (Thr group)	Saline injected (Sham control SC)	Non injected control (NC)
Fertile hatchability %	91.86	88.46	93.07
Hatching weight g	47.67± 0.61[a]	48.44± 0.29[a]	48.22±1.54[a]
Mortality %	8.14	11.54	6.93

[a, b] Different superscripts within row indicate significant difference ($P< 0.05$)
Values are means ±SE (Standard error); Thr group (eggs injected with Threonine); SC (sham control, eggs injected with Saline); NC (non-injected eggs)

Growth Performance

The effect of egg injection and early nutrition with diet supplemented with or without threonine on growth performance are illustrated in Table 4. The group of chicks that was injected with threonine and fed on diet supplemented with threonine (G6) showed insignificant increase in final body weight compared to the other groups. However, it significantly exhibited the least amount of feed consumed during the production period. Considerable increase in feed intake was observed for the chicks fed on the commercial diet and hatched from non-injected eggs compared with those fed on threonine supplemented diet and hatched from non-injected eggs ($P < 0.05$). Furthermore, feed intake was higher for chicks hatched from sham control and fed commercial diet and diet supplemented with Thr than those hatched from non-injected eggs and fed Thr- supplemented diet. The group of chicks injected with threonine and fed diet supplemented with threonine recorded significantly improved FCR compared with the control group. Moreover, amino acid supplementation enhanced body weight in Thr-injected birds expressed by the highest final weight among experimental groups and gained more weight than control at the end of rearing period.

Serum Antioxidant Capacity

Results of serum antioxidant capacity are illustrated in Table 5. Group 3 showed significantly (P < 0.05) the maximum SOD activity compared to the control group (G1). On the other hand, supplementation of the diet with extra Thr (G4 and G5) didn't influence the SOD activity in the serum but showed the least concentration, however G6 (the group injected pre hatch with Thr and fed Thr supplemented diet) had slightly elevated SOD than G4 and G5. The serum MDA concentration was reduced by the inclusion of Thr post hatch of G5. Likewise, Thr supplementation reduced MDA content in the serum of (G5) chicks compared to that of G2. However, serum MDA was not altered by Thr supplementation in the other groups.

Carcass traits

Table 6 shows the results of carcass traits of broiler chicks. Results revealed that pre or post hatch supplementation of L-Thr had no effect on carcass traits (dressing %, breast yield or thigh yield). The carcass traits were not significantly affected.

Immune organs weight

Table 7 summarizes the effect of Thr *in ovo* injection and dietary supplementation on the immune organs relative weight. G6 exhibited a significant increase in relative weight of spleen compared to G3, whereas there was no significant difference in the relative weight of spleen between the other groups. Additionally, the thymus of chicks hatched from none injected eggs and fed extra threonine diet was significantly higher than those of un injected eggs and fed commercial diet.

Intestinal histomorphology

Figures 1 and 2 show the results of the jejunum villus height: crypt depth ratio. There was a significant difference between control group G1 and G3, G4 and G6 thus indicating that post hatch

Table 4. Growth performance, feed consumption, body weight gain and FCR in experimental groups.

Item	Experimental diets[1]					
	G1	G2	G3	G4	G5	G6
Initial body Weight (g)	46.66±0.17^{c}	48.1 ± 0.1 ^{ab}	48.0±0.3^{ab}	47.3±0.3^{bc}	48.59± 0.17^{a}	47.33±0.17^{bc}
Final body weight (g)	1802.6±37.2^{a}	1774.4±22.2^{a}	1767.5±34.6^{a}	1714.5±30.9^{a}	1713.8±25.8^{a}	1812.5±28.5^{a}
Total Weight gain (g)	1755.8 ±37.2^{a}	1726.4±22.2^{a}	1719.6±34.7^{a}	1667.1± 30.9^{a}	1665.2± 25.9^{a}	1765.2±28.5^{a}
Total Feed intake (g)	3354.5±46.9^{a}	3080.3±22.1^{b}	3046.8±9.46^{b}	2893.4± 3.42^{c}	3075.8± 64.3^{b}	2878.7±13.6^{c}
FCR	1.9 ± 0.04^{a}	1.78±0.02^{ab}	1.78±0.03^{ab}	1.74±0.03^{bc}	1.85 ±0.05^{ab}	1.63±0.02^{c}

$^{a, b}$ Different superscripts within row indicate significant difference (P < 0.05)
Values are means ±SE (Standard error); G1 (is non injected group fed basal diet), G2 (saline-injected and fed basal diet), G3 (threonine injected and fed basal diet), G4 (non-injected and fed threonine supplemented diet), G5 (saline injected and fed threonine supplemented diet) and G6 (threonine injected and fed threonine supplemented diet).

Table 5. Serum antioxidants (SOD and MDA) of broilers in experimental groups.

	G1	G2	G3	G4	G5	G6
SOD (U/ml)	12.99±0.91^{bc}	20.05±1.87^{b}	36.60±2.76^{a}	8.65±0.73^{c}	6.82±0.07^{c}	11.13±0.5^{bc}
MDA (nmol/ml)	1.19±0.05^{b}	9.92±0.2^{a}	1.91±0.16^{b}	4.13±0.17^{ab}	0.56±0.01^{b}	5.05±0.34^{ab}

$^{a, b}$ Different superscripts within row indicate significant difference (P < 0.05)
Values are means ±SE (Standard error); G1 (is non injected group fed basal diet), G2 (saline-injected and fed basal diet), G3 (threonine injected and fed basal diet), G4 (non-injected and fed threonine supplemented diet), G5 (saline injected and fed threonine supplemented diet) and G6 (threonine injected and fed threonine supplemented diet).

Table 6. Carcass traits of broilers in experimental groups.

	G1	G2	G3	G4	G5	G6
Dressing %	77.03±0.52^{a}	74.98±0.84^{a}	71.56±1.37^{b}	76.3±0.85^{a}	76.76±0.37^{a}	76.78±0.39^{a}
Breast wt (g)	350.7±19.5^{a}	338.3±30.9^{a}	312.3±30.7^{a}	356.7±23.4^{a}	337.0±13.7^{a}	370.7±10.6^{a}
Breast yield %	30.28±2.01^{a}	27.21±2.30^{a}	29.35±2.53^{a}	30.74± 1.30^{a}	25.65± 1.47^{a}	28.25±0.96^{a}
Thigh wt (g)	487.7 ±31.8^{a}	484.0 ±17.0^{a}	472.67± 6.57^{a}	455.0 ±11.5^{a}	503.0±17.0^{a}	473.0±45.4^{a}
Thigh yield %	42.48±4.18^{a}	38.75±1.88^{a}	44.34 ±0.94^{a}	39.31±0.56^{a}	38.21± 1.18^{a}	35.77± 1.43^{a}

$^{a, b}$ Different superscripts within row indicate significant difference (P < 0.05)
Values are means ±SE (Standard error); G1 (is non injected group fed basal diet), G2 (saline-injected and fed basal diet), G3 (threonine injected and fed basal diet), G4 (non-injected and fed threonine supplemented diet), G5 (saline injected and fed threonine supplemented diet) and G6 (threonine injected and fed threonine supplemented diet).

Table 7. Relative immune organ weight expressed to body weight of experimental birds.

Item	Experimental diets[1]					
	G1	G2	G3	G4	G5	G6
Relative thymus wt %	0.24±0.02^{b}	0.34±0.05^{ab}	0.21±0.03^{b}	0.43±0.03^{a}	0.26 ±0.03^{b}	0.24±0.03^{b}
Bursa relative wt%	0.15±0.01^{a}	0.19±0.03^{a}	0.18±0.02^{a}	0.19±0.02^{a}	0.1±0.01^{a}	0.16±0.02^{a}
Spleen relative wt%	0.16±0.01^{ab}	0.11±0.01^{b}	0.14±0.01^{ab}	0.15±0.01^{ab}	0.16 ±0.01^{ab}	0.19±0.01^{a}

$^{a, b}$ Different superscripts within row indicate significant difference (P < 0.05)
Values are means ±SE (Standard error); G1 (is non injected group fed basal diet), G2 (saline-injected and fed basal diet), G3 (threonine injected and fed basal diet), G4 (non-injected and fed threonine supplemented diet), G5 (saline injected and fed threonine supplemented diet) and G6 (threonine injected and fed threonine supplemented diet).

dietary extra Thr supplementation increased villus height to crypt depth ratio in jejunum. Additionally, groups of chicks prehatch inoculated with Thr displayed best ratio as shown in G3 and G6.

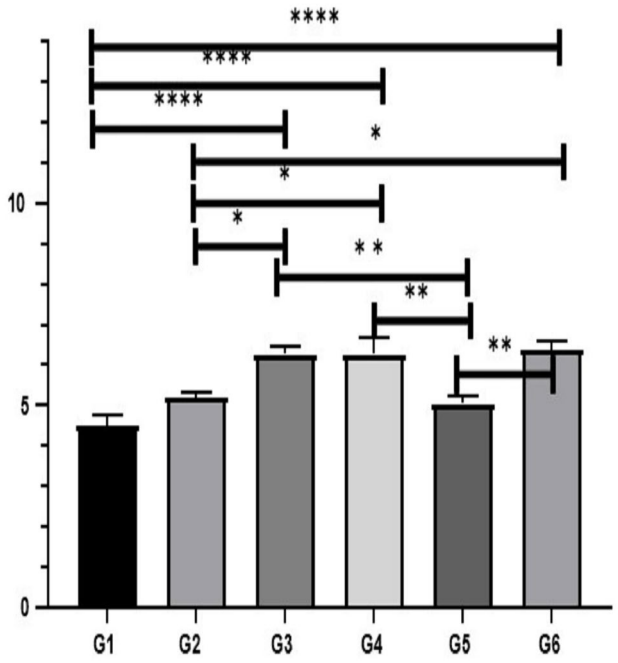

Fig 1. Villus height: crypt depth ratio in jejunum of broiler chicks fed diets supplemented with or without extra threonine and hatched from egg injected with threonine, saline or non-injected.
G1 (is non injected group fed basal diet), G2 (saline-injected and fed basal diet), G3 (threonine injected and fed basal diet), G4 (non-injected and fed threonine supplemented diet), G5 (saline injected and fed threonine supplemented diet) and G6 (threonine injected and fed threonine supplemented diet). (*) means $P \leq 0.05$; (**) means $P \leq 0.01$; (***) means $P \leq 0.001$; (****) means $P \leq 0.0001$

DISCUSSION

Thr is an important amino acid for early embryonic growth (Bhanja and Mandal, 2005), and *in ovo* injection into the amnion transports nutrients into the gut, stimulating hatchling nutritional status and improving hatchability (Uni and Ferket, 2004). In this trial, however, there was no evidence of Thr affecting hatchability. Similarly, Kadam *et al*. (2008) found that injecting graded doses of Thr into the yolk sac on day 14 of incubation had no effect on hatchability. On day 7 of incubation, the same was observed when a mixture of amino acids was injected into the yolk sac of birds (Ohta *et al*., 1999; Ohta and Kidd, 2001). On the other hand, there have been cases of Thr *in ovo* treatment reducing hatchability (Salmanzadeh *et al*., 2011). In general, *in ovo* injecting site and time (Ohta and Kidd, 2001), injection depth (Coskun *et al*., 2014), and injecting material type affect hatchability. As a result, a plausible reason for the discrepancies in the results in the instance of the combination of technical procedure and substance that used for *in ovo* injection.

Elevated final body weight may be a result of administration of the threonine throughout embryonic phase, encouraging higher protein synthesis, and reduced protein breakdown owing to amino acid injection (Bhanja and Mandal, 2005). Dietary amino acids can improve protein synthesis, growth and modulate enterocyte metabolism by supplying cell energy as amino acids have the ability of enhancing mitochondrial respiration, increased intracellular content of pyruvic acid and lactic acid (Xiao *et al*., 2020). That agreed with Tahmasebi and Toghyani (2016) who indicated that *in ovo* Thr. administration improved BW of chicks throughout the entire rearing period which most likely was mediated by its impact on GIT development. Similarly, Bhanja and Mandal (2005) found that injecting various amino acid combinations boosted the BW of chicks on days 7 and 21 of age. In addition, injecting 20 or 30 mg Thr into the yolk of 14-day embryos significantly improved the weight gain of chicks at 21 and 28 days of age (Kadam *et al*., 2008).

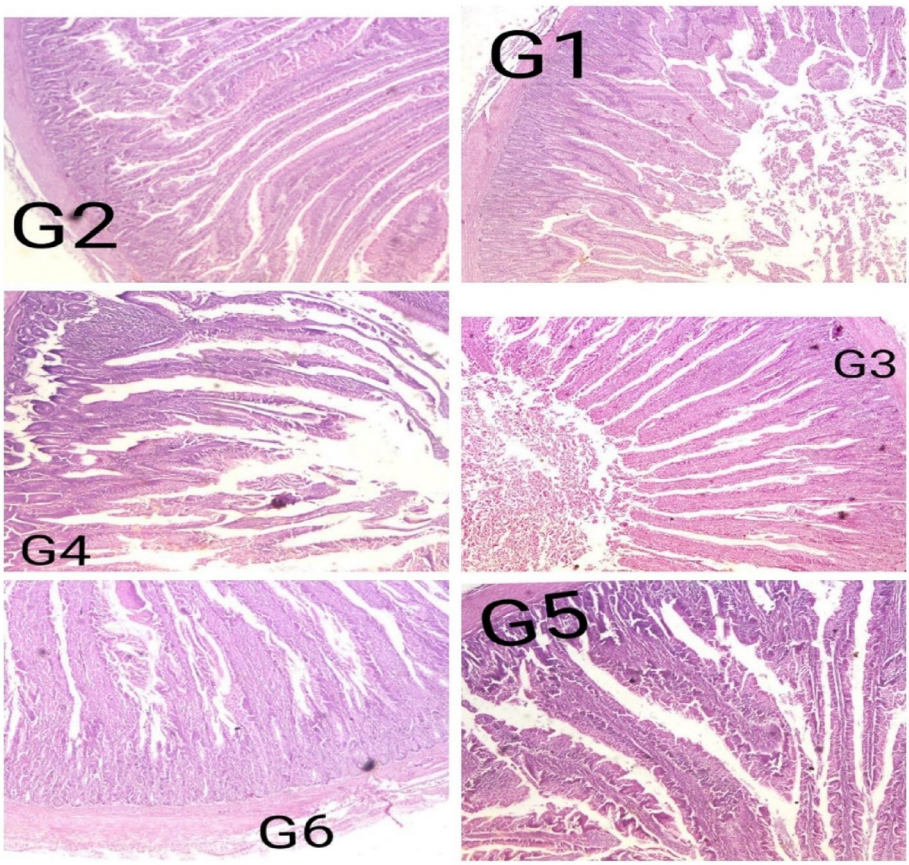

Fig. 2. Photomicrograph showing jejunum of experimental broilers.
G1 (is non injected group fed basal diet), G2 (saline-injected and fed basal diet), G3 (threonine injected and fed basal diet), G4 (non-injected and fed threonine supplemented diet), G5 (saline injected and fed threonine supplemented diet) and G6 (threonine injected and fed threonine supplemented diet).

Considerable increase in total feed intake was observed for the chicks fed commercial diet and hatched from non-injected eggs (G1) compared with those fed threonine supplemented diet and hatched from un injected eggs (G4) ($P < 0.05$). This high total feed intake may be explained as an attempt of the birds due to the poor digestive capacity of them compared with the high requirements of nutrients at early age, so trying to compensate it by increasing feed intake. De Verdal *et al*. (2010) noted that broilers exhibited higher feed intake in response to their low digestive ability. Total feed intake of chicks hatched from SC group and fed commercial diet was higher than those hatched from non-injected eggs and fed Thr supplemented diet. On the other hand, Kadam *et al*. (2008) found that the *in ovo* Thr-administered group had a higher feed intake throughout days 14–21 of age.

The group of chicks from *in ovo* injected eggs with threonine and fed diet supplemented with threonine recorded significantly the best FCR compared with the control group. Moreover, amino acid application enhanced body weight as Thr-injected birds had the highest final weight among experimental groups and gained more weight than control at the end of rearing period. In agreement with, Bhanja and Mandal (2005) noted better feed conversion ratio (FCR) in the chicks injected with amino acids compared with the untreated controls. Also, Kadam *et al*. (2008) found that threonine- *in ovo* injected chicks had insignificant better FCR than the untreated control group, also that group exhibited the highest amount of consumed feed that was attributed to the functional development of the gastrointestinal tract. Meanwhile, Thr is considered as an essential component of mucin and gastric enzymes.

Better FCR in our trial may be attributed to the extra level of dietary threonine. That was consistent with the findings of Chee *et al*. (2010a), who stated that dietary Thr range (from 8.0 to 10.5 g/kg) did not influence weigh gain, feed intake and feed conversion ratio of broilers in a 21-d study. Also, Chen *et al*. (2017) indicated that extra supplementation of L-Thr did not have an impact on the growth performance of broilers during the 21- d study and they attributed the similar growth performance among treatments to the threonine in the basal diet (8 g/kg) which was enough to cover its requirements of broilers according to recommendation of Ross 308 breed manual.

Moreira Filho *et al*. (2019) stated that better performance of birds complemented by *in ovo* injection was probably related to the metabolic changes during embryogenesis. In the last week of incubation, glycogen deposits are mobilized to assist the development and ensure hatching. For this reason, metabolism is transferred to the supply of energy. Beta oxidation of egg yolk lipids is reduced at the end of embryogenesis owing to reduced oxygen supply (De Oliveira *et al*., 2008), the main glucose source for the embryo at that period is probably gluconeogenesis from protein catabolism (Uni and Ferket, 2004). It is probable that Thr *in ovo* injection in the last week of incubation in the current study improved performance post-hatch by supporting amino acid supply for gluconeogenesis and reducing muscle protein catabolism.

Serum antioxidants considered the key parameters of evaluating oxidative status in the enzymatic system. Meanwhile, immune function was strongly associated to antioxidant function which could be a crucial index of immune function. ROS are produced during normal cell metabolism, however, quantities of ROS that surpass the antioxidant protection levels of cells can cause widespread damage to DNA, proteins, and endogenous lipids (Yu, 1994). In this study, dietary Thr did not improve the oxidative status of the broilers. According to Azzam *et al*. (2012), L-Thr supplementation had no effect on serum MDA, GSH, or GSH-Px, but had a substantial impact on plasma SOD activity. Chen *et al*. (2017) discovered that supplementing diets with more Thr (3 g/kg) lowered MDA levels in the serum in broilers.

The increased breast meat output of broilers fed diets containing extra level of Thr is in consistent with previous investigations of (Taghinejad-Roudbaneh *et al*., 2013) in broilers who reported a higher breast meat output (427 vs. 361 g) in broilers fed Thr supplemented diets compared to those fed a diet without Thr supplementation. Thr, like serine, is involved in muscle growth, which results in a better carcass characteristic.

The spleen is the organ responsible for antibody synthesis and immune cell proliferation (Tarantino *et al*., 2013; Pozo *et al*., 2009). The immune system's hub for T-cell proliferation and maturation is the thymus gland (Gordon and Manley, 2011). Under built-up litter conditions, Corzo *et al*. (2007) found that a greater amount of Thr was necessary for the formation and development of the thymus in broilers. Increasing the dietary standardised ileal digestible Thr also enhanced the piglets' relative thymus weight (Ren *et al*., 2014). In line with these findings (Corzo *et al*., 2007; Ren *et al*., 2014), dietary Thr supplementation increased the relative weight of the spleen and thymus in the present study, implying that a higher level of Thr than the Ross 308 breed manual requirements could benefit the growth and development of immune organs in broilers at a young age.

Thr is also important for intestinal mucosa, with the gut retaining around 60% of the Thr and enterocytes using about 80% of the retained Thr (Stoll *et al*., 1998). Thr is implicated in the production of mucin and immunoglobulins, it is important for maintaining barrier integrity (Min *et al*., 2016). Pre hatch (*in ovo*) injection of threonine and post hatch extra Thr supplementation improved the intestinal morphology of broilers in the current study, as demonstrated by higher ratio of villus height to crypt depth. An earlier GIT maturation may have contributed to the improved performance of birds supplemented *in ovo* with Thr in the current study, as supplemented birds had greater intestinal mucosa development at hatch compared to the control group, and the improvement increased linearly with increasing Thr levels in the diet. The nutrients injected into the amniotic fluid are ingested by the embryos before hatching, stimulating the growth of the GIT and boosting the capacity of the chick to digest and absorb nutrients after hatching (Uni and Ferket, 2004). Thr is known to play a role in formation and maintenance of intestinal mucosal integrity (Mao *et al*., 2011; Moreira Filho *et al*., 2015). The sooner the GIT's functional capacity is developed, the greater the bird's ability to correctly use feed nutrients, resulting in improved performance (Uni *et al*., 2006). This is especially important during the first week after hatching, when there are many physiological changes and high energy demands to support rapid GIT and immune system development, thermoregulation adjustment, and rapid body growth (Uni *et al*., 2003; Ferket, 2004). The effects of dietary Thr on intestinal mucin dynamics in broilers could reveal the possible impacts of Thr on intestine development in broilers (Horn *et al*., 2009). Our findings support those Chee *et al*. (2010a, b) who found that supplementation with additional Thr increased the ratio of villus height to crypt depth in the jejunum.

Additionally, Zaefarian *et al*. (2008) and Moreira Filho *et al*. (2015), found that Thr supplementation improved villus height, epithelial thickness, goblet cell quantity, and crypt depth in the three segments of the small intestine. Graded dietary standardised ileal digestible Thr has also been shown to promote intestinal morphology in weaned piglets (Ren *et al*., 2014). In addition, Chen *et al*. (2017) reported that adding Thr to the diet of broilers, especially at 3 g/kg, enhanced villus height in the ileum and the ratio of villus height to crypt depth in the jejunum and ileum. The enhancement of intestinal morphology due to increasing dietary Thr, supposed to be due to the fast growth of broilers fed on Thr-supplemented diets which resulted in higher ratio of villus height to crypt depth and increased villus surface area, enterocyte proliferation, intestinal mucin secretion, and better nutrient absorption.

CONCLUSION

In ovo injection and post hatch dietary supplementation of L-Thr has a positive effect on the growth performance of broiler chickens and enhance the immunity of broiler chickens mediated by the development of immune organs and improve the intestinal morphology of broilers as demonstrated by higher ratio of

villus height to crypt depth. Contrary, the *in ovo* injection or dietary supplementation of threonine had no effect on the serum antioxidant capacities.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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