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MODULATION OF THE INTESTINAL HEALTH BY INCREASING DIETARY THREONINE TO LYSINE RATIO DURING A *SALMONELLA* CHALLENGE IN BROILER CHICKENS

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ABSTRACT

The aim of this study was to assess threonine/lysine ideal ratio for optimum intestinal health of broiler chickens infected with *Salmonella*. Additional Thr (equal or 25% more than breed's threonine/lysine ratio requirement) added to a control threonine deficient and high crude fiber diet, fed to 144 one-d-old male Ross 308 broiler chicks (four replications per treatment). NIR analyze of ingredients amino acids used for starter, grower and experimental finisher rations formulation base on breed's nutritional catalog for 41 days after first 24 h similar prestarter diet. On d-32 of age all chickens of four replications of each treatment were infected orally with equal numbers of *Salmonell aparatyphi* A (5×10^4cfu/bird), individually. To confirm success of challenge, Cloacae swab samples on d-39 and spleen samples on d-42 cultured on XLD agar for isolation of *Salmonella paratyphi* A. Moreover, on d-42 blood samples obtained for widal quantitative tube test to determining *S.* paratyphi A antibody titer. Ileum samples of one chicken per replicate on d-42 obtained for morphological analysis. *Salmonella* cultures of cloacae and spleen samples and widal titer showed success of challenge. A non-significant trend was observed between increased Thr/Lys ratio and increased widal titer. Increase in Thr/Lys ratio did not affect villus width, crypt depth and villus height/villus width ratio but increased Villus height and Villus height/crypt depth ratio. This finding indicated on healthier mucosa in response to increase in Thr/Lys ratio in *S.* paratyphi A infected chicken.

Keywords: Broiler, *salmonella*, Intestinal Morphology, Threonine

INTRODUCTION

Threonine is the third most limiting amino acid in most plant-based broiler diets behind the total sulphur-containing amino acids and lysine **[1]**. Among the essential amino acids, threonine is particularly important for maintenance of gut barrier integrity and has an important role in the structure and function of gastrointestinal tract **[2, 3, 4]**.

Kidd deduced that NRC overestimated the threonine requirement of broilers (Thr/Lys ratio admittedly) for optimum performance **[1, 5]**. Likewise, NRC reported that findings about broilers Thr requirement was insufficient **[5]**. Lysine and protein requirement of modern broiler strains is more than that reported by NRC as a result of their fast growth rates.

Threonine integrates with intestinal health, which is an indispensable, and sometimes limiting, amino acid for poultry **[6]**. Threonine is critical for intestinal growth, development and maintenance due to its importance in the structure of mucin that is the main component of the mucous layer of gut and responsible for its viscous and elastic gel-like properties **[7, 8]**. Mucins are major glycoproteins protecting the epithelium from chemical, enzymatic, physical, and bacterial aggressors that may be present in the gut lumen **[9, 10, 11]**. The fixation of commensal bacteria on the mucus layer prevents colonization by opportunistic pathogens. Fixation restricts free access to the underlying mucosa, causing mucus to act as an impermeable barrier or retention zone. Conversely, the ability of pathogenic bacteria to interact with mucin can be an important step in facilitating colonization of the GIT. If the bacteria are able to bind strongly to the mucus layer, their clearance through motility and abrasive forces of digestion may be delayed and colonization of the GIT may be favored. In addition, the rate of bacterial growth and penetration in the mucus can exceed the natural turnover rate of this layer and, therefore, favor bacterial colonization further **[8]**.

Threonine constitutes up to 11% of the amino acid structure encoded by the mucin 2 (MUC2) gene **[12]**. A threonine deficiency will affect mucin secretion and, gut barrier integrity **[13]**. Therefore, it has been suggested that mucin dynamics may be sensitive to Thr availability especially in digestive infections **[3, 14, 15, 16]**.

Salmonella infects poultry and humans by the oral route through contaminated food or water. *Salmonellae* especially Paratyphoid serovars that are motile make colony on gut epithelium then after damaging mucous layer, transmit across from gut mucosal and immune barrier to blood flow and can cause septicemia or tissues infection and damage

[17]. European Centre for Disease Prevention and Control reported *Salmonella* paratyphi A was the most commonly identified serotype in human cases of paratyphoid fever in EU/EEA countries **[18]**. Human contamination is related to the consumption of poultry products especially broiler chicken products that can induce *Salmonella* to the human food chain **[17]**. *Salmonella* intestinal colonization plays a significant role in carcass contamination during slaughter and processing **[19]**. Indeed, intestine removal has been associated with increasing bacterial counts on processed carcasses **[20, 21]**. Therefore, it is important to target *Salmonella* reduction strategies during the incubation in intestine.

Based on evidence that *Salmonella* influences morphological aspects of the intestine, and noting the importance of threonine in mucin synthesis, we hypothesized that addition of supplemental dietary threonine would benefit gut barrier integrity during an acute Salmonellosis infection. Therefore, the objective of this study was to evaluate the effect of supplemental dietary threonine to modulate intestinal morphology of broiler chickens infected with *Salmonella*.

MATERIALS AND METHODS

Birds and Diets

One-hundred forty-four one day old *salmonella* negative male Ross 308 broiler chicks were used in this experiment. Chicks were raised in wire floor pens (10 chicks/1 m^2 pen) in an environmentally controlled room with continuous lighting. Chicks had free access to feed and water during the experimental period. After 24 h eating similar prestarter pellet, chicks were fed experimental starter (2-10 d), grower (11-24 d) and finisher (25-42 d) rations based on breed's nutritional catalog **[22]**. At beginning of day 2, chicks were weighed and randomized to treatment groups so initial body weights were similar among treatment groups. Four replicate pens of 12 chicks were randomly assigned to each of the 3 treatment combinations of three experimental diets for the 10-dexperiment that was started from day 32 with *Salmonella* challenge.

NIR analyze used to determine amino acids profile of all ingredients by Paya Amin Mehr Co. Ltd., Evonik Animal Nutrition Service in Tehran, Iran. Treatment 1 had no threonine supplement, treatment 2 had threonine supplement to meet Thr/Lys ratio requirement as pointed in Ross 308 broiler nutrition specification and treatment 3 had more threonine supplement to meet Thr/Lys ratio 25% more. Increase in diet's crude fiber can affect intestinal mucosa and consequently digestive tract health that may

lead to gut susceptibility to infections **[8, 23]**. Therefore, with the aim of increasing diet's crude fiber and decreasing threonine content of treatment 1 as far as possible, a basal diet formulated in total amino acids system on base of corn, wheat, barley, wheat bran, rice bran and soybean meal. Basal diets ingredients and nutrient analysis and L-Threonine supplement quantity of experimental diets showed in **Table 1**. The difference between threonine supplement content of treatment 1&2 with treatment 3, correct with adding grind fine sand as filler to treatment 1&2.

Experimental Design and *Salmonella* Challenge

Because of epidemiological importance of *S.paratyphi* Aserovar around the world; *Salmonella enterica* subsp. *Enterica* serovar paratyphi A (ATCC® 9150™) was used for infection induction. This bacterium was obtained from microorganism's bank of Iranian Biological Resource Centre (IBRC), ACECR, Tehran, Iran (IBRC No.: IBRC-M 10668). The challenge organism for this experiment was grown in tryptic soy broth at 37°C then diluted to 5×10^4cfu/ml **[24]**.

All chickens of four replications of each treatment individually infected by oral gavage using an animal feeding needle with equal numbers of *Salmonella* paratyphi A (5×10^4cfu/ml per bird) at the age of 32-d of old in a completely randomized design.

***Salmonella* Recovery**

Three cloacae swab samples from each pen of challenged chickens were cultured on d-39 to confirm success of *salmonella* present in challenged chicken. Also, one chicken per replicate was sacrificed, and submitted to necropsy on d-42. Serosal coverages of spleens were cut by a sterile scalpel. Samples from core of spleens were cultured for isolation of *Salmonella* Paratyphi A.

A resistance again tylosin observed in an antibiogram test that initially done on this *Salmonella* serovar. Cloacae and spleens samples were streaked for isolation onto xylose lysine deoxycholate (XLD) agar plates containing tylosin (20 µg/mL) and incubated for 24 and 48 h at 37°C. Plates were evaluated for the presence or absence of *salmonella*, which grew as red colonies on this selective medium **[25, 26, 27]**.

At the age of 42-d of old blood samples obtained from wing vein of two chicken per replicate after washing skin with distilled water and then drying. Serums were examined by widal quantitative tube test originally designed for the diagnosis of enteric fever for *Salmonella paratyphi* A antibody titer **[28]**. Widal test is an agglutination test for detection of antibodies against *Salmonella typhi* and *Salmonella* paratyphi, the common causal agents of enteric fevers. When serum sample containing antibodies against *S.* paratyphi

A, antibodies mix with respective antigens and agglutination will take place. Serum samples diluted from 1:10 to 1:640 in 7 tube by normal saline with serial dilution method. No serum was added in another tube that was control tube. One drop of specific tube test stained antigen of *S. paratyphi* A was added to all tubes (Remel stained *Salmonella* O and H suspensions, Remel Europe ltd, UK), mixed and rotated for one minute. Antigen was dyed for easy identification of agglutination. Tube incubated at 50°C for 2 hours. Then, the highest dilution of serum that produces the clearly visible positive agglutination in room temperature was taken as titre (reverse of the dilution) **[29, 30]**.

Intestinal Morphology

At the age of 42-d of old five centimeter from the middle of ileum of one chicken per replicate was cut. The sections were placed in buffered formalin (10%) for further processing after 72 h fixation. Chicken were fasted for 12 h to promote emptying of the digestive tract. Then, the sections were stabilized in paraffin and sliced by a microtome with 7 µm thickness. Slices (at least 6 slices per section fixed on a microscope slide) were stained by hematoxylin and eosin stain and then mounted by entellan rapid mounting medium. Sections analyzed by an image analyzing microscope with LEICA QWin 0760 software (Leica Microsystems Imaging Solutions Ltd., Clifton Road. Cambridge CB1 3QH.United Kingdom) to determine villus height, crypt depth, villus width, villus height to crypt depth ratio and villus height to villus width ratio.

Statistical Analysis

The statistical normality of all data were tested in MINITAB® software (confidence level=95%) **[31]**. Then treatments analyzed by ANOVA procedure using the GLM procedure of SAS® software **[32]**. When significant differences among means were found, means were separated using Duncan's Multiple Comparison test (α=5%) for post hoc multiple comparisons.

RESULTS AND DISCUSSION

As showed in **Table 2**, *Salmonella* culture of treatment's cloacae samples showed that only (one chicken equal to 8.333% of) Thr/Lys ratio deficient diet was not completely infected *Salmonella paratyphi* A. *Salmonella* culture of spleen samples showed 25% of spleens in excess Thr/Lys ratio diet were *Salmonella paratyphi* A free. Widal titer of *Salmonella paratyphi* A indicated on *Salmonella* infection in all chickens of all treatments. Increase in Thr/Lys ratio didn't affect percentage of cloacae and spleen *Salmonella* positive samples. A non-significant trend was observed between increased Thr/Lys ratio and increased widal titer. *Salmonella*

paratyphi A culture of cloacae and spleen samples and widal titer results indicated on success of challenge. Relatively low widal titer of Thr/Lys ratio deficient diet may indicate on weak humoral immune responses to presence of *Salmonella paratyphi* A antigen in blood **[33]**.

As showed in Figure 1 and Table 2, increase in Thr/Lys ratio didn't affect villus width, crypt depth and villus height/villus width ratio in *Salmonella* infected chicken. However, villus height/villus width ratio showed a non-significant trend to increase with increase in Thr/Lys ratio. Villus height increased in adequate and excess Thr/Lys ratio diets compare with Thr deficient diet ($P<0.001$). Villus height/crypt depth ratio increased in excess Thr/Lys ratio diet compare with other treatments ($P<0.05$). Increase in Villus height and Villus height/crypt depth ratio indicate on beneficial effect of increasing Thr/Lys ratio in diets of *Salmonella* Paratyphi A infected chickens. In contrast, in a 6 day coccidiosis morphological parameters of infected chicken in response to increase in Thr/Lys ratio didn't affected and relatively were in inverse of present findings **[16]**.This maybe because of different mechanism of creating lesions on mucosa of infections agents or different examined Thr level.

Widal titer in response to increase in Thr/Lys ratio increased non-significantly, nevertheless the morphological results altogether exhibited healthier mucosa in response to increase in Thr/Lys ratio. Healthier mucosa may indicate on less passage of *Salmonella* through mucus layer and less presence of antigen in blood, and then fewer antibodies may be create. But non-significant increase in widal titer in response to increase in Thr/Lys ratio may indicate on better humoral immune responses.

CONCLUSION

Present study showed that a higher Thr/Lys ratio will not totally prevent the severity of *Salmonella* lesions but will improve gut health during infection. Therefore, Thr/Lys ratio requirement in intestinal infections may be more than needs for optimum performance.

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Table 1: Composition of Experimental Diets

Items	Starter 2-10 d	Grower 11-24 d	Finisher 25-42 d
Ingredients (g/kg)			
Corn	**432.91**	**472.21**	**503.00**
Wheat	**100.00**	**105.00**	**105.00**
Barley	**50.00**	**60.00**	**60.00**
Wheat bran	**25.00**	**30.00**	**30.00**
Rice bran	**25.00**	**30.00**	**30.00**
Soybean meal	**311.20**	**247.00**	**209.67**
Soybean oil	**6.02**	**11.45**	**20.60**
Choline chloride 60%	**1.45**	**1.40**	**1.30**
L-Threonine suppl. T-1[1]	**-**	**-**	**-**
L-Threonine suppl. T-2[1]	**0.59**	**0.48**	**0.36**
L-Threonine suppl. T-3[1]	**2.81**	**2.44**	**2.13**
L-Lysine monohydrochloride	**2.45**	**2.20**	**1.93**
DL- Methionine	**2.97**	**2.40**	**2.10**
Limestone	**11.84**	**9.60**	**9.57**
Dicalcium phosphate	**18.15**	**15.90**	**14.90**
Sodium bicarbonate	**4.10**	**3.90**	**3.70**
Salt	**0.60**	**1.00**	**1.10**
Vitamin premix[2]	**2.50**	**2.50**	**2.50**
Mineral premix[2]	**2.50**	**2.50**	**2.50**
Maduramycin 1%	**0.50**	**0.50**	**-**
NutrientsBy analysis			
AME_n (kcal/kg)	**2720**	**2820**	**2920**
CP (%)[3]	**21.07**[3]	**18.78**	**17.34**
Thr T. 1 (%)	**0.790**	**0.697**	**0.641**
Thr T. 2 (%)	**0.846**	**0.743**	**0.675**
Thr T. 3 (%)	**1.057**	**0.929**	**0.843**
Lys (%)	**1.287**	**1.110**	**0.994**
Thr /Lys T.1 (%)	**61.38**	**62.82**	**64.47**
Thr /Lys T.2 (%)	**65.73**	**66.93**	**67.91**
Thr /Lys T.3 (%)	**82.16**	**83.68**	**84.83**
Met + Cys (%)	**0.965**	**0.852**	**0.785**
Val (%)	**0.990**	**0.882**	**0.815**
Ile (%)	**0.871**	**0.761**	**0.693**
Arg (%)	**1.379**	**1.201**	**1.090**
Trp (%)	**0.258**	**0.225**	**0.204**
Crude fiber (%)	**4.108**	**3.89**	**3.70**
Ca (%)	**0.96**	**0.80**	**0.77**
A.P. (%)	**0.45**	**0.40**	**0.38**
Na (%)[4]	**0.15**	**0.17**	**0.16**
Cl (%)[4]	**0.15**	**0.17**	**0.17**
K (%)[4]	**0.87**	**0.78**	**0.71**
DCAD (meq/kg)[4]	**249**	**224**	**207**

[1] L-Threonine supplement of treatment 1&2&3. Feed grade and 98.5% purity; [2] Breed's special supplement made as Ross nutrition catalog suggested (Aviagen, 2009), contain: 4400000 IU/kg of Vit. A, 2000000 IU /kg of Vit.D_3, 30000 IU /kg of Vit.E, 1200 mg/kg of Vit. K (Menadione), 1200 mg/kg of B_1, 3200 mg/kg of B_2, 24000 mg/kg of Nicotinic Acid, 6000 mg/kg of Pantothenic Acid, 1600 mg/kg of B_6, 60 mg/kg of Biotin, 800 mg/kg of Folic Acid, 6 mg/kg of B_{12}; 6400 mg/kg of Copper, 500 mg/kg of Iodine, 16000 mg/kg of Iron, 48000 mg/kg of Manganese, 120 mg/kg of Selenium, 40000 mg/kg of Zinc; [3] All limiting essential Amino acids were supplied in basal diet by increase in ration crude protein content; [4] by calculation

Table 2: Response of *Salmonella* Paratyphi A infected chicken to different Thr/Lys ratio (d-32 to d-42)

Traits	Thr/Lys ratio	*Salmonella* isolated cloacae (%)	*Salmonella* isolated spleens (%)	*Salmonella* widal titer	villus height (µm)	villus width (µm)	crypt depth (µm)	villus height/crypt depth ratio	villus height/villus width ratio
Treatment 1	**deficient**	**91.667**	**100.000**	**46.67**	**441.41[b]**	**121.05**	**134.76**	**3.510[b]**	**4.025**
Treatment 2	**adequate**	**100.000**	**100.000**	**73.33**	**614.48[a]**	**154.79**	**179.80**	**3.537[b]**	**4.068**
Treatment 3	**excess**	**100.000**	**75.000**	**106.67**	**620.22[a]**	**133.52**	**139.45**	**4.737[a]**	**4.694**
SEM[1]		**4.811**	**14.433**	**17.84**	**28.424**	**12.17**	**12.68**	**0.217**	**0.359**
***P*-value[2]**		**0.405**	**0.405**	**0.153**	**0.0005**	**0.162**	**0.058**	**0.044**	**0.668**
CV[3]		**9.89**	**31.49**	**66.79**	**21.30**	**33.16**	**32.99**	**23.74**	**32.60**

[1] Standard error of means; [2] Significance level of calculated F in analysis of variance; [3] Coefficient of variation (%)
[ab] Means without a common superscript letter statistically differ within each part of a column

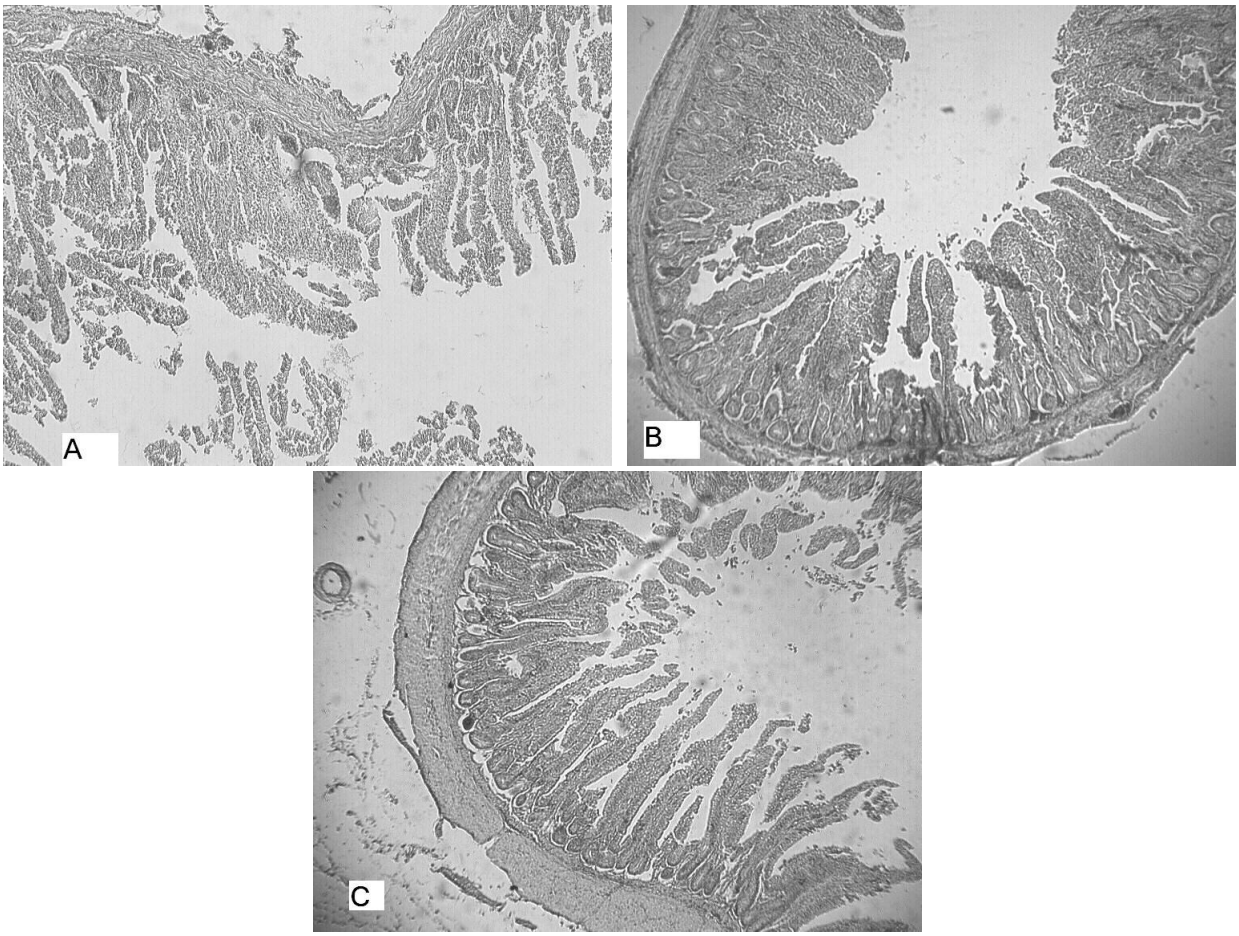

Figure 1: Representative Histological Images of Ileal mucosa from 42-d-old Broiler Chicks Fed Diets Containing Deficient (A), Adequate (B) and Excess (C) Thr/Lys Ratio