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Effects of Glutamine on Growth Performance, Intestinal Morphology and Intestinal Barrier Function of Broilers

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ABSTRACT

The effect of glutamine (Gln) on growth performance, intestinal morphology and intestinal barrier function were evaluated in broilers. A total of 320 birds were divided into a control group (CON) and three experimental groups (Gln 1, Gln 2 and Gln 3). Broilers of group CON received basal diet; broilers in group Gln 1, Gln 2 and Gln 3 were supplemented with 0.5%, 1.0% and 1.5 % Gln, respectively, for 42 days. The results indicated that Gln has no influence on the average daily gain (ADG) among the treatments in the periods of 1 to 21 d, 22 to 42 d and 1 to 42 d ($P > 0.05$). However, Gln improved average daily feed intake (ADFI) and feed intake: average daily gain (F: G), increased the villus height, villus height to crypt depth ratio (V/C) and the activities of sucrose, the ZO1, claudin-1 and occluding mRNA expression levels ($P<0.05$). Moreover, Gln decreased the crypt depth of jejunum and ileum in broilers at days 21 and 42 ($P<0.05$). In conclusion, Gln had a positive effect on growth performance and gut parameters by modifying the function of the intestinal mucosa barrier.

Key words: Broiler, Disaccharides activity, Glutamine, Intestinal morphology, Intestinal barrier function.

INTRODUCTION

Glutamine (Gln) is a nutritionally essential amino acid for animals and humans (Wang *et al.*, 2015), which is mostly used in the small intestine. Accumulating evidence showed that Gln could modulate the growth, development and health of the gut (Deniel *et al.*, 2007), maintain the gut morphology and barrier function by regulating the disaccharides activity (Soltan, 2009), metabolism of intestinal bacteria (Dai *et al.*, 2013), expression of tight junction protein, claudin-1 and occluding *in vitro* and *in vivo* models (DeMarco *et al.*, 2003). In addition, inclusion of Gln in the diet can enhance whole-body and intestinal growth (Haynes *et al.*, 2009), improve small intestinal morphology and structure, prevents intestinal atrophy, decrease the bacterial adherence to the intestinal mucosa and bacterial in piglets and broilers under normal and abnormal conditions (Luquetti *et al.*, 2016). All these results clearly indicate that Gln is a beneficial nutritional additive for the intestinal growth and development, the integrity of the epithelial barrier (DeMarco *et al.*, 2003). The main objective of the present study is to evaluate of the effect of Gln on growth performance, intestinal development, barrier function and health in broiler.

MATERIALS AND METHODS

320 one-day-old Arbor Acres (AA) broilers were assigned to four groups (each group was replicated eight times with 10 birds per replicate): a control group (CON) and three experimental groups (Gln 1, Gln 2 and Gln 3). Broilers of group CON received basal diet; broilers in group Gln 1, Gln 2 and Gln 3 were treated with 0.5%, 1.0% and 1.5 % Gln, respectively, for 42 days. The basal diets (corn and soybean meal based) formulated according to the NRC (1998) recommendations, has been shown in Table 1. Diets and fresh water were offered *ad libitum* throughout the experimental period. All procedures in the present experiments were approved by the Institutional Animal Care and Use Committee of Henan University of Science and Technology.

At 21 and 42 days of age, eight chicks per cage were randomly selected after fasting for 12 h and were humanly euthanized. The jejunum and ileum samples were collected and immediately fixed with formalin solution for gut morphological analysis. Then, each residual intestine segment was opened longitudinally and the mucosa samples were gently scraped with a glass microscope slide and separated into two subsamples. One mucosa subsample were stored quickly at -80°C to assess RNA quality. The other collected mucosa scraping was subsampled, then placed on ice until they were centrifuged for 10 min at 1,500× *g* and the suspension was collected and stored immediately at -80°C for disaccharides activity assays.

Individual body weight was measured at 1, 21 and 42 d of age and then average daily gains (ADG) were

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calculated. The record of feeds offered and feed residues were also taken to determine the average daily feed intake (ADFI) and feed/gain ratio (F/G).

Three cross-sections for each intestinal segment (jejunum and ileum) were processed for paraffin embedding; 5-µm thicknesses were cut and stained with hematoxylin-eosin. A total of 15 intact, well-oriented crypt-villus units were selected in triplicate for each intestinal cross-section. Villus height (VH, µm) and crypt depth (CD, µm) were measured using an image processing and analysis system (version 6.0, Image-Pro Plus, Olympus Optical Co. Ltd., Tokyo, Japan). Villus height: crypt depth (V/C) was also calculated for each intestinal segment.

The activities of glucose, maltase and sucrase in the intestinal mucosa supernatant were determined using corresponding diagnostic kits (Nanjing Jiancheng Bioengineering Institute, China), according to the instructions of the manufacturer. Mucosal protein content in the supernatant was also determined using corresponding diagnostic kit (Nanjing Jiancheng Bioengineering Institute, China).

Total RNA of the intestinal mucosa samples (2 µg) was isolated using Trizol Reagent (TaKaRa Biotechnology. Dalian, Liaoning, PR China) and ground according to the instructions of the manufacturer. The RNA pellets were dried, resuspended. The RNA integrity was electrophoresed by 1% agarose-formaldehyde gel. The amplified RNA quantity was determined by a spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Subsequently, 1 µg of extracted total RNA was immediately reverse transcribed into cDNA with the Prime Script RT Reagent Kit (TaKaRa Biotechnology, Dalian, Liaoning and PR China). Then, labeled cRNAs were disrupted, incubated, diluted and assembled RNA microarray. The PCR was performed in duplicate on each sample. The speciûc primers for ZO-1, occluding and Claudin-1 are summarized in Table 2. Relative mRNA expression levels were indicated with 2 ($2^{-\Delta\Delta ct}$) and normalized to β-actin.

Statistical analyses of data were performed using SPSS version 21.0 (SPSS Inc., Chicago, IL, USA, 2012). Differences among group means were checked for significance using the Duncan multiple range tests at 5% significance. All results were expressed as the mean ± standard deviation (SD).

Table 1: Ingredients and nutrient level of the experimental diet (%).

Item (%)	1-21 d	22-42 d
Corn	28.45	29.50
Soybean meal	33.50	31.5
Wheat	25.70	24.56
Soybean oil	6.70	9.43
Limestone	2.00	1.55
$CaHPO_4$	1.20	1.13
NaCl	0.2	0.2
Premic[a]	2.25	2.13
Nutrient level[b]		
Apparent metabolism energy (MJ/kg)	30.5	30.2
Crude protein (%)	22.85	21.50
Calcium (%)	1.05	0.90
Available phosphorus (%)	0.72	0.68
Lysine	1.45	1.36
Methionine	0.68	0.63
Methionine + cysteine	1.03	0.98

Note:[1]Each kg of premix contained: Fe (from ferrous sulfate), 80 mg; Cu (from copper sulfate), 8 mg; Mn (from manganese sulfate), 100 mg; Zn (Bacitracin Zn), 65 mg; iodine (from calcium iodate), 0.35 mg; Se (from sodium selenite), 0.15 mg. Vitamin A (transretinyl acetate), 12,500 IU; Vitamin D_3 (cholecalciferol), 2,500 IU; Vitamin E,18.5 mg; Vitamin K_3,2.65 mg; thiamine 2.2 mg; riboflavin, 8 mg; nicotinamide, 40 mg; pyridoxine·HCl, 4 mg; biotin, 0.04 mg; folic acid, 1 mg; vitamin B_{12} (cobalamine), 0.013 mg.

RESULTS AND DISCUSSION

Growth performance

No significant differences (P>0.05) occurred in ADG among the treatments in the periods of 1 to 21 d, 22 to 42 d and 1 to 42 d (Table 3). The ADFI and F: G in the Gln group were lower than those of the control group during days 1 to 21, days 22 to 42 and days 1 to 42 (P<0.05).

Intestinal Morphology

The villus height in jejunum and ileum was increased significantly (P<0.05) in Gln treatments compared to the control (CON) group in chickens at 21 and 42 d age (Table 4). The crypt depth of jejunum and ileum in broiler chickens at 21 and 42 d were decreased in Gln supplemented chickens as compared to the CON group (P < 0.05). Gln supplementation was found to be effective for increasing the villus height to crypt depth ratio (V/C) of jejunum and ileum in broiler chickens at 21 and 42 d age (P<0.05).

The mucosa disaccharides activity

The activities of glucose, maltase and sucrase in the jejunum and ileum mucosa were increased (P<0.05) in Gln treatments compared to the control group in chickens at 21 d (Table 5). At 42 d of age, the glucose and sucrase enzyme activity of the jejunum and ileum mucosa were increased in Gln supplemented chickens (P<0.05). No significant

Table 2: The Primer sequences for target and β-actin genes.

Gene Name	*Primer Sequence*	Fragment Size
occludin	5'-CCGTAAGCCCTAGTTGGAT-3'	214
	5'-ATTGAGCCGGGCGTTGATG-3'	
Claudin-1	5'-CCTGATCACCCTCTTGGGAG-3'	145
	5'-GCTGCACTCACTCATTGGCT-3'	
ZO-1	5'-TGTAACCACAGCATGAGGTG-3'	98
	5'-CTGGGATGGCTCCATGTGGT-3'	
β-actin	5'-TTGGTTCGTCAAGCAAGTGG-3'	100
	5'-CCCCCATATACTGGCACCTT-3'	

[1]ZO-1, zonula occludens-1.

difference found in the maltase enzyme activity of jejunum mucosa as well as in ileum mucosa glucose and maltase enzyme activity among Gln treatments and CON group at 42 d (P>0.05).

Tight Junction Proteins (TJP) mRNA Expression Levels in Duodenum, Jejunum and Ileum

Broilers in the Gln treatmen groups had higher ZO1, claudin-1 and occluding mRNA expression levels in the jejunum and ileum as compared to the CON group (P<0.05) on days 21 (Table 6). However, dietary Gln supplementation had no effect on the ZO1, claudin-1 and occluding mRNA expression levels in the jejunum and ileum as compared to the CON group on day 42 (P>0.05).

Glutamine can promote weight gain and feed efficiency, increase the activity of digestive enzyme and improve the intestinal morphology in chickens (Soltan, 2009). In the current study, we observed that glutamine supplementation decreased the ADFI and F:G, but there was no significant difference on ADG among the groups. Moreover in some cases ADG was higher in CON group than some treatment groups. So in this study there was no significant effect of Gln supplementation on growth rate, which may be attributed to the beneficial effect of Gln on intestine function or morphology (Yi *et al.*, 2005). However, other studies have also showed that glutamine did not affect growth performance (Luquetti *et al.*, 2016), or can effective decreased BWG only in 21 days broilers (Yi *et al.*, 2005).

Table 3: Effect of dietary Gln on the growth performance in broilers.

Items	Diet Treatments[1]			
	CON	Gln1	Gln2	Gln3
1 ~ 21 d				
ADG/g	20.85±0.20	20.97±0.31	21.12±0.32	21.05±0.27
ADFI/g	35.10±0.28[b]	33.65±0.34[a]	33.49±0.32[a]	33.15±0.51
F/G	1.68±0.02[b]	1.61±0.01[a]	1.59±0.03[a]	1.57±0.04[a]
22 ~ 42 d				
ADG/g	45.39±0.38	44.96±0.60	44.918±0.37	44.96±0.68
ADFI/g	99.85±1.58[b]	94.87±0.69[a]	93.86±1.01[a]	91.74±1.11[a]
F/G	2.20±0.04[b]	2.11±0.07[a]	2.09±0.04[a]	2.04±0.06[a]
1 ~ 42 d				
ADG/g	33.21±0.61	32.97±0.36	33.35±0.28	33.15±0.38
ADFI/g	67.48±0.95[b]	64.32±0.67[a]	63.78±0.39[a]	62.54±0.28[a]
F/G	2.03±0.04[b]	1.95±0.03[a]	1.91±0.05[a]	1.89±0.03[a]

Note: [1]Control = basal diet; Gln = basal diet with 0.5, 1.0 and 1.5 % glutamine in the starter and grower phases, respectively

[2, a, b]Values within the same row that do not share a common superscript are significantly different at $P < 0.05$; n = 10.

[3]ADG=average daily gain; ADFI = average daily feed intake; F: G = feed intake: average daily gain.

Table 4: Effect of dietary Gln on the intestinal morphology in broilers (μm).

Items		Diet Treatments[1]			
		CON	Gln1	Gln2	Gln3
21 d					
Jejunum	Villus height	612.5±46.4[a]	770.2±28.3[b]	795.0±26.7[b]	754.4±40.2[b]
	Crypt depth	175.7±4.0[b]	148.6±3.2[a]	140.3±6.0[a]	155.7±4.1[a]
	V/C	3.49±0.15[a]	5.18±0.24b	5.67±0.11[b]	4.85±0.23[b]
Ileum	Villus height	356.7±10.3[a]	462.0±11.6[b]	496.2±24.4[b]	488.7±20.1[b]
	Crypt depth	147.2±6.0[b]	125.0±2.1[a]	120.114±1.1[a]	115.4±2.9[a]
	V/C	2.42±0.13[a]	3.69±0.20[b]	4.13±0.34[b]	4.24±0.19[a]
42 d					
Jejunum	Villus height	780.4±5.2[a]	985.3±52.2[b]	1022.8±30.3[b]	1087.0±68.6[b]
	Crypt depth	246.8±3.5[b]	220.6±6.8[a]	215.4±5.2[a]	210.6±9.0[a]
	V/C	3.16±0.12[a]	4.47±0.09[b]	4.75±0.11[b]	5.16±0.29[b]
Ileum	Villus height	488.3±9.9[a]	576.9±12.4[b]	597.9±19.7[b]	621.1±20.2[b]
	Crypt depth	185.3±3.4[b]	137.3±3.3[a]	124.8±6.9[a]	115.7±5.2[b]
	V/C	2.63±0.15[a]	4.20±0.20[b]	4.79±0.31[b]	5.37±0.41[b]

Note: [1]Control = basal diet; Gln = basal diet with 0.5, 1.0 and 1.5 % glutamine in the starter and grower phases, respectively.

[2, a, b]Values within the same row that do not share a common superscript are significantly different at $P < 0.05$; n = 10.

[3]V/C=Villus height / Crypt depth.

These discrepancies are probably due to the age of broiler, the amount of additives, stress status and feeding environment (Xu *et al.,* 2012; Zhang *et al.*, 2012).

The morphology of the small intestine has been used widely to assess intestinal health and function in broilers (Wang *et al.*, 2014). Yi *et al.* (2001) reported that Gln increased villus height of small intestine in broilers. Consistent with those results, our study showed that supplementation of Gln increased the villus height in the intestine. Gln exerts the beneficial effects on small intestinal morphology probably due to its energy providing and metabolism in the small intestinal cells of broilers (Le Bacquer *et al.*, 2003; Wu *et al.*, 2019). These results clearly indicate an improving effect of Gln on the villus height and crypt depth of various intestinal segments, which might be another mechanism responsible for its beneficial effect on the improved the ADFI and F: G as the above mentioned.

Some reports indicated that the intestinal development, structure, function and mitochondrial membrane integrity were generally associated with the activity of disaccharides (Tran *et al.,* 2011). In current study, we observed marked increase in glucose, maltase and sucrase activities in broilers fed Gln diets at 21 d, which are consistent with results of evaluation of intestinal morphology. These are probably due to the fact that Gln could improve the abundance of intestinal disaccharides activities while

Table 5: Effect of dietary Gln on the mucosal disaccharides activity of duodenum, jejunum and ileum in broilers.

Items		Diet Treatments[1]			
		CON	Gln1	Gln2	Gln3
21 d					
Jejunum	Glucose (μmol/g)	3.74±0.38[a]	10.98±1.41[b]	12.43±0.53[b]	15.21±1.57[b]
	Maltase (U/mg)	0.065±0.003[a]	0.074±0.002[b]	0.079±0.001[b]	0.084±0.005[b]
	Sucrase (U/mg)	0.063±0.002[a]	0.076±0.001[b]	0.079±0.004[b]	0.083±0.003[b]
Ileum	Glucose (μmol/g)	2.15±0.12[a]	6.83±0.20b	6.98±0.11[b]	7.21±0.18[b]
	Maltase (U/mg)	0.037±0.001[a]	0.051±0.003[b]	0.059±0.002[b]	0.062±0.004[b]
	Sucrase (U/mg)	0.036±0.002[a]	0.053±0.001[b]	0.054±0.003[b]	0.056±0.002[b]
42 d					
Jejunum	Glucose (μmol/g)	12.47±1.52[a]	20.49±1.74[b]	22.42±0.96[b]	21.84±0.93[b]
	Maltase (U/mg)	0.148±0.005	0.158±0.004	0.160±0.007	0.165±0.005
	Sucrase (U/mg)	0.085±0.002[a]	0.103±0.004[b]	0.110±0.005[b]	0.114±0.010[b]
Ileum	Glucose (μmol/g)	6.34±0.25	6.46±0.31	6.61±0.27	7.01±0.30
	Maltase (U/mg)	0.162±0.003	0.167±0.002	0.171±0.001	0.174±0.004
	Sucrase (U/mg)	0.071±0.003[a]	0.092±0.002[b]	0.096±0.001[b]	0.095±0.004[b]

Note: [1]Control = basal diet; Gln = basal diet with 0.5, 1.0 and 1.5 % glutamine in the starter and grower phases, respectively.

[2, a, b]Values within the same row that do not share a common superscript are significantly different at $P < 0.05$; n = 10.

Table 6: Effect of dietary Gln on the relative expression of tight joint protein mRNA in broilers.

Items		Diet Treatments[1]			
		CON	Gln1	Gln2	Gln3
21 d					
Jejunum	ZO-1	0.95±0.11[a]	2.05±0.14[b]	2.27±0.12[b]	2.86±0.16[b]
	occluding	0.78±0.11[a]	2.19±0.18[b]	2.95±0.16[b]	3.91±0.15[b]
	Claudin-1	2.65±0.18[a]	5.54±0.14[b]	5.97±0.12[b]	6.34±0.13[b]
Ileum	ZO-1	0.62±0.09[a]	1.84±0.11[b]	1.95±0.13[b]	1.89±0.12[b]
	occluding	0.50±0.13[a]	1.86±0.11[b]	1.94±0.16[b]	2.10±0.15[b]
	Claudin-1	2.03±0.12[a]	5.01±0.13[b]	5.19±0.14[b]	5.21±0.11[b]
42 d					
JejunumIleum	ZO-1	1.12±0.61	1.13±0.55	1.33±0.48	1.69±0.81
	occluding	0.48±0.11	0.61±0.13	0.65±0.20	0.74±0.18
	Claudin-1	0.67±0.51	1.15±0.28	1.38±0.31	1.45±0.42
	ZO-1	0.95±0.12	1.16±0.15	1.24±0.13	1.30±0.11
	occluding	0.34±0.11	0.39±0.17	0.42±0.13	0.41±0.15
	Claudin-1	0.52±0.15	0.83±0.42	0.86±0.17	0.91±0.28

Note: [1]Control = basal diet; Gln = basal diet with 0.5, 1.0 and 1.5 % glutamine in the starter and grower phases, respectively.

[2, a, b]Values within the same row that do not share a common superscript are significantly different at $P < 0.05$; n = 10.

modifying intestinal morphology and function and trophic effect on the intestinal mucosa in broilers (Wu *et al.*, 2018).

TJP including ZO1, occluding and claudins proteins play an important role in the maintenance of normal intestinal epithelial barrier function, preventing colonization and invasion of bacteria and toxins to the intestine mucosa and blood circulation (Giancamillo *et al.*, 2013; Zhang *et al.,* 2013). Gln, as a source of fuel for cells, can stimulate intestinal mucosal protein synthesis, which is responsible for the maintenance of the tight junction and the normal intestinal barrier function. Wu *et al.* (2018) has suggested that glutamine could effectively increase the tight junction proteins mRNA expression levels in broilers. In present study, Glutamine supplementation was found to be beneficial in increasing intestinal epithelial tight junction proteins mRNA expression levels in normal broilers, indicating that glutamine is involved directly or indirectly in the protective function of intestinal epithelial barrier. This protective effect are further supported by several *in vivo* studies using the models of intestinal epithelium (Li and Neu 2009). These are probably due to the fact that Gln is related to fuel energy supply to proliferating and differentiating enterocytes. But the underlying molecular protective mechanisms on gut for the Gln should be addressed in future studies involving *in vitro* and *in vivo* experiments.

Overall, our results indicated that administration of Gln was able to improve the morphological development of the small intestine, the activity of intestinal mucosa disaccharides and increase the mRNA expression of ZO-1, claudin-1 and occludin proteins in broilers. It is evident that dietary Gln has the direct or indirect protective effect on the intestinal epithelial barrier function and development of broilers.

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