Dietary L-glutamine supplementation improves growth performance, gut morphology, and serum biochemical indices of broiler chickens during necrotic enteritis challenge

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ABSTRACT Necrotic enteritis (NE) causes significant economic losses in the broiler chicken industry, especially in birds raised without in-feed antibiotics. L-glutamine (Gln) is an amino acid that may compensate for metabolic losses from infection and improve the intestinal development. This study investigated the effects of dietary Gln (10 g/kg) supplementation on growth performance, intestinal lesions, jejunum morphology, and serum biochemical indices of broiler chickens during NE challenge. The study employed a factorial arrangement of treatments with factors: NE challenge, no or yes; dietary Gln inclusion, 0 g/kg in starter (S), d 0 to 10, grower (G) d 10 to 24, and finisher (F) d 24 to 35; 10 g/kg in S, G, F, or 10 g/kg in S, G only. Each treatment was replicated in 6 floor pens with 17 birds per pen as the experimental unit for performance and 2 birds for other measurements. Challenge significantly reduced bird performance, increased incidence of intestinal lesions, and affected intestinal development and serum biochemical indices. Regardless of challenge, Gln supplementation increased gain ($P < 0.05$), feed intake ($P < 0.05$), and decreased FCR ($P < 0.05$) on d 24. On d 35, Gln improved gain ($P < 0.05$) and FCR ($P < 0.001$) whereas withdrawing Gln from finisher tended to diminish the beneficial effect on weight gain but not FCR. Dietary Gln reduced lesion scores in the jejunum ($P < 0.01$) and ileum ($P < 0.01$) in challenged birds. On d 16, Gln increased villus height to crypt depth ratio in unchallenged birds ($P < 0.05$) and reduced crypt depth of challenged birds on d 24 ($P < 0.05$). Regardless of challenge, supplementation with Gln reduced crypt depth on d 16 ($P < 0.05$), and increased villus height ($P < 0.01$) and the villus height to crypt depth ratio ($P < 0.001$) on d 24. Dietary Gln lowered serum uric acid level regardless of challenge ($P < 0.05$). The current study indicates that dietary Gln alleviates adverse effects of NE and may be useful in antibiotic-free diets.

Key words: necrotic enteritis, L-glutamine, intestinal morphology, performance, broiler

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INTRODUCTION

Necrotic enteritis (**NE**) in poultry caused by *Clostridium perfringens* (**Cp**) has been previously controlled by in-feed antibiotics and the ban of in-feed antibiotics in the European Union resulted in an increased incidence of this disease (Shojadoost et al., 2012). The subclinical form of NE persists in flocks without detectable clinical signs and may impair performance due to poor digestion without major signs of disease (Van der Sluis, 2000; Kaldhusdal et al., 2001). It has been estimated that NE causes losses of 6 billion US dollars to the global poultry industry annually (Wade and Keyburn, 2015). Thus, there is current heightened interest in solutions to control NE, and this requires a better understanding of the pathology and metabolic cascade resulting in performance loss. Inflammation is a vital consequence of NE that has been often overlooked. Pro-inflammatory cytokines and chemokines increase dramatically in chickens during NE infection (Lee et al., 2011) and NE also induces strong local inflammatory reactions as seen at the interface of the basal domain of enterocytes and hyperaemic lamina propria (Olkowski et al., 2006). Inflammation is an energy-consuming process and immunologically challenged vertebrates can have increased resting metabolic rates of 8% to 27% (Martin et al., 2003). Jin et al. (1995) found that isolated mitochondria of laboratory rats stimulated in vivo with pro-inflammatory cytokines could undergo a 30% increase in respiration rate. Fuelling such up-regulation requires glucose and L-glutamine (**Gln**) at high levels, leading to a rapid breakdown of the body's reserves of protein to provide Gln as the key substrate of gluconeogenesis (Michie, 1996). As the most

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abundant amino acid (**AA**) in plasma and skeletal muscle, circulating and tissue Gln concentrations drop dramatically during the infection and a moderate infection can lead to 150 to 200% increases in rates of gluconeogenesis in the host resulting in severe wasting of lean tissue (Askanazi et al., 1980; Lochmiller and Deerenberg, 2000).

Traditionally classified as a non-essential AA, Gln is suggested to be conditionally essential during critical infection especially when the gut mucosal barrier becomes compromised (Souba et al., 1990). As the main metabolite and energy source to nourish enterocytes, Gln is responsible for mucosal structure maintenance, through mucin synthesis and the maintenance of a barrier against bacterial attacks (Lacey and Wilmore, 1990; Khan et al., 1999). Reconstitution of damaged intestinal mucosa and enterocyte differentiation benefit from Gln supplementation (Blikslager et al., 2001; Murakami et al., 2007). Bartell and Batal (2007) found that 10 g/kg Gln addition to the feed increased weight gain, intestinal villus heights and serum immunoglobulin A and G concentrations of broiler chickens from d21 posthatch as compared to un-supplemented diets. Similarly, Soltan (2009) observed the highest weight gain and lowest FCR by adding 10 g/kg Gln in the diets comparing to other inclusion rates, 0 g/kg, 0.5 g/kg, 1.5 g/kg, and 2.0 g/kg respectively.

Thus it was hypothesized that 10 g/kg Gln supplementation may provide protection to broiler chickens by maintaining growth performance, gut morphology, serum biochemical indices, and compensating for metabolic loss due to inflammation during NE challenge.

Table 1. Ingredient and nutrient composition of experimental basal diets (per cent unless otherwise noted).

Ingredients (%)	Starter	Grower	Finisher
Wheat	38.1	42.3	46.4
Barley	20.0	20.0	20.0
Soybean meal	30.9	25.9	22.7
Meat and bone meal	5.6	6.0	4.0
Cottonseed oil	3.40	3.80	4.90
Limestone	0.51	0.18	0.57
Sodium chloride	0.09	0.09	0.12
Na bicarbonate	0.20	0.18	0.20
Vitamin mineral premix[1]	0.20	0.20	0.20
Choline Cl 60%	0.08	0.08	0.07
L-lysine HCl	0.30	0.28	0.24
D,L-methionine	0.41	0.36	0.33
L-threonine	0.22	0.19	0.15
TiO2	0.0	0.5	0.0
Nutrient composition			
ME, kcal/kg	2,950	3,000	3,100
Crude protein	24.1	22.4	20.3
Crude fat	5.70	6.10	7.00
Crude fibre	3.00	2.90	2.90
Calcium	0.96	0.87	0.78
Digestible Lys	1.28	1.15	1.02
Digestible M+C	0.95	0.87	0.80
Digestible Thr	0.86	0.77	0.68
Available phosphorus	0.48	0.50	0.39
Sodium	0.18	0.16	0.16
Chloride	0.25	0.22	0.20
Choline, mg/kg	1,700	1,600	1,500
Linoleic acid	2.47	2.65	3.20

[1]Vitamin and mineral concentrate supplied per kilogram diet: retinol, 12,000 IU; cholecalciferol, 5,000 IU; tocopheryl acetate, 75 mg, menadione, 3 mg; thiamine, 3 mg; riboflavin, 8 mg; niacin, 55 mg; pantothenate, 13 mg; pyridoxine, 5 mg; folate, 2 mg; cyanocobalamin, 16 μg; biotin, 200 μg; Cu (sulfate), 16 mg; Fe (sulfate), 40 mg; I (iodide), 1.25 mg; Se (selenate), 0.3 mg; Mn (sulfate and oxide), 120 mg; Zn (sulfate and oxide), 100 mg; cereal-based carrier, 149 mg; mineral oil, 2.5 mg.

MATERIALS AND METHODS

Experimental Design and Bird Husbandry

The Animal Ethics Committee of University of New England (Australia) reviewed and approved all experimental procedures involved in this study. Day old Ross 308 male broiler chicks (N = 612) were obtained from a local hatchery (Baiada Hatchery, Tamworth, NSW, Australia) vaccinated against Marek's disease, infectious bronchitis, and Newcastle disease. Experiment was conducted in floor pens with fresh wood shavings as bedding material, in an environmentally controlled room (Rob Cumming Poultry Innovation Centre at Kirby, University of New England, Australia) that was disinfected prior to bird arrival. Birds were distributed into appropriate pens immediately after arrival with uniform starting pen weights. The lighting and temperature program followed the breeder recommendations (Aviagen, 2014).

The study employed a factorial arrangement of treatments in a completely randomized design with 6 replicate pens per treatment and 17 birds per pen. Factors were: NE challenge, no or yes; dietary Gln inclusion, 0 g/kg in starter (**S**), d 0 to 10, grower (**G**) d 10 to 24, and finisher (**F**) d 24 to 35; 10 g/kg in S, G, F, or 10 g/kg in S, G only. Day 24 results were analyzed as 2 × 2 and d 35 results were analyzed as 2 × 3 per dietary treatments. Diets with Gln addition were supplemented by withdrawing the same amount of wheat. The Gln used was a white crystalline powder with 98.5% plus purity (Fufeng Biotechnologies, Urumqi, China). Free AAs in each diet were analyzed in duplicate by Waters AccQTag Ultra chemistry on a Waters Acquity UPLC (Milford, USA) and results are expressed as an average. Free Gln concentrations in the Gln supplemented diets were 10.7 mg/g, 9.3 mg/g, and 10.6 mg/g in starter, grower, and finisher respectively and no free Gln was detected in un-supplemented diets confirming correct inclusion levels. Diets (Table 1) were based on wheat, soybean meal, barley, meat, and bone meal and were formulated to meet Ross 308 nutrient specifications (Aviagen, 2014). Levels of Gln in basal diets were calculated to be 6.0 g/kg in S, 6.4 g/kg in G, and 6.9 g/kg in F based on ingredient values given in Li et al. (2011). Birds had ad libitum access to feed and water. Pen weight and cumulative pen feed intake were recorded on d 0, 24, and 35 and used to calculate mean bird weight gain (BWG), feed intake (FI), and feed conversion ratio (FCR, corrected for mortality).

Mortality was recorded as it occurred with lesions examined post challenge.

NE Challenge

The challenge model was as described in previous reports by Wu, et al. (2014), M'Sadeq et al. (2015). Briefly, on d 9, each bird in the NE-challenge group was given 1 mL per os wild types of *Eimeria spp.* (Bioproperties Pty Ltd., Sydney, Australia). Each 1 mL inoculum included PBS suspension of approximately 5,000 oocysts each of *E. acervulina* and *E. maxima*, and 2,500 oocysts of *E. brunetti*. To the unchallenged group, 1 mL of sterile PBS was given as control. On d 14, each bird in the NE-challenge group was given 1 mL of Cp suspension at a concentration of 10^8 to 10^9 CFU/mL. A primary poultry isolate of Cp type A strain EHE-NE18 (CSIRO Livestock Industries, Geelong, Australia) was incubated overnight at 39°C in 100 mL of sterile thioglycollate broth (USP alternative, Oxoid, Australia) followed by subsequent overnight incubations of 1 mL of the previous culture in 100 mL of sterilized cooked meat medium (Oxoid, Australia), and then in 500 mL of thioglycollate broth containing starch (10 g/L) and pancreatic digest of casein (5 g/L) to obtain the challenge inoculum. Birds in the unchallenged groups received 1 mL of sterile thioglycollate broth. Unchallenged and challenged groups were physically partitioned to prevent cross contamination.

Lesion Scoring

On d 16, 2 birds per pen were randomly selected and euthanized by cervical dislocation. The entire length of the small intestine (duodenum, jejunum, and ileum) of all sampled birds underwent a lesion scoring process, based on a previously reported lesion scoring system that ranges from 0 to 4 (Prescott et al., 1978; Broussard et al., 1986). Score 0 referred to intestine of healthy appearance, 1 referred to gas-filled intestine with evidence of at least two necrotic lesions, 2 referred to ballooned, friable, foul-smelling intestine with evidence of necrotic lesions, 3 referred to intestines that displayed all the above along with a yellow pseudomembrane (described as having an appearance of "Turkish towel"), and 4 referred to prevalence of ruptures of the intestinal epithelial layer and blood filled intestine. Three experienced personnel, with no knowledge of the trial design, were involved in the scoring process. Scores from selected birds were averaged and pen was the experimental unit for lesion scoring.

Intestinal Morphology and Serum Biochemical Indices

On d 16 post hatch, 2 birds from each replicate pen were randomly selected and euthanized by cervical dislocation for sample collection. This was repeated on d 24. Results were averaged per pen for each day before statistical analysis. Samples were processed for intestinal morphology following the method described as Sayrafi et al. (2011) with slight modifications. Approximately 2 cm tissue samples from the middle point of jejunum were transected and fixed in 10% buffered formalin. Tissues were serial dehydrated by transferring through alcohols with increasing concentrations and embedded in paraffin. Tissue sections (5 to 6 μm) were cut by a microtome and stained with hematoxylin-eosin. Images were taken using a Nanozoomer slide scanner (Nanozoomer 2.0-RS; Hamamatsu Photonics, Hertfordshire, UK) and histomorphometric measurement was performed by employing ImageJ software (US National Institutes of Health, Bethesda, MD). A total of 10 well-oriented, intact villi and crypts were randomly selected in duplicate from each tissue sample and the average of 20 values obtained for each chick was taken.

Blood samples were collected in non-heparinized tubes by puncturing the brachial vein and centrifuged at 2,000 × g for 10 min to obtain serum. The serum was pooled from 2 birds in each replicate pen and analyzed for glutamic-pyruvic transaminase (**SGPT**), total cholesterol, total protein, glucose, and uric acid with a spectrophotometer (Siemens Dimension XP and Plus) using the commercially available kit package. Pen was the experimental unit.

Statistical Analysis

Statistical analysis was conducted using IBM SPSS Statistics package version 22 (Armonk, USA). The main effects of NE challenge, Gln addition and interactions were examined by analysis of variance, using the General Linear Model. Intestinal lesion scoring and mortality data were analyzed by the nonparametric Kruskal-Wallis test, as the data were not normally distributed. Treatment means were separated using Tukey's HSD (honest significant difference) multiple range test where appropriate. Statistical significance was declared at $P < 0.05$.

RESULTS

Performance

At any stage of this experiment, treatment did not affect mortality ($P > 0.05$) and no NE challenge × Gln interactions were observed on performance ($P > 0.05$).

Effects of NE challenge and Gln supplementation on the performance from d 0 to 24 are shown in Table 2. NE challenge reduced FI ($P < 0.001$) and BWG ($P < 0.001$), and increased FCR ($P < 0.001$). Regardless of NE challenge, supplementation with Gln led to higher FI ($P < 0.01$), BWG ($P < 0.001$), and reduced FCR ($P < 0.05$).

From d 0 to 35 as shown in Table 3, the long term effect of NE was observed as lower BWG ($P < 0.01$) and

Table 2. Performance of broilers from d 0 to d 24.

Interaction		Feed Intake g/bird	Weight gain g/bird	FCR	Mortality%
Challenge	Gln				
No	G0	1,588	1,335	1.190	1.17
No	G1	1,671	1,419	1.178	0.00
Yes	G0	1,406	1,154	1.217	1.17
Yes	G1	1,490	1,231	1.210	1.75
SEM		20	19	0.003	0.41
Main effects					
Challenge					
No		1643[a]	1391[a]	1.184[b]	0.00
Yes		1462[b]	1205[b]	1.214[a]	2.00
10 g/kg Gln addition[1]					
G0		1497[b]	1244[b]	1.203[a]	1.00
G1		1581[a]	1325[a]	1.194[b]	1.00
P value					
Challenge		<0.001	<0.001	<0.001	0.15
Gln		0.003	<0.001	0.033	0.74
Challenge × Gln		0.99	0.85	0.58	0.37

[a-b]Means not sharing the same superscripts are significantly different ($P < 0.05$).

[1]G0 (control) = (no Gln addition in starter, grower); G1 = (10 g/kg Gln in starter, grower).

Table 3. Performance of broilers from d 0 to d 35.

Interaction		Feed Intake g/bird	Weight gain g/bird	FCR	Mortality%
Challenge	Gln				
No	G0	3,353	2,476	1.354	1.40
No	G1	3,452	2,584	1.336	1.20
No	G2	3,335	2,523	1.321	1.20
Yes	G0	3,284	2,357	1.393	1.20
Yes	G1	3,440	2,509	1.371	2.30
Yes	G2	3,351	2,441	1.373	2.30
SEM		25	19	0.005	0.50
Main effects					
Challenge					
No		3380	2528[a]	1.337[b]	1.20
Yes		3359	2436[b]	1.379[a]	1.90
10 g/kg Gln addition[1]					
G0		3319	2417[b]	1.374[a]	1.30
G1		3446	2547[a]	1.353[b]	1.80
G2		3343	2482[a,b]	1.347[b]	1.80
P value					
Challenge		0.67	0.007	<0.001	0.43
Gln		0.10	0.010	<0.001	0.86
Challenge × Gln		0.78	0.84	0.38	0.94

[a-b]Means not sharing the same superscripts are significantly different ($P < 0.05$).

[1]G0 (control) = (no Gln addition in starter, grower and finisher); G1 = (10 g/kg Gln in starter, grower, and finisher), G2 = (10 g/kg Gln in starter, grower, no Gln in finisher).

increased FCR by 0.04 units ($P < 0.001$). Birds fed on diets with Gln addition with or without withdrawing Gln in finisher had lower FCR ($P < 0.001$) than controls. Feeding Gln addition throughout the experiment resulted in higher BWG ($P < 0.05$) than control group and numerically higher than the group supplemented with Gln only in starter and grower.

Table 4. Duodenum, jejunum, and ileum NE lesion score at d 16.

Treatments		Duodenum	Jejunum	Ileum
Challenge	Gln			
No	G0	0.25[b]	0.08[b]	0.08[b]
No	G1	0.29[b]	0.00[b]	0.00[b]
Yes	G0	0.50[a,b]	1.25[a]	0.75[a]
Yes	G1	0.92[a]	0.29[b]	0.21[b]
SEM		0.09	0.10	0.06
Main effects				
Challenge				
No		0.24[b]	0.11[b]	0.08[b]
Yes		0.74[a]	0.70[a]	0.44[a]
10 g/kg Gln addition[1]				
G0		0.38	0.67[a]	0.42[a]
G1		0.60	0.15[b]	0.10[b]
P value				
Challenge		0.003	0.002	0.003
Gln		0.24	0.028	0.028
Challenge × Gln		0.013	0.001	0.002

[a-b]Means not sharing the same superscripts are significantly different ($P < 0.05$).

[1]G0 (control) = (no Gln addition in starter, grower); G1 = (10 g/kg Gln in starter, grower).

Pen was the experimental unit. Lesions from 2 birds selected for scoring from each pen were averaged.

Intestinal Lesion Scores

Intestinal lesion scores in the duodenum, jejunum, and ileum measured at d 16 are presented in Table 4. Challenge × L Gln interactions were observed for lesion scores in duodenum, jejunum, and ileum. Supplementation of Gln in starter and grower ameliorated lesions caused by NE challenge in jejunal ($P < 0.001$) and ileal ($P < 0.01$) tissues but not in the duodenal tissue ($P > 0.05$).

Gut Morphology

The effects of NE challenge and Gln addition on jejunal morphology on d 16 and 24 are presented in Table 5. On d 16, birds fed diets with added Gln had reduced jejunal crypt depth compared to control birds fed the control diet regardless of NE challenge ($P < 0.05$). Birds challenged with NE had shorter ($P < 0.01$) villi height and greater ($P < 0.001$) crypt depth. An interaction was observed ($P < 0.05$) between NE challenge and Gln for villus height to crypt depth ratio (V:C ratio). Supplementation of Gln only increased V:C ratio in the birds without NE challenge but not in the NE challenged birds.

On d 24, reduced V:C ratio was observed with NE challenge ($P < 0.001$) and Gln supplemented birds had a greater V:C ratio ($P < 0.001$) regardless of challenge. An interaction between NE challenge and Gln for crypt depth was detected ($P < 0.05$). Supplementation with Gln had no effect on crypt depth in unchallenged birds ($P > 0.05$) but Gln reduced crypt depth in challenged birds ($P < 0.05$). Therefore challenge only increased crypt depth in when Gln was not added to the diet.

Table 5. Villus height (μm), crypt depth (μm), and villus height:crypt depth (V:C ratio) of segments of the jejunum of broiler chickens on d 16 and 24 subjected to Gln supplementation and NE challenge.

		d 16			d 24		
Interaction		Villus height	Crypt depth	V:C ratio	Villus height	Crypt depth	V:C ratio
Challenge	Gln						
No	G0	912	111	8.6[b]	1,339	99[b]	13.7
No	G1	1,115	92	12.7[a]	1,417	91[b]	15.9
Yes	G0	827	215	4.2[c]	1,142	196[a]	6.8
Yes	G1	823	177	4.7[c]	1,415	118[b]	12.3
SEM		34	10	0.7	32	10	0.7
Main effects							
Challenge							
No		1,014[a]	102[b]	10.6[a]	1,378	95[b]	14.8[a]
Yes		825[b]	196[a]	4.5[b]	1,278	157[a]	9.6[b]
10 g/kg Gln addition[1]							
G0		869	163[a]	6.4[b]	1,241[b]	147[a]	10.2[b]
G1		969	134[b]	8.7[a]	1,416[a]	104[b]	14.1[a]
P value							
Challenge		0.003	<0.001	<0.001	0.098	<0.001	<0.001
Gln		0.096	0.023	0.007	0.005	0.009	<0.001
Challenge × Gln		0.085	0.43	0.036	0.11	0.031	0.077

[a-c]Means not sharing the same superscripts are significantly different ($P < 0.05$).
[1]G0 (control) = (no Gln addition in starter, grower); G1 = (10 g/kg Gln in starter, grower).
Villus and crypt measurements from 2 birds selected from each pen were averaged and pen was the experimental unit.

Serum Biochemical Indices

The effects of NE challenge and Gln supplementation on serum biochemical metabolites on d 16 and d 24 are shown in Table 6. On d 16, NE challenge increased SGPT level ($P < 0.01$), and decreased cholesterol ($P < 0.001$) and uric acid ($P < 0.05$) concentrations regardless of Gln addition. On d 24, birds with NE challenge had lower cholesterol level ($P < 0.01$) and higher total protein concentration ($P < 0.05$). Supplementation with Gln reduced the uric acid on d 24 regardless of NE challenge ($P < 0.05$).

Neither NE challenge nor Gln addition affected the glucose level of chickens on d 16 or d 24. No NE challenge × Gln interactions were observed on all the measured serum biochemical indices ($P > 0.05$).

DISCUSSION

No differences in mortality were observed as a result of experimental necrotic enteritis or supplementation with Gln. The low average mortality of 1.6% observed in this study with application of experimental NE is not unusual. Sharma et al., 2017 using the same NE model found <3% overall mortality on d 35 with differences as a result of NE challenge. Jararayaman et al. 2013 reported no mortality as a result of experimental induction of NE using mixed species of *Eimeria* and a confirmed field strain of pathogenic Cp. Induction of lesion causing NE using two confirmed toxigenic strains of Cp with no *Eimeria*, Yang et al., 2016 reported low mortality (3.3%) with no differences as a result of NE challenge. In all studies reductions in bird performance and increased lesions were detected as a result of experimental NE. In the study of M'Sadeq et al., 2015 overall mortality in challenged birds was reported to be 15% on d 35 using the same strains of *Eimeria* and Cp as the current study. Chick quality, brooding conditions and diet may be reasons for these differences.

In this study, supplementation with 10 g/kg Gln improved the performance, jejunum morphology, and reduced serum uric acid levels and the prevalence of intestinal NE lesions of broilers under NE challenge. Previous reports have been inconsistent on whether Gln supplementation in diet can improve broiler performance. Sakamoto et al. (2006) reported that no difference was observed on the performance between 14-d-old broilers fed corn-soy diets with or without supplementation of 10 g/kg Gln, whereas Bartell and Batal (2007) observed a significant improvement in BWG when fed the same amount of Gln. However, effects of Gln are most likely to be pronounced in the presence of stressors as reported by Novak et al. (2002) who suggested Gln may be conditionally essential for broiler health and productivity under critical conditions. Dai et al. (2011), Hu et al. (2015) and Olubodun et al. (2015) found that Gln significantly improved chicken growth during heat stress. For the first time, the current study revealed a positive role of 10 g/kg Gln supplementation in controlling the impacts of NE on the severity of lesion and performance in broiler chickens.

L-Glutamine may provide metabolic fuel to enterocytes thus benefit gut morphology and mucosa (Lacey and Wilmore, 1990; Bartell and Batal, 2007) as the present study showed Gln increased villus height, V:C ratio, and reduced crypt depth. Increased villus height may result in a greater absorptive capability for available nutrients (Caspary, 1992) whereas low crypt depth values indicate decreasing metabolic cost of intestinal epithelium turnover (Floc'h and Sève, 2000) which may

Table 6. Serum biochemical indices of broiler chickens on d 16 and 24 subjected to Gln supplementation and NE challenge.

		d 16					d 24				
Interaction		SGPT (IU/L)	Cholesterol (mmol/L)	Glucose (mg/dL)	Total protein (g/L)	Uric acid (mg/dL)	SGPT (IU/L)	Cholesterol (mmol/L)	Glucose (mg/dL)	Total protein (g/L)	Uric acid (mg/dL)
Challenge	Gln										
No	G0	5.22	3.58	244	24.2	9.04	4.09	4.31	246	28.0	10.89
No	G1	5.53	3.75	247	23.7	9.30	3.02	4.16	239	28.7	9.22
Yes	G0	9.21	3.17	243	24.0	8.04	2.56	3.73	257	30.1	10.92
Yes	G1	9.77	3.07	249	23.2	8.39	2.77	3.36	252	29.1	9.33
SEM		0.67	0.08	2.45	0.22	0.20	0.38	0.11	3.19	0.25	0.35
Main effects											
Challenge											
No		5.43[b]	3.69[a]	246	23.9	9.21[a]	3.55	4.23[a]	242	28.3[b]	10.05
Yes		9.58[a]	3.11[b]	247	23.4	8.30[b]	2.67	3.55[b]	254	29.6[a]	10.12
10 g/kg Gln addition[1]											
G0		7.40	3.38	244	24.1	8.60	3.32	4.02	251	29.0	10.90[a]
G1		7.75	3.41	248	23.4	8.80	2.90	3.76	245	28.9	9.28[b]
P value											
Challenge		0.002	<0.001	0.85	0.53	0.031	0.30	0.002	0.087	0.015	0.92
Gln		0.73	0.80	0.42	0.20	0.47	0.61	0.21	0.35	0.78	0.030
Challenge × Gln		0.92	0.34	0.74	0.79	0.91	0.45	0.61	0.83	0.074	0.96

[a-b]Means not sharing the same superscripts are significantly different ($P < 0.05$).
[1]G0 (control) = (no Gln addition in starter, grower); G1 = (10 g/kg Gln in starter, grower).
Serum was pooled from 2 birds per pen at each time point and pen was the experimental unit.

be reflected by the lower FCR observed in the current study. This may be due to the crypt as the villus factory with deeper crypts indicating faster tissue turnover for renewal of the villus as needed in response to inflammation from pathogens or their toxins (Potten, 1997; Willing and Van Kessel, 2007). Thus, a greater V:C ratio suggests increased nutrient absorption, decreased secretion in the gastrointestinal tract, and improved performance. Furthermore, longer villus height and shorter crypts depths on d 24 may be the evidence that Gln provides beneficial effect on enterocytes to prevent injury as evidenced by higher weight gain and decreased FCR from d 0 to d 24 with added Gln. Another possibility is that Gln improves recovery from the challenge or stress without accelerated enterocyte renewal rate. This is partially supported by Yi et al. (2005) showing feeding 10 g/kg Gln supplementation helped recovery of delayed small intestinal development of broiler chickens that fasted for 48 h post hatch.

The protective effect of Gln on alleviating intestinal lesions may also be associated with enhanced development of the intestinal mucosa. L-glutamine is responsible for retaining the mucosal structure (Khan et al., 1999) and for reconstruction after damage (Rhoads et al., 1997). Souba et al. (1990) suggested glutamine is an important AA for maintenance of gut metabolism, structure, and function especially during critical illness when the gut mucosal barrier compromised based on human research. The current study suggests Gln improved intestinal architecture in the jejunum and ileum during the NE outbreak and recovery and consequently favors intestine function and nutrient absorption. The response of Gln on lesion scores in challenged birds however appeared to differ across the various gut sections. In the jejunum and ileum, lower lesions of 4-fold and 3-fold respectively were noted as a result of dietary Gln in challenged birds. However in the duodenum, lesions were 1.8-fold higher with Gln in challenged birds. This suggests that Gln may be more protective in ileum and jejunum than duodenum. This finding may be due to absorptive, enzymatic, or other metabolic differences including ability of duodenal cells to use Gln for gluconeogenesis. The inflammatory response triggered by NE challenge results in gluconeogenesis to maintain the glucose levels especially during the anorexia observed during the acute phase of NE (Fischer et al., 1995; Scanes, 2009).

In the current study, FI was decreased by 11% as a result of NE challenge on d 24 while serum glucose remained at similar levels in challenged and unchallenged groups. Therefore, Gln will be greatly utilized as the key substrate of gluconeogenesis and uptaken in skeletal muscle, the major repository of Gln (Lacey and Wilmore, 1990; Wu et al., 1991). Karinch et al. (2001) suggested skeletal muscle exhibited a twofold increase in Gln release during infection whereas the intracellular Gln pool depleted indicating release rates exceeded Gln synthesis rates. Supplementation with Gln may compensate this effect and prohibit the lean muscle from exceeding loss. L-glutamine was also found to decrease intestinal nuclear factor kappa B activity and pro-inflammatory cytokine expression in rats (Fillmann et al., 2007; Chen et al., 2008). However, Gln might not play as an anti-inflammatory agent in the present study as total cholesterol level was not affected by Gln supplementation.

Infection and inflammation greatly alter the cholesterol level (Khovidhunkit et al., 2004) and NE

challenge resulted in lower cholesterol levels in the current study that has been also observed in broiler chickens during *Eimeria* challenge (Allen, 1988). Feingold and Grunfeld (2015) suggested "treatment of the underlying disease leading to a reduction in inflammation results in a return of the lipid profile towards normal", whereas there was no such observation in Gln supplemented groups in our study. Furthermore, NE challenge elevated SGPT level indicates liver damage and pathological manifestation of liver dysfunction whereas Gln supplementation did not have significant impact on SGPT level. Liver enlargement and lesions caused by NE infection have been well documented (Løvland and Kaldhusdal, 1999; 2001) and such liver mobility can be mainly associated with systemic inflammation (Lichtman et al., 1990; Tremaroli and Bäckhed, 2012) suggesting that Gln might influence chicken performance without directly suppressing inflammation induced by NE.

It is noteworthy that phasing out Gln supplementation in the finisher diet diminished its beneficial effect on BWG, indicating the requirement of Gln during the recovering from the NE infection. Apart from the significant roles Gln playing as immuno-nutrient and enterocytes fuel, it may also be due to a better AA utilization efficiency, considering Gln is also a vital carrier of nitrogen between tissues (Lacey and Wilmore, 1990). This was reflected by reduced serum uric acid levels in Gln fed groups. Donsbough et al. (2010) suggested serum uric acid could be used as a key indicator of AA utilization in diets and a lower uric acid level indicates an improved AA utilization that is consistent with the improved growth.

CONCLUSION

Supplementation with 10 g/kg Gln partially alleviated the impact of NE in broiler chickens due to positive effects on gut development and compensation for metabolic nutrient losses. Supplementation of birds with Gln improves performance and may help birds recover from NE infection. The current study showed the range and regime of dietary Gln supplementation that may be deployed under antibiotic-free production situations.

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