

Impact of dietary glutamine on amino acid digestibility values and intestinal morphometric parameters in neonate chicks

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Abstract

Two experiments were conducted to investigate the effects of fortification of the pre-starter diet with glutamine in post-hatch broiler chicks. In the first experiment, male chicks were used to study the impact of glutamine supplementation at three levels (0%, 1% and 2%) on performance, apparent faecal digestibility (AFD) and ileal digestibility (AID) of amino acids (AAs), as well as intestinal morphometric parameters from 0 to 14 days old. The best performance was obtained at 1% supplementation. Average daily feed intake and feed conversion ratio (FCR) were affected by treatments. Supplementing diets with glutamine resulted in increase of digestibility values for arginine (Arg), lysine (Lys), isoleucine (Ile) and valine (Val). Glutamine-supplemented diets led to higher villus height (VH) and shallower crypt depth (CD) in the jejunum and ileum. A second experiment was performed to investigate whether the form of glutamine could affect performance and morphometric parameters in newly hatched chickens. A control diet was prepared, based on casein as the sole source of protein-bound AAs. Three diets based on synthetic AAs were used to obtain nutrient values of the control diet, which were supplemented with a synthetic form of glutamine, glutamic acid and a 50 : 50 ratio of glutamine : glutamic acid. Chickens fed the control diet presented higher average daily weight gain and better FCR. None of the AID coefficients of amino acids were influenced by the dietary treatments, except glutamic acid and aspartic acid, which were decreased in chicks fed synthetic AAs. In conclusion, 1% glutamine supplementation improved performance, morphometric parameters and AID and AFD values of cationic AAs. The form in which dietary glutamine was provided could affect performance.

Keywords: Amino acid, broiler, digestibility, intestinal morphology

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Introduction

The post-hatch developmental period represents a critical phase in attaining quality broiler performance at marketing. The importance of dietary protein and amino acid (AA) quality and quantity as significant compounds of diet during this phase of a chicken's life has been documented (Noy & Skalan, 2002; Noy & Uni, 2010; Gous, 2010; Panda *et al.*, 2013). Neonatal broiler chicks are more susceptible to low levels of protein in the diet than to the lack of other macronutrients (Swennen *et al.*, 2010; Hada *et al.*, 2013). Consumption of a high-protein diet, regardless of the differences in bioavailability coefficients of AAs in various sources of protein, is the usual strategy in feed formulation for neonatal chickens. This often ensures optimum growth during and after the critical period of birds' life. However, providing protein above the animal's requirements may not always guarantee performances in neonatal animals.

In high crude protein (CP) diets, the concentration of non-essential AAs increased that may play an effective role after birth. Glutamine (Gln), as one of the non-essential AAs could serve as an oxidative fuel for enterocytes and immune cells, a precursor of purine and pyrimidine synthesis, a modulator of protein turnover or an intermediary for gluconeogenesis, acid-base balance and redox homeostasis. Therefore, Gln is an irreplaceable precursor for certain biologically vital molecules (Souba, 1993). Because of two active nitrogen groups and carbon skeleton, Gln as a nitrogen and carbon transmitter causes rapid turnover of plasma Gln pool (Curthoys *et al.*, 1995). Glutamine tracer kinetic studies in human beings have shown that up to 30% of the Gln pool is used for macromolecule synthesis (Biolo *et al.*, 2005). The Gln molecule is responsible for maintaining the intestinal mucosa structure which is acting as a barrier against bacteria (Khan *et al.*, 1999), in addition to boosting the maturity and integrity of the intestinal flora associated with the

immune system (Yi *et al.*, 2005; Murakami *et al.*, 2007), a Gln supply might improve intestinal conditions for better nutrient absorption. The positive effects of Gln on the performance of broilers in various situations have been documented (Dai *et al.*, 2009; 2011; 2012; Nassiri Moghadam & Alizadeh-Ghamsari, 2013). Nevertheless, little research has been conducted on the effects of Gln supplementation of broiler pre-starter diets on other AA absorption during and post-hatch. In addition, the modality of Gln supplementation as a synthetic free AA or as part of an intact polypeptide or protein molecule and its consequent responses have been less studied.

Because of the obvious effect of Gln on the histo-morphological condition of the epithelial cells of the mucosa, the first experiment was carried out to evaluate the effects of graded levels of dietary Gln on performance, apparent faecal digestibility (AFD) and ileal digestibility (AID) values, and morphology of the intestinal mucosa in newly hatched chicks. The second experiment was conducted to compare the effects of an intact protein-bound source of Gln, casein, and a synthetic source of L-Gln on performance, AID values, and morphological parameters of the intestinal mucosa.

Materials and Methods

Experiment 1 was performed to examine whether the inclusion of dietary Gln might affect histo-morphometric parameters of the intestine and essential AA digestibility in newly hatched chicks. A total of 240 (3*5*16) newly hatched broiler chicks was randomly allotted to 15 four-floor electrically heated battery cages on arrival at the research facility. To minimize the weight range of newly hatched chicks, 3000 eggs of uniform weight (60 ± 1.5 g) were selected from 6500 eggs. Twenty trays of 150 eggs were labelled and followed throughout the incubation period. For the first 18 days of incubation, the temperature was set at 37.8 °C, with 52% relative humidity. Eggs were turned every hour. From day 18 until hatch, the temperature and relative humidity were reduced to 36.5 °C and 48%, respectively. On day 18 of incubation, the eggs were candled. Those with evidence of live embryos were transferred to the hatcher. At the end of incubation period, hatched chickens were sexed and weighed. Therefore, 240 day-old healthy male Ross 308 chicks with relatively similar bodyweights (38 ± 0.3 g) were selected and wing-tagged to be allotted to one of the three dietary treatments. Housing conditions were controlled throughout the experimental period, and chicks had *ad libitum* access to feed and water. The treatment diets were formulated to meet the National Research Council (NRC 1994) minimum recommendations, and were fed from 0 to 28 days old. Soybean meal, as the sole source of intact protein, was sampled before the diet was formulated to determine CP as Kjeldahl nitrogen $\times 6.25$, moisture, ether extract, ash, crude fibre and total AA content (Evonik-Degussa GmbH). The amounts of calcium (method 927.02), phosphorus (method 965.17), potassium (method 969.23), sodium (method 969.23) and chloride (method 943.01) were analysed in duplicate samples by AOAC (2015) procedures in all unpurified feeding ingredients. Diets were formulated to provide constant amount of the mentioned elements in all treatments. All of these elements were maintained constant in all treatments. The experimental diets were formulated to be isoenergetic (Table 1) with cellulose as filler (Table 2). Diets were presented as crumble, and supplemented with complete vitamin and trace mineral mixes.

Experiment 2 was carried out to determine the differences between providing Gln and Glu in the form of casein (protein-bound AA) and as a crystalline form of AA (99.6% purity). Casein was sampled before the diets were formulated to determine nutrient content, including all essential AAs, as described previously. All diets were formulated to be iso-energetic (12.67 MJ/kg of apparent metabolizable energy). The levels of calcium, phosphorus, sodium, potassium and chlorine were maintained equally in all treatments (Tables 3 and 4). Four purified dietary treatments – consisting of various sources of Gln and Glu, including casein, a crystalline form of Gln, a crystalline form of Glu, and 50 : 50 blend of Glu : Gln – were prepared to provide equal total amounts of Gln and Glu (Table 3). For maintaining the total amount of essential AAs equal and above the NRC (1994) recommended levels in all treatments (Table 4), crystalline AAs were added to equal the levels of those in the diet that included casein. Feed grades of L-Lys HCl, DL-Met and L-Thr were used in the diets, whereas for other AAs, reagent grade (minimum 99.5% purity) was provided.

On days 0, 4, 7 and 14 post hatch, five chicks per treatment (with bodyweight close to average) were weighed, stunned electrically, euthanized and the abdominal cavities opened. The small intestine was removed and divided into three segments: duodenum, from the pylorus to entry of the bile and pancreatic ducts; jejunum, from the distal portion of the duodenal loop to Meckel's diverticulum; and ileum, anterior portion of the ileocecal junction.

Segments were cut longitudinally and flushed with cold phosphate-buffered saline (PBS). For morphometric measurement, approximately 3 cm of the middle portions of the duodenum, jejunum and ileum (the apex of the duodenum, 2 cm proximal to Meckel's diverticulum, 8 to 10 cm proximal to ileocecal junction) were cut with a razor blade and fixed in 10% buffered formalin solution for 10 days. Five cross-sections of 70%

Table 1 Nutritional values of the experimental diets (Experiment 1)

Ingredient	Experimental diets		
	0% Glutamine	1% Glutamine	2% Glutamine
Calculated composition			
Apparent metabolizable energy (MJ/kg)		12.67	
Available phosphorus (g/kg)		5.3	
Sodium (g/kg)		2.0	
Chlorine (g/kg)		1.9	
Potassium (g/kg)		8.6	
Analysed composition			
Crude protein (g/kg)	233.6	233.6	233.6
Calcium (g/kg)	10.9	10.9	10.9
Lysine (g/kg)	14.4	14.4	14.4
Methionine + cystine (g/kg)	10.9	10.9	10.9
Threonine (g/kg)	10.3	10.3	10.3
Tryptophan (g/kg)	12.6	12.6	12.6
Arginine (g/kg)	15.4	15.4	15.4
Valine (g/kg)	11.1	11.1	11.1
Isoleucine (g/kg)	9.7	9.7	9.7

ethanol preserved segments for each sample were prepared and embedded in low-melt paraffin (Beçak & Paulete, 1976). Sections were cut from the waxed tissue on a Leitz 1512 microtome. Samples were then stained with haematoxylin and eosin (Uni *et al.*, 1995). Six of the straightest most intact villi and associated crypts were selected for each intestinal cross-section. VH is defined as the length from the villus tip to the valley between villi. CD was measured from the muscular layer to the villus crypt baseline. In addition, the three thickest muscular layers were measured from the submucosal layer to the serosal layer of the intestinal walls. Mean VH (Villus Height), CD (Crypt Depth), VCR (VH : CD ratio) and muscular layer thickness were calculated and used for statistical analysis.

At the end of Experiment 2, blood samples were taken per replicate from the brachial vein and placed in heparinized tubes. Samples were put on ice immediately, and processed within 30 minutes of collection. Plasma was obtained by centrifuging the blood samples at 3000 × *g* for 15 minutes at 4 °C. Plasma aliquots were kept frozen at -20 °C until analysed. Concentrations of free plasma AAs were measured with a model 3A30 Carlo Erba Amino Analyzer (Carlo Erba-Fisons, Rodano, Milan, Italy), as described (Marchesini *et al.*, 1987).

To determine the AFD of AAs, excreta from each cage were collected for 6- to 12-hour periods, depending on the amount of excretion, on day 4 (80 - 92 hours after hatch), day 7 (154 - 166 hours after hatch) and day 14 post hatch. Samples were homogenized using a PT 10/35 polytron mixer (Brinkmann Instruments, Westbury, NY), duplicated, and placed in a -20 °C freezer. After less than a month, the samples were lyophilized and then milled, using a 0.5-mm sieve. Nitrogen content of the samples was then determined using the procedure (Kjeldahl) described by AOAC (1995). Amino acid concentrations in the feed and excreta were determined using ion exchange chromatography following hydrolysis in 6N HCl for 24 hours at 110 °C (Spackman *et al.*, 1958). Analyses of methionine and cystine were conducted after performic acid oxidation (Moore, 1963; Chung *et al.*, 1989). Tryptophan analysis was accomplished after 16 hours' alkaline (4 N LiOH) hydrolysis according to the procedure of Werner (1986).

On days 4, 7 and 14, four birds from each cage from each feeding group were stunned electrically and euthanized by exsanguination. Intestinal tracts were removed immediately, and the contents of the distal half of the ileum were collected by gently flushing with cold PBS into plastic containers. The collected digesta of the birds within a cage were pooled at the sampling time and mixed with 4.5% formic acid. These samples were stored in a freezer maintained at -20 °C in airtight containers until analysed. The samples were then lyophilized and ground through a 0.5-mm sieve, and then divided in two. The following procedure was used: Nitrogen content by Kjeldahl method described by AOAC (1995) and chromic oxide after ashing at 450 °C

Table 2 Composition (% as dry matter basis) of the experimental diets (Experiment 1)

Ingredient	Experimental diets		
	0% Glutamine	1% Glutamine	2% Glutamine
Maize	457	457	457
Soybean meal	384	384	384
Maltodextrin	30	30	30
Calcium carbonate	10	10	10
Soybean oil	38	38	38
Choline chloride	1.7	1.7	1.7
Vitamin premix ¹	2	2	2
Mineral premix ²	2	2	2
Dicalcium phosphate	24.5	24.5	24.5
Sodium chloride	1.7	1.7	1.7
Sodium bicarbonate (NaHCO ₃)	2.4	2.4	2.4
Chromium oxide premix ³	20	20	20
Ethoxyquin	0.2	0.2	0.2
DL-Methionine	3.3	3.3	3.3
L-Lysine HCl	2	2	2
L-Threonine	1.2	1.2	1.2
L-Glutamine	0	10	20
Cellulose	20	10	0
Total	1000	1000	1000

¹ Provided per kilogram of diet: thiamin, 2.3 mg; niacin, 29.5 mg; riboflavin, 6.2 mg; D-Ca-pantothenate, 16.4 mg; vitamin B₁₂, 0.016 mg; pyridoxine-HCL, 3.7 mg; D-biotin, 0.25 mg; folic acid, 0.86 mg; menadione dimethylpyrimidinol bisulfate, 2.65 mg; vitamin A (retinyl acetate), 12500 IU; vitamin D (cholecalciferol), 5000 IU; vitamin E (α -tocopheryl acetate), 70 IU.

² Provided per kilogram of diet: MgSO₄·5H₂O, 3.1 g; ZnCO₃, 0.10 g; ZnSO₄, 0.4 g; CaCO₃, 0.32 g; MnSO₄·H₂O, 0.65 g; MnO, 0.22 g; FeSO₄·7H₂O, 0.42 g; KI, 43 mg; CuSO₄·5H₂O, 30 mg; Na₂MoO₄·2H₂O, 10 mg; H₃BO₃, 8.6 mg; CoSO₄·7H₂O, 1.18 mg; Na₂SeO₃, 0.35 mg.

³ Supplied 5 g chromic oxide per kg of diet. The premix was added at a ratio 1 : 3 of chromic oxide : corn starch.

(Fenton & Fenton, 1979). Samples were analysed for AA concentration as described for faecal samples. Chrome was measured in samples of feed, excreta and ileal content in duplicate.

The AID coefficients of AAs that were evaluated in the experiments were calculated using the method of Ten Doeschate *et al.* (1993) from the formulae:

$$DC_{\text{diet}} = 1 - [(M_{\text{diet}}/M_i) \times (C_i/C_{\text{diet}})]$$

$$DC_{\text{ti}} = (DC_{\text{diet}} \times C_{\text{diet}} - DC_{\text{ref}} \times C_{\text{ref}} \times 0.50) / (C_{\text{diet}} - C_{\text{ref}} \times 0.50),$$

where DC_{diet} and DC_{ti} are digestibility coefficients of an AA in the diet and test ingredient;

M_{diet} and M_i are chromic oxide content of the feed and the ileal digesta;

C_i , C_{diet} , and C_{ref} are the AA content of ileal digesta, the feed, and the reference diet; and

DC_{ref} is the digestibility coefficient for the reference diet, calculated using the same equation as for DC_{diet} .

The digestible AA content was calculated using total nutrient content multiplied by the digestibility coefficients.

The experiments were analysed statistically using the general lineal model ANOVA (version 9.2, SAS Institute Inc., Cary, NC) as a completely randomized design (SAS, 2001). Cage was considered an experimental unit. Means were compared using Duncan's multiple range test, and in all cases significance level was set at $P < 0.05$.

Table 3 Nutrient composition (dry matter basis) of the diets (Experiment 2)

Ingredient (g/kg)	Casein	Glutamic acid	Glutamine	Glutamine + glutamic acid
Dextrose	220	220	220	220
Purified corn starch	330	360	360	360
Maltodextrin	45	45	45	45
Casein	250	0	0	0
L-Glutamic acid	0.00	48.80	0	24.40
L-Glutamine	0.00	0.00	48.80	24.40
Cellulose	73.89	81.31	81.31	81.31
Soybean oil	5.10	5.10	5.10	5.10
Choline chloride	3.30	3.30	3.30	3.30
Purified vitamin premix ¹	1.90	1.90	1.90	1.90
Mineral premix ²	2.20	2.20	2.20	2.20
Calcium carbonate	16.24	12.75	12.75	12.75
Dicalcium phosphate	16.23	26.32	26.32	26.32
L-Arginine	6.48	14.07	14.07	14.07
L-Lysine	0.00	22.46	22.46	22.46
DL-Methionine	3.44	11.02	11.02	11.02
L-Threonine	0.10	9.75	9.75	9.75
L-Isoleucine	0.00	11.66	11.66	11.66
L-Valine	0.00	14.67	14.67	14.67
L-Leucine	0.00	8.68	8.68	8.68
L-Tryptophan	0.00	2.71	2.71	2.71
L-Histidine	0.00	3.62	3.62	3.62
L-Phenylalanine	0.00	11.36	11.36	11.36
Glycine	0.68	5.03	5.03	5.03
L-Alanine	0.00	6.50	6.50	6.50
L-Proline	0.00	24.92	24.92	24.92
L-Aspartate	0.00	15.40	15.40	15.40
Potassium chloride	1.36	0.06	0.06	0.06
Sodium bicarbonate	3.20	8.79	8.79	8.79
Potassium bicarbonate	0.68	2.42	2.42	2.42
Chromic oxide premix ⁴	20.00	20.00	20.00	20.00
Ethoxyquin	0.20	0.20	0.20	0.20
Total	1000	1000	1000	1000

¹ Provided per kilogram of diet: thiamin (thiamin mononitrate), 5.5 mg; niacin, 70 mg; riboflavin, 11 mg; D-Ca-pantothenate, 30 mg; vitamin B₁₂, 0.022 mg; pyridoxine-HCL, 6.5 mg; D-biotin, 0.43 mg; folic acid, 2.5 mg; menadione dimethylpyrimidinol bisulfate, 4 mg; vitamin A (retinyl acetate), 12500 IU; vitamin D (cholecalciferol), 5000 IU; vitamin E (DL- α -Tocopheryl acetate), 70 IU; ascorbic acid, 280 mg.

² Provided per kilogram of diet: MgSO₄·5H₂O, 3.1 g; ZnCO₃, 0.10 g; ZnSO₄, 0.4 g; CaCO₃, 0.32 g; MnSO₄·H₂O, 0.65 g; MnO, 0.22 g; FeSO₄·7H₂O, 0.42 g; KI, 43 mg; CuSO₄·5H₂O, 30 mg; Na₂MoO₄·2H₂O, 10 mg; H₃BO₃, 8.6 mg; CoSO₄·7H₂O, 1.18 mg; Na₂SeO₃, 0.35 mg. The carrier was calcium carbonate, and the premix contained less than 0.7% mineral oil.

³ Crystalline amino acids were assumed to be 100% digestible.

⁴ 5 g chromic oxide per kg of diet. The premix was added at a ratio 1 : 3 of chromic oxide : corn starch.

Table 4 Nutritional composition of diets (Experiment 2)

	Dietary treatments			
	Casein	Glutamic acid	Glutamine	Glutamine + glutamic acid
Analysed composition				
Crude protein (g/kg)	252.4	234.7	252	248.4
Calcium (g/kg)	11.1	11.1	11.0	11.0
Lysine (g/kg)	19.6	19.5	19.5	19.5
Methionine (g/kg)	10.9	10.9	11.6	11.6
Methionine + cystine (g/kg)	11.9	11.9	11.9	11.9
Threonine (g/kg)	10.5	10.5	10.9	10.7
Tryptophan (g/kg)	3.1	3.2	3.0	3.0
Arginine (g/kg)	16.7	16.7	16.6	16.6
Valine (g/kg)	16.2	16.5	16.4	16.3
Isoleucine (g/kg)	12.8	12.7	12.7	12.8
Calculated composition				
Apparent metabolizable energy (MJ/kg)			12.67	

Results

Experiment 1: During days 0 to 14 after hatch, chickens that had *ad libitum* access to the 1% Gln supplemented diet had the highest average daily weight gain and average daily feed intake ($P < 0.05$) (Table 5). The FCR was significantly ($P < 0.05$) better at 0 - 4, 4 - 7 and 7 - 14 days old for chicks fed the 1% Gln-supplemented diet than for chicks fed the control and 2% Gln-supplemented diets. By seven days old, there were higher significant differences ($P < 0.05$) among chicks fed the experimental diets. The worst performances, including ADG and ADI, were recorded for the 2% Gln-supplemented diet.

The AID and AFD coefficients of AAs were determined for the three dietary treatments in Experiment 1 (see Tables 6, 7 and 8). Supplementing diets with Gln increased almost all digestibility coefficients including AFD and AID for Arg, Lys, Ile and Val in chickens at day 4 after hatch ($P < 0.05$). On the other hand, by increasing dietary Gln concentration, a significant drop in AID and AFD values was observed for Glu and Asp. These effects lasted and intensified until on day 7 post hatch an increase in the AID coefficient of Leu was seen for the 1% Gln-supplemented diet. On day 14 post hatch (Table 8), the impact of dietary treatments lessened and the differences were still significant only for Lys, Arg and Glu.

The intestinal morphometric parameters of three segments of small intestine of male broiler chicks in various treatments of Experiment 1 on days 4, 7 and 14 post hatch are presented in Table 9. On days 4 and 7 post hatch, there was a significant difference in the jejunum and ileum in VH and CD among treatments. Compared with the unsupplemented diet, birds fed the 1% and 2% Gln-supplemented diets had higher VH and shallower CD of jejunum and ileum. At day 7 post hatch, the increase in height of VH was observed for both Gln-supplemented diets. The muscular layer thickness, excluded from ileum records on day 4 after hatch, which was higher for Gln supplemented diets, was not affected by dietary treatments. Compared with unsupplemented groups, on day 7 post hatch, birds in groups that Gln had higher VCR in all three segments of the intestine. This impact was observed for the jejunum on days 4 and 14 after hatch only.

Experiment 2: During the 0 - 4, 4 - 7 and 7 - 14 day periods, performance was influenced significantly by dietary treatments (Table 10). In comparison with synthetic AA-based diets, which were fortified with Gln, Glu and a 1 : 1 blend of these two non-essential AAs, broiler chicken groups fed a diet based on casein as the source of intact protein presented higher ADG and better FCR at day 4 post hatch. This effect lasted partially in day 7 and 14 records. Casein-fed birds showed higher ADG and ADI at days 7 and 14 post hatch. For synthetic AA-based diets, lower FCR values were observed at all ages. In Experiment 2, none of the AID coefficient of AAs was influenced by the dietary source of AAs, except Glu and Asp ($P < 0.05$), which decreased clearly in broiler chicks fed Glu, Gln or a blend of the two (Table 11). This effect was not observed for Asp at day 7 of sampling.

Table 5 Effect of dietary glutamine concentration on growth performance measured in 4-, 7- and 14-day-old male broiler chickens (Experiment 1)

Variable	Supplemented glutamine level			SEM
	0%	1%	2%	
0 - 4 d				
ADG (g/d)	15.97 ^c	17.13 ^a	16.89 ^b	0.32
ADI (g/d)	16.60 ^b	17.00 ^a	16.77 ^b	0.36
FCR	1.02 ^a	0.98 ^b	1.01 ^a	0.01
4 - 7 d				
ADG (g/d)	25.23 ^b	28.68 ^a	25.88 ^b	0.39
ADI (g/d)	33.30 ^b	36.30 ^a	33.90 ^b	0.40
FCR	1.30 ^a	1.26 ^b	1.31 ^a	0.01
7 - 14 d				
ADG (g/d)	36.94 ^b	38.74 ^a	34.32 ^c	0.57
ADI (g/d)	54.30	55.36	54.18	0.63
FCR	1.48 ^b	1.41 ^c	1.56 ^a	0.02

^{a,b} Different superscripts within a row indicate significant differences among treatments ($P < 0.05$).
 ADG: average daily gain; ADI: average daily feed intake;
 FCR: feed conversion ratio [(bodyweight-initial body weight) / cumulative feed intake].

Table 6 Effect of dietary glutamine concentration on apparent faecal digestibility (AFD) and apparent ileal digestibility (AID) coefficients (%), mean) of amino acids in a complete diet measured in 4-day-old male broiler chickens (Experiment 1)

Amino acids	Supplemented glutamine level						Pooled SEM for AFD	Pooled SEM for AID
	0%		1%		2%			
	AFD	AID	AFD	AID	AFD	AID		
Lysine	78	79 ^c	79	81 ^b	80	84 ^a	0.80	0.63
Methionine	84	84	84	84	84	84	0.97	0.93
Cysteine	65	68	65	68	65	68	0.85	0.64
Isoleucine	79 ^b	82 ^b	82 ^a	84 ^a	82 ^a	84 ^a	0.72	0.78
Valine	78	81 ^b	79	83 ^a	79	83 ^a	0.90	0.44
Leucine	80	81	80	82	81	82	1.07	0.97
Threonine	77	77	79	80	79	80	1.87	1.29
Tryptophan	78	79	78	79	78	79	0.58	0.55
Histidine	81	81	81	80	82	82	0.86	0.83
Phenylalanine	75	77	75	77	74	77	1.04	0.66
Arginine	73 ^c	75 ^c	75 ^b	79 ^b	78 ^a	82 ^a	0.61	0.46
Proline	75	82	75	81	75	81	1.07	1.12
Glutamate	82 ^a	77 ^a	82 ^a	77 ^a	79 ^b	73 ^b	1.45	1.11
Aspartate	78	79 ^a	79	79 ^a	78	74 ^b	1.77	1.17
Alanine	80	80	80	80	79	79	1.39	1.47
Serine	77	78	77	77	78	77	1.19	0.96

^{a,b} Different superscripts within a row indicate significant differences among treatments ($P < 0.05$).
 AFD and AID records were compared separately.

Table 7 Effect of dietary glutamine concentration on apparent faecal digestibility (AFD) and apparent ileal digestibility (AID) coefficients (%; mean) of amino acids in a complete diet, measured in 7-day-old broiler chickens (Experiment 1)

Amino acids	Supplemented glutamine level						Pooled SEM for AFD	Pooled SEM for AID
	0%		1%		2%			
	AFD	AID	AFD	AID	AFD	AID		
Lysine	85 ^b	85 ^c	87 ^a	88 ^b	88 ^a	91 ^a	0.63	0.52
Methionine	87	85	87	85	87	85	0.43	0.42
Cysteine	73	76	75	76	75	76	0.47	0.40
Isoleucine	85 ^b	85 ^b	87 ^a	88 ^a	87 ^a	88 ^a	0.62	0.68
Valine	84 ^b	87 ^b	86 ^b	89 ^b	85 ^{ab}	88 ^{ab}	0.29	0.28
Leucine	86	88 ^b	87	90 ^a	86	88 ^b	0.48	0.35
Threonine	83	85	83	85	83	85	0.90	0.61
Tryptophan	85	86	85	86	84	86	0.28	0.30
Histidine	83	86	85	86	83	86	0.46	0.33
Phenylalanine	81	85	80	85	80	85	0.68	0.69
Arginine	86 ^c	87 ^c	89 ^b	90 ^b	92 ^a	93 ^a	0.31	0.25
Proline	88	89	88	89	87	89	0.33	0.24
Glutamate	90 ^a	92 ^a	88 ^b	89 ^b	86 ^c	84 ^c	0.57	0.34
Aspartate	86 ^a	88 ^a	83 ^b	84 ^b	83 ^c	81 ^c	0.87	0.41
Alanine	88	90	88	90	88	90	0.30	0.34
Serine	86	87	85	87	85	86	0.66	0.50

^{a,b} Different superscripts within a row indicate significant differences among treatments ($P < 0.05$). AFD and AID records were compared separately.

Discussion

The effects of dietary CP content on the AID values of AA and protein have been reported in broilers (Donkoh & Moughan, 1994), rats (Sarwar & Peace, 1986; Donkon & Moughan, 1994) and growing pigs (Furuya & Kaji, 1989; Keith & Bell, 1991; Fan *et al.*, 1994), indicating that an increase of dietary protein might lead to a greater AID of AAs. Eggum (1973) illustrated that AFD of CP in soybean meal increased curvilinearly with increasing CP concentration in a rat diet. Comparably, an increase in AID values was reported as the dietary CP content increased from 4% to 24% in pigs, in which AID values gradually reached a plateau and became independent of CP (Fan *et al.*, 1994). Fan *et al.*, (1994) reported quadratic plateau relationships between the dietary AA contents and their AID. AID alteration of Glu in response to its dietary content, unlike other AAs, tended to follow a pattern in which, after reaching the plateau, AID of Glu tended to decrease as its dietary level was increased (Fan *et al.*, 1994). This decrease was observed in Experiment 1, in which fortifying a diet with 2% Gln led to a decrease of AID and AFD coefficients of Glu. One of the most common theories to explain the decrease of AA absorption in high-protein diets is the limitation in uptake of free AAs and small peptides by enterocytes (Webb, 1990). In these circumstances, the concentration of free form AAs exceeds the maximum capacity of the intestinal transport system. Nevertheless, this theory did not make complete sense in the observations of the first experiment which, despite consistency of all dietary essential AAs, AID and AFD of Lys, Arg, Val, Ile and Asp, were influenced by dietary Glu level. The presence of free form of AA in the lumen of the intestine, induces AA transport systems (Diamond & Karasov, 1987) and the secretion of pancreatic proteases (Johnson, 2001). Thus, AAs in the lumen of the intestine from crystalline origin might boost the absorption capacity. The results of the second experiment – in which a perceptible amount of crystalline AA was replaced with casein as an intact protein-bound AA, but did not affect AID values apart from Glu and Asp – are conspicuous evidence that the quantity of free form of AA in the intestine lumen does not play a major role in absorption and metabolism of AAs. Otto *et al.* (2003) reported that the presence of crystalline AA in reduced CP diets of growing pigs may raise AID values of all essential and non-essential AAs linearly. In contrast, Chung & Baker (1992) reported

higher digestibility values for protein-bound diets than crystalline AA-based diets in growing pigs and roosters. It remains puzzling that some interactions occur for intestinal absorption between intact protein-bound AA and crystalline ones. The high inclusion of crystalline AA often results in a reduction of performance. This is attributed by some researchers to dwindling appetite (Han & Baker, 1993; Carew *et al.*, 1998; Si *et al.*, 2004; Namroud *et al.*, 2008; 2010). The drastic reduction in feed intake was observed in the second experiment in days 4 - 7 and 7 - 14 post hatch owing to the exclusion of casein from the diet as the source of intact protein-bound AAs, but the AID and AFD values were not affected. The notable point in the present study was that the concentration of all essential AAs plus Gly, Pro, Ala, Asp + Asn and Glu + Gln remained constant, and the only variable was the source of Glu + Gln.

Table 8 Effect of dietary glutamine concentration on apparent faecal digestibility (AFD) and apparent ileal digestibility (AID) coefficients (%; mean) of amino acid in a complete diet measured in 14-day-old broiler chickens (Experiment 1)

Amino acids	Supplemented glutamine level						Pooled SEM for AFD	Pooled SEM for AID
	0%		1%		2%			
	AFD	AID	AFD	AID	AFD	AID		
Lysine	89 ^b	90 ^b	90 ^{ab}	93 ^a	91 ^a	93 ^a	0.54	0.39
Methionine	89	88	89	89	88	88	0.89	0.66
Cysteine	79	80	79	79	80	79	0.93	0.47
Isoleucine	88	90	88	89	87	90	0.99	0.61
Valine	89	89	88	90	89	90	1.05	0.63
Leucine	87	90	87	90	88	89	0.58	0.44
Threonine	88	88	88	89	88	88	1.37	0.77
Tryptophan	90	92	91	91	89	90	0.40	0.32
Histidine	89	89	88	89	88	89	0.46	0.39
Phenylalanine	88	88	86	89	87	88	0.86	0.75
Arginine	90	90 ^b	90	93 ^a	92	94 ^a	1.75	0.39
Proline	92	95	93	93	92	93	0.56	0.28
Glutamate	94	93 ^b	92	95 ^a	94	96 ^a	0.84	0.88
Aspartate	94	97	94	96	93	95	1.15	0.80
Alanine	94	96	92	95	93	95	0.66	0.39
Serine	95	95	95	94	94	93	0.92	0.52

^{a,b} Different superscripts within a row indicate significant differences among treatments ($P < 0.05$). AFD and AID records were compared separately.

With regard to gastrointestinal development, Maiorka *et al.*, (2000) reported that 1% Gln supplementation led to positive effects on VH, CD and the VCR in the duodenum and on VH in the ileum at 7 days of age. According to the current findings, Gln supplementation (Experiment 1) led to overall improvement in intestinal morphology, including VH, CD and VCR, until 14 days old. In partial agreement with these findings, Nassiri Moghaddam & Alizadeh-Ghamsari (2013) reported the beneficial influence of 1% or 1.5% Gln supplementation of diet on increases in VH and villus surface area of the duodenum and jejunum of 21 and 42-day-old broilers. Benefits from Gln supplementation on small intestine morphology of chicks were observed by Khempaka *et al.* (2011). Pohlenz *et al.* (2012) demonstrated the efficient utilization of Gln by intestinal mucosal cells of channel catfish, which led to improvement of intestinal microstructure. However, this did not result in improved growth performance at the end of the trial. The positive influence of

Gln and Arg supplementation on morphological measurements and probable nutrient absorption of the gastrointestinal tract was seen in hybrid striped bass (Cheng *et al.*, 2012). The results of Experiment 1 indicate that, along with better performance, diets supplemented with Gln resulted in better development of the intestinal mucosa in neonate broiler chickens. Longer villi and higher VCR facilitate the absorption of nutrients due to increased absorption area, thereby potentially influencing the digestibility values of AA.

Table 9 Intestinal morphology of the small intestine of broiler chickens on days 4, 7 and 14 post hatch in various treatments (Experiment 1)

Treatment	Villus height (μm)			Crypt depth (μm)			Muscular layer thickness (μm)			VH : CD ratio		
	D	J	I	D	J	I	D	J	I	D	J	I
Day 4 post hatch												
0% Gln	453.5	411.5 ^b	212.8 ^c	100.8	94.4 ^a	77.9 ^a	138	160	103 ^b	4.5	4.4 ^b	2.7
1% Gln	451.5	463.2 ^a	233.9 ^b	100.5	85.0 ^b	76.5 ^a	142	160	133 ^a	4.5	5.4 ^a	3.1
2% Gln	460.1	470.2 ^a	247.5 ^a	102.1	84.3 ^b	70.3 ^b	143	164	134 ^a	4.5	5.6 ^a	3.5
SEM	11.61	10.17	7.05	3.36	2.11	1.42	7.27	8.16	4.58	0.12	0.24	0.18
Day 7 post hatch												
0% Gln	605.9 ^b	530.9 ^b	251.4 ^b	155.4	127.7 ^a	80.2 ^a	175	178	129	3.9 ^b	4.2 ^b	3.1 ^b
1% Gln	688.4 ^a	615.7 ^a	303.8 ^a	153.0	105.6 ^b	75.1 ^b	172	173	134	4.5 ^a	5.8 ^a	4.0 ^a
2% Gln	695.1 ^a	608.1 ^a	310.2 ^a	156.3	103.6 ^b	73.6 ^b	170	177	136	4.4 ^a	5.9 ^a	4.2 ^a
SEM	14.93	12.26	11.07	4.01	2.85	1.48	8.02	12.49	9.41	0.15	0.20	0.21
Day 14 post hatch												
0% Gln	1217.5	936.1 ^b	689.6 ^b	192.7	190.5	96.7	168	178	131	6.3	4.9 ^b	7.2
1% Gln	1231.7	1104.8 ^a	719.8 ^a	188.1	185.4	95.2	174	186	133	6.5	6.0 ^a	7.6
2% Gln	1227.1	955.7 ^b	680.1 ^b	192.2	189.1	93.7	170	183	135	6.4	5.0 ^b	7.3
SEM	24.56	17.44	11.15	5.59	3.67	4.60	11.51	13.30	12.70	0.22	0.28	0.38

^{a,b} Different superscripts in a column of each period indicate significant differences among treatments ($P < 0.05$). Gln: glutamine; D: duodenum; J: jejunum; I: ileum.

An indicative influence of dietary Gln on the AID and AFD values of cationic, branched-chain and anionic AA was found in the first study. Raising the dietary Gln level from 0% to 2% resulted in higher AFD and AID coefficients for cationic AAs, except histidine, but, conversely, led to a lower digestibility coefficient for anionic AAs involving Glu and Asp. The increase in AID and AFD with increasing dietary Gln was found for branched-chain AAs, including Ile, Leu and Val. The results of first study support the effect of dietary Gln on other AA digestibilities. To the authors' knowledge, the effect of Gln on ADI of other AAs has not been studied in poultry, but the reduced impact of high dosage dietary Glu on the AID value of Glu has been reported in pigs (Fan *et al.*, 1994). The results of the second experiment showed that no differences between free and protein-bound forms of dietary Gln and/or Glu impact on AA digestibilities, except for Glu and Asp. The dietary free form of Glu and Gln led to a decrease of AID values of Asp and Glu, which were not seen in the protein-bound form (casein) dietary treatment. The effect of dietary protein quality and quantity on the expression of AA transporter genes in brush border membrane of intestine was reported (Chen *et al.*, 2005; Gilbert *et al.*, 2008). Amino acids, particularly Gln, can be used as source of energy. To complicate matters, enterocytes express transporter molecules with overlapping substrate specificity at a different velocity, which mediates the absorption of AAs (Karasov *et al.*, 1987; Gilbert *et al.*, 2008). The lack of data and complications of transporter responses to affectors, including nutritional parameters, make it difficult to predict transporter regulation in response to certain AAs or imbalances among AAs. Although the effect of Gln on molecular characteristics of the AA transporter was not studied in the present experiment, it may have undefined effects according to its clear influence on digestibility of certain limiting AAs, including Lys, Arg, Ile and Val, and the development of morphometric parameters of the intestine. Greater knowledge of the ways in which dietary Gln may regulate the expression and activity of transporter genes (velocity and specificity)

Table 10 Effect of glutamine (Gln) and glutamic acid source (Glu) on the growth performance measured in 4-, 7- and 14-day old male broiler chickens (Experiment 2)

Variable	Glutamine and glutamic acid source				SEM
	Casein	Gln	Glu	Gln + Glu	
0 - 4 days					
ADG (g/d)	14.84 ^a	13.13 ^b	13.03 ^b	13.38 ^b	0.41
ADI (g/d)	15.04	14.13	14.07	14.31	0.37
FCR	1.01 ^b	1.07 ^a	1.08 ^a	1.07 ^a	0.02
4 - 7 days					
ADG (g/d)	22.39 ^a	19.08 ^b	19.68 ^b	18.63 ^b	0.45
ADI (g/d)	25.47 ^a	19.61 ^b	19.93 ^b	19.80 ^b	0.57
FCR	1.14 ^a	1.02 ^c	1.01 ^c	1.06 ^b	0.02
7 - 14 d					
ADG (g/d)	33.72 ^a	24.39 ^b	25.04 ^b	24.59 ^b	0.52
ADI (g/d)	48.87 ^a	33.06 ^b	32.75 ^b	33.53 ^b	0.69
FCR	1.45 ^a	1.35 ^b	1.31 ^b	1.36 ^b	0.04

^{a,b} Different superscripts within a row indicate significant differences among treatments ($P < 0.05$).

ADG: average daily gain; ADI: average daily feed intake.

FCR: feed conversion ratio = [(bodyweight-initial bodyweight) / cumulative feed intake].

Table 11 Effect of glutamine (Gln) and glutamic acid source (Glu) on apparent ileal digestibility coefficient (% , mean) of amino acids in a completely purified diet (Experiment 2)

	Amino acids															
	Lys	Met	Cys	Ile	Leu	Val	Thr	Trp	His	Arg	Phe	Pro	Glu	Asp	Ala	Ser
Day 4 post hatch																
Casein	90	93	88	94	94	93	90	91	95	90	92	95	94 ^a	95 ^a	90	90
Gln	91	95	88	93	94	93	91	92	94	92	95	93	87 ^b	91 ^b	91	90
Glu	90	95	88	94	94	92	88	92	96	90	95	94	84 ^c	92 ^b	92	90
Gln+Glu	92	95	88	94	92	91	91	93	94	90	93	93	89 ^b	90 ^b	90	90
SEM	0.54	0.79	0.26	0.54	0.58	0.81	1.57	0.42	1.11	0.85	1.32	0.92	0.83	0.66	0.59	0.76
Day 7 post hatch																
Casein	95	95	89	97	96	95	95	96	95	95	93 ^b	94	96 ^a	96	96	94
Gln	97	93	89	95	95	95	95	95	96	94	96 ^a	95	92 ^b	95	96	96
Glu	96	95	90	95	96	96	95	95	96	94	96 ^a	96	93 ^b	95	96	95
Gln+Glu	96	95	90	95	96	95	94	94	95	94	96 ^a	94	92 ^b	95	96	95
SEM	0.97	0.66	0.57	0.94	0.80	0.73	1.41	1.03	0.64	0.86	0.71	1.05	0.93	0.88	0.54	0.99
Day 14 post hatch																
Casein	95	94	93	95	96	96	94	97	96	97	96	97	96 ^a	97 ^a	95	96
Gln	95	94	92	96	97	95	94	98	97	97	96	98	91 ^b	93 ^b	95	96
Glu	96	93	92	96	96	95	94	98	97	98	96	96	89 ^b	93 ^b	96	96
Gln+Glu	95	93	91	96	96	95	93	98	96	97	96	97	91 ^b	94 ^b	94	97
SEM	0.52	0.57	0.78	0.64	0.83	0.70	1.05	0.41	0.59	0.82	0.63	1.26	1.15	1.04	0.80	0.67

^{a,b} Different superscripts within a column of each period indicate significant differences among means ($P < 0.05$).

Lys: lysine, Met: methionine, Cys: cystine, Thr: threonine, Trp: tryptophan, Arg: arginine, Val: valine, Ise: isoleucine, Leu: leucine, His: histidine.

would allow the discovery of ways to manipulate regulatory mechanisms to increase the capacity and efficiency of limiting AA absorption, and, thereby, optimization of dietary AA balance.

Feeding diets with a high level of synthetic AA resulted in increased blood ammonia level and decreased plasma uric acid concentration (Namroud *et al.*, 2008). Noda (1975) confirmed that the blood ammonia level may act as a signal to regulate appetite in rats. Intraportal infusion of a small amount of ammonia salts led to depression of appetite in fasted dogs (Russek, 1971). The current observations in Experiment 2 – including lower daily feed intake and subsequent lower bodyweight gain in newly hatched broiler chickens fed free AA-included diets – was in agreement with the authors' previous study in 2008. According to blood ammonia records in Experiment 2 (not published), aminostatic hypothesis (Leung & Rogers, 1969; Peng & Harper, 1970) may play a role in the control of appetite.

Conclusion

In conclusion, 1% L-Gln supplementation to post-hatch broilers, along with improvement in performance, modified the mucosal integrity of the small intestine, and led to increments in the digestibility values of certain AAs, including L-Lys, L-Arg, L-Ile and L-Val, which could be used to manipulate dietary AA as an economic strategy. Although excluding intact protein-bound AAs and supplementing Gln in crystalline form in diets affected performance and morphometric parameters negatively, they did not influence the digestibility values of other amino acids.

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Authors' Contributions

The research was conducted as part of NFN's PhD thesis in Poultry Nutrition. MS, MZ and OM supervised the whole study. KN and NFN did the statistical analyses. Finally, all authors commented on early and final versions of the manuscript.

Conflict of Interest Declaration

The authors have declared that there is no conflict of interests.

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