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ORIGINAL ARTICLE

Immune response in *Eimeria* sp. and *E. coli* challenged broilers supplemented with amino acids

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ABSTRACT. The aim of this study was to evaluate the immune response of broiler chickens in *Eimeria* sp. and *E. coli* challenged broilers supplemented with glutamine, arginine and threonine. There were six hundred one-day-old male Cobb 500 broiler chickens. The design was completely randomised using a 2 x 3 factorial design (unchallenged and challenged x 3 diets). A commercial diet was used as a control and two other diets were formulated with glutamine (1.5 and 3% Aminogut®), arginine (1 and 2% L-Arginine), and threonine (1 and 2% L-threonine). At day 28, the birds fed the highest level of amino acid showed lower levels of IgA ($P<0.05$) compared to the control group, and resulted in an increased number of goblet cells. In the period of 1 week after the challenge, the challenged birds showed lower measures ($P<0.05$) of the thymus compared to the birds that had not undergone challenge. At day 28, unchallenged birds showed a reduction ($P<0.05$) in splenic measures in the treatment with the highest level of amino acids. The highest measures were correlated to control birds that were not challenged. At day 21, blood urea levels were increased ($P<0.05$) for birds fed amino acids supplementation compared to those which received no supplementation. After 28 days, uric acid levels were similar between the two supplemented diets. These results suggest that diet supplementation with amino acids above the recommended levels for growth may be necessary to improve the immune response against an *Eimeria and E. coli* challenge.

Key words: aminoacid, immune response, coccidiosis, broilers.

RESUMEN. El objetivo del estudio fue evaluar la respuesta inmune de pollos de engorde desafiados con *Eimeria* sp. y *E. coli*, y suplementados con glutamina, arginina y treonina. Se utilizaron 600 pollos machos de 1 día de edad de la línea comercial Cobb 500. Se usó un diseño completamente al azar en un arreglo factorial 2x3 (con y sin desafío y 3 dietas experimentales). Se utilizó una dieta comercial como control y otras dos dietas fueron formuladas con glutamina (1,5 y 3% Aminogut®), arginina (1 y 2% de L-arginina) y treonina (1 y 2% de L-treonina). A los 28 días, las aves que recibieron el mayor nivel de aminoácidos mostraron niveles más bajos de IgA ($P<0,05$) comparadas al grupo control, y aumentaron el número de células caliciformes. Una semana después del desafío, las aves desafiadas mostraron medidas más bajas ($P<0,05$) del timo en comparación con aves no desafiadas. A los 28 días, en aves no desafiadas hubo una reducción ($P<0,05$) en las medidas esplénicas en el tratamiento con mayor nivel de aminoácidos. Las medidas más altas se correlacionaron con aves control no desafiadas. A los 21 días, los niveles de urea en la sangre aumentaron ($P<0,05$) en aves que recibieron suplementación de aminoácidos. La suplementación dietética con aminoácidos en las aves desafiadas influyó en el sistema inmunológico, alterando las mediciones morfométricas del bazo y timo, y resultó en una disminución de los niveles de IgA. Igualmente, aumentaron las concentraciones séricas de ácido úrico, urea y también de la enzima hepática AST, independientemente del desafío.

Palabras clave: aminoácido, respuesta inmune, coccidiosis, pollos.

INTRODUCTION

A health standard is imperative to allow chickens to express their maximum genetic potential. When the immune system is activated, there are physiological and metabolic changes in their organism, which alter food intake and consequently weight gain and feed conversion ratio. In commercial broiler breeding, environmental stress factors such as overcrowding, humidity, low quality of the air and the presence of infectious and stressing agents may alter their metabolism and nutrient absorption (Norup *et al* 2008).

The mucosa epithelium of the digestive tract represents an important barrier against a broad spectrum of significant immunogenic substances within the intestinal lumen and epithelium of intestinal mucosa (Dignass 2001). Under inflammatory conditions of the mucosa, increased turnover of body protein may be the most significant change with greater impact on growth and weight gain (Hill and Hill 1998).

However, the profile of the amino acids released from muscle proteolysis might not meet the specific requirements in these conditions, resulting in excessive use of proteins with high metabolic cost to the organism (Reeds *et al* 1994). Thus, the supply of potentially important amino acids for the immune response could reduce muscle loss, and accelerate recovery in inflammatory processes.

Glutamine (Gln) is considered an essential amino acid in certain species under inflammatory conditions (Newsholme 2001, Wang *et al* 2015). The role of this amino acid is well recognised as the main fuel for the intestinal mucosa and immune cells (Calder 1995, Wu and Morris 1998). In birds, threonine is a precursor of glycine and serine and is involved in the immune response, comprising the immune systems globulins and is necessary in gastro-intestinal mucin production (Lemme 2001, Moghaddam *et al* 2011). The use of threonine has been documented by Wang *et al* (2006) in increasing serum antibody titers as well as the

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concentrations of IgA and IgG and the decrease of IL-6, cytokine associated with decreased appetite, within the mucosa of the jejunum of broiler chickens challenged with *E. coli*. Bartell and Batal (2007) reported that the inclusion of 1% glutamine in the diet of broilers had increased the production of IgA and IgG.

Dietary supplementation of Arg and Glu reduce the presence of pro-inflammatory genes in the small intestine (Fu *et al* 2005, Wang *et al* 2008, Tan *et al* 2014[b]) and increase the concentration of polyamines that have a direct effect on cell division, protein synthesis and tissue growth (Wu and Morris 1998). Arg is considered an essential amino acid for poultry, for the fact that the biochemical cycle of urea is not functional in birds (Austic and Nesheim 1971), so they are dependent on dietary Arg.

Arg can be used for the synthesis of polyamines (putrescine, spermine and spermidine) or proline, it must be hydrolysed into ornithine and urea through arginase (Meijer *et al* 1990, Wu and Morris 1998). Plasma urea is combined with supplementation of Arg, and between 40 and 60% of urea excreted by the birds is the result of ornithine synthesis (Ruiz-Feria *et al* 2001).

In infectious processes, activated cells such as macrophages, neutrophils and endothelial cells secrete NO (nitric oxide) from Arg and reactive oxygen intermediates. However, Hartman *et al* (2006) reported that the cells of birds are less prone to adverse effects from these free radicals by having high circulating levels of uric acid. Uric acid has recently been considered an important antioxidant (Hare and Johnson 2003).

The immune response related to infection with coccidiosis involves non-specific response ensured by the innate response and specificity of the response (Ruff 1999). Immediately after infection by *Eimeria spp*., β cells in the intestinal mucosa begin to produce antibodies. In chickens, the predominant immunoglobulin in intestinal secretions is IgA that is able to cross directly through the epithelial surface (Wallach 2010).

A better understanding of the role amino acids have in the immune response against challenging situations in the intestinal mucosa, and the understanding of the mechanisms involved in the modulation of the immune system is essential to maintain the health, performance and economic results of the poultry production. The objective of this study was to evaluate the immune system of broiler chickens submitted to an experimental model of infection and supplemented with amino acids: glutamine, arginine and threonine.

MATERIAL AND METHODS

The research was conducted in the Experimental Poultry Department of Federal University of Paraná, Palotina, PR, Brazil. All procedures of animal use and biological material collection were approved by the Ethics Committee of Animal Use in Experimentation under the protocol number 04/2012.

Six hundred one-day-old male Cobb broiler chicks were utilized. A completely randomized design was used in a 2 × 3 factorial scheme (unchallenged and challenged x 3 diets), totaling 6 treatments and 5 replications with 20 broilers each. The treatments were: treatment 1) unchallenged + diet 1; treatment 2) unchallenged + diet 2; treatment 3) unchallenged + diet 3; treatment 4) challenged + diet 1; treatment 5) challenged + diet 2; treatment 6) challenged + diet 3.

The diets consisted of the following: Diet 1) commercial diet; Diet 2) commercial diet + Aminogut®(1.5%) + L-arginine (1%) + L-threonine (1%); Diet 3) commercial diet + Aminogut® (3%) + L-arginine (2%) + L-threonine (2%). Aminogut® is a commercial source of glutamine associated with glutamic acid (10% from each other); L-Arginine contains 99% Arg, and L-Threonine contains 99% Thr.

The addition of supplemented levels resulted in 2.340 and 4.680% of digestible Arg, 1.781 and 3.562% of digestible Thr and 4.010 and 4.160% of Glu + Gln digestible in the starter diet 2 and diet 3, respectively. In the growth feed, the final concentrations were 2.230 and 4.460% of digestible Arg, 1.716 and 3.432% of digestible Thr and 3.812 and 3.962% of Glu + Gln digestible, to diet 2 and diet 3, respectively.

At 10 d of age, broilers were fed commercial feed, and after 11 d, experimental diets were provided. The diets, based on corn and soybean meal were formulated according to values of the chemical composition of feedstuffs and the nutritional recommendations adopted by the regional poultry industry (table 1). The feeding program was divided into 3 periods: starter (1 to 10 d), grower (11 to 29 d), and finisher (30 to 42 d).

EXPERIMENTAL CHALLENGE

At 14 days of age, the group of challenged broilers received a commercial vaccine for coccidiosis that targets *Eimeria acervulina, Eimeria maxima, Eimeria praecox, Eimeria tenella,* and *Eimeria mitis*. Prior to the application of the vaccine, sporulation of oocytes was performed by injection of O_2 directly into Falcon tubes with the vaccine, which were quickly sealed and maintained in a BOD oven at 82.4ºF for 48 h. The vaccine was inoculated directly into the gizzard of each broiler at 20 times the dose recommended by the manufacturer (±80,000 oocytes). In previous studies from our laboratory, this dose has been used to induce only moderate intestinal damage, but not birds mortality.

Following coccidial infection, an increased mucosal barrier permeability exposes underlying immune cells to pathogens, further compromising the barrier function. Therefore, after 2 d, an inoculum containing *E. coli* was provided in the drinking water at a concentration of 108 CFU/d/broiler during 2 consecutive d to the same group challenged with the vaccine. Thus, the so-called challenged

Table 1. Percentage and calculated composition of experimental diets of broilers in period of grower (11 to 29 days) and finisher (30 to 42 days).

Ingredient (%)	Grower	Finisher
Corn	41.55	45.78
Soy Bean	39.30	35.10
Soy Oil	8.10	8.20
Salt	0.31	0.38
Calcitic Limestone	1.08	1.03
Dicalcium phosphate	1.72	1.70
Sodium Bicarbonate	0.10	0.00
DL-Methionine 98%	0.35	0.33
L- Lysine 50,7%	0.23	0.23
L-Threonine 98%	0.08	0.07
Cholinechloride 60%	0.08	0.08
Premix mineral and vitaminic[1,2]	0.10	0.10
Kaolin*	0-7	0-7
Calculated values		
Protein, %	21.63	19.98
EM, Kcal/Kg	3,000	3,050
Calcium, %	0.929	0.900
Phosphorus, %	0.440	0.410
Lysine Dig., %	1.200	1.100
Methionine Dig., %	0.625	0.590
Met + Cis Dig., %	0.912	0.859
Threonine Dig., %	0.791	0.726
Tryptophan Dig., %	0.237	0.216
Arginine Dig., %	1.356	1.240

*Replaced by the inclusion of amino acids in the diet 2 (1.5kg Aminogut® of L-Arginine 1 kg and 1 kg of L-Threonine) and diet 3 (3 kg Aminogut®, 2 kg of L arginine and 2 kg of L-Threonine).
[1]Vitamin mixture initial (content per kg of premix): Vit. The UI 7,000,000.00; Vit. D3 2,200,000.00 UI; Vit. E 11,000.00 mg; Vit. K3 1,600.00 mg; Vit. B1 2,000.00 mg; Vit. B2 5,000.00 mg, Vit. B12 12,000.00 mg; Niacin 35,000.00 mg; Pantothenic Acid 13,000.00 mg; Folic Acid 800.00 mg; Antioxidant 100,000.00.
[2]Mineral mixture (content per kg of premix): Iron 10,000.00 mg; Copper 16,000.00 mg; Iodine 2,400.00 mg; Zinc 100,000.00 mg; Manganese 140,000.00 mg; Selenium 400.00 mg.

groups were characterized by *Eimeria* and *E. coli* infection. This strain of E. coli had been isolated from feces of adult broilers and vindicated by biochemical series and PCR, courtesy of the Poultry Medicine Laboratory, Londrina State University. The total viable count of the *E. coli* population was determined using the method of spread plate on tryptone soya agar (TSA) and incubating at 37±1 °C for 48 h. The total bacterial load in inoculum reached 8 log10 CFU/mL.

MUCOSAL SAMPLE ANALYSES AND MORPHOMETRIC MEASUREMENT OF ORGANS

At 21, 28 and 42 days, 2 birds from each experimental unit were sacrificed by cervical dislocation. Approximately 5cm-length jejunum fragments (from the distal portion of the duodenal loop to the Meckel's diverticulum) were obtained, opened, fixed longitudinally on polystyrene plates and washed with saline solution. The samples were fixed in a buffered formaldehyde solution and later were embedded in paraffin (Uni *et al* 1998). Each fragment was subjected to 5 mm thick semi-serial cuts and stained with PAS (Periodic Acid Schiff). To allow the counting of goblet cells, the images were captured by light microscopy (Olympus BX 50), using a computerized image analyzer system (ImagePro-Plus - Version 5.2 - Media Cybernetics) at 40x magnification. The goblet cell count was made in images captured at 40x magnification per mm^2 of villus area.

These same birds had their intestinal mucosa scraped, which was then analyzed for levels of immunoglobulin A (IgA) by the method of enzyme-linked immunosorbent assay (ELISA). To perform the ELISA test, the plate was absorbed with the intestinal mucosa dilution of 1000ng/100ml/well, after determining protein concentration by the Bradford method.

For the morphometric measurement of the cloacal bursa, thymus and spleen, the organs were fixed in a formaldehyde solution. The images were prepared and analyzed by the computer image analyzing software (ImagePro-Plus - Version 5.2 - Media Cybernetics). They were obtained from measurements of the total area of each organ (mm^2), lymphoid tissue area (cloacal bursa) and longitudinal and transverse axis of the spleen and thymus (mm). From these measurements it was possible to obtain the measures of thymic and splenic index by multiplying the longitudinal and transverse axis of each organ, based on the methodology described by Ishibashi (1991).

To ascertain the amount of liver lymphoid clusters, tissue was fixed in buffered formaldehyde and later enclosed in paraffin. Five micrometer slices were performed, stained with the hematoxylin and eosin method (HE). With the aid of an optical microscope, ten fields were observed at a magnification of 400x. The number of lymphoid clusters found in each of the ten fields was added up to reach the total number of lymph clusters in each sample.

BIOCHEMICAL ANALYSIS

At 28 and 35 days of age, one and two weeks post-infection, 2 birds per replicate (10 birds/treatment) had their blood collected to assess the biochemical serum profile. The biochemical analysis was conducted in the chemical analyzer BS-200 Mindray® (Mindray Medical International Limited). Specific DIALAB® brand commercial kits were used to determine the serum concentrations of amino acid metabolism indicators such as: urea and uric acid besides to liver function tests: albumin, AST (Aspartate transaminase) and GGT (Gamma-glutamyltransferase or GAMMA-GT).

STATISTICAL ANALYSIS

The data was submitted to analysis of variance using the GLM procedure of the SAS program (SAS Institute, 2002) with a 5% level of significance, and in case of significant differences, the averages were compared by Tukey's test (5%). For the counting of variable lymphoid clusters, data transformation was performed by logarithmic base ten. To obtain the variable of the lgA mucosa, an analysis was made using generalized models assuming a negative binomial distribution with the standard link function being performed by the Student test.

RESULTS

The involvement of functional amino acids in the immune response of the intestinal mucosa of broilers subjected to experimental infection model was investigated. Table 2 shows the ELISA absorbance values for mucosal IgA of the jejunum. It was observed that a week after the infection, there was a smaller value ($P<0.05$) of IgA in the intestinal mucosa of the challenged birds, regardless of diet. At day 28, the birds that received the highest level of amino acid in diets showed lower levels of IgA ($P<0.05$) compared with the control group. Similarly, regardless of the challenge, IgA levels decreased according to the inclusion of amino acids (table 2).

The number of goblet cells/mm of villus is presented on table 3. Within a week after infection, the birds that were challenged with the coccidiosis vaccine and were inoculated with *E. coli* showed a higher ($P<0.05$) number of goblet cells. This was already expected since goblet cells are secreting glycoprotein mucus. At day 28, the highest level of dietary amino acid supplementation resulted in an increased number of goblet cells compared to the diet with intermediate level of supplementation.

The morphometric measurements of the thymus and spleen are presented in tables 4 and 5, respectively. In the period of 1 week after the challenge (21 days), there was a difference ($P<0.05$) between the thymic area values

Table 2. ELISA absorbance values for IgA on jejunum mucosa of broilers supplemented with amino acids, challenged or not, and collected at 21 and 28 days old.

	21 days			28 days		
	Challenged	Unchallenged	Mean	Challenged	Unchallenged	Mean
Diet 1	0.069	0.074	0.071	0.094	0.085	0.089^{A}
Diet 2	0.057	0.071	0.064	0.086	0.084	0.085^{AB}
Diet 3	0.059	0.076	0.067	0.068	0.083	0.076^{B}
Mean	0.061^{b}	0.073^{a}		0.083	0.084	
CV, %		20.39			21.28	
Interaction		ns			ns	

Diet 1: Control diet; Diet 2: Aminogut® (1,5%) + L-Arginine (1,0%) + L-Threonine (1,0%); Diet 3: Aminogut ® (3,0%) + L-Arginine (2,0%) + L-Threonine (2,0%)
^{a,b}Means followed by different lowercase letters in the same line are different from each other in challenge factor. ^{A,B}Means followed by different lowercase letters in the same column are different from each other in challenge factor.

Table 3. Goblet cell count on jejunum mucosa of broilers supplemented with amino acids and, challenged or not, and collected at 21, 28, and 42 days old.

	21days			28 days			42 days		
	C	NC	Mean	C	NC	Mean	C	NC	Mean
Diet 1	15.15	12.75	13.95	13.95	13.19	13.55^{AB}	16.75	18.98	17.93
Diet 2	16.75	11.78	14.26	12.25	11.75	12.01^{B}	18.22	18.58	18.39
Diet 3	13.55	10.50	12.11	15.50	13.76	14.63^{A}	18.81	16.93	18.29
Mean	15.15^{a}	11.72^{b}		13.89	12.94		18.32	18.12	
CV, %		25.64			21.90			27.17	
Interaction		ns			ns			ns	

C: Challenged with coccidiosis vaccine and E. coli inoculation; NC: unchallenged.
Diet 1: Control diet; Diet 2: Aminogut® (1,5%) + L-Arginine (1,0%) + L-Threonine (1,0%); Diet 3: Aminogut ® (3,0%) + L-Arginine (2,0%) + L-Threonine (2,0%)
^{a,b}Means followed by different lowercase letters in the same line are different from each other in challenge factor. ^{A,B}Means followed by different lowercase letters in the same column are different from each other in challenge factor.

Table 4. Morphometric thymus measurements of broilers fed amino acid addition, challenged or not, and collected at 21 and 28 days old.

	Thymus area, mm^2			Thymus index, mm^2		
	Challenged	Unchallenged	Mean	Challenged	Unchallenged	Mean
			21 days			
Diet 1	1111.84	1397.12	1254.48	1.04	1.74	1.39
Diet 2	1087.41	1626.28	1433.83	1.27	2.07	1.78
Diet 3	1084.21	1454.40	1232.29	1.32	1.76	1.50
Mean	1094.18^{b}	1506.49^{a}		1.21^{b}	1.88^{a}	
CV, %		39.44			44.13	
Interaction		ns			ns	
28 days						
Diet 1	1902.58	2039.91	1976.53	2.29	2.16	2.22
Diet 2	2311.22	1800.10	2055.66	2.14	2.20	2.17
Diet 3	1504.16	1559.74	1526.39	1.85	1.94	1.88
Mean	1905.99	1842.29		2.09	2.12	
CV, %		37.51			39.66	
Interaction		ns			ns	

C: Challenged with coccidiosis vaccine and E. coli inoculation; NC: unchallenged.
Diet 1: Control diet; Diet 2: Aminogut® (1,5%) + L-Arginine (1,0%) + L-Threonine (1,0%); Diet 3: Aminogut ® (3,0%) + L-Arginine (2,0%) + L-Threonine (2,0%)
^{a,b}Means followed by different lowercase letters in the same line are different from each other in challenge factor.

Table 5. Morphometric spleen measurements of broilers fed amino acid addition, challenged or not, and collected at 21 and 28 days old.

	Spleen Area, mm^2			Spleen index, mm^2		
	Challenged	Unchallenged	Mean	Challenged	Unchallenged	Mean
21 days						
Diet 1	1738.62	1827.55	1773.20	2.13	2.31	2.21
Diet 2	1783.03	1540.47	1648.27	2.38	1.99	2.15
Diet 3	1712.26	1713.88	1713.12	2.13	2.16	2.15
Mean	1743.97	1677.79		2.20	2.13	
CV, %		27.42			27.74	
Interaction		ns			ns	
28 days						
Diet 1	2497.98^{bA}	3204.11^{aA}	2777.58	3.05^{bA}	4.25^{aA}	3.51
Diet 2	2206.23^{aA}	2945.61^{aA}	2547.48	2.80^{aA}	3.85^{aA}	3.28
Diet 3	2330.19^{aA}	2014.37^{aB}	2160.14	2.86^{aA}	2.48^{aB}	2.66
Mean	2370.94	2710.16		2.93	3.51	
CV, %		23.37			27.88	
Interaction		*			*	

C: Challenged with coccidiosis vaccine and E. coli inoculation; NC: unchallenged; *: $P<0.05$
Diet 1: Control diet; Diet 2: Aminogut® (1,5%) + L-Arginine (1,0%) + L-Threonine (1,0%); Diet 3: Aminogut ® (3,0%) + L-Arginine (2,0%) + L-Threonine (2,0%)
^{a,b}Means followed by different lowercase letters in the same line are different from each other in challenge factor. ^{A,B}Means followed by different lowercase letters in the same column are different from each other in challenge factor.

and thymic index, however, at 28 days there was no effect ($P>0.05$) for such parameters. The challenged birds showed lower measures of the thymus compared to the birds that had not undergone the challenge.

There was an interaction ($P<0.05$) between dietary and challenge factors for the spleen area data and splenic index at 28 days (table 5). Amongst the unchallenged birds a reduction in splenic measures was seen, but only in the treatment

with the highest level of amino acids. The highest numbers were correlated to control birds that were not challenged.

Table 6 shows the results for the total area of the cloacal bursa, lymphoid tissue area, and the percentage of lymphoid tissue in the total area. At day 21 and 28 there was no difference ($P>0.05$) between treatments, possibly because the immune response to coccidiosis primarily involves cellular immune and humoral response and is not related to the cloacal bursa.

The results for lymphoid clusters count are shown in table 7. At day 28, there was a significant interaction ($P<0.05$) between diets and the experimental challenge. There was no difference in unchallenged birds. However, for the challenged birds there was a significant decrease ($P<0.05$) in lymphoid clusters. This result may be related to the values observed after 28 days, two weeks after infection to the detected IgA in the intestinal mucosa of the challenged birds. The decrease in IgA and lymphoid clusters showed a less aggressive response, and therefore less costly for the organism, but effective.

With regards to the biochemical analysis of urea and serum uric acid (table 8), there was no significant interaction between the studied factors. In the poultry serum examination 7 days after the challenge (21 days), it was found that blood urea levels were increased ($P<0.05$) when comparing the birds which received no supplementation with those fed diets 2 and 3, respectively. At day 28, the urea levels were similar between the two supplemented

Table 6. Morphometric cloacal bursa measurements of broilers supplemented with amino acids, challenged or not, and collected at 21 and 28 days old.

	Total area			Lymphoid tissue area			% Lymphoid tissue		
	C	NC	Mean	C	NC	Mean	C	NC	Mean
					21 days				
Diet 1	751.24	655.52	695.40	677.63	584.85	623.51	90.01	89.68	89.82
Diet 2	751.40	690.77	717.30	666.80	611	636.75	89.39	89.60	89.50
Diet 3	767.26	807.51	780.68	687.12	733.42	702.56	89.29	90.72	89.76
Mean	757.70	701.78		678.21	628.04		89.51	89.88	
CV, %		17.14			18.20			3.85	
Interaction		ns			ns			ns	
					28 days				
Diet 1	798.19	1165.21	1060.34	879.34	1083.51	1046.39	86.56	91.44	90.55
Diet 2	856.67	943.82	894.15	711.08	836.09	767.90	85.82	89.05	87.29
Diet 3	915.73	978.29	940.75	782.35	979.25	810.48	85.93	83.56	85.61
Mean	864.58	1067.60		765.66	994.09		85.97	90.12	
CV, %		27.22			26.91			5.41	
Interaction		ns			ns			ns	

C: Challenged with coccidiosis vaccine and E. coli inoculation; NC: unchallenged.
Diet 1: Control diet; Diet 2: Aminogut® (1,5%) + L-Arginine (1,0%) + L-Threonine (1,0%); Diet 3: Aminogut ® (3,0%) + L-Arginine (2,0%) + L-Threonine (2,0%)

Table 7. Liver lymphoid clusters counting of broilers fed amino acid addition, challenged or not, and collected at 21 and 28 days old.

	Challenged	Unchallenged	Mean	Challenged	Unchallenged	Mean
	21 days			28 days		
Diet 1	2.55	1.28	2	0.11^{Bb}	0.88^{Aa}	0.47
Diet 2	1.25	0.33	0.76	1.13^{Aa}	1.33^{Aa}	1.21
Diet 3	1.33	1.12	1.24	0.66^{ABa}	0.25^{Aa}	0.47
Mean	1.73	0.87		0.62	0.77	
CV, %		148.04			129.85	
Interaction		ns			*	

C: Challenged with coccidiosis vaccine and E. coli inoculation; NC: unchallenged; *: $P<0.05$
Diet 1: Control diet; Diet 2: Aminogut® (1,5%) + L-Arginine (1,0%) + L-Threonine (1,0%); Diet 3: Aminogut ® (3,0%) + L-Arginine (2,0%) + L-Threonine (2,0%)
^{a,b}Means followed by different lowercase letters in the same line are different from each other in challenge factor. ^{A,B}Means followed by different lowercase letters in the same column are different from each other in challenge factor.

diets; however the values were higher when compared to the control diet. These levels were not affected ($P>0.05$) by the experimental challenge.

After 28 days, the uric acid levels are proportional to the addition of amino acids to the diet. In this period there was a mobilization of amino acids for the immune response and the excess started being excreted 2 weeks after the challenge ended.

Table 9 shows the liver function tests to levels of the AST enzyme (aspartate transaminase), a significant difference was observed between the birds fed the control diet and the birds that received supplementation at higher

Table 8. Urea and uric acid concentrations of broilers supplemented with amino acids, challenged or not, and collected at 21 and 28 days old.

	Urea, mg/dL			Uric Acid, mg/dL		
	Challenged	Unchallenged	Mean	Challenged	Unchallenged	Mean
			21 days			
Diet 1	6.650	6.510	6.580^{C}	1.450	1.490	1.470
Diet 2	10.290	9.400	9.845^{B}	1.640	1.330	1.485
Diet 3	14.170	12.560	13.365^{A}	1.578	1.510	1.542
Mean	10.370	9.490		1.555	1.443	
CV, %		19.88			32.28	
Interaction		ns			ns	
			28 days			
Diet 1	11.400	9.667	10.482^{B}	4.500	5.489	5.024^{B}
Diet 2	11.610	12.280	11.945^{AB}	6.670	6.130	6.400^{AB}
Diet 3	14.570	13.130	13.850^{A}	8.650	8.110	8.380^{A}
Mean	12.607	11.762		6.757	6.614	
CV, %		34.08			38.01	
Interaction		ns			ns	

C: Challenged with coccidiosis vaccine and E. coli inoculation; NC: unchallenged.
Diet 1: Control diet; Diet 2: Aminogut® (1,5%) + L-Arginine (1,0%) + L-Threonine (1,0%); Diet 3: Aminogut ® (3,0%) + L-Arginine (2,0%) + L-Threonine (2,0%)
[a,b]Means followed by different lowercase letters in the same line are different from each other in challenge factor. [A,B]Means followed by different lowercase letters in the same column are different from each other in challenge factor.

Table 9. Albumine, AST (Aspartate transaminase) and GGT (Gamma-glutamyltransferase or Gamma-GT) concentrations of broilers supplemented with amino acids, challenged or not, and collected at 21 and 28 days old.

	Albumine, g/dL			AST, UI/L			Gama GT, UI/L		
	C	NC	Mean	C	NC	Mean	C	NC	Mean
					21 days				
Diet 1	1.330	1.360	1.345	203.620	192.900	198.542^{B}	14.310	14.811	14.736
Diet 2	1.300	1.290	1.295	239.930	202.756	222.321^{AB}	13.520	12.933	13.242
Diet 3	1.233	1.290	1.263	233.833	234.378	234.106^{A}	13.856	13.070	13.442
Mean	1.290	1.313		225.517	210.011		13.897	13.586	
CV, %		34.00			18.94			15.06	
Interaction		ns			ns			ns	
					28 days				
Diet 1	1.350	1.250	1.300	208.313	217.763	213.038^{B}	8.400	6.125	7.263^{A}
Diet 2	1.220	1.200	1.211	283.530	261.930	272.730^{A}	4.590	4.930	4.760^{B}
Diet 3	1.322	1.290	1.305	311.960	312.870	312.415^{A}	5.390	7.190	6.290^{AB}
Mean	1.293	1.248		272.193	267.504		5.964	6.079	
CV, %		12.96			22.94			49.41	
Interaction		ns			ns			ns	

C: Challenged with coccidiosis vaccine and E. coli inoculation; NC: unchallenged.
Diet 1: Control diet; Diet 2: Aminogut® (1,5%) + L-Arginine (1,0%) + L-Threonine (1,0%); Diet 3: Aminogut ® (3,0%) + L-Arginine (2,0%) + L-Threonine (2,0%)
[a,b]Means followed by different lowercase letters in the same line are different from each other in challenge factor. [A,B]Means followed by different lowercase letters in the same column are different from each other in challenge factor.

level, which had higher AST levels. At day 28 a change was observed ($P<0.05$) in AST levels, and the birds which received amino acids in diet 2 and 3 had higher AST levels than control birds. For the Gamma GT enzyme, the birds that received supplementation at an intermediate level, showed lower levels than the control birds.

DISCUSSION

The fact that the birds receiving the highest level of amino acid in diets showed lower levels of IgA, might demonstrate a quick and efficient response of the birds supplemented with amino acids on the damage caused by *Eimeria* to the intestinal mucosa. Due to the complex life cycle the parasite develops inside the host, the immune response is also quite complex, involving cell-mediated immunity, antibody and cytokine production. These results agree with the reports of Bartell and Batal (2007), who concluded that supplementing broilers with levels of 1 and 4% glutamine lower levels of mucosal IgA. Likewise, Tan *et al* (2014[a]) found lower values for IgA broilers a week after receiving the coccidiosis vaccine and arginine supplementation in their diet.

Threonine plays an important role in the composition of mucin just as glutamine is necessary for glycosylation, this explains why at 28 days the highest level of dietary amino acid supplementation resulted in an increased number of goblet cells compared to the diet with intermediate level of supplementation. These cells protect the intestinal epithelium from the actions of digestive enzymes and abrasive effects of digest (Robertis and Hib 2001) and aggressors agents. Zhang *et al* (2014) supplemented the diet of Beijing mallards with increasing levels of threonine and found increased mucin secretion but no difference in goblet cell counting. Tan *et al* (2014[a]) found a significant increase in density and number of goblet cells in the jejunum of the birds challenged with the coccidiosis vaccine supplemented with arginine.

The thymus is a primary lymphoid organ responsible for differentiation and maturation of T cells. Increasing evidence shows that cell-mediated immunity plays a major role in the resistance against coccidiosis, including non-specific activation of lymphocytes, macrophages and natural killer cells (NK), such as activation of antigen-specific T cells, which explains the reduction of the organ when involving immune cell situations, as observed in this study with challenged birds showing lower thymus measurements compared to the birds that were not challenged.

Regarding the spleen size, a reduction in splenic measures was observed in the unchallenged birds for the treatment with the highest level of amino acids. The highest numbers were correlated to control unchallenged birds. The spleen represents the largest unit of the mononuclear phagocyte system, constituting the largest lymphoid tissue accumulation in the body and the only organ of this type interposed into the bloodstream. On the other hand, Sakamoto *et al* (2006) supplemented birds with Glu and found a higher relative spleen weight in birds supplemented initial diets. Bartell and Batal (2007), under these same health conditions, added 1 to 4% of Glu into the diet and found no changes in thymus weight, spleen and cloacal bursa.

The immune response related to infection with coccidiosis involves a non-specific response ensured by the innate response and specificity of response, which involves the activity of cytokines, hormones, leukocytes, lymphoid tissues associated to intestine (GALT), macrophages and other antigen presenting cells (Ruff 1999). Perhaps this fact justifies the absence of changes in morphometric measurements of the cloacal bursa, since it is involved with humoral immunity. Fernandes *et al* (2011) also found no significant effect on the relative weight of thymus and cloacal bursa, antibody production and measures of cloacal folds at various ages when supplementing birds with arginine, which had been challenged with vaccine of Gumboro.

Research on avian species supplemented with arginine pointed regulatory actions (Tayade *et al* 2006, Lee *et al* 2002, Tan *et al* 2014[b], Tan *et al* 2014[c]), and improvements in the immune system such as increased nitric oxide production by macrophages (Sung *et al* 1991). However, such improvements have often been achieved with levels much higher (2 to 3% of arginine in the feed) than the recommendations by the NRC (1994), which is 1.25% (Kidd 2004). The immune stimulatory effects are even more impressive in stressed or immune suppressed animals.

For the challenged birds there was a significant decrease in lymphoid clusters in birds of diet 3. This result may be related to the values observed after 28 days, two weeks after infection to the detected IgA in the intestinal mucosa of the challenged birds. The decrease in IgA and lymphoid clusters showed a less aggressive response, and therefore less costly for the organism, but effective. Accordingly, Fernandes *et al* (2014) added Arg to the broilers diet and found that the birds that had not received Arg supplementation in their diet had a higher number of lymphoid clusters when compared to the birds fed supplemented diets.

Several authors have observed the relationship between the supplementation with higher levels of amino acids and its interaction with the immune response. Kidd *et al* (2002) found a mortality reduction in chickens challenged with *Eimeria acervulina, Eimeria tenella e Eimeria maxima* when diets were formulated with a Arg:Lys ratio of 1.3 if compared to birds that received 0.9 and 1.1. Rubin *et al* (2007), while the use of arginine levels of 1.33 and 1.83% for 1 to 21 days and 1.14 to 1.64% from 22 to 42 days reported that the levels used did not influence the performance and immune response of broilers. Kidd *et al* (1997) evaluated the humoral and cellular responses in chicks fed with different threonine levels in their diet (0.68 to 0.86) and found no improvement in immunity. Moreover, Tayade *et al* (2006) demonstrated that the supplementation of 2% of arginine in the diet increased the antibody counting and

protection against the Virus of Bursa`s Infectious Disease and improved intestinal and systemic immune response against infectious bronchitis virus.

Several studies have demonstrated the immunomodulatory effect of nutrient association, for example lysine and threonine (Kidd *et al* 1997), arginine and vitamin E (Abdukalykova and Ruiz-Feria 2006), arginine and tryptophan (Emad *et al* 2011), arginine and methionine (Rubin 2007). These experiments demonstrate the interrelationship between supplemented amino acids and immune response. Thus, the results obtained in this experiment cannot be attributed to a single supplemented amino acid. However, further studies are needed given the complexity of the interaction of amino acids and the components of the cellular and humoral immune response.

The biochemical analysis of urea and serum uric acid showed that the blood urea levels increased when comparing unsupplemented birds with those receiving diets 2 and 3. The increase in urea levels associated with Arg supplementation demonstrates that there was ornithine synthesis. Arg at high levels stimulates the activity of arginase, which degrades Arg into ornithine and urea. About 40 to 60% of urea excreted by birds is the result of ornithine synthesis (Ruiz-Faria *et al* 2001). Ornithine is a precursor of polyamines, which in turn are associated with cell division, protein synthesis and tissue growth (Wu and Morris 1992).

Most amino acids are metabolized in the liver and part of the NH_3 generated is recycled and used in a wide variety of biosynthetic processes being excreted directly over or converted into urea or uric acid (Nelson and Cox 2008). It was possible to observe in this study that this happens because the uric acid levels are proportional to the addition of amino acids to the diet. After 28 days, there was a mobilization of amino acids for the immune response and the excess excretion began 2 weeks after the challenge ended. Uric acid has recently been considered an important antioxidant (Hare and Johnson 2003), specially in challenging infectious situations where high levels of NO are produced.

The enzymes that assess liver function, AST and Gama GT, were analised. The AST enzyme is part of a group of enzymes that catalyze the inter-conversion of amino acids and oxy-acids by transferring the amino group (Hochleithner 1994). This explains the fact that in birds supplemented with amino acids, serum concentrations of this liver enzyme were higher, and also the higher metabolism rate of the amino acids or the effect attributed to imbalance between amino acids (Buttery and D'Mello 1994).

The availability of substrates is currently considered the main tool in maintaining the structure and function of the mucosal barrier (Suchner *et al* 2000). Potential regulatory activities of functional amino acids must be included in determining the nutritional requirements of broilers (Wang *et al* 2015). However, the optimal levels that facilitate the expression of these activities are still not clear. Improving animal health is important not only from an ethical point of view, but for the safety of human consumption and the higher profitability of the poultry chain. The metabolic disorders accompanying an inflammatory process divert the nutrients from important physiological processes for the growth. Therefore, the impact of an inflammatory or infectious response on the diet needs must be considered. Clearly, the type of protein synthesized in response to a disease alters the amino acid requirements. Which and how many amino acids are involved? Many studies are still required to answer this question.

In conclusion, dietary supplementation of broilers with arginine, glutamine and threonine in infectious challenge situation, influenced the immune system by altering the morphometric measurements of the spleen and thymus. Supplementation with the trophic amino acids led to an increase in the uric acid serum concentration, urea and also liver enzyme AST, regardless the challenge.

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