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Glutamine use in feeding juvenile pirarucu, *Arapaima gigas* (Schinz, 1822)

[*Uso da glutamina na alimentação de juvenis de pirarucu,* Arapaima gigas *(Schinz, 1822)*]

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ABSTRACT

This study aimed to evaluate glutamine supplementation effects on variables of growth performance, body composition, intestinal morphology and enzymatic aspects of juvenile *Arapaima gigas.* Research was conducted at the Fish Nutrition and Feeding Laboratory, where 60 examples of pirarucu (initial average weight of 82.12g) were distributed over 15 polyethylene tanks (310L), in a completely randomized design, with five treatments and three repetitions (four fish per experimental unit). Experimental diets were prepared containing five inclusion levels of the amino acid glutamine (0.0, 0.5, 1.0, 1.5 and 2.0%), supplied three times a day for 45 days. Quadratic effect was observed for the variables of growth performance, weight gain, food consumption, food conversion, and specific growth and protein efficiency rates. A significant effect was observed on intestinal villi at the height of the anterior portion and on activity of the enzyme's alkaline proteases, lipase, amylase and aspartate aminotransferase. However, glutamine supplementation had no significant effect on survival rate. Inclusion of 1.02% of glutamine in the diets of juvenile pirarucu improved growth performance and influenced intestinal villi height and activity of important digestive enzymes, favoring nutrient digestion and absorption.

Keywords: amino acid, growth, digestive enzymes, morphology

RESUMO

Objetivou-se avaliar os efeitos da suplementação com glutamina sobre variáveis de desempenho produtivo, composição corporal, morfologia do intestino e aspectos enzimáticos de juvenis de Arapaima gigas. *O experimento foi conduzido no Laboratório de Nutrição e Alimentação de Peixes, onde 60 exemplares de pirarucu (peso médio inicial de 82,12g) foram distribuídos em 15 tanques de polietileno (310L), em delineamento inteiramente ao acaso, com cinco tratamentos e três repetições (quatro peixes por unidade experimental). As dietas experimentais foram confeccionadas contendo cinco níveis de inclusão do aminoácido glutamina (0,0; 0,5; 1,0; 1,5 e 2,0%), fornecidas três vezes ao dia, ao longo de 45 dias. Foi observado efeito quadrático para variáveis de desempenho produtivo: ganho de peso, consumo alimentar, conversão alimentar, taxa de crescimento específico e taxa de eficiência proteica. Observou-se ainda efeito significativo sobre a altura das vilosidades da porção anterior do intestino e a atividade das enzimas: proteases alcalinas, lipase, amilase e aspartato aminotransferase. Entretanto, a suplementação com glutamina não influenciou significativamente a sobrevivência dos animais. A adição de 1,02% de glutamina nas dietas para juvenis de pirarucu melhorou o desempenho produtivo e influenciou a altura das vilosidades intestinais e a atividade de enzimas digestivas importantes, favorecendo a digestão e a absorção de nutrientes.*

Palavras-chave: aminoácido, desempenho, enzimas digestivas, morfologia

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INTRODUCTION

Functional amino acids, especially glutamine, are important regulators of key metabolic pathways (Li *et al*., 2009). This amino acid is fundamental in regulating the acid-base balance of the body and acts as a transporter of nitrogen and ammonia (Darmaun and Humbert, 2000; Li *et al*., 2009). Since glutamine acts on intestinal mucosa and promotes the proliferation and renewal of coating epithelial cells, the morphophysiologic aspects of the digestive system should be considered when developing diets for specific fish species (Pezzato *et al*., 2004).

Knowledge of enzyme activity along the digestive tract of fishes is also important in understanding digestive processes since fish growth depends on digestion and absorption of nutrients, as well as all metabolic interactions. Pirarucu, *Arapaima gigas* (Shinz, 1882), are rapidly growing fish native to the Amazonian basin, able to reach 10kg within the first year of cultivation (Cipriano *et al*., 2016). Pirarucu is highly valued in northern Brazil because of its taste, the quality of its meat and lack of filet bones (Ituassú *et al*., 2005).

Failure to understand the nutritional and dietary requirements of this fish can hinder the sustainable development of commercial pirarucu farming. Consequently, this study aimed to evaluate glutamine supplementation effects on the variables of growth performance, intestine morphology and enzymatic aspects of juvenile *Arapaima gigas*.

MATERIAL AND METHODS

Research was approved by the Committee of Ethics for the Use of Animals (CEUA), of the State University of Santa Cruz - UESC, Protocol No. 001/2016, on May 31, 2016. The experiment was conducted at the Fish Nutrition and Feeding Laboratory (AQUANUT), located in the State University of Santa Cruz - UESC, Ilhéus - Bahia (18° 43'51"S latitude, 44° 53'33"W longitude) for 45 days (August to October 2018). Sixty pirarucu specimens, average initial weight of 82.12±0.25g, were distributed over 15 (310L capacity) circular polyethylene tanks in a closed water recirculation system with constant biological filter aeration. Each tank represented one experimental unit, in which the fish were distributed in a completely randomized design with five treatments and three replicates and a density of four fish per tank.

Water quality was monitored periodically, and temperature, dissolved oxygen and pH were measured daily using a multiparameter (YSI Pro Plus) and ammonia concentration (NH_3) was determined bi-weekly using a bench photocolorimeter (HANNA, model HI83203). Average water temperature during the experimental period was 27.7±0.63°C, dissolved oxygen was 8.05±2.15mg L^{-1}, pH was 6.50±0.66 and ammonia was 1.60±0.67mg L^{-1}.

Experimental diets were formulated using SUPER CRAC® software and prepared by Pratigi Alimentos using conventional ingredients (Table 1). Following the extrusion process, 4mm granulometry diets were supplemented in an "*on top*" form, with glutamine spraying associated with 1.0% oil and five inclusion levels of the glutamine amino acid (0.0, 0.5, 1.0, 1.5 and 2.0%) to replace wheat bran. The fish were allowed to adapt to the laboratory and food management conditions for 10 days, during which they were given extruded commercial diet (4mm granulometry) with 400g kg^{-1} of crude protein. After the adaptation period, the fish were fed with the experimental diets until the point of apparent satiety three times a day (8 a.m., 12 p.m. and 4 p.m.) for 45 days.

For the final biometry, all fish underwent a 24-hour fast to empty the gastrointestinal tract, after which they were counted and individually weighed for each experimental unit. Such data, associated with feed intake during the experimental period, were used to calculate growth performance variables as follows: Weight gain (WG) = (final body weight - initial body weight); Food conversion (FC) = (Feed intake/weight gain); Specific growth rate (SGR) = 100 x [(ln mean final weight - ln mean initial weight)/time]; Protein efficiency rate (PER) = body weight gain/protein consumed; Survival (SR) = [(number of fish at end of the experiment/number of fish at start of the experiment) x 100].

Table 1. Centesimal composition and chemical composition of experimental diets

Ingredients (%)	Levels of inclusion of glutamine (%)				
	0.0	0.5	1.0	1.5	2.0
Soybean meal (45%)	27.40	27.40	27.40	27.40	27.40
Wheat bran (16%)	16.10	15.60	15.10	14.60	14.10
Corn gluten meal (60%)	14.90	14.90	14.90	14.90	14.90
Poultry viscera flour (58%)	11.90	11.90	11.90	11.90	11.90
Meat and bone meal (45%)	11.00	11.00	11.00	11.00	11.00
Corn bran	7.30	7.30	7.30	7.30	7.30
Corn meal Fish flour (55%)	5.00	5.00	5.00	5.00	5.00
Soy oil	4.50	4.50	4.50	4.50	4.50
Hydrolyzed fish soybean oil (15%)	1.00	1.00	1.00	1.00	1.00
Glutamine	0.00	0.50	1.00	1.50	2.00
Premix vit-min[1]	0.50	0.50	0.50	0.50	0.50
Common salt	0.287	0.287	0.287	0.287	0.287
Antifungal	0.080	0.080	0.080	0.080	0.080
Antioxidant BHT[2]	0.013	0.013	0.013	0.013	0.013
Ascorbic acid vit C mon 35	0.020	0.020	0.020	0.020	0.020
Total	100.00	100.00	100.00	100.00	100.00
Chemical composition analyzed					
Gross energy (kcal kg^{-1})	4400	4424	4447	4433	4420
Crude protein (%)	44.32	44.37	44.34	44.47	44.45
Ethereal extract (%)	12.30	12.38	12.05	12.19	12.20
Dry matter (%)	93.16	93.24	93.22	93.30	93.41
Mineral matter (%)	10.37	10.17	10.00	10.08	10.23

[1]Premix vitamin mineral (composition/ kg of product): vit. A = 6000000IU; vit. D3 = 2250000IU; vit. E = 75000mg; vit. K3= 3000mg; vit. thiamine = 5000mg; riboflavin = 10000mg; vit. pyridoxine = 8000mg; Biotin = 2000mg; vit. C = 192.500mg; Niacin = 30.000mg; Folic acid = 3000mg; Fe = 100000mg; Cu = 600mg; Mn = 60000mg; Zn = 150000mg; I = 4500mg; Cu = 15000mg; Co = 2000mg; Se = 400mg.
[2] Butyl hydroxytoluene.

To determine the chemical composition, four specimens were collected at the start of the experiment and one more at each repetition until the end of the experiment. Chemical composition of experimental diets and fish (whole and eviscerated) were analyzed according to AOAC (Official…, 2016). To determine dry matter, samples were lyophilized, milled in a knife mill, passed through a 0.5mm sieve and stored in the refrigerator. Crude energy was determined using a calorimeter pump (IKA C-200). Ethereal extract, crude protein and mineral matter were analyzed at the Forage and Pasture Laboratory of the State University of Southwest of Bahia - UESB, Itapetinga campus.

For enzyme activity and histological analyses, one pirarucu specimen was collected per experimental unit (15 fish) and anesthetized with benzocaine according to the protocol proposed by Ranzani-Paiva *et al.* (2013). Following anesthesia, the fish were euthanized (through the medulla), dissected to remove gut fragments (anterior and middle sections) and fixed in 10% buffered formalin for 24 hours. Histological analyses were performed at the UESC Animal Pathology Laboratory, where samples were subjected to routine techniques that culminated in hematoxylin-eosin staining. Intestinal villi height was measured using Image-Pro Plus for the fish subjected to experimental diets, totaling 90 villi per treatment.

Enzymatic analyses were carried out at the Aquaculture Laboratory of the Federal University of the São Francisco Valley - UNIVASF. Enzymatic activity of amylase, alkaline proteases (nonspecific) and lipase were determined using a homogenization buffer composed of two solutions: Solution I (10mM phosphate buffer, 20mM Tris buffer, pH 7.0) and Solution II (glycerine), at a ratio of 1:1. Amylase activity was estimated according to the modified methodology proposed by Bernfeld (1955). Protease alkaline activity was determined using 50µL of the homogenate in 1.0mL of Tris/HCl buffer (0.1M and pH 8.5) associated with azocasein substrate (1%) (Sarath *et al*., 1989). Non-specific lipase activity was determined according to the method described by Gawlicka *et al.* (2000). Aspartate aminotransferase (AST) enzyme activity was determined using liver fragments through a commercial Labtest AST/GOT LIQUIFORM kit (Ref. 109).

All data obtained when the experiment was completed were subjected to normality and homoscedasticity tests followed by variance analysis at a 5% significance level. Effects of glutamine levels on growth performance variables, body chemical composition, intestinal villi height and enzymatic activity were verified through linear and quadratic regression models using the R Development Core Team (2011) statistical program.

RESULTS

Supplementing the feed with increasing glutamine levels resulted in a quadratic effect for the variables weight gain, food intake, feed conversion, specific growth rate and protein efficiency ratio (Table 2). Survival did not significantly differ among the evaluated treatments.

Table 2. Productive growth variables (Mean±SEM) of juvenile pirarucu subjected to increased glutamine levels

	Levels of glutamine (%)					P-value
	0.0	0.5	1.0	1.5	2.0	
Weight gain (g)*	70.5±6.8	115.3±2.8	129.6±3.4	116.7±2.6	74.7±2.6	0.0000
Food consumption (g)*	215.9±22.7	315.8±5.2	341.1±11.4	296.0±3.2	234.0±7.9	0.0000
Food conversion *	3.1±0.1	2.7±0.1	2.6±0.1	2.5±0.1	3.1±0.1	0.0000
Specific growth rate (%)*	1.4±0.1	2.0±0.0	2.1±0.0	2.0±0.0	1.4±0.0	0.0000
Protein efficiency rate (%)*	1.6±0.2	2.6±0.1	2.9±0.1	2.6±0.1	1.7±0.1	0.0000
Survival rate (%)	88.3±0.2	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0	0.2596

SEM = Standard error of mean. *Quadratic effect ($P \leq 0.05$). Regression analysis. Weight gain - WG= ($Y= -57.386x^2 + 116.73x + 70.686$, $R^2= 0.95$); Food consumption - CON= ($Y= -112.35x^2 + 228.4x + 221.03$, $R^2= 0.86$); Food conversion - FC= ($Y= 0.5866x^2 – 1.1988x + 3.1049$, $R^2= 0.74$); Specific growth rate - SGR= ($Y= -0.715x^2 + 1.4586x + 1.379$, $R^2= 0.94$); Protein efficiency rate - PER= ($Y= -1.2827\ x^2 + 2.609x + 1.5799$, $R^2= 0.95$).

Variables WG, CON, FC, SGR and PER had the best results with the inclusion of 1.02% glutamine, obtained by deriving the quadratic formula. Diet supplementation with increased glutamine levels did not affect ($P>0.05$) the chemical composition of juvenile pirarucu for the analyzed variables (Table 3).

Regarding the histological analysis of different intestinal regions, considering villi height measurements, it was observed that supplementation with increasing glutamine levels had a positive effect ($P \leq 0.05$) on the morphology of intestinal villi (anterior region) of juvenile pirarucu (Table 4). The maximum height of intestinal villi was also obtained at 1.02% inclusion level. However, the average height of intestinal villi *A. gigas* juveniles did not differ significantly.

Table 3. Mean values of body chemical composition (Mean±SEM) of juvenile pirarucu supplemented with increasing levels of glutamine

	Levels of glutamine (%)					P-value
	0.0	0.5	1.0	1.5	2.0	
Dry matter (%)	25.0±1.6	24.8±1.4	24.9±1.6	24.8±1.4	24.9±1.4	0.8287
Crude protein (%)	66.9±1.1	64.3±1.1	68.3±0.2	66.4±2.0	65.5±2.0	0.4249
Gross energy (kcal kg^{-1})	4373±26.2	4363±23.7	4419±36.0	4421±4.6	4413±8.7	0.5768
Ethereal extract (%)	14.9±0.1	14.7±0.2	15.2±0.5	15.3±0.1	15.2±0.2	0.9769
Mineral matter (%)	20.8±0.1	24.3±0.5	20.2±0.1	21.1±0.3	22.0±1.1	0.9496

SEM = Standard error of mean. Significance level (P≤0.05). Regression analysis.

Table 4. Mean values of intestinal villi height (Mean±SEM) of juvenile pirarucu supplemented with increasing levels of glutamine

	Levels of glutamine (%)					P-value
	0.0	0.5	1.0	1.5	2.0	
Anterior Intestine (µm)*	82.67±4.0	90.48±5.8	113.42±3.1	87.72±5.9	86.25±0.4	0.0021
Middle Intestine (µm)	41.41±1.6	37.41±1.0	39.01±0.7	40.75±1.1	39.55±0.9	0.1662

SEM = Standard error of mean. Significance level (P≤0.05). Regression analysis. *Y= $-19.308x^2 + 39.285x + 81.631$, $R^2 = 0.42$.

Supplementation with increasing levels of glutamine significantly influenced digestive enzyme activity that is important for protein and energy metabolism. Alkaline proteases, lipase, amylase and aspartate aminotransferase presented quadratic effect, displaying optimum points at 1.09, 1.09, 1.07 and 1.27% of glutamine inclusion, respectively (Table 5).

Table 5. Mean values of enzymatic activity (Mean±SEM) of juvenile pirarucu submitted to increasing levels of glutamine

(U mg^{-1} of protein)	Levels of glutamine (%)					P-value
	0.0	0.5	1.0	1.5	2.0	
Alkaline proteases *	1.31±0.0	1.70±0.0	1.88±0.0	1.63±0.1	1.53±0.1	0.0001
Lipase*	4.26±0.0	4.55±0.0	4.65±0.1	4.53±0.1	4.41±0.0	0.0003
Amylase*	0.42±0.1	0.72±0.0	0.88±0.1	0.70±0.0	0.56±0.0	0.0001
Aspartate Aminotransferase*	1.67±0.2	0.19±0.1	0.19±0.1	0.21±0.0	0.37±0.2	0.0001

SEM = Standard error of mean. *Quadratic effect. Significance level (P≤ 0.05). Regression analysis. Alkaline proteases = (Y= $-0.4048x^2 + 0.8835x + 1.333$, $R^2 = 0.72$); Lipase= (Y= $-0.2933x^2 + 0.6413x + 4.278$, $R^2 = 0.73$); Amylase= (Y= $-0.3495x^2 + 0.7517x + 0.4292$, $R^2 = 0.78$); Aspartate Aminotransferase= (Y= $0.9457x^2 – 2.4094x + 1.5175$, $R^2 = 0.79$).

DISCUSSION

The highest weight gain of juvenile pirarucu was reached at 1.02% glutamine inclusion. Improvements in the observed weight gain were possibly due to the ability of glutamine to supply nitrogen for synthesis of other important amino acids and, consequently, to protein deposition, consequently decreasing catabolism of skeletal muscle tissue and promoting weight gain and growth (Newsholme *et al*., 2003). Reduction in values up to the 2.0% inclusion level may be due to excessive glutamine supply, which may cause adverse neurotoxic effects in pirarucu. Glutamine is metabolized to glutamate and ammonia, both of which have neurological effects (Garlick, 2001). Ammonia plays an important role in maintaining nitrogen homeostasis in organisms, but it is extremely toxic in high concentrations, especially to the central nervous system (Cooper and Plum, 1987).

Results obtained for weight gain corroborate the findings of Cheng *et al.* (2011) in a study of *Sciaenops ocellatus*, a predatory marine fish (6.9g mean initial weight). After the feed was supplemented with glutamine (0.0, 1.0 and 2.0%) and arginine, these authors confirmed that dietary inclusion of glutamine improved weight gain and efficiency of such species.

For the variable food consumption, an increase in consumption was observed until the level of 1.02% glutamine, followed by a drop to 2.0%. Differences in consumption for specimens in differing treatments are possibly due to the beneficial effect of this amino acid up to a certain inclusion level since toxicity related to an excess of glutamine negatively influenced fish fed with diets containing higher levels and caused a reduction in consumption.

Feed conversion had a positive relation with glutamine supplementation, reaching lower indexes at the 1.02% inclusion level, ranged from 2.5 to 3.1. The worse results were obtained from the control group and the diet containing 2.0% supplementation. As feed conversion is a result of consumption and weight gain, such results also fluctuated. In this case, the worst values were observed for specimens supplemented with higher levels of glutamine, which also exhibited lower intake and weight gain.

Results obtained in this study for feed conversion corroborate those found by Yan and Qiu-Zhiu (2006), who observed that glutamine supplementation up to 1.2% improved feed conversion of juvenile common carp (*Cyprinus carpio*), an omnivorous species. The differences in the results of this study and other researchers may be due to the use of different species, leading to the different digestive tract morphologies and metabolisms in which glutamine is harnessed differently (Cheng *et al.*, 2011).

In this study, specific growth rate obtained its best average value (2.1%) for the 1.02% glutamine inclusion level. The lowest values were observed in the controlled diet (1.4%) and in the diet containing 2.0% glutamine inclusion (1.4%). This result was expected because the specific growth of the species is directly related to weight gain, so the specimens with greater weight gain also exhibited better specific growth rates.

Protein efficiency rate had the best results at 1.02% glutamine inclusion, while the worst values were also observed in the control treatments and with 2.0% supplementation. The protein efficiency rate indicates how much crude dietary protein was converted to body protein. Therefore, the results suggest that fish supplemented with glutamine obtained better protein utilization at 1.02% amino acid inclusion, which corroborates the weight gain values and reveals the beneficial effect of glutamine up to a certain level. The survival rate varied from 88.3 to 100.0% and mortality was observed only in the controlled treatment, without inclusion of glutamine. Although supplementation with this amino acid did not significantly influence survival, it is possible that the absence of mortality in other treatments is related to the capacity of glutamine to enhance the immunity of species (Newsholme *et al*., 2003).

The lack of any significant effect on the chemical composition of the bodies of juvenile pirarucu can be related to the similar protein and energy levels in the experimental diets, taking into account the nutritional requirements for such species. The results obtained in this study for the chemical composition of the bodies of juvenile pirarucu resemble those found by Coutinho *et al.* (2016) in a study of juvenile *Sparus aurata*, a predatory species, supplemented with glutamine (0.0, 0.5, 1.0 and 2.0%), in which no significant effect was found for the body composition of such species.

Glutamine supplementation positively influenced ($P \leq 0.05$) intestinal villi height of the anterior region. The best value was obtained at 1.02% glutamine inclusion, followed by reduction in values up to 2.0% inclusion of the amino acid. Increased villus most likely occurred because glutamine is an important energy source for enterocytes, thus providing nitrogen for nucleotide biosynthesis necessary for replication of intestinal mucosal cells (Yan and Qiu-Zhou, 2006). However, reduction in intestinal villi height at 2.0% glutamine inclusion may have been caused by the neurotoxic effects promoted through the excess of such amino acid.

The positive effect of glutamine on the height of intestinal villi of the anterior region led to improved growth performance. Once the contact surface increases, digestion and the absorption processes of nutrients also intensify, leading to

better weight gain of specimens (Silva *et al.*, 2010). Such results are similar to those observed by Yan and Qiu-Zhou (2006) in a study of juvenile common carp. The authors observed that fish fed with 1.2% glutamine exhibited an increase of intestinal villi height and an improvement in intestinal function.

Morphometric analysis of the middle intestines of juvenile pirarucu demonstrated that glutamine supplementation does not significantly influence villi height in such a region. Histologically, it is possible to observe a decrease in intestinal villi complexity from the proximal to distal region, thus, the middle intestine has little participation in digestion and absorption of nutrients (Gonçalves *et al.*, 2012), which explains the absence of the effect of glutamine on this parameter.

An increase was observed in enzymatic activity of alkaline (nonspecific) proteases and lipase up to 1.09% glutamine inclusion, followed by a reduction until the level of 2.0%. Such results suggest food consumption influenced activity of such enzymes since the specimens studied with highest enzymatic activity also exhibited higher consumption, leading to higher amounts of substrate for enzyme performance. The positive effects of glutamine up to a certain level may also be attributed to improved exocrine pancreas activity, which is diffused in the liver (Yan and Qiu-Zhou, 2006).

Results obtained for alkaline proteases and lipase corroborate those found by Yan and Qiu-Zhou (2006), in a study with common carp supplemented with glutamine (0.0, 0.4, 0.8, 1.2, 1.6 and 2.0%), in which activities of alkaline proteases and lipase were positively related to glutamine supplementation and increasing levels up to 1.2% and higher did not differ significantly.

Glutamine supplementation promoted a significant increase in amylase activity at up to 1.07% inclusion, with a reduction at the final inclusion level. As in cases of alkaline proteases and lipase, specimens exhibiting higher food intake also exhibited higher amylohydrolytic activity. Liu *et al.* (2015) also observed a significant effect on the enzymatic activity of post-larval amylases of *Cynoglossus semilaevis* Günther (mean weight of 10.64mg) supplemented with glutamine (0.0, 0.5, 1.0 and 2.0%). According to these authors, specimens fed with diets containing 2.0% of the additive had significantly higher amylase activity than in other treatments.

Aspartate aminotransferase (AST) activity also showed a quadratic effect with glutamine supplementation. Highest AST activity was observed in the controlled group, after which activity was reduced at 1.27% and, from this inclusion level, enzyme activity increased. Results for AST suggest that activity of the enzyme for the control treatment (0.0% glutamine) and containing 2.0% glutamine were affected when the protein efficiency rate was lower.

The increase in AST activity for specimens supplemented with 2.0% of the amino acid may also be related to toxicity effects from excessive glutamine since high ammonia concentration generated by high levels of glutamine may lead to depletion of some intermediates of the citric acid cycle (Bombardelli *et al.*, 2003). Therefore, specimens would also be using the protein to generate intermediate compounds and, consequently, energy.

CONCLUSION

Supplementation with 1.02% glutamine helped improve the growth performance variables (weight gain, food intake, feed conversion rate, specific growth rate and protein efficiency rate) as well as intestinal mucosa structure (anterior region).

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