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Supplementation of diets for piglets with L-Arginine and powdered whole milk

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Abstract

The objective of this study was to evaluate the supplementation of L-Arginine in diets with and without dairy products for piglets weaned at 21 days old. Thirty-two animals with initial mean weight of 5.16 ± 1.92 kg and from the same commercial lineage were allocated in a randomized block design to four diets. The experimental diets consisted of a corn and soybean meal-based diet (NDD), the same diet supplemented with 0.6% L-Arginine (NDDA), the diet supplemented with powdered whole milk (DD), and the diet supplemented with powdered whole milk and 0.6% of L-Arginine (DDA). At 43 days old, 16 animals were slaughtered. The productive performance, incidence of diarrhoea, serum parameters, relative organ weight, morphometry and intestinal health were evaluated. Supplementation with 0.6% of L-Arginine increased (P <0.05) the daily and final weight gain of the piglets at 32 days old and reduced the incidence of diarrhoea. DDA promoted a higher villi to crypt ratio (P <0.05). There was a lower rate of mitosis and apoptosis in the jejunum of animals fed DD and DDA. The non-dairy diet supplemented with 0.6% arginine (NDDA) increased serum immunoglobulin A (IgA) and Immunoglobulin G (IgG) concentrations compared with NDD (P <0.05). Thus, supplementation with 0.6% L-Arginine increased immunological activity, improved intestinal integrity, and reduced the incidence of diarrhoea.

Keywords: diarrhoea, intestinal, immunoglobulin A, immunoglobulin G, integrity, swine
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Introduction

Piglet performance in the weaning phase is critical to success in the later stages of rearing. However, the weaning period is challenging for piglets owing to stressors, which include a change in social environment, mixing litters, and a new diet rich in carbohydrates of plant origin. Thus, it is common to observe a lack of appetite for the first days after weaning, which can affect the digestive system, including changes in the level of the intestinal mucosa, and the immune system (Lallès *et al.*, 2004). Severe diarrhoea and reduced food intake may occur, with consequent decreased performance. Thus, the maintenance and enhancement of the immunological status of these animals is important to minimize or prevent the low performance, morbidity, and mortality of piglets in the nursery (Pluske *et al.*, 2018).

Historically antibiotics were used widely as a feed additive in diets for piglets to prevent digestive disorders and to increase feed efficiency. However, the European Community banned the use of antimicrobials in animal feed owing to the risk of microbial resistance, which might affect the health of consumers. The increased scale of production in pig farms and global pressure to restrict the use of antimicrobials were major obstacles to maintaining the intestinal health of piglets, which affects their performance in the nursery Phase (Yi *et al.*, 2018). Given this scenario, alternative feedstuffs have been used in animal feeds in place of antibiotics.

The use of amino acids with functional properties may be an interesting proposition, because they regulate important metabolic pathways that improve growth, health, and survival (Wu, 2013). Research indicated that functional amino acids improve digestion and maintain intestinal integrity, improving nutrient absorption and immune response in weanling piglets (Roth, 2008: Wang *et al.*, 2008; Wu *et al.*, 2010; Silva, 2015; Yi *et al.* 2018),

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Arginine, an amino acid that is classified as 'conditionally essential' for piglets, has been shown to be functional when added to the diet of newly weaned piglets (Wu *et al*., 2013; Wu *et al*., 2015). Many cell types appear to use arginine as a precursor of nitric oxide (NO), which is essential for vasodilation, immune response, neurotransmission and adhesion of platelets and leukocytes (Mateo *et al.*, 2008). Thus, arginine is deemed a functional amino acid in animal nutrition and health. This study aimed to evaluate dietary supplementation with 0.6% L-Arginine in diets with and without powdered whole milk for weaned piglets.

Materials and Methods

All experimental procedures were approved by the Committee on Ethics in the Use of Animals of the Federal University of Paraíba (CEUA/UFPB), under protocol CEUA No. 7991180418. The experiment was carried out with 32 piglets, weaned at 21 ± 2 days old and belonging to the same commercial lineage, which were obtained from a commercial pig farm in the municipality of Belo Jardim, Pernambuco State, Brazil. After weaning, the animals were transported to the experimental nursery of the Swine Laboratory of the Centre for Human, Social and Agrarian Sciences of UFPB, Paraíba State, Brazil. To control initial differences in live weight (5.16 kg ± 1.92), the animals were distributed in a randomized block design with four treatments and four replicates, each of which consisted of one castrated male piglet and one female. The piglets were housed in suspended nursery cages with a plastic floor, pacifier drinking troughs, feeding troughs, and heating system with 70 W incandescent lamps. The maximum and minimum temperatures and relative humidity were recorded twice daily at 30.6 ± 0.38 °C, 25.4 ± 0.56 °C, and 78.3 ± 4.69%, respectively, during the experiment.

The experimental diets were formulated to meet the minimum nutritional requirements of piglets from 21 to 32 days old (Phase I) and from 33 to 42 days old (Phase II) (Rostagno *et al.*, 2017). No antibiotics or growth promoters were used. Phase I diets (Table 1) provided 3400 kcal/kg of metabolizable energy and contained 21.42% crude protein. The contents of digestible methionine + cysteine (0.813%), lysine (1.451%), threonine (0.972%), valine (1.001%), and tryptophan (0.276%) were constant across the experimental diets. Isoleucine levels were 0.789% in the NDD and NDDA diets and 0.815% in DD and DDA . In the NDD and DD diets the arginine levels was 1.451% and in the NDDA and DDA diets it was 1.840%. The leucine levels were 1.583% in the NDD and NDDA diets and 0.815% in the DD and DDA diets. All Phase I diets contained 1.068% calcium, 0.528% available phosphorus and 0.250% chlorine. The NDD and NDDA diets contained 0.798% potassium, whereas DD and DDA contained 0.826% potassium.

Phase II diets (Table 2) provided 3375 kcal/kg of metabolizable energy and contained 19.870% crude protein. The contents of digestible methionine + cysteine (0.754%), lysine (1.346%), threonine (0.902%), valine (0.929%) and tryptophan (0.256%) were constant. Isoleucine levels were 0.721% in the NDD and NDDA diets and 0.729% in DD and DDA. In the NDD and DD diets the arginine levels was 1.346% and in the NDDA and DDA diets it was 1.723%. The leucine levels were 1.497% in the NDD and NDDA diets and 1.556% in DD and DDA. . All Phase II diets contained 1.068% calcium, 0.528% available phosphorus and 0.250% chlorine. The NDD and NDDA diets contained 0.736% potassium, whereas the DD and DDA diets contained 0.755% potassium.

The animals were weighed at the beginning and end of each phase. Feed and orts were weighed, and the daily feed intake (DFI), daily weight gain (DWG), and feed conversion (FC) were determined. The faecal scores of piglets were surveyed twice a day during the first 19 days of the experiment. Faecal consistency was assessed visually at 08h00 and 17h00 by one observer. The data were recorded as 1 = normal faeces, 2 = pasty faeces, and 3 = aqueous faeces. Scores 1 and 2 were considered non-diarrhoeic faeces, and 3 indicated diarrhoea. Before slaughter, blood samples were collected from the jugular vein in tubes without anticoagulant. The tubes were then centrifuged at 958 x g for 10 minutes. The serum was transferred into microtubes and cooled for biochemical analysis.

The serum was processed in the Laboratory of Chromatography and Atomic Absorption Spectrometry of the Postgraduate Programme in Agro-Food Technology (PPGTA), Campus III of UFPB. Serum concentrations of IgA, IgG, gamma-glutamyl transferase (GGT), aspartate aminotransferase (AST), creatinine, urea, and total protein were measured in a spectrophotometer with commercial Labtest® kits (Labtest Diagnóstica, Lagoa Santa, Minas Gerais, Brazil), namely IgA (Turbiquest, ref. 358), IgG (Turbiquest, ref. 359), GGT (Liquiform, ref. 105), AST/GOT (Liquiform, ref. 109), creatinine K (Liquiform, ref. 96), urea UV (Liquiform, ref. 104), and total protein (ref. 99).

One animal from each pair that was closest to the mean live weight of the replicate was slaughtered at 43 days old. They were stunned by electronarcosis and bled. Afterwards, their abdomens were opened, using tweezers and scalpel, for sample collection. The samples of duodenum, jejunum and liver were immediately immersed in methacarn solution and sent to the Histology Laboratory of the Veterinary Sciences Department of the Agrarian Sciences Centre, Campus of Areia, UFPB, where the slides were prepared, and morphometric analyses of the intestinal epithelium were conducted using light microscopy. To prepare the

slides, the samples remained in the methacarn solution for 12 hours. They were then washed in running water and 70% ethyl alcohol to remove the fixative. Subsequently, they were dehydrated in a series of alcohol baths that increased in concentration from 70% to 100%, diaphanized in xylol, and embedded in paraffin. Eight semi-serial 5 µm thick slices of each segment were made for each animal. Histological slides were read through an Olympus BX41 light microscope, coupled to an Olympus DP11-N image capture system and image analyser system, using Image Pro-Plus® 4.1 software (Olympus Corp., Shinjuku City, Tokyo, Japan).

Table 1 Composition of experimental diets for 21 - 32 day old piglets weighing 5 to 9 kg

Ingredients, %	NDD	NDDA	DD	DDA
Corn grain, 8.8%	54.690	54.690	54.790	54.062
Soybean meal, 45%	34.080	34.080	29.170	29.320
Soybean oil	5.000	5.000	0.966	1.208
Powdered whole milk	-	-	10.000	10.000
Dicalcium phosphate	2.385	2.385	2.111	2.111
Calcitic limestone	0.910	0.910	0.878	0.877
L-Arginine	0.182	0.600	0.258	0.600
L-Lysine	0.616	0.616	0.543	0.540
DL-Methionine	0.242	0.242	0.219	0.219
L-Threonine	0.330	0.330	0.296	0.296
L-Tryptophan	0.058	0.058	0.056	0.055
L-Valine	0.142	0.142	0.093	0.093
Mineral supplement[1]	0.150	0.150	0.150	0.150
Vitamin supplement[2]	0.250	0.250	0.250	0.250
Common salt	0.308	0.308	0.193	0.194
Butylated hydroxytoluene	0.020	0.020	0.020	0.020
Washed sand	0.622	0.204	0.000	0.000

NDD: corn-soy-based diet, NDDA: corn-soy-based diet supplemented with 0.6% L-Arginine, DD: corn-soy-based diet supplemented with powdered whole milk, DDA: corn-soy-based diet supplemented with powdered whole milk and 0.6% of L-Arginine

[1]Iodine: 140 µg, selenium: 300 µg, manganese: 10 mg, zinc: 100 mg, copper: 10 mg, iron: 99 mg

[2] Vitamin A: 4,000 IU, vitamin D_3: 220 IU, vitamin E: 22 mg, vitamin K: 0.5 mg, vitamin B_2: 3.75 mg, vitamin B_{12}: 20 mg, calcium pantothenate: 12 mg, niacin: 20 mg, choline: 400 mg

Villus height (VH), crypt depth (CD), villus to crypt ratio (VH/CD), mucosal thickness, villus width, and goblet cell index were evaluated (Moreira Filho *et al*., 2015). The absorptive area was determined by Silva's (2015) method.

The histological slides were prepared according to the same procedure. The primary antibody Caspase-3 (Abcam®, Cambridge, United Kingdom) was used to determine the rate of apoptosis in the villi of the duodenal and jejunal mucosa. Anti-PCNA (Abcam) was used to determine the rate of mitosis in the mucosa crypts of the duodenum initial portion and jejunum middle portion. The protocol to detect cell death and reveal proliferating cell nuclear antigen (PCNA) was based on Luna *et al.* (2014). To evaluate the mitosis rate, randomized measurements were made until 10,000 µm (linear) of epithelium was reached per treatment. These epithelia were quantified as the number of anti-PCNA+ nuclei. All readings were performed by the same evaluator using a 40x objective lens.

The rates of apoptosis were evaluated by cytoplasmic antibody positivity, using six photomicrographs from each animal, totalling 24 (4 animals x 6 photomicrographs) samples for each treatment. Thus, positivity scores were assigned to each photomicrograph, namely 0 (absence of positivity -), 1 (little positivity +), 2 (moderate positivity ++), and 3 (intense positivity +++), following the semi-quantitative score (Ishak *et al.*, 1995) with modifications. All readings were performed by the same evaluator, using a 40x objective lens.

Table 2 Composition of experimental diets for 33- to 42-day-old piglets weighing 9 to 15 kg

Ingredients, %	NDD	NDDA	DD	DDA
Corn grain, 8.8%	60.000	60.000	60.000	60.000
Soybean meal, 45%	29.730	29.730	26.306	26.306
Soybean oil	4.224	4.224	1.424	1.424
Powdered whole milk	-	-	7.000	7.000
Dicalcium phosphate	2.164	2.164	1.973	1.973
Calcitic limestone	0.841	0.841	0.818	0.818
L-Arginine	0.194	0.600	0.248	0.600
L-Lysine	0.608	0.608	0.556	0.556
DL-Methionine	0.215	0.215	0.199	0.199
L-Threonine	0.311	0.311	0.287	0.287
L-Tryptophan	0.060	0.060	0.059	0.055
L-Valine	0.136	0.136	0.101	0.101
Mineral supplement[1]	0.150	0.150	0.150	0.150
Vitamin supplement[2]	0.250	0.250	0.250	0.250
Common salt	0.235	0.235	0.154	0.154
Butylated hydroxytoluene (BHT)	0.020	0.020	0.020	0.020
Washed sand	0.852	0.447	0.020	0.097

NDD: corn-soy-based diet, NDDA: corn-soy-based diet supplemented with 0.6% L-Arginine, DD: corn-soy-based diet supplemented with powdered whole milk, DDA: corn-soy-based diet supplemented with powdered whole milk and 0.6% of L-Arginine

[1]Iodine: 140 µg, selenium: 300 µg, manganese: 10 mg, zinc: 100 mg, copper: 10 mg, iron: 99 mg

[2] vitamin A: 4,000 IU, vitamin D_3: 220 IU, vitamin E: 22 mg, vitamin K: 0.5 mg, vitamin B_2: 3.75 mg, vitamin B_{12}: 20 mg, calcium pantothenate: 12 mg, niacin: 20 mg, choline: 400 mg

Data were subjected to analysis of variance using the GLM procedure in SAS (SAS Institute Inc., Cary, North Carolina, USA). The means were compared with the Student-Newman-Keuls (SNK) test and differences were declared significant at the 5% level of probability. The normality of the errors was tested by the Cramer-von Mises test. Non-parametric statistics was used to evaluate the incidence of diarrhoea, and the means were compared by the Cochran-Mantel-Haenszel test.

Results and Discussion

Supplementation with 0.6% L-Arginine increased weight gain and final weight at 32 days old (P <0.05), regardless of whether the diet contained powdered whole milk (Table 3). In Phase II, FC of the NDD diet was worse than in the other treatments (P <0.05).

Animals fed NDDA and DD had reduced incidence of diarrhoea in comparison with NDD (P <0.05). Piglets fed with DDA had the lowest incidence of diarrhoea compared with the other treatments (Figure 1).

Table 3 Effect of L-Arginine supplementation in diets with and without powdered whole milk for weaned piglets on daily feed intake, daily weight gain, feed conversion and weight at 32 and 42 days old

Performance measure	Experimental diets				Mean square error	*P*-value
	NDD	NDDA	DD	DDA		
Phase I (from 21 to 32 days)						
Daily feed intake, kg	0.256	0.294	0.261	0.286	0.031	0.311
Daily weight gain, kg	0.089[b]	0.195[a]	0.133[b]	0.155[ab]	0.046	0.057
Feed conversion	3.181	1.589	2.133	1.947	0.768	0.078
Final weight, kg	5.957[b]	7.226[a]	6.498[b]	6.725[ab]	0.518	0.042
Phase II (from 21 to 42 days)						
Daily feed intake, kg	0.247	0.294	0.291	0.287	0.036	0.300
Daily weight gain, kg	0.124	0.174	0.184	0.178	0.041	0.216
Feed conversion	2.351[a]	1.774[b]	1.641[b]	1.670[b]	0.329	0.043
Final weight, kg	7.574	8.732	8.895	8.749	0.843	0.843

NDD: corn-soy-based diet, NDDA: corn-soy-based diet supplemented with 0.6% L-Arginine, DD: corn-soy-based diet supplemented with powdered whole milk, DDA: corn-soy-based diet supplemented with powdered whole milk and 0.6% of L-Arginine

[a,b] Within a row, means with a common superscript were not different with probability *P* =0.05

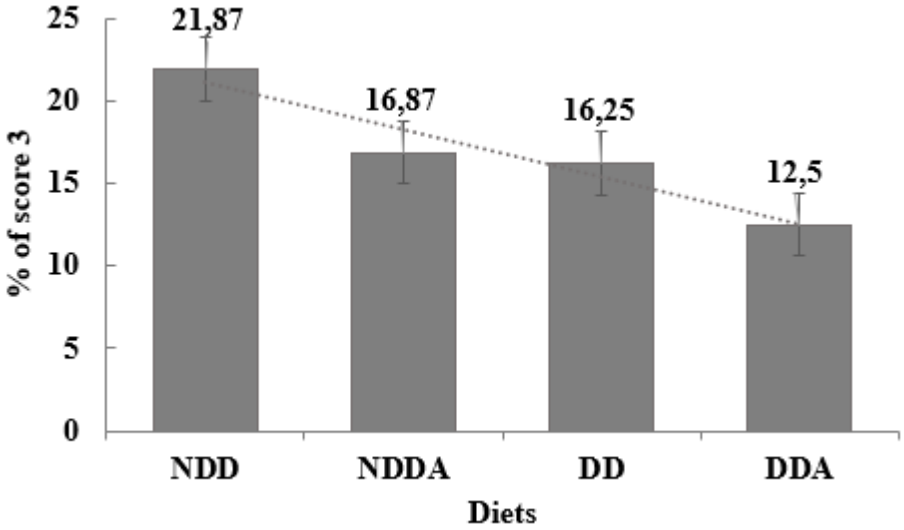

Figure 1 Incidence of diarrhoea in piglets fed with diets with and without dairy product supplemented with L-Arginine

NDD: corn-soy-based diet, NDDA: corn-soy-based diet supplemented with 0.6% L-arginine, DD: corn-soy-based diet supplemented with powdered whole milk, DDA: corn-soy-based diet supplemented with powdered whole milk and 0.6% of L-Arginine

The diets had no effect (*P* <0.05) on serum concentrations of gamma-glutamyltransferase, aspartate aminotransferase, creatinine, urea, and total protein (Table 4), which were all within the normal reference levels for pigs (Kaneko *et al*., 2008). However, the diets influenced the serum concentrations of IgA and IgG (*P* <0.05). The animals fed with NDD and NDDA had higher serum IgA and IgG concentrations higher than NDD. The animals fed with DD and DDA had serum IgA and IgG concentrations that were higher than those fed with ND and NDD and supplementation with 0.6% arginine in this diet provided no significant difference (*P* <0.05).

Table 4 Effects of L-Arginine supplementation in diets for weaned piglets with and without powdered whole milk on serum concentrations of immunoglobulin A, immunoglobulin G, gamma-glutamyltransferase, aspartate aminotransferase, creatinine, urea, and total protein

	NDD	NDDA	DD	DDA	Mean square error	*P*-value
Immunoglobulin A, mg/dL	40.7[c]	48.0[b]	55.9[a]	58.6[a]	2.48	0.0001
Immunoglobulin G, mg/dL	472.5[c]	517.3[b]	549.0[a]	550.6[a]	6.76	0.0001
γ-Glutamyl transferase, U/L	79.8	80.7	78.4	79.3	4.28	0.8871
Aspartate aminotransferase, U/L	81.8	84.5	81.2	80.8	3.82	0.5412
Creatinine, mg/dL	1.97	2.07	2.00	2.17	0.16	0.3615
Urea, mg/dL	13.7	16.5	13.7	17.5	5.64	0.9246
Total protein, g/dL	4.74	4.52	4.55	4.85	0.48	0.8185

NDD: corn-soy-based diet, NDDA: corn-soy-based diet supplemented with 0.6% L-Arginine, DD: corn-soy-based diet supplemented with powdered whole milk, DDA: corn-soy-based diet supplemented with powdered whole milk and 0.6% of L-Arginine
[a,b.c] Within a row, means with a common superscript were not different with probability *P* =0.05

The experimental diets influenced the relative weight of spleen, with the highest value being observed in animals fed NDDA, which differed (*P* <0.05) from those fed with DDA (Table 5). No significant differences were observed for the relative weights of intestine, liver, and pancreas. Under these conditions, the animals fed with NDDA had a higher spleen relative weight than DDA, though both were supplemented with 0.6% arginine. There was no significant difference (*P* <0.05) in the diets without arginine supplementation (NDD and DD).

Table 5 Effects of L-Arginine supplementation in diets for weaned piglets with and without powdered whole milk on their relative weights of intestine, liver, spleen, and pancreas

Organ	NDD	NDDA	DD	DDA	Mean square error	*P*-value
Intestine	5.141	6.990	6.409	5.727	1.047	0.139
Liver	0.973	1.300	1.181	1.113	0.253	0.377
Spleen	0.063[ab]	0.077[a]	0.054[ab]	0.046[b]	0.011	0.026
Pancreas	0.086	0.097	0.113	0.083	0.024	0.345

NDD: corn-soy-based diet, NDDA: corn-soy-based diet supplemented with 0.6% L-Arginine, DD: corn-soy-based diet supplemented with powdered whole milk, DDA: corn-soy-based diet supplemented with powdered whole milk and 0.6% of L-Arginine
[a,b] Within a row, means with a common superscript were not different, with probability *P* =0.05

Morphometric characteristics in the duodenum and jejunum were influenced by the diets (*P* <0.05) (Table 6). In the duodenum, VH was greater in pigs fed DD and DDA. In NDDA, supplemental L-Arginine reduced the adverse effect of DD and provided a larger absorptive area. NDDA and DD produced greater depth of crypts in the duodenum, evidencing a higher cellular turnover. The VH/CD ratio and VD increased in animals fed DDA, again demonstrating the synergistic effect of the ingredients.

Table 6 Effects of L-Arginine supplementation in diets for weaned piglets with and without powdered whole milk on villus height, crypt depth, villus to crypt ratio, mucosal thickness, villus width, absorptive area, and goblet cell number in the duodenum

	NDD	NDDA	DD	DDA	Mean square error	*P*-value
Villus height, µm	191.0^{c}	221.8^{b}	277.0^{a}	272.0^{a}	32.23	0.0001
Crypt depth, µm	192.28^{b}	215.1^{a}	216.3^{a}	198.3^{b}	28.81	0.0001
Villus height: crypt depth	1.00^{c}	1.03^{c}	1.28^{b}	1.39^{a}	0.16	0.0001
Villus width, µm	47.60^{d}	64.13^{c}	78.06^{b}	92.49^{a}	9.48	0.0001
Absorptive area, µm²	9093^{d}	14215^{c}	21670^{b}	25177^{a}	3417	0.0001
Goblet cell number	11.10	11.35	12.80	11.70	2.54	0.1663

NDD: corn-soy-based diet, NDDA: corn-soy-based diet supplemented with 0.6% L-Arginine, DD: corn-soy-based diet supplemented with powdered whole milk, DDA: corn-soy-based diet supplemented with powdered whole milk and 0.6% of L-Arginine
^{a,b,c,d} Within a row, means with a common superscript were not different with probability *P* =0.05

In the jejunum, VH was lower (*P* <0.05) in animals fed NDDA than in NDD or DD. Animals fed DDA had intermediate VH, which was not different from those fed the other diets. However, because NDD resulted in a higher VH, it promoted an increased CD and a lower VH/CD ratio (*P* <0.05). DDA provided a higher villus height to crypt ratio, greater villus width, and larger absorptive area. The diets had no influence on the number of goblet cells in the duodenum or jejunum (*P* >0.05).

Table 7 Effects of L-Arginine supplementation in diets for weaned piglets with and without powdered whole milk on villus height, crypt depth, villus to crypt ratio, mucosal thickness, villus width, absorptive area, and goblet cell number in the jejunum

	NDD	NDDA	DD	DDA	Mean square error	*P*-value
Villus height, µm	229.6^{a}	215.3^{b}	228.9^{a}	220.4^{ab}	30.35	0.0111
Crypt depth, µm	245.4^{a}	194.5^{b}	196.5^{b}	181.9^{b}	34.40	0.0001
Villus height to crypt depth	0.94^{c}	1.12^{b}	1.16^{ab}	1.24^{a}	0.19	0.0056
Villus width, µm	47.60^{d}	64.13^{c}	78.06^{b}	92.49^{a}	8.94	0.0001
Absorptive area, µm²	109321^{d}	137910^{c}	17824^{b}	20327^{a}	2817	0.0001
Goblet cell number	11.95	11.45	12.65	12.60	3.17	0.5853

NDD: corn-soy-based diet, NDDA: corn-soy-based diet supplemented with 0.6% L-Arginine, DD: corn-soy-based diet supplemented with powdered whole milk, DDA: corn-soy-based diet supplemented with powdered whole milk and 0.6% of L-Arginine
^{a,b,c,d} Within a row, means with a common superscript were not different, with probability *P* =0.05

Animals fed NDD and NDDA had a higher (*P* <0.05) rate of mitosis in the duodenum cells compared with those fed with DD and DDA, regardless of L-Arginine supplementation (Table 8). In the jejunum, animals fed with NDDA and DD had a higher (*P* >0.05) cellular mitosis rate compared with those fed NDD and DDA.

In the duodenum, the positivity of cytoplasmic labelling to anti-caspase, which occurs mainly at the apex of the villi, where cell death and desquamation of enterocytes normally occur, was higher (*P* <0.05) in animals fed NDD and NDDA than those fed DD and DDA. In the jejunum, the rate of apoptosis was higher (*P* <0.05) in animals fed NDDA and DDA, with the effect being more intense in those animals fed DDA.

Table 8 Effects of L-Arginine supplementation in diets with and without powdered whole milk on rate of cellular mitosis and apoptosis in the duodenum and jejunum of weaned piglets

Intestinal segment	Experimental diets				Mean square error	*P*-value
	NDD	NDDA	DD	DDA		
	Rate of mitosis, cell count					
Duodenum	48.80[a]	47.75[a]	41.15[b]	42.25[b]	7.90	0.0044
Jejunum	38.10[b]	42.70[a]	42.00[a]	37.55[b]	7.03	0.0450
	Positivity score for rate of apoptosis					
Duodenum	1.33[a]	1.25[a]	0.71[b]	0.96[b]	0.14	0.0090
Jejunum	0.21[c]	0.83[b]	0.33[c]	1.09[a]	0.09	0.0001

NDD: corn-soy-based diet, NDDA: corn-soy-based diet supplemented with 0.6% L-Arginine, DD: corn-soy-based diet supplemented with powdered whole milk, DDA: corn-soy-based diet supplemented with powdered whole milk and 0.6% of L-Arginine

[a,b,c] Within a row, means with a common superscript were not different with probability *P* =0.05

When weaned at 21 days old, the digestive system of piglets is not adapted to digest a vegetable diet, since from birth its enzymatic profile is adapted to the digestion of milk, and ingredients of plant origin often contain anti-nutritional factors. In this study, the performance of piglets was affected negatively in the first few days after weaning when they were fed a diet based on corn and soybean meal. Animals fed NDDA had higher DWG and final weight in Phase I compared with those fed with DD. In Phase II, except for FCR (P <0.05), there was no difference among diets in the performance of the piglets. Feed conversion was improved by supplementation of L-arginine, powdered whole milk, or these products in combination. Thus, including powdered whole milk in the diet might be an alternative to supplementation with L-Arginine.

Increased expression of arginine transporters (CAT-1, CAT-2, and CAT-3 genes) in the jejunum might indicate an increase in demand for arginine in response to oxidative stress (Zheng *et al.*, 2017) and catabolic disease states (Pan *et al*., 2004). Stress associated with weaning probably necessitated a higher intake of arginine owing to inadequate de novo synthesis by the intestinal-renal axis.

The reduced incidence of diarrhoea in piglets fed with dairy diets may be associated with a lactose effect that produced lower stomach pH and facilitated digestion (Kummer *et al.*, 2009). In this study, the similarity of NDDA and DD (P >0.05) and their difference (P <0.05) from NDD indicated positive effects of supplementation with either L-Arginine or powdered whole milk. The further reduction in diarrhoea when the ingredients were fed in combination might indicate a degree of synergy between them. Proline (an arginine metabolite) is a key component of the collagen extracellular matrix and is crucial for angiogenesis and vascular remodelling (Yao *et al.*, 2011). Thus, dietary supplementation with arginine may contribute to reduced microvascular endothelial dysfunction, which would affect nutrient absorption and the incidence of diarrhoea.

On the other hand, the proliferation of pathogens, which can adhere to the intestinal mucosa and trigger inflammatory processes, may damage the epithelium and affect the absorptive capacity of nutrients and cause diarrhoea. M1 macrophages, which act in defence against microbial invaders and produce proinflammatory cytokines, are induced by microbial products to activate the individual nitric oxide synthase enzyme (NOS2), which uses NADPH and oxygen to act on L-Arginine and produce large amounts of nitric oxide (nitrogen monoxide (NO)). Nitric oxide binds to enzymes containing metals, such as ribonucleotide reductase, and prevents DNA synthesis and microbial proliferation, making possible the efficient destruction of bacteria, fungi, protozoa, some helminths, and tumour cells (Tizard, 2014). Han *et al.* (2009) stated that NO can kill microorganisms, and arginine would have an effect analogous to antibiotics.

Immunoglobulin production is stimulated by the cellular response differentiation of T helper lymphocytes into Th2, from antigen-presenting cells, co-stimulating the maturation of B cells for antibody production, constituting the humoral immune response (Tizard, 2014). Possibly, in this study, the dairy diets contributed to a greater differentiation of Th2 responses by T lymphocytes, increasing the serum IgA and IgG concentrations (Table 4) because of a factor intrinsic to milk.

Likewise, the supplementation of 0.6% arginine in DDA increased the serum IgA and IgG concentrations, with a positive effect on immunity. Nitric oxide plays an important role in Th1/Th2 balance (Viana *et al.*, 2010). It is possible that arginine supplementation promoted the production of antibodies,

modulating the differentiation of Th2 responses in T lymphocytes, stimulating the maturation of B lymphocytes.

Animals fed with NDDA showed a higher spleen relative weight, which may be associated with an immunological state, since the spleen is a lymphoid organ associated with the circulatory and immune systems, acting in the blood filtering process by removing antigenic and microbial particles. Tan *et al.* (2009), who investigated the immune status of early weaned piglets, found changes in the spleen relative weight, which was higher in animals supplemented with 0.2% and 0.8% arginine compared to those that were not. The higher spleen relative weight in animals fed with NDDA may be associated with increased blood capillary and increased blood exposure to the population of immune cells, T and B lymphocytes, with an improved vascular development and vasodilatory capacity of the arginine (Yao *et al.*, 2011), and of immune modulation (Wu *et al.*, 2009).

Animals fed with NDA had a higher VH/CD ratio in the jejunum compared with those fed with ND. Villus height was higher in the duodenum, indicating a possible relationship with arginine supplementation. Arginine is already known as an important vasodilator that regulates vascular tone and blood dynamics. In the pig small intestine, micro-vessels are found mainly in the mucosa and submucosa, and the villi development depends on an appropriate supply of nutrients through the blood by micro-vessels, and the intestine by enteral feeding (Yao *et al.*, 2011). This explains the increased absorptive areas in the jejunum and duodenum as a result of arginine supplementation. Zheng *et al.* (2017) indicated that supplementation of arginine reduced the CD in the jejunum significantly, with or without induced oxidative stress. A similar reduction was observed in the present study, in which there was a reduction in the CD in the jejunum in DDA.

The jejunum VH was higher in animals fed with NDD. Exposure to a predominantly vegetable diet may have increased the need for cell proliferation to maintain the villi, as the highest CD and mitotic rate (Table 8) resulted from this diet. The high demand for cell proliferation, despite increased villus height, resulted in crypts so deep that this led to a worse VH/CD ratio.

There was a higher mitosis rate in the duodenum, in animals fed with NDD and NDDA (P <0.05). This may be a compensatory response to the damage caused by these diets, since it promoted a higher apoptosis rate than DD and DDA (Table 8). However, the animals had higher villi as a consequence of the lower apoptosis rate, indicating greater integrity of villus. Regarding the jejunum, animals fed with NDDA or DD had a higher mitosis rate than those fed NDD or DDA (P <0.05). The effect of NDDA could be associated with a compensatory effect provided by the diet because animals fed with DD had higher VH than those fed with NDDA (Table 7).

The lower apical cell death in the duodenal villi in DD and DDA may be related to a higher serum IgA concentration in these groups (Table 4). Although the results correspond to what was observed in the blood serum, this immunoglobulin is secreted by plasmocytes (B cells) in the mucosa, such as the intestinal mucosa. Immunoglobulins bind and label antigens, providing more efficient immunological activity (Tizard, 2014), which may have provided better protection against possible microorganisms that are pathogenic to enterocytes.

Animals fed with NDA and DDA had a higher rate of cellular apoptosis. Animals fed with NDDA had an apoptosis rate higher than those fed with NDD and a higher mitosis rate in the jejunum, indicating a higher cellular turnover. However, animals fed with DDA, which had a higher jejunal apoptosis rate compared with the other diets (P >0.05) had a different behaviour regarding the mitosis rate (Table 8) in the jejunum. However, this caused no significant damage to the intestinal epithelium, since DDA had better FC from 21 to 42 days old (Table 3), and had no major intestinal disorders, as was observed in the incidence of diarrhoea.

Conclusions

Dietary supplementation of 0.6% L-Arginine promoted an increase in immunological activity in piglets weaned at 21 days old, improving their intestinal integrity and reducing the incidence of diarrhoea in the nursery phase, offsetting the detrimental effect of predominantly vegetable diets for piglets.

Authors' Contributions

FAMB and LAFP were responsible for project execution; LAFP, JHVS, and TDDM designed the project; RRG was responsible for the histomorphological analyses; JLSA, JMSA and JMMB conducted the zootechnical analyses; FAMB, LAFP and RRG edited the text.

Conflict of Interest Declaration

The authors declare that there was no conflict of interest.

References

Han, J., Liu, Y.L., Fan, W., Chao, J., Hou, Y.Q., Yin, Y.L., Zhu, H.L., Meng G.Q. & Che Z.Q., 2009. Dietary L-Arginine supplementation alleviates immunosuppression induced by cyclophosphamide in weaned. Amino Acids 37(4), 643-651. https://doi.org/10.1007/s00726-008-0184-9

Ishak, K, Baptista, A., Bianchi, L., Callea, F., De Groote, J., Gudat, F., Denk, H., Desmet, V., Korb, G., MacSween, R.N.M., Phillips, M.J., Portmann, B.G., Poulsen, H., Scheuer, P.J., Schmid, M. & Thaler, H., 1995. Histological grading and staging of chronic hepatitis. Journal of Hepatology 22, 696-699. https://doi.org/10.1016/0168-8278(95)80226-6

Kaneko, J., Harvey, J. & Bruss, M. (ed.), 2008. Clinical biochemistry of domestic animals, 6th edition. Academic Press, Burlington, Massachusetts, USA.

Kummer, R., Gonçalves, M.A.D., Lippke, R.T., Prado Marques, B.M.F.P. & Mores, T.J., 2009. Fatores que influenciam no desempenho dos leitões na fase de creche. Acta Scientiae Veterinaria 37 (Suppl 1), 195-209.

Lallès, J-P., Bosi, P., Smidt, H. & Stokes, C.R., 2007. Weaning - A challenge to gut physiologists. Livestock Science 108, 82-93. https://doi.org/10.1016/j.livsci.2007.01.091

Liu, Y., Huang, J., Hou, Y., Zhu, H., Zhao, S., Ding, B., Yin, Y., Yi, G., Shi, J. & Fan W., 2008. Dietary arginine supplementation alleviates intestinal mucosal disruption induced by *Escherichia coli lipopolysaccharide* in weaned pigs. Brit. J. Nutr. 100(3), 552-560. DOI: 10.1017/S0007114508911612

Luna, A.C.L., Passos, C.C., Ferreira, A.O., Bateman, A., Miglino. A.M. & Guerra, R.R., 2014. Expression of progranulin during the first stages of liver development in rat Fischer 344. Brazilian J. Vetr. Res. Anim. Sci. (Impresso), 50, 270-278. https://doi.org/10.11606/issn.2318-3659.v50i4p270-278

Mateo, R.D., Wu, G., Moon, H.K., Carroll, J.A., & Kim, S.W. 2008. Effects of dietary arginine supplementation during gestation and lactation on the performance of lactation primiparous sows and nursing piglets. J. Anim. Sci. 86(4), 827-835. https://doi.org/10.2527/jas.2007-0371

Moreira Filho, A.L., de Oliveira, C.J.B., de Oliveira, H.B., Campos, D.B., Guerra, R.R., Costa, F.G.P. & Givisiez, P.E.N., 2015. High incubation temperature and threonine dietary level improve ileum response against post hatch *Salmonella enteritidis* inoculation in broiler chicks. PloS One. https://doi.org/10.1371/journal.pone.0131474

Pan, M., Choudry, H.A., Epler, M.J., Meng, Q., Karinch, A., Lin, C. & Souba, W., 2004. Arginine transport in catabolic disease states. Journal of Nutrition 134(10), 2826S-2819S. https://doi.org/10.1093/jn/134.10.2826S

Pluske, J.R., Kim, J.C. & Black, J.L., 2018. Manipulating the immune system for pigs to optimise performance. Animal Production Science 58(4), 666-680. https://doi.org/10.1071/AN17598

Rostagno, H.S., Albino, L.F.T., Donzele, J.L., Gomes, P.C., de Oliveira, R.F., Lopes, D.C., Ferreira, A.S., Barreto S.L.T., & Euclides, R.F., 2017. Tabelas Brasileiras para Aves e Suínos: composição dos alimentos e exigências nutricionais. 4th ed. UFV, Viçosa. Pp. 443-444.

Roth, E., 2008. Non-nutritive effects of glutamine. J. Nutr. 138(10), 2025-2031.https://doi.org/10.1093/jn/138.10.2025S

Sharma, K.K., Mediratta, P.K., Reeta, K.H. & Mahajan, P., 2004. Effect of L-Arginine on restraint stress induced modulation of immune responses in rats and mice. Pharmacological Research 49(4), 455.460. https://doi.org/10.1016/j.phrs.2003.11.009

Silva, D.R.P. 2015. Adição de L-glutamina + ácido glutâmico e L-arginina na dieta de leitões recém desmamados. Dissertação (Mestrado em Zootecnia). Programa de Pós-Graduação em Zootecnia. UFPB. Areia-PB.

Tan, B., Li, X.G., Kong, X., Huang, R., Ruan, Z., Yao, K., Deng, Z., Xie, M., Shinzato, I., Yin, Y. & Wu, G., 2009. Dietary L-Arginine supplementation enhances the immune status in early-weaned piglets. Amino Acids 37(2), 321-331. https://doi.org/10.1007/s00726-008-0155-1

Tizard, I.R., 2014. Imunologia veterinária. 9 ed. Elsevier, Rio de Janeiro.

Viana, M.L., Rosana G.C., Santos, R.G.C., Generoso, S.V., Arantes, R.M.E., Correia, M.I.T.D. & Cardoso, V.N., 2010. Pre-treatment with arginine preserves intestinal barrier integrity and reduces bacterial translocation in mice. Nutrition 26, 218-223. https://doi.org/10.1016/j.nut.2009.04.005

Wang, J., Chen, L., Li, P., Li, X., Zhou, H., Wang, F., Li, D., Yin, Y. & Wu, G., 2008. Gene expression is altered in piglet small intestine by weaning and dietary glutamine supplementation. Journal of Nutrition 138 (6), 1025-1032. https://doi.org/10.1093/jn/138.6.1025

Wu, G., 2009. Amino acids: Metabolism, functions, and nutrition. Amino Acids 37, 1-17. https://doi.org/10.1007/s00726-009-0269-0

Wu, G., 2013. Functional amino acids in nutrition and health. Amino Acids 45, 407-411. https://doi.org/10.1007/s00726-013-1500-6

Wu, L., Liao, P., He, L., Feng, Z., Ren, W., Yin, J., Duan, J., Li, T. & Yin, Y., 2015. Dietary L-Arginine supplementation protects weanling pigs from deoxynivalenol-induced toxicity. Toxins 7(4), 1341-1354. https://doi.org/10.3390/toxins7041341

Wu, X., Ruan, Z., Gao, Y., Yin, Y., Zhou, X., Wang, L., Geng, M., Hou Y. & Wu, G., 2010. Dietary supplementation with L-Argininеor N- carbamy l-glutamate enhances intestinal growth and heat shock protein-70 expression in weanling pigs fed a corn- and soybean meal-based diet. Amino Acids 39, 831-839. https://doi.org/10.1007/s00726-010-0538-y.

Yao, K., Guan, S., Li, T., Huang, R., Wu, G., Ruan, Z. & Yin, Y., 2011. Dietary L-Arginine supplementation enhances intestinal development and expression of vascular endothelial growth factor in weanling piglets. 105(5), 703-709. DOI: 10.1017/S000711451000365X

Yeh, C.L., Yeh, S.L., Lin, M.T. & Chen, W.J., 2002 Effects of arginine-enriched total parenteral nutrition on inflammatory-related mediator and T-cell population in septic rats. Nutrition 18 (7-8), 631-635. https://doi.org/10.1016/S0899-9007(02)00809-2

Yi, D., Li, B., Hou, Y., Wang, L., Zhao, D., Chen, H., Wu, T., Zhou, Y., Ding, B. & Wu, G., 2018. Dietary supplementation with an amino acid blend enhances intestinal function in piglets. Amino Acids 50(8), p.1089-1100. DOI: 10.1007/s00726-018-2586-7

Zheng, P., Yu, B., He, J., Tian, G., Luo, Y., Mao, X., Zhang, K., Che, L. & Chen, D., 2013. Protective effects of dietary arginine supplementation against oxidative stress in weaned piglets. Brit. J. Nutr. 109(12), 2253-2260. DOI: 10.1017/S0007114512004321

Zheng, P., Yu, B., He, J., Yu, J., Mao, X., Luo, Y., Luo, J., Huang, Z., Tian, G., Zeng, Q., Che, L. & Chen, D., 2017. Arginine metabolism and its protective effects on intestinal health and functions in weaned piglets under oxidative stress induced by diquat. Brit. J. Nutr. 117(11), 1495-1502. https://doi.org/10.1017/S0007114517001519