CSIRO PUBLISHING

Animal Production Science, 2019, **59**, 1880–1885
https://doi.org/10.1071/AN17630

Supplementation of diets with glutamine and glutamic acid attenuated the effects of cold stress on intestinal mucosa and performance of weaned piglets

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Abstract. In this study we investigated the effect of glutamine and glutamic acid inclusion in the diet of weaned piglets subjected to cold stress and thermoneutral environment. Sixty-four weaned piglets were assessed from 28 to 65 days of age. A completely randomised design consisting of a 2 × 2 factorial arrangement was tested – environments (thermoneutral and cold stress) and diets (control and L-glutamine + L-glutamic acid (G + GA)). Performance, relative organ weight and carcass yield, and morphology of the intestinal mucosa were assessed. Supplementing the diets with G + GA reduced feed intake under both environments. This was associated with a decline in growth rate for piglets in the thermoneutral environment but not in the cold environment ($P < 0.002$). Feed efficiency was lower for piglets offered the control diets in the cold environment, but was significantly improved (24.6%) by G + GA supplementation in the cold but not the thermoneutral environment ($P < 0.001$). G + GA supplementation decreased small intestinal length and altered intestinal morphology with the highest villus/crypt depth ratio observed in piglets offered the G + GA supplemented diet in the cold environment. In summary, glutamine and glutamic acid diets mitigated the effects of cold stress on the intestinal mucosa and performance of weaned piglets.

Additional keywords: animal stress, digestion and feeding, nutrition, pigs, temperature.

Received 24 January 2017, accepted 9 April 2019, published online 15 August 2019

Introduction

Weaning is a period of challenges for the piglets due to abrupt separation from the sow, transportation to a new physical environment, cold stress, a different food source, commingling with piglets from other litters, greater exposure to pathogens and aversive handling practices such as restraint and vaccination (Campbell *et al.* 2013). Among these stressors, the thermal environment has been reported to adversely affect health and piglet's development (Jones *et al.* 2001; Brown-Brandl *et al.* 2004; Carroll *et al.* 2012). In cold environments, production efficiency is often compromised because metabolism is markedly altered to provide energy for greater thermogenesis at the expense of tissue accumulation and growth (Heath 1983). Cold stress reduces the availability of glucose for normal tissue development, accompanied by changes in the metabolic profile and disturbances of gastrointestinal integrity (Johnson *et al.* 2015). In addition, cold stress can also be considered as a predisposing factor that increases the incidence of diarrhoea in post-weaning piglets (van Beers-Schreurs *et al.* 1992).

For these reasons, special care should be taken in weaning piglets and providing high-quality diets to minimise the risk of diseases and reinforce gastrointestinal and immune systems (Martínez-Miró *et al.* 2016). To this end, feed additives such as enzymes and dietary acids may be used to improve the digestibility of the diet. Dietary supplementation of glutamine and glutamic acid is highlighted by Burrin *et al.* (2008): these substances have trophic action on the intestinal mucosa and may support better piglet performance due to the greater

capacity to digest and absorb nutrients from the diet. In addition, these amino acids may be precursors for the synthesis of other amino acids, nucleotides, nucleic acids, amino sugars and proteins (Sakiyama *et al.* 2009; Molino *et al.* 2012).

Some studies have demonstrated the benefits of glutamine supplementation on swine performance in challenging situations (Wu *et al.* 1996; Kitt *et al.* 2003; Yi *et al.* 2005; Zhong *et al.* 2011; Molino *et al.* 2012). Yi *et al.* (2005) stated that glutamine supplementation in feed minimised growth depression and intestinal mucosa villous atrophy of piglets challenged with *Escherichia coli* K88+. However, the aforementioned studies were conducted with pigs challenged in a thermoneutral environment, and not with piglets in a cold environment that are at greater risk of compromised health and development. In this sense, glutamine supplementation associated with glutamic acid seems to be an alternative to improve the development of the intestinal mucosa of weaned piglets in cold stress conditions.

The objective of this study was to investigate the effects of glutamine and glutamic acid supplemented in the diet on the performance, carcass and organ weights, and intestinal morphology of piglets managed in thermoneutral and cold stress environments.

Material and methods

Animal care and use

The study was reviewed and approved by the Federal University of Paraíba Animal Care and Use Committee.

Animals and experimental design

The experiment was conducted in air-conditioned rooms in Bananeiras city, Paraiba, Brazil (06°45′00″S, 35°38′00″W, 520 m altitude). Sixty-four piglets (32 castrated males and 32 females) from Agroceres commercial line, weaned at 25 days (d) of age and with average initial weight of 7.00 ± 0.58 kg, and final live weight of 30.20 ± 2.82 kg (mean ± s.e.m.) were used. There was a 3 days adaptation period to the facilities, and the experimental period was 37 days. One male and one female were randomly assigned to 1.0 × 1.3 m cages, suspended 1 m from the ground and equipped with a slatted floor, feeders and nipple drinkers. A completely randomised design was used with four treatments (factorial arrangement 2 × 2; environments (thermoneutral and cold stress) and diets (control and G + GA)), with 16 replicates. Each experimental unit consisted of one castrated male and one female.

Management

Corn and soybean meal-based diets (Table 1) were offered *ad libitum* twice a day. They were formulated as recommended by Rostagno *et al.* (2011), in order to meet the nutritional requirements of pre-initial (7–15 kg) and initial (15–30 kg) phases. The commercial product used was AminoGut, composed of 10% L-glutamine + 10% L-glutamic acid (AminoGut, AJINOMOTO, Ajinomoto of Brazil Industry and Food Trade Ltda, São Paulo, Brazil). AminoGut was used to replace 1.0% of washed sand in the control diets, whereas G and GA were included at 0.1% or 1 kg/tonne.

The environmental temperature in the two rooms was adjusted to thermoneutral or cold stress, according to the age of the piglets. In the thermoneutral environment, air temperature was 25°C from 28 to 49 days of age, and 20°C from 50 to 65 days of age. In the cold stress environment, the temperature was kept 5°C lower than the thermoneutral. Temperature and relative humidity were monitored daily at 0900 and 1500 hours (Table 2) with a digital thermo hygrometer (accuracy ± 0.35°C temperature, and ± 0.25% relative humidity; model 766401000 Incoterm, Cotronic Technology Ltd, Guangdong, China).

Table 1. Composition and nutrient content of the control diets
CP, crude protein; ME, metabolisable energy

	Control diets	
	28–49 days	50–65 days
Ingredient (%)		
Corn	45.91	60.94
Soybean meal (46% CP)	33.04	28.89
Whey powder	11.42	4.28
Soybean oil	5.03	1.70
Dicalcium phosphate	1.45	1.13
Inert[B]	1.00	1.00
Limestone	0.69	0.77
Premix[C]	0.50	0.50
Sodium chloride	0.23	0.31
Antioxidant[D]	0.02	0.02
L-lysine HCl (78%)	0.41	0.29
DL-methionine (99%)	0.08	0.06
L-threonine (98%)	0.17	0.07
L-tryptophan (98%)	0.01	0.00
Nutrient[A]		
ME (Mcal/kg)	3.37	3.32
CP (%)	21.00	19.24
Lactose (%)	8.00	3.00
Digestible lysine (%)	1.33	1.09
Digestible methionine (%)	0.37	0.34
Digestible threonine (%)	0.83	0.68
Digestible tryptophan (%)	0.24	0.20
Calcium (%)	0.82	0.76
Available phosphorus (%)	0.45	0.38

[A]Nutritional composition was calculated according to Rostagno *et al.* (2011).
[B]Washed sand or AminoGut (commercial product containing 10% L-glutamine + 10% L-glutamic acid; >95%), according to the treatment.
[C]Premix: folic acid 120 mg/kg, pantothenic acid 3200 mg/kg, biotin 40 mg/kg, choline 60 g/kg, niacin 6000 mg/kg, vitamin A 2 000 000 IU/kg, vitamin B_1 300 mg/kg, vitamin B_2 1200 mg/kg, vitamin B_6 400 mg/kg, vitamin B_{12} 4000 mg/kg, vitamin D3 400 000 IU/kg, vitamin E 8000 IU/kg, vitamin K 3400 mg/kg; minerals: cobalt 92 mg/kg, copper 4000 mg/kg, iron 20 g/kg, iodine 200 mg/kg, manganese 14 g/kg, selenium 80 mg/kg, zinc 20 g/kg.
[D]Butylated hydroxytoluene.

Table 2. Air temperature and relative humidity (mean ± s.e.m.) during the experimental period

Piglet age	Environments	Air temperature (°C)	Relative humidity (%)
28–49 days	Thermoneutral	25.3 ± 1.050	72.5 ± 3.900
	Cold	19.7 ± 1.220	69.7 ± 4.800
50–65 days	Thermoneutral	20.2 ± 0.780	73.9 ± 2.850
	Cold	15.4 ± 1.180	64.8 ± 4.450

Performance and carcass traits

Daily feed intake (ADFI; kg), daily weight gain (ADG; kg) and feed efficiency (gain : feed (G : F); kg/kg) were calculated for the total period. At the end of the experimental period, animals were fasted for 12 h and weighed before slaughter. The carcasses were eviscerated and weighed, as well as the following organs: heart, liver, lung, spleen and small intestine. The small intestine length was determined with a measuring tape. Yield data (%) was calculated as the ratios between carcass weight and final liveweight at slaughter (kg/kg), organ weight and carcass weight (kg/kg), and intestine length and carcass weight (cm/kg).

Sample collection and morphological analyses

To assess intestinal mucosa morphology, tissue fragments of ~3 cm were sampled in the middle portion of the duodenum of 16 animals (four animals in each treatment), washed in 0.9% NaCl saline solution and stored in 10% formaldehyde for 24 h. Samples were processed routinely for histological analyses in the Laboratory of Animal Products of the Centre for Agricultural Sciences of the Federal University of Paraiba. Briefly, after washing in 70% ethanol, tissue samples were subjected to dehydration in an increasing series of ethanol concentrations (70, 80, 90 and 100%), cleared with xylene, and included in paraffin. Later, 5-μm sections were semi-serially cut and placed onto glass slides to be stained with hematoxylin and eosin. Photomicrographs were taken using a digital camera model (Sony Cyber-shot DSC-W230 Digital Camera, 12.1 Megapixel, San Diego, CA, USA) attached to a microscope, with 2.6 zoom and ×20 lens.

The morphometric study was conducted using image analyser software (Image J, National Institutes of Mental Health, Bethesda, MD, USA). Villous height and crypt depth were determined in addition to the villus height/crypt depth ratio, with 10 readings per slide. Villus height was determined from its basal region, which coincides with the surface of the crypt to its apex. Crypt depth was measured from the crypt villus transition region to its base. Villus/crypt ratio was determined from villus height divided by crypt depth. One tissue fragment from each slaughtered piglet (four per treatment) was removed, and two glass slides were made. Photomicrographs were made on each slide, with 10 readings per slide; totalling 320 readings per analysed histological variable.

Statistical analyses

The data were analysed by the least-squares method and averages were compared by Tukey's test at 5% probability. For data related to organ weights, fasting bodyweight was used as covariate. The mathematical model can be described as:

$$Y_{ijkl} = \mu + D_i + E_j + I_{ij} + \beta(X_{ij} - X) + \varepsilon_{ijkl}$$

where Y_{ijk} is observed value, μ is overall average, D_i is diet treatment fixed effect, E_k is environment fixed effect, I_{ij} is diet and environment interaction; X_{ij} is covariate, and ε_{ijk} is residual term, inclusive of the random error.

All statistical analyses were performed using SAS 9.2 (SAS Institute Inc., Cary, NC, USA).

Results

Performance and carcass

Performance results are shown in Table 3. ADFI was ($P < 0.001$) reduced by G + GA supplementation in both environments. For ADG and G : F there were interactions ($P < 0.05$) between environment and diet. In the thermoneutral environment G + GA supplementation reduced ADG but it was unaffected in the cold environment. G : F was lower for piglets offered the control diets and housed in the cold environment but was significantly increased by G + GA supplementation in this environment but not in the thermoneutral environment.

There were no interactions ($P > 0.05$) between environment and diet for carcass measurements (Table 4). Piglets in cold environment had a lower ($P = 0.005$) carcass weight and increased ($P = 0.002$) heart, ($P = 0.003$) liver, ($P = 0.004$) lung and ($P = 0.006$) spleen weights when compared with the thermoneutral environment. The piglets fed the supplemented diet had an increased ($P = 0.003$) spleen weight and had a less ($P = 0.002$) small intestine.

Morphological characteristics

Duodenum morphology is shown in Table 5. There was an interaction ($P < 0.001$) between environment and diet; the

Table 3. Effects of the thermal environment and dietary supplementation with L-glutamine (G) and L-glutamic acid (GA) on the performance of piglets from 28 to 65 days of age

Treatment means in the same row followed by a different lowercase letter and in the same column followed by a different uppercase letter differ significantly by Turkey's test at 5% probability. D, diet; E, environment

Variable	Diets		s.e.m.	*P*-value		
	Control	G + GA[A]		D	E	D × E
			Daily weight gain (kg)			
Thermoneutral	0.455aA	0.384bB	6.423	0.004	0.048	0.002
Cold	0.393aA	0.400aA				
			Daily feed intake (kg)			
Thermoneutral	0.682aA	0.567bA	9.242	<0.001	0.979	0.777
Cold	0.687aA	0.561bA				
			Gain : feed (kg/kg)			
Thermoneutral	0.667aA	0.677aA	7.098	<0.001	0.004	<0.001
Cold	0.572bB	0.713aA				

[A]G and GA included at 0.1% (1 kg/t).

Table 4. Effects of the thermal environment and dietary supplementation with L-glutamine (G) and L-glutamic acid (GA) on the carcass and organ yield and length of small intestine of piglets at 65 days of age

Treatment means in the same row followed by a different lowercase letter and in the same column followed by a different uppercase letter differ significantly by Turkey's test at 5% probability. D, diet; E, environment

Variable	Environments	Diets		s.e.m.	*P*-value		
		Control	G + GA[A]		D	E	D × E
Carcass (%)	Thermoneutral	74.78aA	75.30aA	0.430	0.531	0.005	0.683
	Cold	73.68aB	73.99aB				
Heart (%)	Thermoneutral	0.70aB	0.70aB	0.010	0.883	0.002	0.888
	Cold	0.78aA	0.76aA				
Liver (%)	Thermoneutral	3.54aB	3.75aB	0.150	0.920	0.003	0.807
	Cold	3.96aA	4.13aA				
Lung (%)	Thermoneutral	1.44aB	1.53aB	0.080	0.204	0.004	0.545
	Cold	2.11aA	1.83aA				
Spleen (%)	Thermoneutral	0.24bB	0.29aB	0.010	0.003	0.006	0.837
	Cold	0.32bA	0.37aA				
Small intestine (cm kg^{-1})	Thermoneutral	13.89aA	12.49bA	0.330	0.002	0.101	0.958
	Cold	12.93aA	11.47bA				

[A]G and GA included at 0.1% (1 kg/t).

Table 5. Effects of the thermal environment and dietary supplementation with L-glutamine (G) and L-glutamic acid (GA) on the villus height, crypt depth and villus/crypt ratio in the duodenum of piglets at 65 days of age

Treatment means in the same row followed by a different lowercase letter and in the same column followed by a different uppercase letter differ significantly by Turkey's test at 5% probability. D, diet; E, environment

Variable (μm)	Diet		s.e.m.	*P*-value		
	Control	G + GA[A]		D	E	D × E
		Villus height				
Thermoneutral	349.63bA	432.17aA	1.230	<0.001	<0.001	<0.001
Cold	326.34bB	434.98aA				
		Crypt depth				
Thermoneutral	94.64bB	101.76aA	1.090	<0.001	<0.001	<0.001
Cold	99.67aA	98.87aA				
		Villus/crypt ratio				
Thermoneutral	3.72bA	4.25aB	0.080	<0.001	<0.001	<0.001
Cold	3.30bB	4.40aA				

[A]G and GA included at 0.1% (1 kg/t).

data are discussed within each factor. Villus height increased ($P < 0.001$) when G + GA were supplemented in the diet in both environments. The cold stress decreased ($P < 0.001$) villus height in piglets fed the control diet, but was not different between thermoneutral and cold-stressed piglets that were fed G + GA.

Crypt depth increased ($P < 0.001$) in cold-stressed piglets fed the control diet, but no difference between environments was seen in G + GA supplemented piglets. Villus/crypt ratio increased ($P < 0.001$) with supplementation in both environments. In the cold stress piglets, control diet decreased ($P < 0.001$) villus/crypt ratio, while in G + GA supplemented animals, villus/crypt ratio was increased.

Discussion

Results of the present study showed that supplementation of the diet with G + GA mitigated the effects of cold stress on piglet performance, and markedly improved feed efficiency. Supplementation of the diet had little effect on organ weights except for the spleen and the small intestine, and the effects were similar across environments. However, supplementation did affect intestinal development, with piglets in the cold environment overcoming the lower villus height evident between the two environments when piglets were offered the control diet and exhibiting a lower and higher villus/crypt ratio than their warmer counterparts on the control and supplemented diets respectively. These results suggest that cold stress likely inhibits mucosal development and nutrient absorption, but that this is overcome by increasing the supply G + GA. Glutamine is one of the major fuel sources for the small intestinal mucosal cells. The two amino acids have high availability and are fully utilised in the small intestine (Stoll *et al.* 1998). In addition, improved cell turnover, results in increased absorption efficiency and utilisation of nutrients derived from food (Amorim *et al.* 2017). Several studies also observed improved performance in weaned piglets that were supplemented with glutamine (Yi *et al.* 2005; Abreu *et al.* 2010; Molino *et al.* 2012).

The literature indicates that in cold conditions the animal increases the consumption of food to compensate the energetic costs of thermoregulation. In our study, piglets in both

environments ate similar amounts of feed and both reduced their intake when the diet was supplemented with G and GA. During the course of the experiment it was observed that the piglets spent much of the time lying together, mainly in the cold environment. The piglets may have chosen to stay warm (lying together) avoiding exposing through displacement, thus decreasing the search for food, which may be why ADFI was not increased by the lower ambient temperature (Dividich and Noblet 1983; Herpin *et al.* 2002, 2005). According to Ferreira (2005), piglets naturally like to stay grouped in the cold.

The increase in organ weights in the cold environment was in line with expectations that under cold stress activity is increased in the more metabolically active organs (e.g. heart, liver, lung and spleen) to increase endogenous heat production and maintain body temperature (Saraiva *et al.* 2003; Manno *et al.* 2005). The increase in the weight of the spleen elicited by G and GA supplementation may reflect the role of the two amino acids (or the organ) in the immune system. Moreover, glutamine is essential for the growth, survival and physiological health of actively dividing cells such as enterocytes, fibroblasts and lymphocytes (Calder and Newsholme 2002; Fuchs and Bode 2006).

The addition of glutamine in the diet promotes improvements in the villus height and crypt depth, and increasing intestinal wall thickness (Stoll *et al.* 1998). The higher villus height exhibited by piglets offered the G + GA diets in the cold environment was likely associated with the fact that glutamine hydrolysis generates products such as fumarate and aspartate, which can directly enter the Krebs cycle and produce ATP, showing that glutamine is the primary energy source for piglet enterocytes (Tucci *et al.* 2011). Thus, the fast oxidative metabolism of enterocytes will be supplied by the energy required to maintain the process of differentiation and cell proliferation, in addition to stimulating the defence mechanism of the intestinal mucosa (Blachier *et al.* 2009).

Our observations on the effect G + GA supplementation on the villus/crypt height ratio are in line with the results of Wu *et al.* (1996), Liu *et al.* (2002) and Silva (2009). Wu *et al.* (1996) reported that, in addition to substantial improvements in the performance of pigs, glutamine dietary inclusion (1.0%) was able to prevent atrophy of the jejunum villus in piglets, both in the first and in the second week after weaning. Our findings on villi height and crypt depth are also in agreement with the reports by Maiorka *et al.* (2002) and Kumar *et al.* (2007). These studies showed that the action of glutamine associated with glutamic acid relates to cellular turnover rate of epithelial cells, which brings benefits to mucosal integrity, improves villi height and crypt depth and consequently improves feed efficiency and performance. To date, there are no reports on the effects of cold stress on the intestinal development of weaned piglets. However, there is evidence that cold stress results in a compromised intestinal epithelial cell proliferation rate in rats (Kaushik and Kaur, 2005), and also affects the intestinal development in poultry (Tsiouris *et al.* 2015). In this context, future experiments should aim to further investigate the effects of cold on the growth, development and intestinal health of weaned piglets, as this is a crucial stage in the pigs development and affects life time performance.

Conclusions

Glutamine and glutamic acid supplementation (0.1%) overcame reduced intestinal development in the cold environment resulting in improved feed efficiency and performance of weaned piglets.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

We gratefully acknowledge the Bioclimatology, Behaviour and Animal Welfare Group of the Universidade Federal da Paraíba, and Animal Science Graduate Program of the Universidade Federal da Paraíba. The study was financially supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

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Handling editor: Kate Plush