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Methionine + cystine levels and vitamin B_6 supplementation on performance and enzyme expression of methionine metabolism of gilts from 75 to 100 kg

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ABSTRACT - This study was carried out to evaluate the effect of different levels of standardized ileal digestible (SID) methionine + cystine (Met+Cys) and vitamin B_6 supplementation on the performance, blood variables, and gene expression of enzymes involved in methionine metabolism in female pigs between 75 and 100 kg. Fifty six female pigs were used (Talent × Topigs 20), averaging 75.06±1.68 kg in initial weight, allotted in a completely randomized block design arranged in a 2 × 4 factorial scheme, composed of two vitamin B_6 supplementation levels (1.58 and 3.58 mg/kg) and four levels of SID Met+Cys (0.370, 0.470, 0.570, and 0.670%), with seven replicates and one animal per experimental unit. No interactions between vitamin B6 supplementation and SID Met+Cys levels were observed. The levels of SID Met+Cys and vitamin B_6 supplementation did not affect animal performance. Triacylglycerols showed a quadratic response to the SID Met+Cys levels, in which the lowest plasma concentration was estimated as 0.575%. Treatments did not affect the expression of the methionine synthase and cystathionine-γ-lyase enzymes or serum homocysteine levels. The SID Met+Cys requirement for female pigs from 75 to 100 kg is equal to or lower than 10.60 g/day, which corresponds to the level of 0.370% Met+Cys in the diet and a relationship 0.48% with the SID lysine.

Key Words: blood variable, cystathionine-γ-lyase, homocysteine, methionine synthase, performance, pigs

Introduction

Methionine is an essential amino acid for pigs and participates in several metabolic pathways. In addition to its protein deposition function, methionine is the precursor for other amino acids, including cysteine, which is also used for body protein synthesis (Brosnan and Brosnan, 2006). As S-adenosylmethionine, methionine is an important donor of methyl groups ($-CH_3$) for a number of body substances (such as creatine) and is also involved in polyamine synthesis (Nelson and Cox, 2014). Cysteine, in its turn, is involved in the synthesis of the coat protein and other major body components, such as glutathione (Stipanuk and Ueki, 2011).

Nowadays, the relationship of hyperhomocysteinemia with the development of cardiovascular disease has aroused the interest of researchers in studying homocysteine, which is a metabolite of the methionine cycle. In general, metabolism maintains the blood homocysteine concentrations at low levels, but their elevation has multifactorial causes, the most reported of which are related to nutrition, including the deficiency of vitamins involved in methionine metabolism (B_6, B_{12}, and folate) or high dietary methionine levels (Shoveller et al., 2004; França et al., 2006; Zhang et al., 2009).

Vitamin B_6 (pyridoxine) plays an important role in methionine metabolism and controls homocysteine levels. It acts like a cofactor for three enzymes linked to methionine metabolism: serine hydroxymethyl transferase, cystathionine β-synthase, and cystathionine-γ-lyase; the latter two related to the transsulfuration pathway, which is considered the major route for the elimination of excess homocysteine (Brosnan and Brosnan, 2006).

However, knowledge of the mechanisms underlying methionine metabolism requires more detailed information, such as the mechanisms by which specific nutrients regulate the expression of certain genes related to the metabolism of this amino acid. These interactions are studied by genomic nutrition, a science known as nutrigenomics (Gonçalves et al., 2009). Thus, gene expression related to

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methionine metabolism, associated with the concentrations of metabolites such as homocysteine, can improve our understanding of animal physiology across different nutritional strategies.

In this way, this study was carried out to evaluate different levels of standardized ileal digestible (SID) methionine + cystine (Met+Cys) and vitamin B_6 supplementation on the performance, blood variables, and gene expression of enzymes involved in methionine metabolism in female pigs from 75 to 100 kg.

Material and Methods

The experiment was carried out in Maringá, Paraná, Brazil (23 ° 21'S, 52 ° 04'W, 564 m altitude). The experimental procedures were approved by the local Animal Use and Ethic Committee, protocol No. 164/2014).

Fifty-six female pigs were used (Talent × Topigs 20), averaging an initial weight of 75.06±1.68 kg. The pigs were housed in an open-sided finishing barn, in pens with 2.20 × 1.00 m divided in half by iron bars (1.10 m^2 each), with a solid cement floor and shallow pool area (0.10 m deep), and equipped with semi-automatic individual feeders and nipple drinkers (free access to feed and drinking water) located in a masonry building. The facilities were previously washed and disinfected, keeping sanitary break of seven days before the animal housing. During the experiment, the sanitary management consisted in the washing of installations and water renewal of water blade every 48 h. The temperature and humidity were monitored with the aid of a data logger (Hobbo U10®), installed in the center of the experimental building, and data were collected every 30 min during the experimental period.

The animals were allotted in a completely randomized block design arranged in a 2 × 4 factorial scheme, with seven replicates per treatment and one animal per experimental unit. Treatments were composed of two vitamin B_6 supplementation levels (1.58 and 3.58 mg/kg) and four levels of SID Met+Cys (0.370, 0.470, 0.570, and 0.670%). These SID Met+Cys levels were established according to a literature review, considering the genotype used in the experiment.

The experimental diets were formulated using corn, soybean meal, vitamins, minerals, and additives (Table 1) to meet the requirements proposed by the NRC (2012) for female pigs from 75 to 100 kg, except for SID Met+Cys, which ranged from 0.370 to 0.670%.

The amino acid compositions of corn and soybean meal used in the diets were analyzed by reflectance spectrophotometry technique on near infrared and then the ileal digestibility coefficients were applied as proposed by Rostagno et al. (2011) to estimate the SID amino acid values. DL-methionine was added to the experimental diets to meet the levels of 0.470, 0.570, and 0.670% of SID Met+Cys. Glutamic acid was used to keep the nitrogen levels consistent across all experimental diets. The vitamin premix secured the supplementation of 1.58 mg/kg of vitamin B_6, according to the manufacturer's recommendation, and the level of 3.58 mg/kg was achieved through the synthetic vitamin B_6 (pyridoxine hydrochloride).

Table 1 - Centesimal, chemical, and energetic composition of experimental diets (as feed basis) containing different levels of standardized ileal digestible (SID) methionine + cystine (Met+Cys)

Item	SID Met+Cys (%)			
	0.370	0.470	0.570	0.670
Ingredient (%)				
Corn	84.34	84.34	84.34	84.34
Soybean meal	11.35	11.35	11.35	11.35
Oil	1.07	1.04	1.03	1.00
Limestone	0.99	0.99	0.99	0.99
Dicalcium phosphate	0.70	0.70	0.70	0.70
Salt (NaCl)	0.20	0.20	0.20	0.20
L-lysine-HCl (78.4%)	0.39	0.39	0.39	0.39
DL-methionine (99.0%)	-	0.10	0.20	0.30
L-threonine (98.0%)	0.11	0.11	0.11	0.11
L-tryptophan (98%)	0.03	0.03	0.03	0.03
L-valine (98.5%)	0.03	0.03	0.03	0.03
Glutamic acid	0.31	0.21	0.10	-
Inert[1]	-	0.03	0.05	0.08
Antioxidant[2]	0.01	0.01	0.01	0.01
Growth promoter[3]	0.02	0.02	0.02	0.02
Mineral premix[4]	0.05	0.05	0.05	0.05
Vitamin premix[5]	0.40	0.40	0.40	0.40
Calculated composition (%)				
Metabolizable energy (kcal/kg)	3,300	3,300	3,300	3,300
Total nitrogen (%)	2.01	2.01	2.01	2.01
Calcium (%)	0.56	0.56	0.56	0.56
Available phosphorus (%)	0.26	0.26	0.26	0.26
Sodium (%)	0.10	0.10	0.10	0.10
Potassium (%)	0.45	0.45	0.45	0.45
Chlorine (%)	0.25	0.25	0.25	0.25
SID lysine (Lys) (%)	0.770	0.770	0.770	0.770
SID methionine (%)	0.174	0.274	0.374	0.474
SID Met+Cys (%)	0.370	0.470	0.570	0.670
SID threonine (%)	0.480	0.480	0.480	0.480
SID tryptophan (%)	0.130	0.130	0.130	0.130
SID valine (%)	0.510	0.510	0.510	0.510
SID isoleucine (%)	0.411	0.411	0.411	0.411
SID leucine (%)	1.046	1.046	1.046	1.046
SID histidine (%)	0.298	0.298	0.298	0.298
SID phenylalanine (%)	0.522	0.522	0.522	0.522
SID arginine (%)	0.668	0.668	0.668	0.668
SID Met+Cys:Lys	0.48	0.61	0.74	0.87

[1] Sand.
[2] Butylated hydroxytoluene (BHT).
[3] Tylosin phosphate (25%).
[4] Content/kg of diet: iron, 50.00 g; cooper, 5.00 mg; cobalt, 0.50 mg; manganese, 20.00 mg; zinc, 50.00 mg; iodine, 0.75 mg; selenium, 0.30 mg.
[5] Content/kg of diet: vitamin A, 4,400 IU; vitamin D_3, 960 IU; vitamin E, 25.60 IU; vitamin B_1, 0.640 mg; vitamin B_2, 2.13 mg; vitamin B_6, 1.58 mg; vitamin B_{12}, 16.00 mcg; nicotinic acid, 19.34 mg; pantotenic acid, 12.16 mg; vitamin K_3, 1.92 mg; folic acid, 0.192 mg; biotin, 0.064 mg; and choline, 127.31 mg.

At the beginning and end of the study, the body weight of each pig was recorded to determine the average daily gain (ADG). The experimental diets were weighed when offered to the animals to determine the average daily feed intake (ADFI) and the daily intake of SID Met+Cys, and the feed:gain ratio (F:G) was then calculated.

At the end of the experimental period (100.11±3.52 kg body weight) the animals were subjected to a 6-h fasting and then blood sampling was performed from the jugular vein (Cai et al., 1994) and samples were transferred into glass tubes containing ethylenediamine tetraacetic acid to analyze urea, total proteins, creatinine, and triacylglycerols. To determine glucose in the plasma, glass tubes containing fluoride were used. After sampling, blood was centrifuged at 3,000 × *g* for 15 min to obtain the blood plasma (Moreno et al., 1997), which was placed in eppendorf-type microtubes.

The analysis of glucose, urea, total proteins, creatinine, and triacylglycerol was carried out by the colorimetric method using commercial kits (Gold Analisa, Belo Horizonte, Minas Gerais, Brazil), following the manufacturer's recommendations. The amount of each blood component was measured by absorbance readings, using a BIOPLUS® spectrophotometer (model 2000).

Blood samples were also collected in tubes containing a gel without physicochemical properties, chilled, and sent to the laboratory for homocysteine analysis, which was determined by the IMMULITE unit (Siemens) using chemiluminescence (Demuth et al., 2004).

Immediately after slaughter, samples of liver (left medial lobe) and *longissimus dorsi* (left half carcass, last thoracic vertebra) were collected for gene expression analysis. All materials used in the collection were pretreated with RNase inhibitor (RNase Zap®, Life Technologies, Brazil). The samples were placed in microcentrifuge tubes containing RNAlater® (Life Technologies, Brazil), chilled for 24 h at 2-4 ºC, and then stored in a freezer at –18 °C until RNA extraction.

Total RNA was extracted using Trizol® (Invitrogen, Carlsbad CA, USA), according to the procedures described by the manufacturer (1 mL per 100 mg of tissue), and quantified using a spectrophotometer at 260-nm wavelength. The integrity of RNA was analyzed using a 1-% agarose gel stained with 10% ethidium bromide and visualized under ultraviolet light. The RNA samples were treated with DNase I (Invitrogen, Carlsbad, CA, USA) to remove possible genomic DNA contamination. The synthesis of complementary DNA was performed using SuperScriptTM III First-Strand Synthesis Super Mix kit (Invitrogen Corporation, Brazil) according to the manufacturer's specifications and stored at –20 ° C until use.

The gene expression of the enzyme methionine synthase and cystathionine γ-lyase were measured by quantitative real-time polymerase chain reaction using the fluorescent dye SYBR Green and the LightCycler® 96 (Roche, Basel, Switzerland). All analyses were performed in duplicate, each in a volume of 20 µL. The cycling was performed according to the manufacturer's instructions.

The primers used in the methionine synthase and cystathionine γ-lyase amplification reactions were designed based on the gene sequences available for pigs (*Sus scrofa*) at NCBI GenBank (www.ncbi.nlm.nih.gov) using the website www.idtdna.com (Table 2). Endogenous controls (ß-actin, GAPDH, and HPRT1) were tested and ß-actin was selected due to a more efficient amplification.

The results of gene expression were recorded as threshold cycle (Ct) values and adjusted by the equation proposed by Coble et al. (2011), as follows:

Adjusted Ct = 40 – [(average Ct of target gene) + (median Ct of the endogenous control – average Ct of the endogenous control) × (regression coefficient of the target gene / regression coefficient of the endogenous control)].

The UNIVARIATE procedure of SAS (Statistical Analysis System, version 9.0) was applied to assess the presence of outliers. Data on performance, blood variables, and gene expression were subjected to ANOVA and the effects of block, SID Met+Cys, vitamin B_6 supplementation, and interaction between SID Met+Cys and vitamin B_6 were included in the mathematical model.

The initial weight was used as a covariate for the performance analysis and was withdrawn from the model since it did not produce a significant effect. The F test was applied for the vitamin B_6 supplementation. The degrees of freedom relating to SID Met+Cys levels were deployed in orthogonal polynomials to obtain the regression equations. All statistical tests were performed through the GLM procedure of SAS, adopting a significance level of 5% ($P \leq 0.05$).

Results

No interactions ($P>0.05$) were observed between vitamin B_6 supplementation and SID Met+Cys levels for any evaluated parameters (Tables 3-5). The levels of SID Met+Cys and vitamin B_6 supplementation did not affect the ADFI, ADG, and F:G (Table 3). The intake of SID Met+Cys increased linearly ($P<0.01$) according to increasing dietary SID Met+Cys levels.

The highest vitamin B_6 supplementation in the diet (3.58 mg/kg) induced a high plasma total protein (P = 0.05) concentration (Table 4). Triglycerides showed a quadratic response (P<0.01), in which the minimum value (33.91 mg/dL) was estimated at 0.575% of SID Met+Cys. The other studied parameters were not affected (P>0.05) by SID Met+Cys levels or vitamin B_6 supplementation.

SID Met+Cys levels and vitamin B_6 supplementation did not affect (P>0.05) the expression of methionine synthase or cystathionine γ-lyase in liver or muscle and the serum homocysteine concentrations (Table 5).

Discussion

Considering the room temperature (20.95±3.37 ºC) and the relative humidity (69.2±13.13%) recorded during the trial period, the animals were not subjected to extreme environmental conditions, because Ferreira (2005) considers the temperatures of 5 and 27 °C as the minimum critical and maximum, respectively, and the ideal relative humidity between 50 and 70% for finishing pigs. According to Le Bellego and Noblet (2002) and Moura et al. (2006), the possible amino acid imbalance caused by a deficiency or

Table 2 - Primer sequences used to detect methionine synthase (MS), cystathionine γ-lyase (CGL), and ß-actin by quantitative real-time polymerase chain reaction

Gene	Gene ID[1]	Primer sequence (5'-3')	Amplicom (bp)	At (ºC)
MS	XM_001927058.2	D: CACGGATGGCTTGGTCAATATC R: AGGTGGAACTCTGGGCTTATAG	106	60
CGL	NM_001044585	D: CTCTGCAATCGAGGTCTGAAG R: GAGGGCAACCCAGGATAAATAA	128	60
β-actina	XM_003124280.3	D: CTTCTAGGCGGACTGTTAGTTG R: AGCCATGCCAATCTCATCTC	86	60

At - annealing temperature.
[1] Access number in NCBI GenBank.

Table 3 - Performance of female pigs from 75 to 100 kg fed diets containing different levels of standardized ileal digestible (SID), methionine + cystine (Met+Cys), and vitamin B_6 supplementation

Item	SID Met+Cys (%)											P-value			
	0.370	0.470	0.570	0.670	Mean	0.370	0.470	0.570	0.670	Mean	SE	Met+Cys × B_6	B_6	Regression	
	1.58 mg B_6/kg of diet					3.58 mg B_6/kg of diet								L	Q
ADFI (kg)	2.75	2.82	2.64	2.86	2.77	2.97	2.67	2.83	2.82	2.83	0.041	0.37	0.49	0.89	0.42
DISID Met+Cys (g)[1]	10.19	13.27	15.06	19.15	14.42	11.00	12.57	16.13	18.93	14.66	0.215	0.45	0.57	<0.01	0.20
ADG (kg)	0.94	0.98	0.97	1.05	0.99	1.07	0.96	1.01	1.01	1.01	0.019	0.42	0.45	0.56	0.33
F:G	2.92	2.87	2.74	2.75	2.82	2.79	2.81	2.85	2.80	2.82	0.031	0.45	0.82	0.36	0.66

ADFI - average daily feed intake; DISID Met+Cys - daily intake of SID Met+Cys; ADG - average daily gain; F:G - feed:gain ratio; SE - standard error (n = 56); L - linear; Q - quadratic.
[1] Linear effect (Y = –0.1043 + 28.1370X (R^2 = 0.99)).

Table 4 - Plasma levels of glucose, urea, creatinine, triglycerides, and total protein in female pigs from 75 to 100 kg fed diets containing different levels of standardized ileal digestible (SID) methionine + cystine (Met+Cys) and B_6 vitamin supplementation

Item	SID Met+Cys (%)											P-value			
	0.370	0.470	0.570	0.670	Mean	0.370	0.470	0.570	0.670	Mean	SE	Met+Cys × B_6	B_6	Regression	
	1.58 mg B_6/kg of diet					3.58 mg B_6/kg of diet								L	Q
Glucose (mg/dL)	52.43	58.93	58.50	58.57	57.11	60.00	61.07	57.57	64.57	60.80	1.073	0.50	0.09	0.16	0.38
Urea (mg/dL)	21.71	23.81	19.57	22.05	21.79	23.76	21.67	22.81	23.86	23.02	0.623	0.46	0.33	0.88	0.80
Creatinine (mg/dL)	1.44	1.71	1.65	1.43	1.56	1.84	1.61	1.49	1.69	1.66	0.045	0.10	0.30	0.45	0.76
Triglycerides (mg/dL)[1]	43.93	41.58	36.29	36.92	39.68	53.43	34.36	31.33	37.21	39.08	1.517	0.24	0.89	0.01	<0.01
Total protein (g/dL)[2]	6.39	6.24	6.03	5.68	6.08b	6.39	6.35	6.40	6.28	6.36a	0.067	0.41	0.05	0.08	0.16

SE - standard error (n = 56); L - linear; Q - quadratic.
[1] Quadratic effect (Y = 150.0684 – 404.1946X + 351.6267X^2 (R^2 = 0.96)).
[2] Means followed by different letters in the row differ by the F test (P≤0.05).

Table 5 - Gene expression[1] of the methionine synthase (MS) and cystathionine-γ-lyase (CGL) enzymes in the liver (l) and muscle (m) and serum levels of homocysteine in female pigs from 75 to 100 kg fed diets containing different levels of standardized ileal digestible (SID) methionine + cystine (Met+Cys) and vitamin B_6 supplementation

Item	SID Met+Cys (%)											P-value			
	0.370	0.470	0.570	0.670	Mean	0.370	0.470	0.570	0.670	Mean	SE	Met+Cys × B_6	B_6	Regression	
	1.58 mg B_6/kg of diet					3.58 mg B_6/kg of diet								L	Q
MSm	8.16	7.83	8.24	7.39	7.90	8.28	8.54	7.15	7.60	7.89	0.204	0.47	0.97	0.13	0.32
CGLm	7.88	7.99	9.01	7.21	8.02	8.08	8.59	7.84	8.64	8.29	0.234	0.30	0.58	0.55	0.10
MSl	9.20	9.38	9.24	9.88	9.42	10.34	9.80	9.82	9.74	9.93	0.178	0.65	0.18	0.97	0.51
CGLl	11.24	10.77	11.25	15.24	12.12	14.95	13.60	13.14	11.56	13.31	0.711	0.29	0.42	0.89	0.46
Homocysteine (µmol/L)	29.00	28.70	27.93	26.40	28.01	19.47	27.63	23.81	27.31	24.55	0.819	0.20	0.08	0.43	0.72

SE - standard error (n = 56); L - linear; Q - quadratic.
[1] Expressed as arbitrary unity.

excess of SID Met+Cys in the diet negatively influences the voluntary food intake of the animals. Despite the varying SID Met+Cys levels (0.370-0.670%) evaluated in this study, the ADFI of the animals was not affected. Thus, the highest studied Met+Cys level (0.670%) did not represent an excess enough to reduce the feed intake, once methionine excess may cause a decrease in the feed intake (Edmonds et al., 1987). Indeed, the intake of SID Met+Cys increased according to increasing SID Met+Cys levels in the diets and this response was expected, as the ADFI did not change.

Methionine is considered a limiting amino acid for pigs, acting in several metabolic pathways mainly involved in protein synthesis (as a primer in the translation process and as the precursor of cysteine, which is also used for protein synthesis). S-adenosylmethionine is the most important CH_3 donor in the body and is involved in the biosynthesis of important components for the growth and development of pigs, such as creatine, carnitine, and polyamines (Brosnan and Brosnan, 2006; Nelson and Cox, 2014). Thus, methionine deficiency can limit the maximum performance of these animals; however, the evaluated SID Met+Cys levels did not influence the ADG and F:G of the animals, demonstrating that the level of 0.370%, corresponding to a daily intake of 10.60 g SID Met+Cys and a 0.48% ratio with the SID Lys, was sufficient to meet these requirements.

There are a few studies evaluating SID Met+Cys levels in female finishing pigs. Loughmiller et al. (1998), using female pigs (72 to 104 kg) observed that the SID Met+Cys requirement is 0.350% for the high ADG and to improve F:G, corresponding to a 0.50% ratio with the SID lysine (Lys). In another study, Knowles et al. (1998) obtained a total Met+Cys:Lys ratio ranging from 0.40 to 0.47%, based on performance and carcass characteristics of female pigs with a live weight from 74 to 110 kg. According to these authors, the optimal Met+Cys:Lys ratio required to achieve maximum performance was not higher than 0.47%, corresponding to 0.306% Met+Cys in the diet. These results support our findings that the optimum SID Met+Cys ratio with SID lysine is lower than 0.48%.

Methionine has been referenced as a major amino acid precursor of immunoglobulins and, according to Machado and Fontes (2006), it is the amino acid used in inflammatory processes for the synthesis of antioxidant components such as glutathione. Therefore, it can be deduced that the animals of this study were under low sanitary challenge, which may partly explain the lack of methionine supplementation effect on the performance variables.

Since the increased intake of SID Met+Cys did not adversely influence the performance of pigs, it can be assumed that methionine intake above the level of 0.370% may have been used for other physiological functions, rather than protein deposition, as suggested by Chung and Baker (1992) and Vaz et al. (2005). One of the possible metabolic fates of methionine could be related to methyl group donation for the synthesis of biomolecules such as carnitine, which is essential for lipid metabolism as it is involved in the transportation of long-chain fatty acids in mitochondrial membrane. The acyl-fatty acid binds to carnitine to form the acyl-fatty carnitine, enabling its transport from the cytosol to the mitochondrial matrix, where it is oxidized to generate energy (Stephens et al., 2007; Strijbis et al., 2008; Apple et al., 2011). Therefore, carnitine can decrease the number of free fatty acids available for lipid biosynthesis, which may partially explain the decrease in plasma triglyceride concentration with increasing SID Met+Cys levels.

Plasma total protein concentration was higher in animals that received the largest vitamin B_6 supplementation (3.58 mg/kg). These results may be related to the action of vitamin B_6, such as pyridoxal phosphate coenzyme in reactions involving amino acid metabolism. Conventionally,

pyridoxal phosphate acts as an intermediate carrier of amino groups in the transamination reactions, acting at the active site of transaminases. Thus the pyridoxal phosphate is converted into its aminated medium (pyridoxamine-phosphate), but then gives an amine radical (NH_3) to a specific α-keto acid (Nelson and Cox, 2014). These authors also reported that vitamin B_6 plays important roles in the organism to maintain energy metabolism, especially in a situation of low blood glucose (fasting, for example). The pyridoxal phosphate is cofactor of the enzyme glycogen phosphorylase, responsible for the cleavage of glycogen to release glucose (glycogenolysis). Nevertheless, no changes were observed in ADG and F:G due to the highest vitamin B_6 supplementation, indicating that the lowest level (1.58 mg/kg) met the animal requirements. Rostagno et al. (2011) suggested a vitamin B_6 supplementation of 1.2 mg/kg (for 70-100 kg BW) and the NRC (2012) recommended 1 mg/kg (for 75-100 kg body weight).

Serum homocysteine concentrations were not changed by the increased SID Met+Cys levels, suggesting a possible metabolism of the excess concentration in the blood stream, because the remethylation and transsulfuration can prevent homocysteine excess. Through remethylation, the homocysteine is reconverted into methionine by the action of methionine synthase and/or betaine-homocysteine methyl transferase; already at transsulfuration, the homocysteine is irreversibly converted to cysteine by the enzymes cystathionine β-synthase and cystathionine γ-lyase. The allosteric control of S-adenosylmethionine is responsible for regulating these pathways. Increasing methionine levels leads to a high S-adenosylmethionine concentration, reducing the number of methyl groups in the cycle by inhibiting the activities of enzymes involved in the homocysteine remethylation, such as betaine-homocysteine methyl transferase and methylene tetrahydrofolate reductase, as well as stimulating the activity of cystathionine β-synthase, which is responsible for the irreversible homocysteine loss. However, when the methionine concentration is low, S-adenosylmethionine concentrations decrease and cystathionine β-synthase activity is restored to normal levels, conserving homocysteine for remethylation (Prudova et al., 2006; Nijhout et al., 2006). The deficiency of some vitamins (B_6, B_{12}, and folate) that act as enzymatic cofactors may also change the activity of the enzymes involved in methionine metabolism, impairing the control of homocysteine concentrations (Lima et al., 2006; Zhang et al., 2009).

The vitamin B_6 in its active form (pyridoxal phosphate) is an enzyme cofactor for three methionine metabolism enzymes: serine hydroxymethyl transferase, cystathionine β-synthase, and cystathionine γ-lyase, the latter two related to transulfuration, considered the major elimination route of homocysteine excess (Brosnan and Brosnan, 2006; Stipanuk and Ueki, 2011). Thus, the low vitamin B_6 supplementation (1.58 mg/kg feed) was effective to metabolize homocysteine through the transulfuration pathway. This result is consistent with the study carried out by Miller et al. (1994), who observed that mice with vitamin B_6 deficiency had blood homocysteine levels similar to the non-deficient mice; however, a 30-fold increase in homocysteine concentration was observed when mice were given a methionine excess, compared with animals receiving the control treatment.

However, no interactions between vitamin B_6 supplementation and SID Met+Cys levels were observed on methionine synthase and cystathionine γ-lyase expression in the liver or muscle and the respective vitamin B_6 supplementation and SID Met+Cys level did not affect these parameters. These results differ from those found by Sato et al. (1996), who observed an increase in the renewal rate of the cystathionine γ-lyase enzyme in rodents subjected to vitamin B_6 deficient diets. However, Zhang et al. (2009) did not observe changes in hepatic gene expression of the cystathionine β-synthase and cystathionine γ-lyase enzymes when evaluating extreme dietary levels (0 and 3 mg/kg diet) of vitamin B_6 in weanling pigs. Thus, the results obtained in our study provide evidence that the gene expression of the cystathionine γ-lyase and methionine synthase enzymes in pigs is only slightly influenced by the vitamin B_6 levels and SID Met+Cys evaluated in this study.

In the study of Zhang et al. (2009), though vitamin B_6 levels did not influence the hepatic gene expression of the cystathionine β-synthase and cystathionine γ-lyase enzymes, deficiency of this vitamin reduced the activity of cystathionine β-synthase, cystathionine γ-lyase, and serine hydroxymethyl transferase (pyridoxal phosphate–dependent); this developed a severe hyperhomocysteinemia that was attributed mainly to lower activity of the enzymes cystathionine β-synthase and cystathionine γ-lyase, which are responsible for eliminating homocysteine excess by transsulfuration. According to the authors, the blood homocysteine levels can be used as an index to indicate the state of the vitamin B_6 supply in pigs. In accordance with the performance data, the requirement of SID Met+Cys for female pigs, from 75 to 100 kg, is equal to or lower than 0.370%, corresponding to an intake of 10.60 g/day. This intake is lower than the 14.50 g/day suggested by Rostagno et al. (2011) for female pigs, from 70 to 100 kg, but is greater than the 10.55 g of Met+Cys/day presented by the NRC (2012) for female pigs from 75 to 100 kg. Considering the methionine:cysteine ratio of 0.50% presented by the NRC

(2012) and the principle that two cysteine molecules are required to form cystine (Lewis, 2003), it is recommended that the sulfur amino acid requirements be expressed as methionine + cysteine, since a methionine molecule can be converted to a cysteine molecule in a 1:1 ratio. When expressing the sulfur amino acid requirements as methionine + cystine, a knowledge about the cystine deficit is necessary to proceed with methionine supplementation to meet a 2:1 ratio, since two methionine molecules are required to synthesize one cystine. Furthermore, the NRC (2012) already shows the sulfur amino acid requirements as methionine + cysteine.

Conclusions

The standardized ileal digestible methionine + cystine requirement for female pigs from 75 to 100 kg is equal to or lower than 10.60 g/day, corresponding to 0.370% methionine + cystine in the diet and a 0.48% ratio with the of standardized ileal digestibl lysine. The low vitamin B_6 supplementation (1.58 mg/kg feed) is sufficient to metabolize homocysteine through the transulfuration pathway.

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