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Gene expression, serum amino acid levels, and growth performance of pigs fed dietary leucine and lysine at different ratios

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ABSTRACT. We examined 96 pigs (28.1 ± 0.83 kg) to analyze the effect of Leu:Lys ratios on expression of the cationic amino acid transporters $b^{0,+}$ and CAT-1 in the jejunum and liver as well as myosin expression in 2 muscles to estimate the optimum standardized ileal digestible (SID) Leu:Lys ratio for growth rate and efficiency. A wheat- and wheat bran-based diets were formulated to meet the requirements of SID amino acids other than Leu (0.70%) and Lys (0.80%). L-Leu was added to the basal diet in 5 SID Leu:Lys ratios (88, 100, 120, 140, and 160% in diets 1-5). Tissue samples were collected from 8 pigs with ratios of 88, 120, and 160%. Relative expression of $b^{0,+}$, CAT-1, and myosin was analyzed. $b^{0,+}$ expression in the jejunum was higher but lower in the liver of pigs with the 120% ratio compared to those with the 88 or 160% ratio; myosin expression in longissimus dorsi was also higher in pigs with the 120% ratio ($P < 0.05$). CAT-1 was lower in the jejunum and longissimus dorsi of pigs with 120 or 160% ratios than in pigs with 88%. Serum concentration of nearly all amino acids decreased with excess dietary Leu ($P < 0.05$). The SID Leu:Lys of 104 and 109%

optimized average daily gain and feed conversion ratio, respectively. Thus, the dietary Leu:Lys ratio affects the expression of genes coding for amino acid transporters and myosin, the availability of Lys, and the growth rate and efficiency in pigs.

Key words: Gene expression; Leu:Lys ratio; Pigs; Serum amino acids

INTRODUCTION

Leucine (Leu) is considered a functional amino acid (AA) because it stimulates protein synthesis in skeletal muscle by activating the mammalian target of rapamycin (mTOR) and enhancing the rates of mRNA translation (Anthony et al., 2000). Leu acutely stimulates muscle protein synthesis in fasted piglets by modulating the activation of mTOR and components of translation initiation (Suryawan et al., 2011), but only when other amino acids are supplied to maintain appropriate aminoacidemia (Wilson et al., 2010). Leu is also involved in the absorption of cationic AA (Bröer, 2008), which may affect the availability of lysine (Lys) for protein synthesis. Lys is transported across the apical and basolateral membranes of enterocytes through the transporters $b^{0,+}$ and cationic amino acid transporter-1 (CAT-1), respectively (Majumder et al., 2009). The $b^{0,+}$ system, which exchanges Leu for Lys (Torras-Llort et al., 2001), is the most important Lys transporter in the small intestine, and the absorption of Lys by $b^{0,+}$ is coupled with Leu efflux (Pineda et al., 2004). However, excess levels of dietary Leu appear to reduce Lys availability by decreasing the expression of $b^{0,+}$ (García-Villalobos et al., 2012). Leu stimulates muscle protein synthesis in pigs, and this stimulation is dependent on amino acid availability, but excess Leu may affect Lys absorption. Because typical pig diets formulated to meet Lys requirements (first limiting AA) contain excess Leu, the dietary Lys:Leu ratio is an important issue.

Studies regarding the optimal dietary Leu:Lys ratio for pigs are limited, although true digestible ratios of 100% (Chung and Baker, 1992; Augspurger and Baker, 2004) to 110% (Wang and Fuller, 1989) have been reported to be adequate in diets for 10-, 20-, and 30-kg pigs, using performance and nitrogen balance variables as indicators. These Leu:Lys ratios were determined before the Lys and Leu interaction for absorption and the Leu stimulation effect on protein synthesis stimulation were identified. Understanding the mechanism regarding how dietary Leu:Lys ratios affect the availability of all AA can be used to prevent negative interactions and to improve muscle protein synthesis. We hypothesized that the Leu:Lys ratio affects AA availability and muscle protein synthesis, which may affect both the rate and efficiency of growth in pigs. Lys, threonine, methionine, tryptophan, and valine are currently available in crystalline form and can be supplemented in the diet to eliminate excess AA such as Leu. Because myosin is the most abundant protein in muscle (Czerwinski and Martin, 1994), its expression can be used as indicator of protein synthesis in pigs. The objective of the present study was to analyze the effect of different Leu:Lys ratios on the expression of the cationic AA transporters $b^{0,+}$ and CAT-1 in the jejunum, liver, longissimus dorsi (LD) muscle, and semitendinosus (ST) muscle, the expression of myosin in LD and ST, and the availability of AA (serum concentration, SC). In addition, the optimum standardized ileal digestible (SID) Leu:Lys ratio for maximizing the growth rate and feed efficiency in growing pigs was estimated.

MATERIAL AND METHODS

Animals and housing

The pigs used in the experiment were cared for in accordance with the guidelines established in the Official Mexican Regulations on Animal Care (NOM-062-Z00-1999, 2001). A total of 96 crossbred pigs (Large White x Duroc) with initial body weight (BW) of 28.1 ± 0.83 kg were used. The trial was run over 2 periods. In each period, there were 2 pigs (1 barrow and 1 gilt) per pen and 4 replicate pens for each dietary treatment (24 pens/period), for a total of 8 replicates per treatment. Pigs were assigned randomly to pens, but littermates were housed separately. The initial BW was balanced across pens. Pigs were housed in iron mesh-raised floor metabolism pens (1.2 x 1.2 x 1.0 m) equipped with a stainless-steel self-feeder and a nipple water drinker in a temperature-controlled room (22°-24°C). Feed was provided *ad libitum*. Pigs and feed disappearance were weighed on a weekly basis to calculate average daily weight gain (ADG), feed intake (ADFI), and feed conversion ratio (FCR). The average BW of the pigs at the end of the 3-week study was 41.6 ± 0.95 kg.

Dietary treatments

Five wheat- and wheat bran-based experimental diets were formulated using analyzed AA contents of ingredients and published SID coefficients (AminoDat® 4.0, Evonik Industries, Hanau, Germany), to meet the requirements of AA other than Leu and Lys (NRC, 1998; Table 1). The SID Leu:Lys ratios in treatments 1-5 were 88, 100, 120, 140, and 160. These Leu:Lys ratios were obtained by adding different levels of corn gluten meal and free Leu to the diets. Corn gluten meal contains as much as 10X more Leu than wheat; in turn, wheat is one of the few feed ingredients whose Leu content is lower than the estimated requirement (NRC, 1998).

Table 1. Composition of the experimental diets.

Diets	1	2	3	4	5
SID Leu:Lys ratio (%)	88	100	120	140	160
Ingredients (%)					
Wheat	80.26	80.26	80.00	78.00	79.52
Wheat bran	12.00	12.00	11.18	11.41	10.00
Corn starch	3.00	2.90	3.00	3.00	3.00
Corn gluten meal	-	-	2.50	3.50	3.50
Dicalcium phosphate	0.35	0.35	0.25	0.36	0.37
Soybean oil	0.94	0.94	-	0.64	0.36
Limestone	1.33	1.33	1.36	1.32	1.31
Salt	0.27	0.27	0.27	0.27	0.27
Mineral-vitamin Premix*	0.20	0.20	0.20	0.20	0.20
L-lysine · HCl	0.65	0.65	0.63	0.62	0.62
L-leucine	-	0.10	0.011	0.10	0.257
L-threonine	0.32	0.32	0.26	0.25	0.25
DL-methionine	0.16	0.16	0.11	0.10	0.10
L-tryptophan	0.05	0.05	0.05	0.05	0.05
L-isoleucine	0.16	0.16	0.10	0.10	0.10
L-valine	0.18	0.18	0.12	0.10	0.10
L-phenylalanine	0.09	0.09	0.01	-	-
L-histidine	0.04	0.04	0.02	0.01	0.01

*Supplied per kg of diet: vitamin A = 4800 IU; vitamin D3 = 800 IU; vitamin E = 4.8 IU; vitamin K3 = 1.6 mg; riboflavin, 4 mg; D-pantothenic acid = 7.2 mg; niacin = 16 mg; vitamin B12 = 12.8 mg; Zn = 64 mg; Fe = 64 mg; Cu = 4 mg; Mn = 4 mg; I = 0.36 mg; Se = 0.13 mg.

The Lys level was set at 0.80% SID Lys, which corresponds to approximately 92% of the Lys requirement for the genotype and BW of pigs used in the study (NRC, 1998). Thus, Leu was first limiting and Lys second limiting in the basal diet to avoid underestimation of the Leu to Lys ratio. Supplemental methionine, threonine, tryptophan, isoleucine, valine, phenylalanine, and histidine were added to the basal diet to exceed (+7%) the recommended AA ratio, but were the same across diets for other AA (AminoDat® 4.0) relative to the adequate level of Lys (0.85% SID basis). Surpluses of isoleucine and valine were avoided, but sufficient levels (NRC, 1998) of these AA were supplied in free form to eliminate any potential effect of the interactions between branch-chained AA. The net energy level was similar among diets (Table 2). Amino acid contents in feed ingredients, diets, and serum were determined by Evonik Industries (Llames and Fontaine, 1994).

Table 2. Nutrient composition of the experimental diets.

	SID Leu:Lys ratio (%)				
	88	100	120	140	160
Calculated NE, Ca, and available P contents					
NE (MJ/kg)	10.40	10.39	10.30	10.34	10.36
Total Ca (%)	0.61	0.61	0.60	0.61	0.61
Available P (%)	0.23	0.23	0.23	0.25	0.23
Analyzed CP and total AA contents (%)					
CP	13.92	14.03	15.19	15.63	15.99
Arginine	0.65	0.64	0.70	0.69	0.70
Histidine	0.35	0.35	0.37	0.36	0.36
Isoleucine	0.57	0.55	0.59	0.60	0.61
Leucine	0.84	0.92	1.12	1.30	1.48
Lysine	0.84	0.84	0.87	0.86	0.90
Methionine	0.34	0.32	0.33	0.33	0.34
Phenylalanine	0.65	0.64	0.68	0.70	0.70
Threonine	0.66	0.60	0.66	0.62	0.63
Tryptophan	0.20	0.21	0.20	0.21	0.21
Valine	0.72	0.69	0.74	0.72	0.74

Tissue collection

Four pigs each from treatments 1, 3, and 5 (Leu:Lys ratio, 88, 120, and 160%, respectively) were fasted overnight for 9 h; at the end of the 21-day trial, each pig was allowed to consume 650 g feed. Next, 2.5 h after the last feeding, all pigs were euthanized by electrical stunning and exsanguination to collect samples from small intestine (jejunum) mucosa, liver, and the LD and ST muscles. Samples of mucosa scratched from middle jejunum, liver, LD, and ST (approximately 0.5-1.0 g) were collected into 2-mL Eppendorf microtubes and immediately frozen in liquid nitrogen. Blood samples from the carotid artery (10 mL) were collected to analyze the SC of free AA. All samples were stored at -82°C until analysis.

Total RNA extraction and purification

Samples of the jejunum mucosa, liver, LD, and ST were treated to extract total RNA using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) as reported by Méndez et al. (2011). Purified RNA was then eluted with 30 µL RNase-free water and stored at -82°C. The concentration of total RNA was determined spectrophotometrically (Helios β, Thermo Electron Co.,

Waltham, MA, USA) at 260 nm, and RNA purity was assessed by using the A_{260}/A_{280} ratio, which ranged from 1.8-2.0 (Sambrook and Russell, 2001). The integrity of total RNA was evaluated by gel electrophoresis on 1% agarose gels. All RNA samples were of good quality with a 28S:18S rRNA ratio of approximately 2.0:1 (Sambrook and Russell, 2001).

Reverse transcription

Approximately 2 µg total RNA was treated with 1 U DNase I (1 U/µL; Invitrogen) and 6 µL 5X reverse transcription buffer in a 30-µL reaction completed with diethylpyrocarbonate-treated water; the reaction was carried out for 15 min at room temperature and another 15 min at 70°C to stop the reaction. Reverse transcription was initiated with DNase-treated RNA samples, adding 1 µL random primers (150 ng/µL; Invitrogen) and 1 µL dNTPs (10 µM each). The reaction was incubated at room temperature and then chilled on ice for 1 min. Next, 3 µL dithiothreitol (0.1 M), 1 µL ribonuclease inhibitor (40 U/µL; RNase OUT, Invitrogen), and 2 µL 5X reverse transcription buffer were added to the reaction and incubated at 42°C for 2 min to stabilize the reaction before adding 1 µL reverse transcriptase enzyme (200 U/µL; RT-Superscript III, Invitrogen). The reaction was incubated at 42°C for 50 min. The mixture was incubated at 70°C for 15 min and then chilled on ice to stop the reaction. cDNA samples were quantified spectrophotometrically and diluted to a final concentration of 50 ng/µL.

Real-time polymerase chain reaction (PCR)

Specific primers for the AA transporters $b^{0,+}$, CAT-1, and myosin (heavy chain 4 of isoform IIB) mRNA were designed based on their published sequences at GenBank (Table 3). Type IIB fibers account for approximately 80% of the total fibers in some pig muscles (Czerwinski and Martin, 1994; Davoli et al., 2003) and are extensively expressed in the LD and ST of pigs (Lefaucheur et al., 2002). Additionally, a housekeeping 18S rRNA gene was used as an endogenous control to normalize variations in mRNA levels. End-point PCR was carried out to standardize the amplification conditions for each pair of primers. To confirm the specificity of the PCR products related to its mRNA, a sample of each PCR product was purified using a commercial kit (PureLink, PCR Purification kit; Invitrogen) and sequenced at the Davis Sequencing Facility (Davis, CA, USA).

Table 3. Primers for the real-time PCR analyses of cDNA derived from cationic amino acid transporters mRNA, myosin mRNA, and 18S ribosomal RNA.

mRNA	Primer	Location (bp) on the template	Sequence	Amplicon size (bp)
CAT-1 (GenBank: AY371320)				
	Forward	4239-4258	5'-GTCGGTTGCAAAGACCATTT-3'	329
	Reverse	4548-4567	5'-GAGCGGTGCTGACAACAGTA-3'	
$b^{0,+}$ AT (SLC7A9) (GenBank: EF127857)				
	Forward	1-19	5'-CGGAGAGAGGATGAGAAGT-3'	562
	Reverse	545-562	5'-GCCCGCTGATGATGATGATGA-3'	
Myosin, heavy chain 4 (GenBank: NM_001123141)				
	Forward	4582-4599	5'-AGATTTCTGACCTGACTG-3'	320
	Reverse	4904-4921	5'-TCTCCCTCCATCTTCTTC-3'	
18S rRNA (GenBank: AY265350)				
	Forward	236-255	5'-GGCCTCACTAAACCATCCAA-3'	295
	Reverse	511-530	5'-TAGAGGGACAAGTGGCGTTC-3'	

Expression of $b^{0,+}$, CAT-1, and myosin was estimated by quantitative PCR (qPCR) assays, using the Maxima SYBR Green/ROX qPCR Master Mix (Fermentas, Corp., Vilnius, Lithuania) into a Chromo 4-DNA Engine with the MJ Opticon Monitor 3.1 software (Bio-Rad, Hercules, CA, USA). The equipment was calibrated using a standard curve using the 18S rRNA cloned into a TOPO vector 4.0. The standard curve was obtained using known concentrations of 100-fold serial dilutions of cDNA. The qPCRs contained 50 ng cDNA, 0.5 µM of each specific primer, 12.5 µL 2X SYBR green/ROX qPCR Master Mix, and DNase/RNase-free water to a final volume of 25 µL. 18S rRNA was used as a housekeeping gene to normalize the amount of amplified DNA. PCR conditions used for the amplification and quantification were an initial denaturing stage (95°C for 1 min), followed by 45 cycles of amplification (denaturing at 95°C for 30 s; annealing at 56°C for 15 s; and extension at 72°C for 30 s); and a melting curve program (60°-90°C). Fluorescence was measured at the end of each cycle and at every 0.2°C during the melting program.

Analysis of free amino acids in serum

Blood samples were centrifuged at 500 *g*, 4°C for 10 min to separate serum from blood cells, and serum was deproteinized using an Ultrafree-MC 10,000 nominal molecular weight limit filter unit (Millipore, Bedford, MA, USA) at 5000 *g*, 4°C for 30 min (Sunde et al., 2003). The supernatant filtrate was derivatized using Waters AccQ-Tag reagent. AA analysis was performed by high-performance liquid chromatography according to the method described by Llames and Fontaine (1994).

Statistical analysis

Analyses of variance were performed using the general linear model of SAS (Statistical Analysis System 9.1, SAS Institute, Cary, NC, USA). Three contrasts were constructed to compare the effect of Leu:Lys ratios on gene expression and serum AA (C_1, 88 *vs* 120; C_2, 120 *vs* 160; C_3, 88 *vs* 160). For growth performance variables, the pen was the experimental unit. For the best possible fit to the response data, a two-slope linear broken-line regression (Robbins et al., 2006) was used to estimate the optimum dietary Leu:Lys ratio at which the performance (ADG and FCR) response was maximized. Probability levels of $P \leq 0.05$, and $0.05 < P \leq 0.10$ were defined as significant differences and tendencies, respectively.

RESULTS

One pig from treatment 2 died from non-experimental-related reasons; the remaining animals were healthy throughout the experiment. Real-time qPCR assays were validated by sequencing the final products of $b^{0,+}$, CAT-1, myosin, and 18S rRNA, which showed 100% homology with their expected sequences acquired from GenBank. 18S rRNA expression was very stable, and its content was used as an endogenous control to normalize the expression of other genes in response to various stimuli (Liao et al., 2009). Thus, in this study, we also normalized the relative expression of $b^{0,+}$, CAT-1, and myosin mRNA to 18S rRNA expression, using the 18S rRNA expression levels in the small intestine epithelia and muscles.

The relative expression values of the cationic AA transporters and myosin are presented

in Table 4. The expression of $b^{0,+}$ in the jejunum was higher in pigs fed the 120% compared to the 88% Leu:Lys ratio (P = 0.013) and 160% Leu:Lys ratio (P = 0.045). No difference was observed between the 88 and 160% Leu:Lys ratios (P = 0.575). In the liver, the expression of $b^{0,+}$ was lower in pigs fed the 120% compared to both the 88% (P = 0.002) and 160% (P = 0.005) Leu:Lys ratios; however, there was no difference between the 88 and 160% ratios (P = 0.595). The expression of $b^{0,+}$ in LD and ST was not affected (P > 0.10). Generally, the highest and lowest expression levels of $b^{0,+}$ in pigs were observed in jejunum and the ST muscle, respectively. CAT-1 expression in the jejunum was lower in pigs fed the 120% (P = 0.009) or the 160% (P = 0.020) Leu:Lys ratio compared to the 88%; but no difference was found between the 120 and 160% Leu:Lys ratios (P = 0.743). The Leu:Lys ratio did not affect CAT-1 expression in the liver and ST (P > 0.10). In the LD, CAT-1 expression was higher in pigs fed the 88% Leu:Lys ratio, as compared with those fed the 120% (P = 0.003), and was generally higher than in pigs fed the 160% ratio (P = 0.099); pigs fed the 120% ratio expressed more CAT-1 than those fed the 160% ratio (P = 0.050). In general, the highest CAT-1 expression was observed in the LD muscle, whereas expression was lowest in the jejunum. Myosin expression in the LD was higher in pigs fed the 120% Leu:Lys ratio compared with those fed the 88% (P = 0.044) or 160% ratio (P = 0.041), but no difference was observed between the 88 and 160% ratios. In the ST, myosin expression was generally higher in pigs fed the 120% compared to those fed the 160% Leu:Lys ratio (P = 0.086), but no difference was observed between the 88 and 120% (P = 0.266) or 160% (P = 0.441) ratios.

Table 4. Effect of dietary SID Leu:Lys ratios on the relative expression of genes coding for $b^{0,+}$ and CAT-1 in the jejunum, liver, and the longissimus (LD) and semitendinosus (ST) muscles, and myosin in the LD and ST muscles of pigs (arbitrary units; ratio of $b^{0,+}$ mRNA:18S rRNA)[1].

AA transporter	SID Leu:Lys (%)			SE	P values[2]		
	88	120	160		C_1	C_2	C_3
$b^{0,+}$							
Jejunum	0.081	0.760	0.224	0.178	0.013	0.045	0.575
Liver	0.162	0.033	0.145	0.021	0.002	0.005	0.595
LD	0.015	0.015	0.004	0.007	0.963	0.311	0.332
ST	0.003	0.003	0.002	0.001	0.891	0.682	0.586
CAT-1							
Jejunum	0.278	0.109	0.130	0.044	0.009	0.743	0.020
Liver	0.051	0.021	0.076	0.025	0.416	0.152	0.495
LD	1.283	0.178	0.783	0.192	0.003	0.050	0.099
ST	0.007	0.011	0.022	0.006	0.703	0.219	0.121
Myosin							
LD	8.546	19.534	8.338	3.055	0.044	0.041	0.963
ST	1.874	3.293	0.919	0.818	0.266	0.086	0.441

[1]Each mean represents 8 replicate pigs per treatment. [2]Contrasts for Leu:Lys ratios: C_1, 88 *vs* 120; C_2, 120 *vs* 160; C_3, 88 *vs* 160.

Serum values of free AA are shown in Table 5. Increasing the Leu:Lys ratio from 88 to 120% increased serum Arg (P = 0.039), Leu (P = 0.014), and Phe (P = 0.012), decreased valine (P = 0.050), and tended to reduce isoleucine (P = 0.068) and Met (P = 0.076). A further increase from 120 to 160% decreased serum Arg (P = 0.002), Lys, and Phe (P = 0.001). Serum isoleucine (P = 0.044), Lys (P = 0.008), and valine (P = 0.030) were lower, Arg tended to be lower (P = 0.086), and serum Leu was higher (P = 0.003) in pigs fed the 160% compared to those fed the 88% ratio.

Table 5. Effect of dietary SID Leu:Lys ratios on the concentration of free amino acids in serum of growing pigs[1].

Amino acid	SID Leu:Lys (%)			SE	P values[2]		
	88	120	160		C_1	C_2	C_3
Arginine	4.00	4.79	3.37	0.23	0.039	0.002	0.086
Isoleucine	3.50	2.20	2.02	0.44	0.068	0.793	0.044
Leucine	2.01	3.67	4.26	0.39	0.014	0.310	0.003
Lysine	4.38	4.90	3.05	0.28	0.225	0.001	0.008
Methionine	1.25	0.90	0.72	0.21	0.076	0.319	0.014
Phenylalanine	2.30	2.82	2.04	0.16	0.012	0.001	0.145
Threonine	6.09	5.32	5.44	0.31	0.136	0.286	0.208
Valine	9.69	4.88	4.15	1.53	0.050	0.742	0.030

[1]Each mean represents 8 replicate pigs per treatment. [2]Contrasts for Leu:Lys ratios: C_1, 88 *vs* 120; C_2, 120 *vs* 160; C_3, 88 *vs* 160.

The performance results are presented in Table 6. There was a linear and quadratic ($P < 0.01$) response in the ADG of pigs as the SID Leu:Lys ratio increased from 88 to 160%. As a result, the final BW increased in a linear and quadratic ($P < 0.01$) manner with an increasing Leu:Lys ratio. In contrast, ADFI decreased linearly and the FCR showed a quadratic response ($P < 0.01$) with an increased Leu:Lys ratio. The optimum dietary SID Leu:Lys ratio for ADG and FCR were estimated by using the two-slope linear broken-line regression analysis and found to be 104% ($r^2 = 0.93$; Figure 1) and 109% ($r^2 = 0.96$; Figure 2), respectively.

Table 6. Effect of dietary SID Leu:Lys ratios on average daily gain (ADG), daily feed intake (ADFI), and feed conversion ratio (FCR) of pigs[1].

Item	SID Leu:Lys (%)					SE	P values	
	88	100	120	140	160		Linear[2]	Quad.[3]
Body weight (kg)								
Initial	28.58	27.41	28.24	27.75	28.25	1.308	0.935	0.682
Final	41.68	42.73	42.52	41.05	40.72	0.325	0.001	0.002
ADG (kg)	0.644	0.698	0.689	0.618	0.603	0.016	0.003	0.002
ADFI (kg)	1.40	1.42	1.36	1.30	1.31	0.034	0.008	0.778
FCR (g/g)	2.17	2.03	1.97	2.11	2.16	0.054	0.698	0.006

[1]Each mean represents 16 replicate pens (1 barrow, 1 gilt) per treatment. [2]Contrast P values for linear effect. [3]Contrast P values for quadratic effect.

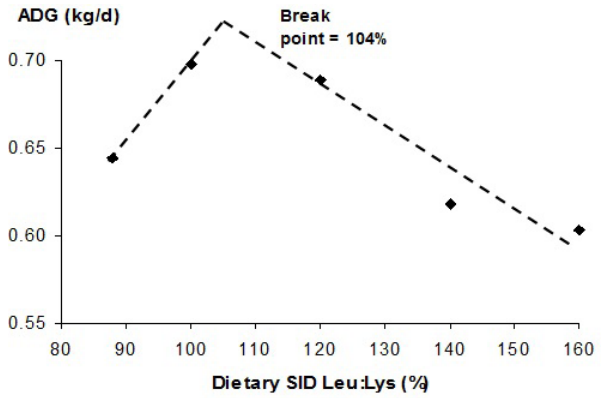

Figure 1. Fitted two-slope linear broken-line of average daily gain (ADG) as a function of standardized ileal digestible (SID) Leu:Lys in 25- to 45-kg pigs. Data points (lozenges) represent treatment means (N = 8 observations per treatment mean). The optimum SID Leu:Lys determined by two-slope broken-line model was 104% [$Y = 714.7 - 4.5 (104 - x)^2 - 2.15 (x - 104)$; $r^2 = 0.93$].

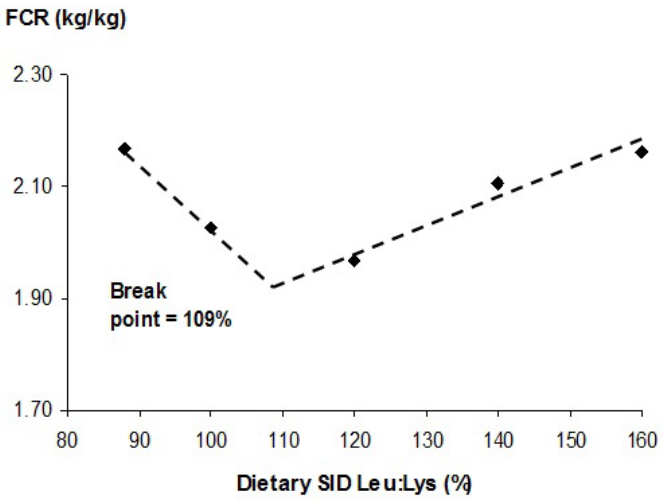

Figure 2. Fitted two-slope linear broken-line of feed conversion ratio (FCR) as a function of standardized ileal digestible (SID) Leu:Lys in 25- to 45-kg pigs. Data points (lozenges) represent treatment means (N = 8 observations per treatment mean). The optimum SID Leu:Lys determined by two-slope broken-line model was 109% [$Y = 1.927 + 0.012 (109 - x)^2 - 0.005 (x - 109)$; $r^2 = 0.96$].

DISCUSSION

We examined whether variations in the dietary Leu:Lys ratio affect the expression of 2 cationic AA transporters ($b^{0,+}$ and CAT-1) and myosin as well as the SC of AA, as indicators of AA availability in muscle protein synthesis. In addition, the SID Leu:Lys ratio that maximizes the growth rate and feed efficiency in growing pigs was estimated.

Lys is transported across the cell membrane by $b^{0,+}$ and CAT-1 (Majumder et al., 2009). The $b^{0,+}$ system exchanges Leu for Lys (Torras-Llort et al., 2001), and the intestinal absorption of Lys by $b^{0,+}$ is coupled with Leu efflux (Pineda et al., 2004). As reported in several studies (Hatzoglou et al., 2004; Bröer, 2008) and proposed by Liao et al. (2009), the expression of cationic AA transporters represents their functional activity. Thus, expression values of the genes analyzed in the present experiment are thought to reflect the abundance of their respective encoded proteins. In this study, pigs fed a diet with the 120% Leu:Lys ratio increased $b^{0,+}$ expression in the jejunum by approximately 9- and 3-fold compared with those fed the 88 or 160% Leu:Lys ratio. Similarly, García-Villalobos et al. (2012) reported that excess Leu in a wheat-based diet with a 160% Leu:Lys ratio and adequate Lys content reduced $b^{0,+}$ expression by approximately 70% in the jejunum. These results and those of García-Villalobos et al. (2012) indicate that either a deficiency or excess of Leu in the diet negatively affects Lys absorption. In contrast to the jejunum, $b^{0,+}$ expression in the liver was lower in pigs fed the 120% Leu:Lys ratio compared with the 88 and 160% ratios. Because $b^{0,+}$ was more highly expressed in the jejunum of pigs fed the 120% ratio, increased Lys flow from the small intestine to the liver was expected. Supply of AA reduces but low delivery increases the activity of the transporter (adaptive regulation theory; Hatzoglou et al., 2004). Therefore, the expected high Lys supply to the liver of pigs fed the 120% ratio may explain the reduced $b^{0,+}$ expression in the liver. Regarding $b^{0,+}$ expression in the LD and ST, Vekony et al. (2001) reported that $b^{0,+}$ is

mainly expressed in epithelial cells. In this study, the expression of $b^{0,+}$ in these muscles was less than 1% of that in the jejunum.

The CAT-1 transporter is a high-affinity Na-independent transporter of L-arginine and L-Lys whose expression level is modulated by growth factors, hormones, and nutrients (Hatzoglou et al., 2004). CAT-1 gene expression increases when AA are limited and reduces in the abundance of AA; this regulation takes place at the level of mRNA synthesis and mRNA stability (Majumder et al., 2009). Thus, mRNA abundance in pigs fed different dietary AA levels appears to reflect transporter abundance. In the present study, pigs fed the 120% Leu:Lys ratio showed the lowest expression of CAT-1 in the jejunum, which coincides with the highest expression of $b^{0,+}$ in the jejunum. Based on the adaptive theory (Hatzoglou et al., 2004), the reduced CAT-1 expression suggests higher extracellular availability of Lys in pigs fed the 120% ratio. In agreement with these results, myosin expression in the LD of pigs fed the 120% ratio was more than 2-fold higher compared to that in pigs fed either the 88 or 160% ratio. CAT-1 is primarily expressed in non-epithelial cells (Vekony et al., 2001). Accordingly, in the present study, the average expression of CAT-1 in LD muscle was approximately 400-fold that in the jejunum. Notably, CAT-1 expression in LD muscle was more than 50-fold higher than in ST muscle, suggesting the existence of not only tissue-related differences, but also muscle type-related differences in the expression of cationic AA transport systems.

Myosin is the major component of thick filaments and the most abundant of all muscle proteins (Czerwinski and Martin, 1994). According to Lefaucheur et al. (2002), type IIB fibers, which account for approximately 80% of the total fibers in some pig muscles, are extensively expressed in the ST and LD of pigs. Gunawan et al. (2007) reported higher expression of myosin IIB isoform in the LD compared with the ST in pigs. In this study, myosin was expressed in both muscles, but the expression in LD was approximately 6-fold higher compared to that in ST. Furthermore, pigs fed the diet containing the 120% Leu:Lys ratio expressed approximately 2-fold more myosin in both muscles compared to pigs fed the 160% ratio. Leu that is either infused (Wilson et al., 2010) or ingested (Rieu et al., 2007) appears to stimulate muscle protein synthesis, but only if sufficient amounts of the other essential AA are available (Wilson et al., 2010). Although pigs that consumed the 160% ratio diet ingested more Leu, their myosin expression was lower than those that consumed the 120% ratio diet, suggesting reduced availability of other AA such as Lys. Notably, pigs fed the diet containing the 120% Leu:Lys ratio showed the highest expression of myosin in both muscles as well as $b^{0,+}$ in jejunum. According to Drummond et al. (2010), the expression of AA transporters is a unique regulatory mechanism associated with the muscle protein anabolic response after AA availability increases. These results suggest an association between the dietary Leu:Lys ratio, the absorption of Lys, and the synthesis of protein in muscle.

Exogenous protein is a principal source of increased free amino acids in plasma collected within the first 4 h post-prandial in humans (Adibi and Mercer, 1973); thus, the level of dietary AA appears to reflect their SC values. In the current study, blood samples used for AA analyses were collected 2.5 h post-prandial. The SC of Lys did not differ between pigs fed the diet containing the 88 or 120% Leu:Lys ratio, but SC was approximately 40% lower in pigs with the 160% than in those with the 120% ratio. A reduction in serum Lys can be explained as the result of either lower Lys intake, higher protein accretion that increases cellular Lys uptake, or impaired Lys absorption. Lys intake of pigs fed the 120% ratio did not differ from that of pigs fed the 160% Leu:Lys ratio, and thus a lower Lys intake does not explain the lower serum Lys in pigs fed the 160% ratio. Myosin expression in the LD and ST of pigs fed the 160%

ratio was approximately 40 and 30%, respectively, of that in pigs fed the 120% ratio, so the higher protein accretion in pigs fed the 160% ratio was also discharged. In contrast, expression of $b^{0,+}$ was lower in pigs fed the 160% compared to the 120% ratio. Therefore, lower serum Lys appears to result from the negative effect of dietary excess Leu on the absorption of Lys.

Serum Leu in weaned pigs fed excess Leu diets (Edmonds and Baker, 1987; Langer and Fuller, 2000; Wiltafsky et al., 2010) are higher than in pigs fed non-excess Leu diets. In agreement with the results of previous studies, we found that the SC of Leu was 80 and 120% higher in pigs with the 120 or 160% Leu:Lys ratio, respectively, compared with pigs fed the 88% ratio. In contrast, the SC of isoleucine and valine in pigs fed the 120 and 160% Leu:Lys ratios decreased to less than 50% of that in pigs fed the diet with the 88% ratio. These results are also consistent with previous reports (Edmonds and Baker, 1987; Wiltafsky et al., 2010), resulting from increased activity of the enzyme complex that catabolizes all 3 branched chain amino acids in pigs fed diets with excess Leu (Block et al., 1987; Wiltafsky et al., 2010). Furthermore, competitive inhibition for absorption among branched chain amino acids has been documented (Hagihira et al., 1961). Therefore, excess Leu is expected to increase the catabolism or inhibit the absorption of isoleucine and valine, explaining the reduced SC of these AA when excess Leu was provided in pigs fed the 120 and 160% Leu:Lys ratios. These data are critical because most ingredients used to formulate practical diets contain excess Leu, and low-protein diets may be limiting in isoleucine and valine.

The ADG was maximized at an SID Leu:Lys of 100 to 120% and FCR was maximized at an SID Leu:Lys of 120%. Interestingly, the highest ADG and best FCR of pigs in the current study coincided with the highest expression of $b^{0,+}$ in the jejunum and myosin in LD, as well as the highest serum Lys. Furthermore, pigs fed the 160% ratio had lower ADG, SC of Lys, and expression $b^{0,+}$ in the jejunum and myosin in LD. Because Lys was first limiting in all diets, combining the results of performance, expression of $b^{0,+}$ and myosin, and the SC of Lys supports the hypothesis that the dietary Leu:Lys ratio affects the availability of Lys for growth. In contrast, increased catabolism of isoleucine and valine caused by excess Leu reduces the amount of available isoleucine and valine (Harper et al., 1984). Accordingly, in the present study, excess Leu reduced serum isoleucine and valine in pigs fed the 160% ratio diet; however, isoleucine and valine were not limiting in this diet because their serum values in pigs fed the 120 or 160% ratio did not differ, but ADG was higher in the 120% ratio pigs. Therefore, the level of Lys in typical diets containing excess Leu appears to be critical.

Excess dietary Leu appears to reduce ADFI (Edmonds and Baker, 1987; Wiltafsky et al., 2010) by decreasing the formation of serotonin (a neurotransmitter involved in feed intake regulation), as Leu also competes with Trp for transport across the blood-brain barrier into the brain (Henry et al., 1996). Furthermore, Ropelle et al. (2008) found that Leu decreased AMP-dependent kinase and increased mTOR activity in the hypothalamus, leading to inhibition of neuropeptide Y, and concluded that AMP-dependent protein kinase and mTOR interact in the hypothalamus to regulate feed intake. In the present study, the linear decrease in the ADG as the Leu:Lys ratio increased from 100 to 160%, was closely associated with the linear reduction in ADFI. However, based on the analyzed AA content of these diets, there was no difference between intake of the first limiting AA (Lys, Thr, Met, isoleucine, and valine). Thus, availability of AA (particularly Lys) rather than ADFI appears to explain the low ADG in pigs fed the excess Leu diets.

The optimum dietary SID Leu:Lys ratio was estimated to be 104% for ADG and 109% for FCR in the present study using 28- to 43-kg pigs. Using purified diets, the dietary optimal

Leu:Lys has been estimated to be 100% for 10- to 20-kg pigs (Chung and Baker, 1992) and 110% for 25- to 50-kg growing pigs (Wang and Fuller, 1989). Augspurger and Baker (2004), using corn-peanut meal-whey-SBM-based diets, determined the SID Leu requirement for 10- to 20-kg pigs to be approximately 1.05%, which corresponds to an SID Leu:Lys of 100%. In a study by Wiltafsky et al. (2010), the calculated SID Leu:Lys ratio in wheat-barley-soybean meal diets for 8- to 20-kg pigs that produced the best growth response was from 105-107%. Based on these and our current results, the optimal SID Leu:Lys of 100-110% appears to be adequate for growing pig diets, regardless of the diet composition.

CONCLUSIONS

The dietary Leu:Lys ratio selectively affects the expression of genes coding for cationic AA transporters ($b^{0,+}$ and CAT-1) and myosin, the SC of nearly all essential AA, and the performance (ADG and FCR) of growing pigs. Although the Leu:Lys ratio also affected ADFI, daily AA intake did not differ. There appears to be a close relationship between the expression of $b^{0,+}$ in the jejunum and myosin in the LD, the SC of Lys, the ADG and FCR, and the Leu:Lys ratio. Lys availability appears to be the limiting factor in excess Leu diets for growing pigs. Therefore, Lys intake and availability should be considered when the diets for pigs contain excess Leu (>140% SID Leu:Lys).

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