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Effects of reducing dietary protein on the expression of nutrition sensing genes (amino acid transporters) in weaned piglets*

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Abstract: The effects of crude protein (CP) levels in the diet on the mRNA expression of amino acid (AA) transporters were studied in a 45-d trial. Eighteen piglets with an initial body weight (BW) of 9.57 kg were assigned to three groups (14%, 17%, and 20% CP in the diet) in a completely randomized design (six replicates per treatment). Diets were supplemented with crystalline AA to achieve equal standardized ileal digestible contents of Lys, Met plus Cys, Thr, and Trp, and were provided *ad libitum*. After 45 d, all piglets were slaughtered to collect small intestine samples. Compared with the values in the 14% CP group, the expressions of *ASCT2*, *4F2hc*, and ATB^0 mRNA in the jejunum were increased by 23.00%, 12.00%, 6.00% and 48.00%, 47.00%, 56.00% in the 17% and 20% CP groups, respectively. These results indicate that a 14% CP diet supplemented with crystalline AA may not transport enough AA into the body and maintain growth performance of piglets. However, a reduction of dietary 17% CP may reduce the excretion of nitrogen into the environment while supporting the development of piglets. Therefore, the 17% CP level is more suitable than 14% CP level.

Key words: Crude protein, Amino acid balance, Amino acid transporters
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1 Introduction

It has been well documented that low crude protein (CP), amino acid (AA)-supplemented diets may reduce feed costs and nitrogen excretion (Kerr *et al.*, 2003; Zarate *et al.*, 2003; Fairbrother *et al.*, 2005; Deng *et al.*, 2007a; 2007b; Lallès *et al.*, 2007; Hou *et al.*, 2008; Kang *et al.*, 2008; Yue and Qiao, 2008; Deng *et al.*, 2009; Opapeju *et al.*, 2009; Gallo *et al.*, 2014; Gloaguen *et al.*, 2014; Liu *et al.*, 2014; Recktenwald *et al.*, 2014). Therefore, the CP level in the diet of weaned pigs has been suggested to be one of the main factors that affect their growth performance (Gallo *et al.*, 2014), feed efficiency, and gastrointestinal health (Yin and Tan, 2010; Acciaioli *et al.*, 2011). An increase in the entry of nutrients (e.g. dipeptides, tripeptides, arginine, lysine, and histidine) from the lumen of the small intestine into the enterocyte can enhance tissue protein synthesis and improve the efficiency of utilization of dietary nutrients, and it can selectively modulate the gene expression of AA transporters in intestinal cells (Wang *et al*., 2009). Some studies have reported that improving the efficiency of protein use and maintaining performance can be achieved by reducing the CP content of the diet while ensuring the AA balance (Kerr and Easter, 1995; Le Bellego and Noblet, 2002; Kerr *et al.*, 2003; Opapeju *et al.*, 2008; Gloaguen *et al.*, 2014). However, Nyachoti *et al*. (2006) demonstrated that piglet performance may be impaired when dietary CP levels are reduced from 230 to 190 or 170 g/kg, as the AA

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deficiency may change the efficiency of AA absorption and transportation (Tuitoek *et al.*, 1997; Tan *et al.*, 2009; 2011; Gallo *et al.*, 2014). The present study investigated the effects of reducing dietary CP while supplementing with essential AA on the gene expression of AA transporters in weaned piglets.

2 Materials and methods

2.1 Animals and experimental treatment

The experimental protocol was approved by the Protocol Management and Review Committee of the Institute of Subtropical Agriculture, Chinese Academy of Sciences (CAS). Pigs were cared for according to the guidelines of the Institute of Subtropical Agriculture on Animal Care, CAS.

Eighteen piglets (Duroc×Landrace×Yorkshire, female), from the experimental field of the animal observation station in southern center of China, were weaned at 28 d of age, but the experiment started after a 7-d adaptation period when the piglets were weighed to determine an initial mean body weight (BW; mean±standard deviation (SD), (9.57±0.64) kg), and randomly assigned to three different groups (six pigs per group). Each of the groups was fed diets with different levels of CP (14%, 17%, and 20%), respectively. Their ingredients are shown in Tables 1 and 2. The dietary treatment met the National Research Council (NRC; 2012) nutrient specifications for 11 to 20 kg BW pigs. All of the animals were housed individually in cages, and had free access to feed and drinking water at all times throughout the experimental period.

2.2 Relative quantification of mRNA expression of AA transporters

Primers for the selected genes (Table 3) were designed using Oligo 6.0 software (Molecular Biology Insights, CO, USA). After the quantity of complementary DNA (cDNA) was determined by a NanoDrop® ND-1000 (NanoDrop Technologies, Rockland, DE, USA), real-time quantitative polymerase chain reaction (PCR) analyses were performed on an ABI 7900HT Fast Real-Time PCR System (Applied Biosystems, CA, USA) with a total volume of 10 μl containing 5 ng of cDNA, 5 μl SYBR Green mix, 0.2 μl ROX Reference Dye (50×), and 0.2 μl each of the forward and reverse primers (Yao *et al.*, 2012). The following protocol was used: (1) pre-denaturation (30 s at 95 °C); (2) amplification and quantification, repeated 40 cycles (5 s at 95 °C, 30 s at 60 °C); and (3) a melting curve program (extension at 72 °C). *β-Actin* was used as an internal reference gene to normalize target gene transcript levels. The efficiency of the real-time reverse-transcription PCR was determined by the amplification of a dilution series of cDNA according to the equation $10^{(-1/\text{slope})}$. Target mRNA

Table 1 Composition and nutrient levels of diets[a]

Component	Content (%)[b]		
	14% CP	17% CP	20% CP
Ingredient			
Corn	71.80	66.50	63.70
Soybean meal	13.40	18.80	19.80
Whey powder	4.40	4.30	4.30
Fish meal	1.50	4.00	9.00
Soybean oil	4.10	2.60	0.80
Lys	0.88	0.62	0.38
Met	0.27	0.19	0.10
Thr	0.33	0.21	0.09
Trp	0.08	0.04	0.01
Calcium hydrophosphate	1.15	0.74	0.00
Limestone	0.79	0.70	0.52
Salt	0.30	0.30	0.30
1% premix compound[c]	1.00	1.00	1.00
Total	100.00	100.00	100.00
Calculated nutrient content			
DE (MJ/kg)	14.60	14.60	14.60
CP	14.00	17.00	20.00
Total Ca	0.70	0.71	0.69
Total P	0.53	0.55	0.57
Arg	0.75	0.91	1.09
His	0.34	0.40	0.46
Ile	0.49	0.60	0.70
Leu	1.15	1.32	1.49
Lys	1.23	1.23	1.23
Met+Cys	0.68	0.68	0.68
Phe	0.59	0.69	80
Thr	0.73	0.73	0.73
Trp	0.20	0.20	0.20
Val	0.53	0.65	0.77
EAA/NEAA	0.90	0.80	0.70
Recommendation rate of NRC (2012)	0.80	0.80	0.80

[a] Diets contain 14%, 17%, and 20% CP, respectively, with appropriate crystalline AA supplementation. [b] The values are expressed as percentage (%), except for digestible energy (DE; MJ/kg), essential AA (EAA)/nonessential AA (NEAA), and recommendation rate of NRC (2012). [c] Premix provided these amounts of vitamins and minerals per kilogram on an as-fed basis: vitamin A, 10800 IU; vitamin D_3, 4000 IU; vitamin E, 40 IU; vitamin K_3, 4 mg; vitamin B_1, 6 mg; vitamin B_2, 12 mg; vitamin B_6, 6 mg; vitamin B_{12}, 0.05 mg; biotin, 0.2 mg; folic acid, 2 mg; niacin, 50 mg; D-calcium pantothenate, 25 mg; Fe, 100 mg as ferrous sulfate; Cu, 150 mg as copper sulphate; Mn, 40 mg as manganese oxide; Zn, 100 mg as zinc oxide; I, 0.5 mg as potassium iodide; and Se, 0.3 mg as sodium selenite

Table 2 CP content and AA composition of feed ingredients used in formulating the experiment diets[a]

Component	Content (%)[b]		
	14% CP	17% CP	20% CP
CP	14.14	17.32	20.27
EAA			
Arg	0.71	0.93	1.09
His	0.30	0.37	0.44
Ile	0.46	0.60	0.71
Leu	1.11	1.32	1.52
Lys	1.26	1.25	1.26
Met	0.41	0.42	0.40
Met+Cys	0.63	0.65	0.62
Phe	0.56	0.70	0.81
Thr	0.76	0.75	0.76
Trp	0.20	0.20	0.20
Tyr	0.41	0.50	0.59
Val	0.54	0.64	0.72
NEAA			
Ala	0.75	0.90	1.07
Asp	1.15	1.49	1.76
Cys	0.22	0.23	0.22
Glu	2.28	2.78	3.15
Gly	0.53	0.71	0.92
Pro	0.90	1.04	1.17
Ser	0.60	0.74	0.85
EAA	6.29	7.18	7.91
NEAA	6.84	8.40	9.74
EAA/NEAA	0.92	0.85	0.81

[a] Diet contain 14%, 17%, and 20% CP, respectively, with appropriate crystalline AA supplementation. [b] The values are expressed as percentage (%), except for essential AA (EAA)/nonessential AA (NEAA)

and *β-actin* mRNA were amplified with comparable efficiencies (Wang *et al.*, 2009; He *et al.*, 2013). Negative controls, in which cDNA was replaced by water, were also tested (Liu *et al.*, 2012).

2.3 Statistical analysis

All data were subjected to a one-way analysis of variance (ANOVA; SAS Version 9.2) and data were presented as mean±standard error of the mean (SEM). Differences with P values of <0.05 were considered to be statistically significant.

3 Results

3.1 Growth performance of piglets fed diets with different levels of CP (internal data)

The growth performance of weaned piglets fed diets with different CP levels is unpublished data. After the animals were fed different diets for 45 d, significant differences were noted in the average daily gain (ADG) among the three groups (P<0.01), and the 20% CP group had a significantly higher ADG than the other groups (P<0.01). Average daily feed intake (ADFI) showed the same trend as ADG: the 20% CP group had the highest ADFI and ADG, and 14% CP group had the lowest ADFI and ADG. However, there was no significant difference in ADFI between the 17% and 20% CP groups. While the 20% CP group had the lowest feed to gain (F/G) ratio, there were no significant differences between the 14% and 17% CP groups.

Table 3 Primers used for real-time PCR analysis

Gene	Primer sequence (5'→3')	Length (bp)	Accession No.
ASCT2	F: GCTTCCGAGAGCCAAGAACT	152	XM_003127238.3
	R: TCCTTAACGCCTGGAAGCTG		
EAAC1	F: GCTTCCTTCTTCCAGGGTCC	148	NM_001164649.1
	R: CTGGCCAATGTGGCTTGTTC		
$B^{0+}AT$	F: GAGAGGTTTGGTCTTACTGCG	96	XM_003353809.2
	R: GCTATGACCAAGACGGAGCG		
$y^{+}LAT1$	F: TTTGTCTGACCGGCTCTTCC	286	XM_005666262.1
	R: GAGATCTCCTGCTGTCCTGG		
4F2hc	F: CTCGAACCCACCAAGGAC	174	NM_001110171.1
	R: GAGGTGAGACGGCACAGAG		
ATB^{0}	F: TGGGGCATTTTGATTGCAGC	238	NM_001166042.1
	R: CTCCCCAGTCAGGGTATGGA		
β-Actin	F: GGATGCAGAAGGAGATCACG	130	DQ845171
	R: ATCTGCTGGAAGGTGGACAG		

F: forward; R: reverse

Table 4 Effects of diets with different levels of CP on jejunal intestinal *ASCT2*, *EAAC1*, $B^{0+}AT$, y^+LAT1, *4F2hc*, and ATB^0 mRNA relative abundance in weaned piglets (n=6)

mRNA	Relative abundance			SEM	P-value
	14% CP	17% CP	20% CP		
ASCT2	1.00	1.23	1.48	0.14	0.0340
EAAC1	1.00	0.94	1.41	0.15	0.0270
$B^{0+}AT$	1.00	1.49	1.44	0.16	0.0160
y^+LAT1	1.00	0.87	0.92	0.04	0.7369
4F2hc	1.00	1.12	1.47	0.14	0.0846
ATB^0	1.00	1.06	1.56	0.18	0.0142

Diets contain 14%, 17%, and 20% CP, respectively, with appropriate crystalline AA supplementation

3.2 mRNA expression of AA transporters

Changes in the abundance of *ASCT2*, *EAAC1*, $B^{0+}AT$, y^+LAT1, *4F2hc*, and ATB^0 mRNA in the jejunum of the piglets are shown in Table 4. While the abundance of *ASCT2*, *EAAC1*, $B^{0+}AT$, and ATB^0 mRNA significantly differed among the three groups (P<0.05), there were no differences in the expression of y^+LAT1 and *4F2hc* mRNA (P>0.05). Compared with the values in the 14% CP group, the expression of *ASCT2*, *4F2hc*, and ATB^0 mRNA in the jejunum was increased by 23.00%, 12.00%, 6.00% and 48.00%, 47.00%, 56.00% in the 17% and 20% CP groups, respectively. The 17% CP group showed the lowest expression of *EAAC1* mRNA, and the highest expression of $B^{0+}AT$ mRNA. Furthermore, while the expression of y^+LAT1 in the 17% and 20% CP groups was decreased by 13.00% and 8.00%, respectively, compared with that in the 14% CP group, there were no significant differences among the three groups (P>0.05).

4 Discussion

AAs regulate key metabolic pathways that are crucial for the maintenance, health, and growth of animals (Wu, 1998; Hu *et al.*, 2008). The absorption of AA requires many transporter systems that differ with respect to their substrate specificity and driving force (Wang *et al.*, 2009). It has been reported that some genes (such as *ASCT2*, *EAAC1*, $B^{0+}AT$, y^+LAT1, *4F2hc*, and ATB^0) that are involved in the control of growth or AA metabolism are regulated by AA availability (Wu, 1998; Lallès *et al.*, 2007; Zhang *et al.*, 2012; Abdulhussein and Wallace, 2014). In our study, we found that, with an increase in the dietary CP level, the expression of AA transporter genes (*ASCT2*, *EAAC1*, $B^{0+}AT$, *4F2hc*, and ATB^0) tended to increase, while an opposite trend was seen related to the abundance of y^+LAT1 mRNA. A possible reason was that y^+LAT1 and *EAAC1* have differential expression in our study because the diets with different CP levels related with absorption in piglets. The transport of AA in the small intestine is critical for the supply of AA to all tissues and the homeostasis of serum AA levels (Zhang *et al.*, 2012). Since dietary CP can provide an ideal AA level, the genes of AA transporters that regulate protein metabolism in piglets should be more activated. The present results indicate that the expression of *ASCT2*, *EAAC1*, $B^{0+}AT$, and ATB^0 mRNA in the jejunum was influenced by dietary CP levels. Our results regarding the expression of y^+LAT1 mRNA were similar to those of He *et al.* (2013). Under conditions of nutritional stress, the expression of some AA transporters (y^+LAT1) may be increased to maintain normal growth of animals under adverse conditions (He *et al.*, 2013). Skalli *et al.* (2014) suggest that dietary protein levels regulate the expression of AA transporter genes by a complex regulatory system, which may also affect the energy balance and endocrine system. AAs and hormones can play an important role in the regulation of gene expression. Overall, the use of low-CP (14%) AA-supplemented diets in piglets may decrease the transport capacity of AA in the small intestine. However, the absorption and transport of AA is a complex physiological process that is influenced by many factors (Wang *et al.*, 2009; He *et al.*, 2013;

Zhang *et al.*, 2013; Chen *et al.*, 2014), including the dietary CP level and the AA balance. Thus, the transport of these nutrients is a key regulatory step in the use of dietary protein by weaned piglets. An excess decrease in dietary CP with AA supplementation resulted in significant reductions in the expression of AA transporter genes in the jejunum. This result suggests that there is an appropriate range within which CP in the diet may be reduced, and any further decrease may dramatically reduce the efficiency of energy and AA utilization in the small intestine (Jensen, 1998). This understanding may also make it possible to increase productivity and reduce the impact of livestock on the environment (Franklin *et al.*, 2002). While this finding has important practical implications, further research will be required to elucidate the underlying mechanisms.

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Compliance with ethics guidelines

Li WU, Liu-qin HE, Zhi-jie CUI, Gang LIU, Kang YAO, Fei WU, Jun LI, and Tie-jun LI declare that they have no conflict of interest.

All institutional and national guidelines for the care and use of laboratory animals were followed.

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中文概要

题　目：降低日粮粗蛋白水平对断奶仔猪营养感受体基因（氨基酸转运载体）表达的影响

目　的：验证日粮中粗蛋白质浓度对断奶仔猪氨基酸转运载体的影响效应。

创新点：从营养物质感应体的角度分析采用低氮日粮的可行性，并探索营养限制对营养转运体的影响及它们间的相互作用。

方　法：十八头初始体重 9.57 公斤的断奶仔猪被随机分为三组（每组 6 个重复），分别饲喂含有 14%、17%和 20%的粗蛋白日粮 45 天。按照理想蛋白质模

型，日粮分别添加赖氨酸、蛋氨酸+半胱氨酸、苏氨酸和色氨酸来满足断奶仔猪的需要，整个试验期自由采食。试验结束后，屠宰仔猪并采集小肠样品。与 14%粗蛋白组相比，空肠中 *ASCT2*、*4F2hc* 和 ATB^{0} mRNA 表达在 17%和 20%粗蛋白水平组分别上升 23.00%、12.00%、6.00%和 48.00%、47.00%、56.00%。

结　论：结果表明，14%粗蛋白水平组外源添加合成氨基酸并不能促进氨基酸转运载体增加氨基酸的吸收来满足断奶仔猪的生长性能。然而，17%粗蛋白水平组可以减少氮排放到环境中，同时又能满足此阶段断奶仔猪的生长发育。因此，17%粗蛋白水平组对这个阶段断奶仔猪最合适。

关键词：粗蛋白；氨基酸平衡；氨基酸转运载体