Amino Acids (2016) 48:21–30
DOI 10.1007/s00726-015-2059-1

ORIGINAL ARTICLE

Low-protein diets affect ileal amino acid digestibility and gene expression of digestive enzymes in growing and finishing pigs

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Received: 19 March 2015 / Accepted: 17 July 2015 / Published online: 26 July 2015

Abstract The objective of this study was to evaluate effects of dietary crude protein (CP) intake on ileal amino acid digestibilities and expression of genes for digestive enzymes in growing and finishing pigs. In Experiment 1, 18 growing pigs (average initial BW = 36.5 kg) were assigned randomly into one of three treatments (n = 6/treatment group) representing normal (18 % CP), low (15 % CP), and very low (12 % CP) protein intake. In Experiment 2, 18 finishing pigs (average initial BW = 62.3 kg) were allotted randomly into one of three treatments (n = 6/treatment group), representing normal (16 % CP), low (13 % CP) and very low (10 % CP) protein intake. In both experiments, diets with low and very low CP were supplemented with crystalline amino acids to achieve equal content of standardized ileal digestible Lys, Met, Thr, and Trp, and were provided to pigs ad libitum. Daily feed intake, BW, and feed/gain ratios were determined. At the end of each experiment, all pigs were slaughtered to collect pancreas, small-intestine samples, and terminal ileal chymes. Samples were used for determining expression of genes for digestive enzymes and ileal amino acid digestibilities. Growing pigs fed the 12 % CP and 15 % CP diets had lower final body weight ($P < 0.01$) and ADG ($P < 0.0001$) when compared with pigs fed the 18 % dietary CP diet. Growing pigs fed with the 12 % CP diet showed higher digestibilities for CP ($P < 0.05$), DM ($P < 0.05$), Lys ($P < 0.0001$), Met ($P < 0.01$), Cys ($P < 0.01$), Thr ($P < 0.01$), Trp ($P < 0.05$), Val ($P < 0.05$), Phe ($P < 0.05$), Ala ($P < 0.05$), Cys ($P < 0.01$), and Gly ($P < 0.05$) than those fed the 18 % CP diet. Finishing pigs fed the 16 % CP diet had a higher ($P < 0.01$) final body weight than those fed the 10 % CP diet. mRNA levels for digestive enzymes (trypsinogen, chymotrypsin B, and dipeptidases-II and III) differed among the three groups of pigs ($P < 0.05$), and no difference was noted in the genes expression between control group and lower CP group. These results indicated that a reduction of dietary CP by a six-percentage value limited the growth performance of growing–finishing pigs and that a low-protein diet supplemented with deficient amino acids could reduce the excretion of nitrogen into the environment without affecting weight gain.

Keywords Low nitrogen · Crude protein · Amino acid · Digestive enzyme · Pig

Abbreviations
AA Amino acids
BW Body weight

L. He and L. Wu made equal contributions to this study, so they are joint first authors.

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CP Crude protein
DE Digestible energy

Introduction

There is growing interest in amino acid (AA) nutrition to enhance the efficiency of global animal production (Wu et al. 2014a, b). Dietary protein is an important source of amino acids for livestock species and, therefore, inadequate protein intake results in their suboptimal growth and health (Noblet et al. 2001; Orlando et al. 2007; Wang et al. 2015b). On the other hand, sufficient provision of dietary protein is necessary to supply balanced amounts of various AA and small peptides in the gastrointestinal tract (Portejoie et al. 2004; Rezaei et al. 2013a, b; Toledo et al. 2014). Adequate understanding of the digestive network for the regulation of protein and AA metabolism in animals (including pigs) is crucial for designing the new generation of diets to feed them. Although studies have been conducted to investigate effects of dietary protein and AA intake on weight gains, little is known about the impact of supplemental AA in diets on AA digestibilities and expression of genes for digestive enzymes in growing–finishing animals.

A reduction in dietary protein level results in a concomitant decrease in the intake of AA and nitrogen (Portejoie et al. 2004; Wang et al. 2012; Zollitschstelzl 1992), thereby limiting de novo synthesis of AA and impairs digestive function (Orlando et al. 2007; Wu et al. 2014c). The digestive tract is principally responsible for the terminal digestion and absorption of nutrients, including protein (Wu 2013a). The digestive network of animals is a process of complex metabolic transformations, which include glucose and AA utilization, intracellular protein turnover and fat deposition, as well as their regulation by hormones and other factors (Jobgen et al. 2006; Navarro-Guillen et al. 2015; Perez-Jimenez et al. 2009). To efficiently extract nutrients from the ingested food, a repertoire of digestive enzymes is required to break-down macronutrients in the diet into a form that can be readily absorbed (Kaji et al. 2013). It is known that proteolytic enzymes are mainly produced by stomach (pepsin), pancreas (trypsin, chymotrypsin, and elastase), and intestine (membranous and cytosolic enzymes) (Infante and Cahu 2007).

Previous studies with pigs have shown that there exists a close relationship between AA nutrition and efficiency of nutrient utilization (Sharma et al. 2014; Wu 2014; Wu et al. 2014c). However, expression of genes for digestive enzymes has been reported to be regulated by the nature and molecular forms of dietary nutrients (e.g., protein and AA) (Bartelt et al. 2002; Wu 2009). Therefore, the objective of the present study was to determine the effect of low-protein diet on ileal AA digestibilities and expression of genes encoding digestive enzymes in growing and finishing pigs.

Materials and methods

Experimental diets and procedure

This study was conducted and approved by the Animal Welfare Committee of the Institute of Subtropical Agriculture, The Chinese Academy of Sciences. Cross-bred pigs (Duroc × Landrace × Yorkshire) were randomly assigned randomly into one of three dietary treatments representing normal (group C), low (group B) and very low (group A) intake of dietary crude protein (CP). Pigs were housed individually in metabolism cages and there were 6 pigs per treatment group. Titanium dioxide (TiO_2), which served as a digestion indicator/marker, was added to all the experimental diets. There were 2 separate studies involving eighteen growing pigs (Experiment 1) and eighteen finishing pigs (Experiment 2). In both experiments, diets were formulated according to the National Research Council (NRC 2012) to meet nutrient requirements for growing and finishing pigs. There was a 3-day acclimatization period prior to the commencement of each experiment. Pigs had free access to feed and drinking water throughout the experimental period. Body weight of each pig was recorded at the beginning and end of the study to compute weight gains. Feed intake was calculated on a daily basis as the difference between the feed offered and the feed remained in the feeder.

Experiment 1

Eighteen cross-bred (Duroc × Landrace × Yorkshire) growing pigs with the average body weight of 36.47 ± 0.20 kg were assigned randomly into one of three dietary CP levels: normal (18 % CP; group C), low (15 % CP; group B), and very low (12 % CP; group A) CP. Experimental diets designated as Groups A and B were supplemented with some AA that are not synthesized in the body (L-lysine, L-methionine, L-threonine, and L-tryptophan) to meet the requirements of growing pigs (NRC 2012).The experiment lasted 30 days. The composition of the experimental diets used in this study is shown in Table 1.

Experiment 2

Eighteen cross-bred (Duroc × Landrace × Yorkshire) finishing pigs with the average body weight of 62.30 ± 0.07 kg were assigned randomly into one of three dietary CP levels: normal (16 % CP; group C), low (13 % CP; group B) and very low (10 % CP; group A) CP. Experimental diets fed to pigs in Groups A and B were supplemented with different levels of L-lysine, L-methionine, L-threonine, and L-tryptophan) to meet the requirements

Table 1 Feedstuff ingredients and nutrient composition in experimental diets for 30- to 60-kg growing pigs (%)

Items	12 % CP	15 % CP	18 % CP
Feed ingredient			
Corn	77.60	67.50	58.60
Soybean meal	10.00	19.50	29.00
Wheat bran	5.06	6.94	7.80
Soybean oil	3.00	2.38	1.55
Lys	0.74	0.46	0.18
Met	0.17	0.09	0.00
Thr	0.26	0.14	0.01
Trp	0.07	0.02	0.00
$CaHPO_3$	0.90	0.78	0.69
Rock-powder	0.90	0.89	0.87
Salt	0.30	0.30	0.30
1 % Premix[a]	1.00	1.00	1.00
Total	100.00	100.00	100.00
Nutrient composition			
DE (MJ/kg)	14.20	14.20	14.20
CP	12.35	15.16	18.27
Lys	0.94	0.97	0.97
Met + Cys	0.55	0.56	0.57
Thr	0.60	0.61	0.61
Trp	0.17	0.17	0.17
His	0.25	0.33	0.41
Ile	0.35	0.49	0.64
Leu	0.94	1.14	1.35
Phe	0.46	0.62	0.77
Val	0.44	0.56	0.66
Total Ca	0.61	0.63	0.60
Total P	0.45	0.48	0.51
Starch	49.87	44.16	38.96
NDF	10.09	11.09	11.87
ADF	3.53	4.14	4.68

[a] Premix provided these amounts of vitamins and minerals per kilogram on an as-fed basis: vitamin A, 10,800 IU; vitamin D3, 4,000 IU; vitamin E, 40 IU; vitamin K3, 4 mg; vitamin B1, 6 mg; vitamin B2, 12 mg; vitamin B6, 6 mg; vitamin B12, 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 sulfate; 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

of finishing pigs (NRC 2012). This experiment lasted for 50 days. The composition of experimental diets used in this study is shown in Table 2.

Sample preparation

At the end of each experiment, pigs were anesthetized with an intravenous injection of sodium pentobarbital (50 mg/kg BW) and then euthanized. The entire intestine and viscera for each pig was rapidly removed (Wang et al. 2014). The digesta samples were obtained from the ileum for determining the digestibilities of energy (DE), dry matter (DM), crude protein (CP), and AA. Meanwhile, duodenum, jejunum (approximately 3 g from the mid-point of each segment), and pancreas section samples were collected, immediately frozen in liquid nitrogen and stored at −80 °C for subsequent analysis of gene expression.

Analysis of conventional index

Ileal digesta samples were pooled for each pig and homogenized in a blender (Waring Commercial, Torrington, CT), sub-sampled, and freeze-dried. Ileal digesta samples were finely ground in a coffee grinder (CBG5 Smart Grind, Applica Consumer Products Inc., Shelton, CT) and thoroughly mixed for analysis. All samples and experimental diets were analyzed for DM, DE, nitrogen (N), and the content of AA. Dry matter was determined according to the method of AOAC (1990; method 925.09) and gross energy (GE) was determined using an adiabatic oxygen bomb calorimeter (Parr Instrument Co., Moline, IL). Nitrogen was determined using an N analyzer (Model CNS-2000, Leco Corporation, St. Joseph, MI). Samples for AA analysis were prepared by acid hydrolysis according to the method of AOAC (1984; method 982.30) as modified by Mills et al. (Mills et al. 1989). Briefly, approximately 100 mg of each sample was digested in 4 mL of 6 N HCl for 24 h at 110 °C, followed by neutralization with 4 mL of 25 % (wt/vol) NaOH and further cooled to room temperature. The mixture was then equalized to a 50-mL volume with sodium citrate buffer (pH 2.2) and analyzed using an AA analyzer (Sykam, Eresing, Germany). Samples for analysis of S-containing AA (Met and Cys) were subjected to performic acid oxidation before acid hydrolysis. However, tryptophan was not determined, as it was destroyed during acid hydrolysis (Dai et al. 2014).

Quantification of mRNA and cDNA synthesis by real-time PCR analysis

Primers were designed with the use of Primer 5.0 according to the gene sequence of pigs (http://www.ncbi.nlm.nih.gov/pubmed/) to produce amplification products (Table 3). β-Actin was used as a housekeeping gene to normalize target gene transcript levels. Total RNA was isolated from liquid nitrogen-frozen and ground jejunal tissue with the TRIZOL reagents (Invitrogen, USA) and then treated with DNase I (Invitrogen, USA), according to the manufacturer's instructions. β -Actin was used as an internal control to normalize target gene transcript levels. Real-time PCR was performed as previously described (He et al. 2013). Briefly, 1 μL cDNA template was added to a total volume of 10 μL assay solution containing 5 μL SYBR Green

Table 2 Feedstuff ingredients and nutrient composition in experimental diets for 60- to 100-kg finishing pigs (%)

Items	10 % CP	13 % CP	16 % CP
Feed ingredient			
Corn	87.40	78.36	67.00
Soybean meal	5.50	15.00	23.76
Wheat bran	2.00	3.00	6.00
Soybean oil	1.71	0.90	0.88
Lys	0.55	0.27	0.01
Met	0.09	0.00	0.00
Thr	0.19	0.06	0.00
Trp	0.06	0.01	0.00
CaHPO3	0.65	0.55	0.50
Rock-powder	0.55	0.55	0.55
Salt	0.30	0.30	0.30
1 % Premix[a]	1.00	1.00	1.00
Total	100.00	100.00	100.00
Nutrition composition			
DE (MJ/kg)	14.20	14.20	14.20
CP	10.26	13.17	16.30
Lys	0.73	0.72	0.72
Met + Cys	0.43	0.42	0.50
Thr	0.49	0.50	0.56
Trp	0.13	0.13	0.17
His	0.22	0.31	0.39
Ile	0.30	0.45	0.60
Leu	0.91	1.13	1.32
Phe	0.41	0.57	0.71
Val	0.36	0.50	0.61
Total Ca	0.51	0.50	0.52
Total P	0.38	0.40	0.45
Starch	55.22	49.97	43.71
NDF	9.37	10.18	11.33
ADF	3.14	3.69	4.34

[a] Premix provided these amounts of vitamins and minerals per kilogram on an as-fed basis: vitamin A, 10,800 IU; vitamin D3, 4,000 IU; vitamin E, 40 IU; vitamin K3, 4 mg; vitamin B1, 6 mg; vitamin B2, 12 mg; vitamin B6, 6 mg; vitamin B12, 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 sulfate; 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

mix, 0.2 μL Rox, 3 μL deionized H_2O, and 0.4 μmol/L each of forward and reverse primers. We used the following protocol: (i) pre-denaturation (10 s at 95 °C); (ii) amplification and quantification, repeated 40 cycles (5 s at 95 °C, 20 s at 60 °C); (iii) melting curve construction (60–99 °C with heating rate of 0.1 °C S-1 and fluorescence measurements). The relative level of a target gene was expressed as a ratio of the target gene to the control gene using the formula $2^{-(\Delta\Delta Ct)}$, where $\Delta\Delta Ct = (Ct_{Target} - Ct_{\beta\text{-actin}})_{treatment} - (Ct_{Target} - Ct_{\beta\text{-actin}})_{control}$. The relative expression of target genes in the control group was set to be 1.0.

Statistical analysis

Data were analyzed using the one-way analysis of variance and the Student–Newman–Keuls multiple comparison test, as described by Assaad et al. (2014). P values <0.05 were considered statistically significant.

Results

Growth performance

As shown in Table 4, growing pigs fed the 12 % CP and 15 % CP diets had a lower BW ($P < 0.01$) and ADG ($P < 0.0001$), when compared with pigs fed the 18 % CP diet. Growing pigs fed the control diet had a better ($P < 0.0001$) ratio of feed to gain than those fed the 12 and 15 % CP diets. However, the ADFI of growing pigs was not affected ($P > 0.05$) by altering dietary CP levels.

Finishing pigs fed the control diet had a higher ($P < 0.01$) final BW than those fed the 10 % CP diet. The ADG of finishing pigs was reduced ($P < 0.0001$) from 782 g/d in pigs fed the control diet to 634.33 g/d in pigs fed the 10 % CP diet. Finishing pigs fed the control diet or the 13 % CP diet had a similar ratio of feed to gain, which was higher ($P < 0.0001$) than that for pigs fed the 10 % CP diet. Dietary treatment had no effect ($P > 0.05$) on the ADFI of finishing pigs.

Ileal terminal digestibilities of AA

As shown in Table 5, the low CP diet had no effect ($P > 0.05$) on its digestible energy in growing pigs. Pigs fed the 13 % CP diet showed higher digestibilities of CP ($P < 0.05$), DM ($P < 0.05$), Lys ($P < 0.0001$), Met ($P < 0.01$), Cys ($P < 0.01$), Thr ($P < 0.01$), Trp ($P < 0.05$), Val ($P < 0.05$), Phe ($P < 0.05$), Ala ($P < 0.05$), Cys ($P < 0.01$), and Gly ($P < 0.05$) than pigs fed the 18 % CP diet. Lys digestibility increased ($P < 0.0001$) from 72.93 % in pigs fed the 18 % CP diet to 80.87 % in pigs fed the 13 % CP diet. Similar results were obtained for Met. However, there were no differences ($P > 0.05$) in the digestibilities of other AA between the 15 % CP diet and the control diet (Table 5).

Data on ileal AA digestibilities in finishing pigs are summarized in Table 6. There were no differences ($P > 0.05$) on Leu, Phe, Asp, and His digestibilities among the three groups of finishing pigs. Pigs fed the 10 % protein

Table 3 Primers used for real-time PCR analysis

Accession no.	Gene	Primer sequence
XM_003355779.3	Dipeptidase-II (DPP-II)	F:TGTGGCAGATCACTTCGACC
		R:CTCTTCCTCACTCCAGCCAC
XM_003355778.2	Dipeptidase-II (DPP-II)	F:CCTCACAGAATGCAGCCCTT
		R:CAGGTTACTGGCCTCAGCAG
XM_005657730.1	Maltase	F:GCACAGATCAGCCGATGAGA
		R:CAAATGACCGTCCAGCTCCT
XM_005657098.1	Sucrase	F:TGGTGGCACTGTTATCCGAC
		R:GAGCAGGCTCTTGACATGGT
XM_005668172.1	Enterokinase	F:TCTCCATACGGAGGAAGCCA
		R:TGGGCCAGTCATCCCATTTC
NM_001177912.2	Pancrelipase	F:AAGGTGGAGAGCGTGAACTG
		R:TCCAGCCCTGTGATTCGTTC
NM_001244379.1	Chymotrypsin C	F:GCGGCACCTTAATCACCTCT
		R:GGCAGGCATAACACCTGGAT
XM_003472038.1	Chymotrypsin B	F:AACAGGCTTCCACTACTGCG
		R:TGGTCAGTAGCAAAGGGCAG
NM_214169.1	Pancreatic carboxypeptidase B1	F:AGGTGAGAAGGTGTTCCGTG
		R:TGCGAGAGATGAGGTCTGGA
NM_001162891.1	Trypsinogen	F:AGCAATTCATCAATGCCGCC
		R:CAGGAGCGAAGGGTAGCTG
NM_214195.1	Pancreatic α-amylase 2B	F:TGCTCTTGAATGTGAGCGGT
		R:TACGGACGCCAACGTTGTTA
XM_001929136.5	Pancreatic α-Amylase 2A	F:TAAGCACATGTGGCCTGGAG
		R:AAGGGCTCTATCAGAGGGCA

Table 4 Effects of low CP diets on the growth performance of growing and finishing pigs

Items	Group A	Group B	Group C	SEM	*P* value
Growing pigs (Experiment 1)					
Initial body weight (kg)	36.53	36.43	36.60	1.47	0.983
Final bodyweight (kg)	57.3^{b}	58.73^{b}	64.33^{a}	2.32	0.002
Average daily gain (ADG, g/d)	692.2^{b}	742.8^{b}	924.3^{a}	123.6	<0.0001
Average daily feed intake (ADFI, g/d)	1755.2	1849.5	2008.8	192.3	0.069
The ratio of feed and gain (F:G)	2.54^{a}	2.49^{a}	2.17^{b}	0.19	<0.0001
Finishing pigs (Experiment 2)					
Initial body weight (kg)	62.28	62.30	62.33	2.02	0.990
Final body weight(kg)	94.01^{b}	97.88^{ab}	101.43^{a}	4.57	0.009
Average daily gain (ADG, g/d)	634.3^{c}	711.7^{b}	782.0^{a}	73.16	<0.0001
Average daily Feed intake (ADFI, g/d)	2547.5	2614.7	2819.3	212.4	0.059
The ratio of Feed and Gain (F:G)	4.02^{a}	3.67^{b}	3.60^{b}	0.23	<0.0001

Data are means with the pooled SEM, $n = 6$/treatment group. Within a row, values with different superscript letters differ ($P < 0.05$)

Experiment 1: Group A = 12 % CP (very low CP level); Group B = 15 % CP (low CP level); Group C = 18 % CP (normal CP level). Groups A and B were supplemented with L-lysine, L-methionine, L-threonine, and L-tryptophan (see Table 1)

Experiment 2: Group A = 10 % CP (very low CP level); Group B = 13 % CP (low CP level); Group C = 16 % CP (normal CP level). Groups A and B were supplemented with L-lysine, L-methionine, L-threonine, and L-tryptophan (see Table 1)

diet showed higher digestibilities of DE ($P < 0.01$), CP ($P < 0.01$), DM ($P < 0.01$), Ile ($P < 0.01$), Lys ($P < 0.05$), Met ($P < 0.05$), Thr ($P < 0.0001$), Trp ($P < 0.0001$), Val ($P < 0.05$), Ala ($P < 0.001$), Arg ($P < 0.05$), Cys ($P < 0.001$), Glu ($P < 0.05$), Gly ($P < 0.05$), Ser ($P < 0.01$), Tyr ($P < 0.01$), and Pro ($P < 0.05$) than those fed the control

Table 5 Effects of low CP diets on the ileal digestibilities of DE, CP, DM, and IDAA in growing pigs (Experiment 1)

Items	Group A	Group B	Group C	SEM	*P* value
Energy	79.25	77.36	76.18	0.89	0.317
Protein	80.15^{a}	78.41^{ab}	76.53^{b}	1.05	0.048
DM	88.36^{a}	86.92^{ab}	84.16^{b}	1.23	0.032
Ile	72.87	71.73	70.06	0.82	0.347
Leu	73.84	73.36	71.95	0.57	0.526
Lys	80.87^{a}	75.51^{b}	72.93^{c}	2.34	<0.0001
Met	80.05^{a}	78.23^{a}	72.35^{b}	2.32	0.002
Thr	82.46^{a}	78.74^{b}	77.13^{b}	1.58	0.005
Trp	81.12^{a}	79.95^{ab}	78.96^{b}	0.62	0.025
Val	77.10^{a}	73.84^{b}	73.16^{b}	1.22	0.014
Phe	81.25^{a}	80.38^{ab}	78.79^{b}	0.72	0.046
Ala	74.17^{a}	72.01^{ab}	71.26^{b}	0.87	0.048
Arg	86.64	85.82	84.12	0.74	0.340
Asp	84.18	83.39	84.11	0.25	0.856
Cys	83.39^{a}	80.43^{ab}	77.94^{b}	1.58	0.007
Glu	86.75	86.63	85.27	0.47	0.558
Gly	79.16^{a}	75.53^{b}	76.12^{b}	1.12	0.014
His	76.23	75.92	76.47	0.16	0.945
Ser	75.82	74.36	74.02	0.55	0.428
Tyr	80.26	78.15	77.97	0.74	0.390
Pro	78.34	77.27	76.05	0.66	0.342

Data are means with the pooled SEM, $n = 6$/treatment group. Within a row, values with different superscript letters differ ($P < 0.05$)

Group A = 12 % CP (very low CP level); Group B = 15 % CP (low CP level); Group C = 18 % CP (normal CP level). Groups A and B were supplemented with L-lysine, L-methionine, L-threonine, and L-tryptophan (see Table 1)

Table 6 Effects of -CP diets on the ileal digestibilities of DE, CP, DM, and IDAA in finishing pigs (Experiment 2)

Items	Group A	Group B	Group C	SEM	*P* value
Energy	80.52^{a}	78.28^{ab}	75.14^{b}	1.56	0.008
Protein	81.74^{a}	80.36^{a}	76.95^{b}	1.42	0.005
DM	87.26^{a}	85.97^{a}	82.13^{b}	1.54	0.008
Ile	74.72^{a}	75.28^{a}	70.36^{b}	1.56	0.009
Leu	76.25	74.89	73.16	0.89	0.131
Lys	82.18^{a}	80.36^{ab}	78.91^{b}	0.95	0.048
Met	80.47^{a}	78.93^{ab}	76.56^{b}	1.14	0.044
Thr	83.86^{a}	80.14^{b}	76.68^{c}	2.07	<0.0001
Trp	82.73^{a}	78.45^{b}	76.12^{c}	1.94	<0.0001
Val	75.87^{a}	73.53^{ab}	71.14^{b}	1.37	0.014
Phe	80.13	79.67	77.38	0.85	0.103
Ala	76.62^{a}	74.13^{ab}	70.85^{b}	1.67	0.005
Arg	87.13^{a}	87.34^{a}	84.18^{b}	1.02	0.020
Asp	85.72	84.13	82.65	0.89	0.165
Cys	83.25^{a}	80.76^{b}	75.24^{c}	2.37	<0.0001
Glu	86.14^{a}	84.36^{ab}	80.84^{b}	1.56	0.025
Gly	79.25^{a}	77.74^{ab}	73.26^{b}	1.8	0.011
His	75.64	74.35	73.85	0.53	0.511
Ser	82.74^{a}	80.53^{ab}	75.26^{b}	2.22	0.009
Tyr	81.24^{a}	77.21^{ab}	75.13^{b}	1.79	0.005
Pro	75.38^{a}	74.53^{a}	70.15^{b}	1.62	0.039

Data are means with the pooled SEM, $n = 6$/treatment group. Within a row, values with different superscript letters differ ($P < 0.05$)

Group A = 10 % CP (very low CP level); Group B = 13 % CP (low CP level); Group C = 16 % CP (normal CP level). Groups A and B were supplemented with L-lysine, L-methionine, L-threonine, and L-tryptophan (see Table 1)

diet. Ileal digestibilities of protein, DM, Arg, Ile, Thr, Trp, and Pro in finishing pigs did not differ ($P > 0.05$) between the 13 % CP and 10 % CP groups.

Gene expression of digestive enzymes

During the growing period, pigs fed the 12 % CP diet showed lower mRNA levels for trypsinogen ($P < 0.001$), pancreatic α-amylase 2A ($P < 0.05$), chymotrypsin B ($P < 0.01$), chymotrypsin C ($P < 0.01$), duodenal enterokinase ($P < 0.01$), jejunal dipeptidase-II ($P < 0.05$), and jejunal dipeptidase-III ($P < 0.01$), when compared to pigs fed the 18 % CP diet. However, there were no differences in the expression of trypsinogen, pancreatic α-amylase 2A, chymotrypsin B, chymotrypsin C, duodenal enterokinase, jejunal dipeptidase-II, or jejunal dipeptidase-III between the 15 and 18 % CP groups. The mRNA levels for pancreatic α-amylase 2B, pancrelipase, jejunal maltase, or jejunal sucrase did not differ among the three groups of growing pigs ($P > 0.05$).

During the finishing period, mRNA levels for trypsinogen ($P < 0.05$), pancreatic α-amylase 2B ($P < 0.05$), jejunal dipeptidase-II ($P < 0.01$), and jejunal dipeptidase-III ($P < 0.05$) were decreased in pigs fed the 12 % CP diet, compared with pigs fed the 16 % CP diet. The lowest mRNA levels for trypsinogen ($P < 0.05$), jejunal dipeptidase-II ($P < 0.01$), and jejunal dipeptidase-II ($P < 0.05$) were observed in pigs fed the 10 % CP diet (Table 7). mRNA levels for pancreatic α-amylase 2A, pancreatic carboxypeptidase B, pancrelipase, jejunal maltase, or jejunal sucrase did not differ ($P > 0.05$) among the three groups of finishing pigs.

Discussion

A reduction in the N excretion of livestock is a critical issue of environmental protection. Reducing dietary CP intake is an effective way to decrease N excretion of growing and finishing pigs (Dourmad and Jondreville 2007; Gallo et al. 2014). Substantial reductions of protein in diets can be implemented if diets are supplied with nutritionally indispensable AA (Hernandez et al. 2011; Hinson et al. 2009;

Table 7 Effects of low CP diets on mRNA levels for digestive enzymes in growing and finishing pigs

Items	Group A	Group B	Group C	SEM	*P* value
Growing pigs (Experiment 1)					
Trypsinogen	0.61[b]	0.89[a]	1.00[a]	0.19	<0.0001
Pancreatic α-amylase 2A	0.75[b]	0.94[ab]	1.00[a]	0.1	0.048
Pancreatic α-amylase 2B	0.97	1.12	1.00	0.05	0.317
Pancreatic carboxypeptidase B	0.76	0.94	1.00	0.09	0.084
Chymotrypsin B	1.47[a]	1.06[b]	1.00[b]	0.1	0.004
Chymotrypsin C	1.59[a]	1.04[b]	1.00[b]	0.11	<0.0001
Pancrelipase	0.96	0.86	1.00	0.04	0.374
Duodenal enterokinase	1.39[a]	0.94[b]	1.00[b]	0.1	0.006
Jejunal Maltase	0.88	0.74	1.00	0.09	0.194
Jejunal Sucrase	0.96	0.77	1.00	0.07	0.115
Jejunal Dipeptidase-II	0.74[b]	0.91[ab]	1.00[a]	0.11	0.016
Jejunal Dipeptidase-III	0.79[b]	1.03[a]	1.00[a]	0.09	0.009
Finishing pigs (Experiment 2)					
Trypsinogen	0.78[b]	0.98[a]	1.00[a]	0.09	0.013
Pancreatic α-amylase 2A	0.80	1.07	1.00	0.10	0.117
Pancreatic α-amylase 2B	0.84[b]	1.03[a]	1.00[ab]	0.07	0.015
Pancreatic carboxypeptidase B	0.80	1.06	1.00	0.10	0.135
Chymotrypsin B	1.33[a]	1.04[ab]	1.00[b]	0.08	0.036
Chymotrypsin C	1.04	0.95	1.00	0.03	0.446
Pancrelipase	1.05	0.88	1.00	0.05	0.348
Duodenal enterokinase	1.15	0.89	1.00	0.07	0.091
Jejunal Maltase	0.91	0.97	1.00	0.03	0.796
Jejunal Sucrase	1.02	0.95	1.00	0.02	0.833
Jejunal Dipeptidase-II	0.85[b]	1.03[a]	1.00[a]	0.07	0.007
Jejunal Dipeptidase-III	0.78[b]	0.98[a]	1.00[a]	0.09	0.013

Data are means with the pooled SEM, $n = 6$/treatment group. Within a row, values with different superscript letters differ ($P < 0.05$)

Experiment 1: Group A = 12 % CP (very low CP level); Group B = 15 % CP (low CP level); Group C = 18 % CP (normal CP level). Groups A and B were supplemented with L-lysine, L-methionine, L-threonine, and L-tryptophan (see Table 1)

Experiment 2: Group A = 10 % CP (very low CP level); Group B = 13 % CP (low CP level); Group C = 16 % CP (normal CP level). Groups A and B were supplemented with L-lysine, L-methionine, L-threonine, and L-tryptophan (see Table 1)

Kim et al. 2009). However, maximal growth performance and feed efficiency of pigs depend on adequate provision of AA that can be synthesized by animals (Hou et al. 2013, 2015; Wu et al. 2013). In our current study, we hypothesized that addition of certain AA to a low CP diet may improve the growth performance of the growing and finishing pigs. We sought to evaluate the effect of 3- or 6-percentage reduction in dietary protein when supplemented with crystalline AA on the weight gain and digestible ability of the swine. Based on the results from the current study, a reduction in dietary CP by a 3-percentage value with a concomitant addition of "nutritionally essential" AA would support similar growth performance and feed efficiency in finishing pigs but not in growing pigs. However, a further reduction of dietary CP by a 6-percentage value had a negative impact on growth performance in both growing and finishing pigs. Our results are consistent with previous findings (Gallo et al. 2014). Obviously, despite supplementation with nutritionally essential AA, reducing dietary CP by a 3-percentage value reduces ADG, feed efficiency, and the final BW of growing pigs because such a diet cannot provide sufficient "nutritionally nonessential AA" (Hou et al. 2015; Wang et al. 2014, 2015a; Wu et al. 2013, Wu 2014). These findings further underscore the important role for synthesizable AA in maintaining growth and development of growing pigs (Wu 2014).

In previous experiments with pigs fed an "ideal" ration, growth was unaffected by ad libitum feeding experiments until a 100-kg BW (Hinson et al. 2009; Kerr et al. 2003b; Ruusunen et al. 2007). A common feature of these studies was to reduce dietary CP content, while maintaining the same dietary concentration of nutritionally essential AA per kilogram of feed by supplementing with crystalline AA. In this regard, Galassi et al. (2010) found that growth performance and feed efficiency of restricted-fed pigs were unaffected by diets providing only 120 or 99 g CP/kg feed when both diets supplied 6.5, 2.1, 4.5, and 1.4 g/kg feed of total Lys, Met, Thr, and Trp, respectively (Galassi et al. 2010). However, the component of weight gains in growing and finishing pigs was not determined in these studies. It is not known whether the maintenance of weight gains in pigs fed the low CP diet resulted from an increase in whole-body fat with a concomitant decrease in the percentage of protein in the carcass. To date, no studies have been performed to evaluate the effects of low-protein diets with supplemental AA on growing and finishing pigs raised under an ad libitum feeding regimen. Based on the results of our present research and those from other investigators (Gallo et al. 2014; Xiccato et al. 2005), we propose that a reduction in dietary CP along with concomitant supplementation

of appropriate amounts of free AA (including synthesizable AA) can provide an effective strategy to improve feed efficiency in swine production while reducing the excretion of urinary and fecal nitrogen from the animals to the environment. This can be applied to both male and female pigs (Guzman-Pino et al. 2014; Hansen et al. 2014; Noblet et al. 2001; Orlando et al. 2007).

The AA content in the diets used in this experiment is consistent with the requirement of growing and finishing pigs (NRC 2012). Designing a low CP diet for growing and finishing pigs requires the use of crystalline AA to maintain an optimal efficiency in the utilization of dietary protein for protein deposition in the body (Kerr et al. 2003a, b). As the costs of crystalline AA (including "nutritionally nonessential AA") are lowered in the future, low CP diets will be economically attractive to farmers and also environmentally desirable.

The values for the ileal digestibility of CP and some AA in the diets used in our current study differed significantly. Reduction of dietary CP by a 6-percentage value showed the highest ileal digestibility of CP and AA. These results are in agreement with values previously reported by Agyekum et al. (2014). The difference in the response of CP and AA ileal digestibility to diets with different CP levels may be due to relative increases in the rates of protein digestion and absorption of the resultant products (i.e., free AA, dipeptides, and tripeptides) in the small intestine. Feed-grade crystalline AA are fully available to the small intestine, whereas not all AA in dietary protein are released by digestive proteases in the gut lumen (Wu, 2014). As noted previously, it is beneficial to feed a low CP diet to growing and finishing pigs when such a diet is supplemented with crystalline AA. This notion is also supported by a number of published studies (Abbasi et al. 2014; Awad et al. 2014; Hansen et al. 2014; Kulthe et al. 2014; Rodriguez-Gonzalez et al. 2014; Sharma et al. 2014; Xu and Pan 2014). Therefore, our findings raise the possibility that the nutritional needs of swine can be met when dietary CP is reduced by a 3-percentage value, and the low-protein diet is supplemented with adequate amounts of AA. However, when dietary CP is reduced by a 6-percentage value and the low-protein diet is supplemented only with nutritionally essential AA, the growth performance and feed efficiency of growing and finishing pigs cannot be maintained even though the AID of AA are improved. Growth is a complex phenomenon that relies on the digestive capabilities of animals (Gomez-Requeni et al. 2013). Several studies on pigs have suggested that the activity of the main digestive enzymes and their responses to different dietary compositions determine how effectively a given diet may promote whole-body growth (Guzman-Pino et al. 2014; Perez-Jimenez et al. 2009). The digestion of dietary CP and carbohydrates in pigs is accomplished by many different kinds of digestive enzymes in the different parts of the gastrointestinal tract: pancreatic α-amylase 2A, pancreatic α-amylase 2B, pancreatic carboxypeptidase B1, chymotrypsin B, chymotrypsin C, pancrelipase, trypsinogen, enterokinase; maltase, sucrase, dipeptidase-II, and dipeptidase-III. In the current study, the mRNA levels for trypsinogen, pancreatic α-amylase 2A, chymotrypsins B and C, duodenal enterokinase, and jejuna dipeptidase-II and III differed markedly in growing pigs fed the three experimental diets. Likewise, mRNA levels for trypsinogen, pancreatic α-amylase 2B, chymotrypsin B, jejuna dipeptidases-II and III differed among the three groups of finishing pigs. Of note, the values for pigs fed the 13 or 16 % CP diet were higher than the pigs fed the 10 % CP diet. Thus, our results indicate that intestinal expression of the genes for protein digestion is reduced in response to a low-protein diet.

Pancreatic enzymes have been extensively studied (Cahu et al. 2004). Their expression is increased with age. In pigs, the secretion of pancreatic enzymes in the intestinal lumen increases during the first several weeks of postnatal life. This process characterizes the normal maturation of the pancreas and is controlled by cholecystokinin, which in turn is indirectly and positively regulated by the dietary protein level and other nutrients (Abbasi et al. 2014). Intestinal peptide hydrolases are found in two main subcellular locations, the cytosol and the brush border membrane of enterocyte (Wu 2013a, b). Cytosolic enzymes are mainly di- and tripeptidases located in the enterocyte cytosol, completing protein hydrolysis by reducing peptides to free amino acids (Hu et al. 2015; Navarro-Guillen et al. 2015). Protein hydrolysates generated from the diet stimulate the activities of these cytosolic peptidases (Infante and Cahu 2007) and consequently facilitate the utilization of dietary protein by pigs. Interestingly, we observed an increase in intestinal mRNA levels for chymotrypsins B and C as well as duodenal enterokinase in growing and finishing pigs fed low CP diets. It should be borne in mind that expression of digestive enzymes is subjected to complex regulation at both transcriptional and translational levels by a variety of factors, including the composition and balance of dietary AA. Future studies are required to determine protein abundances of digestive enzymes in pigs fed low CP and adequate CP diets.

In summary, low-protein diets supplemented with crystalline AA can maintain expression of digestive enzymes and ileal amino acid digestibility in growing and finishing pigs, and support weight gains and feed efficiency in finishing pigs when dietary CP is reduced by a 3-percentage value. Such low CP diets with supplementation with nutritionally essential AA alone cannot maintain weight gains or feed efficiency in growing pigs, indicating the nutritional importance of adequate amounts of synthesizable AA in animal nutrition. These novel findings have important

implications for the development of new interventions to ameliorate dietary protein shortage and environmental pollution from livestock production. Further research is warranted to understand how dietary AA affects the digestible network in growing and finishing pigs.

Acknowledgments This work was supported by National Basic Research Program of China (2013CB127301, 2013CB127306), National Scientific and Technology Support Project (2013BAD21B04), the Chinese Academy of Sciences through its Hundred Talent Program to Kan Yao, and Texas A&M AgriLife Research (H-8200).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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