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Effects of low-protein diets supplemented with indispensable amino acids on growth performance, intestinal morphology and immunological parameters in 13 to 35 kg pigs

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The objective of this study was to determine if a moderate or high reduction of dietary CP, supplemented with indispensable amino acids (IAA), would affect growth, intestinal morphology and immunological parameters of pigs. A total of 40 barrows (initial BW = 13.50 ± 0.50 kg, 45 ± 2 day of age) were used in a completely randomized block design, and allocated to four dietary treatments containing CP levels at 20.00%, 17.16%, 15.30% and 13.90%, respectively. Industrial AA were added to meet the IAA requirements of pigs. After 4-week feeding, blood and tissue samples were obtained from pigs. The results showed that reducing dietary CP level decreased average daily gain, plasma urea nitrogen concentration and relative organ weights of liver and pancreas ($P < 0.01$*), and increased feed conversion ratio (*$P < 0.01$*). Pigs fed the 13.90% CP diet had significantly lower growth performance than that of pigs fed higher CP at 20.00%, 17.16% or 15.30%. Moreover, reducing dietary CP level decreased villous height in duodenum (*$P < 0.01$*) and crypt depth in duodenum, jejunum and ileum (*$P < 0.01$*). The reduction in the dietary CP level increased plasma concentrations of methionine, alanine (*$P < 0.01$*) and lysine (*$P < 0.05$*), and decreased arginine (*$P < 0.05$*). Intriguingly, reducing dietary CP level from 20.00% to 13.90% resulted in a significant decrease in plasma concentration of IgG (*$P < 0.05$*), percentage of $CD3^+T$ cells of the peripheral blood (*$P < 0.01$*), also down-regulated the mRNA abundance of innate immunity-related genes on toll-like receptor 4, myeloid differentiation factor 88 (*$P < 0.01$*) and nuclear factor kappa B (*$P < 0.05$*) in the ileum. These results indicate that reducing dietary CP level from 20.00% to 15.30%, supplemented with IAA, had no significant effect on growth performance and had a limited effect on immunological parameters. However, a further reduction of dietary CP level up to 13.90% would lead to poor growth performance and organ development, associated with the modifications of intestinal morphology and immune function.*

Keywords: amino acids, protein level, immune function, intestinal morphology, pig

Implications

Reducing dietary CP level is an effective way to improve the efficiency of N utilization, while maintaining normal growth performance of pigs once the indispensable amino acids (IAA) requirements are met. The results of this study suggest that not only IAA, but also the intact protein, dispensable amino acids and/or nitrogen should be taken into consideration in extremely low-CP diet to maintain normal growth and health of pigs.

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Introduction

Reducing the CP level in diets has been recognized as an effective strategy to reduce the incidence of diarrhea in piglets (Yue and Qiao, 2008; Heo *et al.*, 2010). It is well documented that reducing dietary CP level from 16% to 12%, supplemented with indispensable amino acids (IAA), could maintain similar growth performance as pigs fed control diet (Figueroa *et al.*, 2002 and 2003; Kerr *et al.*, 2003). In addition, a moderate reduction of dietary CP level has been shown to decrease the harmful microbial metabolites in the digesta such as ammonia N (Nyachoti *et al.*, 2006; Opapeju *et al.*, 2008), as well as improving the villous morphology of weaned piglets (Gu and Li, 2004;

Opapeju *et al.*, 2008). In contrast, several studies have shown that pig growth performance can decrease when the dietary CP level is reduced by more than 4% , even though supplemented with IAA (Powell *et al.*, 2011; Gloaguen *et al.*, 2014), moreover, poor intestinal growth and morphology, reduced disaccharidase activities in small intestine were observed in pigs receiving extremely low-CP diets (Guay *et al.*, 2006; Yue and Qiao, 2008). However, there is little information whether there are alterations on immunological traits of pigs receiving extremely low-CP diet supplemented with IAA.

We hypothesized that feeding a moderate reduction of dietary CP level supplemented with IAA would maintain normal growth and immune function of pigs, while a high reduction of dietary CP level have a negative effect on growth performance and immune function. Therefore, the objective of the current study was to evaluate the effects of both a moderate and a high reduction of dietary CP level, supplemented with IAA, on growth performance and immune function of pigs.

Material and methods

All experimental protocols for the present study were approved by the Animal Care and Use Committee of Sichuan Agricultural University.

Experimental diets

Four diets based on corn, soybean meal, rapeseed meal and cottonseed meal were used in the current study. Diets differed in CP level but contained the same amount of net energy and other nutrients which met or exceeded the requirements recommended by National Research Council (2012) for 11- and 25-kg pigs, industrial AA were added to the four diets to cover the requirements of standardized ileal digestible (SID) AA. Low-CP diets were formulated by mainly reducing the soybean meal but increasing the cornstarch contents. Feed ingredients contributing to AA were analyzed for CP and AA contents, the analyzed values were used in dietary formulation. Dietary composition and analysis is presented in Table 1. In the 20.00% CP diet, lysine, methionine, threonine, tryptophan and valine were limiting; thus, industrial L-lysine.HCl, DL-methionine, L-threonine, L-tryptophan and L-valine were included. In the 17.16% CP diet, additional isoleucine was limiting; thus, industrial L-isoleucine was included. In the 15.30% and 13.90% CP diets, nine IAA with the exception of arginine were limiting and they were included in their industrial form.

Pigs and housing

A total of 40 crossbred barrows (Duroc × (Landrace × Yorkshire)), with an initial BW of 13.50 ± 0.50 (mean ± SEM) kg and aged at 45 ± 2 days, were randomly assigned into four groups in a randomized complete block design. Pigs were individually housed in metabolic cages (1.5 × 0.7 × 1.0 m). Each cage was equipped with a feeder and a nipple drinker. Pigs had free access to feed and water over the 4-week study. The room temperature was maintained at 22–24°C. BW and feed intake were monitored weekly, average daily feed intake (ADFI), average daily gain (ADG) and feed conversion ratio (FCR) were calculated.

Blood sampling

On day 28, blood samples were collected by jugular venipuncture in the morning (0800 h) after an overnight fast according to our previous study (Hu *et al.*, 2015). Approximately 1 ml of the blood was injected into ethylene–diamine–tetraacetic acid-coated tubes for the determination of peripheral blood T-cells subsets. The rest were contained in heparinized tubes, followed by 3000 × **g** at 4°C for 15 min, plasma was separated and then immediately stored at −80°C for later analysis.

Chemical analysis

The dry matter, ether extract and crude fiber were determined according to AOAC (2003) procedures. Nitrogen content (CP = 6.25 × N) was determined using the Kjeldahl methodology. The ADF content was determined following the procedures of Van Soest *et al.* (1991). The starch of ingredients was analyzed using the method of McCleary *et al.* (1994). The AA contents in ingredients and diets were determined by ion exchange chromatography using an auto amino acid analyzer (L-8800; Hitachi, Tokyo, Japan) after hydrolysis of the samples in 6 N HCl for 24 h at 110°C under a nitrogen atmosphere, whereas methionine and cystine were measured after performic acid oxidation, and the tryptophan content was determined after alkaline hydrolysis (AOAC, 2003).

Tissue sample collection and histological assessment

At the end of week 4, eight pigs with similar BW were selected from each treatment and sedated with an intravenous injection of sodium pentobarbital (80 mg/kg of BW) before jugular exsanguination. After the abdomen was exposed, heart, spleen, pancreas, kidney and liver were quickly removed and weighed. All tissue samples were rinsed by saline water (0.9% NaCl), pieces of spleen and intestinal ileum tissues were immediately frozen in liquid nitrogen and stored at −80°C until analysis. Another portion of intestinal tissue in duodenum, jejunum and ileum (~2 cm long, respectively) was collected and stored in 4% methanol solution for histological measurement.

The duodenal, jejunal and ileal samples were embedded in paraffin. The villous height and crypt depth were determined according to our previous study (Hu *et al.*, 2015). Briefly, each tissue sample was used to prepare five slides and each slide had three sections (5 μm thick), which were stained with hematoxylin and eosin for intestinal morphology analysis of 20 intact well-oriented crypt–villus units each section (Scion Image software, Version 4.02, 2004).

Table 1 *Ingredients composition and nutrient levels of the experimental diets (as-fed basis)*

	Dietary CP level (%)			
Item	20.00	17.16	15.30	13.90
Ingredient (%)				
Corn (8.0% CP)	67.41	67.48	67.81	67.44
Soybean meal (45.2% CP)	14.60	10.56	6.47	2.49
Rapeseed meal (38.0% CP)	3.00	2.17	1.33	0.51
Cottonseed meal (43.5% CP)	3.00	2.17	1.33	0.51
Soy protein concentrate (66.3% CP)	4.00	4.00	4.00	4.00
Fish meal (66.2% CP)	3.00	3.00	3.00	3.00
Corn starch	–	4.24	8.03	12.28
Rice bran	–	1.14	2.29	3.43
Soy oil	1.50	1.20	0.80	0.40
$_{L}$-Lysine·HCl (98.5%)	0.41	0.57	0.73	0.89
$_{DL}$-Methionine (99%)	0.18	0.24	0.31	0.37
$_{L}$-Threonine (98.5%)	0.14	0.22	0.30	0.37
$_{L}$-Tryptophan (98%)	0.03	0.06	0.08	0.11
$_{L}$-Valine (98.5%)	0.01	0.12	0.22	0.32
$_{L}$-Isoleucine (99%)	–	0.06	0.16	0.25
$_{L}$-Leucine (99%)	–	–	0.11	0.27
$_{L}$-Phenylalanine (99%)	–	–	0.12	0.31
$_{L}$-Histidine·HCl (99%)	–	–	0.06	0.13
Limestone	0.82	0.83	0.84	0.84
Dicalcium phosphate	0.50	0.54	0.61	0.68
Salt	0.30	0.30	0.30	0.30
Choline chloride (50%)	0.10	0.10	0.10	0.10
Vitamin and mineral premix[1]	1.00	1.00	1.00	1.00
Calculated composition				
NE (Kcal/kg)[2]	2422	2439	2439	2438
CP (%)	19.71	17.60	15.72	13.92
Ca (%)	0.70	0.70	0.70	0.70
Available P (%)	0.33	0.33	0.33	0.33
Standardized ileal digestible AA (%)[3]				
Lysine	1.23	1.23	1.23	1.23
Methionine + cysteine	0.68	0.68	0.69	0.69
Threonine	0.73	0.73	0.74	0.73
Tryptophan	0.20	0.20	0.20	0.20
Valine	0.78	0.78	0.78	0.78
Isoleucine	0.66	0.63	0.63	0.63
Leucine	1.42	1.27	1.23	1.23
Phenylalanine	0.83	0.72	0.73	0.73
Phenylalanine + tyrosine	1.40	1.20	1.15	1.15
Histidine	0.49	0.43	0.42	0.42
Arginine	1.11	0.94	0.78	0.61
Analyzed composition (%)				
CP	20.00	17.16	15.30	13.90
Lysine	1.35	1.33	1.35	1.31
Methionine	0.49	0.51	0.55	0.58
Methionine + cysteine	0.79	0.78	0.77	0.76
Threonine	0.85	0.83	0.81	0.82
Tryptophan	0.23	0.24	0.21	0.22
Valine	0.93	0.93	0.87	0.89
Isoleucine	0.74	0.67	0.69	0.68
Leucine	1.66	1.44	1.39	1.37
Phenylalanine	0.92	0.85	0.81	0.79
Histidine	0.55	0.48	0.48	0.47
Arginine	1.18	1.01	0.82	0.66
Alanine	1.00	0.89	0.84	0.70
Aspartate	1.80	1.52	1.22	0.98

Table 1 *Continued*

Item	Dietary CP level (%)			
	20.00	17.16	15.30	13.90
Cysteine	0.30	0.26	0.22	0.18
Glutamate	3.58	3.15	2.67	2.10
Glycine	0.80	0.72	0.60	0.52
Proline	1.03	0.92	0.86	0.77
Serine	0.89	0.80	0.69	0.53
Tyrosine	0.67	0.59	0.48	0.43

NE = net energy; SID = standardized ileal digestible.
[1]Supplied the following per kg of diet: 8000 IU, vitamin A; 2400 IU, vitamin D_3; 20 mg, vitamin E; 15 mg, pantothenic acid; 5 mg, vitamin B_6; 0.3 mg, biotin; 3 mg, folic acid; 0.03 mg, vitamin B_{12}; 40 mg, ascorbic acid; 120 mg, Fe; 25 mg, Cu; 20 mg, Mn; 20 mg, Zn; 0.5 mg, I; 0.30 mg, Se.
[2]Values for net energy were calculated according to Noblet *et al.* (1994), the contents of ether extract, starch, CP and ADF were analyzed.
[3]Values for standardized ileal digestible (SID) concentrations of amino acids for the diets were estimated using SID coefficients for the various ingredients provided by NRC (2012).

Plasma urea nitrogen, total protein and AA analysis

Plasma urea nitrogen (PUN) and total protein (TP) were measured using a biochemistry analyzer (Beckman CX4, Beckman Coulter Inc., Fullerton, CA, USA) and commercial kits (Sino-German Beijing Leadman Biotech Ltd, Beijing, China) according to the manufacturer's instructions. Plasma AA contents were determined as previously described (Kong *et al.*, 2009). Briefly, 1 ml of plasma and 2.5 ml of 7.5% trichloroacetic acid were mixed thoroughly and centrifuged at 12 000 × **g** at 4°C for 15 min. The supernatant was analyzed for amino acids using an auto amino acid analyzer (L-8800; Hitachi, Tokyo, Japan).

Immunogloblins and peripheral blood T-cells subsets analysis

Plasma concentrations of IgA, IgG and IgM were measured using the commercially available ELISA kits from R&D system (Minneapolis, MN, USA). The methods were referred to the manufacturer's instructions. Each plasma sample was analyzed in duplicates. The plate wells were added with 50 μl of standard and plasma samples. Each well was then added with 50 μL of horseradish peroxidase, sealed and mixed prior to incubation at 37°C for 60 min and then completely washed 5 times using a washing buffer. Afterwards, 50 μl of Chromogen Solution A and 50 μl of Chromogen Solution B were added to each well and incubated at 37°C in the dark for 15 min, subsequently added 50 μl of stop solution. After completely mixed, the optical density (OD) values of standards and plasma samples were determined at 450 nm within 15 min (MuLtisKan MK3; Thermo Labsystems, Milford, MA, USA). The concentration of the plasma sample was quantified against the standard curve which was drawn from the concentration and absorbance of standards. The minimal detection limit was 3.12 μg/ml for IgA, 15.6 μg/ml for IgG and 2.5 μg/ml for IgM, respectively.

The peripheral blood of eight pigs each group were used to determine the percentages of $CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$T cells and $CD4^+/CD8^+$ratio by the flow cytometry method, as described by our previous study (Hu *et al.*, 2015). Total peripheral blood lymphocytes were separated from anti-clotting peripheral blood by separation medium. The cells were stained with mouse anti-porcine CD3 (Cat. NO. 561485; BD Pharmingen, Franklin Lakes, NJ, USA), mouse anti-porcine CD4 (Cat. NO. 561472; BD Pharmingen) and mouse anti-porcine CD8 (Cat. NO. 551303; BD Pharmingen). PBS (1×; Gibco, Carlsbad, CA, USA) and 1.0% bovine serum albumin (ICN Biomedicals, Aurora, OH, USA) were used as diluent and washing buffer. Flow cytometry analysis was performed on a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA, USA).

Total RNA extraction and RT reaction

Total RNA was isolated from tissue samples of spleen and ileum using TRIzol Reagent (TaKaRa Biotechnology Dalian Co., Ltd, Dalian, China) according to the manufacturer's instructions. The quality and purity of RNA samples were verified by both agarose gel (1.0%) electrophoresis and nucleic acid analyzer (Beckman DU-800; Beckman Coulter Inc.). Both genomic DNA elimination and RT were performed using PrimeScript RT reagent kit with gDNA eraser (TaKaRa Biotechnology Dalian Co., Ltd) according to the manufacturer's guidelines.

Real-time PCR

Real-time PCR was performed using SYBR Premix Ex Taq (Tli RNaseH Plus) qPCR kit (TaKaRa Biotechnology Dalian Co., Ltd) with ABI 7900HT (Applied Biosystems, Foster City, CA, USA). The PCR reaction consisted of 5.0 μl SYBR Premix Ex Taq (2×), 0.4 μl forward primer (10 μM), 0.4 μl reverse primer (10 μM), 0.2 μl ROX Reference Dye (50×), 1.0 μl cDNA and 3.0 μl double distilled water in a total volume of 10 μl. Cycling conditions were 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 34 s. The primer pairs used are shown in Table 2. At the end of amplification, melting curve analysis was performed to identify amplification specificity. The $2^{-\Delta\Delta C_t}$ method was used to process the real-time PCR results according to Livak and Schmittgen (2001), with β-actin as the housekeeping gene. All samples were run in triplicate.

Table 2 *Primer sequences of the target and reference genes*

Gene[1]	Primer sequence (5′–3′)	Product (bp)	GenBank accession
TLR-4	Forward: AGAAAATATGGCAGAGGTGAAAGC Reverse: CTTCGTCCTGGCTGGAGTAGA	64	GQ304754
TLR-9	Forward: AATCCAGTCGGAGATGTTTGCT Reverse: GACCGCCTGGGAGATGCT	79	AY859728
MyD88	Forward: GTGCCGTCGGATGGTAGTG Reverse: TCTGGAAGTCACATTCCTTGCTT	65	NM001099923
TRAF-6	Forward: GCTGCATCTATGGCATTTGAAG Reverse: CCACAGATAACATTTGCCAAAGG	70	AJ606305.1
NF-κB	Forward: TGCTGGACCCAAGGACATG Reverse: CTCCCTTCTGCAACAACACGTA	60	AK348766.1
TOLLIP	Forward: CCCGCGCTGGAATAAGG Reverse: CATCAAAGATCTCCAGGTAGAAGGA	74	AK239879.1
IL-1β	Forward: TCTGCCCTGTACCCCAACTG Reverse: CCAGGAAGACGGGCTTTTG	64	NM214055.1
β-Actin	Forward: GGCGCCCAGCACGAT Reverse: CCGATCCACACGGAGTACTTG	66	DQ845171.1

[1]Gene abbreviations: TLR = toll-like receptor; MyD88 = myeloid differentiation factor 88; TRAF-6 = TNF receptor-associated factor 6; NF-κB = nuclear factor kappa B; TOLLIP = toll-interacting protein; IL-1β = interleukin-1 beta.

Statistical analysis

All data were analyzed by ANOVA, using the GLM procedure of the SPSS 17.0 (Chicago, IL, USA), as a completely randomized block design with dietary treatments as main effects. Results were expressed as means with their standard errors. Duncan's multiple range test was used to compare differences among the four treatment groups. Each animal was considered as an experimental unit. Effects were considered significant at $P < 0.05$, whereas $0.05 < P < 0.10$ was considered a tendency.

Results

Growth performance

The effects of dietary CP level on growth performance are summarized in Table 3. BW was markedly reduced on day 14, 21 and 28 ($P < 0.05$) as dietary CP level decreased. With the reduction of dietary CP level, ADG was markedly decreased during week 2 and overall (day 0 to 28) ($P < 0.01$), and tended to decrease during week 1 ($P = 0.06$) and week 4 ($P = 0.07$). No significant differences were observed for the ADFI among dietary treatments. As dietary CP level decreased, FCR significantly increased during week 1, 2 and the overall study ($P < 0.01$).

Relative organ weights and intestinal morphology

There were no significant effects of dietary treatment on relative organ weights of heart, spleen and kidney (expressed as a percentage of final BW). However, the relative weights of liver and pancreas decreased as the dietary CP level was reduced ($P < 0.05$) (Table 4). There was a significant reduction of the villous height in duodenum ($P < 0.01$) as dietary CP level was decreased. In addition, the crypt depth was markedly reduced ($P < 0.01$) in duodenum, jejunum and ileum. As a result, the ratio of villous height to crypt depth (VCR) was increased in the jejunum ($P < 0.05$), ileum ($P < 0.01$) and tend to increase ($P = 0.08$) in the duodenum.

Table 3 *Growth performance of pigs fed low-CP diets supplemented with various AA*[1]

	Dietary CP level (%)					
Item	20.00	17.16	15.30	13.90	SEM	*P*-Value
BW (kg)						
Day 0	13.57	13.85	13.89	13.75	0.50	0.80
Day 7	18.48	18.26	18.52	17.46	0.76	0.20
Day 14	23.38[a]	22.82[a]	22.69[a]	21.19[b]	0.94	0.02
Day 21	28.78[a]	27.80[a]	27.69[a]	25.79[b]	1.13	<0.01
Day 28	34.80[a]	34.16[a]	33.78[a]	31.42[b]	1.32	<0.01
ADG (kg/day)						
Day 0 to 7	0.70	0.63	0.66	0.53	0.09	0.06
Day 7 to 14	0.70[a]	0.65[ab]	0.60[bc]	0.53[c]	0.06	<0.01
Day 14 to 21	0.77	0.71	0.71	0.66	0.07	0.17
Day 21 to 28	0.86	0.91	0.87	0.80	0.05	0.07
Day 0 to 28	0.76[a]	0.73[a]	0.71[a]	0.63[b]	0.04	<0.01
ADFI (kg/day)						
Day 0 to 7	0.87	0.85	0.91	0.85	0.08	0.69
Day 7 to 14	1.21	1.08	1.14	1.10	0.10	0.27
Day 14 to 21	1.42	1.34	1.40	1.31	0.13	0.66
Day 21 to 28	1.52	1.53	1.51	1.47	0.13	0.92
Day 0 to 28	1.19	1.14	1.18	1.13	0.09	0.68
FCR						
Day 0 to 7	1.24[b]	1.41[b]	1.39[b]	1.62[a]	0.14	<0.01
Day 7 to 14	1.75[bc]	1.67[c]	1.93[ab]	2.08[a]	0.14	<0.01
Day 14 to 21	1.84	1.91	1.96	2.01	0.13	0.25
Day 21 to 28	1.77	1.69	1.73	1.83	0.12	0.45
Day 0 to 28	1.57[b]	1.57[b]	1.66[b]	1.78[a]	0.06	<0.01

ADG = average daily gain; ADFI = average daily feed intake; FCR = feed conversion ratio.
[a,b,c]Means in a row without common superscripts differ ($P < 0.05$).
[1]Values are means with pooled SEM, $n = 10$ pigs/group.

Table 4 *Relative organ weights and small intestinal morphology of pigs fed low-CP diets supplemented with various AA*[1]

Item	Dietary CP level (%)				SEM	*P*-Value
	20.00	17.16	15.30	13.90		
Organ weights (% final BW)						
Heart	0.46	0.48	0.48	0.45	0.04	0.61
Liver	2.62[a]	2.58[a]	2.40[b]	2.37[b]	0.12	0.01
Spleen	0.19	0.19	0.20	0.18	0.02	0.74
Kidney	0.58	0.55	0.52	0.55	0.04	0.21
Pancreas	0.24[a]	0.23[ab]	0.21[bc]	0.20[c]	0.02	<0.01
Intestinal morphology (μm)						
Duodenum						
Villous height	427[b]	459[a]	429[b]	369[c]	17.30	<0.01
Crypt depth	349[a]	330[a]	317[ab]	285[b]	21.75	<0.01
VCR	1.24	1.40	1.37	1.31	0.08	0.08
Jejunum						
Villous height	420	403	372	374	30.65	0.10
Crypt depth	304[a]	287[a]	245[b]	248[ab]	24.68	<0.01
VCR	1.39[c]	1.42[bc]	1.54[a]	1.52[ab]	0.07	0.02
Ileum						
Villous height	332	358	349	330	30.15	0.55
Crypt depth	351[a]	224[b]	269[b]	255[b]	42.79	<0.01
VCR	0.96[c]	1.61[a]	1.36[ab]	1.34[b]	0.16	<0.01

VCR = villous height to crypt depth ratio.
[a,b,c] Means in a row without common superscripts differ ($P < 0.05$).
[1]Values are means with pooled SEM, $n = 8$ pigs/group.

Plasma urea nitrogen, total protein and AA

As shown in Table 5, the PUN concentration decreased ($P < 0.01$) as dietary CP level reduced. However, there were no dietary effects on plasma concentration of TP. The reduction in the dietary CP level increased plasma concentrations of methionine, alanine and lysine ($P < 0.05$) and decreased concentration of arginine ($P < 0.05$).

Immunoglobulins and peripheral blood T-cells subsets

The plasma concentrations of IgA, IgG and IgM are reported in Table 6. During the experimental period, there were no significant differences for plasma concentrations of IgA and IgM among dietary treatments. However, plasma concentration of IgG was decreased with the reducing dietary CP level ($P < 0.05$). As shown in Table 7, low-CP diets markedly decreased the percentage of $CD3^+$T cells ($P < 0.01$). However, there were no significant differences on the percentages of $CD3^+CD4^+$, $CD3^+CD8^+$Tcells and the $CD4^+/CD8^+$ratio among dietary treatments.

Gene expression in the intestine and spleen

The Low-CP diets decreased the mRNA abundance of TLR-4, MyD88 and NF-κB ($P < 0.05$), but increased the mRNA abundance of TOLLIP ($P < 0.05$) in the ileum (Table 8). However, no significant difference was observed for the innate immunity-related gene expression of spleen (date not shown).

Discussion

The current results showed that pig growth performance was similar when dietary CP level was reduced from 20.00% to 15.30%, however, a further decrease from 15.30% to 13.90% caused a lower ADG and higher FCR compared with pigs receiving 20.00% CP diet. Consistently, a recent study by Powell *et al.* (2011) demonstrated that growth performance of pigs (20–50 kg) could be maintained when dietary CP level was reduced by ~5% units with the supplementation of IAA. Moreover, Wu (2014) emphasized both IAA and DAA should be taken into consideration in the formulation of balanced diets to maximize protein accretion in animals. It was reported that generation of DAA in pigs would be reduced by a deficiency of total N in low-CP diet (Columbus *et al.*, 2012), and become a limiting factor for normal growth of pigs (Gloaguen *et al.*, 2014). In this study, the decreasing growth performance of pigs fed 13.90% CP diet may be partially resulting from insufficient contents of total N, which was 30.5% lower than that of pigs fed 20.00% diet. Accordingly, the ratio between N from IAA and N from DAA ($IAA_N : DAA_N$) was 62 : 38 in the 13.90% CP diet, it is much higher than the optimal ratio of $IAA_N : DAA_N$ (50 : 50) reported in growing pigs (Lenis *et al.*, 1999). Furthermore, low-CP diet supplemented with IAA would cause a deficiency of intact protein and related release of peptides (Deng *et al.*, 2007). These intact protein and small peptides could be more effective than a diet containing free AA for protein synthesis of animals (Boza *et al.*, 2000).

Table 5 *Plasma urea nitrogen, total protein and AA of pigs fed low-CP diets supplemented with various AA*[1]

	Dietary CP level (%)					
Item	20.00	17.16	15.30	13.90	SEM	*P*-Value
PUN (mg/dl)	7.58[a]	2.99[b]	2.53[b]	1.24[c]	0.78	<0.01
TP (g/l)	54.26	56.78	56.21	56.17	2.23	0.36
AA (ng/μl)						
Lysine	24.45[b]	25.01[b]	29.19[ab]	33.40[a]	3.76	<0.05
Methionine	5.44[b]	6.03[b]	7.51[a]	8.29[a]	0.76	<0.01
Threonine	13.51	13.72	17.57	16.37	2.62	0.10
Valine	20.20	19.84	19.74	22.23	2.65	0.57
Isoleucine	9.58	9.63	8.87	9.00	0.76	0.40
Leucine	17.80	17.73	17.92	17.10	1.75	0.93
Phenylalanine	8.79	8.55	8.48	8.28	0.78	0.81
Histidine	7.00	6.87	5.99	6.64	0.97	0.45
Arginine	12.80[a]	12.29[a]	11.72[a]	9.78[b]	1.18	0.02
Alanine	29.60[c]	40.52[b]	39.48[b]	49.98[a]	4.46	<0.01
Aspartate	6.37	6.51	6.40	6.51	0.51	0.97
Cysteine	2.31	2.21	2.16	1.95	0.76	0.93
Glutamate	34.73	32.70	33.81	35.52	3.82	0.78
Glycine	94.56	95.67	89.97	85.85	5.78	0.10
Proline	22.55	25.50	24.54	25.36	2.00	0.13
Serine	11.90	13.70	12.31	13.10	1.48	0.29
Tyrosine	15.58	14.70	13.27	12.40	2.09	0.18

PUN = plasma urea nitrogen; TP = total protein.
[a,b,c] Means in a row without common superscripts differ ($P < 0.05$).
[1]Values are means with pooled SEM, $n = 8$ pigs/group.

Table 6 *Plasma immunoglobulin concentrations of pigs fed low-CP diets supplemented with various AA*[1]

	Dietary CP level (%)					
Item	20.00	17.16	15.30	13.90	SEM	*P*-Value
IgA (μg/ml)	69.00	65.57	64.94	67.01	6.68	0.83
IgG (μg/ml)	366.50[a]	354.49[a]	365.16[a]	319.84[b]	20.93	0.02
IgM (μg/ml)	69.54	66.01	72.23	70.20	7.15	0.67

IgA = immunoglobulin A; IgG = immunoglobulin G; IgM = immunoglobulin M.
[a,b]Means in a row without common superscripts differ ($P < 0.05$).
[1]Values are means with pooled SEM, $n = 8$ pigs/group.

Table 7 *Peripheral blood $CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$T-cells percentages and $CD4^+/CD8^+$ratio of pigs fed low-CP diets supplemented with various AA*[1]

	Dietary CP level (%)					
Item	20.00	17.16	15.30	13.90	SEM	*P*-Value
$CD3^+$(%)	70.22[a]	66.38[ab]	64.84[bc]	61.29[c]	3.11	<0.01
$CD3^+CD4^+$(%)	27.38	26.98	24.50	23.74	2.74	0.20
$CD3^+CD8^+$(%)	36.85	36.88	36.04	36.83	3.91	0.99
$CD4^+/CD8^+$	0.75	0.74	0.68	0.66	0.10	0.48

[a,b,c] Means in a row without common superscripts differ ($P < 0.05$).
[1]Values are means with pooled SEM, $n = 8$ pigs/group.

Table 8 *The mRNA abundance of innate immunity-related genes in the ileum of pigs fed low-CP diets supplemented with various AA*[1]

	Dietary CP level (%)					
Item	20.00	17.16	15.30	13.90	SEM	*P*-Value
TLR-4	1.00[a]	0.90[a]	0.71[ab]	0.50[b]	0.20	<0.01
TLR-9	1.00	0.95	0.72	0.94	0.27	0.43
MyD88	1.00[a]	0.88[ab]	0.72[bc]	0.60[c]	0.15	<0.01
TRAF-6	1.00	0.91	1.12	0.86	0.21	0.38
NF-κB	1.00[a]	0.79[ab]	0.94[a]	0.63[b]	0.16	0.02
TOLLIP	1.00[b]	0.99[b]	1.06[b]	1.42[a]	0.21	0.02
IL-1β	1.00	1.02	0.97	0.50	0.38	0.18

TLR = toll-like receptor; MyD88 = myeloid differentiation factor 88; TRAF-6 = TNF receptor-associated factor 6; NF-κB = nuclear factor kappa B; TOLLIP = toll-interacting protein; IL-1β = interleukin-1 beta.
[a,b,c] Means in a row without common superscripts differ ($P < 0.05$).
[1]Values are means with pooled SEM, $n = 8$ pigs/group.

The decreasing dietary CP level decreased the relative organ weights of liver and pancreas, which is in agreement with the results by Kerr *et al.* (1995) and Heo *et al.* (2010). The reduction in pancreas weight of pigs fed low-CP diets may suggest a reduced amount of pancreatic protease, which would be required to digest the smaller quantity of consumed protein (Heo *et al.*, 2010). Besides, the liver weight has been reported to decrease with decreasing dietary protein level, the lower liver may be due to an adaptation of liver to less AA metabolism in pigs receiving low-CP diet (Kerr *et al.*, 1995). The small-intestinal morphology is usually used to reflect intestinal development. The shortening of the villous height may imply the decreased surface area for nutrient absorption, and a deeper crypt may suggest a faster turnover of new villous cells (Xia *et al.*, 2005). In the current study, the villous height in duodenum and crypt depth in duodenum, jejunum and ileum were both significantly decreased, but VCR was increased in all three intestinal segments when the dietary CP level was reduced from 20.00% to 13.90%. A consistent report showed that a reduction in villous height and crypt depth in the upper small intestine, when dietary CP level was decreased from 24.9% to 6.3% (Gu and Li, 2004), while VCR was increased in the jejunum of pigs receiving low-CP diets (Guay *et al.*, 2006). These results may be ascribed to the lower peptide-bound AA in low-CP diets, which decreased mucosal protein content in the small intestine (Guay *et al.*, 2006). However, intestinal morphology was not affected when dietary CP level was reduced from 25.10% to 19.20% (Heo *et al.*, 2010), in which 19.20% CP diet may have proper intact protein or peptide-bound AA for pigs, compared with the other studies (Gu and Li, 2004; Guay *et al.*, 2006).

The decreasing PUN concentration in pigs fed low-CP diets suggests that AA were excessive in the higher CP diets. PUN concentration is mostly dependent on the amounts and balance of AA (Nyachoti *et al.*, 2006; Yue and Qiao, 2008). Previous studies have also reported that there were reductions in PUN concentration by feeding low-CP diets

(Figueroa *et al*., 2002; Nyachoti *et al*., 2006). Besides, reducing dietary CP level from 20.00% to 13.90% resulted in higher plasma concentrations of lysine, methionine and alanine, this response is in agreement with previous reports that the higher protein degradation of pigs occurred in pigs fed low-CP diet, thus increased the plasma concentration of some free AA (Guay and Trottier, 2006). On the other hand, the higher plasma concentrations of lysine and methionine may be related to the higher supplementation of industrial lysine and methionine in low-CP diet (Figueroa *et al*., 2003). The lower plasma concentration of arginine may reflect the lower dietary provision of arginine in pigs fed low-CP diet. In addition, pigs can synthesize arginine with precursors such as glutamine, glutamate and proline (Wu, 1997), the lower level of these precursors may be responsible for the lower plasma concentration of arginine. It is implied that dietary protein level can affect immune function of animals and humans (Ruth and Field, 2013). In this study, plasma concentration of immunoglobulins did not markedly differ when dietary CP level was reduced to 15.30%, however, plasma IgG concentration of pigs fed 13.90% CP diet was significantly lower than that of pigs fed 15.30%, 17.16% and 20.00% CP diets, even though all IAA met the requirement of pigs during this stage. The poor humoral immune response may be related to the deficiency of DAA in the 13.90% CP diet, because some DAA (e.g. glutamate and aspartate) have been demonstrated to regulate immune function (Wu, 2014). In this study, glutamate and aspartate were only 59% and 54% of that in control diet, respectively. Dietary glutamate has been reported to improve immune status of rats recovering from methotrexate treatment (Lin *et al*., 1999). In addition, glutamate is a specific precursor for the intestinal synthesis of glutathione, which is required to protect the intestinal mucosa and optimize immune cell function (Ruth and Field, 2013).

In this study, we also observed the low-CP diet affected cellular immunity-related parameters. The percentage of $CD3^+$T cells was decreased along with the decreasing dietary CP level. $CD3^+$molecules are the surface marker of mature T cells and represent the population of mature T cells (Deng *et al*., 2011). The decreasing $CD3^+$T cells in pigs fed low-CP diet suggested there were lower number of mature T cells in peripheral blood. In the present study, furthermore, we found that the mRNA abundance of TLR-4, MyD88 and NF-κB were markedly decreased in the ileum of pigs fed low-CP diets. Toll-like receptors (TLRs) are typical pattern recognition receptors that play an important role in the activation of the innate immune system following infection (Uematsu and Akira, 2006). The TLR4-Myd88-NF-κB signal pathway is involved in the inflammatory response (Kawai and Akira, 2009). The abnormal expression of TLR-4, MyD88 and NF-κB indicate that feeding extremely low-CP diets may affect the intestinal innate immunity, even though all IAA met the requirement of pigs. It is also reported that some of the traditionally classified DAA (e.g. glutamate and arginine) play important roles in maintaining normal immunity, which is indeed lower in the low-CP diets (Wu, 2014). Particularly, 30–50% of AA (e.g. glutamate, aspartate, glutamine and proline) in the diet are utilized by the small intestine during the first pass metabolism (Wu, 1998), therefore, the lower DAA resulting from the low-CP diet may be responsible for the changed intestinal innate immunity. In addition, low-CP diet, supplemented with IAA, has been shown to alter intestinal bacterial community (Opapeju *et al*., 2009), which could be a factor to sensitize the intestinal immune system.

Conclusions

Our results demonstrate that the reduction of dietary CP level from 20.00% to 15.30%, supplemented with industrial AA, had no significant effect on growth performance and limited effect on immunological parameters of 10 to 35 kg pigs. However, poor growth performance and altered immunological parameters were observed when reducing dietary CP level up to 13.90%, even though industrial AA were added to meet IAA requirements of pigs. The lack of intact protein, DAA and/or nitrogen in low-CP diets are all potential factors leading to poor growth performance and immune function.

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