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Effects of protein sources and levels in antibiotic-free diets on diarrhea, intestinal morphology, and expression of tight junctions in weaned piglets

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ABSTRACT

This study examined effects of dietary protein sources and levels on intestinal health of 21 to 35 d-old weaned piglets fed antibiotics-free diets. A total of 150 weaned piglets (21 d of age) were allotted to 5 dietary treatment groups. Diets were formulated, based on corn-soybean meal, with different protein sources (fish meal and soy protein concentrate) to provide different dietary CP levels. Piglets within 5 dietary treatments were fed diets as follows, respectively: 1) control diet of 17% CP (control); 2) 19% CP diets formulated with more soy protein concentrate (SPC19); 3) fish meal (FM19); 4) 23.7% CP diets formulated with more soy protein concentrate (SPC23); 5) fish meal (FM23). The results showed that piglets from control group had higher ADG and lower incidence of diarrhea compared with those of other groups ($P < 0.05$). The incidence of diarrhea of piglets in FM19 group was lower than those from SPC23 group and FM23 group ($P < 0.05$). With the higher CP levels, villous height and villous height to crypt depth ratio of piglets in the duodenum and jejunum were decreased ($P < 0.05$), but crypt depth was increased ($P < 0.05$). Comparing control group and other groups, we found the expression of inflammatory cytokines interleukin-1β (IL-1β) and interferon-γ (IFN-γ) were increased ($P < 0.05$) in the jejunum and colon of piglets, as did cystic fibrosis transmembrane conductance regulators (CFTR) in the distal colon. The relative transcript abundance of Zonula occludens-1 (ZO-1) in the jejunum, and occludin in the jejunum and ileum of piglets fed 23.7% CP diets were reduced compared with those fed control diet ($P < 0.05$). In conclusion, the 17% CP diet without in-feed antibiotics helped improve growth performance and relief of diarrhea of 21 to 35 d-old weaned piglets. Dietary CP level, rather than its source (either fish meal or soy protein concentrate), has more significant impacts on the growth performance and intestinal health of 21 to 35 d-old weaned piglets when fed antibiotics-free diets.

1. Introduction

Early weaning of piglets is often associated with gut disorders such as mucosal inflammation (Pié et al., 2004), intestinal barrier dysfunction (Wijtten et al., 2011) and diarrhea (Caspary, 1992; van Beers Schreurs et al., 1992). In-feed antibiotics in weaning diets have been used as preventative measures to alleviate these problems for decades (Cromwell, 2002). Increased concerns about negative effects of antibiotics (Chen et al., 2005; Jensen, 2006), such as antibiotic-resistant bacteria have led to a partial and then a total ban on the preventive use of antibiotics in feed (Wierup, 2001; Gallois et al., 2009). Accordingly, the control of post-weaning

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diarrhea requires alternative strategies other than dietary antibiotics.

Both dietary protein levels and sources are important causes of diarrhea in weaned piglets (Pluske et al., 2002). Evidence shows that feeding a low-protein diet (< 18% CP) in the post-weaning period improves fecal consistency and intestinal health (Ball and Aherne, 1982; Wellock et al., 2006; Kong et al., 2007), However, these results are based on antibiotic diets. Despite the protective effects of lower-protein diet, lower-protein diet-fed pigs compromised growth performance compared with pigs fed a higher-protein diet (Nyachoti et al., 2006; Wellock et al., 2006; Yue and Qiao, 2008). Previous studies showed that feeding diets containing 19 to 23% CP could help meet the growth needs of weaning piglets (Htoo et al., 2007; Opapeju et al., 2009). Therefore, it is important to determine whether performance and intestinal health would be affected in piglets if dietary CP is increased from 17.3% without in-feed antibiotics. In addition, weaned piglets fed animal protein sources appear to have a superior feeding value than plant protein sources, which partly due to the plant proteins are less digestible than animal proteins (Yu et al., 2002; Yue and Qiao, 2008). For that reason, this study investigated the effects of dietary CP levels (17, 19, and 23.7%) and protein sources on the growth performance and intestinal health in weaned piglets without feeding any antibiotics growth promoters.

2. Materials and methods

2.1. Animals, diets, and housing

Procedures performed in this experiment were approved by the Animal Care and Use Committee of the Guangdong Academy of Agricultural Sciences.

A total of 150 male weaned piglets (Duroc × Landrace × Large White, 21 d of age, initial BW 5.99 ± 0.14 kg) were randomly assigned in the balance of BW to 1 of 5 treatments, each with 6 replicates (pens) of 5 piglets.

Diets (Table 1) were formulated to provide 17% CP (control), 19% CP (Chinese recommended level Ministry of Agriculture of the People's Republic of China, 2004), and 23.7% CP (NRC recommended level NRC, 2012), respectively, with increasing dietary percentages of soy protein concentrate (SPC19 and SPC23) or fish meal (FM19 and FM23) for the latter two higher CP diets. Control diet contained 17.5% soybean meal and 3% fish meal. All diets were pelleted without any in-feed antibiotics growth promoters and the levels of all essential amino acids met or exceeded the standard of NRC (2012).

The experiment lasted for 2 weeks. The experiment was conducted during summer with an average room temperature of 31 ± 2°C. Water and feed were provided ad libitum throughout the 14-d study. Piglets were weighed at the beginning and the end of the experiment and daily feed intake was recorded with each replicate. Appearance and behaviour of the animals and occurrence of diarrhea were checked daily to evaluate the health status of piglets. Fecal consistency was assessed visually and classified at 4 levels as described previously (Liu et al., 2010): 0, normal; 1, pasty; 2, semi-liquid; and 3, liquid. The piglets were considered to have diarrhea when the fecal consistency was at level 2 or 3, and the incidence of diarrhea was calculated using the number of pig days with diarrhea in each pen as percentage of total pig days during that time interval.

2.2. Slaughter procedure and sampling

On the final day of the experiment, one piglet with BW close to the average weight was chosen from each pen, fasted overnight, blood sampled from the anterior vena cava into heparinised vacutainers, then killed by an intravenous injection of sodium pentobarbital (50 mg/kg BW, Sigma). Blood was centrifuged (800 × *g*, 10 min, 4°C) and plasma samples were held at −80°C until analysis.

Following killing and a midline abdominal incision, the pyloric valve, ileocaecal junction and distal colon were tied to prevent mixing of digesta, and digesta was collected from the distal colon.

The separation of intestinal tract was according to the methods described by Yang et al. (2014) with slight modifications. Briefly, the entire intestinal tract was removed and divided into 4 segments: duodenum, to about 10 cm distal to the pylorus; jejunum, the middle portion; ileum, about 5 cm proximal to the ileocaecal junction; colon, the distal section. The segments were cut longitudinally to expose mucosa and washed three times with ice-cold phosphate buffered saline (PBS). Mucosa from the jejunum, ileum and colon was scraped with glass slides, snap-frozen in liquid nitrogen and stored at −80°C until for further use. Finally, three 2 × 2 cm sections from consistent locations in the duodenum, jejunum, and ileum were fixed in 10% neutral formalin for morphometric analysis.

Table 1
Composition and nutrient level of the experimental diets (as dry-matter basis).[1]

Item	Control	SPC19	FM19	SPC23	FM23
Ingredients, %					
Corn	62.09	59.67	60.22	53.33	53.71
Extruded soybean meal	17.50	17.50	17.50	17.50	17.50
Fish meal	3.00	3.00	7.37	3.00	16.20
Soy protein concentrate		4.36		13.41	
Whey powder	10.00	10.00	10.00	10.00	10.00
Soybean oil	2.85	1.75	2.00		1.35
L-Lys·HCl	0.70	0.48	0.41	0.04	
DL-Met	0.22	0.15	0.12		
L-Thr	0.23	0.13	0.11		
L-Trp	0.06	0.02	0.03		
L-Ile	0.16	0.03	0.04		
L-Val	0.20	0.08	0.06		
L-His	0.03				
L-Phe	0.05				
Premix[2]	0.16	0.16	0.16	0.16	0.16
Dicalcium phosphate	0.79	0.73	0.06	0.61	
Limestone	0.96	0.94	0.92	0.90	0.08
Acidifier	0.30	0.30	0.30	0.30	0.30
TiO_2	0.40	0.40	0.40	0.40	0.40
NaCl	0.15	0.15	0.15	0.15	0.15
$NaHCO_3$	0.15	0.15	0.15	0.15	0.15
Total	100.00	100.00	100.00	100.00	100.00
Calculated nutrient levels, %					
Crude protein (analyzed)	17.00	19.00	19.00	23.70	23.70
DE, MJ/kg	3.40	3.40	3.40	3.40	3.40
Ca	0.80	0.80	0.80	0.80	0.80
Available P	0.40	0.40	0.40	0.40	0.63
Lys	1.36	1.35	1.35	1.36	1.48
Met + Cys	0.77	0.77	0.76	0.76	0.82
Thr	0.86	0.87	0.86	0.95	0.99
Trp	0.25	0.24	0.25	0.30	0.28
Arg	0.94	1.08	1.10	1.37	1.42
Val	0.92	0.93	0.92	1.09	1.12
Leu	1.40	1.59	1.60	1.98	1.98
Ile	0.74	0.73	0.73	0.95	0.91
His	0.43	0.47	0.47	0.61	0.61
Phe	0.81	0.89	0.87	1.15	1.08

SPC = soy protein concentrate; FM = fish meal.

[1] Control = control diet of 17% CP; SPC19 = 19% CP diets formulated with more soy protein concentrate; FM19 = 19% CP diets formulated with more fish meal; SPC23 = 23.7% CP diets formulated with more soy protein concentrate; FM23 = 23.7% CP diets formulated with more fish meal.

[2] Provided per kilogram of diet: vitamin A, 11,000 IU; vitamin D_3, 1,100 IU; vitamin E, 250 IU; vitamin K, 2.5 mg; vitamin B_{12}, 87.5 μg; vitamin B_1, 5 mg; vitamin B_2, 16.5 mg; nicotinamide, 75 mg; pantothenate, 50 mg; folic acid, 1.5 mg; vitamin B_6, 50 mg; biotin, 250 μg; choline chloride, 2.5 mg; Fe ($C_4H_2FeO_4$), 100 mg; Cu ($CuSO_4 \cdot 5H_2O$), 6 mg; Mn ($MnSO_4 \cdot H_2O$), 4 mg; Zn ($ZnSO_4 \cdot H_2O$), 100 mg; I [Ca (IO_3)$_2$], 0.14 mg; Se (Na_2SeO_3), 0.30 mg; Co ($CoSO_4 \cdot 7H_2O$), 6 mg.

2.3. Gut morphological analysis

Fixed tissues were sectioned at 5 μm thickness and stained with haematoxylin and eosin using standard paraffin embedding procedures. At least ten intact, well-oriented crypt-villus units were selected for measurement of villous height and crypt depth using a light microscope fitted with an image analysis system (AxioScope A1, Carl Zeiss, Jena, Germany).

2.4. Plasma urea nitrogen

The concentrations of plasma urea nitrogen (PUN) were determined using a commercial kit (BioAssay System, Hayward, CA), according to the manufacturer's instructions.

2.5. Concentration of chloride ions in colonic contents

The samples were prepared according to Htoo et al. (2007) with some modifications. Digesta (1 g) was mixed thoroughly with 10 mL deionized water, and centrifuged at 2,500 × *g* for 10 min at 4°C. The concentration of Cl^- in the supernatant was determined using a commercial kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

2.6. Intestinal gene expression

Total RNA was extracted from mucosa of jejunum, ileum and colon using TRIzol reagent (Invitrogen, USA). The quality of the RNA was assessed using both 1% agarose gel electrophoresis and a NanoDrop-ND1000 spectrophotometer (Thermo Fisher Scientific Inc., Walldorf, Germany) according to Lackeyram et al. (2010). cDNA was prepared using a PrimeScript RT reagent kit with gDNA eraser (TaKaRa Biotechnology Co., Dalian, China) to remove genomic DNA contaminations.

Real-time PCR was performed in LightCycler 480II System (Roche, Mannheim, Germany) using a SYBR Premix Ex Taq ǁ qPCR kit (TaKaRa Biotechnology Co., Dalian, China). Marker gene transcripts for proinflammatory responses, interleukin-1β (IL-1β) and interferon-γ (IFN-γ), tight junction proteins (Claudin-1, Occludin, ZO-1) and colonic cystic fibrosis transmembrane conductance regulators (CFTR) were quantified using β-actin as a reference transcript. Primer sequences are shown in Table 2. Primers were designed using Primer premier 5.0 software and were synthesised by Sangon Biotech Co. (Shanghai, China). All samples were run in duplicate and relative mRNA abundances of the target genes were determined using the $2^{-\Delta\Delta CT}$ method as ΔCT = CT (target gene) – CT (β-actin), ΔΔCT = ΔCT (treated group) – ΔCT (control group) (Livak and Schmittgen, 2001).

2.7. Statistical analysis

The pen (replicate) of pigs was the experimental unit. The effects of diet were assessed by ANOVA using the GLM procedure of SAS 9.2 (SAS Inst., Inc., Cary, NC). When appropriate, 1 df orthogonal contrasts were examined: the basal (control) diet was compared with all supplemented diets; the 2 × 2 factorial of higher CP diets (source, level; SPC19, FM19, SPC23, FM23) and interaction were compared. Data are presented as least-square means with the SEM derived from the error mean square of each ANOVA for $n = 6$. $P < 0.05$ were considered statistically significant.

3. Results

3.1. Growth performance and diarrhea

Final BW of piglets in control group and FM19 group did not differ (Table 3), but piglets from SPC19, SPC23 and FM23 groups weighed less (84 to 88%, $P = 0.001$) compared with the piglets from control group. There were no differences in ADFI of piglets among groups. Piglets fed the control diet achieved higher ADG than those fed the SPC19, SPC23 and FM23 diets ($P = 0.003$). Furthermore, the G:F of piglets in the control group was higher than those in FM23 group ($P = 0.011$).

Piglets receiving the control diet had lower incidence of diarrhea than those fed all other diets ($P = 0.001$) and incidence was increased further between 19 and 23.7% CP diets ($P = 0.001$), but there were no differences between the protein sources.

3.2. Plasma urea nitrogen

Piglets in the control group had lower PUN concentration than those in all other groups (Table 4; $P = 0.001$) and there were significant increases from 19 to 23.7% CP diets ($P = 0.001$). But there were no significant differences in PUN concentration of piglets between the protein sources within the same CP level.

3.3. Intestinal morphology

The intestinal morphometric results are shown in Table 5. The villous heights of duodenum and jejunum of piglets in control group (17% CP) were significantly higher than those fed SPC19, SPC23 and

Table 2
Primer sequences of the target and reference genes.

Gene	Primer sequence (5′ to 3′)	Product, bp	Gene Bank accession No.
β-actin	F: CGGGACATCAAGGAGAAGC R: ACAGCACCGTGTTGGCGTAGAG	273	DQ845171
IL-1β	F: TCCACCGCAAATGCTTCTAG R: TGCTGTCACCTTCACCGTTC	132	NM_214055.1
IFN-γ	F:GCTCTGGGAAACTGAGTGAC R: TTTCGGTAGTCACTTGAGTA	99	JF906510
Claudin-1	F:GATTTACTCCTACGCTGGTGAC R: CACAAAGATGGCTATTAGTCCC	199	AJ318102
Occludin	F: GTAGTCGGGTTCGTTTCC R: GACCTGATTGCCTAGAGTGT	167	NM_001163647.2
ZO-1	F:CTCTTGGCTTGCTATTCG R: AGTCTTCCCTGCTCTTGC	256	XM_003353439.2
CFTR	F: TTCCTCGTAGTCCTCGCC R: GGTCAGTTTCAGTTCCGTTTG	162	AY585334.1

IL-1β = interleukin-1β; IFN-γ = interferon-γ; ZO-1 = zonula occludens-1; CFTR = cystic fibrosis transmembrane conductance regulators.

Table 3
Effect of different dietary protein sources and levels in antibiotic-free diets on growth performance and incidence of diarrhea of weaned piglets.[1]

Variable	Control	SPC19	FM19	SPC23	FM23	SEM	*P*-value[2]				
							Diets	Basal	Source	Level	Source × level
Initial BW, kg	5.98	6.00	5.99	5.97	5.98	0.14	0.999	0.951	0.974	0.886	0.949
Final BW, kg	7.48^{a}	6.57^{b}	6.73a^{b}	6.33^{b}	6.28^{b}	0.19	0.001	0.001	0.758	0.077	0.580
ADG, g	107^{a}	39.71^{b}	53.15a^{b}	37.46^{b}	25.60^{b}	13	0.003	0.001	0.956	0.303	0.380
ADFI, g	197	169	174	178	155	13	0.293	0.073	0.491	0.734	0.294
G:F	0.56^{a}	0.30a^{b}	0.29a^{b}	0.24a^{b}	0.18^{b}	0.07	0.011	0.001	0.659	0.249	0.752
Diarrhea incidence, %	24.6c	47.2a^{b}	40.1^{b}	53.6^{a}	56.2^{a}	1.99	0.001	0.001	0.272	0.001	0.021

SPC = soy protein concentrate; FM = fish meal.
^{a,b,c}Within a row, values without a common superscript differ ($P < 0.05$), comparisons are from orthogonal contrasts.

[1] Control = control diet of 17% CP; SPC19 = 19% CP diets formulated with more soy protein concentrate; FM19 = 19% CP diets formulated with more fish meal; SPC23 = 23.7% CP diets formulated with more soy protein concentrate; FM23 = 23.7% CP diets formulated with more fish meal.
[2] Diets, all five diets; Basal, control compared to supplemented diets; Source, additional soy or fish protein; Level, 19 and 23.7%. Values are least squares means for 6 replicate pens per treatment.

Table 4
Effect of different dietary protein sources and levels in antibiotic-free diets on the plasma urea nitrogen concentration of weaned piglets.[1]

Variable	Control	SPC19	FM19	SPC23	FM23	SEM	*P*-value[2]				
							Diets	Basal	Source	Level	Source × level
PUN, mM	1.87^{c}	3.65^{b}	3.66^{b}	4.96^{a}	5.13^{a}	0.33	0.001	0.001	0.791	0.001	0.819

SPC = soy protein concentrate; FM = fish meal; PUN = plasma urea nitrogen.
^{a,b,c}Within a row, values without a common superscript differ ($P < 0.05$), comparisons are from orthogonal contrasts.

[1] Control = control diet of 17% CP; SPC19 = 19% CP diets formulated with more soy protein concentrate; FM19 = 19% CP diets formulated with more fish meal; SPC23 = 23.7% CP diets formulated with more soy protein concentrate; FM23 = 23.7% CP diets formulated with more fish meal.
[2] Diets, all five diets; Basal, control compared to supplemented diets; Source, additional soy or fish protein; Level, 19 and 23.7%. Values are least squares means for 6 replicate pens per treatment.

FM23 diets ($P < 0.05$). Piglets fed SPC23 diets had higher villous height in the jejunum than those fed FM19 diets ($P < 0.05$).

The level of dietary protein had a significant effect in crypt depth of duodenum and jejunum of piglets ($P < 0.05$). Specifically, the crypt depths of duodenum and jejunum of piglets fed SPC23 and FM23 diets were significantly higher than those fed control (17% CP) and FM19 diets ($P < 0.05$).

The villous height to crypt depth ratios in the duodenum and jejunum of piglets fed higher CP diets (SPC19, FM19, SPC23, and FM23) were increased compared with those fed control (17% CP) diet ($P < 0.05$). Furthermore, piglets fed FM23 diets had lower villous height to crypt depth ratio in the duodenum and jejunum than those fed FM19 diets ($P < 0.05$).

There were no differences between diets for any of the indices measured in the ileum ($P > 0.05$). Moreover, there were no difference in villous height, crypt depth, and villous height to crypt depth ratio of piglets fed different dietary protein sources-based diets ($P > 0.05$).

3.4. Concentration of chloride ions in colonic contents

The concentration of Cl^{-} in the contents of the terminal colon of piglets fed the control diet was the lowest among all groups

Table 5
Effects of different dietary protein sources and levels in antibiotic-free diets on the intestinal morphology of weaned piglets.[1]

Variable	Control	SPC19	FM19	SPC23	FM23	SEM	*P*-value[2]				
							Diets	Basal	Source	Level	Source × level
Villous height, μm											
Duodenum	379^{a}	299^{b}	330^{ab}	298^{b}	321^{b}	11	0.001	0.001	0.016	0.621	0.660
Jejunum	406^{a}	305^{bc}	350^{ab}	283^{c}	297bc	14	0.001	0.001	0.041	0.011	0.263
Ileum	365	330	335	316	328	11	0.072	0.235	0.464	0.335	0.748
Crypt depth, μm											
Duodenum	189^{c}	228^{ab}	208^{bc}	248^{a}	247^{a}	9	0.001	0.001	0.224	0.002	0.280
Jejunum	184^{c}	221^{abc}	206^{bc}	247^{ab}	258^{a}	14	0.006	0.004	0.891	0.009	0.336
Ileum	199	223	213	227	228	9	0.128	0.251	0.579	0.272	0.487
Villous height: crypt depth											
Duodenum	2.01^{a}	1.32^{c}	1.59^{b}	1.21^{c}	1.31^{c}	0.05	0.001	0.001	0.001	0.001	0.072
Jejunum	2.25^{a}	1.39^{bc}	1.71^{b}	1.16^{c}	1.15^{c}	0.09	0.001	0.001	0.071	0.001	0.082
Ileum	1.73	1.46	1.58	1.40	1.41	0.09	0.111	0.229	0.499	0.220	0.567

SPC = soy protein concentrate; FM = fish meal.
^{a,b,c}Within a row, values without a common superscript differ ($P < 0.05$), comparisons are from orthogonal contrasts.

[1] Control = control diet of 17% CP; SPC19 = 19% CP diets formulated with more soy protein concentrate; FM19 = 19% CP diets formulated with more fish meal; SPC23 = 23.7% CP diets formulated with more soy protein concentrate; FM23 = 23.7% CP diets formulated with more fish meal.
[2] Diets, all five diets; Basal, control compared to supplemented diets; Source, additional soy or fish protein; Level, 19 and 23.7%. Values are least squares means for 6 replicate pens per treatment.

Table 6
Effects of different dietary protein sources and levels in antibiotic-free diets on Cl^- ion concentration of terminal colon contents in weaned piglets.[1]

Variable	Control	SPC19	FM19	SPC23	FM23	SEM	*P*-value[2]				
							Diets	Basal	Source	Level	Source × level
Cl^-, mmol/kg	18.70^c	47.79^b	34.81^b	72.14^a	74.23^a	3.32	0.001	0.001	0.117	0.001	0.035

SPC = soy protein concentrate; FM = fish meal.
[a,b,c]Within a row, values without a common superscript differ ($P < 0.05$), comparisons are from orthogonal contrasts.
[1] Control = control diet of 17% CP; SPC19 = 19% CP diets formulated with more soy protein concentrate; FM19 = 19% CP diets formulated with more fish meal; SPC23 = 23.7% CP diets formulated with more soy protein concentrate; FM23 = 23.7% CP diets formulated with more fish meal.
[2] Diets, all five diets; Basal, control compared to supplemented diets; Source, additional soy or fish protein; Level,19 and 23.7%. Values are least squares means for 6 replicate pens per treatment.

Table 7
Effects of different dietary protein sources and levels in antibiotic-free diets on the mRNA abundances of intestinal mucosa cytokines, tight junction proteins and CFTR in weaned piglets.[1]

Variable	Control	SPC19	FM19	SPC23	FM23	SEM	*P*-value[2]				
							Diets	Basal	Source	Level	Source × level
Jejunum											
IL-1β	1.00^b	1.36^{ab}	1.37^{ab}	2.28^a	2.19^a	0.23	0.005	0.008	0.876	0.002	0.831
IFN-γ	1.00^c	3.01^{ab}	2.40^b	3.57^a	3.40^{ab}	0.22	0.001	0.001	0.096	0.003	0.345
ZO-1	1.00^a	0.46^c	0.77^{ab}	0.37^c	0.56^{bc}	0.06	0.001	0.001	0.001	0.018	0.300
Occludin	1.00^a	0.46^b	0.71^{ab}	0.32^b	0.41^b	0.10	0.001	0.001	0.092	0.037	0.410
Claudin1	1.00	0.65	0.72	0.61	0.59	0.17	0.457	0.457	0.425	0.384	0.545
Ileum											
IL-1β	1.00^b	2.31^a	2.29^a	2.32^a	2.42^a	0.24	0.004	0.001	0.873	0.784	0.803
IFN-γ	1.00	1.44	1.29	1.52	1.53	0.13	0.057	0.057	0.589	0.219	0.562
ZO-1	1.00^a	0.66^b	0.86^{ab}	0.65^b	0.62^b	0.09	0.031	0.007	0.395	0.199	0.261
Occludin	1.00^a	0.60^b	0.72^{ab}	0.56^b	0.56^b	0.06	0.001	0.001	0.377	0.120	0.311
Claudin1	1.00	0.65	0.70	0.61	0.60	0.11	0.142	0.325	0.844	0.524	0.820
Colon											
IL-1β	1.00^b	1.53^{ab}	1.20^b	2.36^a	1.72^{ab}	0.16	0.024	0.003	0.012	0.002	0.342
IFN-γ	1.00^c	2.53^{ab}	1.70^{bc}	2.76^a	2.78^a	0.18	0.001	0.001	0.044	0.003	0.036
CFTR	1.00^b	1.52^{ab}	1.49^{ab}	1.95^a	1.91^a	0.18	0.013	0.003	0.832	0.029	0.967

SPC = soy protein concentrate; FM = fish meal; IL-1β = interleukin-1β; IFN-γ = Interferon-γ; ZO-1 = Zonula occludens-1; CFTR = cystic fibrosis transmembrane conductance regulators.
[a,b,c]Within a row, values without a common superscript differ ($P < 0.05$), comparisons are from orthogonal contrasts.
[1] Control = control diet of 17% CP; SPC19 = 19% CP diets formulated with more soy protein concentrate; FM19 = 19% CP diets formulated with more fish meal; SPC23 = 23.7% CP diets formulated with more soy protein concentrate; FM23 = 23.7% CP diets formulated with more fish meal.
[2] Diets, all five diets; Basal, control compared to supplemented diets; Source, additional soy or fish protein; Level, 19 and 23.7%. Values are least squares means for 6 replicate pens per treatment.

(Table 6; $P < 0.05$). There was a significant decrease in colonic Cl^- concentration of piglets fed 19% CP diets compared with those fed 23.7% CP diets. No significant differences were observed in colonic Cl^- concentration of piglets between the protein sources.

3.5. Gene expression analysis

As shown in Table 7, the mRNA abundances of IFN-γ in the jejunum and colon of piglets in control group were lower compared with those in other groups ($P < 0.05$). The expression of IL-1β in the jejunum, ileum and colon of piglets fed 23.7% CP diets was increased compared with piglets fed control diet ($P < 0.05$). There were no significant differences in these tissues between the protein sources.

Table 7 also shows that the expression of ZO-1 and Occludin in jejunum and ileum of piglets were reduced in the 23.7% diets compared with piglets fed the control diet ($P < 0.05$). No significant differences were observed in the expression of tight junction protein of piglets fed 19 and 23.7% CP diets, or between the protein sources.

Compared with piglets fed the control diets, the colonic expression of CFTR of piglets fed 23.7% diets was increased ($P < 0.05$). For the protein levels, there were no significant differences in this gene expression of piglets fed 19 and 23.7% CP diets, so did between the protein sources.

4. Discussion

Due to the increasing concerns on resistance of antibiotics and future bans of in-feed antibiotic use in livestock production in China, finding effective alternative strategies to control post-weaning growth retardation and diarrhea is very important for swine production. Previous investigations have shown the possible associations between the incidences of diarrhea of piglets and dietary CP sources as well as levels (Nyachoti et al., 2006). However, few have examined the effects of different protein sources and levels on growth performance and intestinal health of piglets fed antibiotics-free diets.

Generally, animal protein sources appear to have a better feeding value than plant protein sources (Yu et al., 2002). For example, compared with dried skim milk, piglets fed soybean meal diet had a lower rate of gain (Li et al., 1990). Piglets of feeding animal protein source diets with antibiotics, such as whey protein concentrate and fish meal, had superior growth performance compared with the soybean meal, fermented soy protein and rice protein concentrate diets-fed pigs (Yun et al., 2005). In our study, the growth performance and incidence of diarrhea of piglets were not affected by dietary protein sources, probably due to the absence of any in-feed antibiotics. But the incidence of diarrhea of piglets in FM19 group was slightly lower than that of those from SPC19 group. The possible explanation for this could be soy allergens in diets, since in our study

all diets contained 17.5% soybean meal and varied percentages of soy protein concentrates. Notably, the antigenic effects associated with soy proteins cause intestinal allergic reactions and thus result in diarrhea of piglets (Li et al., 1990, 1991; Salgado et al., 2002).

Manipulation of dietary CP level has been suggested as an important nutritional strategy for reducing scours in weaned piglets when use antibiotics-free diets (Stein and Kil, 2006). Numerous studies have reported that the growth performance of weaning piglets was decreased with reduced dietary protein levels (Rodriguez et al., 1982; Nyachoti et al., 2006; Htoo et al., 2007). In European, the most efficient way of reducing the incidence of diarrhea in newly weaned pigs is feeding diets containing only 17 to 18% CP in the absence of any in-feed antibiotic (Stein, 2002). In addition, piglets receiving a lower-protein diet supplemented with essential amino acids showed comparable growth performance to piglets fed a higher-protein diet (Lordelo et al., 2008; Heo et al., 2009; Nørgaard and Fernández, 2009). The present study confirms that piglets received lower-protein diet (control) formulated with essential amino acids had reduced incidence of diarrhea and the incidence of diarrhea of other piglets were significantly increased with higher levels of dietary CP in absence of any in-feed antibiotics. This is in accordance with earlier study that the growth performance of 5 to 23 kg weaned piglets was not improved with dietary protein above 18% (Young and Jamieson, 1970). Consistently, our results showed that the growth performance of weaned piglets was not improved with dietary protein level above 17%.

In various species, including pigs, PUN can be used to quantify N utilization and excretion rate (Kohn et al., 2005), with high PUN concentrations indicating low utilization of dietary protein or AA (Waguespack et al., 2011). In the present study, without in-feed antibiotics, the concentration of PUN in the piglets was increased, showing that N utilization was decreased with increased dietary levels of CP.

Morphometric indices of the small intestine, including villous height, crypt depth and their ratio, reflect gut health status in piglets (Han et al., 2013). Increased villous height implies increased absorptive area (Caspary, 1992), whereas deeper crypts indicates more rapid villous epithelial turnover in response to normal desquamation or pathogenic inflammation (Yason et al., 1987). Previous study showed that feeding animal protein sources (for example fish meal) to piglets resulted in better intestinal morphology compared with plant protein sources (for example soybean meal and fermented soy protein) (Yun et al., 2005). However, our study was not consistent with this, perhaps due to the antigenic effects as we mentioned above. In addition, our study showed that villous height increased and villous height to crypt depth ratio of duodenum and jejunum decreased with increased dietary CP, consistent with a previous study suggesting that the intestinal mucosa of piglets fed a high level of CP was more easily damaged (van Beers-Schreurs et al., 1998).

Cytokines play important roles in the immune and inflammatory responses and participate in regulation of the integrity of the intestinal barrier (Al-Sadi et al., 2009). In piglets for example, weaning is associated with up-regulation of inflammatory cytokines in the intestine, and this early inflammatory response may contribute to both anatomical and functional intestinal disorders (Pié et al., 2004). Many pro-inflammatory cytokines, such as IL-1β and IFN-γ, can increase intestinal epithelial permeability (Youakim and Ahdieh, 1999; Al-Sadi and Ma, 2007). When the pro-inflammatory cytokines are generated excessively, the function of epithelial cells (including permeability to macromolecules and nutrients, ion transport) can be greatly affected (Oswald, 2006). According to Opapeju et al. (2010), low-protein diet (170 g/kg CP) reduced inflammatory responses. Jejunal expression of IL-1β and IFN-γ of piglets was increased significantly with higher dietary CP levels in the present study, indicating that pro-inflammatory cytokines induced by higher dietary CP levels may contribute to the diarrhea by changing intestinal epithelial permeability of piglets. The higher expression of IFN-γ in the jejunum and colon of piglets in SPC19 group than that of piglets in FM19 group paralleled the incidence of diarrhea.

Diarrhea is associated with changes in colonic ion channels and concentrations of ions in the luminal contents, among which Cl^- ions and the CFTR are closely related with diarrhea (Berger et al., 2005). The present results showed that the concentration of Cl^- ions in colonic contents of piglets was increased with increasing level of dietary CP and was numerically higher with SPC19 than FM19. Similarly, compared with control diets, colonic expression of CFTR of piglets were increased 50 and 100% in piglets fed 19 and 23.7% CP diets, respectively, but did not differ between the protein sources. In addition to the likelihood of increased CFTR in colonic epithelium with higher CP diets, there could be differences in channel activation and resultant efflux of Cl^- ions (Berger et al., 2005), as downstream actions of inflammatory mediators such as the pro-inflammatory cytokines involve activating PKA, and phosphorylation of CFTR (Kunzelmann and Mall, 2002; Field, 2003). This explanation is consistent with the present finding of higher dietary CP resulting in increased colonic expression of IL-1β and IFN-γ, changes in luminal Cl^- concentrations, and the incidence of diarrhea. The trends shown in each of these variables between 19% CP diets suggest that higher content of soy protein could lead to diarrhea by activating the CFTR ion channel.

To further examine possible underlying mechanisms of the diarrhea, the expression of TJ proteins, as affected by dietary CP, was determined. Over-production of pro-inflammatory cytokines has an adverse effect on intestinal integrity (Liu et al., 2008; Al-Sadi et al., 2013). Recent studies had indicated that most pro-inflammatory cytokines, such as IL-1β, INF-γ, TNF-α, could induce the opening of intestinal TJ barrier and increase permeability of intestinal epithelia (Al-Sadi and Ma, 2007; Brun et al., 2007; Al-Sadi et al., 2009; Pearce et al., 2013). The intestinal barrier is mainly formed by a layer of epithelial cells joined together by TJ (Li et al., 2012), and the proteins ZO-1, occludin and claudin-1 are major and critical components of TJ (Kim et al., 2012). The up-regulation of pro-inflammatory cytokine may induce alterations of TJ protein expression, as study showed that pro-inflammatory cytokines could down-regulate TJ protein expression (Al-Sadi et al., 2009). In the present study, with higher dietary protein, IL-1β expression increased in all tissues as did jejunal INF-γ, while there was decreased expression of ZO-1 and Occludin in jejunum and ileum of piglets, in agreement with Al-Sadi et al. (2008). It is speculated that compromised integrity of intestinal barrier would have contributed to diarrhea.

5. Conclusion

In conclusion, in the absence of in-feed antibiotics, diet containing 17% CP provided better growth performance and decreased incidence of diarrhea of 21 to 35 d weaned piglets compared to higher (19 or 23.7%) dietary CP groups regardless of protein sources. The decline in growth performance of piglets by increase of dietary CP levels may be associated with impairments of intestinal integrity and function, possibly due to provoking intestinal inflammatory responses, altered expression of TJ proteins, enhanced expression of colonic CFTR, and greater efflux of Cl^- ions, and higher occurrence of diarrhea.

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