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Original Research Article

Different dietary protein sources influence growth performance, antioxidant capacity, immunity, fecal microbiota and metabolites in weaned piglets

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ABSTRACT

The inclusion of high-quality proteins are commonly used in swine production. Our research investigated the effects of hydrolyzed wheat protein (HWP), fermented soybean meal (FSBM), and enzyme-treated soybean meal (ESBM) on growth performance, antioxidant capacity, immunity, fecal microbiota and metabolites of weaned piglets. A total of 144 piglets (weaned at 28 d) were allotted to 3 dietary treatments with 6 replicate pens per treatment and 8 piglets per pen. This study included 2 periods: d 0 to14 for phase 1 and d 15 to 28 for phase 2. Dietary treatments contained 15.90% HWP, 15.80% FSBM, and 15.10% ESBM in phase 1, and 7.90% HWP, 7.80% FSBM, and 7.50% ESBM in phase 2, respectively. The ADG of piglets in ESBM was increased ($P < 0.05$) compared with HWP and FSBM during d 1−28. Compared with HWP and FSBM, ESBM increased ($P < 0.05$) the ferric reducing ability of plasma (FRAP), and the serum level of superoxide dismutase (SOD) in piglets on d 14, as well as increased ($P < 0.05$) the serum FRAP level in piglets on d 28. ESBM decreased ($P < 0.05$) serum levels of DAO and IL-1β in piglets compared with HWP on d 28. ESBM enhanced ($P < 0.05$) the relative abundance of Bacteroidetes, Oscillospiraceae and Christensenellaceae, as well as reduced the relative abundance of Clostridiaceae in the feces compared with HWP and FSBM. The PICRUSt analysis revealed that the number of gene tags related to degradation of valine, leucine and isoleucine, as well as lysine degradation in ESBM were lower ($P < 0.05$) than that in HWP and FSBM. ESBM increased ($P < 0.05$) the fecal butyrate level in piglets compared with FSBM, and ESBM tended to decrease ($P = 0.076$) the fecal cadaverine level. Overall, ESBM had advantages over HWP and FSBM in improving antioxidant status, immune function, fecal bacteria and metabolites for weaned piglets.

1. Introduction

In the pig industry, weaning induces intestinal disturbances and results in continuous impairment of the intestinal barrier function and oxidative injury (Yin et al., 2014; Hu et al., 2020). Weaned piglets are suddenly forced to undergo a transition from a highly digestible milk to solid diets including complex protein (Ma et al., 2019a, b), which can result in diarrhea or even death. High-quality proteins should be added in diets to alleviate the weaning stress of piglets. Animal protein sources including fish meal are considered as a source of readily digestible, high-quality protein for weaned piglets. However, the high price, limited supply, and unstable quality of animal protein sources have become important reasons for limiting its addition in diets of weaned piglets. Soybean meal (SBM) is the most widely used plant protein source for weaned piglets, but it contains various anti-nutritional factors including glycinin, β-conglycinin and trypsin inhibitor, which would lead to digestive disorders, immune responses and poor performance (Zheng et al., 2017; Ma et al., 2019a). Fermentation or enzymolytic bioprocessing of SBM could decrease the content of

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antinutritional factors including trypsin inhibitor, glycinin, and β-conglycinin, as well as improve the growth rate of weaned piglets (Ma et al., 2019a). Moreover, hydrolyzed wheat protein (HWP) is a functional protein source with a variety of biologically active wheat peptides, which could promote growth performance of weaned piglets (Wang et al., 2011; Han et al., 2017).

The shortage of dietary protein resources and serious environmental contamination have been the main restrictive factors in the sustainable development of pig industry. Recent research showed that in comparison with traditional diets, adding crystalline amino acids to diets could save protein resources, decrease nitrogen emission and lower feed costs of pigs as well as reduce the incidence of intestinal injury without influencing pig performance (Zhou et al., 2016; Wang et al., 2018a,b; Li et al., 2019b). There is a diverse microbial community in the gastrointestinal tract of pigs, which could provide a variety of physiological functions, such as digestion and absorption of nutrients, production of short-chain fatty acids (SCFA) and improvement of intestinal health (Kamada et al., 2012; Holman et al., 2017; Zhang et al., 2019). Dietary protein is decomposed into peptides and free amino acids in the foregut, mainly under the catalysis of proteases and peptidases, and the fermentation of undigested and endogenous protein takes place in the hindgut (Rist et al., 2013; Fan et al., 2015). The degradation of undigested protein in the hindgut could produce a variety of beneficial metabolites including SCFA or branched-chain fatty acids (BCFA) (Neis et al., 2015), and some potentially toxic products such as ammonia, biogenic amines, phenolic and indolic compounds (Portune et al., 2016). Therefore, better digestibility of protein could decrease the transfer of the undigested protein into the hindgut and reduce the production of potentially toxic products from microbial metabolism.

Although plant protein sources including HWP, FSBM and ESBM have been widely used, the effects of HWP, FSBM and ESBM on immune function and protein fermentation in the hindgut of piglets remain unclear. The purpose of this study was to investigate the effects of HWP, FSBM and ESBM on immunity, fecal microbiota and metabolites of weaned piglets.

2. Materials and methods

All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee of China Agricultural University (Beijing, China). The 3 protein sources used in this study were HWP (hydrolyzed wheat protein; JPC56, Joosten, Weert, The Netherlands), FSBM (fermented soybean meal; Yuanyao Biotechnology Co., LTD, Jiangsu, China) and ESBM (enzyme-treated soybean meal; HP300, Hamlet Protein, Horsens, Denmark). The analyzed nutrient composition of HWP, FSBM and ESBM is presented in Table 1.

2.1. Experimental animals, diets, and design

In this experiment, a total of 144 weaned piglets (Duroc × Landrace × Large White; weaned at 28 d; initial BW = 7.8 ± 1.0 kg) were allotted to 3 dietary treatments with 6 replicate pens per treatment and 8 piglets (4 barrows and 4 gilts) per pen. Our study was divided into phase 1 (d 0 to 14) and phase 2 (d 15 to 28). The experimental diets included: (1) HWP group, 15.90% JPC56 in phase 1 and 7.90% JPC56 in phase 2; (2) FSBM group, 15.80% FSBM in phase 1 and 7.80% FSBM in phase 2; (3) ESBM group, 15.10% HP300 in phase 1 and 7.50% HP300 in phase 2. The percentage of crude protein in the diets was 18.0%, and other nutrients met or exceeded the nutrient requirements of weaned piglets recommended by National Research Council (NRC, 2012).

Table 1
Analyzed nutrient composition of HWP, FSBM and ESBM (%, as-fed basis).

Item	HWP	FSBM	ESBM
Metabolizable energy[1], kcal/kg	3732	3720	3902
Dry matter	92.35	91.56	93.16
Crude protein	56.00	55.00	56.00
Crude fibre	3.20	4.50	4.00
Calcium	0.22	0.29	0.35
Phosphorus	0.55	0.80	0.75
Total starch	0.30	0.50	0.60
Lysine	4.29	3.40	3.34
Methionine	1.53	0.86	0.07
Threonine	1.94	2.29	2.15
Tryptophan	0.63	0.81	0.07

HWP = hydrolyzed wheat protein; FSBM = fermented soybean meal; ESBM = enzyme-treated soybean meal.
[1] Metabolizable energy of HWP, FSBM or ESBM is measured.

The ingredients and nutrient composition of the experimental diet are shown in Table 2.

Piglets were raised in commercial flat-deck pens equipped with duckbill drinkers, adjustable feeders and plastic slatted floors. The room humidity and temperature were maintained at 60% to 70% and 24 to 26 °C, respectively. On d 14 and 28, each piglet was weighed after fasting to calculate growth performance including average daily gain (ADG), average daily feed intake (ADFI) and feed-to-gain ratio (F:G). The fecal score was determined by clinical signs of fecal consistency every day based on the methods described by Pan et al. (2016) and a scoring system was as follows: 1 = hard

Table 2
Ingredients and nutrient composition of the experimental diet (%, as-fed basis).

Item	Phase 1 (d 0 to 14)			Phase 2 (d 15 to 28)		
	HWP	FSBM	ESBM	HWP	FSBM	ESBM
Ingredients						
Corn	39.28	38.97	39.91	36.96	36.87	37.29
Wheat	20.00	20.00	20.00	20.00	20.00	20.00
Soybean meal	5.00	5.00	5.00	16.00	16.00	16.00
HWP	15.90			7.90		
FSBM		15.80			7.80	
ESBM			15.10			7.50
Whey powder	12.00	12.00	12.00	12.00	12.00	12.00
Soybean oil	3.60	3.80	3.40	3.60	3.70	3.50
Dicalcium phosphate	1.50	1.30	1.35	1.10	1.00	1.00
Limestone	0.85	0.95	0.90	0.75	0.80	0.79
Sodium chloride	0.30	0.30	0.30	0.30	0.30	0.30
L-Lysine hydrochloride	0.50	0.73	0.70	0.40	0.52	0.50
DL-Methionine	0.02	0.15	0.25	0.06	0.11	0.17
L-Threonine	0.25	0.23	0.21	0.16	0.15	0.14
L-Tryptophan	0.05	0.02	0.13	0.02	0.00	0.06
Chromic oxide	0.25	0.25	0.25	0.25	0.25	0.25
Premix[1]	0.50	0.50	0.50	0.50	0.50	0.50
Nutrient levels						
Metabolizable energy, kcal/kg	3,399	3,399	3,399	3,349	3,350	3,350
Dry matter[2]	90.06	89.97	90.02	89.93	89.86	89.91
Crude protein[2]	17.99	18.02	18.01	18.01	18.01	18.04
Crude fibre	2.10	2.29	2.21	2.44	2.54	2.49
Total starch	36.09	35.92	36.53	34.97	34.93	35.20
SID lysine	1.35	1.35	1.35	1.23	1.23	1.23
SID methionine	0.39	0.39	0.39	0.36	0.36	0.36
SID threonine	0.79	0.79	0.79	0.73	0.73	0.73
SID tryptophan	0.22	0.22	0.22	0.20	0.20	0.20

HWP = hydrolyzed wheat protein; FSBM = fermented soybean meal; ESBM = enzyme-treated soybean meal; SID = standardized ileal digestible.
[1] Premix provided the following per kilogram of diet: vitamin A, 12,000 IU; vitamin E, 30 IU; vitamin K_3, 3.0 mg; vitamin B_{12}, 12 μg; riboflavin, 4.0 mg; pantothenic acid, 15 mg; niacin, 40 mg; choline chloride, 400 mg; folacin, 0.7 mg; thiamine, 1.5 mg; vitamin B_6, 3.0 mg; biotin, 44 μg; Mn, 30 mg; Fe, 90 mg; Zn, 80 mg; Cu, 10 mg; I, 0.35 mg; Se, 0.3 mg.
[2] Analyzed value.

feces; 2 = slightly soft feces; 3 = soft, partially formed feces; 4 = loose, semiliquid feces; and 5 = watery, mucous-like feces.

2.2. Sample collection

On d 14 and 28, blood samples (5 mL) from 1 piglet per pen were collected from the jugular veins using vacuum tubes (Becton–Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA). All samples were centrifuged at 3,000 × *g* for 10 min at 4 °C, and then stored at −20 °C. During d 26 to 28, feces (200 g) were collected from each pen and then dried at 65 °C for 72 h. On d 28, 6 fresh fecal samples (one piglet per pen) from each treatment were rapidly frozen in liquid nitrogen, and then stored at −80 °C.

2.3. Chemical analysis

All experimental diets and collected feces were determined in duplicate for dry matter (DM, method 934.01) and crude protein (CP, method 990.03) based on the Association of Official Analytical Chemists (AOAC, 2006). Gross energy (GE) was analyzed using an Automatic Energy Analyzer (Parr 1281, Moline, IL, USA). The content of chromium (Cr) in diets and feces was analyzed using an Atomic Absorption Spectrophotometer (Z-5000; Hitachi, Tokyo, Japan) based on the methods of Williams et al. (1962). The apparent total tract digestibility (ATTD) of nutrients was calculated according to the equation: ATTD (%) = 100 − [(Cr_{diet} × $Nutrient_{feces}$)/(Cr_{feces} × $Nutrient_{diet}$)] × 100. The serum contents of advanced oxidation protein products (AOPP) and ferric reducing ability of plasma (FRAP) were detected by ELISA test kits (Zhongshang Boao Biotechnology Co., Ltd., Beijing, China). The serum contents of malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px), diamine oxidase (DAO) and endotoxin were determined by assay kits (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China). Serum D-lactate concentration was analyzed using an ELISA kit (Luyuan Byrd Biotechnology Co., Ltd., Beijing, China). The serum levels of immunoglobulin A (IgA), immunoglobulin G (IgG), and immunoglobulin M (IgM) were measured by assay kits (Leadman Biochemistry Co., Ltd., Beijing, China). The serum contents of interleukin (IL)-1β, IL-6 and tumour necrosis factor (TNF)-α were determined by ELISA kits (Kangjia Hongyuan Biotechnology Co., Ltd., Beijing, China).

2.4. Analysis of microbial community in feces

Total DNA of each fecal sample was extracted using the DNA Kit (Omega Bio-tek, Norcross, GA, USA). Microbial DNA was used as a template to amplify the V3–V4 region of 16S rRNA gene with universal primers 338F (5′-barcode-ACTCCTRCGGGAGGCAGCAG-3′) and 806R (5′-GGACTACCVGGGTATCTAAT-3′). The PCR products were detected by agarose gel electrophoresis and then purified. The purified amplified fragments were pooled and sequenced using the Illumina MiSeq platform (Illumina Inc., San Diego, CA, USA). Operational taxonomic units (OTU) were clustered with a similarity of 97% using UPARSE software. The taxonomy of each 16S rRNA gene sequence was determined by the RDP Classifier (http://rdp.cme.msu.edu/) with confidence greater than 70%. Data processing and analysis were performed using Majorbio cloud platform (Majorbio Bio-pharm Technology Co., Ltd., Shanghai, China).

2.5. Predictive metabolic functions of microbial communities

Predictive metabolic functions of fecal microbial community were estimated by Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) based on 16S rRNA gene sequencing (Langille et al., 2013). Operational taxonomic units were used to predict metabolic functions of fecal microbial communities by referencing the Kyoto Encyclopedia of Genes and Genome (KEGG) orthology database (Kanehisa et al., 2014).

2.6. Measurement of SCFA and BCFA by high performance ion chromatography

The contents of SCFA (acetate, propionate and butyrate) and BCFA (isobutyrate, valerate and isovalerate) in fecal samples were analyzed based on the methods of He et al. (2018). Fecal samples (0.5 g) were dissolved in 8 mL of ultrapure water, sonicated for 30 min, and then centrifuged at 3,000×*g* for 5 min. The collected supernatants were diluted (1:50) using ultrapure water and then filtered into an injection vial through a 0.22 μm membrane. Each sample was detected using a high-performance ion chromatography system (DIONEX ICS-3000, Thermo Fisher, Waltham, MA, USA). The contents of SCFA and BCFA were expressed as mg/g of feces.

2.7. Analysis of ammonia nitrogen in feces

The content of ammonia nitrogen (NH_3–N) was analyzed based on the methods of Chen et al. (2018). In short, fecal samples (0.5 g) were dissolved in 5 mL of ammonia-free water and centrifuged at 5,000×*g* for 15 min. The supernatants (1 mL) were collected and then mixed with 1 mL of potassium sodium tartrate and 19 mL of ammonia-free water in a 50-mL sterile tube, followed by the addition of 1.5 mL of Nessler's reagent. The absorbance was determined at 420 nm against ammonia-free water using a UV–vis Spectrophotometer (MAPADA, Shanghai, China).

2.8. Analysis of biogenic amines in feces

The concentrations of biogenic amines (putrescine, cadaverine and spermine) were analyzed using high performance liquid chromatography according to the methods of Li et al. (2019a) with modification. Briefly, fecal samples (0.5 g) were mixed with 1 mL of trichloroacetic acid and centrifuged at 3,600 × *g* for 10 min. The supernatants were mixed with the same volume of n-hexane and vortexed for 5 min. The extracts were mixed with 20 mL of internal standard, followed by the addition of 1 mL of sodium hydroxide, 1 mL of dansyl chloride, and 1.5 mL of saturated sodium bicarbonate. The mixture was heated at 60 °C for 45 min with occasional shaking, then mixed with 100 μL of ammonia and kept at 40 °C in a water bath under N_2 condition. Finally, the analyzed sample was produced by adding acetonitrile to the residue. Each sample was analyzed by Agilent HPLC 1200 series equipped with a reversed-phase ZORBAX 80 A Extend-C18 column (250 mm × 4.6 mm i.d.; 5 μm particle size, Agilent, Santa Clara, USA). The wavelength, flow rate and column temperature were set at 254 nm, 1.0 mL/min, and 40°C, respectively. The concentrations of biogenic amines were expressed as micrograms per gram of feces.

2.9. Statistical analysis

All data were analyzed by One-Way ANOVA using the GLM procedure of SAS 9.4 (SAS Inst. Inc., Cary, NC), with results presented as mean ± SEM. The χ^2 contingency test was used to analyze differences in the fecal score. Statistical differences among treatments were separated by Student-Neuman-Keul's multiple range tests. Differences in fecal bacterial abundance of piglets at the phylum and family levels were analyzed by the Kruskal–Wallis rank sum test. The abundances of differential bacteria were classified using LEfSe analysis, if LDA score of the fecal microbiota

exceeded 2. Significant differences were defined as $P < 0.05$, and tendency was defined as $0.05 \leq P < 0.10$.

3. Results

3.1. Growth performance and fecal score

As shown in Table 3, ESBM increased ($P < 0.05$) BW of piglets on d 14 and 28 compared with HWP and FSBM. The ADG of piglets in the ESBM group was higher ($P < 0.05$) than HWP and FSBM during d 1 to 14 and 1 to 28, and the ADG of piglets in the ESBM group was higher ($P < 0.05$) than HWP during d 15 to 28. The ADFI of piglets in the HWP group was lower ($P < 0.05$) than FSBM and ESBM. ESBM decreased ($P < 0.05$) F:G of piglets compared with HWP and FSBM during d 1 to 14, and decreased ($P < 0.05$) F:G of piglets compared with FSBM during d 1 to 28. No difference was observed for fecal score among the 3 dietary treatments ($P > 0.05$).

3.2. Nutrient digestibility

Piglets fed with ESBM or HWP had higher ($P < 0.05$) ATTD of CP than those fed with FSBM (Fig. 1). No difference was observed for ATTD of GE among the 3 dietary treatments ($P > 0.05$).

3.3. Serum oxidative status

ESBM increased ($P < 0.05$) FRAP content in serum of piglets compared with HWP and FSBM on d 14 and 28 (Table 4). Serum activity of SOD was highest in piglets fed with ESBM diet on d 14 and 28. No differences were observed for AOPP, MDA, CAT, and GSH-Px among the 3 dietary treatments ($P > 0.05$).

3.4. Serum DAO, endotoxin and D-lactate

ESBM decreased ($P < 0.05$) DAO content in serum of piglets compared with HWP on d 28 (Table 5). No differences were observed for endotoxin and D-lactate among the 3 dietary treatments ($P > 0.05$).

Table 3
Effects of dietary protein sources on growth performance in piglets.[1]

Item	HWP	FSBM	ESBM	*P*-value
Initial BW, kg	7.75 ± 0.42	7.76 ± 0.42	7.76 ± 0.42	0.157
Day 14 BW, kg	11.18 ± 0.54^{b}	11.40 ± 0.54^{b}	11.75 ± 0.60^{a}	0.002
Day 28 BW, kg	16.77 ± 0.67^{b}	17.20 ± 0.86^{b}	18.19 ± 0.84^{a}	0.001
Day 1 to 14				
ADG, g/d	244.75 ± 9.66^{b}	259.77 ± 10.31^{b}	285.97 ± 13.64^{a}	0.002
ADFI, g/d	433.96 ± 19.74^{b}	456.88 ± 23.35^{a}	461.67 ± 25.88^{a}	0.025
F:G	1.78 ± 0.05^{a}	1.76 ± 0.04^{a}	1.61 ± 0.03^{b}	0.030
Fecal score	2.32 ± 0.10	2.37 ± 0.06	2.29 ± 0.07	0.732
Day 15 to 28				
ADG, g/d	399.66 ± 16.72^{b}	416.25 ± 24.92^{ab}	459.66 ± 20.28^{a}	0.047
ADFI, g/d	666.64 ± 41.37^{b}	731.96 ± 47.79^{a}	756.73 ± 37.07^{a}	0.023
F:G	1.67 ± 0.07	1.76 ± 0.03	1.65 ± 0.03	0.170
Fecal score	2.24 ± 0.03	2.20 ± 0.05	2.24 ± 0.15	0.958
Day 1 to 28				
ADG, g/d	322.20 ± 11.07^{b}	337.54 ± 15.93^{b}	372.81 ± 16.01^{a}	0.001
ADFI, g/d	550.30 ± 30.02^{b}	594.42 ± 34.57^{a}	609.20 ± 30.78^{a}	0.006
F:G	1.70 ± 0.05^{ab}	1.76 ± 0.03^{a}	1.63 ± 0.02^{b}	0.009
Fecal score	2.28 ± 0.05	2.29 ± 0.02	2.26 ± 0.08	0.958

HWP = hydrolyzed wheat protein; FSBM = fermented soybean meal; ESBM = enzyme-treated soybean meal; BW = body weight; ADG = average daily gain; ADFI = average daily feed intake; F:G = feed-to-gain ratio.
[a, b] Within a row, means without a common superscript differ at $P < 0.05$.
[1] Values are given as means ± SEM, $n = 6$.

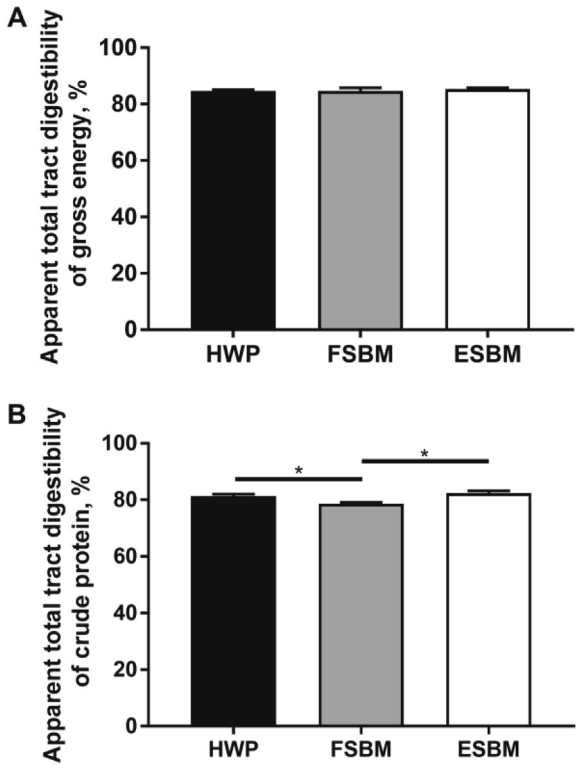

Fig. 1. Effects of dietary protein sources on apparent total tract digestibility of nutrients in piglets. (A) Gross energy. (B) Crude protein. HWP = hydrolyzed wheat protein; FSBM = fermented soybean meal; ESBM = enzyme-treated soybean meal. Values are given as means ± SEM, $n = 6$. * represents significant difference ($P < 0.05$).

Table 4
Effects of dietary protein sources on oxidative status in serum of piglets.[1]

Item	HWP	FSBM	ESBM	*P*-value
Day 14				
AOPP, pmol/L	91.93 ± 3.37	85.94 ± 4.60	90.28 ± 1.93	0.413
FRAP, mmol/L	0.27 ± 0.01^{b}	0.26 ± 0.01^{b}	0.30 ± 0.01^{a}	0.001
MDA, nmol/mL	3.29 ± 0.10	3.08 ± 0.23	2.71 ± 0.13	0.094
SOD, U/mL	127.19 ± 2.80^{b}	132.59 ± 4.25^{b}	142.89 ± 2.28^{a}	0.017
CAT, U/mL	4.32 ± 0.31	4.47 ± 0.31	4.61 ± 0.20	0.746
GSH-Px, U/mL	300.53 ± 13.29	309.12 ± 14.49	307.72 ± 17.08	0.930
Day 28				
AOPP, pmol/L	79.95 ± 6.66	78.55 ± 3.60	82.65 ± 8.90	0.246
FRAP, mmol/L	0.24 ± 0.01^{b}	0.24 ± 0.01^{b}	0.27 ± 0.01^{a}	0.013
MDA, nmol/mL	2.91 ± 0.15	3.13 ± 0.09	2.72 ± 0.15	0.211
SOD, U/mL	138.87 ± 2.71^{ab}	133.67 ± 4.25^{b}	145.64 ± 2.28^{a}	0.039
CAT, U/mL	4.48 ± 0.37	4.42 ± 0.30	4.70 ± 0.36	0.838
GSH-Px, U/mL	303.16 ± 14.65	262.46 ± 22.45	290.53 ± 10.28	0.283

HWP = hydrolyzed wheat protein; FSBM = fermented soybean meal; ESBM = enzyme-treated soybean meal; AOPP = advanced oxidation protein products; FRAP = ferric reducing ability of plasma; MDA = malondialdehyde; SOD = total superoxide dismutase; CAT = catalase; GSH-Px = glutathione peroxidase.
[a, b] Within a row, means without a common superscript differ at $P < 0.05$.
[1] Values are given as means ± SEM, $n = 6$.

3.5. Concentrations of inflammatory cytokines and immunoglobulins in serum

As shown in Table 6, ESBM tended to decrease concentrations of IL-1β ($P = 0.060$) and IL-6 ($P = 0.070$) in serum of piglets on d 14. ESBM decreased ($P < 0.05$) IL-1β level in serum of piglets compared with HWP on d 28. No differences were observed for IgA, IgG or IgM among the 3 dietary treatments ($P > 0.05$).

Table 5
Effects of dietary protein sources on levels of DAO, endotoxin and D-lactate in serum of piglets.[1]

Item	HWP	FSBM	ESBM	*P*-value
Day 14				
DAO, U/L	17.91 ± 0.50	17.56 ± 0.76	16.57 ± 0.57	0.257
Endotoxin, EU/mL	15.68 ± 1.06	16.89 ± 1.27	16.01 ± 1.01	0.704
D-lactate, μmol/L	163.52 ± 7.70	164.50 ± 10.38	174.19 ± 9.44	0.621
Day 28				
DAO, U/L	14.50 ± 0.37[a]	13.23 ± 0.59[ab]	12.75 ± 0.42[b]	0.044
Endotoxin, EU/mL	12.74 ± 0.52	12.69 ± 0.49	12.83 ± 0.56	0.983
D-lactate, μmol/L	218.57 ± 16.89	223.37 ± 13.25	211.59 ± 11.21	0.731

HWP = hydrolyzed wheat protein; FSBM = fermented soybean meal; ESBM = enzyme-treated soybean meal; DAO = diamine oxidase.
[a, b] Within a row, means without a common superscript differ at $P < 0.05$.
[1] Values are given as means ± SEM, $n = 6$.

Table 6
Effects of dietary protein sources on inflammatory cytokines and immunoglobulins in serum of piglets.[1]

Item	HWP	FSBM	ESBM	*P*-value
Day 14				
IL-1β, pg/mL	17.58 ± 0.53	17.57 ± 1.05	14.94 ± 0.20	0.060
IL-6, pg/mL	107.15 ± 4.09	102.29 ± 3.56	92.51 ± 3.52	0.070
TNF-α, pg/mL	77.10 ± 6.63	69.57 ± 6.76	71.37 ± 3.49	0.699
IgA, g/L	1.05 ± 0.08	1.11 ± 0.05	1.19 ± 0.08	0.461
IgG, g/L	7.20 ± 0.61	7.11 ± 0.44	7.70 ± 0.76	0.817
IgM, g/L	0.58 ± 0.03	0.69 ± 0.07	0.70 ± 0.05	0.286
Day 28				
IL-1β, pg/mL	20.20 ± 0.54[a]	18.86 ± 0.91[ab]	16.45 ± 0.87[b]	0.027
IL-6, pg/mL	86.33 ± 7.34	95.23 ± 6.06	84.96 ± 6.48	0.624
TNF-α, pg/mL	77.91 ± 5.42	74.91 ± 3.62	71.52 ± 5.27	0.694
IgA, g/L	1.21 ± 0.12	1.22 ± 0.08	1.16 ± 0.10	0.775
IgG, g/L	9.52 ± 0.59	9.92 ± 0.29	10.30 ± 0.58	0.474
IgM, g/L	0.71 ± 0.07	0.77 ± 0.05	0.83 ± 0.05	0.409

HWP = hydrolyzed wheat protein; FSBM = fermented soybean meal; ESBM = enzyme-treated soybean meal; IL-1β = interleukin-1β; IL-6 = interleukin-6; TNF-α = tumour necrosis factor-α; IgA = immunoglobulin A; IgG = immunoglobulin G; IgM = immunoglobulin M.
[a, b] Within a row, means without a common superscript differ at $P < 0.05$.
[1] Values are given as means ± SEM, $n = 6$.

3.6. Fecal bacterial community

The OTU Venn analysis identified 47, 34 and 63 unique OTU in the HWP, FSBM and ESBM groups, respectively (Fig. 2A). The Shannon index was higher ($P < 0.05$) in fecal samples of the ESBM group compared with the HWP and FSBM groups, and the Simpson index was decreased ($P < 0.05$) in fecal samples of the ESBM group compared with the FSBM group (Fig. 2B). Principal component analysis (PCA) showed that greater variations were detected in fecal samples of the ESBM group compared with the HWP and FSBM groups, and the HWP and FSBM groups had similar microbial communities (Fig. 3A). The UPGMA tree showed significant differences in the structure of fecal microbiota among the 3 dietary treatments, indicating that the protein sources had important impacts on fecal microbial communities (Fig. 3B).

At the phylum level, Firmicutes and Bacteroidetes were the dominant bacteria, which accounted for 90% (Fig. 4A). At the family level, the predominant families within the Firmicutes phylum consisted of Christensenellaceae, Clostridiaceae, Erysipelotrichaceae, Lachnospiraceae, Lactobacillaceae, Oscillospiraceae, Peptostreptococcaceae, and Streptococcaceae, while Muribaculaceae was the dominant family in the Bacteroidetes phylum (Fig. 4B). The abundance of Bacteroidetes in fecal samples of the ESBM group was increased ($P < 0.05$) compared with HWP and FSBM (Fig. 5A). The relative abundance of Clostridiaceae in fecal samples of the ESBM group was decreased ($P < 0.05$) compared with HWP and FSBM, and piglets in the ESBM group had higher ($P < 0.05$) abundances of Oscillospiraceae and Christensenellaceae than HWP and FSBM (Fig. 5B).

LEfSe analysis was performed to identify different bacteria that were specific for piglets among the 3 dietary treatments (Fig. 6). The

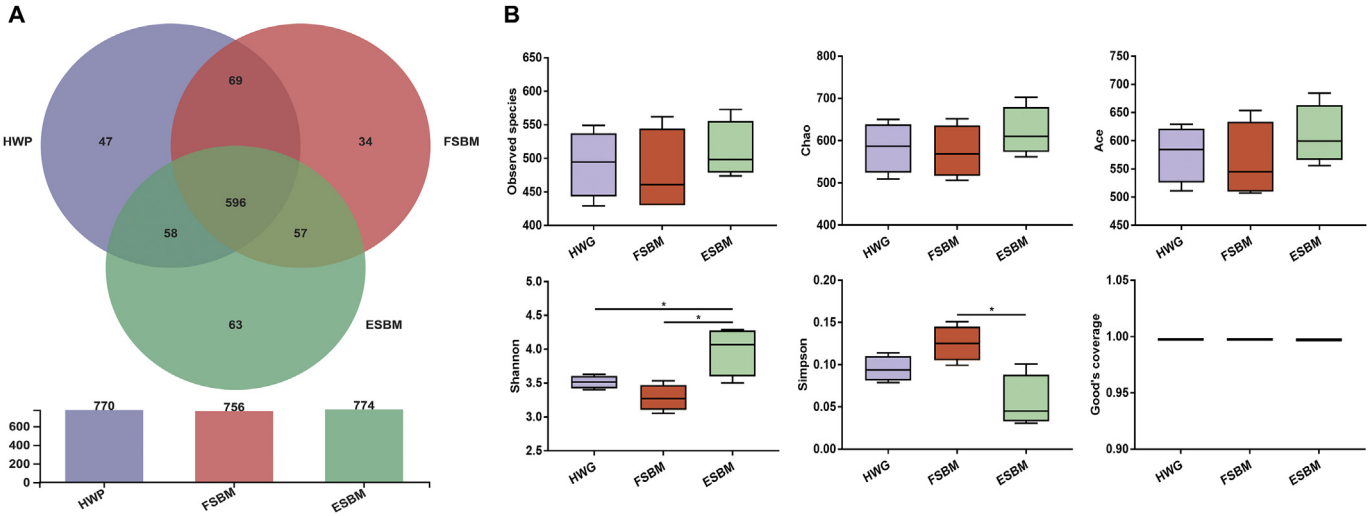

Fig. 2. Fecal microbiota richness and diversity. (A) OTU Venn of 3 dietary treatments. (B) Comparison of α-diversity indices among 3 dietary treatments. HWP = hydrolyzed wheat protein; FSBM = fermented soybean meal; ESBM = enzyme-treated soybean meal. Values are given as means ± SEM, $n = 4$. * represents significant difference ($P < 0.05$).

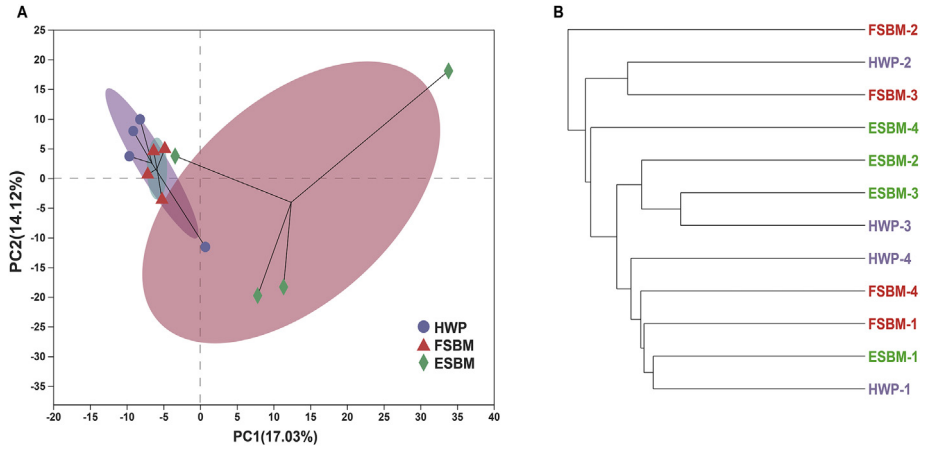

Fig. 3. Comparison of fecal microbiota structure by β-diversity based on the OTU level. (A) Principal component analysis (PCA). (B) UPGMA tree. HWP = hydrolyzed wheat protein; FSBM = fermented soybean meal; ESBM = enzyme-treated soybean meal. $n = 4$.

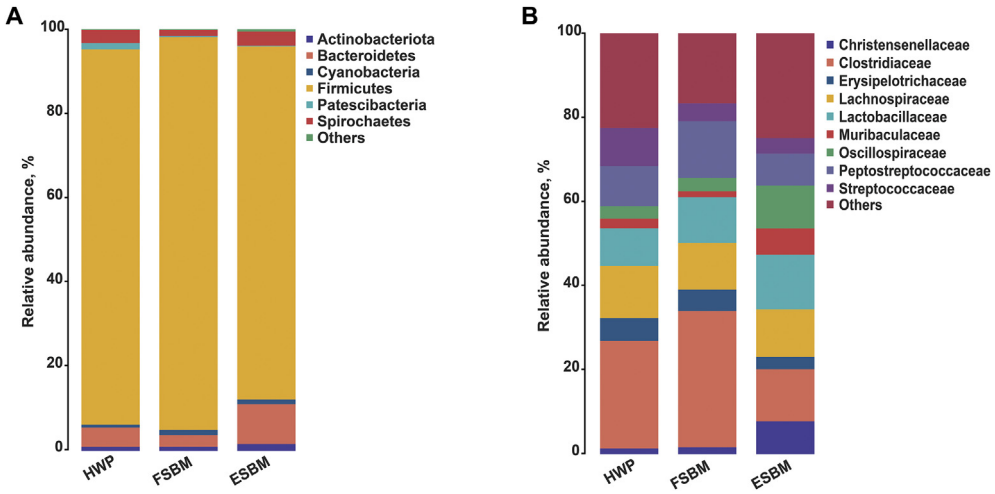

Fig. 4. Relative abundance of fecal microbiota at the (A) phylum and (B) family levels. HWP = hydrolyzed wheat protein; FSBM = fermented soybean meal; ESBM = enzyme-treated soybean meal. $n = 4$.

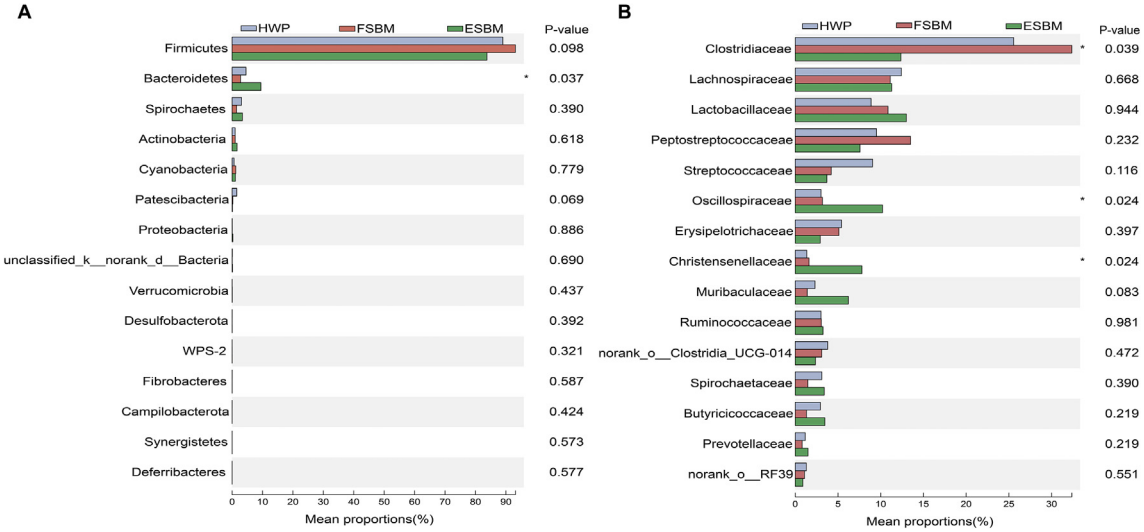

Fig. 5. Differences in the fecal microbiota compositions at the (A) phylum and (B) family levels based on a contribution degree at top 15. HWP = hydrolyzed wheat protein; FSBM = fermented soybean meal; ESBM = enzyme-treated soybean meal. $n = 4$. * represents significant difference ($P < 0.05$).

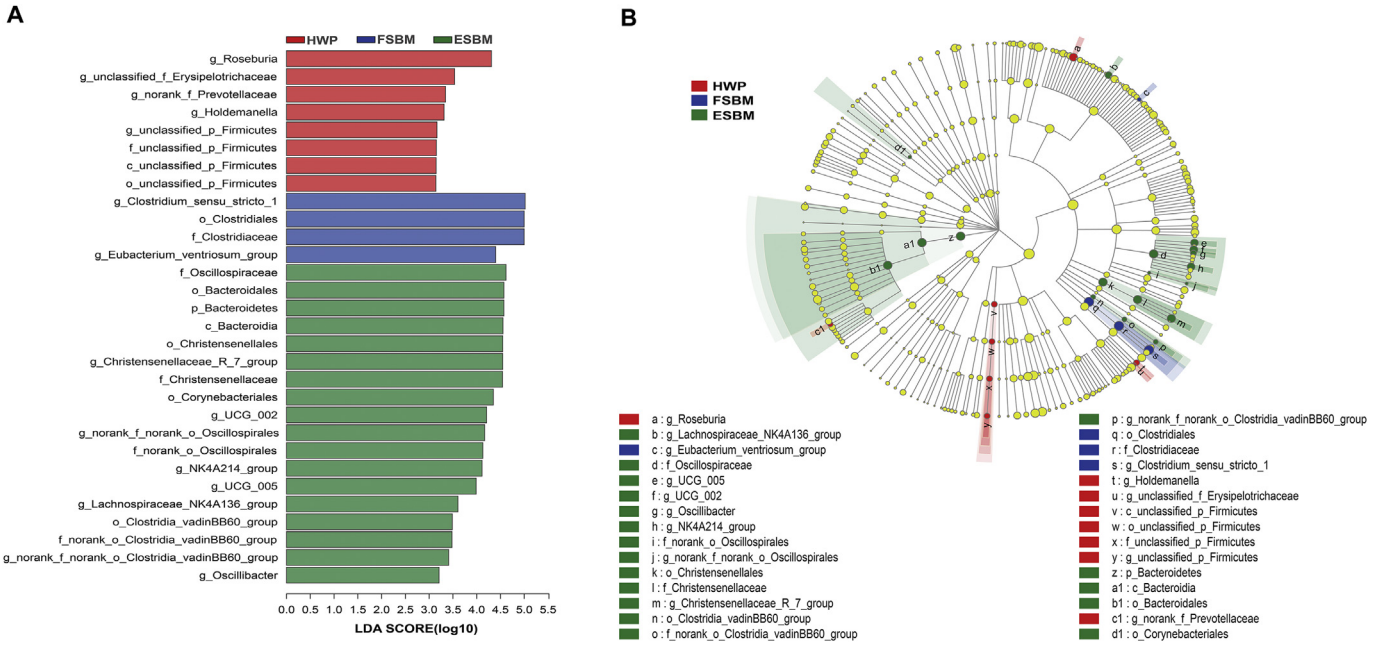

Fig. 6. LefSE analysis of fecal microbiota among 3 dietary treatments. (A) Linear discriminant analysis (LDA) score of the fecal microbiota, and the score ≥2 means significant. (B) Cladogram of LEfSe shows taxonomic profiling from the phylum to genus level, the yellow node represents no difference, but other color nodes represent significant difference. HWP = hydrolyzed wheat protein; FSBM = fermented soybean meal; ESBM = enzyme-treated soybean meal. $n = 4$.

results showed 30 different OTU among treatments (Fig. 6A). Among the different OTU, 8 of these OTU were characteristic for the HWP group, 4 of these OTU were characteristic for the FSBM group, and 18 of these OTU were characteristic for the ESBM group. A great abundance of *Roseburia*, *Holdemanella*, *unclassified_f_Erysipelotrichaceae*, *unclassified_p_Firmicutes*, and *norank_f_Prevotrllaceae* in the HWP group, *Eubacterium_ventriosum_group*, Clostridiales, Clostridiaceae, and *Clostridium_sensu_stricto_1* in the FSBM group, Lachnospiraceae_*NK4A136_group*, Oscillospiraceae, *UCG_005*, *UCG_002*, *Oscillibacter*, *NK4A214_group*, norank_o_Oscillospirales, Christensenellales, Christensenellaceae, Christensenellaceae_*R_7_group*, Clostridia_vaclinBB60_group, norank_o_Clostridia_vadinBB60_group, *norank_f_ norank_o_Clostridia_vadinBB60_group*, Bacteroidetes, Bacteroidia, Bacteroidales, and Corynebacteriales in the ESBM group were observed (Fig. 6B).

3.7. Prediction on amino acid metabolism of bacterial communities using PICRUSt

Ten pathways associated with amino acid metabolism are shown in Fig. 7. Piglets in the ESBM group had lower numbers of the genes associated with valine, leucine, isoleucine and lysine degradation than HWP and FSBM. No differences were observed for other pathways associated with amino acid metabolism among the 3 dietary treatments ($P > 0.05$).

3.8. Fermentation metabolites of fecal samples

The contents of SCFA and BCFA in fecal samples of piglets among the 3 dietary treatments are shown in Table 7. Butyrate content in fecal samples of the ESBM group was increased ($P < 0.05$) compared with FSBM. As shown in Table 8, no difference was observed for NH_3–N among all dietary treatments ($P > 0.05$). Cadaverine content in fecal samples of the ESBM group tended to be lower ($P = 0.076$) than other groups.

4. Discussion

4.1. Growth performance and nutrient digestibility

In the pig industry, plant protein sources such as HWP, FSBM and ESBM have been widely used in the diets of weaned piglets to improve performance (Wang et al., 2011; Han et al., 2017; Ma et al., 2019b). In this experiment, we found that piglets fed with ESBM had higher ADG than that of piglets fed with HWP and FSBM, indicating that piglets in the ESBM group had a higher growth rate compared with other diets. However, there were different conditions for lower ADG in piglets fed with HWP or FSBM compared with ESBM. Our result showed that no difference was observed for F:G between HWP and ESBM groups, but piglets fed with HWP had lower ADFI than that of piglets fed with ESBM during the whole period, indicating that lower ADFI in HWP may contribute to lower ADG in HWP compared with ESBM group. The decrease in ADFI of HWP group may be related to lower palatability compared with ESBM, which needs to be further explored. In addition, no difference was observed for ADFI between the FSBM and ESBM groups, but piglets fed with FSBM had higher F:G than that of piglets fed with ESBM, suggesting that the FSBM group had lower ADG than the ESBM group. Enzymatic hydrolysis of SBM could increase the proportions of multiple small peptides alongside the depression of most antinutritional factors (Zhou et al., 2011), which has great potential to improve the efficiency of growth in weaned piglets. However, Ma et al. (2019a) showed that no significant difference was observed for F:G between the FSBM and ESBM groups. The different experimental conditions, diet compositions, and inclusion levels may explain the inconsistency. The digestion and absorption of dietary nutrients in the gastrointestinal tract provide animals

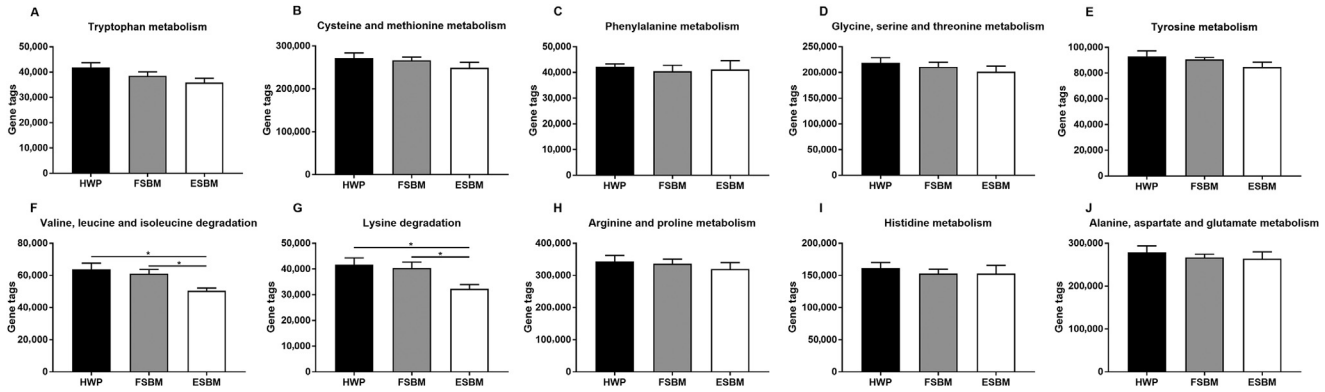

Fig. 7. Prediction on amino acid metabolism of fecal microbiota using PICRUSt. HWP = hydrolyzed wheat protein; FSBM = fermented soybean meal; ESBM = enzyme-treated soybean meal. PICRUSt = Phylogenetic Investigation of Communities by Reconstruction of Unobserved States. Values are given as means ± SEM, $n = 4$. * represents significant difference ($P < 0.05$).

Table 7
Effects of dietary protein sources on concentrations of SCFA and BCFA in feces of piglets (mg/g).[1]

Item	HWP	FSBM	ESBM	*P*-value
Acetate	5.68 ± 0.42	5.27 ± 0.38	5.74 ± 0.36	0.625
Propionate	3.19 ± 0.29	2.68 ± 0.24	2.95 ± 0.15	0.359
Butyrate	1.71 ± 0.10[ab]	1.59 ± 0.07[b]	1.94 ± 0.10[a]	0.029
SCFA	10.59 ± 0.74	9.54 ± 0.59	10.63 ± 0.52	0.375
Isobutyrate	0.37 ± 0.03	0.30 ± 0.04	0.37 ± 0.03	0.247
Isovalerate	0.39 ± 0.04	0.32 ± 0.05	0.37 ± 0.03	0.559
Valerate	0.58 ± 0.05	0.51 ± 0.09	0.49 ± 0.08	0.702
BCFA	1.35 ± 0.06	1.13 ± 0.17	1.23 ± 0.14	0.577
Total SCFA	11.93 ± 0.78	10.67 ± 0.71	11.85 ± 0.64	0.342

HWP = hydrolyzed wheat protein; FSBM = fermented soybean meal; ESBM = enzyme-treated soybean meal; SCFA = short-chain fatty acids; BCFA = branched-chain fatty acids.
[a, b] Within a row, means without a common superscript differ at $P < 0.05$.
[1] Values are given as means ± SEM, $n = 6$.

Table 8
Effects of dietary protein sources on concentrations of NH_3–N and biogenic amine in feces of piglets (μg/g).[1]

Item	HWP	FSBM	ESBM	*P*-value
NH_3–N	151.56 ± 15.10	154.35 ± 10.93	162.49 ± 11.14	0.827
Putrescine	3.33 ± 0.28	3.19 ± 0.50	2.10 ± 0.48	0.267
Cadaverine	3.08 ± 0.43	3.21 ± 0.46	2.01 ± 0.26	0.076
Spermine	0.25 ± 0.02	0.19 ± 0.05	0.27 ± 0.03	0.406

HWP = hydrolyzed wheat protein; FSBM = fermented soybean meal; ESBM = enzyme-treated soybean meal.
[1] Values are given as means ± SEM, $n = 6$.

with energy and amino acids for maintenance and growth via biological oxidation and metabolism (Chwalibog et al., 2004). In this study, piglets fed with FSBM had higher ADFI than that of piglets fed with HWP. However, no difference was observed for ADG between the HWP and FSBM groups. Interestingly, piglets fed with HWP had greater ATTD of CP than FSBM, which may explain why there was no difference for ADG between the HWP and FSBM groups.

4.2. Serum oxidative status

Weaning can induce oxidative stress (Ma et al., 2019a), which is an imbalance process between the production of endogenous reactive oxygen species and their elimination by the antioxidant system (Bhat et al., 2015). Serum antioxidant capacity could reflect the host's resistance to endogenous oxidative injury, and a higher antioxidant capacity has beneficial effects on alleviating oxidative stress (Wang et al., 2018a,b). Diets alter endogenous antioxidant capacity by adjusting the ability to scavenge reactive oxygen species (Seifried et al., 2007). Protein source is the only variable in the present study. In order to determine the differential effects of HWP, FSBM and ESBM on antioxidant capacity in weaned piglets, we analyzed serum oxidative status of weaned piglets among 3 dietary treatments. Ferric reducing ability of plasma is considered as the total antioxidant power of plasma, which reflects the combined antioxidant status of exogenous and endogenous non-enzymatic compounds in biological fluids (Benzie and Strain, 1996). In this study, piglets fed with ESBM had higher serum FRAP level than that of piglets fed with HWP or FSBM, indicating that ESBM had an advantage over HWP and FSBM in improving non-enzymatic antioxidant defense. Weaning stress could reduce activities of GSH-Px and SOD, as well as increase contents of hydrogen peroxide (H_2O_2) and MDA in serum of piglets (Zhu et al., 2012). Superoxide dismutase is considered as the first line of defense against excessive oxidative radicals and could catalyze the transformation of superoxide radicals into H_2O_2, which is decomposed into H_2O and O_2 by GSH-Px and CAT (Bai et al., 2018). In our study, ESBM increased SOD activity in serum of piglets compared with HWP or FSBM, suggesting that ESBM had an advantage over HWP and FSBM in improving enzymatic antioxidant defenses. The possible reason is that different protein sources have greatly different amino acid compositions and bioactive substances including small peptides and enzymes (Zhou et al., 2011; Shi et al., 2018), which may affect antioxidant capacity in weaned piglets. Collectively, ESBM could have a higher ability to improve antioxidant capacity by activating nonenzymatic and enzymatic antioxidant defense components compared with HWP or FSBM, and is more suitable to be used as a protein source for piglets in order to prevent oxidative stress induced by the weaning process.

4.3. Biomarkers of intestinal permeability in serum and inflammatory status

The intestinal epithelial barrier is primarily composed of a layer of enterocytes and intercellular multiprotein complexes that could prevent the flow of harmful substances such as toxins, antigens, and pathogens from the intestinal lumen into the circulating system (Li et al., 2019b; Shang et al., 2019). Weaning stress can induce

intestinal injury, which is related to higher intestinal permeability, resulting in translocation of pathogens through the intestinal epithelial cells into systemic circulation (Li et al., 2019b). DAO, endotoxin and D-lactate are biomarkers associated with intestinal permeability and their serum levels could reflect the integrity of the intestinal barrier (Xiong et al., 2019). In this study, we found that ESBM decreased serum DAO concentration on d 28 compared with HWP. This result indicated that piglets in the ESBM group had an advantage in decreasing intestinal permeability and enhancing intestinal barrier function over HWP, and this is one of the potential reasons for higher growth performance of piglets fed with ESBM than piglets fed with HWP. Weaning stress could also induce intestinal inflammation and the production of pro-inflammatory cytokines, which results in growth retardation and intestinal disorders in piglets (Bomba et al., 2014). Pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α participate in the regulation of inflammatory response and their levels could reflect the inflammatory status in the body (Xiong et al., 2019). Our results showed that ESBM decreased the contents of IL-1β and IL-6 in the serum of piglets compared with HWP and FSBM, indicating that ESBM was superior to HWP and FSBM in alleviating weaning stress-induced inflammatory response. This may be because differential impacts of dietary protein are associated with its sources and different bioprocessing techniques, which was supported by Ma et al. (2019a).

4.4. Fecal bacterial community

Diets provide available substrates including carbohydrates and protein for the intestinal microbiota, and influence microbial structure and metabolism, which could promote growth performance and intestinal health of piglets (Li et al., 2019a). Normally, dietary nutrients are digested and absorbed in the foregut, then the undigested ingredients and endogenous substances are fermented by intestinal microbiota in the hindgut (Zhu et al., 2017; Li et al., 2019b). Carbohydrate fermentation is beneficial to intestinal epithelial cells due to the production of SCFA (Wong et al., 2006; Li et al., 2019a), while protein fermentation produces potentially toxic metabolites including ammonia, biogenic amines, and aromatic compounds that have adverse impacts on intestinal health (Li et al., 2019a). The α-diversity could be used as an indicator of the functional resilience of the gut microbial ecosystem, including species richness (Observed species, Chao, and Ace) and species diversity (Shannon, Simpson, and Good's coverage) (Zhang et al., 2020). In this study, no changes were observed for species richness of the fecal samples in piglets fed with HWP, FSBM, and ESBM. However, ESBM increased Shannon index in the fecal samples of piglets compared with HWP and FSBM, and ESBM decreased Simpson index in the fecal samples of piglets compared with FSBM, revealing that ESBM had an advantage over HWP and FSBM in improving fecal bacterial diversity and maintaining gut immune homeostasis in weaned piglets. The β-diversity by PCA and UPGMA tree analysis showed that fecal microbiota responded differently to dietary protein sources. The reason for greater variations in the fecal microbiota structure of ESBM compared with other groups was unclear, but the fecal microbiota structure of HWP and FSBM was similar, which may partly explain the similar growth performance of piglets. At the phylum level, Firmicutes and Bacteroidetes were the main species in fecal samples, which is consistent with the results conducted by Zhao et al. (2018). In this study, ESBM markedly enhanced Bacteroidetes abundance in fecal samples of piglets compared with HWP and FSBM. The phylum Bacteroidetes are considered as important bacteria for degrading plant polysaccharides and other recalcitrant organic carbon and nitrogen sources (Yu et al., 2017). The higher abundance of Bacteroidetes are beneficial to shape the metabolic environment of the gut ecosystem and prevent diarrhea because they could participate in the regulation of immune system in the intestine, indicating the relationship between early bacterial colonization and immune maturation after weaning (Jakobsson et al., 2014). Although no difference was observed for fecal score among different protein sources, the higher abundance of Bacteroidetes in piglets fed with ESBM could have contributed to an improvement in the intestinal immunity and further promoted growth performance of piglets. Down to the family level, ESBM decreased Clostridiaceae abundance and increased relative abundances of Oscillospiraceae and Christensenellaceae in fecal samples compared with HWP and FSBM. These findings were confirmed by LEfSe analysis, which can identify unique high-dimensional biomarkers for analyzed microbial communities (Segata et al., 2011). Certain bacterial species, from the family Clostridiaceae, were implicated in poor performance (Li et al., 2019a). In addition, *Clostridium perfringens*, belonging to the family Clostridiaceae, does not necessarily harm the health of the pig, but may induce zoonotic diseases if excreted in the form of feces (Baer et al., 2013). In previous studies, Oscillospiraceae and Christensenellaceae are potentially beneficial microorganisms and promote the production of secondary bile acids that are known to protect against infection with *Clostridium difficile* (Konikoff and Gophna, 2016; Chernevskaya et al., 2020). Butyrate is considered to be an important source of energy for colonocytes and could promote the proliferation and differentiation of intestinal epithelium (Morrison and Preston, 2016). Moreover, Christensenellaceae is butyric acid-producing bacteria and has a beneficial effect on maintaining intestine structure and function (Shang et al., 2019), which could partly explain the higher butyrate content in fecal samples of piglets fed with ESBM. Collectively, ESBM had an advantage on modulating the intestinal microbial community over HWP and FSBM such as an increase in the abundance of beneficial bacteria and a decrease in the abundance of potential pathogenic bacteria, which may explain the higher growth rate of piglets fed with ESBM. However, the underlying mechanism behind this process needs further research.

4.5. Fermentation metabolites in feces

Short-chain fatty acids are derived from the fermentation of dietary carbohydrates and protein, and BCFA are derived from the deamination of valine, leucine, and isoleucine (Fan et al., 2017). In this study, no difference was observed for BCFA among all dietary treatments. However, the PICRUSt program showed that the number of gene tags associated with the degradation of valine, leucine, and isoleucine in the ESBM group were significantly lower than the HWP and FSBM groups. In this study, the reason for this result may be that the gene tags are primarily related to decarboxylation, and it is necessary to further investigate the relationship between dietary protein sources and valine, leucine, and isoleucine degradation. The microbial fermentation of amino acids in the hindgut generates ammonia, biogenic amines, indoles, and phenols, which could induce an increase of pathogenic microorganisms and impair growth performance of pigs (Rist et al., 2013; Portune et al., 2016). The protein restriction in diets supplemented with crystalline amino acids inhibits formation of harmful substances in the large intestine without affecting the growth of pigs (Wang et al., 2018a,b). NH_3—N is a toxic metabolite produced by the deamination of amino acids, which can interfere with metabolism of epithelial cells and impair intestinal health of pigs (Chen et al., 2018; Li et al., 2019a). Our results showed that no difference was observed for NH_3—N content in the fecal samples among all dietary treatments, indicating that microbial deamination of dietary and endogenous protein was not altered by the addition of HWP, FSBM,

and ESBM. However, ESBM tended to decrease fecal cadaverine content compared with HWP and FSBM, which revealed a decline in protein fermentation. Cadaverine is produced by microorganisms through the decarboxylation of lysine in the large intestinal lumen (Davila et al., 2013; Luo et al., 2015). The PICRUSt analysis revealed that ESBM reduced the number of gene tags related to lysine degradation compared with HWP and FSBM, which may contribute to explaining the decreased fecal cadaverine content of piglets fed with ESBM.

5. Conclusions

Different protein sources could differentially modulate antioxidant capacity, immune function, and fecal bacterial community and metabolites of weaned piglets. ESBM had advantages over HWP and FSBM in promoting performance of weaned piglets, which was mainly reflected by the increased ADG. ESBM improved antioxidant status and immunity via increased serum levels of FRAP and SOD and decreased serum concentrations of DAO and IL-1β compared with HWP and FSBM. Moreover, ESBM modulated fecal microbiota composition and metabolites including a lower abundance of Clostridiaceae and higher abundances of Bacteroidetes, Oscillospiraceae and Christensenellaceae, and enhanced fecal butyrate content and reduced protein fermentation compared with HWP and FSBM.

Author contributions

Lianhua Zhang: Investigation, Data curation, Formal analysis, Writing – original draft preparation, Conceptualization, Methodology, Software, Writing – review & editing. **Xiangshu Piao**: Conceptualization, Methodology, Writing – review & editing, Supervision.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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