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Original Research Article

Dietary crude protein time-dependently modulates the bacterial community and metabolites and changes dietary nutrient efficiency in growing pigs

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ABSTRACT

The reduced nutrient digestibility of low-protein (LP) diets has been shown to be caused by the weakened fermentative capacity of the post-gut flora. The dynamic regulation of dietary protein contents on post-gut microbial population and fermentative metabolism is unclear. Twelve growing barrows (19.9 ± 0.8 kg) fitted with a T-cannula at the blind end of the cecum were randomly administered a high-protein (HP, 21.5% crude protein [CP]) diet or an LP (15.5% CP) diet for 28 d. The cecal content and feces were collected at d 1, 14, and 28 of the experiment for microflora structures and metabolite concentrations analysis. The nutrient digestibility coefficient and plasma biochemical parameters were also determined. Compared with the HP treatment, the LP treatment showed decreased plasma urea nitrogen concentration and apparent total tract digestibility of dry matter, gross energy, and CP ($P < 0.01$). In addition, urinary nitrogen losses, total nitrogen losses, and daily nitrogen retention in the LP treatment were lower than those in the HP treatment ($P < 0.01$), and the nitrogen retention-to-nitrogen intake ratio in the LP treatment was increased ($P < 0.01$). The HP group showed increased cecal total short-chain fatty acids (SCFA) concentration and fecal propionate, butyrate, and total SCFA concentrations ($P < 0.05$) on d 14 and 28, which may be mainly related to the elevated abundance of SCFA-producing bacteria, such as *Ruminococcus*, *Lactobacillus*, and *Prevotella* ($P < 0.05$). Probiotics, such as *Bifidobacterium*, *Bacteroidales S24-7*, and *Rikenella*, enriched in the LP treatment possibly contributed to reduced plasma endotoxin content. The differences in the abundances of almost all the above-mentioned flora appeared on d 28 but not d 14. Likewise, differences in the Simpson and Shannon indices and clustering patterns of the microbiota between treatments were also only observed on d 28. To sum up, in a time-dependent manner, the LP diet increased probiotics with gut-improving functions and decreased SCFA-producing bacteria, which may cause enhanced intestine health and reduced nutrient digestibility.

1. Introduction

With crystalline amino acid supplementation, low-protein (LP) diets can satisfy the demand of livestock and poultry for amino acids while saving feed costs and decreasing nitrogen excretion (Wang et al., 2018). These attributes facilitate efficient husbandry. However, the growth performance of pigs fed LP diets can be impaired even when limiting amino acid requirements are met (Powell et al., 2011) which may result from reduced nutrient utilization (Wang et al., 2020). Firstly, decreased protein intake

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reduces stimulation of digestive enzyme secretion, leading to impaired chemical digestion (He et al., 2016). Moreover, the lessened amount of nitrogen entering the hindgut hinders the growth and proliferation of microbes and then suppresses the fermentative digestion of nutrients by microorganisms (Diether and Willing, 2019).

Our group previously suggested that reduced dietary crude protein (CP) content does not negatively impact the digestion of nutrients in the foregut of pigs (Wang et al., 2020). It can elucidate that more indigestible protein content from the small intestine transports into the large intestine in pigs fed high-protein (HP) diets compared to LP diets. According to the results of Sung et al. (2023), the direction of microbiota shift induced by the high indigestible protein may reduce the fiber utilization in the hindgut. Therefore, dietary protein content can significantly modulate the microflora (Le Sciellour et al., 2018), and consequently change the fermentative digestion of nutrients by microorganisms.

Previous researchers have investigated extensively the influence of dietary CP content on microflora in the small intestine, large intestine, and feces (Fan et al., 2015; Duarte and Kim, 2022). However, in these studies, samples from the foregut and hindgut were collected at slaughter. Due to cost and animal welfare, it is difficult to sample at different experimental time points to explore the dynamics of bacterial flora. Additionally, although feces can be collected continuously, fecal microbes may not be representative of hindgut microbes. The colon and cecum are the primary sites for microbial fermentation, and microbial populations functional characteristics of microbes in the cecum and colon are similar (Gresse et al., 2019). Therefore, the objective of this study was to test the hypothesis that dietary protein content dynamically regulates the microbiota community and then changes the nutrients metabolism in the large intestine of pigs. Pigs fitted with T-cannulas at the blind end of cecum were used as the animal model in this study.

2. Materials and methods

2.1. Animal ethics statement

All experimental supplies and pigs were offered by the FengNing Swine Research Unit of China Agricultural University (Chengdejiuyun Agricultural and Livestock Co., Ltd., Hebei, China). Animal experiments were permitted by the China Agricultural University Animal Care and Use Committee (Beijing, AW11102202-1-1), and this work was performed in accordance with the ARRIVE guidelines.

2.2. Experimental design

Twelve crossbred (Duroc × Landrace × Yorkshire) barrows (19.9 ± 0.8 kg BW) fitted with a T-cannula at the blind end of the cecum randomly consumed 1 of 2 treatment diets to provide 6 observations per treatment. Dietary treatments were a HP diet and a LP diet. Experimental diets were formulated to satisfy the nutrient requirement of pigs outlined by National Research Council (NRC, 2012, Table 1).

Barrows were housed individually in stainless steel holding crates (1.4 m × 0.7 m × 0.6 m). Daily feed supplied to each pig was divided equally into 2 meals offered at 09:00 and 16:00. Daily feed intake of each pig was restricted to be 2.8 times greater than the maintenance energy requirements (197 kcal of ME/kg $BW^{0.6}$; NRC, 2012). Room temperature was maintained at 23 ± 2 °C.

A T-cannula was installed in the blind end of the cecum of each pig 14 d before the commencement of the experiment (Fig. 1). After recovery, the pigs were assigned to 2 dietary treatments based on their body weight block, with 6 replicate pigs per diet. The experiment lasted 28 d. On d 1, 14, and 28 of the experiment, fresh cecal digesta and fecal samples were collected into sterile centrifuge tubes, flash-frozen in liquid nitrogen, and stored in a freezer at −20 °C for subsequent determination of microbial populations and short-chain fatty acids (SCFA). On d 28, blood samples of pigs were collected from the jugular vein into anticoagulant tubes (Becton, Dickinson & Co., NJ, USA) after overnight fasting.

Urine and feces were collected from each pig from d 24 through d 28 of the experiment. Fresh feces were placed into plastic bags as soon as they appeared in the metabolism crates and stored at −20 °C. Urine was collected into a bucket containing HCl (10 mL; 6 mol/L) located under the metabolism crates. A 10% aliquot of urine was filtered through gauze and stored at −20 °C. At the end of the 5-d collection period, feces and urine were pooled within each

Table 1
Composition and nutrient concentrations of experimental diets (%, as-fed basis).

Item	HP diet	LP diet
Ingredients		
Corn	58.21	72.13
Soybean meal	34.87	15.26
Wheat bran	2.99	8.10
Soybean oil	0.95	0.29
Limestone	0.88	0.90
Dicalcium phosphate	0.70	0.86
Salt	0.30	0.30
L-Lys·HCl	0.08	0.62
DL-Met	0.02	0.17
L-Thr	–	0.22
L-Trp	–	0.07
L-Val	–	0.08
Premix[1]	1.00	1.00
Total	100.00	100.00
Analyzed nutrient concentrations		
Dry matter	89.69	90.51
Gross energy, kcal/kg	4037	3096
Crude protein	21.48	15.54
Ether extract	2.69	2.43
Ash	4.52	4.20
Acid detergent fiber	3.89	3.70
Neutral detergent fiber	10.45	11.06
Arg	1.34	0.93
His	0.54	0.41
Ile	0.90	0.63
Leu	1.97	1.56
Lys	1.15	1.10
Met	0.36	0.44
Met + Cys	0.68	0.69
Phe	1.10	0.79
Thr	0.83	0.83
Trp	0.25	0.23
Val	1.00	0.79
Calculated nutrient concentrations[2]		
Net energy, kcal/kg	2400	2400
Standardized ileal digestible amino acids		
Lys	1.00	1.00
Met + Cys	0.59	0.59
Thr	0.63	0.63
Trp	0.21	0.21
Val	0.82	0.66

HP = high-protein; LP = low-protein.
[1] Premix provided the following per kilogram of diets: vitamin A as retinyl acetate, 8250 IU; vitamin D_3 as cholecalciferol, 825 IU; vitamin E as DL-alpha-tocopherol acetate, 40 IU; vitamin K_3 as menadione nicotinamide bisulfite, 4 mg; thiamine as thiamine mononitrate, 1 mg; riboflavin, 5 mg; pantothenic acid as DL-calcium pantothenate, 15 mg; pyridoxine as pyridoxine hydrochloride, 2 mg; vitamin B_{12}, 28 μg; niacin, 36 mg; biotin, 4 mg; folacin, 2 mg; choline chloride, 600 mg; Mn as MnO, 25 mg; Fe as $FeSO_4·H_2O$, 80 mg; Zn as $ZnSO_4$, 100 mg; Cu as $CuSO_4·5H_2O$, 50 mg; I as KI, 0.5 mg; Se as Na_2SeO_3, 0.15 mg.
[2] Values for net energy content and standardized ileal digestible amino acids of ingredients were referred to China National Standard (GB/T 39235-2020).

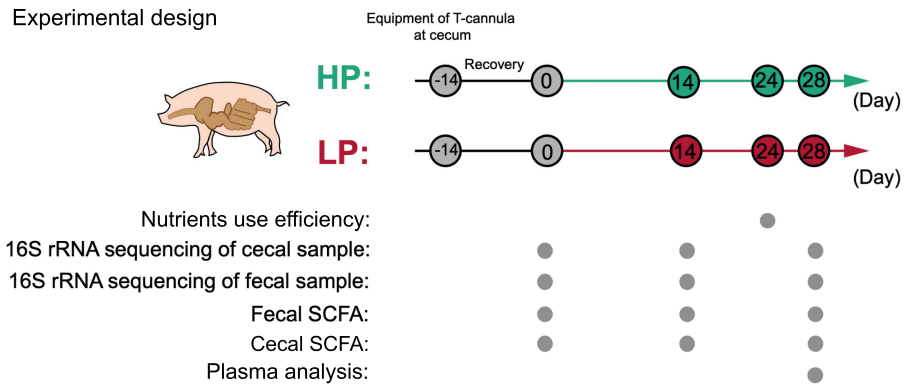

Fig. 1. Experimental design. Black lines, adaptation and recovery period; green lines, high-protein diet feeding period; red lines, low-protein diet feeding period; gray points, sample collection. HP = high-protein diet; LP = low-protein diet; SCFA = short-chain fatty acids.

pig. Subsamples (approximately 250 g) of feces were dried for 72 h at 65 °C and ground through a 1-mm screen. Feces and urine samples were stored at −20 °C for subsequent analysis.

2.3. Chemical analyses

Diets and fecal samples were analyzed for dry matter (DM) (AOAC, 2006; method 930.15) and CP (AOAC, 2006; method 984.13). Ether extract and ash in the diets were analyzed according to method 920.39 and 942.05 (AOAC, 2006), respectively. Determinations of neutral detergent fiber (NDF) and acid detergent fiber (ADF) were performed using a filter bag and fiber analyzer (Ankom, NY, USA; Van Soest et al., 1991). An automatic oxygen bomb calorimeter (Model 6400, Parr, USA) was used to measure the gross energy (GE) of feed and feces. Samples of each diet were analyzed for the specific array and concentration of amino acids according to the method described previously (Yao et al., 2008).

2.4. Plasma amino acid and biochemical parameter analyses

Concentrations of plasma amino acids were determined using an S-433D Amino Acid Analyzer (Sykam, Munich, Germany), as described previously (Zhou et al., 2022a). Concentrations of plasma total cholesterol (TC), triglyceride (TG), total protein (TP), endotoxin, and urea nitrogen (UN) were determined using a biochemical analyzer (Bayer, Manufactured Bayer Diagnostics Manufacturing) with commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The activity of plasma glutamic-pyruvic transaminase (GPT), glutamic-oxalacetic transaminase (GOT), alkaline phosphatase (ALP), and lipase was determined using a CX-4 automatic biochemical analyzer (Beckman Institute, California, USA).

2.5. Short-chain fatty acid analyses

Concentrations of SCFA in fermentation supernatants were determined using the methods reported by Dunkley et al. (2007). Briefly, cecum digesta and feces samples (approximately 1 g) were diluted with ultrapure water (8 mL) and homogenized in sterile tubes (10 mL). Subsequently, samples were incubated in an ultrasonic bath for 30 min. Afterward, samples were centrifuged at 10,000 × *g* for 10 min for supernatant collection. Finally, concentrations of SCFA in the supernatant were measured using gas chromatography (Ion Chromatography System; Dionex ICS-3000, Sunnyvale, CA, USA). The sum of acetate, propionate, butyrate, and valerate contents was considered to be the total SCFA content in the present study, due to these 4 fatty acids account for more than 95 % of SCFA in the caecum and feces of growing pigs (Brestenský et al., 2017).

2.6. Bacterial microbial population analyses

The DNA of bacteria in the cecum and feces was extracted using a kit (Omega Bio-Tek, Norcross, GA, USA) according to the manufacturer's instructions. The 16S rRNA V3–V4 hypervariable region was amplified using a thermocycler polymerase chain reaction system (GeneAmp 9700, ABI, USA). The Illumina HiSeq 2500 platform (San Diego, CA, USA) was applied to purify, quantify, pool, and sequence the resulting amplicons. UCHIME was used for the definition and removal of nonnormal gene sequences.

2.7. Calculations

The apparent total tract digestibility (ATTD) of nutrients was calculated according to the following equation (Wang et al., 2020):

$$\text{ATTD}\ (\%) = [(N_{in} - N_{out})/N_{in}] \times 100.$$

In this equation, N_{in} is the nutrient intake in diet (gram in DM basis), and N_{out} is the nutrient output in feces (gram in DM basis).

2.8. Statistical analysis

The PROC MIXED procedures of SAS version 9.4 (SAS Institute, Cary, NC) was used to perform data analysis. All data were evaluated for normal distribution and homogeneous variance using the UNIVARIATE procedure. Data for nutrient digestibility, nitrogen balance, plasma biochemical parameters, amino acid levels, and SCFA contents of the cecum and feces were analyzed using independent-sample *t*-tests. Dietary treatment was the fixed effect and individual pig was regarded as a random effect and served as a replicate. Data obtained by *t*-tests are shown as the Lsmeans and SEM in tables and are presented as the means ± SD in figures. A *P*-value < 0.05 was considered significant, and 0.05 ≤ *P*-value < 0.10 was considered a tendency.

The α diversity of the bacterial community in cecal content and feces was analyzed using the Mann–Whitney U and Kruskal–Wallis tests. QIIME software package based on Bray–Curtis distance metrics was applied to conduct a principal coordinate analysis of microbial compositions between treatments. Based on 97% similarity, UPARSE software was used to perform OTU clustering of sequences, and RDP Classifier software was used to compare similarity with the GreenGene database to obtain

microbial taxonomic information at the phylum, class, order, family, and genus levels.

3. Results

3.1. Nutrient digestibility and nitrogen balance

Compared with the HP treatment, the LP diet decreased ATTD of DM, GE, and CP ($P < 0.01$; Table 2). Nitrogen intake, urinary nitrogen losses, total nitrogen losses, and nitrogen retention of pigs fed LP were all lower than those of HP-fed pigs ($P < 0.01$). Ratio of nitrogen retention to nitrogen intake for LP-fed pigs was higher than that of HP-fed pigs ($P < 0.01$). The fecal nitrogen losses for HP-fed pigs tended to be greater than pigs fed LP diet ($P = 0.06$).

3.2. Plasma biochemical parameters and amino acids

Plasma concentrations of TC, TG, TP, GPT, GOT, ALP and lipase were not different between treatments (Table 3). Compared with the HP diet, the LP diet decreased plasma concentrations of UN and endotoxins ($P < 0.01$).

Compared to HP-fed pigs, LP-fed pigs presented decreased plasma concentrations of His, Ile, Ala, Val, and Cit ($P < 0.05$; Table 4). Plasma concentrations of Lys, Met, and Trp in LP-fed pigs were higher than those in HP-fed pigs ($P < 0.05$). In addition, the LP diet tended to increase plasma concentrations of Glu and Tau ($0.05 \leq P < 0.10$).

3.3. SCFA concentrations in cecal content and feces

Concentrations of SCFA in the cecal contents and feces did not differ between treatments at the beginning of the experiment (Tables 5 and 6). On d 14, acetate concentration of cecal contents in LP-fed pigs was lower than that in the HP-fed pigs ($P < 0.05$) but only tended to be different between treatments in feces. There was no difference in propionate, butyrate, valerate, and total SCFA concentrations of the cecal content between treatments but propionate, butyrate, and valerate concentrations of feces from HP-fed pigs were higher than those from LP-fed pigs ($P < 0.05$). Additionally, the total SCFA concentration of feces from HP-fed pigs was greater than that from LP-fed pigs ($P < 0.01$).

On d 28, HP-fed pigs presented increased acetate and total SCFA concentrations in the cecum compared with LP-fed pigs ($P < 0.05$). Acetate, propionate, and butyrate concentrations of feces from HP-fed pigs were greater than from LP-fed pigs ($P < 0.05$). Similarly, the HP diet increased total SCFA concentration in feces from HP vs. LP-fed pigs ($P < 0.01$).

Table 2
Effects of the dietary crude protein content on the apparent total tract digestibility of nutrients and nitrogen balance in growing pigs.

Item	HP diet	LP diet	SEM	*P*-value
Nutrient digestibility, %				
Dry matter	89.20	87.74	0.420	<0.01
Gross energy	88.44	86.57	0.458	<0.01
Crude protein	90.80	88.82	0.482	<0.01
Nitrogen balance, g/d				
Nitrogen intake	40.61	30.18	0.503	<0.01
Fecal nitrogen losses	3.74	3.37	0.173	0.06
Urinary nitrogen losses	17.36	9.28	0.359	<0.01
Total nitrogen losses	21.10	12.65	0.314	<0.01
Nitrogen retention	19.52	17.53	0.682	<0.01
Nitrogen retention/intake, %	48.02	58.05	1.270	<0.01

HP = high-protein; LP = low-protein; SEM = standard error of the mean.

Table 3
Effects of dietary crude protein content on plasma biochemical traits of growing pigs.

Item	HP diet	LP diet	SEM	*P*-value
TC, mmol/L	2.15	2.25	0.112	0.37
TG, mmol/L	0.53	0.50	0.068	0.71
TP, g/L	57.43	58.23	1.164	0.50
GPT, U/L	61.77	53.83	5.671	0.19
GOT, U/L	37.20	31.84	6.870	0.45
ALP, U/L	193.05	195.50	18.853	0.90
Lipase, U/L	385.95	344.91	41.879	0.35
UN, mmol/L	5.97	3.05	0.625	<0.01
Endotoxin, EU/L	138.64	93.54	8.688	<0.01

HP = high-protein; LP = low-protein; SEM = standard error of the mean; ALP = alkaline phosphatase; GOT = glutamic oxalacetic transaminase; GPT = glutamic-pyruvic transaminase; TC = total cholesterol; TG = triglyceride; TP = total protein; UN = urea nitrogen.

Table 4
Effects of dietary crude protein content on plasma amino acid concentrations of growing pigs (μmol/L).

Item	HP diet	LP diet	SEM	*P*-value
Essential amino acids				
Arg	161.18	151.47	14.289	0.51
His	85.62	62.92	9.701	0.04
Ile	115.55	94.32	7.078	0.01
Leu	204.28	185.48	15.798	0.26
Lys	80.28	117.88	13.429	0.02
Met	27.23	34.68	2.888	0.03
Phe	72.68	69.43	4.779	0.51
Thr	178.53	204.25	20.908	0.25
Trp	34.67	44.15	3.525	0.02
Val	265.15	190.08	16.178	<0.01
Nonessential amino acids				
Ala	519.65	423.10	37.134	0.03
Asp	19.13	18.88	3.125	0.94
Cit	73.27	49.57	10.487	0.04
Glu	178.22	194.72	23.913	0.50
Gln	424.18	461.67	18.108	0.07
Gly	939.73	967.50	111.067	0.81
Orn	96.03	84.50	10.984	0.32
Ser	111.02	114.10	9.069	0.74
Tau	80.22	101.73	10.008	0.06
Tyr	114.57	97.58	10.887	0.15

HP = high-protein; LP = low-protein; SEM = standard error of the mean.

Table 5
Effects of dietary crude protein content on SCFA concentrations in the cecum of growing pigs (mg/g).

Item	HP diet	LP diet	SEM	*P*-value
Day 1				
Acetate	3.92	3.83	0.153	0.54
Propionate	2.53	2.42	0.370	0.78
Butyrate	0.90	1.01	0.260	0.69
Valerate	0.35	0.41	0.106	0.57
Total SCFA	7.70	7.67	0.786	0.96
Day 14				
Acetate	4.17	3.61	0.181	0.01
Propionate	2.36	2.46	0.196	0.61
Butyrate	1.32	1.19	0.189	0.51
Valerate	0.58	0.67	0.176	0.60
Total SCFA	8.43	7.93	0.419	0.32
Day 28				
Acetate	4.05	3.56	0.222	0.04
Propionate	2.63	2.41	0.206	0.45
Butyrate	1.12	1.10	0.136	0.90
Valerate	0.70	0.65	0.098	0.64
Total SCFA	8.50	7.72	0.247	0.02

HP = high-protein; LP = low-protein; SCFA = short-chain fatty acids; SEM = standard error of the mean.

Table 6
Effects of dietary crude protein content on SCFA concentrations in the feces of growing pigs (mg/g).

Item	HP diet	LP diet	SEM	*P*-value
Day 1				
Acetate	3.49	3.63	0.237	0.55
Propionate	1.80	1.81	0.305	0.97
Butyrate	0.84	0.83	0.085	0.86
Valerate	0.53	0.55	0.037	0.64
Total SCFA	6.66	6.82	0.429	0.74
Day 14				
Acetate	3.61	2.91	0.338	0.07
Propionate	1.80	1.39	0.167	0.04
Butyrate	1.06	0.84	0.093	0.04
Valerate	0.53	0.40	0.045	0.02
Total SCFA	7.00	5.54	0.246	<0.01
Day 28				
Acetate	3.65	2.80	0.271	0.01
Propionate	1.86	1.43	0.167	0.03
Butyrate	1.01	0.68	0.126	0.02
Valerate	0.59	0.52	0.052	0.23
Total SCFA	7.11	5.43	0.265	<0.01

HP = high-protein; LP = low-protein; SCFA = short-chain fatty acids; SEM = standard error of the mean.

3.4. Bacterial community

There were no significant differences in diversity and richness of the bacterial communities in cecal contents and feces on d 1 and 14 between the pigs receiving HP or LP diets (Fig. 2). On d 28, the Simpson index in the cecal content and Shannon, Simpson, and CHAO indices in the feces of LP-fed pigs were greater than those of HP-fed pigs ($P < 0.05$).

Phylum-level analysis demonstrated that the microbiota composition in cecal contents was consistently dominated by Firmicutes (46.89%; Fig. 3), Bacteroidetes (49.28%), and Actinobacteria (2.20%) on d 14 and Firmicutes (47.93%), Bacteroidetes (47.54%), and Actinobacteria (2.79%) on d 28. At the family level, Prevotelaceae (47.00%), Veillonelaceae (12.96%), and Lachnospiraceae (12.19%) were the dominant bacteria in cecal contents on d 14, and Prevotellaceae (43.83%), Lactobacillaceae (12.83%) and Lachnospiraceae (10.29%) were the dominant bacteria in cecal contents on d 28.

For fecal microbiota, Firmicutes (45.11%), Bacteroidetes (42.08%), and Actinobacteria (4.32%) were dominant phyla on d 14, and Firmicutes (46.22%), Bacteroidetes (34.50%), and Actinobacteria (11.61%) were also dominant phyla on d 28. At the family level, Prevotellaceae (30.48%), Veillonelaceae (15.64%) and Lachnospiraceae (7.07%) were the dominant bacteria on d 14, and Prevotellaceae (20.02%), Veillonelaceae (16.60%) and Lachnospiraceae (11.61%) were the dominant bacteria on d 28.

Assessment of difference in β-diversity based on operational taxonomic unit (OTU) in cecal contents and fecal microbiota is illustrated in Fig. 4. PERMANOVAs of the unweighted UniFrac distances revealed distinct clustering patterns in cecal contents ($P = 0.64$ on d 1; $P = 0.67$ on d 14; $P = 0.37$ on d 28) and fecal ($P = 0.74$ on d 1; $P = 0.15$ on d 14; $P = 0.02$ on d 28) microbiota between the HP and LP treatments. Notably, microbial communities of HP and LP treatments differed significantly on Axis 2 of the cecal content (41.01%; $P < 0.05$ on d 1 and 14) and on Axis 1 of feces (48.65%; $P < 0.05$ on d 28).

Differences in the microbial community among dietary treatments are shown in Fig. 5. To screen for differential microorganisms in cecal contents and feces under different dietary protein contents, microorganisms with a relative abundance of OTU higher than 0.1% on d 28 were analyzed by linear discriminant analysis effect size (LEfSe). There were 17 and 56 differential OTUs identified in cecal contents and feces between treatments, respectively ($P < 0.05$). Four common differential OTUs were observed: OTU_78, OTU_218, OTU_880, and OTU_37, which were annotated by QIIME and found to be *unclassified_f_Lachnospiraceae* in Lachnospiraceae, *Parabacteroides* in Porphyromonadaceae, *Anaerovibrio* in Veillonellaceae, and *Ruminococcaceae_UCG*-014 in Ruminococcaceae.

There was one differential OTU with an abundance greater than 1% in cecal contents (OTU_859). After annotation, it was found that the abundance of *Bifidobacterium* in LP-fed pigs was greater than in HP-fed pigs ($P < 0.05$). There were 9 different OTUs with abundances greater than 1% in feces (OTU_771, OTU_1066, OTU_1091, OTU_761, OTU_297, OTU_963, OTU_423, OTU_630, and OTU_438). After annotation, *Prevotella_1*, *Prevotellaceae_UCG-003*, *Rikenellaceae_RC9_gut_group*, *Treponema_2*, and *Ruminococcus* were enriched in the LP group ($P < 0.05$), and the abundance of *Lactobacillus* was greater in HP-fed compared with LP-fed pigs ($P < 0.05$).

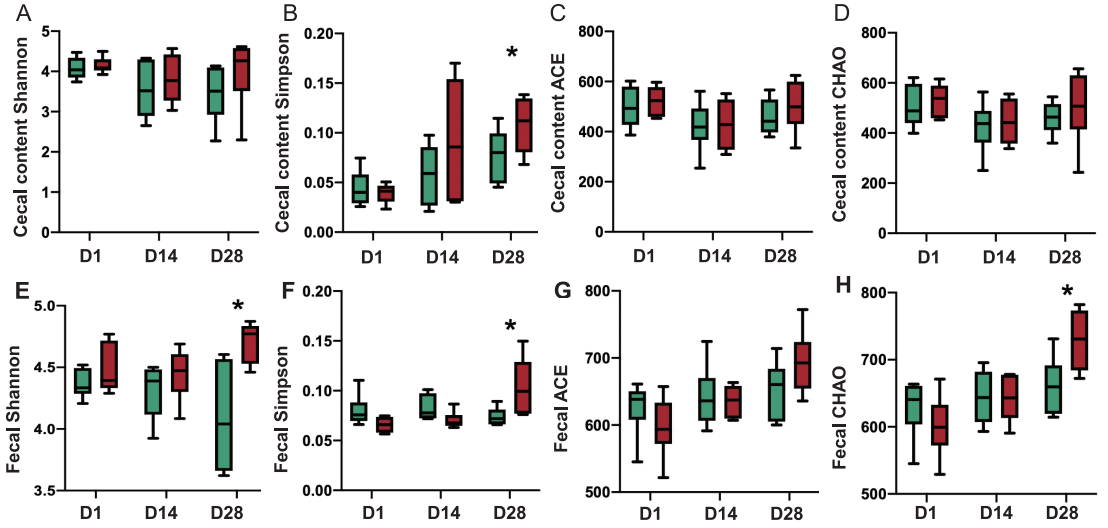

Fig. 2. The α diversity of cecal contents and fecal bacterial communities. Shannon index (A), Simpson index (B), ACE index (C) and CHAO index (D) of the cecal bacterial community and Shannon index (E), Simpson index (F), ACE index (G) and CHAO index (H) of the fecal bacterial community. * Significant differences between treatments within sampling day ($P \leq 0.05$). Green box, high-protein diet treatment; red box, low-protein diet treatment.

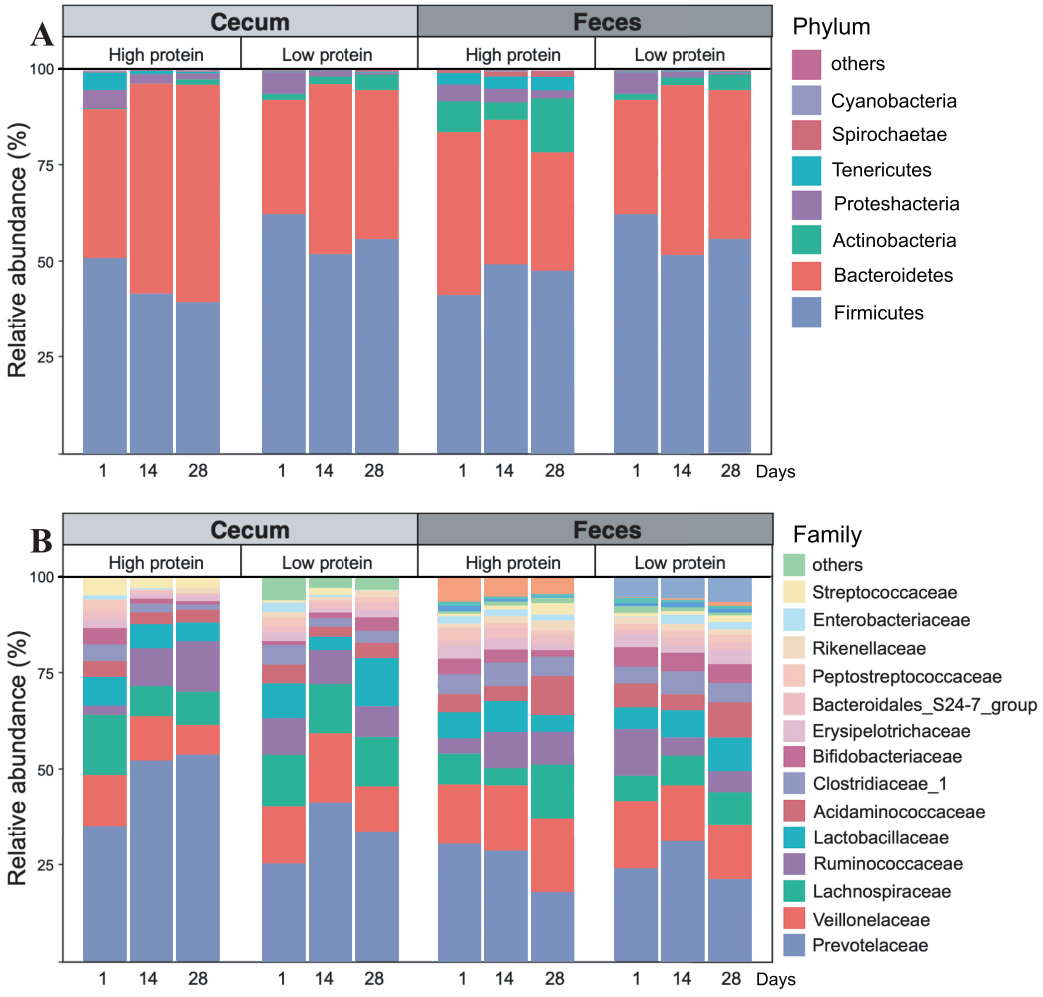

Fig. 3. Cecal and fecal bacterial communities at phylum and family levels in cecal contents and feces. Microbial community bar plots of phyla with an abundance of 0.015% or greater (A) and families with a proportion of 0.015% or higher (B).

4. Discussion

Nitrogen efficiency is an important evaluator of whether dietary amino acid content satisfies nutritional requirements of pigs. There have been extensive summaries of nitrogen efficiency in pigs with different dietary CP contents, and most of the results suggest no obvious change in nitrogen deposition when dietary protein content is reduced by 4 percentage points or less (Le Bellego and Noblet, 2002; Zhang et al., 2013). Results of the present experiment showed that reducing dietary CP content by 6 percentage points caused a reduction in daily nitrogen retention. However, the nitrogen efficiency was enhanced in pigs fed LP diet, which mainly due to the lower nitrogen losses in urine, but not in feces. These results implied that reducing dietary CP content and supplemented with crystalline amino acid led to a balance of amino acid, and there is no need to degrade the excess nitrogen to ammonia and then excreted through urinary nitrogen losses (Wang et al., 2020).

Plasma UN is the product of protein metabolism. Amino acid deamination causes an increase in plasma UN content. Generally, decreased plasma UN content suggests an improved balance of dietary amino acids, weakened urea synthesis in the liver and kidney, and enhanced dietary protein utilization efficiency (Heo et al., 2009). Concentration of plasma UN is correlated negatively with muscle growth (Liu et al., 2015). In the present experiment, plasma UN content of pigs in the LP treatment was lower than that of pigs in the HP treatment indicating that the LP diet may have improved the amino acid balance of pigs, and enhanced nitrogen efficiency. Endotoxin is a secretion released by gram-negative bacteria. Elevated plasma endotoxin content reflects impaired intestinal barrier function, increased intestinal permeability, and enhanced metabolic activity of microorganisms (Deitch et al., 1987; Mani et al., 2012). In the current experiment, plasma endotoxin concentration was reduced in pigs fed the LP diet, which suggests improved intestinal barrier function.

Plasma amino acid concentration is regulated by dietary composition, amino acid transport, first pass consumption, and gut microbiota metabolism. In the present study, the LP diet was supplemented with the first 5 limiting amino acids to satisfy the dietary demand of pigs. Dietary Met content was greater in the LP diet than in the HP diet (0.44% vs. 0.36%). This difference may have contributed to the increased plasma Met concentration in LP-fed pigs. Dietary Lys and Trp content were similar between treatments. Crystalline amino acids present an improved absorption rate and efficiency compared with these amino acids contained in intact protein (crystalline amino acids can be directly absorbed without digestion; the rapid absorption rate lessens the

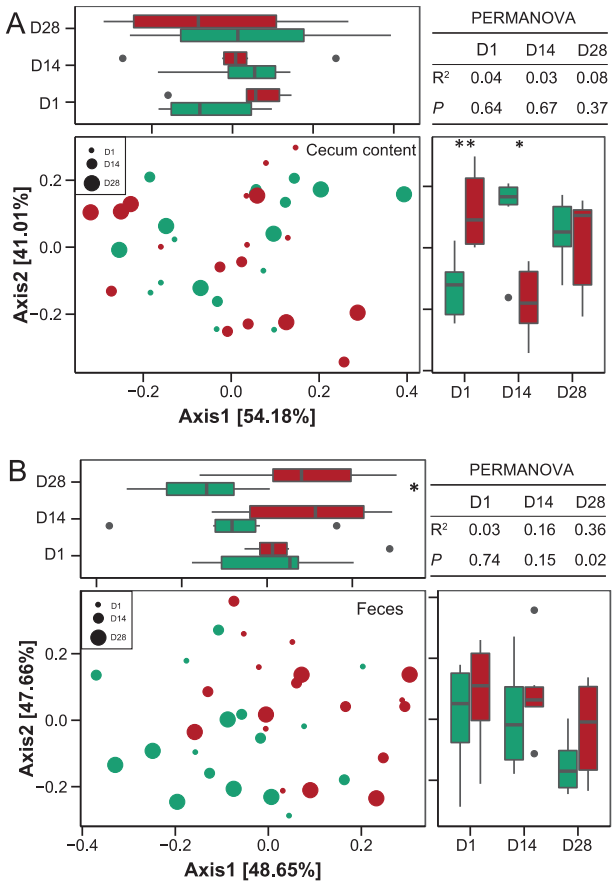

Fig. 4. Principal coordinate analysis. Principal coordinate analysis of the microbiota from cecal contents (A) and feces (B) of pigs fed a high-protein diet (green points and green boxes) or a low-protein diet (red points and red boxes); $n = 6$. Distances between the symbols on the ordination plot reflect the relative dissimilarities in the communities. ** $P < 0.01$, * $P < 0.05$.

consumption by microorganisms; Nolles et al., 2009), which maybe contribute to elevated plasma concentrations of these 2 amino acids in pigs fed LP diet containing more crystalline Lys and Trp. Notably, Thr is an important amino acid for intestinal mucosal repair and can be extensively metabolized by intestinal cells (Wang et al., 2018). Therefore, even though an increased proportion (26.5% vs. 0%) of Thr in the LP group was present in the crystalline state, first-pass consumption of Thr may not differ between treatment groups, which led to similar plasma concentrations.

Adequate digestion is the basis for efficient utilization of dietary nutrients. Digestion of dietary nutrients in pigs includes 2 procedures: chemoenzymatic digestion, which mainly occurs in the fore-gastrointestinal tract, and microbial digestion, which mainly occurs in the hindgut (Ji et al., 2022). Digestion efficiency of nutrients hinges on factors such as the type and amount of nutrients ingested and the segment of the gastrointestinal tract where digestion occurs (Schop, 2020). Generally, reducing dietary protein content impairs nutrient digestibility (Xue et al., 2017). This observation was confirmed in the present experiment, where a 6% reduction in dietary CP obviously reduced ATTD of DM, GE, and CP. Because we previously demonstrated that compromised nutrient digestibility of LP diets does not occur in the foregut (Zhou et al., 2022b), the current experiment investigated time-dependent changes in the population and metabolites of the gut microbiota in pigs fed a LP diet to identify key reasons for reduced digestibility.

Bacterial Simpson indices of feces and cecal contents were improved after 28-d of feeding the LP diet, but this improvement did not appear on d 14. Additionally, differences in clustering patterns of microorganism in feces between treatments only appeared on d 28. These data confirmed that reduced dietary protein content can effectively promote diversity of gut microbiota, and this effect only happens after a period of treatment. Notably, no significant difference in microflora clustering patterns of cecal contents between treatments was observed in this experiment. This may be because the posterior end of the colon is the primary site of protein microbial fermentation (Windey et al., 2012), and the decrease in dietary protein content cannot largely change the population of the microbes in the proximal colon.

SCFA are produced mainly by hindgut bacteria that ferment carbohydrates which cannot be digested and absorbed in the foregut (Cummings and Englyst, 1987). SCFA manufactured by colonic microbes provide 6% to 10% of the total energy requirements in humans (Bergman, 1990). SCFA can also contribute to energy homeostasis and intestinal health (Den Besten et al., 2013). In the current experiment, elevated concentrations of SCFA in cecal contents and feces in the HP group may be related to increased nitrogen entering the hindgut to nourish crucial SCFA-producing bacteria. OTU37, annotated as a taxonomic unit in *Ruminococcaceae_UCG_14*, was enriched in the cecal contents and feces of the HP group. *Ruminococcus* is an autochthonous and benign species that primarily inhabits the cecum and colon (Donaldson et al., 2016). As a SCFA producer, *Ruminococcus* has abundant and diverse carbohydrate-degrading enzymes, is highly specialized for degradation of complex plant material, and is associated with utilization of resistant starch (Biddle et al., 2013; Newman et al., 2018). Beef cattle with high feed efficiency had an elevated relative abundance of *Ruminococcus* (Li and Guan, 2017), and increased weight gain and ruminal SCFA concentrations were found in cattle yaks with enriched *Ruminococcus* (Dai et al., 2021); in addition, *Ruminococcus* is significantly positively correlated with intestinal acetate and propionate concentrations (Xie et al., 2022). Therefore, improved nutrient digestibility and intestinal SCFA concentrations of pigs fed HP diet may be partly attributed to enrichment of *Ruminococcus*.

Lactobacillus can produce SCFA through fermenting carbohydrate end-products such as pyruvate, which is generated during the glycolytic pathway and through the phosphoketolase route in hetero-fermenting conditions (Pessione, 2012). Moreover, *Lactobacillus* spp. can also promote proliferation of SCFA-producing bacteria (Wang et al., 2018), prevent acute intestinal inflammation (Lee et al., 2012), strengthen the intestinal barrier and modulate gut microbiota to relieve enterotoxigenic *Escherichia coli* (ETEC)-induced diarrhea (Yang et al., 2014), and reduce expression and secretion of interleukin (IL)-6, tumor necrosis factor-α (TNF-α) and interleukin (IL)-8 (Wang et al., 2018). In the present study, enriched *Lactobacillus* in HP-fed pigs may have supported adequate fermentation of carbohydrates and improved intestinal function, which enhanced DM and GE digestibility coefficients and increased SCFA production.

Prevotella participates in glucose metabolism with acetate as its main fermentation product. Almost all microorganisms that break down polysaccharide are derived from *Prevotella* (Heinritz et al., 2016). In addition, *Prevotella* is a key factor in modulating inflammation and caspase-8-mediated IL-1β maturity and play important roles in metabolism of amino acids, energy, coenzymes, and vitamins by the host (Lukens and Kanneganti, 2014). In the current experiment, *Prevotella* was the dominant bacteria in cecal contents. In addition, *Prevotella* was particularly prevalent at the genus level in HP-fed pigs, which may contribute to degradation and fermentation of undigested carbohydrates in the hindgut to produce SCFA

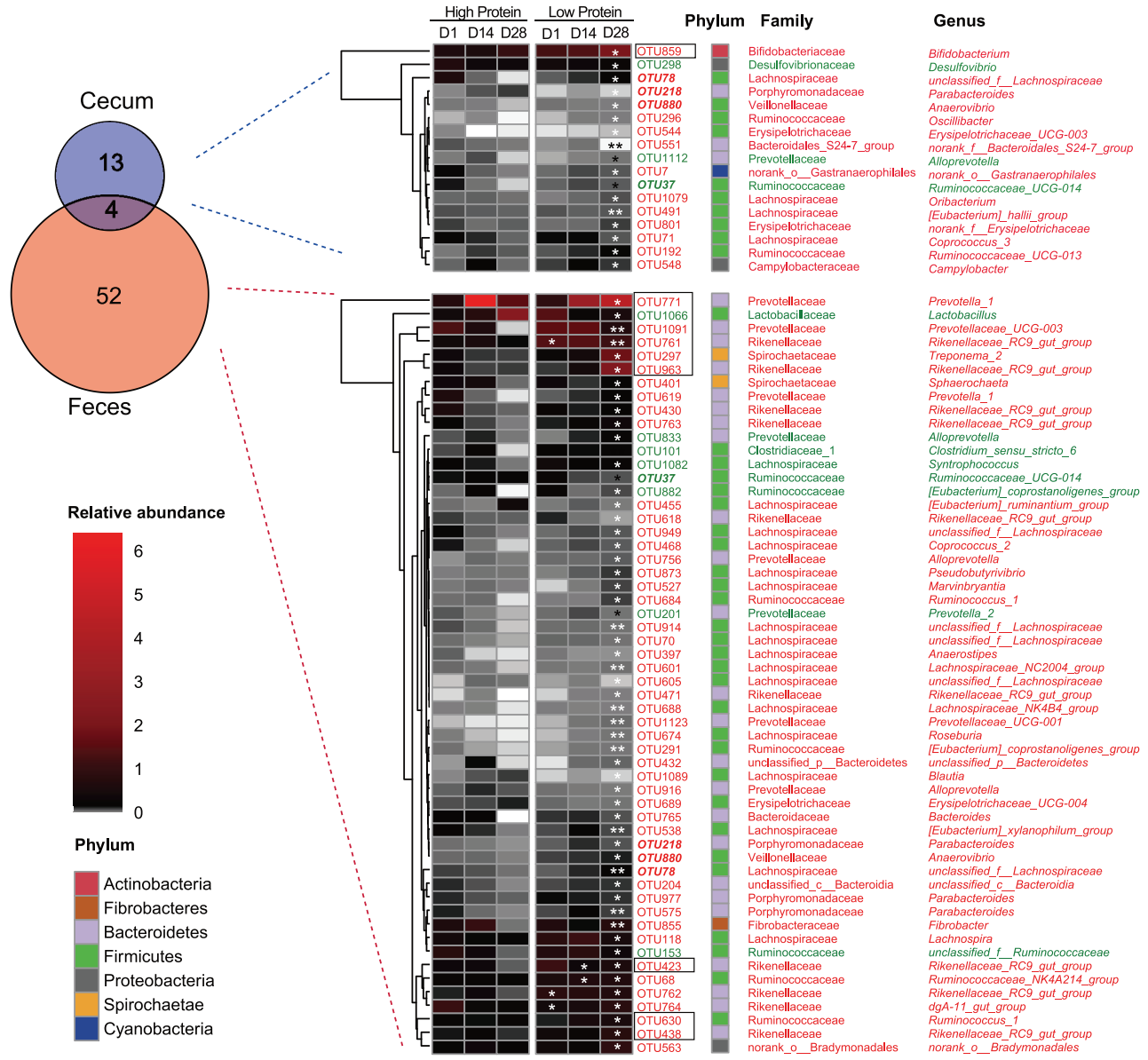

Fig. 5. Analysis of all differentially abundant bacteria. Cladogram of linear discriminant analysis effect size demonstrates taxonomic profiling for the distinct bacteria among cecal content and feces of pigs fed a high-protein diet (green) or a low-protein diet (red). $n = 6$. ** $P < 0.01$, * $P < 0.05$.

(especially acetate) production that may benefit nutrient digestibility (Tan et al., 2018).

Numerous studies have demonstrated LP diets can improve gut health, reduce harmful fermentation in the hindgut, and optimize microbiota population, which was confirmed in the present study (Zhou et al., 2020, 2022b). LEfSe analysis revealed that abundance of *Bifidobacterium*, which was greater than 1%, increased with decreasing dietary protein content. *Bifidobacterium* possesses a variety of probiotic functions, such as relieving intestinal diseases caused by immune system disorders, inhibiting invasion of pathogenic bacteria, and alleviating oxidative loss (Heinritz et al., 2016). Additionally, the *Bacteroidales S24-7* was enriched in LP-fed pigs. A recent metagenomic analysis described the ability of the *Bacteroidales S24-7* to protect against oxidative stress, suggesting that they behave as anaerobes capable of growing under marginally oxic conditions (Ormerod et al., 2016). In addition, this bacterial can repair barrier in inflamed mouse intestines (Osaka et al., 2017) and promote healthy metabolic states (Lippert et al., 2017). Feeding the LP diet decreased abundance of *Clostridium_sensu_stricto_6*. *Clostridium* spp. is a very large group of bacteria present in the gastrointestinal tract of pigs in the present study. Some bacteria in this group belong to *Clostridium sensu stricto*, which are generally considered as pathogenic (Rajilić-Stojanović and De Vos, 2014). However, members of the endospore-forming *Clostridium sensu stricto* (Wiegel et al., 2006) can employ the phosphotransbutyrylase/butyrate kinase pathway to produce SCFA, especially butyrate. This phenomenon may be a reason why concentration of SCFA decreased in LP-fed pigs. In addition, some bacteria such as *Rikenella* (Lippert et al., 2017) is positively related to health, which were enriched in LP-fed pigs. Enrichment of these species commonly used as probiotics in LP-fed pigs may inhibit the proliferation of harmful bacteria, thereby promoting gut health, which may also account for the observed reduction in plasma endotoxin concentrations.

5. Conclusion

The LP diet reduced daily nitrogen retention but increased nitrogen efficiency in pigs. Modulation of dietary protein contents on microbiota populations of the gut and metabolite concentrations was clearly time-dependent. The HP diet increased the abundance of SCFA-producing bacteria, such as *Ruminococcus, Lactobacillus*, and *Prevotella*, and increased SCFA concentrations in the cecum and feces, while the LP diet enriched gut health-related bacteria, such as *Bifidobacterium*, *Bacteroidales S24-7*, and *Rikenella,* which may have contributed to improved intestinal health and decreased plasma endotoxin concentrations.

Author contributions

Yuming Wang: Conceptualization, Methodology, Project administration, Formal analysis, Funding acquisition, Writing - original draft. **Junyan Zhou**: Conceptualization, Data curation, Formal analysis, Writing – review & editing. **Ning Cao**: Data curation, Investigation, Writing-review & editing. **Lu Wang**: Investigation, Project administration. **Jiayu Tu**: Project administration, Visualization. **Xiangfang Zeng**: Investigation, Supervision. **Shiyan Qiao**: Conceptualization, Supervision, Writing - review & editing.

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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