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Original Research Article

Effect of fermented rapeseed meal on growth performance, nutrient digestibility, and intestinal health in growing pigs

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ABSTRACT

To explore the effects of fermented rapeseed meal (FRSM) on growth performance and intestinal health, a total of 30 growing pigs were randomly allotted to three treatments consisting of corn-soybean meal diet (CSD), rapeseed meal diet (RSD), and fermented rapeseed meal diet (FRSD). Results showed that compared with RSD, FRSD feeding increased the average daily gain and final body weight in pigs ($P < 0.01$). Compared with RSD feeding, FRSD feeding elevated the apparent digestibility of crude protein, acid detergent fiber, and ether extract in pigs ($P < 0.01$). Moreover, the FRSD group exhibited greater apparent ileal digestibility of His, Thr, Lys, and Ser than the RSD group ($P < 0.01$). The digestible energy, metabolic energy, and nitrogen utilization were higher in the FRSD and CSD groups than in the RSD group ($P < 0.01$). As compared to the RSD, FRSD feeding decreased the serum concentration of leptin but significantly increased the concentrations of immunoglobulin (Ig) A, IgG, ghrelin, and enzyme activities of amylase, lipase, and trypsin in the pancreas ($P < 0.05$). Interestingly, the villus height, the ratio of villus height to crypt depth, and the activities of brush border enzymes (e.g., maltase and sucrase) in the small intestine were higher in the CSD and FRSD groups than in the RSD group ($P < 0.05$). As compared to the RSD, the FRSD feeding not only increased the expression level of the occludin in the small intestinal epithelium ($P < 0.05$) but also elevated the expression levels of claudin-1, *MUC1*, and *PepT1* genes in the duodenum, and elevated the expression levels of *SGLT1* and *CAT1* genes in the jejunum ($P < 0.05$). Importantly, FRSD feeding significantly decreased the abundance of *Escherichia coli*, but increased the abundance of *Lactobacillus* and the content of butyrate in the cecum and colon ($P < 0.05$). These results indicated that compared with rapeseed meal, fermented rapeseed meal exhibited a positive effect on improving the growth performance and intestinal health in growing pigs, and the results may also help develop novel protein sources for animal nutrition and the feed industry.

1. Introduction

Soybean meal (SBM) is the most widely used plant-protein source in animal nutrition and the feed industry as it contains relatively high concentrations of protein and well-balanced amino acids (Eklund et al., 2012; Stein et al., 2008). Currently, the annual consumption of SBM worldwide is up to 244,098 kilotons, and the price of SBM has continuously increased in the last decade of the decline in soybean production (FAOSTAT, 2022). Meanwhile, there was a competition for feed ingredients between humans and monogastric animals, leading to a shortage of feed ingredients (Adeniji and Azeez, 2008). For economic and sustainable reasons, alternatives to conventional protein sources such as SBM have attracted considerable research interest worldwide.

Rapeseed meal (RSM) is a by-product of rapeseed oil production, mainly consisting of protein, fiber, and minerals (Bell, 1984). RSM is abundant in sulfur-containing amino acids and was found to have an excellent balance of essential amino acids (Feng and Zhu, 2010).

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However, the nutrient digestibility of RSM for monogastric animals is poor due to its high content of anti-nutritional factors such as glucosinolates, phytic acid, and fiber (Khajali and Slominski, 2012; Lin et al., 2010; McDonnell et al., 2010; Torres-Pitarch et al., 2015; Zhu et al., 2018). Also, loss of appetite, decrease of dietary net energy and impaired functions of the thyroid and kidney were observed after RSM ingestion because of the crude fiber and glucosinolates (Jha and Berrocoso, 2015; Noblet and Goff, 2001; Tripathi and Mishra, 2007). Moreover, several studies have demonstrated that the diet containing RSM impaired intestinal morphology and intestinal integrity (Liu et al., 2020; Qaisrani et al., 2014; Wu et al., 2022).

Currently, there are two major approaches to improving the nutritional value of the RSM. The first is microbial fermentation, which has been considered as one of the most efficient approaches to eliminate those anti-nutritional factors (e.g., glucosinolates, phytic acids, and fibers) and improve the nutritional value of RSM (Al Juobori et al., 2014; Żuchowski et al., 2013). Another approach is the pre-treatment of the RSM by industrial enzymes. For instance, pre-treating the RSM with fiber-degrading enzymes significantly increased the concentration of glucose and fructose and therefore increased the availability of nutrients in RSM (Kequan et al., 2011). Previous studies indicated that bacterial strains used in microbial fermentation may lack some extracellular hydrolytic enzymes (e.g., cellulase and pectinase), leading to incomplete removal of the anti-nutritional factors (Hu et al., 2015; Wang et al., 2010). To overcome this problem, enzymes and microorganisms were jointly utilized to improve the quality of RSM fermentation (Zhu et al., 2021). For instance, the content of small-molecular proteins was increased by 81.7%, and the content of glucosinolates was reduced by 30.06% in RSM upon joint fermentation by enzymes and microorganisms (Tie et al., 2020). Fermented rapeseed meal (FRSM) treated with fiber-degrading enzymes and Lactobacilli increased the in vitro digestibility of CP and DM by 23% and 20%, respectively (Zhu et al., 2021).

FRSM was used in the diet of pigs in 1976 for the first time, and in the following decades, studies reported that the supplementation of FRSM elevated the performance of animals compared with RSM (Borgida and Tollier, 1976; Czech et al., 2023; Shi et al., 2016). Furthermore, recent studies showed that FRSM exhibited a positive effect on the absorption of intestines, such as increasing the villus height to crypt depth ratio (V:C ratio) and digestive enzyme activity, which improved nutrient digestibility and performance of piglets (Ashayerizadeh et al., 2019; Czech et al., 2021). The activity of digestive enzymes is related to intestinal morphology, and they are vital indicators reflecting the digestive capacity of animals (Attia et al., 2011; Helm et al., 2020). Potential beneficial effects of feeding FRSM on the digestibility of nutrients and intestinal health may be due to its influence on digestive enzymes, nutrient transporters, intestinal integrity, and intestinal morphology. Therefore, the objective of the present study was to determine the effects and mechanism of pre-treated RSM (joint fermentation by *Bacillus subtilis* and enzyme complex) on growth performance and intestinal health in growing pigs. It was hypothesized that the adverse effects of RSM on performance and intestinal health could be ameliorated by joint fermentation with *B. subtilis* and enzymes.

2. Materials and methods

2.1. Animal ethics statement

All experimental protocols in the current study were reviewed and approved by the Animal Experimental Committee of Sichuan Agricultural University (No. 20181105). The experiment was conducted at the Animal Experiment Center of Sichuan Agricultural University.

2.2. Preparation of fermented rapeseed meal

Bacillus subtilis (CICC21095) was purchased from the China Center of Industrial Culture Collection (CICC). Commercial complex enzyme preparations (cellulase activity ≥500 U/g; xylanase activity ≥10,000 U/g; α-amylase activity ≥200 U/g; pectinase activity ≥100 U/g) were purchased from Baiyin Sino Biological Technology Co., Ltd. (Gansu, China). Basal substrate (BS) containing 90% rapeseed and 10% wheat bran was mixed and inoculated with *B. subtilis* of 10% (v/w), added 1.25 g/kg complex enzyme preparations, and then fermented in a closed fermenter at 45 °C for 48 h. The BS to distilled water ratio was 1:0.9. Freshly fermented samples were dried at 55 °C for 2 d. The nutrient composition of the FRSM is presented in Table S1.

2.3. Experimental diets

Three experimental diets were used for this experiment. The energy value of SBM, RSM and FRSM was calculated according to a formula given by Li et al. (2015) and Zhang et al. (2020). The experimental diets were in meal form, including a corn-soybean diet (CSD) and two experimental diets in which the 35% CP of the control diet was replaced by rapeseed meal (RSD) or fermented rapeseed meal (FRSD) (Table 1). Vitamins and minerals in experimental diets were in accordance with NRC (2012). The cost of the three diets is presented in Table S2.

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

Ingredient	CSD[1]	RSD[2]	FRSD[3]
Corn starch	–	7.00	6.52
Corn	67.30	57.80	60.00
SBM	22.00	11.08	10.62
RSM	–	13.63	–
FRSM	–	–	12.24
Sugar	5.00	5.00	5.00
Soy oil	2.00	2.00	2.00
Choline chloride	0.15	0.15	0.15
$CaCO_3$	0.70	0.80	0.80
$CaHPO_4$	1.40	0.90	1.00
NaCl	0.30	0.30	0.30
L-Lys	0.30	0.49	0.52
DL-Met	0.10	0.10	0.10
L-Thr	0.10	0.10	0.10
Vitamin premix[4]	0.05	0.05	0.05
Mineral premix[5]	0.20	0.20	0.20
Cr_2O_3	0.40	0.40	0.40
Total	100.00	100.00	100.00
Calculated nutrient level			
Digestible energy, MJ/kg	14.26	14.26	14.26
Crude protein	15.54	15.54	15.54
Calcium	0.66	0.66	0.66
Total phosphorus	0.57	0.55	0.56
Available phosphorus	0.32	0.31	0.33
Chemical analysis			
Dry matter	85.74	86.55	86.87
Crude fiber	2.51	4.43	4.01
Neutral detergent fiber	8.61	12.96	11.64
Total glucosinolate, μmol/g	–	6.24	2.82

SBM = soybean meal; RSM = rapeseed meal; FRSM = fermented rapeseed meal.
[1] CSD = corn-soybean meal diet.
[2] RSD = rapeseed meal diet.
[3] FRSD = fermented rapeseed meal diet.
[4] Vitamin premix provided following per kilogram of diet: vitamin A, 15,000 IU; vitamin D_3, 5000 IU; vitamin E, 24 IU; vitamin K_3, 5.0 mg; vitamin B_1, 5.0 mg; vitamin B_2, 12.5 mg; vitamin B_6, 6.0 mg; vitamin B_{12}, 0.3 mg; niacin, 50.0 mg; pantothenic acid, 25.0 mg; folic acid, 2.5 mg; biotin, 0.25 mg.
[5] The premix provided following per kg diet: Fe ($FeSO_4 \cdot H_2O$) 60.0 mg; Cu ($CuSO_4 \cdot 5H_2O$) 4.0 mg; Zn ($ZnSO_4$) 60.0 mg; Mn ($MnSO_4$) 2.0 mg; I (KI) 0.14 mg; Se (Na_2SeO_3) 0.2 mg.

2.4. Animal trial

A total of 30 barrows (Duroc × Landrace × Yorkshire, DLY) with initial body weight (IBW) of 17.46 ± 1.97 kg were used for the trial. The pigs were randomly assigned to one of three dietary treatments, with ten replicate pigs per diet. Pigs were housed in metabolic crates (2.5 m × 1.8 m × 0.8 m) with a nipple drinker, feeder, and slatted floor. The average ambient temperature was maintained at 24 °C. The experiment was conducted for 30 d in 3 phases, consisting of an adaptation period (d 1 to 5), a feeding period (d 6 to 26), and a metabolism period (d 27 to 30). Feed was freely supplied to pigs during the feeding period. In the metabolism phase, the feed intake was equal to their average daily intake in the feeding period. The body weight of each pig was measured in the mornings of d 6 and 27. Feed intake and residue were weighed and recorded daily for 21 d during the feeding period. The average daily gain (ADG), average daily feed intake (ADFI), and the ratio of feed to gain (F:G) were calculated. Daily feeds were offered at 08:00, 14:00, and 20:00.

2.5. Performance measurement and sampling

In the metabolism period, feces were weighed and samples from 4 consecutive days were collected in sample bags, and stored at −20 °C immediately after collection. Urine collections were initiated on d 26 at 09:00 and ceased on d 29 at 09:00. Urine buckets were placed under the metabolic crates and emptied twice daily, and a preservative of 50 mL of sulfuric acid was added to each bucket when emptied. The collected urine was weighed and a 10% subsample was stored at −20 °C. At the end of the experiment, the feces and urine were mixed according to treatment groups. Feces were oven-dried at 60 °C to a constant weight, then smashed to pass through a 1.0-mm screen for chemical analysis. On 31 d, the blood samples were collected from the jugular vein, centrifuged at 3,500 × *g* at 4 °C for 15 min, and the serum samples were stored at −20 °C until analysis. After blood collection, pigs were anesthetized with an intravenous injection of sodium pentobarbital (200 mg/kg BW) before being slaughtered and the tissue of the pancreas was immediately collected and stored at −80 °C. Section of duodenum, jejunum, and ileum was immediately isolated (approximately 4 cm of each part) and gently flushed with ice-cold phosphate-buffered saline and then fixed in 4% paraformaldehyde solution for morphological analysis. The mucosa samples were scraped with a scalpel blade from the duodenum, jejunum, and ileum segments and preserved at −80 °C until analysis. The digesta samples from the end of the ileum were collected in sample bags and stored at −20 °C. The samples were lyophilized and smashed before analysis. Digesta from cecum and colon were collected in sterilized cryopreservation tubes and stored at −80 °C until analysis.

2.6. Chemical analysis

The feces and diets were analysed for dry matter (DM) (Method 934.01; AOAC, 2007), crude protein (CP) (Method 976.05; AOAC, 2007), crude fiber (CF), acid detergent fiber (ADF), neutral detergent fiber (NDF) (Van Soest et al., 1998), ether extract (EE) (Method 922.06; AOAC, 2007) and ash (Method 961.14; AOAC, 2007). Urine samples were analysed for CP and gross energy (GE). The GE of all samples was determined using an adiabatic oxygen bomb calorimeter (Parr Instrument Co., Moline, IL). The amino acid (AA) profile of digesta, ingredients and diets were analysed using an AA analyser (L8800; Hitachi, Tokyo, Japan). Chromic oxide in diets and ileum digesta was determined by spectrophotometry at 440 nm after ashing at 450 °C overnight (Fenton and Fenton, 1979).

2.7. Serum parameter analysis

The serum immunoglobulin (Ig) G, IgM, IgA, ghrelin, leptin, and glucagon-like peptide-1 (GLP-1) were measured using the corresponding ELISA kits (Jiangsu Meimian Industrial Co., Ltd., Jiangsu, China). All procedures were carried out according to the instructions of the kits. The absorbance of each reaction was determined using an ultraviolet–visible spectrophotometer (SFZ1606017568, Shanghai Lengguang Technology Co., Ltd, China) at a wavelength of 450 nm.

2.8. Enzyme activity

The duodenal, jejunal, ileal mucosa, and tissue of the pancreas were homogenized in chilled normal saline at a ratio of 1:9 (w/v) for 5 min. Then centrifuged at 3,500 × *g* at 4 °C for 10 min, the supernatant was used to measure enzyme activities, including the α-amylase, trypsin, lipase, lactase, maltase, and sucrase. The enzyme activities were determined using the assay kit purchased (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Enzyme activity was defined as the hydrolysis of 1 mol of the substrate per milligram of protein tissue per minute under the condition of 37 °C, pH = 6.0.

2.9. Intestinal morphology analysis

Tissue samples from mid-duodenum, jejunum and ileum were fixed in 10% buffered formalin, dehydrated in graded ethanol and xylene baths, embedded in paraffin wax, sliced into tissue sections, stained with hematoxylin and eosin (H&E) staining, covered with coverslips and sealed with resin for further assessment. All morphological features (villus height and crypt depth) were analysed using a microscope (BX41, Olympus Corporation, Tokyo, Japan) and an analysis system (Media Cybernetics, Bethesda, MD, USA). Ten replicate measurements for each of the 10 pigs per dietary treatment were taken and averaged for statistical analysis. Villus height was calculated from the tip of the villus to the villus–crypt junction. Crypt depth was measured from the base to the villus–crypt junction. The V:C ratio was calculated by villus height dividing crypt depth.

2.10. Intestinal gene expression

The frozen duodenal, jejunal, and ileal mucosa samples (about 0.1 g) were homogenized in 1 mL Trizol Reagent (TaKaRa, Beijing, China) to extract total RNA. Then, the concentration and purity of total RNA were assayed by a spectrophotometer (Nan Drop, Gene Company Limited, Guangzhou, China) at 260 and 280 nm, and samples whose OD260/OD280 ratio ranged from 1.8 to 2.0 were deemed appropriate. Subsequently, the RNA samples were prepared for transcription into cDNA. This procedure is based on the PrimeScript RT reagent kit protocol with gDNA Eraser (Takara Biotechnology (Beijing) Co., Ltd., Beijing, China). The transcription process consisted of two steps: (1) 37 °C for 15 min, (2) 85 °C for 5 s. The expression level of the target gene in intestinal mucosa was quantified using the CFX-96 real-time PCR detection system (Bio-Rad Laboratories, California, USA) and SYBR Premix Ex Taq II (Tli RNaseH Plus) reagents (Takara Biotechnology (Beijing) Co., Ltd., Beijing, China). The oligonucleotide primers sequences for intestinal barrier (*ZO-1*, occludin, claudin-1) and functional genes (*MUC1*, *SGLT-1*, *GLUT-2*, *PepT1*, *EAAC1*, *CAT1*) that were used in q-PCR are presented in Table S3. The 10 μL PCR reaction volume, which contained 5 μL of SYBR Premix Ex Taq II (Tli RNaseH Plus), 0.4 μL forward and reverse primer, 1 μL of the cDNA sample and 3.2 μL of RNase-Free water, was run following the q-PCR protocol: 95 °C for

30 s, followed by 40 cycles at 95 °C for 5 s and 60 °C for 30 s. The generated gene-specific amplification products were confirmed by melting curve analysis after each real-time quantitative PCR assay. The housekeeping gene β-actin was used to standardize the mRNA expression level of target genes, which was calculated based on the $2^{-\Delta\Delta Ct}$ method (Fleige et al., 2006).

2.11. Microbiota composition of digesta

The content of total bacteria, *Lactobacillus*, *Escherichia coli*, *Bacillus*, and *Bifidobacterium* in cecal and colonic digesta were determined with the q-PCR technique. The 20 μL PCR reaction volume, which contained 10 μL 2× SuperReal PreMix (Probe), 1 μL forward and reverse prime, 0.8 μL fluorescence probe, 1 μL DNA sample and 6.2 μL RNase-Free ddH_2O, was run following the q-PCR protocol 95 °C for 10 s, followed by 50 cycles at 95 °C for 5 s, 57 °C for 25 s and 72 °C for 30 s. Primer sequences and probes of cecal and colonic digesta used for real-time PCR are presented in Table S4.

2.12. Volatile fatty acids analysis

The content of acetate, propionate, and butyrate were determined by gas chromatograph (GC) (VARIAN CP-3800, California, USA). For this determination, thawed cecal and colonic digesta (0.5 g) were homogenized in 1.3 mL ultrapure water, allowed to stand for 30 min, and then centrifuged at 10,000 × *g* at 4 °C for 15 min. One milliliter supernatant was mixed with 0.2 mL of 25% (w/v) metaphosphoric acid solution and 23.3 μL 10 mmol/L crotonic acid solution before being placed at 4 °C for 30 min and then centrifuged at 10,000 × *g* at 4 °C for 10 min. Briefly, 0.9 mL chromatographic methanol was added to 0.3 mL of supernatant and then centrifuged at 10,000 × *g* at 4 °C for 5 min. The supernatant was filtered through a 0.22-μm filter membrane for the following analysis. One microliter prepared sample was injected into a column of GC for measurement.

2.13. Calculations

Apparent digestibility, nitrogen availability, and apparent ileal digestibility (AID) of diets were calculated using the following equations (Austin, 2000), and Cr_2O_3 was used as an indigestible marker:

$$\text{AID}\ (\%) = \left(1 - \frac{Cr_2O_3\ \text{in diet}\ (\%)}{Cr_2O_3 \text{in digesta}\ (\%)} \times \frac{\text{nutrient in digesta}\ (\%)}{\text{nutrient in diet}\ (\%)}\right) \times 100.$$

The energy voided in feces and urine were calculated for each diet, and the digestible energy (DE) and metabolic energy (ME) in each of the 3 treatments were also calculated as follows:

$$\text{DE}\ (\text{MJ}/\text{kg}) = \left(\frac{\text{gross energy intake} - \text{energy in feces}}{\text{feed intake}}\right),$$

$$\text{ME}\ (\text{MJ}/\text{kg}) = \left(\frac{\text{gross energy intake} - \text{energy in feces} - \text{energy in urine}}{\text{feed intake}}\right).$$

2.14. Statistical analysis

Data were analysed using SAS software 9.2 (SAS Inst. Inc., Cary, NC, USA). The homogeneity of the variance among treatments was verified using Levene's test. Treatment means were compared by Duncan's multiple comparisons. All results are expressed as means and standard error of the mean (SEM). Statistical significance was considered at $P < 0.05$, and $0.05 < P < 0.10$ was considered a tendency.

3. Results

3.1. Growth performance and apparent nutrient digestibility

The IBW did not differ among the three treatments; however, pigs fed with FRSD showed a higher FBW than the pigs fed with RSD (Table 2). The ADG and ADFI were higher in the FRSD and CSD groups than in the RSD group ($P < 0.01$). As compared to the RSD group, the FRSD group had a lower F:G ($P = 0.02$). The digestibility of CP and ADF was higher in the CSD group than in FRSD group ($P < 0.01$). The digestibility of CP, EE, NDF, and ADF was higher in the FRSD group than in RSD group ($P < 0.01$).

3.2. Ileal apparent digestibility of amino acids

As compared to the CSD group, RSD feeding significantly decreased the AID of CP and essential amino acids such as the His, Ile, Leu, Lys, Phe, Thr, and Val ($P < 0.01$). However, compared with RSD, FRSD feeding significantly increased their AID and also improved the AID of nonessential amino acids such as the Pro and

$$\text{Apparent digestibility}\ (\%) = \left(\frac{\text{nutrient intake} - \text{nutrient voided in feces}}{\text{nutrient intake}}\right) \times 100,$$

$$\text{Nitrogen availability}\ (\%) = \left(\frac{\text{nitrogen intake} - \text{nitrogen in feces} - \text{nitrogen in urine}}{\text{nitrogen intake}}\right) \times 100,$$

Table 2
Effect of FRSM on growth performance and apparent digestibility (as-fed basis).[1]

Item	CSD[2]	RSD[3]	FRSD[4]	SEM[5]	*P*-value
IBW, kg	17.53	17.39	17.46	0.36	0.99
FBW, kg	35.00[a]	31.40[c]	33.30[b]	0.42	<0.01
ADG, kg/day	0.83[a]	0.67[b]	0.75[a]	0.02	<0.01
ADFI, kg/day	1.39[a]	1.18[c]	1.26[b]	0.02	<0.01
F:G	1.63[b]	1.85[a]	1.64[b]	0.04	0.02
DM, %	95.27[a]	93.18[b]	94.03[ab]	0.03	<0.01
CP, %	94.50[a]	86.75[c]	90.41[b]	0.69	<0.01
CF, %	83.73[a]	72.25[b]	75.53[ab]	1.09	<0.01
NDF, %	80.67[a]	72.90[b]	80.14[a]	0.92	<0.01
ADF, %	81.87[a]	70.16[c]	75.84[b]	1.07	<0.01
EE, %	89.24[a]	86.63[b]	89.06[a]	0.34	<0.01

FRSM = fermented rapeseed meal; IBW = initial body weight; FBW = final body weight; ADG = average daily gain; ADFI = average daily feed intake; F:G = feed to gain ratio; DM = dry matter; CP = crude protein; CF = crude fiber; NDF = neutral detergent fiber; ADF = acid detergent fiber; EE = ether exact.
[a, b, c] Different superscripts represents a significant difference ($P < 0.05$).
[1] Data are shown as mean and SEM.
[2] CSD = corn-soybean meal diet.
[3] RSD = rapeseed meal diet.
[4] FRSD = fermented rapeseed meal diet.
[5] SEM = standard error of the mean.

Ser ($P < 0.05$) (Table 3). There were no differences in AID of CP, Lys, Met, Phe and Pro between the CSD and FRSD group.

3.3. Availability of energy and nitrogen

As shown in Table 4, the DE and ME were higher in the FRSD group (14.86 and 14.66 MJ/kg, respectively) than in the RSD group (14.49 and 14.25 MJ/kg, respectively), and the DE of FRSD was lower than that of CSD ($P < 0.01$). There were no differences in the ME value between the FRSD and CSD groups (Table 4). The nitrogen retention and apparent availability of RSD and FRSD were lower than those of CSD ($P < 0.01$). However, compared with RSD feeding, FRSD feeding significantly elevated nitrogen retention and nitrogen apparent availability ($P < 0.01$) (Table 4).

Table 3
Effect of FRSM on nutrient AID in growing pigs (%, as-fed basis). [1]

Item	CSD[2]	RSD[3]	FRSD[4]	SEM[5]	*P*-value
CP	73.50[a]	62.16[b]	69.86[a]	1.24	<0.01
Essential AA					
Arg	81.51	74.97	77.97	1.10	0.05
His	78.59[a]	67.74[c]	73.36[b]	1.10	<0.01
Ile	78.32[a]	69.57[b]	69.45[b]	1.25	<0.01
Leu	80.50[a]	72.26[b]	75.19[b]	1.02	<0.01
Lys	83.04[a]	75.87[b]	80.03[a]	0.85	<0.01
Met	86.04	84.42	85.99	0.53	0.41
Phe	79.51[a]	72.26[b]	75.95[ab]	0.93	<0.01
Thr	74.39[a]	63.19[c]	68.67[b]	1.29	<0.01
Val	75.08[a]	64.82[b]	69.64[b]	1.25	<0.01
Nonessential AA					
Ala	74.58[a]	66.07[b]	68.08[b]	1.25	0.01
Asp	73.64[a]	66.32[b]	69.02[b]	0.94	<0.01
Cys	76.48[a]	62.36[b]	65.55[b]	1.71	<0.01
Glu	77.33	73.75	75.64	0.86	0.27
Gly	42.07	35.06	45.53	3.03	0.35
Pro	70.44[a]	50.67[b]	59.99[ab]	2.61	<0.01
Ser	75.70[a]	59.66[c]	65.69[b]	1.62	<0.01
Tyr	73.55[a]	59.18[b]	60.05[b]	1.70	<0.01
Total AA	72.89	64.81	69.36	1.35	0.05

FRSM = fermented rapeseed meal; AID = apparent ileal digestibility; CP = crude protein; AA = amino acids.
[a,b,c] Different superscripts represents a significant difference ($P < 0.05$).
[1] Data are shown as mean and SEM.
[2] CSD = corn-soybean meal diet.
[3] RSD = rapeseed meal diet.
[4] FRSD = fermented rapeseed meal diet.
[5] SEM = standard error of the mean.

Table 4
Effect of FRSM on energy and nitrogen availability (as-fed basis) .[1,2]

Item	CSD[3]	RSD[4]	FRSD[5]	SEM[6]	*P*-value
Diet intake, kg/d	1.48[a]	1.28[b]	1.40[a]	0.02	<0.01
GE intake, MJ/d	23.26[a]	19.68[b]	22.07[a]	0.38	<0.01
GE in feces, MJ/d	1.00[b]	1.17[ab]	1.33[a]	0.02	<0.01
DE in diet, MJ/kg	15.03[a]	14.49[c]	14.86[b]	0.05	<0.01
GE in urine, MJ/d	0.29	0.29	0.27	0.01	0.69
ME in diet, MJ/kg	14.84[a]	14.25[b]	14.66[a]	0.05	<0.01
Nitrogen intake, g/d	35.55[a]	30.71[b]	33.54[a]	0.55	<0.01
Nitrogen in feces, g/d	1.30[b]	1.99[a]	2.00[a]	0.08	<0.01
Nitrogen in urine, g/d	2.23	2.08	1.93	0.10	0.54
Nitrogen retention, g/d	32.02[a]	26.63[c]	29.61[b]	0.57	<0.01
Nitrogen apparent digestibility in diet, %	94.50[a]	86.75[c]	90.41[b]	0.69	<0.01
Nitrogen apparent availability in diet, %	85.68[a]	74.22[c]	80.73[b]	1.13	<0.01

FRSM = fermented rapeseed meal; GE = gross energy; DE = digestible energy; ME = metabolic energy.
[a, b, c] Different superscripts represents a significant difference ($P < 0.05$).
[1] Data are shown as mean and SEM.
[2] Diet intake, feces output, and urine output were based on 4 d collection.
[3] CSD = corn-soybean meal diet.
[4] RSD = rapeseed meal diet.
[5] FRSD = fermented rapeseed meal diet.
[6] SEM = standard error of the mean.

3.4. Serum parameter and pancreatic enzyme activity

The serum concentrations of IgA, IgG, and ghrelin were higher in FRSD and CSD groups than in the RSD group ($P < 0.05$). The RSD group had a higher concentration of leptin than the FRSD and CSD groups (Table 5). The pancreas enzyme activities of α-amylase and trypsin were higher in the FRSD group than in the RSD group ($P < 0.01$). The lipase activity of FRSD was higher than that of CSD and RSD, and the α-amylase activity was lower than that of CSD ($P < 0.05$).

3.5. Intestinal morphology and mucosa enzyme activity

As compared to the CSD group, RSD feeding significantly decreased the villus height and V:C ratio in the duodenum and jejunum ($P < 0.05$; Table 6; Fig. 1). However, compared with RSD, FRSD feeding not only increased the villus height and the V:C ratio in the duodenum and jejunum, but also increased the V:C ratio in the ileum ($P < 0.05$). The enzyme activities of maltase and sucrase

Table 5
Effect of FRSM on serum parameters and pancreatic enzyme activities in growing pigs[1].

Item	CSD[2]	RSD[3]	FRSD[4]	SEM[5]	*P*-value
Serum parameters					
IgA, μg/mL	28.11[a]	25.18[b]	26.72[a]	0.31	<0.01
IgG, μg/mL	283.42[a]	264.27[b]	285.55[a]	3.41	0.01
IgM, μg/mL	26.59[a]	23.69[b]	25.41[ab]	0.42	0.01
Ghrelin, ng/L	1612.37[a]	1503.81[b]	1724.11[a]	33.68	0.02
Leptin, ng/L	1198.2[b]	1277.32[a]	1211.56[b]	13.90	0.04
GLP-1, pmol/L	2.60	2.67	2.55	0.04	0.47
Pancreas enzymes					
Alpha-amylase, U/mgprot	149.11[a]	77.06[c]	113.84[b]	8.60	<0.01
Lipase, U/gprot	10.25[b]	8.97[b]	13.63[a]	0.64	0.01
Trypsin, U/mgprot	809.39[a]	586.65[b]	771.79[a]	30.03	<0.01

FRSM = fermented rapeseed meal; IgA = immunoglobulin A; IgG = immunoglobulin G; IgM = immunoglobulin M; GLP-1 = glucagon like peptide-1.
[a, b, c] Different superscripts represents a significant difference ($P < 0.05$).
[1] Data are shown as mean and SEM.
[2] CSD = corn-soybean meal diet.
[3] RSD = rapeseed meal diet.
[4] FRSD = fermented rapeseed meal diet.
[5] SEM = standard error of the mean.

Table 6
Effect of FRSM on intestinal morphology and brush border enzyme activity[1].

Item	CSD[2]	RSD[3]	FRSD[4]	SEM[5]	*P*-value
Duodenum					
Villus height, μm	622.71[a]	500.01[b]	617.19[a]	19.57	0.01
Crypt depth, μm	411.41	372.75	352.47	10.62	0.06
V:C ratio	1.55[b]	1.36[c]	1.81[a]	0.05	<0.01
Lactase, U/mgprot	6.07	5.06	6.07	0.36	0.39
Maltase, U/mgprot	119.44[a]	90.18[b]	100.03[a]	4.07	<0.01
Sucrase, U/mgprot	9.31[a]	5.71[b]	7.78[a]	0.45	<0.01
Jejunum					
Villus height, μm	649.24[a]	516.41[c]	580.23[b]	15.91	<0.01
Crypt depth, μm	311.19[a]	304.96[a]	264.92[b]	7.74	0.03
V:C ratio	2.17[a]	1.78[b]	2.16[a]	0.05	<0.01
Lactase, U/mgprot	83.05[b]	72.15[b]	113.52[a]	6.55	0.02
Maltase, U/mgprot	746.68[a]	484.66[b]	688.91[a]	32.83	<0.01
Sucrase, U/mgprot	37.45[a]	14.39[b]	35.97[a]	2.63	<0.01
Ileum					
Villus height, μm	450.19[a]	364.79[b]	360.37[b]	9.67	<0.01
Crypt depth, μm	241.63[a]	226.77[a]	201.60[b]	5.51	0.01
V:C ratio	1.93[a]	1.66[b]	1.83[a]	0.04	<0.01
Lactase, U/mgprot	0.92[a]	0.46[b]	1.04[a]	0.08	0.01
Maltase, U/mgprot	90.20[a]	73.58[b]	93.00[a]	2.98	0.01
Sucrase, U/mgprot	18.66[a]	11.20[b]	20.55[a]	1.48	0.02

FRSM = fermented rapeseed meal; V:C ratio = villus height to crypt depth ratio.
[a, b, c] Different superscripts represents a significant difference ($P < 0.05$).
[1] Data are shown as mean and SEM.
[2] CSD = corn-soybean meal diet.
[3] RSD = rapeseed meal diet.
[4] FRSD = fermented rapeseed meal diet.
[5] SEM = standard error of the mean.

in the small intestinal mucosa were higher in the FRSD and CSD groups than in the RSD group (Table 6). As compared to the RSD group, the FRSD feeding also increased the enzyme activity of lactase in the jejunal and ileal mucosa ($P < 0.05$).

3.6. Expression levels of critical functional genes in the intestinal epithelium

As compared to the RSD group, the FRSD feeding significantly elevated the expression levels of occludin and *ZO-1* in the jejunal and ileal mucosa ($P < 0.05$). Compared with CSD and RSD, FRSD feeding also elevated the expression levels of claudin-1 and occludin in the duodenum (Fig. 2). As compared to the CSD group, RSD feeding decreased the expression levels of *MUC1* and *CAT1* in the duodenum and decreased the expression levels of *SGLT1* and *PepT1* in the jejunum; however, compared with RSD feeding, FRSD feeding significantly elevated their expression levels ($P < 0.05$). Moreover, the FRSD feeding also elevated the expression levels of *PepT1* in the duodenum and *CAT1* in the duodenum and ileum compared with RSD feeding (Fig. 2).

3.7. Abundance of intestinal microbiota and microbial metabolites

As shown in Table 7, the abundances of *Lactobacillus* in the cecum and colon were higher in the FRSD group than in the RSD and CSD groups ($P < 0.01$). The CSD group had a higher abundance

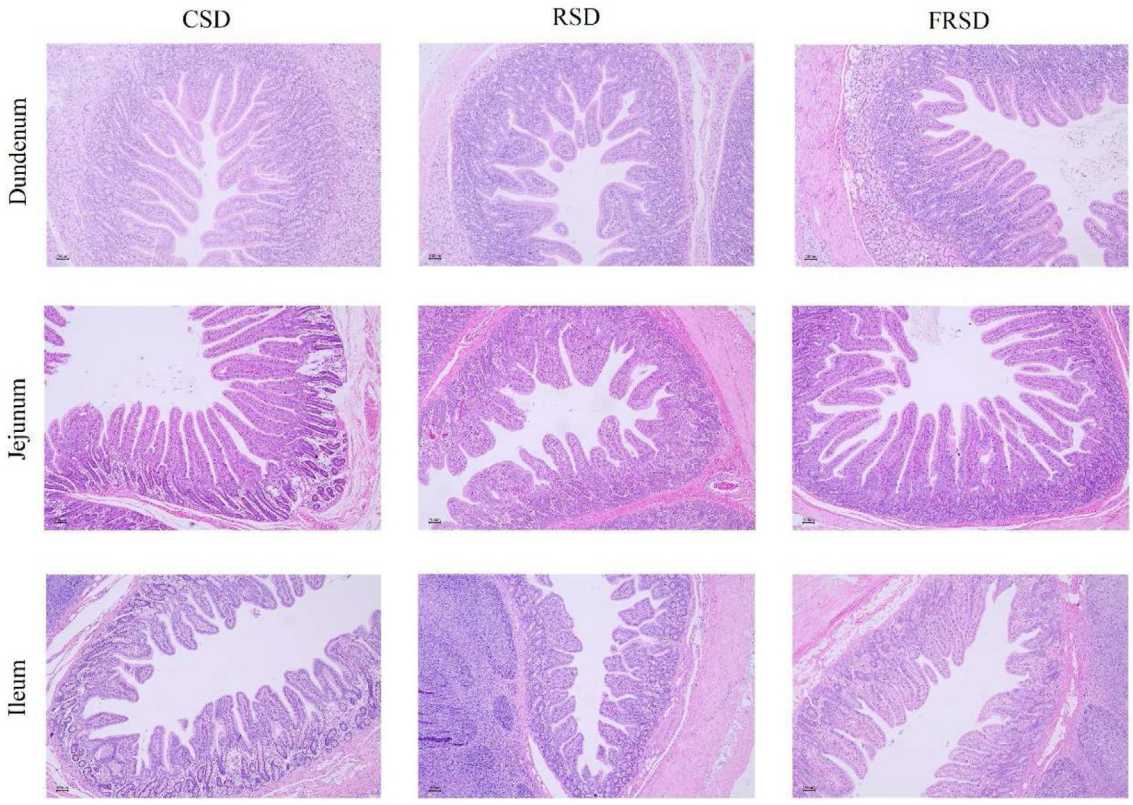

Fig. 1. Effect of FRSM on intestinal morphology in pigs (H&E staining, 40×). FRSM = fermented rapeseed meal; CSD = corn-soybean meal diet; RSD = rapeseed meal diet; FRSD = fermented rapeseed meal diet.

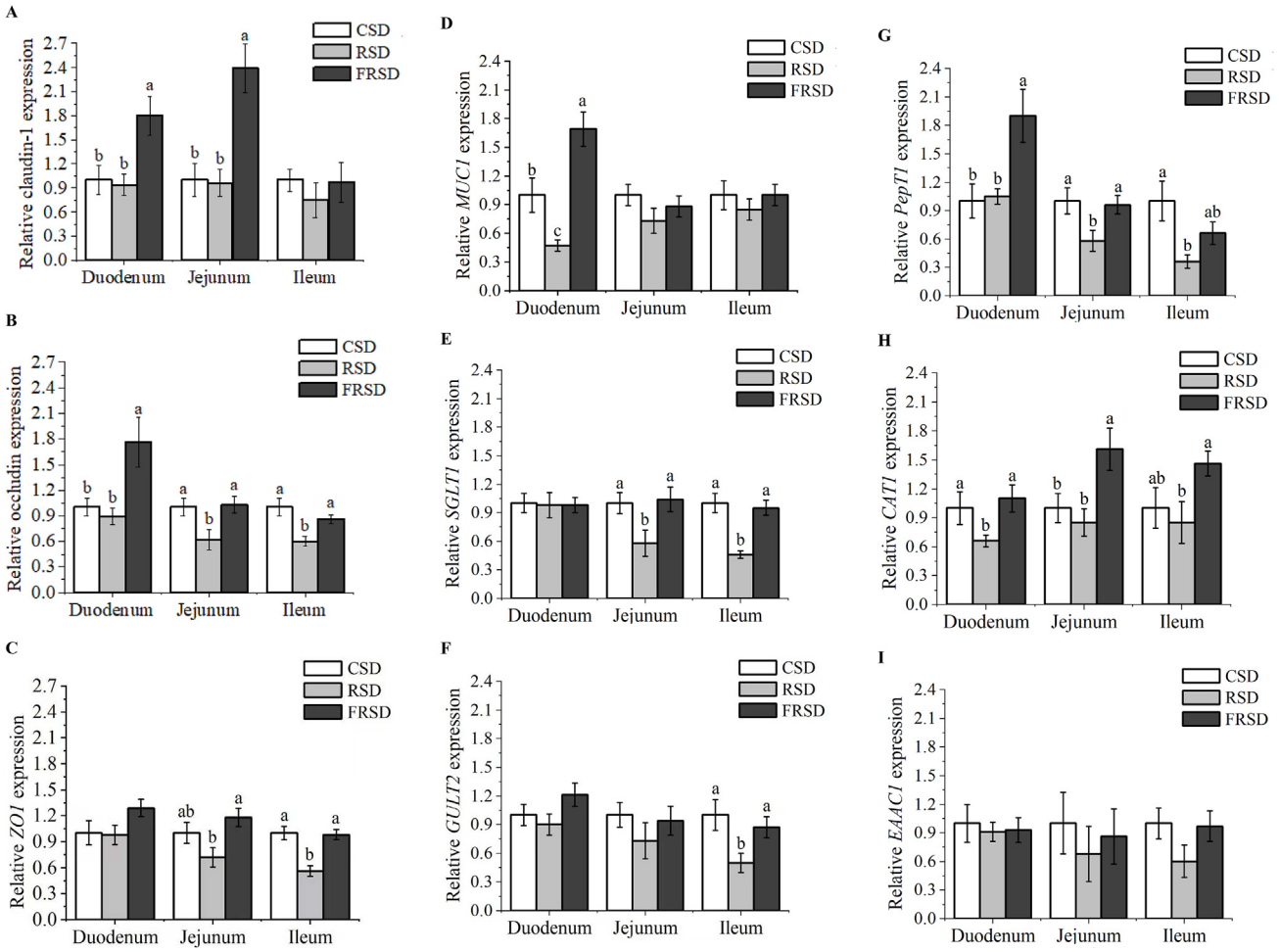

Fig. 2. Effect of FRSM on expressions of critical functional genes in pigs. (A) Relative expression of claudin-1 in three intestinal segments. (B) Relative expression of occludin in three intestinal segments. (C) Relative expression of *ZO-1* in three intestinal segments. (D) Relative expression of *MUC1* in three intestinal segments. (E) Relative expression of *SGLT1* in three intestinal segments. (F) Relative expression of *GULT2* in three intestinal segments. (G) Relative expression of *PepT1* in three intestinal segments. (H) Relative expression of *CAT1* in three intestinal segments. (I) Relative expression of *EAAC1* in three intestinal segments. FRSM = fermented rapeseed meal; CSD = corn-soybean meal diet; RSD = rapeseed meal diet; FRSD = fermented rapeseed meal diet; *ZO-1* = zonula occludens-1; *MUC1* = mucin-1; *SGLT1* = sodium/glucose cotransporter-1; *GULT2* = glucose transporter-2; *PepT1* = peptide transporter-1; *CAT1* = cationic amnio acid transporter-1; *EAAC1* = ex-citatory amino acid carrier-1. [a, b] Mean values with different letters on vertical bars indicate significant differences ($P < 0.05$).

of *Bifidobacterium* in the cecum than the RSD and FRSD groups ($P < 0.05$). Interestingly, the abundances of *E. coli* in the cecum and colon were lower in the FRSD group than in the RSD and CSD groups ($P < 0.05$). The FRSD group had higher butyrate content than the RSD and CSD groups in the cecum ($P < 0.05$). As compared to the RSD group, the FRSD feeding significantly increased the contents of acetate, and butyrate in the colon ($P < 0.05$).

4. Discussion

RSM has long been considered an ideal animal protein source as it contains a large quantity of proteins and essential amino acids (e.g., Met and Cys) (Grela et al., 2019). Woyengo et al. (2014) indicated that the SBM in the diets of pigs could be partially substituted by the RSM. However, the large-scale application of RSM has been limited because of its low digestibility compared to the SBM (McNeill et al., 2004; Wulf and Sudekum, 2005). Therefore, avenues to improve the digestibility of RSM have attracted considerable research interest worldwide. In the present study, the RSM was pretreated using joint fermentation by *B. subtilis* and enzyme complex, and the results showed that the RSD group with 6.24 μmol/g glucosinolates had lower ADG, ADFI, and higher F:G than FRSD group with 2.82 μmol/g glucosinolates. Previous studies indicated that FRSD has a positive effect on the growth of pigs compared with RSD, however, the mechanism remained unclear (Czech et al., 2021; Shi et al., 2016). Bell et al. (1999) indicated that dietary glucosinolates reduce feed intake. Clark and Slavin (2013) reported that dietary fiber has an appetite-reducing effect. According to Dimidi et al. (2019), fermentation is a great approach to enhancing the taste and texture of food, which can remove bitter compounds in the substrate. It was hypothesized that the increase in feed intake likely reflected the positive effects of fermentation on feed taste and appetite regulation. Interestingly, in the present study, FRSD feeding increased the serum concentration of appetite stimulator ghrelin but decreased the appetite-inhibiting regulator leptin concentration, which contributed to the elevated ADFI in the FRSD group. The improvement in growth performance may also relate to the energy level and nutrient digestibility. In the present study, the actual energy levels of the three diets were different, FRSD had 0.37 MJ/kg of DE higher than RSD. According to Meffeja et al. (2006), the different DE levels (2600, 2800, and 3000 kcal/kg) had no significant effect on ADFI, ADG, F:G in growing pigs, and only 3200 kcal/kg diet exhibited lower F:G than others, indicating that the improved feed efficiency in FRSD may attribute to a

Table 7
Effect of FRSM on intestinal microbiota (log_{10} cfu/g) and microbial metabolites (μmol/g)[1].

Item	CSD[2]	RSD[3]	FRSD[4]	SEM[5]	*P*-value
Cecum					
Total bacteria	10.41	10.60	10.56	0.05	0.23
Bifidobacterium	4.63[a]	3.54[b]	3.83[b]	0.17	0.02
Bacillus	4.82[b]	5.20[a]	5.22[a]	0.06	<0.01
Lactobacillus	5.84[c]	6.53[b]	7.18[a]	0.14	<0.01
E. coli	5.85[a]	6.02[a]	4.81[b]	0.20	0.03
Acetate	30.30	28.87	27.89	0.84	0.51
Propionate	15.38	14.34	15.50	0.54	0.61
Butyrate	6.42[b]	6.74[b]	10.01[a]	0.48	<0.01
Total SCFA	54.22	53.08	56.22	1.40	0.68
Colon					
Total bacteria	10.88[b]	11.07[a]	10.85[b]	0.03	<0.01
Bifidobacterium	4.87	4.12	4.10	0.19	0.17
Bacillus	5.23[b]	5.52[a]	5.55[a]	0.05	<0.01
Lactobacillus	6.34[c]	6.95[b]	7.48[a]	0.12	<0.01
E. coli	5.98[a]	6.15[a]	4.64[b]	0.22	0.01
Acetate	16.97[a]	12.64[b]	16.33[a]	0.71	0.02
Propionate	6.50	6.19	8.86	0.52	0.08
Butyrate	3.54[b]	3.91[b]	5.56[a]	0.28	<0.01
Total SCFA	31.92	24.09	31.52	1.49	0.05

FRSM = fermented rapeseed meal; SCFA = short chain fatty acids.
[a, b, c] Different superscripts represents a significant difference ($P < 0.05$).
[1] Data are shown as mean and SEM.
[2] CSD = corn-soybean meal diet.
[3] RSD = rapeseed meal diet.
[4] FRSD = fermented rapeseed meal diet.
[5] SEM = standard error of the mean.

positive effect of FRSM on nutrient digestion and the increase in DE of FRSM. Meanwhile, the efficiency of energy utilization by growing animals depends on the ingredients and energy availability in the diet. Previous studies indicated that the high levels of anti-nutritional factors (e.g., crude fiber) in RSM significantly decreased its energy value (Gädeken et al., 1985; Chibowska et al., 2000). In this study, the DE and ME values were significantly elevated in the RSM upon joint fermentation, indicating that the anti-factors were eliminated. The result is consistent with a previous study on growing pigs (Shi et al., 2015). Furthermore, there are some indications that FRSD may positively affect intestinal morphology, digestive enzyme secretion, and the digestion and absorption of dietary nutrients (Chiang et al., 2009; Li et al., 2022; Shi et al., 2016). This effect was also observed in this experiment. The FRSD feeding elevated the apparent digestibility of DM, CP, EE, ADF and elevated the nitrogen retention and nitrogen availability in pigs compared with RSD feeding, likely reflecting the increase in feed efficiency. According to Khajali and Slominski (2012), fiber content was negatively correlated with energy utilization and nutrient digestibility, and high fiber content would aggravate the emptying of the digestive tract and reduce the digestion time. The increase in the digestion ratio of CP, DM, and EE may attribute to the decrease in fiber content in the FRSD. Moreover, the digestibility of fiber increased with the destruction of cellular mechanical structures such as lignin and hemicellulose (Wilson and Mertens, 1995). The increase in fiber digestibility may be related to the cellulose, pectin, and lignin of RSM that were broken down during the fermentation and the longer digestion time of the FRSD. Previous studies indicated that the RSM contains a large quantity of anti-hydrolytic proteins (e.g., cruciferin), which decrease the nitrogen availability of RSM both in vivo and in vitro (Delisle et al., 1983; Savoie et al., 1988). The improvement of CP and nitrogen availability in FRSD may be due to the macromolecular proteins in RSM being degraded into peptides during the fermentation process. Moreover, the increase in nutrient digestibility may reflect the improvement of the digestion capacity and intestinal health of pigs.

Digestive enzymes such as amylase, lipase, and trypsin are secreted by the pancreas and play critical roles in digestion, such as decomposing starch, fat, and protein (Owsley et al., 1986). And, disaccharidases on the brush border membrane of the small intestine are responsible for carbohydrate decomposition and absorption (Nichols et al., 2003; Urriola and Stein, 2012). According to Scholten et al. (1999), pigs fed fermented diets have a higher absolute lactic acid concentration in the small intestine, which may increase pancreatic juice secretion and lower gastrointestinal pH, positively affecting digestive enzymes. In the present experiment, this resulted in an elevation in enzyme activities of α-amylase, lipase, trypsin, sucrase and maltase in the FRSD group than in the RSD group. Such a relationship has been reported by Canibe and Jensen (2012). Furthermore, the digestion and absorption capacity of the intestine is assumed to largely depend on the integrity of the morphology and intestinal barrier (Pluske et al., 1996). ZO-1 and occludin are essential proteins for maintaining intestinal barrier integrity and can selectively penetrate macromolecular substances (Turner, 2006; Weiler et al., 2005). And, mucin is secreted by goblet cells in the intestinal epithelium and is related to the mucus barrier in the small intestine (Corfield et al., 2000). Importantly, it can serve as a sensor of extracellular alterations such as cell damage or pH changes (Turner, 2009; Singh and Hollingsworth, 2006). Scholten et al. (1999) suggested that fermented diets are beneficial for mucosal structure and digestive capacity. The longer villus height, greater V:C ratio in the duodenum and jejunum, higher *ZO-1* and occludin expressions in the jejunal epithelium, and higher *MUC1* expression in the duodenum in the FRSD group than in the RSD group suggested that FRSM improved the digestive capacity of pigs. The expression of critical transporters for nutrients also reflected the capacity of digestion and absorption. Compared to the RSD, FRSD feeding significantly elevated *SGLT1*, *PepT1*, and *CAT1* expression levels in the jejunal epithelium. SGLT1 is an active glucose transport involved in glucose transportation (Johansson et al., 2013). PepT1 and CAT1 are critical transporters involved in transporting oligo-peptides and amino acids, respectively (Rosensweig et al., 1969; Turner, 2006). The results discussed above suggested that FRSM may improve growth performance by elevating intestinal health, digestive enzyme activities, and the expression of nutrient transporters.

A balanced gut flora is beneficial for the proper function of the digestive system (Song et al., 2010). Previous studies reported that fermented diets improved gastrointestinal function, decreased counts of *E. coli* and increased the counts of *Lactobacillus* (Elbaz et al., 2023; Grela et al., 2018, 2022). This was confirmed in this experiment. The FRSD feeding increased the abundance of *Lactobacillus* but decreased the abundance of *E. coli* in the caecum and colon compared with the CSD and RSD feeding. It can be speculated that the lowered pH, increased short-chain fatty acids (SCFA) in the intestinal contents, and changes in fiber structure may lead to the growth of specific microbes and inhibit harmful bacteria (Grela et al., 2019; Long and Venema, 2020). Previous studies also suggested that the degradability of dietary fiber affected SCFA production, and the reduction in Enterobacteriaceae was also related to SCFA concentration, yet the correlations remain unclear (Burnett and Hanna, 1963; Mathew et al., 1998). Notably, the colonial acetate, and total SCFA contents were elevated in the FRSD group compared with RSD. FRSD also notably increased butyrate content in the hindgut compared with CSD and RSD. SCFA were reported to have a positive effect on intestinal barrier function and modulate intestinal immune response (de Clercq et al., 2016; D'Souza et al., 2017). For instance, butyrate could upregulate the expression of mucins to reinforce the mucus layer in the intestine (Willemsen et al., 2003). This is confirmed by the elevated intestinal integrity in pigs from the FRSD group.

5. Conclusion

Joint fermentation of the RSM by enzyme complex and *B. subtilis* significantly improved its nutrient availability, as indicated by elevated growth performance, nutrient digestibilities, and intestinal epithelium functions, as well as the improved microbiota in growing pigs. The results may be helpful for developing novel protein sources for animal nutrition and the feed industry.

Author contributions

Changyi Shuai: Methodology, Formal analysis, Writing – Original Draft. **Daiwen Chen:** Funding acquisition, Project administration. **Bing Yu:** Supervision. **Yuheng Luo:** Software. **Ping Zheng:** Data Curation. **Zhiqing Huang:** Visualization. **Jie Yu:** Validation. **Xiangbing Mao:** Resources. **Hui Yan:** Validation, Software. **Jun He:** Funding acquisition, Project administration, Methodology, 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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Appendix A. Supplementary data

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