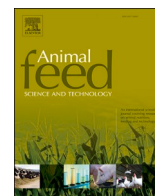




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Effects of dietary supplementation with fermented flaxseed meal on the growth performance, immune function, and intestinal microbiota of growing pigs

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ABSTRACT

Flaxseed meal is a highly nutritious feed resource rich in protein and unsaturated fatty acids. However, the presence of antinutritional factors such as cyanogenic glycoside limits its practical use. Fermentation has been demonstrated to degrade these antinutritional factors. However, little research has been conducted to investigate the effects of fermented flaxseed meal (FFSM) on the growth performance of growing pigs. This study thus aims to examine the effects of supplementing with 10 %, 15 %, and 20 % FFMSM on the growth performance, immune function, and intestinal microbiota in growing pigs. Utilizing a completely randomized design, ninety-six growing pigs, each averaging 39.57 ± 0.63 kg, were allocated into four dietary groups with eight replicates per group and three pigs per replicate. The dietary treatments included: a control group (corn-soybean meal-based diet without FFMSM) and basal diets supplemented with 10 %, 15 %, and 20 % FFMSM. The trial lasted for 30 days. Results showed that including FFMSM at 10 %, 15 %, and 20 % did not affect the average daily gain and average daily feed intake of pigs. However, the gain-to-feed ratio exhibited a linear decrease as the level of FFMSM increased. Furthermore, FFMSM supplementation linearly increases neutral detergent fiber (inclusive of residual ash) and acid detergent fiber digestibility ($P < 0.05$). Investigating immune parameters and metabolites revealed a linear increase in immunoglobulins (IgA and IgM) and total bile acid in serum with increasing levels of FFMSM supplementation ($P < 0.05$). Delving deeper into the gut microbiota's complex ecosystem reveals that the quadratic response in *Lactobacillus* abundance and linear increase in *Bacillus* abundance ($P < 0.05$), while a linear and quadratic decrease in

Abbreviations: ATTD, apparent total tract digestibility; ADF, acid detergent fiber; ALT, alanine transaminase; ASV, amplicon sequence variants; AST, aspartate transaminase; CG, cyanogenic glycosides; CP, crude protein; Cr, chromium; DM, dry matter; EE, ether extract; G:F, gain-to-feed ratio; FSM, flaxseed meal; FFMSM, fermented flaxseed meal; HDL-C, high-density lipoprotein-cholesterol; IgA, immunoglobulin A; IgM, immunoglobulin M; LDL-C, low-density lipoprotein-cholesterol; ANDF, neutral detergent fiber assayed with a heat-stable amylase and expressed inclusive of residual ash; PCoA, principal coordinates analysis; SCFA, short-chain fatty acid; TBA, total bile acid; TC, total cholesterol; TG, triglycerides.

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Clostridium_sensu_stricto_1 abundance with increasing FFSM addition ($P < 0.05$). Additionally, the concentrations of butyric acid and total short-chain fatty acids in feces changed quadratically with increasing proportions of FFSM ($P < 0.05$). The collective findings underscore the valuable role of microbial fermentation in enhancing the nutritional profile and utilization of flaxseed meal in the diets of growing pigs. Incorporation of FFSM not only augments the immune functionality of pigs but also ameliorates the balance of the intestinal microecology.

1. Introduction

Flaxseed meal (FSM), a by-product of flaxseed oil extraction, is not only rich in protein, boasting a content of up to 35 %, but also abundant in polyunsaturated fatty acids, dietary fiber, and various other nutrients (Xu et al., 2022). However, FSM is also characterized by high levels of cyanogenic glycosides (CG). These CG, when ingested, are metabolized by the β -glucosidase enzyme present in FSM to release toxic hydrogen cyanide. Prolonged exposure to elevated levels of CG has been linked to chronic toxicity, enlargement of the thyroid gland, neurological complications, and inhibited growth, among other deleterious effects in animals (Vetter, 2000). Furthermore, studies have highlighted that excessive FSM in pig diets can adversely influence feed intake and growth performance (Batterham et al., 1991; Ndou et al., 2017). As a result, the use of FSM as a plant-based protein source in animal feed formulations has been circumscribed.

Fermentation is now considered a premier strategy for the efficient reduction of CG levels in FSM, while concurrently maintaining its inherent nutritional attributes. In one study, an M-2 strain capable of producing β -glucosidase, which facilitates the degradation of hydrogen cyanide, was isolated from cow feces (Li et al., 2019). When utilized in fermented flaxseed meal (FFSM), this strain brought about a remarkable 89 % reduction in CG content and a significant 44 % increase in protein levels (Li et al., 2019). Another study employing Lactobacillaceae bacteria for flaxseed fermentation reported the complete eradication of CG within 72 h (Huang et al., 2023a). Although these results are promising, the integration of FFSM into poultry and livestock feed regimens remains limited. To date, there has been only a single study in ducks that suggests FFSM may improve dietary utilization, adjust lipid profiles, and facilitate cecal development (Zhai et al., 2019). Consequently, there is a pressing need for more comprehensive research to assess the potential benefits and applications of FFSM in the diets of growing pigs.

Building on our previous findings asserting that fermentation by *Bacillus subtilis* remarkably reduces the CG content in FFSM and favorably appraises its nutritional value for growing pigs (Wei et al., 2024). In addition, a previous study reviewed the potential benefits of fermented feeds on porcine intestinal health and growth performance. The study emphasized the degradation of anti-nutritional factors in the feed and the production of probiotics and their metabolites. These processes were found to contribute to improved gut microbial balance, increase nutrient digestibility, and enhance immune function (Wang et al., 2018). Therefore, we hypothesized that adding FFSM to feed at an appropriate level can improve the growth performance, immunity, and gut microbiota of growing pigs. To test this hypothesis, we investigated the effects of dietary supplementation with different levels of FFSM on growth performance, nutrient utilization, plasma biochemical and immune parameters, and fecal microbiome in growing pigs. The main aim of the study was to provide theoretical underpinnings and insights for applying FFSM in pig diets.

2. Materials and methods

2.1. Animal welfare statement

The animal procedures in this study were approved by the Institute Animal Care and Use Committee of the Institute of Feed Research of the Chinese Academy of Agricultural Sciences. The trial was conducted at the Tianpeng experimental farm, located in Langfang, Hebei province (IFR-CAAS20221025).

2.2. Preparation of FFSM

Flaxseed meal was purchased from Hebei Kaikuo Food Group Co., LTD (Zhangjiakou, China). The fermentation process for FFSM followed the protocol outlined in our previous study (Wei et al., 2024). The initial step in preparing the FSM was to grind the meal into a finely milled powder and then sift it through a 40-mesh screen to ensure a consistent particle size. Subsequently, a mixture was prepared at a ratio of 1:0.6 of FSM to water. To this mixture, molasses was added to constitute 2 % of the total blend. The mix was then inoculated with a 4 % volume of *Bacillus subtilis* culture at a concentration of 1.8×10^8 CFU/mL. Following a thorough mixing to ensure homogeneity, the resulting blend was packed into fermentation bags. These bags were hermetically sealed to prevent contamination and then stored for a fermentation period of 14 days at a constant temperature of 37 °C. For this study, the utilized *Bacillus subtilis* strain, known as *Bacillus subtilis* FRI, showcased a high capability for degrading complex glycans in FSM. This particular strain was sourced from the Pig Nutrition and Feed Innovation Team at the Feed Research Institute of the Chinese Academy of Agricultural Sciences. It has been cataloged in the China General Microbiological Culture Collection Center under the accession number CGMCC No. 28734. The FFSM incorporated in this experiment is sourced from the identical batch employed in our preceding research. The detailed nutritional components of FSM and FFSM are shown in Table 1.

2.3. Experimental animals and diet

Utilizing a completely randomized design, ninety-six (Duroc × Landrace × Large White) growing pigs (BW = 39.6 ± 0.63 kg) were randomly allocated to 32 pens (three pigs/pen) located in the same room and fed one of the four experimental diets (Table 2). The room temperature was controlled at 25 °C. The experimental design included four groups (eight pens/group, n = 8). One served as the control group (CON), receiving the basal diet devoid of FFSM. The three experimental groups were each provided with the basal diet supplemented with FFSM at varying concentrations: 10 % (FFSM10), 15 % (FFSM15), and 20 % (FFSM20), respectively. Furthermore, all experimental diets were supplemented with 0.3 % chromium oxide as an indigestible marker. All pigs had free access to water and were fed *ad libitum*. The feeding trial lasted for 30 days. The experimental diets were formulated according to the nutritional requirements of growing pigs (NRC, 2012).

2.4. Sample collection

The experimental pigs were weighed in the morning on days 0 and 30, while feed intake was recorded for each pen during the entire course of the experiment; and the average daily feed intake, average daily gain, and average gain-to-feed ratio (G:F) were calculated. On days 28–30 of the experiment, fresh feces were collected from the anus of each pig and dried in the oven for 72 h at 65 °C. Then, these drying fecal samples were crushed and stored at 4 °C for further nutrient determination. At the end of the trial, one pig from each pen (replicate) was randomly selected for anterior vena cava blood and fresh feces sampling. The blood sample was centrifuged at 3000 g for 10 min at 4 °C to obtain the plasma sample and was stored at –20 °C for analysis. The fresh feces were snap-frozen and stored at –80 °C for microbiological and short-chain fatty acid (SCFA) analysis.

The nutritional composition of the FSM, FFSM, the formulated diets, and fecal samples was assayed following the Official Methods of Analysis by AOAC International (AOAC 2006). The following parameters were analyzed: Dry matter (DM) was quantified using method 930.15, crude protein (CP) was measured by method 990.03, ether extract (EE) was determined using method 920.39, calcium was assessed by method 967.30, phosphorus was evaluated using method 965.17, and ash was estimated following method 942.05. The quantification of neutral detergent fiber assayed with a heat-stable amylase and expressed inclusive of residual ash (aNDF) and acid detergent fiber (ADF) was performed following the procedure from previous research (Van Soest et al., 1991). The energy content in fecal samples was measured employing a bomb calorimeter (Parr 6300 Calorimeter, Moline, IL, USA). The amino acids (Park et al., 2021) and chromium (Cr) levels (Williams et al., 1962) were determined according to the recommended methods. According to the

Table 1
Chemical components of flaxseed meal (FSM) and fermented flaxseed meal (FFSM) (as-DM basis).

Items	FSM	FFSM
DM basis (g/kg)		
DM	904.0	859.1
CP	290.9	300.2
EE	89.1	71.1
aNDF	320.5	214.7
ADF	169.0	149.9
Ca	3.5	3.9
P	9.0	10.2
GE, MJ/kg	19.74	20.10
CG, mg/kg	184.94	84.91
Essential amino acids, g/kg		
Arginine	21.7	24.9
Histidine	6.8	7.5
Isoleucine	9.9	10.3
Leucine	20.4	22.4
Lysine	13.3	16.8
Methionine	5.5	7.3
Phenylalanine	13.3	13.8
Threonine	12.0	12.0
Tryptophan	5.5	6.5
Valine	13.7	13.7
Non-essential amino acids, g/kg		
Alanine	13.2	13.7
Asparagine	27.8	27.8
Cystine	5.7	6.0
Glutamine	64.2	64.5
Glycine	14.9	16.0
Serine	13.9	15.8
Tyrosine	7.5	7.7
Total amino acids	269.4	286.7

GE = gross energy; DM = dry matter; CP = crude protein; EE = ether extract; aNDF = neutral detergent fiber assayed with a heat-stable amylase and expressed inclusive of residual ash; ADF = acid detergent fiber; P = phosphorus; Ca = calcium; CG = cyanogenic glycosides.

Table 2
Ingredient composition and nutritional level of the diets (as-fed basis).

Items	Dietary treatments ^a			
	CON	FFSM10	FFSM15	FFSM20
Ingredient (g/kg)				
Corn	680.0	632.7	609.0	585.6
Soybean meal, 46 % CP	180.0	125.5	98.0	70.5
Wheat bran	95.7	96.4	95.7	95.7
Fermented flaxseed meal	0	100.0	150.0	200.0
Soybean oil	5.3	6.7	7.8	8.6
Dicalcium phosphate	7.0	5.0	5.0	4.3
Limestone	13.2	13.5	13.5	13.5
L-Lysine HCl, 65 %	6.6	8.0	8.7	9.5
DL-Methionine	0.7	0.6	0.6	0.6
L-Threonine	1.5	1.8	2.0	2.1
L-Tryptophan	0.5	0.3	0.2	0.1
Salt	4.0	4.0	4.0	4.0
Phytase ^b	0.2	0.2	0.2	0.2
Premix ^c	2.3	2.3	2.3	2.3
Chromic oxide	3.0	3.0	3.0	3.0
Analyzed nutrient levels (g/kg)				
GE, MJ/kg	15.91	16.15	16.20	16.27
DM	884.6	884.6	882.9	881.9
CP	143.1	145.1	142.4	145.7
Ash	59.9	56.6	68.5	69.3
EE	18.1	16.0	17.6	19.9
Ca	7.9	7.1	7.0	7.7
P	4.0	3.6	4.1	4.4
aNDF	112.3	121.3	119.6	117.8
ADF	41.9	49.2	44.8	58.3

DM = dry matter; CP = crude protein; Ca = calcium; P = phosphorus; aNDF = neutral detergent fiber assayed with a heat-stable amylase and expressed inclusive of residual ash; ADF = acid detergent fiber; EE = ether extract; GE = gross energy.

^a CON, FFSM10, FFSM15, and FFSM20 diets were prepared by adding different levels of 0 %, 10 %, 15 %, and 20 % fermented flaxseed meal (FFSM), respectively.

^b The phytase (Yidoli Biotechnology Co., Ltd., Neimenggu, China) provided 2000 FTU of enzymatic activity per kg of the experimental diets.

^c Premix supplied per kg of diet: vitamin A, 35.2 mg; vitamin D₃, 7.68 mg; vitamin E, 128 mg; vitamin K₃, 8.16 mg; vitamin B₁, 4 mg; vitamin B₂, 12 mg; vitamin B₆, 8.32 mg; vitamin B₁₂, 4.8 mg; niacin, 38.4 mg; calcium pantothenate, 25 mg; folic acid, 1.68 mg; biotin, 0.16 mg; zinc (ZnSO₄·H₂O), 110 mg; copper (CuSO₄·5 H₂O), 125 mg; iron (FeSO₄·H₂O), 171 mg; cobalt (CoCl₂), 0.19 mg; manganese (MnSO₄·H₂O), 42.31 mg; iodine (Ca(IO₃)₂), 0.54 mg; selenium (Na₂SeO₃), 0.19 mg.

research method described by [Stein et al. \(2007\)](#), the apparent total tract digestibility (ATTD) of each nutrient was calculated using the following equation:

$$\text{ATTD of nutrients} = 1 - [(\text{Nutrient}_{\text{feces}} / \text{Nutrient}_{\text{diet}}) \times (\text{Cr}_{\text{diet}} / \text{Cr}_{\text{feces}})],$$

in which Nutrient_{feces} and Cr_{feces} represent the concentrations of nutrient and Cr in the feces (g/kg DM). Nutrient_{diet} and Cr_{diet} represent the concentration of nutrients and Cr in the diet (g/kg DM).

2.5. Determination of immunoglobulins and biochemical indexes in plasma

The serum levels of insulin-like growth factor 1, growth hormone, total bile acid (TBA), immunoglobulin A (IgA), immunoglobulin G, and immunoglobulin M (IgM) were assessed using commercial ELISA kits (Enzyme-linked Biotechnology Co., Ltd., Shanghai, China), following the recommended analysis procedures. The serum levels of alanine transaminase (ALT), aspartate transaminase (AST), glucose, total protein, albumin, low-density lipoprotein-cholesterol (LDL-C), high-density lipoprotein-cholesterol (HDL-C), total cholesterol (TC), and triglycerides (TG) were measured using commercial kits (Maccura Biotechnology Co., Ltd., Chengdu, China) by using the fully automatic analyzer (Erba Biochemistry Analyzer XL-200, Germany).

2.6. The 16 S rRNA sequence of microbes in feces

The fecal samples were cryopreserved at -80°C and shipped to Major Bio (Shanghai, China) for DNA extraction. High-throughput sequencing technology was employed for 16 S rRNA gene sequencing. The DNA concentration was assessed using a Nanodrop-1000 instrument (Thermo Scientific, Waltham, MA, USA), and DNA quality was evaluated via 1 % agarose gel electrophoresis. The V3–V4 hypervariable regions of the bacterial 16S rRNA gene were targeted using specifically designed barcode fusion primers (338F: 5'-ACTCCTACGGGAGGCAGCAG-3', 806R: 5'-GGACTACHVGGGTWTCTAAT-3') to amplify the extracted genomic DNA. The PCR amplification products were subjected to 2 % agarose gel electrophoresis for visualization, and distinct bands corresponding to the desired products were meticulously excised and subsequently purified using the AxyPrep DNA Gel Recovery Kit (Axygen Biosciences, Union City, CA, USA). Post-electrophoresis quantification provided preliminary insights into the concentration of the PCR products,

which were then precisely quantified employing the QuantiFluor™-ST Blue Fluorescence Quantification System (Promega, Madison, WI, USA). These purified amplicons served as the foundation for constructing MiSeq libraries that were then sequenced using the sophisticated MiSeq sequencing platform (San Diego, CA, USA), with sequencing services carried out at Major Bio (Shanghai, China). The sequencing data were meticulously analyzed, with sequences being assigned to unique amplicon sequence variants (ASV) anchored upon a 99 % similarity threshold to ensure a high accuracy in identifying bacterial taxa. Statistical analyses of the community structure were then conducted based on these aggregated ASV results, yielding detailed insights into the microbial composition. Bioinformatic analysis of fecal microbiota was conducted using the Majorbio cloud platform (<https://cloud.majorbio.com>). All the raw sequencing data from the samples were diligently archived and are publicly accessible in the NCBI Sequence Read Archive database, under the assigned accession number SRP486008.

2.7. SCFA determination

The concentrations of SCFA were determined through ion chromatography following the methodology established in the previous study (Cai et al., 2023). Briefly, an appropriate amount of fecal samples was dissolved in pre-cooled dilution water containing $ZnSO_4 \cdot 7 H_2O$ and $K_4Fe(CN)_6 \cdot 3 H_2O$. The mixture was shaken for 30 min, followed by centrifugation at 10,000 rpm for 10 min at 4 °C. Subsequently, the samples were filtered, and the supernatants were further diluted with distilled water at a ratio of 1:4. Finally, the resulting supernatant was subjected to SCFA detection using a 940 Professional IC Vario Ion Chromatograph (IC; Metrohm, Switzerland).

2.8. Statistical analyses

The data were analyzed using IBM SPSS Statistics 25.0 software (SPSS Inc., Chicago, IL, USA). The statistical model included dietary treatment as a fixed effect and initial body weight and sex as random effects. Growth performance and ATTD were calculated using the pen as the experimental unit. For each selected pig, changes in plasma parameters, fecal microbiota composition at the phylum and genus levels, and fecal SCFA levels were assessed as the experimental unit. The differences in the abundance of the feces microbiota were assessed using polynomial testing, as analyzed with the IBM SPSS Statistics 25.0 software. Polynomial contrasts were used to calculate the linear and quadratic effects of different levels of FFSM inclusion in the diet. It was considered significant when $P < 0.05$ and significant tendency when $0.05 \leq P < 0.10$.

3. Results

3.1. Growth performance

As shown in Table 3, when compared to the CON group, dietary supplementation with FFSM showed no significant effects on average daily gain and average daily feed intake. However, a linear decrease in the G:F was observed as the dietary FFSM levels increased ($P = 0.04$).

3.2. Apparent total tract digestibility of energy and nutrient

The effects of supplementing diets with different levels of FFSM on the ATTD of nutrients in growing pigs are presented in Table 4. There is a linear increase in the ATTD of aNDF, ADF, EE, and ash ($P < 0.05$). Notably, as the FFSM level in the diet increased, the ATTD of aNDF showed a quadratic response, while the ATTD of CP demonstrated a quadratic response ($P < 0.05$).

3.3. Plasma parameters

The impact of different levels of FFSM on the serum biochemical parameters in growing pigs is shown in Table 5. The levels of insulin-like growth factor 1 were linearly and quadratically decreased by the addition of FFSM to the diet ($P < 0.05$). Furthermore,

Table 3

Effects of dietary supplementation fermented flaxseed meal (FFSM) on growth performance of growing pigs (n = 8).

Items	Dietary treatments ^a				SEM ^b	Linear	Quadratic
	CON	FFSM10	FFSM15	FFSM20			
d 0 BW (kg)	39.6	39.6	39.6	39.6	1.33	0.997	0.994
d 30 BW (kg)	66.5	66.9	65.8	65.4	1.59	0.534	0.806
ADG (g/d)	897	911	872	860	20.0	0.125	0.477
ADFI (g/d)	2057	2035	2076	2051	70.0	0.970	0.998
G:F	0.44	0.45	0.42	0.42	0.01	0.040	0.419

BW = body weight; ADG = average daily gain; ADFI = average daily feed intake; G:F = gain-to-feed ratio.

^a CON, FFSM10, FFSM15, and FFSM20 diets were prepared by adding different levels of 0 %, 10 %, 15 %, and 20 % FFSM, respectively.

^b SEM stands for the standard error of the mean.

Table 4

Effects of dietary supplementation fermented flaxseed meal (FFSM) on the apparent total tract digestibility of nutrients (g/kg) in growing pigs (n = 8).

Items	Dietary treatments ^a				SEM ^b	Linear	Quadratic
	CON	FFSM10	FFSM15	FFSM20			
DM	0.910	0.890	0.917	0.902	0.009	0.924	0.764
CP	0.909	0.858	0.903	0.893	0.010	0.976	0.040
aNDF	0.491	0.573	0.606	0.557	0.022	0.034	0.009
ADF	0.438	0.481	0.528	0.511	0.023	0.014	0.209
EE	0.730	0.681	0.757	0.784	0.019	0.015	0.076
Ash	0.768	0.887	0.871	0.910	0.024	0.012	0.237
Ca	0.586	0.593	0.618	0.560	0.016	0.474	0.052
P	0.520	0.493	0.546	0.536	0.026	0.408	0.755
GE	0.931	0.906	0.938	0.928	0.007	0.479	0.332

DM = dry matter; CP = crude protein; aNDF = neutral detergent fiber assayed with a heat-stable amylase and expressed inclusive of residual ash; ADF = acid detergent fiber; EE = ether extract; Ca = calcium; P = phosphorus; GE = gross energy.

^a CON, FFSM10, FFSM15, and FFSM20 diets were prepared by adding different levels of 0 %, 10 %, 15 %, and 20 % FFSM, respectively.

^b SEM stands for the standard error of the mean.

dietary supplementation with FFSM results in a linear increase in plasma TBA levels ($P < 0.001$). The levels of serum ALT, TC, LDL-C, and HDL-C had linear effects ($P < 0.05$), and TG had a linear effect ($P < 0.001$) associated with escalating doses of FFSM in the diet. As presented in Table 6, we observed a linear increase in serum IgA and IgM with dietary supplementation of FFSM ($P < 0.05$).

3.4. The fecal microbiota

The results of alpha diversity indices, which include ACE, Chao1, Shannon, and Simpson index are shown in Table 7. The Ace, Chao1 index responded linearly and the Simpson index quadratic increased with increased FFSM level in diet ($P < 0.05$).

The beta diversity index utilizing Principal Coordinates Analysis (PCoA) based on Bray-Curtis dissimilarity at the ASV level can be found in Fig. 1A. The PCoA analysis highlighted that the distributions of FFSM10, FFSM15, and FFSM20 groups were dispersed compared to the CON group, indicating the differences were statistically significant ($P = 0.017$).

The relative abundance of fecal microorganisms at the phylum level, with the top five phyla being Firmicutes, Bacteroidota, Proteobacteria, Spirochaetota, and Actinobacteriota is presented in Fig. 1B and Table 8. Among these, Firmicutes and Bacteroidota were the most prevalent. The proportions of Firmicutes across the control and FFSM10, FFSM15, FFSM20 groups were 77.44 %, 79.44 %, 78.84 %, and 74.76 %, respectively; whereas Bacteroidota comprised 17.88 %, 16.01 %, 14.64 %, and 14.37 % in control and FFSM10, FFSM15, FFSM20 groups.

The top 30 genera are shown in Fig. 1C. The dominant genera across all treatment groups included *Lactobacillus*, norank_f_Muribaculaceae, *Streptococcus*, Christensenellaceae_R-7_group, and norank_f_norank_o_Clostridia_UCG-014. Within the CON (control) group, the relative abundance of the top five species is *Lactobacillus* (6.21 %), norank_f_Muribaculaceae (8.54 %), *Streptococcus* (5.14 %), Christensenellaceae_R-7_group (7.13 %), norank_f_norank_o_Clostridia_UCG-014 (6.67 %). In the FFSM10, FFSM15, and FFSM20 groups, the relative abundances were as follows: *Lactobacillus* (15.27 %, 12.47 %, 2.82 %), norank_f_Muribaculaceae (7.90 %, 6.05 %, 6.86 %), *Streptococcus* (4.60 %, 9.33 %, 7.76 %), Christensenellaceae_R-7_group (6.21 %, 5.19 %, 6.40 %),

Table 5

Effects of dietary supplementation fermented flaxseed meal (FFSM) on plasma biochemical indices of growing pigs (n = 8).

Items	Dietary treatments ^a				SEM ^b	Linear	Quadratic
	CON	FFSM10	FFSM15	FFSM20			
IGF-1, µg/L	18.0	23.8	16.9	13.8	1.92	0.028	0.029
GH, µg/L	16.9	19.8	20.3	19.2	2.48	0.524	0.463
TBA, mol/L	4.41	6.25	6.47	7.49	0.41	< 0.001	0.364
ALT, IU/L	51.3	40.5	41.8	37.3	3.03	0.007	0.330
AST, IU/L	38.5	33.9	36.1	37.1	3.64	0.909	0.467
GLU, mmol/L	5.54	6.02	5.92	5.77	0.35	0.709	0.380
TC, mmol/L	2.44	2.21	2.11	1.97	0.10	0.004	0.722
TG, mmol/L	0.37	0.43	0.54	0.54	0.03	< 0.001	0.321
LDL-C, mmol/L	1.44	1.29	1.28	1.21	0.07	0.039	0.557
HDL-C, mmol/L	1.02	0.95	0.89	0.80	0.06	0.032	0.870
TP, g/L	59.5	58.7	60.1	52.9	2.20	0.078	0.162
ALB, g/L	34.1	34.7	37.2	33.2	1.53	0.974	0.138

IGF-1 = Insulin-like growth factor 1; GH = growth hormone; TBA, total bile acid; ALT = alanine transaminase; AST = aspartate transaminase; GLU = glucose; TC = total cholesterol; TG, total triglyceride; LDL-C = low-density lipoprotein-cholesterol; HDL-C = high-density lipoprotein-cholesterol; TP = total protein; ALB = albumin.

^a CON, FFSM10, FFSM15, and FFSM20 diets were prepared by adding different levels of 0 %, 10 %, 15 %, and 20 % FFSM, respectively.

^b SEM stands for the standard error of the mean.

Table 6

Effects of dietary supplementation fermented flaxseed meal (FFSM) on immune indexes in plasma of growing pigs (n = 8).

Items	Dietary treatments ^a				SEM ^b	Linear	Quadratic
	CON	FFSM10	FFSM15	FFSM20			
IgG, mg/mL	8.50	9.26	9.21	8.61	0.64	0.924	0.308
IgA, µg/mL	151	189	191	281	23.4	0.002	0.325
IgM, mg/mL	2.46	2.77	5.12	4.75	0.67	0.008	0.637

IgA = Immunoglobulin A; IgG, = Immunoglobulin G; IgM = Immunoglobulin M.

^a CON, FFSM10, FFSM15, and FFSM20 diets were prepared by adding different levels of 0 %, 10 %, 15 %, and 20 % FFSM, respectively.^b SEM stands for the standard error of the mean.**Table 7**

Effects of dietary supplementation fermented flaxseed meal (FFSM) on the alpha diversity of the bacterial community at the amplicon sequence variants (ASV) level in feces of growing pigs (n = 8).

Items	Dietary treatments ^a				SEM ^b	Linear	Quadratic
	CON	FFSM10	FFSM15	FFSM20			
Ace	936	876	750	770	37.7	0.001	0.307
Chao1	917	861	737	758	35.9	0.001	0.302
Shannon	5.12	4.92	4.68	4.92	0.11	0.101	0.061
Simpson	0.021	0.041	0.054	0.025	0.01	0.490	0.008

^a CON, FFSM10, FFSM15, and FFSM20 diets were prepared by adding different levels of 0 %, 10 %, 15 %, and 20 % FFSM, respectively.^b SEM stands for the standard error of the mean.

norank_f_norank_o_Clostridia_UCG-014 (5.01 %, 4.66 %, 6.16 %).

The microbial communities within the samples were assessed using the polynomial contrasts, revealing notable differences at the genus level as shown in Table 8. The results showed a quadratic increase in the relative abundance of *Lactobacillus* (P = 0.006) with increasing FFSM addition, a linear increase in the relative abundance of *Bacillus*, and linear and quadratic effects in the relative abundance of *Clostridium_sensu_stricto_1* (P < 0.05) with increasing FFSM addition.

3.5. The concentration of SCFA in feces

The impact of adding FFSM to the diet was reflected in the SCFA profile in feces (Table 9). The data showed that butyric acid levels rose quadratic in response to increasing amounts of FFSM in the diet (P = 0.006). Additionally, the total concentration of SCFA demonstrated a quadratic effect as the level of FFSM in the diet enhanced (P = 0.049).

4. Discussion

Microbial fermentation has been verified as an effective method for reducing anti-nutritional factors in FSM, thereby enhancing its nutritive value for pigs, which is crucial for their growth and overall health (Wei et al., 2024). Nonetheless, investigations into the use of FFSM as a component of feed for growing pigs are limited. Our study reveals that while incorporating FFSM into the diet does not negatively impact the overall growth performance of pigs, it results in a linear decrease in G:F and ATTD of CP with a quadratic decrease as the level of FFSM in the diet increases. Even though FFSM contains approximately 30 % of CP, the balance of amino acid may not be optimal for all stages of pig growth, which could affect protein utilization and overall feed efficiency. Furthermore, we also observed the ATTD of aNDF, ADF, EE, and ash linearly increased in tandem with escalating levels of FFSM supplementation, with aNDF also exhibiting a quadratic response. These findings are in line with previous studies (Chiang et al., 2009; Chen et al., 2010; Shi et al., 2017), which point to fermented feeds possessing enhanced nutrient digestibility. Furthermore, a meta-analysis suggests that fermented feed had a positive effect on pig performance by increasing nutrient availability and reducing anti-nutrient factors (Xu et al., 2020). Post-fermentation degradation of intricate macromolecular organic substances, such as cellulose, starch, and proteins, into smaller fragments more easily digested by animals, is plausible. The presence of *Bacillus subtilis*, which can produce cellulases and xylanases (Li et al., 2022), may aid in the further breakdown of fibers, which in turn increases the digestibility of fibers like aNDF and ADF. In concert with higher levels of FFSM additions, the concentration of TBA showed a linear elevation. Existing literature confirms that TBA facilitates the digestion and absorption of fats in the intestine (Cao et al., 2021), potentially elucidating the observed improvement in EE digestibility. Additionally, through the fermentation process of FSM, we have reduced the content of the primary anti-nutritional factor CG, by about 55 %. CG, during the digestion process in animals, produces toxic hydrogen cyanide, which has a detrimental effect on the digestive system of animals. Previous research has shown that the addition of an excessive amount of FSM to the diet (exceeding 100 g/kg) reduces the feed intake of animals and harms the growth performance of pigs (Batterham et al., 1991; Ndou et al., 2017). This is primarily due to the presence of CG, which in turn reduces the digestibility of nutrients. In our study, the reduction of CG may contribute to an increase in nutrient absorption. To sum up, the act of fermentation refines the nutritive profile of feedstuff by reducing CG and fiber content; it concomitantly amplifies the digestibility of nutrients.

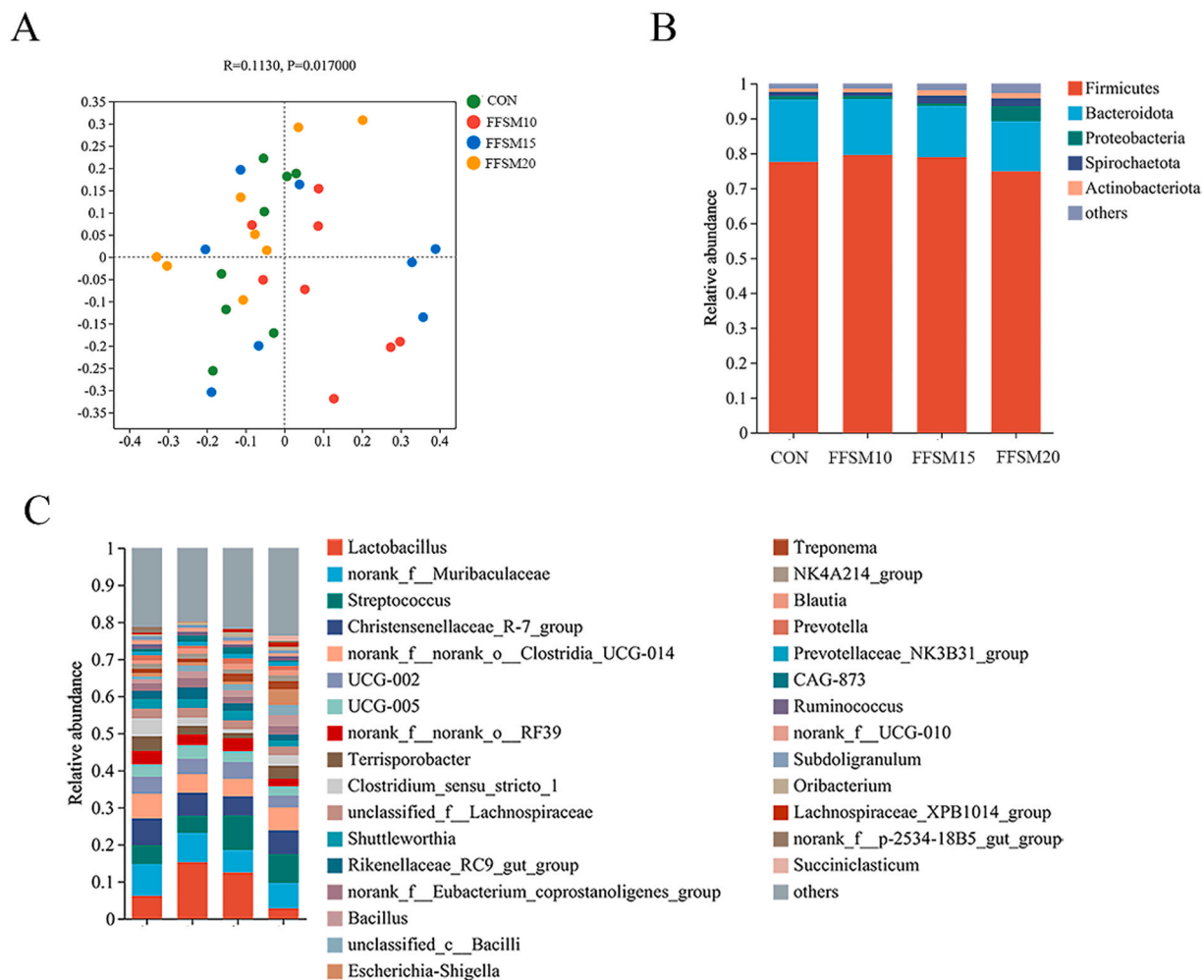


Fig. 1. Effect of dietary supplementation fermented flaxseed meal (FFSM) on the fecal microbiota composition in growing pigs ($n = 8$). (A) Microbial beta-diversity based on the amplicon sequence variants level analysis. (B) The relative abundance of fecal microbiota at the phylum level. (C) The relative abundance of fecal microbiota at the genus level. CON, FFSM10, FFSM15, and FFSM20 diets were prepared by adding different levels of 0 %, 10 %, 15 %, and 20 % FFSM, respectively.

Serum biochemical parameters constitute a multifaceted gauge of metabolic processes related to nutrition, thereby mirroring the overarching well-being of animals. Previous studies have substantiated the capacity of probiotics to modulate lipids, notably by diminishing TC and LDL-C levels (Huang et al., 2023b), while also decreasing concentrations of ALT (Zhang et al., 2017; Cao et al., 2019), which are steps that collectively endorse the overall health of the host. Activities of ALT and AST are recognized as pivotal indicators of liver function (Xu et al., 2017), with increases in their levels typically signaling potential hepatic impairment consequent to toxic insults (Ramesh et al., 2012). Parameters such as TC, TG, LDL-C, and HDL-C are integral to the spectrum of blood lipids, and intricately linked with lipid metabolism and cardiovascular health (Kwiterovic 2000; Liu et al., 2023). In the scope of this experiment, we observed a linear diminution in serum ALT, TC, and LDL-C concentrations alongside incremented doses of FFSM. This was consistent with prior research narratives (Huang et al., 2023b; Zhang et al., 2017; Cao et al., 2019). Flaxseed is rich in alpha-linolenic acid and other active ingredients, a study explored the effects of dietary flaxseed in a rat model of non-alcoholic fatty liver disease and found that including flaxseed in the diet led to an improved lipid composition in the liver and significantly reduced biomarkers of tissue injury, despite consuming a high-fat, high-sucrose diet. Specifically, the flaxseed-supplemented diet significantly lowered liver weight and plasma levels of ALT and AST in obese male rats (Parikh et al., 2024). Therefore, the inclusion of flaxseed meal in the diet of growing pigs may beneficially influence ALT levels and lipid metabolism through analogous mechanisms, thereby exerting a protective effect on liver functionality. Insulin-like growth factor 1 is a vital biomarker intricately linked to growth performance in animals (Slifierz et al., 2013). During our experiment, we noted a linear decrease in insulin-like growth factor 1 serum concentrations corresponding to the escalated inclusion rates of FFSM in the diet. Moreover, it warrants attention that the insulin-like growth factor 1 levels in the group receiving 20 % FFSM were notably lower compared to all the treatment groups. Simultaneously, we also noted a linear increase in the G:F ratio with the escalating levels of FFSM in the feed. This observation suggested higher inclusion of FFSM of

Table 8

Effect of dietary supplementation fermented flaxseed meal (FFSM) on the microbiota composition at the phylum and genus level in feces of growing pigs (n = 8).

Items	Dietary treatments ^a				SEM ^b	Linear	Quadratic
	CON	FFSM10	FFSM15	FFSM20			
Phylum							
Firmicutes	77.4	79.4	78.8	74.8	2.85	0.526	0.322
Bacteroidota	17.9	16.0	14.6	14.4	2.61	0.329	0.768
Proteobacteria	0.95	0.86	0.73	4.30	0.90	0.101	0.172
Spirochaetota	1.21	1.06	2.20	2.21	0.75	0.265	0.923
Actinobacteriota	1.00	1.07	1.61	1.48	0.27	0.115	0.720
Others	0.28	0.21	0.22	0.23	0.06	0.581	0.543
Genus							
g_Lactobacillus	6.21	15.27	12.47	2.82	2.78	0.370	0.006
g_norank_f_Muribaculaceae	8.54	7.90	6.05	6.86	1.83	0.416	0.698
g_Streptococcus	5.14	4.60	9.33	7.76	2.05	0.227	0.823
g_Christensenellaceae_R-7_group	7.13	6.21	5.19	6.40	1.78	0.688	0.554
g_norank_f_norank_o_Clostridia_UCG-014	6.67	5.01	4.66	6.16	0.81	0.612	0.064
g_UCG-002	4.63	4.18	4.54	3.18	0.67	0.230	0.533
g_UCG-005	3.29	3.65	2.90	2.51	0.40	0.099	0.357
g_norank_f_norank_o_RF39	3.64	2.86	3.52	2.06	0.54	0.146	0.588
g_Terrisporobacter	3.96	2.33	1.42	3.50	0.91	0.601	0.066
g_Clostridium_sensu_stricto_1	4.80	2.21	1.03	2.82	0.71	0.046	0.008
g_unclassified_f_Lachnospiraceae	2.59	2.63	2.38	2.34	0.41	0.579	0.929
g_Shuttleworthia	2.55	2.37	2.58	1.62	0.96	0.558	0.690
g_Rikenellaceae_RC9_gut_group	2.24	3.18	2.02	1.64	0.39	0.119	0.124
g_norank_f_Eubacterium_coprostanoligenes_group	2.04	2.52	1.76	2.28	0.43	0.986	0.962
g_Bacillus	1.07	1.80	1.76	2.94	0.39	0.007	0.598

^a CON, FFSM10, FFSM15, and FFSM20 diets were prepared by adding different levels of 0 %, 10 %, 15 %, and 20 % FFSM, respectively.^b SEM stands for the standard error of the mean.**Table 9**

Effects of dietary supplementation fermented flaxseed meal (FFSM) on short-chain fatty acids (SCFAs) in feces of growing pigs (n = 8).

Items	Dietary treatments ^a				SEM ^b	Linear	Quadratic
	CON	FFSM10	FFSM15	FFSM20			
Acetic acid, $\mu\text{mol/g}$	75.1	82.4	81.6	81.0	3.96	0.358	0.343
Propionic acid, $\mu\text{mol/g}$	30.2	34.9	35.1	33.2	2.17	0.362	0.152
Isobutyric acid, $\mu\text{mol/g}$	3.55	4.32	4.44	4.09	0.45	0.420	0.245
Butyric acid, $\mu\text{mol/g}$	42.9	47.9	50.1	34.7	3.14	0.150	0.006
Isovaleric acid, $\mu\text{mol/g}$	5.06	5.33	5.70	5.05	0.81	0.930	0.588
Valeric acid, $\mu\text{mol/g}$	3.94	5.70	5.21	4.05	0.76	0.964	0.065
Total SCFA, $\mu\text{mol/g}$	161	181	182	162	9.37	0.896	0.049

^a CON, FFSM10, FFSM15, and FFSM20 diets were prepared by adding different levels of 0 %, 10 %, 15 %, and 20 % FFSM, respectively.^b SEM stands for the standard error of the mean.

more than 20 % could potentially reduce the growth performance of pigs.

Immunoglobulins serve as pivotal markers for assessing the immune functionality in growing pigs, with their serum levels offering insights into the immunological health of the animal; elevated levels often signify an enhanced immune state. In our investigation, we recorded a proportional rise in the levels of IgA and IgM concurrent with the increased inclusion of FFSM into the diet. These outcomes are consistent with findings from previous studies (Zhu et al., 2017; Lu et al., 2019), demonstrating that pigs consuming fermented soybean meal exhibit significant increases in their serum immunoglobulin G and IgM levels. As a beneficial microbial strain, *Bacillus subtilis* produces metabolites during feed fermentation, especially SCFA, which have been shown to activate the host immune system. For example, butyric acid, one of the SCFA, can activate immune cells and enhance immune responses via the GPR43 receptor, thus potentially promoting immunoglobulin production (Siddiqui and Cresci, 2021). In this study, butyric acid content increased quadratically with the addition of FFSM. Therefore, the observed increase in IgA and IgM may be related to metabolites produced during the fermentation process.

Intestinal flora plays an important role in pig health and production. The alpha diversity indices and PCoA result suggested that the microbial composition of the treatment groups differs significantly from that of the control group. Specifically, in this study, with increasing levels of FFSM in the diets, there was a linear decrease in the ACE and Chao1 indices and a quadratic increase in the Simpson's index, which suggests a decrease in microbial diversity as well as community homogeneity. This may be due to the presence of incompletely eliminated antinutritional factors in FFSM as well as the possibility that FFSM may affect the metabolic pathways of intestinal microorganisms, altering metabolites such as SCFA and thereby affecting the diversity of the intestinal flora. At the genus level, the impact of FFSM was notably on the prominence of *Lactobacillus*, *Bacillus*, and *Clostridium_sensu_stricto_1*. In this study, the

abundance of *Lactobacillus* increased quadratically with the addition of FFSM. Species of *Lactobacillus* are recognized for their beneficial role in gut health, such as inhibiting pathogenic adhesion to the gut lining and enhancing immune modulation (Walter, 2008). Le et al. (2015) reported that there was a positive correlation between the number of *Lactobacillus* and the concentration of SCFA. SCFA results have delineated a significant positive relationship between escalating dosages of FFSM and the fecal concentrations of the total SCFA population, exhibiting quadratic amplifications, which is consistent with the results of this experiment. It is noteworthy that, despite *Lactobacillus* not constituting the primary producers of SCFA, this discovery underscores an interactive relationship between *Lactobacilli* and other bacteria proficient in SCFA production. Furthermore, the *Lactobacillus* makes a significant contribution to the overall content of SCFA in fecal matter. However, aside from the quadratic elevation in the total concentration of SCFA, the butyric acid content also demonstrates a quadratic increase with the escalating dosage of FFSM. Recognized as a boon to host wellness, butyric acid promotes the proliferation of mucosal epithelial cells and has immune-modulating and anti-inflammatory effects (Vita et al. 2017). Interestingly, supplementation with 20 % FFSM reduced the butyric acid concentration. In correspondence with the observed decrease in butyric acid concentration, there is also a reduction abundance of *Lactobacillus* with the FFSM20. This suggests that while an increase in the dosage of FFSM can enhance the concentration of butyric acid, an excessively high dosage, such as 20 %, may lead to a diminution in butyric acid levels and a decrease in *Lactobacillus*, potentially exerting a detrimental effect on gut health. A wide array of research has suggested that dietary inclusion of fermented feed holds the potential for managing the intestinal microbiome equilibrium, safeguarding against the establishment of pathogenic microbes, and bolstering the immune functions in pigs (Wang et al., 2018). This evidence supports the use of FFSM as means to enhance immune function and intestinal health in pig nutrition by promoting the production of SCFA (particularly butyric acid) through beneficial microbial communities.

Multiple studies have highlighted a significant association between the proliferation of *Clostridium_sensu_stricto_1* and the elevation of pro-inflammatory cytokines (such as TNF- α , IL-1 β , and IL-6), in addition to markers indicative of liver damage (ALT and AST) (Yi et al., 2021; Erdenebileg et al., 2023). *Clostridium_sensu_stricto_1* has also been implicated in the increased incidence of diarrhea in piglets, and previous research has shown that its abundance can be diminished by feeding them fermented soybean meal (Zhu et al., 2018). Consistent with previous findings, our experiments showed a linear and quadratic decrease in the relative abundance of *Clostridium_sensu_stricto_1* with the addition of FFSM. Correspondingly, the patterns observed for serum ALT levels were consistent with past research (Yi et al., 2021; Le Roy et al. 2015; Xu et al. 2020). Furthermore, we also found that increased linearly the relative prevalence of *Bacillus* with the addition of FFSM. Many studies have demonstrated the probiotic role of *Bacillus* in reducing the colonization of animal pathogens (Mingmongkolchai, Panbangred 2018; Grant et al., 2018). The data support the hypothesis that a balanced supplementation of fermented flaxseed meal could elevate beneficial genera such as *Lactobacillus* and *Bacillus*, potentially outcompeting and thus reducing the prevalence of the potentially pathogenic *Clostridium_sensu_stricto_1*. This shift in microbial populations is thought to contribute to a more balanced gut microbiome, which may protect against liver damage and support overall health in growing pigs.

5. Conclusions

This study demonstrated that incorporating fermented flaxseed meal into the corn-soybean meal diets of growing pigs at levels of 10 %, 15 %, and 20 % does not affect average daily gain or feed intake. However, the gain-to-feed ratio exhibited a linear decline with increasing fermented flaxseed meal levels. The inclusion of fermented flaxseed meal improved the digestibility of aNDF and ADF and elevated serum immunoglobulin levels (IgA and IgM). Furthermore, fermented flaxseed meal supplementation increased the abundance of beneficial bacteria such as *Lactobacillus* and *Bacillus*, while reducing the abundance of *Clostridium_sensu_stricto_1*. It also enhanced the concentration of butyric acid and total short-chain fatty acids in feces. Therefore, incorporating fermented flaxseed meal into pig diets can sustainably and efficiently support the health and growth of pigs.

CRedit authorship contribution statement

Yanpin Li: Writing – review & editing, Methodology. **Wenjuan Sun:** Writing – review & editing. **Lianghua Ma:** Writing – review & editing, Resources. **Jingjiao Yan:** Writing – review & editing, Resources. **Yu Pi:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Data curation, Conceptualization. **Lei Xu:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Xilong Li:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Xianren Jiang:** Writing – review & editing. **Long Cai:** Writing – review & editing. **Baozhu Guo:** Writing – review & editing, Resources, Methodology. **Yao Guo:** Writing – review & editing, Investigation, Data curation. **Zixi Wei:** Writing – review & editing, Investigation, Data curation.

Declaration of Competing Interest

The authors declare no conflicts of interest.

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Ethics approval

The experimental design and procedures were approved by the Institute Animal Care and Use Committee of the Institute of Feed Research of Chinese Academy of Agricultural Sciences (IFR-CAAS20221025).

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