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Impact of mannanase on broiler performance, intestinal health, and energy utilization with varying soybean meal levels

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Abstract

Mannanase specifically degrades mannan, increases the efficiency of energy utilization, and improves intestinal health in broilers. The interaction effects between mannanase and soybean meal (SBM) have not been extensively explored. Therefore, the present study aimed to determine effects of adding mannanase to diets with different SBM content on broilers, and to explore interaction effects between mannanase and SBM. This study was conducted on Arbor Acres broilers. Under low-energy conditions (metabolizable energy reduced by 50 kcal/kg), a 3 × 2 factorial design was used with three SBM content diets (control group, 50%, or 25% of the SBM content of control) and with two levels of mannanase (0 or 100 mg/kg) respectively. In experiment 1, growth performance and intestinal health were determined. Experiment 2 measured energy metabolism in broilers by respiratory calorimetry, while feces were collected to determine nutrient digestibility. Results indicated that low SBM diets supplemented with degossypolled cottonseed protein and corn gluten meal significantly reduced broiler growth performance during d 0–42. However, mannanase supplementation in diets containing 35.66% and 17.83% SBM significantly improved growth performance from d 0 to 21, reduced respiratory quotient at 21 d, and improved intestinal health ($p < 0.05$). In the 35.66% SBM diet, mannanase also enhanced energy metabolism by improving nitrogen retention and protein energy utilization ($p < 0.05$). However, mannanase showed limited efficacy when SBM content was reduced to 8.92%. Microbiological analyses showed that mannanase significantly reduced *Escherichia coli* and promoted 2-Oxocarboxylic acid metabolism in cecal microbes. In conclusion, there was a reciprocal relationship between mannanase and SBM content, with mannanase still exerting the above beneficial effects at the SBM level of 17.83%.

Introduction

Soybean meal (SBM) is the main source of vegetable protein (VP) in poultry feed, containing about 17%–27% non-starch polysaccharides (NSP)[1], which limits its nutritional value[2]. NSP is a common anti-nutritional factor (ANF) in poultry feed that stimulates the animal's innate immune system and leads to energy loss[3]. Hemicellulose, the second most abundant polymer in nature, is usually found in plant cell walls alongside lignin and cellulose[4]. Mannan, as a major component of hemicellulose in legumes, is relatively the most important type of ANF in SBM. It is a polysaccharide composed of mannose units linked by β-1,4-bonds[5]. NSP in SBM mainly exists as glucomannan and galactomannan[6]. It has been shown that mannan can increase the viscosity of chyme[7,8], induce feed-induced immune response (FIIR), cause energy loss[9], disrupt microbial community structure[10], and affect animal growth performance[11].

Mannanase is widely found in animals, plants, and microorganisms[12]. It can act specifically upon the β-1,4-D-mannosidic bond within mannans[13,14]. Mannanase has strict substrate specificity, allowing it to produce mannooligosaccharides (MOS) while degrading mannan, thus maximizing its energy potential[3]. Studies have shown that mannanase can lower the viscosity of intestinal chyme[15], promote the digestion and absorption of feed nutrients[16,17], and improve the nutritional value of SBM[18]. Additionally, mannanase can regulate intestinal flora structure[19,20], maintain the integrity of intestinal epithelium[21,22], enhance immune function[6,23], reduce the energy loss from FIIR[24], increase the efficiency of energy utilization[25,26], and improve the growth performance of poultry[27–29]. Poultry do not have the enzymes required to break down mannan. Thus, the exogenous supply of mannanase has increasingly emerged as an effective strategy to improve growth performance.

Studies have indicated that adding mannanase to low-energy diet increases N-corrected apparent metabolizable energy (AMEn)[24], and improves the growth performance of broilers[30]. The role played by mannanase depends mainly on the type of substrate and the amount of mannan in the diet[31]. In summary, many studies have confirmed that mannanase specifically degrades mannan, increases energy utilization efficiency, and improves intestinal health in broilers. However, there has been limited research on the interaction between mannanase and SBM. In addition, previous findings have shown that a 50 kcal/kg reduction in dietary energy levels can lead to reduced growth performance and impaired intestinal health in broilers. The addition of mannanase can improve broilers' growth performance by alleviating

intestinal inflammatory response, regulating bacterial flora structure, and increasing the efficiency of feed nutrient utilization[32]. Therefore, the aim of this study was to determine the effects of mannanase supplementation in diets with different SBM content on the growth performance, intestinal health, and effective energy value of broilers under the condition that the metabolizable energy (ME) level of the diets was reduced by 50 kcal/kg, and the effects of the interaction between mannanase and SBM content was also preliminarily explored and revealed.

Materials and methods

Experimental animals and diets

This study was conducted on Arbor Acres (AA+) broilers and the feed formulation was designed following the nutritional demands of broilers as suggested by NY/T33-2004. Corn-SBM diet was formulated under low-energy conditions, reducing the ME by 50 kcal/kg. Diets were formulated with degossypolled cottonseed protein (DCP), and corn gluten meal (CGM) instead of SBM while ensuring that the energy and crude protein (CP) levels were consistent across treatment groups. The feeding management and nutritional supply of the birds were in accordance with the guidelines and regulations presented in the Feeding Management Manual.

Experimental design

This study was randomized into six treatments using a 3 × 2 factorial design. Under low-energy conditions (ME reduced by 50 kcal/kg), broilers were fed diets containing different levels of SBM (0–21 d: 35.66%, 17.83%, and 8.92%; 22–42 d: 30.58%, 15.29%, and 7.65%) with two levels of mannanase (0 or 100 mg/kg). The composition and nutrient levels of the test diets are presented in Table 1, while Table 2 shows the measured values for each treatment group. All the diets were provided in pellet form.

The broiler chickens were randomly divided into each treatment group. In experiment 1, 576 1-day-old AA+ broilers were selected, with eight replicates of 12 chickens per treatment for 42 d. The trial was conducted in two rearing phases at the Zhuozhou Teaching Experimental Farm of China Agricultural University (Hebei, China). In experiment 2, 180 AA+ broilers aged 17 d were selected, with six replicates of five chickens per treatment, and the testing period lasted from 17 to 24 d of age. This trial was completed in three phases, with two replicates as one phase of 7 d each. All experimental broilers were reared in the experimental chicken house of the Animal Husbandry Science Branch of the Jilin Academy of Agricultural Sciences (Jilin, China).

Respiration chambers

In experiment 2, an open-circuit indirect calorimetry respiration chamber designed for poultry was used to assess the impacts of adding mannanase to diets with different SBM levels on nutrient digestibility, nitrogen (N) balance, and energy metabolism in broilers. This equipment was developed by the Institute of Animal Nutrition and Feed Research of Jilin Academy of Agricultural Sciences, and the overall equipment consists of multi-channel gas analysis, multi-channel data acquisition system, respiratory metabolism chamber, multi-gas path driven by vortex fan, and heating and cooling equipment. Wherein the sensor for determining

Table 1. Composition and proportions of experimental diets[a] (%, as is basis).

Item	Day 0 to 21			Day 22 to 42		
	35.66% SBM group	17.83% SBM group	8.92% SBM group	30.58% SBM group	15.29% SBM group	7.65% SBM group
Composition ratio (%)						
Corn (7.8% CP)	54.00	59.82	59.91	58.17	62.78	65.73
SBM (44% CP)	35.66	17.83	8.92	30.58	15.29	7.65
DCP		10.00	12.00		9.65	13.18
CGM	3.24	6.00	10.44	3.17	5.15	7.11
Soybean oil	2.76	1.15	1.02	4.46	3.20	2.25
L-lysine hydrochloride (98.5%)	0.12	0.45	0.60	0.16	0.37	0.50
Calcium hydrogen phosphate	1.84	1.96	2.10	1.51	1.55	1.53
Stone powder	1.25	1.36	1.34	1.26	1.32	1.37
NaCl	0.39	0.39	0.43	0.24	0.24	0.25
Trace mineral feed[b]	0.20	0.20	0.20	0.10	0.10	0.10
Choline chloride (50%)	0.20	0.20	0.20	0.10	0.10	0.10
DL-Methionine (99%)	0.21	0.22	0.17	0.14	0.13	0.11
L-Tryproan		0.10	0.12			
L-Threonine		0.20	0.21			
Antioxidants	0.05	0.05	0.05	0.05	0.05	0.05
Vitamin premix[c]	0.03	0.03	0.03	0.02	0.03	0.03
Phytase	0.02	0.02	0.02	0.02	0.02	0.02
Zeolite	0.02	0.02	2.24	0.02	0.02	0.02
Calculated nutrient levels						
ME, Mcal/kg	2.95	2.95	2.95	3.10	3.10	3.10
CP	22.13	22.13	22.12	20.19	20.19	20.19
Ca	1.01	1.05	1.05	0.92	0.93	0.93
Available phosphorus	0.45	0.47	0.49	0.39	0.40	0.40
Lysine	1.20	1.25	1.22	1.10	1.11	1.12
Methionine	0.56	0.59	0.56	0.46	0.47	0.47
Tryproan	0.18	0.25	0.24			
Threonine	0.72	0.86	0.85			

[a] The feed was in granular form. [b] The analytical values for each kg of trace mineral feed ingredients were presented as follows: Cu 8 g; Fe 40 g; Zn 55 g; Mn 60 g; I 750 mg; Se 150 mg; Co 250 mg; moisture ≤ 10%. [c] The analytical values for each kg of vitamin premix composition were presented as follows: vitamin A 50 million IU; vitamin D_3 12 million IU; vitamin E 100,000 IU; vitamin K_3 10 g; vitamin B_1 8 g; vitamin B_2 32 g; vitamin B_6 12 g; vitamin B_{12} 100 mg; nicotinamide 150 g; D-pantothenic acid 46 g; folic acid 5 g; biotin 500 mg; moisture ≤ 6%.

Table 2. Measured values of effective energy and digestible essential amino acid contents of experimental diets from 0 to 21 d (%).

Items	35.66% SBM	17.83% SBM	8.92% SBM
ME (Mcal/kg)	2.94	2.94	2.97
NE (Mcal/kg)	2.23	2.35	2.22
Digestible lysine	1.20	1.18	1.17
Digestible methionine	0.50	0.55	0.53
Digestible threonine	0.77	0.82	0.84
Digestible tryptophan	0.17	0.25	0.24
Digestible arginine	1.32	1.37	1.30
Digestible leucine	1.76	1.71	1.78
Digestible isoleucine	0.69	0.64	0.61
Digestible histidine	0.42	0.45	0.43
Digestible phenylalanine	0.91	0.92	0.95
Digestible glycine	0.37	0.38	0.37
Digestible cystine	0.26	0.30	0.30
Digestible valine	0.77	0.79	0.78
Digestible tyrosine	0.46	0.50	0.52

the oxygen (O_2) concentration is a zirconia sensor (Model 65-4-20, The Advanced Micro Instruments, USA), and the sensor for determining carbon dioxide (CO_2) concentration is an infrared sensor (AGM10, Sensors Europe GmbH, Germany). Additionally, the data acquisition system can provide real-time displays of test data and the operating status of the equipment. The remote control software can automatically calculate O_2 consumption, CO_2 production, and respiratory quotient (RQ) of broilers, and record the temperature and humidity data inside and outside the respiratory chamber, which can be viewed on the control interface of computerized data acquisition. The equipment was tested for functionality and air-tightness before the experiment.

Measurement of growth performance

On d 21 and 42, feed intake (FI) and body weight (BW) were measured for each replicate of each group in experiment 1. Average daily feed intake (ADFI), average daily gain (ADG), and feed conversion rate (FCR) were respectively computed for the periods of 0–21, 22–42, and 0–42 d.

Respiratory calorimetry

On d 20, two healthy broilers were randomly chosen from each replicate of each treatment group in experiment 2 and placed in the metabolic chamber of the respiratory calorimetry device. Broilers were acclimatized for 1 d and then measured for 3 d. The BW of the test chickens at 21 d and 42 d were recorded respectively, and the relevant indexes were calculated according to the following formula:

$$\text{Gross energy intake (GEI)} = \text{The gross energy (GE) of feed} \times \text{EI}$$

$$\text{Gross excretory energy (GEE)} = \text{Fecal energy (FE)} \times \text{Fecal output}$$

$$\text{Apparent metabolizable energy intake (AMEI)} = \text{GEI} - \text{GEE}$$

$$\text{Apparent metabolizable energy (AME)} = \frac{\text{AMEI}}{\text{FI}}$$

$$\text{AMEn} = \frac{\text{AMEI} - \text{N deposition (RN)} \times 34.39}{\text{FI}}$$

$$\text{Net energy (NE)} = \frac{\text{AMEI} - \text{Heat increment (HI)}}{\text{FI}}$$

where, RN is the amount of N (kg) deposited by broilers per 1 kg of feed consumed and 34.39 is the correction factor.

$$\text{Heat production (HP)} = 16.18 \times O_2 \text{ consumption } (VO_2) + 5.02 \times CO_2 \text{ excretion } (VCO_2)$$

$$\text{Fasting heat production (FHP)} = \text{Metabolic weight } (BW^{0.70}) \times 450$$

$$RQ = \frac{VCO_2}{VO_2}$$

$$HI = HP - FHP$$

$$\text{Energy deposition (RE)} = \text{AMEI} - \text{HP}$$

$$\text{N deposition (TRN)} = \text{Ingested N } (N_{int}) - \text{Excreted N } (N_{exc})$$

$$\text{Protein deposition energy } (RE_{pro}) = TRN \times 6.25 \times 23.84$$

$$\text{Fat deposition energy } (RE_{fat}) = RE - RE_{pro}$$

where, 6.25 is the conversion coefficient of protein to N and 23.8 is the energy contained in 1 kg of protein (MJ).

Measurement of nutrient digestibility

In experiment 2, excreta were collected concurrently with the respiratory calorimetry, and the apparent total tract digestibility (ATTD) of nutrients was determined using the Total Collection Method. At 21 to 24 d, two broilers were randomly chosen from each replicate of each treatment group for collecting fecal samples. The initial weight of the mixed fecal sample was recorded, and then it was dried in an oven at 65 °C for 72 h until a constant weight was achieved. The dried sample was allowed to cool at room temperature for 24 h, weighed again, and crushed. Dry matter (DM), CP, GE, and amino acid (AA) in diets and feces were determined. DM, CP, and GE were detected by GB/T 6435-2014, GB/T 6432-1994, and ISO 9831:1998, respectively. AA was determined according to GB/T 18246-2019. The ME content in the feed was calculated according to the formula: ME of feed (MJ/kg) = GE of feed × ATTD of GE.

The ATTD was calculated using the following formula:

$$\text{The ATTD of nutrients (\%)} = \frac{\text{Nutrient content of ingested feed} - \text{Nutrient content of feces}}{\text{Nutrient content of ingested feed}}$$

Sample collection

On d 21 and 42, a healthy broiler was randomly chosen from each replicate in experiment 1, weighed, and blood was collected. The molecular and tissue samples were collected from the jejunum and ileum after the execution of broilers. The intestinal chyme was carefully rinsed with normal saline. The molecular samples were promptly flash-frozen in liquid nitrogen, while the tissue samples intended for morphological analysis were fixed in a 4% paraformaldehyde solution. Additionally, samples of ileal and cecal chyme were collected and rapidly flash-frozen in liquid nitrogen for further study.

Measurement of jejunal chyme viscosity

An Ostwald viscosimeter (1831-2; 0.55 mm; Huanguang Glass Instruments Co., Ltd., Taizhou, Zhejiang, China) was used to determine the chyme viscosity[32].

Measurement of intestinal barrier integrity

This test was performed using chicken diamine oxidase (DAO) and D-lactic acid (D-LA) ELISA kits produced by Shanghai Guduo Biotechnology Co., LTD. (Shanghai, China) to measure DAO and D-LA contents in serum. A chicken endotoxin (ET) test LAL kit provided by Xiamen Bioendo Technology Co., Ltd. (Xiamen, China) was used to evaluate ET levels in serum.

Measurement of intestinal morphology

After paraffin embedding and fixation of ileal tissues, sections were prepared. The intestinal tissues were stained with HE. Then, these stained tissues were observed using a Leica microscope (Wetzlar, Germany, ModelDMi8) to determine the morphology of the intestinal epithelium[32].

Measurement of gene expression related to intestinal immunity and intestinal barrier

Total RNA from the jejunum and ileum was extracted using TRIzol reagent. The concentration of total RNA was measured with a NanoDrop 2000 microspectrophotometer. In accordance with the instructions included with each kit, reverse transcription and fluorescence quantification were carried out separately. Using *β-actin* as an internal reference gene, the expression levels of genes related to intestinal immunity and intestinal barrier were measured by an ABI 7500 real-time fluorescence quantitative PCR instrument. Table 3 lists the primers that were employed for real-time fluorescent quantitative PCR in this trial. The relative expression of the mRNA of each target gene was calculated using the $2^{-\Delta\Delta Cr}$ method.

16S rRNA sequencing of microorganisms in the ileum and cecum

DNA isolation and high-throughput sequencing operations

To further investigate the effect of mannanase addition to diets containing 17.83% SBM on intestinal microorganisms of broilers at 21 d, 16S rRNA gene sequencing was conducted on chyme samples from both the 17.83% SBM group and the mannanase addition group in experiment 1. DNA of microorganisms was extracted from the contents of the ileum and cecum. The extraction and concentration of DNA were determined. After the amplification of the bacterial DNA, the PCR products were purified. Then the library was constructed and quantified[32]. After the library met the required standards, HiSeq2500-PE250 was used for the sequencing process. The sequencing analysis was carried out by Shenzhen Weike Meng Technology Group Co., Ltd.

Table 3. Sequences of oligonucleotide primers[a] for real-time quantitative fluorescence PCR.

Gene[b]	Sequences of primers (5'-3')[c]	Serial No.
Claudin-1	F: CATACTCCTGGGTCTGGTTGGT R: GACAGCCATCCGCATCTTCT	NM_001013611.2
β-actin	F: CAACACAGTGCTGTCTGGTGGTAC R: CTCCTGCTTGCTGATCCACATCTG	NM_205518.1
Occludin	F: ACGGCAGCACCTACCTCAA R: GGGCGAAGAAGCAGATGAG	NM_205128.1
ZO-1	F: CTTCAGGTGTTTCTCTTCCTCCTC R: CTGTGGTTTCATGGCTGGATC	XM_015278981.1
MUC 2	F: TTCATGATGCCTGCTCTTGTG R: CCTGAGCCTTGGTACATTCTTGT	XM_421035
IL-1β	F: ACTGGGCATCAAGGGCTA R: GGTAGAAGATGAAGCGGGTC	NM_204524.1
IL-6	F: CGCCCAGAAATCCCTCCTC R: AGGCACTGAAACTCCTGGTC	XM_015281283.1
IL-8	F: ATGAACGGCAAGCTTGGAGCTG R: TCCAAGCACACCTCTCTTCCATCC	XM_015301388.1
IL-10	F: GCTGCCAAGCCCTGTT R: CCTCAAACTTCACCCTCA	NM_001004414.2
IL-18	F: TGATGAGCTGGAATGCGATG R: ACTGCCAGATTTCACCTCCTG	NM_204608.3
TNF-α	F: GAGCGTTGACTTGGCTGTC R: AAGCAACAACCAGCTATGCAC	XM_204267
MyD88	F: TGCAAGACCATGAAGAACGA R: TCACGGCAGCAAGAGAGATT	NM_001030962.5
NF-κB	F: GTG TGA AGA AAC GGG AAC TG R: GGC ACG GTT GTC ATA GAT GG	NM_205129.1
TLR-4	F: CCACTATTCGGTTGGTGGAC R: ACAGCTTCTCAGCAGGCAAT	NM_001030693.1

[a] Primers were designed using Primer Express software (Sangon Biotech Co., LTD., Shanghai, China). [b] Zonula occludens-1 (*ZO-1*); mucin2 (*MUC 2*); interleukin-1*β* (*IL-1β*); interleukin-6 (*IL-6*); interleukin-8 (*IL-8*); interleukin-10 (*IL-10*); interleukin-18 (*IL-18*); tumor necrosis factor α (*TNF-α*); myeloid differentiation factor 88 (*MyD88*); nuclear factor kappa B (*NF-κB*); toll-like receptor-4 (*TLR-4*). [c] F for forward; R for reverse.

Processing of the sequences and analyses in bioinformatics

Effective tags were clustered to obtain amplicon sequencing variants (ASVs) using Divisive Amplicon Denoising Algorithm 2 (DADA 2). The sequenced samples were bipartite sequenced using the Illumina NovaSeq platform, and the obtained sequencing data were split by barcode to obtain valid sequence information. The entire raw sequences of all samples were filtered using the DADA 2 plugin of QIIME2 software, followed by denoising and merging, with chimeras removed to form operational taxonomic units (OTUs). Based on the principles of algorithmic design, the representative sequence was annotated with species. The Greengenes Database 13_8 database was used for species annotation analysis and based on the species annotation information, OTUs, and their contained sequences were checked. Based on the absolute abundance and the annotation information of OTUs, the number of sequences per sample at seven taxonomic levels were counted as a proportion of the total number of sequences to effectively assess the resolution of species annotation of samples. UniFrac distances of samples were calculated using QIIME software to construct the Unweighted Pair-Group Method with Arithmetic mean (UPGMA) clustering trees. Based on the Latent Dirichlet Allocation (LDA), Linear discriminant analysis Effect Size (LEfSe) was used to identify the biomarkers that showed statistically significant differences among different groups (bacterial categories with significant differences in relative abundance). R software (Version 2.15.3) was used to make Venn diagrams, coverage index plots, PCA and PCoA plots, along with Analysis of similarities (ANOSIM). T-tests and Kruskal Wallis tests between each group were performed using R software for microorganisms with relative abundance > 0.001. Finally, PICRUSt2 was utilized to predict and conduct an analysis of the function of the metagenome.

Statistical analyses

In this study, the data from each group in the two experiments were statistically analyzed by SPSS Statistics V22.0. A general linear model (univariate analysis) was employed for statistical analysis, and differences between treatments were analyzed by Duncan's multiple comparison test. All differences that were considered statistically significant were determined based on a p value < 0.05. Additionally, p values between 0.05 and 0.1 were categorized as trends.

Results

Growth performance

As shown in Table 4, there was a significant reciprocal effect of mannanase and SBM content on BW of broilers at 21 d ($p < 0.05$) and an approaching significance in the reciprocal effect on ADG ($p = 0.069$) and FCR of broilers from 0 to 21 d ($p = 0.055$). Adding mannanase to the diets significantly reduced the FCR of broilers at d 0 to 42 ($p < 0.01$) and d 22 to 42 ($p < 0.05$). A reduction in SBM content negatively impacted broiler growth performance. Specifically, compared with the SBM control group, when the SBM content was reduced to 50% and 25% of the control group, the BW of broilers at 42 d and ADG at d 0 to 42 were significantly decreased, and the FCR of broilers at d 0 to 42 was significantly increased ($p < 0.01$). In addition, when the SBM content was reduced to 25% of the control group, it significantly reduced the ADFI of broilers from 0 to 42 d compared to the other two groups ($p < 0.05$). The addition of mannanase significantly improved the growth performance of broilers from 0 to 21 d, with increasing the BW, and ADG, and reducing the FCR in both the control diet group and the 17.83% SBM group. However, mannanase showed limited efficacy when SBM content was reduced to 8.92%.

Table 4. Effect of mannanase addition to diets with different SBM content on growth performance of broilers.

SBM content	Mannanase	Day 0 to 21				Day 22 to 42				Day 0 to 42		
		BW (kg)	ADG (kg)	ADFI (kg)	FCR	BW (kg)	ADG (kg)	ADFI (kg)	FCR	ADG, kg	ADFI, kg	FCR
Control group	0 mg/kg	0.832^{bc}	0.037^{b}	0.054	1.427^{bc}	2.690	0.088	0.151	1.711	0.063	0.102	1.569
	100 mg/kg	0.877^{a}	0.040^{a}	0.055	1.375^{d}	2.852	0.094	0.153	1.631	0.067	0.104	1.503
50% of SBM of control	0 mg/kg	0.822^{cd}	0.037^{b}	0.054	1.450^{b}	2.625	0.086	0.154	1.797	0.061	0.104	1.623
	100 mg/kg	0.862^{ab}	0.039^{a}	0.055	1.412^{c}	2.678	0.087	0.153	1.765	0.063	0.104	1.589
25% of SBM of control	0 mg/kg	0.804^{cd}	0.036^{b}	0.054	1.493^{a}	2.475	0.080	0.149	1.879	0.058	0.102	1.686
	100 mg/kg	0.798^{d}	0.036^{b}	0.054	1.496^{a}	2.427	0.077	0.143	1.848	0.057	0.098	1.672
SEM		0.006	0.000	0.000	0.008	0.029	0.001	0.001	0.015	0.001	0.007	0.010
Main effect												
Mannanase	0 mg/kg	0.819	0.037^{b}	0.054	1.457^{a}	2.597	0.085	0.151	1.795^{a}	0.061	0.103	1.626^{a}
	100 mg/kg	0.846	0.038^{a}	0.054	1.428^{b}	2.652	0.095	0.150	1.748^{b}	0.062	0.102	1.588^{b}
SBM content	control group	0.855	0.039^{a}	0.054	1.401^{c}	2.771^{a}	0.091^{a}	0.152^{a}	1.671^{c}	0.065^{a}	0.103^{a}	1.536^{c}
	50% of SBM of control	0.842	0.038^{a}	0.054	1.431^{b}	2.652^{b}	0.086^{b}	0.153^{a}	1.781^{b}	0.062^{b}	0.104^{a}	1.606^{b}
	25% of SBM of control	0.801	0.036^{b}	0.054	1.494^{a}	2.451^{c}	0.078^{c}	0.146^{b}	1.863^{a}	0.057^{c}	0.100^{b}	1.679^{a}
p-value												
Mannanase		0.003	0.004	0.269	0.003	0.211	0.469	0.422	0.022	0.154	0.658	< 0.001
SBM content		< 0.001	< 0.001	0.762	< 0.001	< 0.001	< 0.001	0.036	< 0.001	< 0.001	0.041	< 0.001
Mannanase × SBM		0.036	0.069	0.526	0.055	0.161	0.267	0.339	0.515	0.140	0.292	0.120

[a, b, c] The data in the same row with shoulder labels containing different letters indicate significant differences ($p < 0.05$).

Table 5. Effect of mannanase addition to diets with different SBM content on the respiratory metabolism and N balance of broilers at 21 d.

SBM content	Mannanase	VO_2 (L)	VCO_2(L)	RQ	N_{int} (g/d)	N_{exc} (g/d)	TRN (g/d)
35.66% SBM	0 mg/kg	35.407	36.399	1.028^{a}	3.470	1.413	2.057^{b}
	100 mg/kg	34.152	34.300	1.011^{bc}	3.640	1.094	2.546^{a}
17.83% SBM	0 mg/kg	32.990	33.632	1.020^{ab}	3.494	1.634	1.861^{b}
	100 mg/kg	32.771	32.432	1.003^{c}	3.521	1.530	1.992^{b}
8.92% SBM	0 mg/kg	36.165	36.354	1.006^{bc}	3.462	1.573	1.889^{b}
	100 mg/kg	31.563	32.075	1.013^{abc}	3.532	1.576	1.957^{b}
SEM		0.510	0.516	0.002	0.019	0.045	0.053
Main effect							
Mannanase	0 mg/kg	34.854^{a}	35.462^{a}	1.018	3.476^{b}	1.540^{a}	1.936^{b}
	100 mg/kg	32.829^{b}	32.936^{b}	1.009	3.565^{a}	1.400^{b}	2.165^{a}
SBM content	35.66% SBM	34.780	35.350	1.020	3.555	1.256^{b}	2.302^{a}
	17.83% SBM	32.880	33.032	1.012	3.508	1.582^{a}	1.926^{b}
	8.92% SBM	33.864	34.215	1.010	3.497	1.574^{a}	1.923^{b}
p-value							
Mannanase		0.039	0.010	0.043	0.012	0.061	0.005
SBM content		0.270	0.141	0.147	0.328	0.001	< 0.001
Mannanase × SBM		0.154	0.388	0.037	0.213	0.196	0.068

[a, b, c] The data in the same row with shoulder labels containing different letters indicate significant differences ($p < 0.05$).

Respiratory metabolism and N balance

As shown in Table 5, there was a significant reciprocal effect of mannanase and SBM content on RQ in broilers at 21 d ($p < 0.05$), and there was a relative trend in the reciprocal effect on TRN ($p = 0.068$). Adding mannanase to the diet significantly increased TRN in broilers ($p < 0.01$), reduced VO_2 and VCO_2 ($p < 0.05$) and increased N_{int} in broilers at 21 d ($p < 0.05$), with a relative trend to decrease N_{exc} ($p = 0.061$). The effect of SBM content on the N balance of broilers at 21 d was specifically shown that compared with the 35.66% SBM group, when the SBM content was reduced to 17.83% and 8.92%, the N_{exc} was significantly increased and TRN was significantly decreased in broilers at 21 d ($p < 0.01$). Adding mannanase significantly reduced the RQ of broilers at 21 d in both the control diet group and the 17.83% SBM group. However, mannanase showed limited efficacy when SBM content was reduced to 8.92%.

Energy metabolism

As shown in Table 6, there was a relative trend in the reciprocal effect of mannanase and SBM content on RE_{pro} ($p = 0.068$). Mannanase significantly reduced HP and HI in broilers at 21 d ($p < 0.05$), significantly increased RE and NE:ADG ($p < 0.05$), and also highly significantly increased NE value of the feed, NE:AME, and RE_{pro} in broilers at 21 d ($p < 0.01$). The effect of SBM content on energy metabolism in broilers at 21 d was mainly reflected in the fact that when the SBM content was reduced to 17.83% and 8.92%, the RE_{pro} in broilers at 21 d was significantly decreased. The 17.83% SBM group significantly increased the RE_{fat}. When SBM content was 35.66%, adding mannanase significantly increased the RE_{pro} in broilers at 21 d. However, mannanase showed limited efficacy when SBM content was reduced to 8.92%.

The ATTD of nutrients

As shown in Table 7, there was a relative trend in the reciprocal effect of mannanase and SBM content on the ATTD of CP in broiler chickens at 21 d ($p = 0.093$). Adding mannanase to diets significantly increased the ATTD of CP in broilers at 21 d ($p < 0.05$). The effects of SBM content on the ATTD of nutrients of broilers at 21 d were mainly shown as follows: compared with the 35.66% SBM group, the 17.83% and 8.92% SBM

Table 6. Effect of mannanase addition to diets with different SBM content on energy metabolism in broilers at 21 d.

SBM content	Mannanase	THP (KJ)	HI (KJ)	RE (KJ)	RE_{pro} (KJ)	RE_{fat} (KJ)	AME (MJ/kg)	AMEn (MJ/kg)	NE (MJ/kg)	NE : AME	AME : ADG (KJ/g)	NE : ADG (KJ/g)
35.66% SBM	0 mg/kg	755.609	318.010	553.430	306.545^{b}	246.884	12.307	11.641	9.330	0.758	12.667	9.607
	100 mg/kg	724.772	267.340	647.049	379.367^{a}	267.682	12.527	11.803	10.153	0.812	12.602	10.213
17.83% SBM	0 mg/kg	702.607	262.826	608.921	277.277^{b}	331.644	12.323	11.723	9.841	0.798	12.690	10.130
	100 mg/kg	693.039	249.293	634.557	296.747^{b}	337.811	12.454	11.811	10.116	0.812	12.633	10.261
8.92% SBM	0 mg/kg	767.646	333.854	553.408	281.412^{b}	271.996	12.438	11.827	9.294	0.748	12.977	9.696
	100 mg/kg	671.706	263.332	649.486	291.569^{b}	357.916	12.485	11.849	9.993	0.800	13.094	10.478
SEM		10.776	10.552	15.626	7.846	13.337	0.077	0.071	0.109	0.008	0.084	0.107
Main effect												
Mannanase	0 mg/kg	741.954^{a}	304.897^{a}	571.920^{b}	288.412^{b}	283.508	12.356	11.730	9.488^{b}	0.768^{b}	12.778	9.811^{b}
	100 mg/kg	696.506^{b}	259.988^{b}	643.697^{a}	322.561^{a}	321.136	12.489	11.821	10.087^{a}	0.808^{a}	12.776	10.317^{a}
SBM content	35.66% SBM	740.191	292.675	600.240	342.956^{a}	257.283^{b}	12.417	11.722	9.741	0.785	12.635	9.910
	17.83% SBM	697.823	256.060	621.739	287.012^{b}	334.728^{a}	12.389	11.767	9.978	0.805	12.662	10.195
	8.92% SBM	719.676	298.593	601.447	286.491^{b}	314.956^{ab}	12.462	11.838	9.643	0.774	13.035	10.087
p-value												
Mannanase		0.028	0.030	0.025	0.005	0.135	0.426	0.551	0.005	0.006	0.993	0.017
SBM content		0.230	0.178	0.810	< 0.001	0.040	0.935	0.820	0.377	0.189	0.111	0.508
Mannanase × SBM		0.193	0.494	0.566	0.068	0.379	0.911	0.931	0.503	0.431	0.883	0.398

$^{a, b}$ The data in the same row with shoulder labels containing different letters indicate significant differences ($p < 0.05$).

Table 7. Effect of mannanase addition to diets with different SBM content on the ATTD of DM, CP, and GE of broilers at 21 d (%).

SBM content	Mannanase	ATTD of DM	ATTD of CP	ATTD of GE
35.66% SBM	0 mg/kg	67.062	59.355^{b}	72.360
	100 mg/kg	69.497	71.307^{a}	73.988
17.83% SBM	0 mg/kg	68.646	53.074^{b}	74.507
	100 mg/kg	69.777	56.517^{b}	76.043
8.92% SBM	0 mg/kg	69.744	54.575^{b}	76.982
	100 mg/kg	70.157	55.347^{b}	75.970
SEM		0.541	1.417	0.560
Main effect				
Mannanase	0 mg/kg	68.484	55.668^{b}	74.616
	100 mg/kg	69.810	61.057^{a}	75.334
SBM content	35.66% SBM	68.280	65.331^{a}	73.174^{b}
	17.83% SBM	69.211	54.795^{b}	75.275^{ab}
	8.92% SBM	69.950	54.961^{b}	76.476^{a}
p-value				
Mannanase		0.239	0.015	0.508
SBM content		0.474	< 0.001	0.053
Mannanase × SBM		0.752	0.093	0.527

$^{a, b}$ The data in the same row with shoulder labels containing different letters indicate significant differences ($p < 0.05$).

groups significantly decreased the ATTD of CP ($p < 0.01$) and also had a relative tendency to increase the ATTD of GE when the SBM content was reduced to 8.92% ($p = 0.053$). When the SBM content was 35.66%, adding mannanase significantly increased the ATTD of CP at 21 d.

As shown in Table 8, the addition of mannanase to the diets significantly increased the ATTD of essential amino acids (EAA) such as Lysine (Lys), Valine (Val), and Isoleucine (Ile) in broilers at 21 d, and significantly increased the ATTD of nonessential amino acid such as Alanine (Ala) ($p < 0.05$), with a relative trend to increase the ATTD of Leucine (Leu) ($p = 0.079$) and the ATTD of Phenylalanine (Phe) in broilers at 21 d ($p = 0.072$). The effect of SBM content on the ATTD of AA in broilers at 21 d were mainly expressed in the following ways: compared with the 35.66% SBM group, the 17.83% and 8.92% SBM groups significantly reduced the ATTD of Lys, Ile, and Histidine (His), and increased the ATTD of Tryptophan (Trp) in broilers at 21 d ($p < 0.01$).

Chyme viscosity of the jejunum

As shown in Table 9, there was a highly significant reciprocal effect of mannanase and SBM content on the chyme viscosity of jejunum in broilers at 21 d. Mannanase significantly reduced the chyme viscosity of jejunum ($p < 0.01$). The effects of SBM content on jejunal chyme viscosity were mainly manifested by the fact that the 17.83% and 8.92% SBM reduction groups significantly reduced the chyme viscosity of the jejunum in broilers at 21 d compared with the 35.66% SBM diet ($p < 0.05$). The addition of mannanase to the diets significantly reduced the jejunal chyme viscosity of broilers at 21 d, and when the SBM content was 30.58%, mannanase could highly significantly reduce chyme viscosity of broilers at 42 d.

Intestinal barrier integrity

As shown in Table 10, there was a highly significant interaction effect of mannanase and SBM content on serum D-LA levels in broiler chickens at 21 d. Adding mannanase to the diets significantly reduced DAO and ET in the serum of broilers at 21 d ($p < 0.05$). The levels of ET in the serum of broilers at 21 d decreased with a reduction in SBM content and was significantly reduced in the 17.83% and 8.92% SBM groups compared to the 35.66% SBM diet ($p < 0.01$). Adding mannanase significantly reduced the serum D-LA levels of broilers at 21 d in the control diet and the 17.83% SBM group. However, mannanase showed limited efficacy when SBM content was reduced to 8.92%.

Intestinal morphology

As shown in Table 11, there was a significant reciprocal effect of mannanase and SBM content on the VH and CD of ileal epithelium in broilers at 21 d ($p < 0.05$). At the same time, it had a highly significant reciprocal effect on the V:C ($p < 0.01$). When the SBM content was 35.66% and 17.83%, adding mannanase significantly increased the VH and V:C of ileal epithelium in broilers at 21 d. When the SBM content was 8.92%, mannanase highly significantly increased the V:C of ileal epithelium in broilers at 21 d, but it did not improve the VH of ileal epithelium.

Relative expression of genes related to the intestinal barrier

Relative expression of jejunal barrier-related genes

As shown in Table 12, mannanase highly significantly increased the relative expression of Claudin-1 in the jejunum of broilers at 21 d ($p < 0.01$). There was no significant effect of SBM content on the relative

Table 8. Effect of mannanase addition to diets with different SBM content on the ATTD of AA of broilers at 21 d (%).

SBM content	Mannanase	Threonine	Lysine	Tryptophan	Methionine	Arginine	Leucine	Valine	Isoleucine	Phenylalanine
35.66% SBM	0 mg/kg	0.830	0.894	0.852	0.935	0.937	0.890	0.829	0.851	0.897
	100 mg/kg	0.838	0.907	0.845	0.942	0.943	0.899	0.860	0.876	0.914
17.83% SBM	0 mg/kg	0.817	0.872	0.877	0.929	0.934	0.872	0.815	0.826	0.884
	100 mg/kg	0.830	0.881	0.905	0.934	0.934	0.879	0.826	0.834	0.891
8.92% SBM	0 mg/kg	0.836	0.870	0.872	0.928	0.932	0.883	0.828	0.832	0.898
	100 mg/kg	0.840	0.886	0.903	0.931	0.933	0.897	0.836	0.847	0.904
SEM		0.004	0.003	0.008	0.002	0.002	0.003	0.004	0.005	0.003
Main effect										
Mannanase	0 mg/kg	0.828	0.879^{b}	0.867	0.931	0.934	0.882^{b}	0.824^{b}	0.836^{b}	0.893^{b}
	100 mg/kg	0.836	0.892^{a}	0.884	0.936	0.937	0.891^{a}	0.840^{a}	0.852^{a}	0.903^{a}
SBM content	35.66% SBM	0.834	0.901^{a}	0.848^{b}	0.939	0.940	0.894^{a}	0.845^{a}	0.864^{a}	0.905^{a}
	17.83% SBM	0.824	0.877^{b}	0.891^{a}	0.931	0.934	0.876^{b}	0.820^{b}	0.830^{b}	0.888^{b}
	8.92% SBM	0.838	0.881^{b}	0.888^{a}	0.929	0.933	0.890^{a}	0.832^{ab}	0.840^{b}	0.901^{a}
p-value										
Mannanase		0.299	0.005	0.222	0.170	0.488	0.079	0.039	0.037	0.072
SBM content		0.310	< 0.001	0.034	0.101	0.300	0.019	0.047	0.003	0.031
Mannanase × SBM		0.879	0.789	0.475	0.918	0.813	0.850	0.444	0.612	0.668

SBM content	Mannanase	Histidine	Glycine	Aspartic acid	Glutamic acid	Cystine	Alanine	Serine	Proline	Tyrosine
35.66% SBM	0 mg/kg	0.875	0.484	0.832	0.896	0.757	0.831	0.836	0.844	0.858
	100 mg/kg	0.892	0.549	0.861	0.907	0.781	0.862	0.861	0.866	0.892
17.83% SBM	0 mg/kg	0.846	0.483	0.823	0.894	0.768	0.828	0.828	0.832	0.874
	100 mg/kg	0.860	0.497	0.832	0.900	0.790	0.842	0.837	0.842	0.872
8.92% SBM	0 mg/kg	0.853	0.502	0.832	0.904	0.780	0.856	0.845	0.851	0.871
	100 mg/kg	0.852	0.543	0.835	0.908	0.802	0.867	0.851	0.857	0.890
SEM		0.005	0.012	0.005	0.003	0.006	0.004	0.004	0.004	0.004
Main effect										
Mannanase	0 mg/kg	0.858	0.490^{b}	0.829	0.898	0.768^{b}	0.839^{b}	0.836^{b}	0.842^{b}	0.867^{b}
	100 mg/kg	0.868	0.530^{a}	0.843	0.905	0.791^{a}	0.857^{a}	0.850^{a}	0.855^{a}	0.885^{a}
SBM content	35.66% SBM	0.884^{a}	0.516	0.846	0.902	0.769	0.847^{ab}	0.849	0.855	0.875
	17.83% SBM	0.853^{b}	0.490	0.827	0.897	0.779	0.835^{b}	0.832	0.837	0.873
	8.92% SBM	0.853^{b}	0.523	0.833	0.906	0.791	0.862^{a}	0.848	0.854	0.880
p-value										
Mannanase		0.243	0.096	0.119	0.203	0.062	0.015	0.083	0.089	0.020
SBM content		0.005	0.470	0.202	0.450	0.329	0.019	0.154	0.106	0.665
Mannanase × SBM		0.612	0.665	0.444	0.865	0.998	0.450	0.522	0.631	0.124

[a, b] The data in the same row with shoulder labels containing different letters indicate significant differences ($p < 0.05$).

Table 9. Effect of mannanase addition to diets with different SBM content on jejunal chyme viscosity in broilers (P).

SBM content	Mannanase	21 d	42 d
Control group	0 mg/kg	1.083	1.120^{a}
	100 mg/kg	1.059	1.079^{b}
50% of SBM of control	0 mg/kg	1.070	1.113^{a}
	100 mg/kg	1.033	1.096^{ab}
25% of SBM of control	0 mg/kg	1.048	1.096^{ab}
	100 mg/kg	1.046	1.124^{a}
SEM		0.004	0.005
Main effect			
Mannanase	0 mg/kg	1.067^{a}	1.110
	100 mg/kg	1.046^{b}	1.110
SBM content	Control group	1.071^{a}	1.099
	50% of SBM of control	1.051^{b}	1.104
	25% of SBM of control	1.047^{b}	1.110
p-value			
Mannanase		0.004	0.251
SBM content		0.015	0.604
Mannanase × SBM		0.108	0.008

[a, b] The data in the same row with shoulder labels containing different letters indicate significant differences ($p < 0.05$).

Table 10. Effect of mannanase addition to diets with different SBM content on intestinal barrier integrity of broilers at 21 d.

SBM content	Mannanase	DAO (ng/mL)	D-LA (nmol/L)	ET (EU/ml)
35.66% SBM	0 mg/kg	24.711	73.848^{a}	0.219
	100 mg/kg	24.171	68.059^{c}	0.188
17.83% SBM	0 mg/kg	25.079	68.146^{c}	0.152
	100 mg/kg	22.858	63.183^{d}	0.135
8.92% SBM	0 mg/kg	24.433	69.186^{bc}	0.170
	100 mg/kg	23.430	72.881^{ab}	0.151
SEM		0.295	0.785	0.007
Main effect				
Mannanase	0 mg/kg	24.741^{a}	70.393	0.180^{a}
	100 mg/kg	23.486^{b}	68.041	0.158^{b}
SBM content	35.66% SBM	24.441	70.953	0.204^{a}
	17.83% SBM	23.969	65.664	0.160^{b}
	8.92% SBM	23.932	71.034	0.143^{b}
p-value				
Mannanase		0.036	0.069	0.048
SBM content		0.728	0.001	< 0.001
Mannanase × SBM		0.480	0.006	0.850

[a, b, c, d] The data in the same row with shoulder labels containing different letters indicate significant differences ($p < 0.05$).

Table 11. Effect of mannanase addition to diets with different SBM content on morphological indexes of ileal epithelium in broilers at 21 d.

SBM content	Mannanase	VH (μm)	CD (μm)	V:C
35.66% SBM	0 mg/kg	656.073^{b}	111.063^{a}	5.441^{c}
	100 mg/kg	729.529^{a}	109.180^{ab}	6.695^{a}
17.83% SBM	0 mg/kg	664.777^{b}	113.061^{a}	5.879^{b}
	100 mg/kg	712.769^{a}	108.930^{ab}	6.492^{a}
8.92% SBM	0 mg/kg	666.053^{b}	100.126^{c}	6.005^{b}
	100 mg/kg	679.013^{b}	104.606^{bc}	6.546^{a}
SEM		5.584	0.889	0.079
Main effect				
Mannanase	0 mg/kg	662.301	108.084	5.775
	100 mg/kg	707.103	107.572	6.578
SBM content	35.66% SBM	692.801	110.122	6.068
	17.83% SBM	688.773	110.996	6.185
	8.92% SBM	672.533	102.366	6.276
p-value				
Mannanase		< 0.001	0.703	< 0.001
SBM content		0.124	< 0.001	0.220
Mannanase × SBM		0.018	0.032	0.007

[a, b, c] The data in the same row with shoulder labels containing different letters indicate significant differences ($p < 0.05$).

Table 12. Effect of mannanase addition to diets with different SBM content on the relative expression of genes associated with the jejunal barrier in broilers at 21 d.

SBM content	Mannanase	*ZO-1*	Occludin	Claudin-1	*MUC-2*
35.66% SBM	0 mg/kg	1.000	1.000	1.000	1.000
	100 mg/kg	1.070	1.579	1.081	1.118
17.83% SBM	0 mg/kg	0.884	1.143	0.748	0.933
	100 mg/kg	0.916	1.321	1.539	1.049
8.92% SBM	0 mg/kg	0.660	1.500	0.641	1.004
	100 mg/kg	1.111	1.770	1.330	0.968
SEM		0.060	0.125	0.082	0.052
Main effect					
Mannanase	0 mg/kg	0.848	1.215	0.796^{b}	0.979
	100 mg/kg	1.032	1.556	1.316^{a}	1.045
SBM content	35.66% SBM	1.035	1.289	1.040	1.059
	17.83% SBM	0.900	1.232	1.143	0.991
	8.92% SBM	0.886	1.635	0.985	0.986
p-value					
Mannanase		0.127	0.183	0.001	0.550
SBM content		0.529	0.378	0.667	0.831
Mannanase × SBM		0.289	0.794	0.109	0.805

[a, b] The data in the same row with shoulder labels containing different letters indicate significant differences ($p < 0.05$).

expression of genes associated with the jejunal barrier in broilers at 21 d. Mannanase could highly significantly increase the relative expression of Claudin-1 in the jejunum of broilers at 21 d.

Relative expression of ileal barrier-related genes

As shown in Table 13, there was no significant reciprocal effect of mannanase and SBM content on the mRNA expression of genes related to the ileal barrier in broilers at 21 d ($p > 0.05$). The addition of mannanase to diets significantly increased the relative expression of occludin and Claudin-1 in the ileum of broilers at 21 d ($p < 0.05$), with a tendency to increase the relative expression of *ZO-1* ($p = 0.062$). SBM content had no significant effect on the relative expression of ileal barrier-related genes in broilers at 21 d ($p > 0.05$). Mannanase significantly increased the relative expression of occludin and Claudin-1, etc. in the ileum of broilers at 21 d, and there was no significant reciprocal effect of mannanase and SBM content on the relative expression of genes associated with the ileal barrier at 21 d.

Table 13. Effect of mannanase addition to diets with different SBM content on the relative expression of genes associated with the ileal barrier in broilers at 21 d.

SBM content	Mannanase	*ZO-1*	Occludin	Claudin-1	*MUC-2*
35.66% SBM	0 mg/kg	1.000	1.000	1.000	1.000
	100 mg/kg	1.363	1.336	1.332	1.227
17.83% SBM	0 mg/kg	0.922	0.793	0.912	0.978
	100 mg/kg	0.935	0.925	1.094	1.177
8.92% SBM	0 mg/kg	0.701	0.733	1.020	1.226
	100 mg/kg	1.254	1.135	1.865	1.004
SEM		0.083	0.067	0.111	0.081
Main effect					
Mannanase	0 mg/kg	0.875^{b}	0.842^{b}	0.977^{b}	1.068
	100 mg/kg	1.184^{a}	1.132^{a}	1.430^{a}	1.136
SBM content	35.66% SBM	1.182	1.168	1.166	1.113
	17.83% SBM	0.928	0.859	1.003	1.077
	8.92% SBM	0.978	0.934	1.442	1.115
p-value					
Mannanase		0.062	0.029	0.040	0.691
SBM content		0.405	0.133	0.247	0.979
Mannanase × SBM		0.392	0.672	0.420	0.481

[a, b] The data in the same row with shoulder labels containing different letters indicate significant differences ($p < 0.05$).

Relative expression of genes related to intestinal immunity

As shown in Table 14, there was a highly significant reciprocal effect of mannanase and SBM content on the relative expression of *IL-1β* and *IL-18* in ileum at 21 d ($p < 0.01$), and there was a relative trend in the reciprocal effect of mannanase and SBM content on the relative expression of *NF-κB* ($p = 0.052$). Adding mannanase highly significantly reduced the relative expression of *MyD88* ($p < 0.01$), *IL-6*, and *TLR-4* in the ileum of broilers at 21 d ($p < 0.05$). Moreover, mannanase tended to increase the relative expression of *IL-10* ($p = 0.071$) and decrease the relative expression of *NF-κB* ($p = 0.068$). The effects of SBM content on the relative expression of genes associated with the ileal inflammatory response of broilers at 21 d were mainly manifested in the following aspects: compared with 35.66% SBM, when the SBM content was reduced to 17.83% and 8.92%, both of them significantly reduced the relative expression of *IL-8* in the ileum of broilers at 21 d ($p < 0.01$). Furthermore, compared to the 35.66% SBM, when the SBM content was reduced to 17.83%, the relative expression of *NF-κB* ($p < 0.01$) and *TLR-4* ($p < 0.05$) in the ileum of broilers at 21 d was significantly reduced. It also had a relative tendency to reduce the relative expression of *MyD88* ($p = 0.078$). When the SBM content was 35.66%, mannanase significantly reduced the relative expression of genes related to the ileal inflammatory response of broilers at 21 d, such as *NF-κB* and *IL-1β*, to alleviate intestinal inflammation. However, mannanase showed limited efficacy when SBM content was reduced to 17.83% and 8.92%.

16S rRNA sequencing of microorganisms in the ileum

Species screening and basic statistics

As is clear from Fig. 1b, the top five species in terms of relative abundance at the phylum level of microorganisms in ileal chyme of broiler chickens at 21 d in the 17.83% SBM group and the 17.83% SBM diet supplemented with 100 mg/kg mannanase were Firmicutes, Bacteroidetes, Proteobacteria, Tenericutes, and Actinobacteria. As is clear from Fig. 1c, the top 10 species in terms of relative abundance at the genus level were *Lactobacillus*, unclassified, *Bacteroides*, *Barnesiella*, *Streptococcus*, *Faecalibacterium*, *Ruminococcus*, *Candidatus_Arthromitus*, *Campylobacter*, and *Oscillospira*. The results of the Kruskal Wallis test showed that mannanase tended to decrease the relative abundance of Firmicutes at the phylum level ($p = 0.064$) and to increase the relative abundance of *Ruminococcus* at the genus level ($p = 0.082$)

Table 14. Effect of mannanase addition to diets with different SBM content on the relative expression of inflammatory response genes in the ileum of broilers at 21 d.

SBM content	Mannanase	*IL-1β*	*IL-6*	*IL-8*	*IL-10*	*IL-18*	*TNF-a*	*MyD88*	*NF-kB*	*TLR-4*
35.66% SBM	0 mg/kg	1.000^{a}	1.000	1.000	1.000	1.000^{a}	1.000	1.000	1.000^{a}	1.000
	100 mg/kg	0.546^{b}	0.302	0.658	1.274	0.547^{b}	0.829	0.754	0.612^{bc}	0.751
17.83% SBM	0 mg/kg	0.434^{b}	0.709	0.385	0.617	0.414^{bc}	0.867	0.685	0.575^{bc}	0.612
	100 mg/kg	0.436^{b}	0.345	0.269	0.843	0.226^{c}	0.809	0.520	0.330^{c}	0.555
8.92% SBM	0 mg/kg	0.434^{b}	0.734	0.337	0.690	0.295^{bc}	0.912	0.941	0.541^{bc}	0.885
	100 mg/kg	0.768^{ab}	0.647	0.426	1.141	0.579^{b}	0.700	0.556	0.679^{b}	0.575
SEM		0.052	0.076	0.055	0.088	0.054	0.062	0.053	0.051	0.048
Main effect										
mannanase	0 mg/kg	0.623	0.815^{a}	0.574	0.769^{b}	0.570	0.926	0.876^{a}	0.705^{a}	0.832^{a}
	100 mg/kg	0.583	0.431^{b}	0.451	1.086^{a}	0.451	0.779	0.610^{b}	0.540^{b}	0.627^{b}
SBM content	35.66% SBM	0.773	0.651	0.829^{a}	1.137	0.774	0.914	0.877^{a}	0.806^{a}	0.876^{a}
	17.83% SBM	0.435	0.527	0.327^{b}	0.730	0.320	0.838	0.603^{b}	0.453^{b}	0.584^{b}
	8.92% SBM	0.601	0.691	0.382^{b}	0.915	0.437	0.806	0.749^{ab}	0.610^{ab}	0.730^{ab}
p-value										
Mannanase		0.650	0.010	0.173	0.071	0.159	0.258	0.009	0.068	0.023
SBM content		0.011	0.626	< 0.001	0.164	< 0.001	0.779	0.078	0.008	0.031
Mannanase × SBM		0.003	0.229	0.150	0.852	0.003	0.879	0.646	0.052	0.470

[a, b, c] The data in the same row with shoulder labels containing different letters indicate significant differences ($p < 0.05$).

compared to the 17.83% SBM reduction group. As is clear from Fig. 1d, the number of species common to both groups was 52 in the 17.83% SBM group and the 17.83% SBM group supplemented with mannanase, with the number of species unique to the 17.83% SBM group being 134 and the number of species unique to the mannanase group being 183.

Statistically significant differences

As can be seen in Fig. 1e, the microorganism with a relative trend of difference between the two groups at the phylum level was *Tenericutes* ($p = 0.056$). As can be seen in Fig. 1f, the microorganism with a significant difference at the genus level was *Weissella* ($p < 0.05$) and the microorganism with a relative trend of difference was *Campylobacter* ($p = 0.052$). As is clear from Fig. 1g–k and Fig. 1l, m, the addition of mannanase to the 17.83% SBM group had no significant effect on the diversity of microorganisms in the ileum of broilers at 21 d. As can be seen in Fig. 1n, *Lactobacillus* showed a highly significant negative correlation with *Streptococcus* ($p < 0.01$) and significant negative correlation with *Candidatus_Arthromitus* ($p < 0.05$). *Achromobacter* was significantly positively correlated with *Bacteroides* and *Campylobacter* ($p < 0.05$).

Functional forecasting and variance analysis

As can be seen in Fig. 1o and p, the addition of mannanase to a diet with 17.83% SBM content significantly affected the microbial Indole alkaloid biosynthesis and Platinum drug resistance at the KEGG-L3 level, as corrected by Bonferroni. Specifically, compared to the 17.83% SBM group, mannanase promoted microbial Indole alkaloid biosynthesis and Platinum drug resistance. The analysis based on the CAZymes showed that mannanase could significantly affect the activities of enzymes associated with microbial metabolism and other pathways. Specifically, the addition of mannanase could highly significantly decrease the activities of (R,R)-butanediol dehydrogenase, Diacetyl reductase ((R)-acetoin forming), and Carboxypeptidase Taq, and significantly increase the activities of Aromatic-L-amino-acid decarboxylase and Protoporphyrinogen oxidase, among others.

16S rRNA sequencing of microorganisms in the cecum

Species screening and basic statistics

As is clear from Fig. 2b, the five microorganisms with the highest relative abundance at the phylum level in the cecal chyme of broiler chickens at 21 d in the 17.83% SBM group and the 17.83% SBM diet supplemented with 100 mg/kg mannanase were *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Tenericutes,* and *Actinobacteria*. As is clear from Fig. 2c, the ten microorganisms with the highest relative abundance at the genus level were *Lactobacillus*, unclassified, *Bacteroides*, *Barnesiella*, *Streptococcus*, *Faecalibacterium*, *Ruminococcus*, *Candidatus_Arthromitus*, *Campylobacter,* and *Oscillospira*. The results of the Kruskal Wallis test showed that mannanase significantly reduced the relative abundance of *Odoribacter*, *Bacteroides,* and *Lachnospiraceae_Clostridium* in the cecal microbiota of broilers at 21 d compared to the 17.83% SBM group ($p < 0.05$), with a concomitant tendency to increase the relative abundance of *Coprococcus* ($p = 0.074$). As is clear from Fig. 2d, the number of species common to both groups was 490 in the 17.83% SBM group and the 17.83% SBM diet supplemented with mannanase, with the number of species unique to the 17.83% SBM group being 876 and the number of species unique to mannanase group being 851.

Statistically significant differences

As can be seen in Fig. 2e, the microorganism with a significant difference between the two groups at the phylum level was *Firmicutes* ($p < 0.05$), and the microorganism with a relative trend of difference was *Actinobacteria* ($p = 0.063$). As is clear from Fig. 2f, the microorganisms with a highly significant difference at the genus level were *Escherichia*, *Lachnospiraceae_Clostridium*, *Coprococcu,* and *Subdoligranulum* ($p < 0.01$), and the species with significant differences were *Sutterella*, *Odoribacter,* and *Erysipelotrichaceae_Clostridium* ($p < 0.05$). The microorganisms with relative trends of differences were *Anaerotruncus* ($p = 0.060$), *Barnesiella* ($p = 0.083$), *Enterococcus* ($p = 0.090$), and *Clostridium* ($p = 0.077$). As is evident in Fig. 2g–k and Fig. 2l, m, the addition of mannanase did not exert a significant influence on the microbial diversity in the cecum of broilers at 21 d. As can be seen in Fig. 2n, *Faecalibacterium* showed a significant negative correlation with *Ruminococcaceae_Ruminococcus*, and *Lactobacillus* showed a significant negative correlation with *Ruminococcaceae_Ruminococcus* ($p < 0.05$). In addition, the addition of mannanase to the diet with 17.83% SBM content also significantly reduced the relative abundance of *Escherichia coli* in the cecum of broilers.

Functional forecasting and variance analysis

As is clear from Fig. 2o, the addition of mannanase to the diet with 17.83% SBM content highly significantly affected the microbial PI3K-Akt signaling pathway, cysteine and methionine metabolism, and glutathione metabolism at the KEGG-L3 level, as corrected by Bonferroni ($p < 0.01$), and significantly affected the microbial cell cycle-Caulobacter, protein processing in the endoplasmic reticulum,

histidine metabolism, lysine biosynthesis, carbapenem biosynthesis, and 2-Oxocarboxylic acid metabolism. Specifically, compared to the 17.83% SBM group, mannanase promoted the microbial 2-Oxocarboxylic acid metabolism and carbapenem biosynthesis. It also inhibited the glutathione metabolism and glyoxylate and dicarboxylate metabolism. The analysis based on the CAZymes showed that

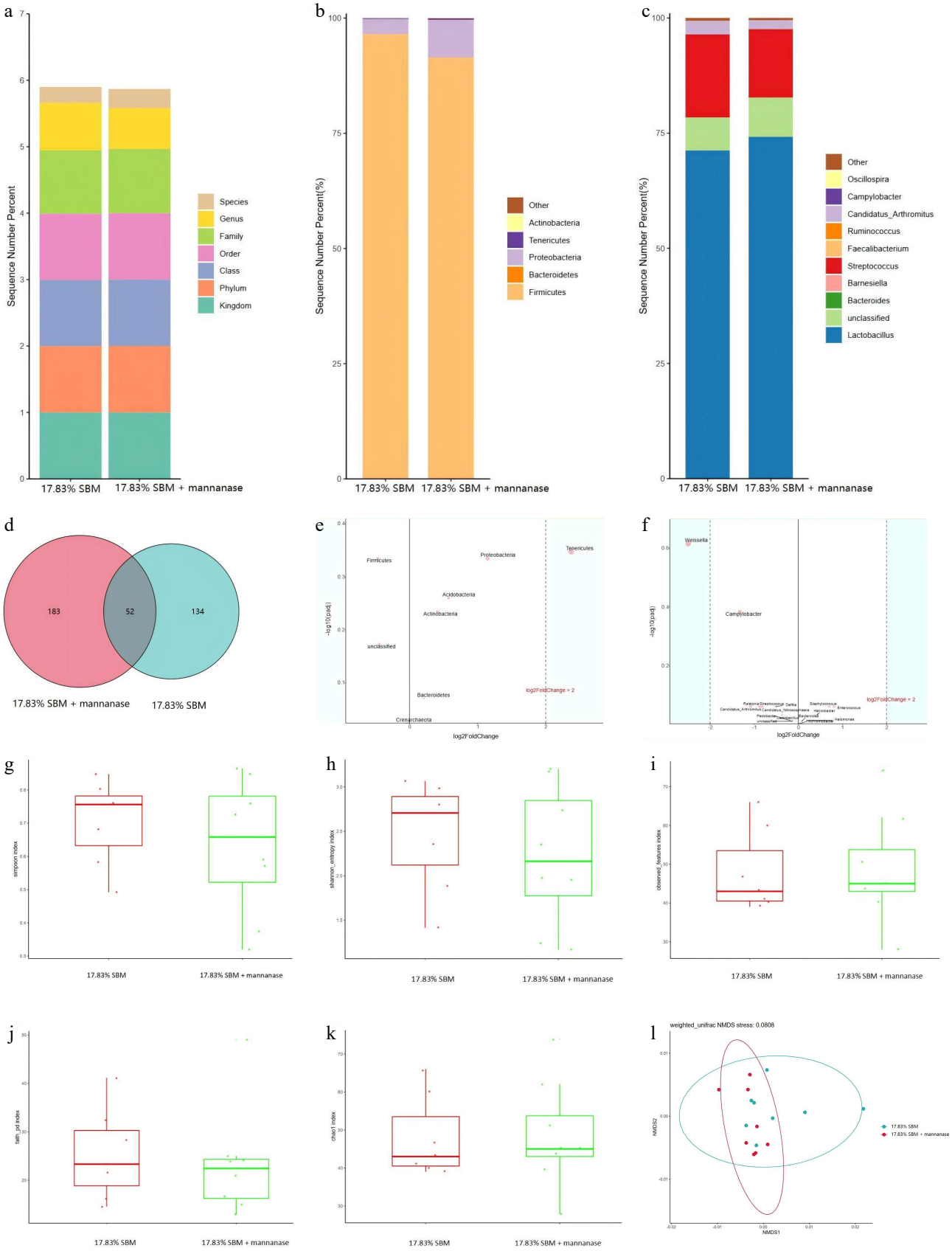

Fig. 1 (to be continued)

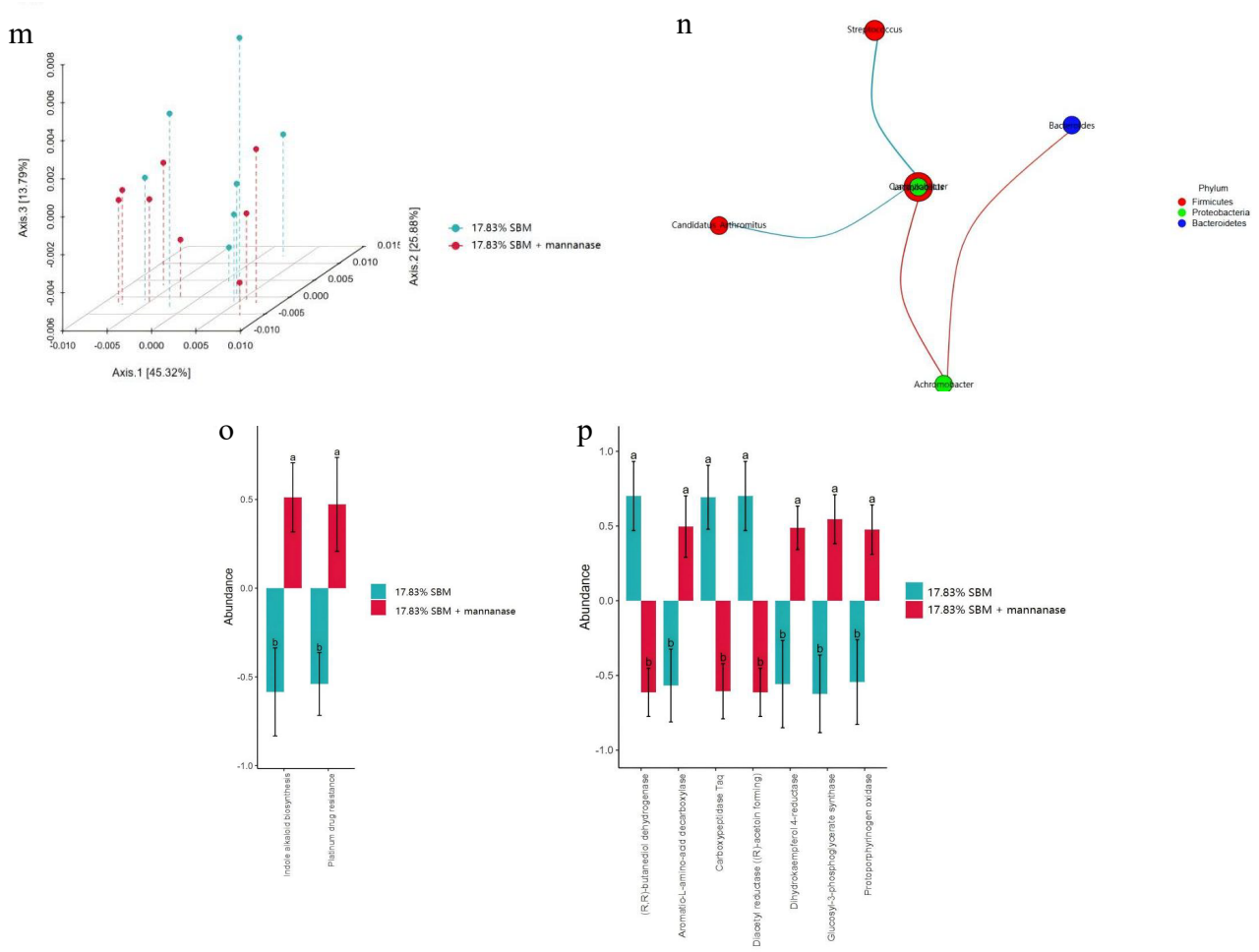

Fig. 1 Effect of mannanase addition to the diet with 17.83% SBM content on ileal microflora of broilers at 21 d. (a) In this study, the Greengenes database was used as the basis for species-level classification of ASVs using sklearn, followed by species screening to retain Bacteria and Archaea. The top 5 species in terms of relative abundance at the phylum level and the top 10 species in relative abundance at the genus level were analyzed in the form of (b), (c) bar charts; and (d) venn diagrams were used to represent the two groups of endemic and shared features. (e), (f) The relative abundance of differential ASVs at the phylum level and genus level of ileum was screened by volcano plots from DESeq2 analysis using 17.83% SBM group as control and 17.83% SBM + 100 mg/kg mannanase as the treatment group, respectively. (g)–(k) The alpha diversity was expressed as simpson's index, shannon index, observed_features, faith_pd and Chao1, and the microorganisms were subjected to (l), (m) NMDS and PCoA based on weighted Unifrac distances to express the beta diversity, respectively. (n) The correlation coefficients of the features were calculated and the nodes with significantly correlated features were connected to plot the network graphs of the Spearman's correlation analysis at the genus level. (o), (p) Based on the relative abundance and sequence of ASVs in the samples, PICRUSt2 was used to predict the macrogenomic results at the level of the KEGG-L3 and CAZymes, respectively, while the comparative analyses were performed to analyze the differences between the groups.

mannanase could significantly affect the activities of enzymes associated with microbial metabolism pathways in the cecum of broilers. Specifically, when the SBM content in the feed was reduced to 17.83%, the addition of mannanase could highly significantly reduce the activities of 2-deoxy-D-gluconate 3-dehydrogenase, increase the activities of glycerate dehydrogenase, and significantly decrease the activities of nitric oxide dioxygenase.

Discussion

As the first line of host defense against pathogens, Toll-like receptors (TLRs) can recognize different pathogen-associated molecular patterns (PAMPs) and play key roles in inflammation and the regulation of immune cells. TLRs trigger a complex inflammatory response through both MyD88-dependent and MyD88-independent pathways, activating NF-κB, and promoting the expression of genes such as *TNF-a*, *IL-1β*, and *IL-6*[33]. During the onset of the inflammatory response, the activation of the TLR/NF-κB and JAK/STAT signaling pathways promotes the release of a variety of pro-inflammatory factors and stimulates immune cells to release additional cytokines[34]. Pro-inflammatory cytokines like IL-1*β* and TNF-a are particularly potent, inducing the production of many pro-inflammatory mediators and playing an important role in the development of the immune system[35–39]. IL-8 is a multifunctional cytokine that stimulates the proliferation of immune cells and is involved in inflammatory diseases[40]. In this study, when ME was reduced by 50 kcal/kg, the diet with 35.66% SBM content triggered an inflammatory response in broilers. In contrast, the addition of mannanase significantly reduced the relative expression of *IL-1β* and *IL-18* in the ileum of broilers at 21 d, and it also tended to reduce the relative expression of *NF-κB*, alleviating the above adverse effects. In addition, the reduction of SBM content to 17.83% significantly reduced the relative expression of *NF-κB*, *TLR-4*, and *IL-8*, with a tendency to lower the relative expression of *MyD88*, compared with the 35.66% SBM group. As a surface component of several pathogens, mannan is the relatively most important ANF in SBM, which stimulates the immune system, triggers an inflammatory response, and causes energy depletion[9,41–43]. Studies have shown that there is a reciprocal effect between mannanase and SBM content[44], showing that adding mannanase to diets reduces the incidence of

inflammatory reactions[3], decreases immune stimulation[24], and improves the immune system of broilers[6]. The results mentioned above are in accordance with our findings and further suggest that mannanase alleviates the inflammatory response in broiler chickens by lowering the expression of the *NF-κB* and lessening the release of pro-inflammatory factors.

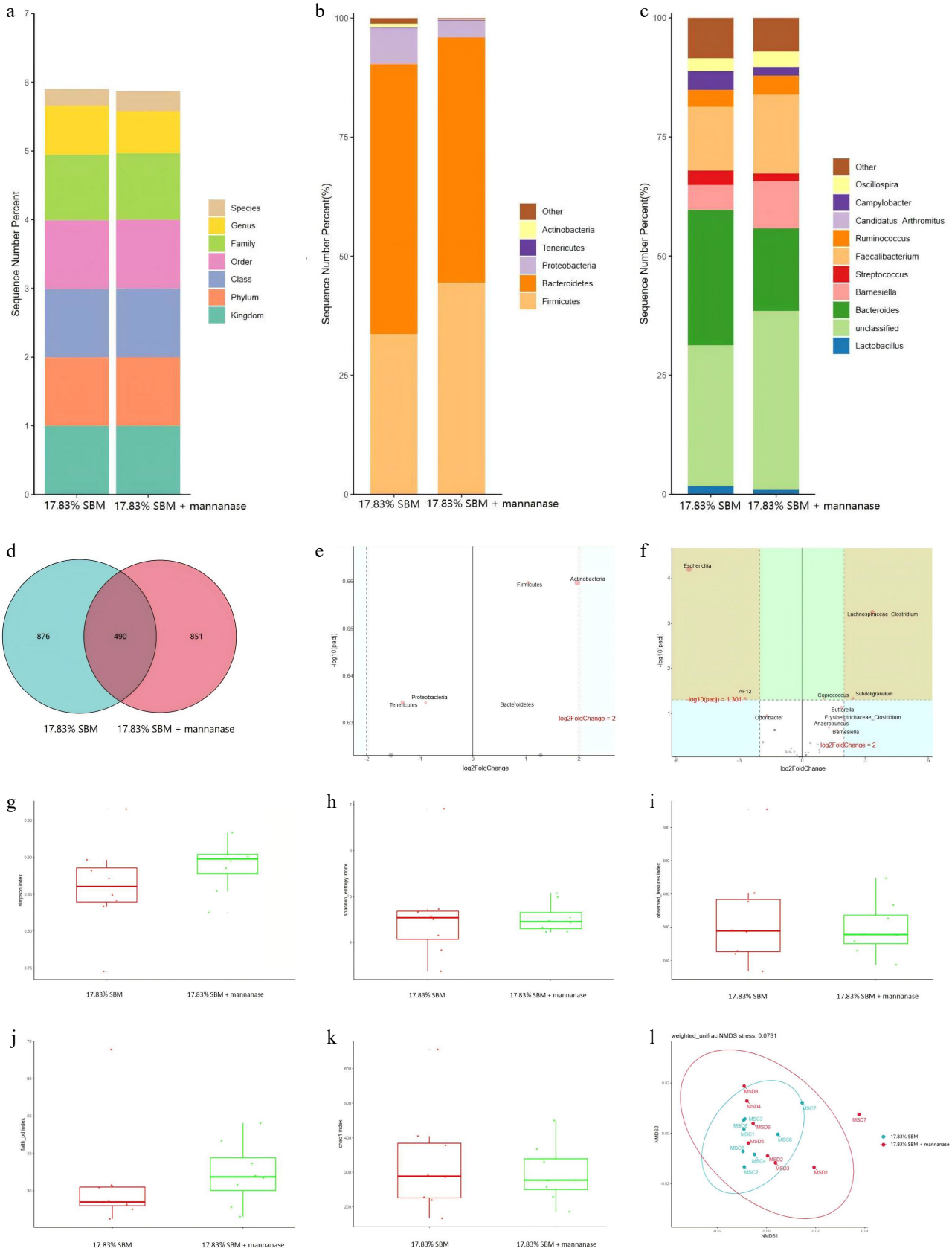

Fig. 2 (to be continued)

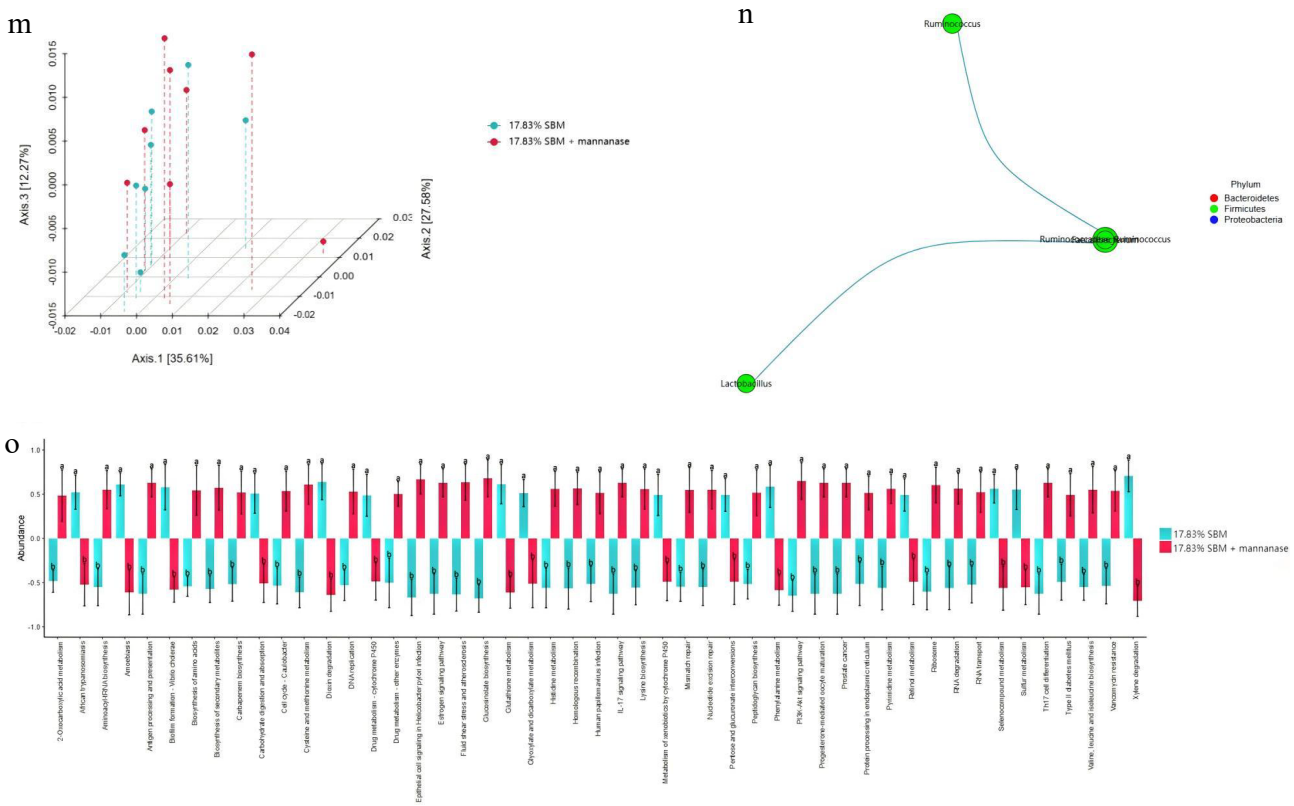

Fig. 2 Effect of mannanase addition to the diet with 17.83% SBM content on cecal microflora of broilers at 21 d. (a) In this study, the Greengenes database was used as the basis for species-level classification of ASVs using sklearn, followed by species screening to retain Bacteria and Archaea. (b), (c) The top five species in terms of relative abundance at the phylum level and the top 10 species in relative abundance at the genus level were analyzed in the form of bar charts; and (d) venn diagrams were used to represent the two groups of endemic and shared features. (e), (f) The relative abundance of differential ASVs at the phylum level and genus level of cecum was screened by volcano plots from DESeq2 analysis using 17.83% SBM group as control and 17.83% SBM + 100 mg/kg mannanase as the treatment group, respectively. (g) - (k) The alpha diversity was expressed as simpson's index, shannon index, observed_features, faith_pd, and Chao1, and the microorganisms were subjected to (l), (m) NMDS and PCoA based on weighted Unifrac distances to express the beta diversity, respectively. (n) The correlation coefficients of the features were calculated and the nodes with significantly correlated features were connected to plot the network graphs of the Spearman's correlation analysis at the genus level. (o) Based on the relative abundance and sequence of ASVs in the samples, PICRUSt2 was used to predict the macrogenomic results at the level of the KEGG-L3 and CAZymes, respectively, while the comparative analyses were performed to analyze the differences between the groups.

Activation of multiple inflammatory pathways and release of inflammatory mediators can affect intestinal barrier function, which will have an impact on the growth performance and intestinal health of broilers[45]. An intact intestinal barrier is mainly composed of a physical barrier, a biochemical barrier, and an immune barrier[46], of which tight junctions (TJs), as an important component of the intestinal barrier, are the determining factors affecting the intestinal barrier function of animals[47,48]. Occludin, claudin-1, and zonula occluden-1 (ZO-1) are the relatively most prominent TJs[49]. As a major component of mucins, MUC 2 is mainly produced by goblet cells. MUC 2 is an important component of the intestinal mucosal layer and plays an important role in separating the intestinal epithelium from microorganisms[50,51]. Mannanase has been shown to regulate intestinal barrier integrity by affecting the expression of TJs and other proteins in the broiler intestine[3]. In this experiment, adding mannanase significantly increased the relative expression of Claudin-1 in the jejunum at 21 d, significantly increased the relative expression of occludin and Claudin-1 in the ileum, and also had a tendency to increase the relative expression of *ZO-1* in the ileum.

When the integrity of the intestinal barrier is impaired, pathogenic microorganisms and toxins can pass through the intestinal epithelium, which may cause a systemic inflammatory response[52–54]. Serum DAO[55], D-LA, and ET[56] are important indicators for evaluating mucosal integrity and intestinal barrier function in broilers. In this study, reducing the ME by 50 kcal/kg in a diet containing 35.66% SBM led to increased levels of D-LA in serum and disruption of intestinal epithelial barrier in broilers at 21 d. Mannanase alleviated the increased intestinal permeability due to lower energy levels and maintained the intestinal barrier function by reducing the levels of DAO and ET. In addition, there was a reciprocal effect between mannanase and SBM content. The diets were formulated with DCP and CGM instead of SBM. When SBM content in the diet was reduced to 17.83%, adding mannanase still tended to reduce serum D-LA in broilers at 21 d. The reduction of serum DAO, D-LA, and ET levels in broilers further demonstrated that mannanase could improve intestinal barrier function and maintain barrier integrity.

There is a close relationship between intestinal morphology and immune regulation. The function of the intestinal barrier and absorption function can be visualized by the microscopic structure of intestinal morphology[57]. The intestinal VH, CD, and V:C are important for measuring the morphological and structural integrity, as well as the functional status of the intestinal mucosa. Mannanase improves intestinal epithelial morphology in broilers by increasing intestinal VH and V:C[21], and decreasing CD[58]. Consistent with our findings, the diet containing 35.66% SBM significantly reduced ileal VH and V:C and disrupted the villus structure in broilers at 21 d when dietary ME was reduced by 50 kcal/kg. In addition, there was an interaction between mannanase and SBM content on the morphology of ileum

epithelium at 21 d. When SBM content was reduced to 17.83%, mannanase still improved ileal morphology and alleviated intestinal damage in broilers by increasing the VH and V:C.

The immune system interacts with intestinal microorganisms to maintain homeostasis of the host's internal environment and regulate intestinal health[59–62]. Gut microbiota composition is an important indicator for evaluating gut health, playing a key role in maintaining gut integrity, energy metabolism, and immune function[63,64]. The diversity of the intestinal flora maintains the stability of the internal environment against the invasion of pathogenic microorganisms[65]. In this study, there was no significant effect of mannanase on the gut microbiota diversity of broilers at 21 d. It has been shown that mannanase can regulate intestinal flora structure by promoting the multiplication of beneficial bacteria like *Lactobacilli*[21], reducing the number of intestinal *Escherichia coli*[7,66], and *Salmonella*[67,68]. At the same time, it also can enhance barrier functions and ultimately improve the intestinal health of broilers. *Ruminococcus* is an anaerobic Gram-positive bacterium related to the class Clostridia[69], which degrades polysaccharides, provides nutrients for the host[70], and is strongly implicated in the development of several diseases[71–73]. *Escherichia coli* is a gram-negative bacterium that can act as a pathogen causing intestinal diseases[74,75]. In this study, in terms of relative abundance of microorganisms, adding mannanase to the diet with 17.85% SBM content tended to decrease the relative abundance of Firmicutes and increase *Ruminococcus* in the ileum of broilers at 21 d. In addition, mannanase also significantly reduced *Lachnospiraceae_Clostridium* and *Escherichia coli* in the cecum of broilers at 21 d. In summary, when SBM content was 17.83%, adding mannanase improved intestinal health by regulating the intestinal flora structure.

A reciprocal relationship between mannanase and substrate type on the viscosity of chyme has been reported. Adding mannanase to poultry feed reduces intestinal chyme viscosity, while higher levels of mannan in diets lead to increased chyme viscosity[15,18]. In the present study, we observed that the addition of mannanase to the diets highly significantly decreased the viscosity of the jejunal chyme in broilers at 21 d, and the chyme viscosity decreased with the decrease in SBM content. Specifically, compared to the 35.66% SBM group, when the SBM content was reduced to 17.83% and 8.92%, the chyme viscosity of broilers at 21 d was also significantly reduced. In addition, there was a reciprocal effect between mannanase and SBM content on the chyme viscosity of the jejunum at 42 d. The effect of mannanase was significant when the SBM content was 35.66%. The addition of mannanase to poultry feed has been reported to promote nutrient absorption, increase nutrient digestibility[8,11,24,31], and improve the nutritive value of soybean meal-based diets[17]. Consistent with our results, adding mannanase to the low-energy diet significantly increased the ATTD of CP and AA such as Lys, Ile, and Ala in broilers at 21 d.

Energy is an important component of poultry feed and plays a central role in poultry nutrition. Compared to ME, NE expresses energy loss in the form of calories which provides a more accurate assessment of energy utilization by broilers[76]. Studies have shown that adding mannanase to diets improves energy utilization efficiency[7,18] by increasing AMEn[10] and decreasing N_{exc}[24,77]. However, studies by respiratory calorimetry to determine the effect of mannanase on N balance and energy metabolism in broilers have not been reported. In this study, we found that adding mannanase to the diets affected N balance by decreasing VO_2 and VCO_2 and increasing TRN in broiler chickens at 21 d. There was a reciprocal effect of mannanase and SBM content on TRN in broilers at 21 d. In addition, we also observed that mannanase supplementation significantly reduced HP and HI in broilers at 21 d, and increased the NE value of feed and NE:AME. There was a significant reciprocal effect of mannanase and SBM content on the RE_{pro} in broilers at 21 d. When SBM content was reduced to 17.83%, it significantly reduced the RE_{pro} and increased RE_{fat} in broilers at 21 d, affecting energy metabolism.

The reduction in feed energy levels and the changes in SBM content affect the growth performance of poultry[41]. Mannanase may ameliorate the decline in the growth performance of broilers due to energy deficiency by reducing FCR and increasing BW and ADG[41], and these results are consistent with our findings. Additionally, it has been shown that there is a reciprocal effect between mannanase and the type of substrate on the growth performance of broilers[66,78] and that the action of mannanase is mainly dependent on the type of substrate and the amount of NSP contained in the feed[79]. In this study, we observed that a 50 kcal/kg reduction in ME led to decreased growth performance in broilers as SBM content was lowered. However, the addition of mannanase mitigated the above harmful effects by increasing BW and ADG of broilers at 21 d and decreasing FCR. Moreover, there was a significant reciprocal effect of mannanase and SBM content on the growth performance of broilers from 0 to 21 d of age. When the SBM content was reduced to 17.83%, the addition of mannanase still significantly improved growth performance of broilers from 0 to 21 d of age. The improved growth performance of broilers in this study also provides further evidence that mannanase can mitigate the negative effects due to lower levels of energy and the mannan content of SBM by improving intestinal barrier function and flora structure. Our findings demonstrate that mannanase supplementation enhances broiler growth performance and intestinal health, particularly in diets containing 35.66% and 17.83% SBM.

Conclusions

Adding mannanase to a low-energy diet can improve the intestinal health of broilers by enhancing intestinal barrier function, balancing intestinal flora structure, maintaining intestinal integrity, and alleviating intestinal inflammatory responses. Mannanase also increases nutrient digestibility and regulates energy metabolism by reducing HI, increasing the NE value of the feed, and NE:AME to improve broilers' growth performance. Additionally, there was a reciprocal effect between mannanase and SBM content. Under conditions where the ME of diets was reduced by 50 kcal/kg, mannanase still exerted the above beneficial effects when the SBM content was reduced to 17.83%.

Ethical statements

All procedures were reviewed and preapproved by the Beijing Experimental Animal Management Regulations and the Experimental Animal Welfare and Animal Experimentation Ethics Review Committee of China Agricultural University, identification number: AW22503202-1-2, approval date: 22/05/2023. The research followed the 'Replacement, Reduction, and Refinement' principles to minimize harm to animals. This article provides details on the housing conditions, care, and pain management for the animals, ensuring that the impact on the animals is minimized during the experiment.

Author contributions

The authors confirm contribution to the paper as follows: conceptualization, data curation, methodology, and writing - draft manuscript preparation: Zhang X; software and resources: Wang B; essential equipment and materials: Ban Z; funding acquisition: Wang M, Wang Y, Wang X; writing - review & editing: Guo Y. All authors reviewed the results and approved the final version of the manuscript.

Data availability

All data generated or analyzed during this study are included in this published article.

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Conflict of interest

The authors declare that they have no conflict of interest.

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