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Analysis of the effects of β-mannanase on immune function and intestinal flora in broilers fed the low energy diet based on 16S rRNA sequencing and metagenomic sequencing

Xiaodan Zhang,* Yanhong Chen,* Zengpeng Lv,* Liangjuan Zhou,† and Yuming Guo*,[1]

*State Key Laboratory of Animal Nutrition and Feeding, College of Animal Science and Technology, China Agricultural University, Beijing 100193, China; and †Beijing Strowin Biotechnology Co. Ltd., Beijing, 100094, China

ABSTRACT As an enzyme, β-mannanase (**BM**) can be widely used as feed additive to improve the growth performance of animals. This experiment aimed to determine the effect of the addition of BM to low-energy diet on the immune function and intestinal microflora of broiler chickens. In this study, 384 one-day-old Arbor Acres broilers were randomly divided into 3 groups (8 replicates per group): positive control (**PC**, received a corn-soybean meal basal diet), negative control (**NC**, received a low-energy diet with Metabolizable Energy (**ME**) reduced by 50 kcal/kg) and NC + BM group (NC birds + 100 mg/kg BM). All birds were raised for 42 d. The results showed that BM mitigated the damage of immune function in peripheral blood of broilers caused by the decrease of dietary energy level by increasing the Concanavalin A (**Con A**) index of stimulation (**SI**) and macrophages phagocytic activity in the peripheral blood of broilers at 42 d ($P < 0.05$). The analysis of cecum flora showed that the low-energy diet significantly reduced the observed_species index ($P < 0.01$), Chao1 index and ACE index ($P < 0.05$), which reduced the abundance and evenness of species in the cecum of broilers at 21 d. It also significantly reduced the relative abundance of *Candidatus_Arthromitus* and significantly increased the relative abundance of *Pseudomonas* in the cecum of broilers at 21 d, while also significantly increasing the relative abundance of *Monoglobus* at 42 d. BM significantly increased the relative abundance of *Lachnospiraceae_UCG-001* and *Lachnospiraceae_bacterium_615* in the cecum of broilers at 21 d. In addition, BM inhibited microbial Fatty acid degradation by decreasing the activity of glutaryl-CoA dehydrogenase. Collectively, BM could improve intestinal health by enhancing the immune function of broilers, promoting the proliferation of beneficial bacteria and reducing the number of harmful bacteria, regulating intestinal flora, thereby alleviating the adverse effects of lower dietary energy levels.

Key words: low-energy diet, mannanase, immunity, metagenome, broiler

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INTRODUCTION

Energy is an important component of poultry diets, and the nutrient requirements of poultry are expressed on the basis of energy. It has been shown that the lower energy level of the diet could lead to a decrease in the immune function of the broilers (Yang et al., 2015; Hu et al., 2019; Vangroenweghe, 2021), disruption of intestinal microbiota (Yang et al., 2022) and damage to intestinal health. Intestinal flora can regulate animal health and is linked to the development of various diseases (Spor et al., 2011).

Soya bean meal (**SBM**) is the most widely used protein sources in animal feeds due to its high protein content and better amino acid balance. SBM contains approximately 17 to 27% non-starch polysaccharides (**NSP**) (Vangroenweghe, 2021). As the second largest component of hemicellulose, mannans are relatively major antinutritional factors (**ANFs**) in corn-soybean meal type feeds. Studies have shown that mannans, as a kind of immunostimulant (Zou et al., 2006), can produce intestinal stress (Hsiao et al., 2006) and anaerobic fermentation of microorganisms in the lower part of the digestive tract, causing disruption of the microbial community (Kim et al., 2017).

β-Mannanase (**BM**) is an endo-hydrolytic enzyme, which can act on the β-1,4-D-mannosidic bonds of mannans and iso-mannans (galactomannans, glucomannans and galactoglucomannans) (Dhawan and Kaur, 2007). While degrading mannans, BM can produce mannan

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[1]Corresponding author: guoyum@cau.edu.cn

oligosaccharides (**MOS**) (Arsenault et al., 2017) to enhance the immune function of broilers (Zou et al., 2006; Zangiabadi and Torki, 2010; Ferreira et al., 2016) and regulate the flora structure by promoting the proliferation of beneficial bacteria such as *Lactobacillus* and *Bifidobacterium* and reducing the colonization of harmful bacteria such as *Salmonella* and *Escherichia coli* in the intestinal tract to improve intestinal health (Al-Ghazzewi and Tester, 2012; K., 2020; Bhaturiwala et al., 2021; Mohammadigheisar et al., 2021). Poultry are unable to secrete BM, so exogenous BM could be an effective tool to improve the intestinal health and growth performance of chickens fed with soybean meal (Hickmann et al., 2021). However, relatively few studies have been conducted on the effects of β-mannanase supplementation on immunity and intestinal microorganisms in broiler chickens fed low-energy diet. Therefore, this experiment was carried out to investigate the effects of adding BM into low-energy diet on the immune function and intestinal microorganisms of broiler chickens.

MATERIALS AND METHODS

Animals and Treatments

A total of 384 one-day-old Arbor Acre broilers were randomly divided into 3 groups (8 replicates per group and 16 broilers per replicate). A one-way experimental design was used for this trial. The groups were as follows: 1) positive control group (PC, corn-soybean meal-based diet); 2) negative control group (NC, metabolizable Energy (ME) below 50 kcal/kg of the basal diet); 3) experimental group (NC + BM, low-energy diet supplemented with 100 mg/kg BM). BM (Shengmei Enzyme, has a BM activity of 3,000 U/g, derived from the deep liquid fermentation of *Bacillus lentus*) was purchased from Beijing Strowin Biotechnology Co., LTD. (Beijing, China).

The corn-soybean meal based-diet was formulated according to the nutritional requirements of broiler chickens as recommended in NY/T33-2004. Compared to the basal diet, the ME of the low-energy diet was reduced by 50 kcal/kg. Table 1 shows the composition and nutrients levels of the test diets. And the experimental diets were pellets. All experimental broilers were housed at the Zhuozhou Experimental Poultry Farm, China Agricultural University (Hebei, China). Broilers were provided with 24 h of lighting and had access to the appropriate food and water throughout the experiment. The initial temperature in the house was set at 33°C and was gradually reduced to 24°C on day 21.

The broilers were managed and fed in accordance with the recommendations and regulations of the Feeding Management Manual. All animal management procedures were carried out in accordance with the Beijing Experimental Animal Management Regulations and approved by the Experimental Animal Welfare and Experimental Animal Ethics Review Committee of China Agricultural University. Animal ethics project numbers: AW02403202-1-1.

Table 1. Ingredient and chemical composition of the test diets [a] (% of dry matter).

Items	PC		NC	
	0−21 d	22−42 d	0−21 d	22−42 d
Composition ratio %				
Corn (7.8% CP)	52.95	57.69	54.60	57.77
SBM (44% CP)	35.33	29.00	34.34	30.58
Corn protein powder	3.60	4.29	4.00	3.17
Soybean oil	3.70	5.10	2.60	4.56
L-Lysine hydrochloride (98.5%)	0.17	0.19	0.21	0.16
Calcium hydrogen phosphate	1.85	1.47	1.76	1.51
Stone dust	1.32	1.30	1.41	1.26
NaCl	0.40	0.22	0.40	0.24
trace mineral premixes [b]	0.20	0.10	0.20	0.10
Choline chloride (50%)	0.15	0.10	0.15	0.10
DL-Methionine (99%)	0.21	0.13	0.21	0.14
Antioxidant	0.05	0.05	0.05	0.05
Vitamins Mineral premixes [c]	0.03	0.02	0.03	0.02
Phytase	0.02	0.02	0.02	0.02
Zeolite powder	0.02	0.02	0.02	0.02
Cr_2O_3	0.00	0.30	0.00	0.30
Total	100.00	100.00	100.00	100.00
Calculation of nutritional levels [d]				
ME kcal/kg	3000.00	3150.00	2950.00	3100.00
Crude protein	22.18	20.16	22.16	20.15
Calcium	1.03	0.92	1.04	0.92
Available phosphorus	0.45	0.38	0.44	0.39
Lysine	1.23	1.10	1.24	1.10
Methionine	0.56	0.46	0.56	0.46

[a]The feed is in pellet form.
[b]Analytical values per kg of trace mineral premixes: copper 8 g, iron 40 g, zinc 55 g, manganese 60 g, iodine 750 mg, selenium 150 mg, cobalt 250 mg, moisture ≤ 10%.
[c]Analytical values per kg of vitamins mineral premixes: 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, vitamin B_3 150 g, vitamin B_5 46 g, vitamin B_9 5 g, vitamin B_7 500 mg, moisture ≤ 6%.
[d]Values calculated from the analysis of the test diets.

Sample Collection

At 21 and 42 d, one healthy broiler was randomly selected from each replicate, weighed and then their blood was collected from the wing veins. Centrifuged at 3,000 *g* for 15 min at 4°C. The thymus, spleen and bursa of Fabricius were removed intact and weighed. Tissue samples (approximately 1.5 cm) were taken from the middle of the jejunum and ileum and fixed in 4% paraformaldehyde. The mucous membranes of the jejunum and ileum were scraped, and chyme from both ileum and cecum were collected and then, frozen in liquid nitrogen. The serum, all mucous membranes and chyme samples were transferred to -80°C and stored for examination.

The Immune Organ Index

The surfaces of the immune organs (thymus, spleen and bursa of Fabricius) were dried with filter paper and

then defatted and weighed while the data were recorded. The formula was calculated as follows:

The immune organ index (g/kg)
= Weight of immune organs (g)/Weight of broilers (kg)

Immune Function of Peripheral Blood

In this test, lymphocytes were cultured in vitro and the proliferation rate of lymphocytes in the peripheral blood of broiler chickens was determined by the Cell Counting Kit-8 (**CCK-8**) method. In addition, the phagocytic activity of macrophages was determined by the neutral red uptake assay. The specific test methods were as follows, and the kits and experimental reagents were purchased from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China).

Isolation of peripheral blood mononuclear cells (PBMCs). Under sterile conditions, 2 mL of Hank's balanced salt solution without phenolphthalein and without calcium and magnesium ions was added to the anticoagulated blood to dilute the blood and facilitate the separation of lymphocytes. Two milliliter of lymphocyte separation medium was aspirated and added to a centrifuge tube. Then 2 mL of anticoagulated blood was aspirated and added slowly along the tube wall to cover the surface of lymphocyte separation medium and form a stratification. Centrifuged at 3,000 *g* for 15 min at 20° C. The middle layer of leukocytes was slowly aspirated and transferred to another centrifuge tube without touching the lower layer of erythrocytes. Centrifuged at 3,000 *g* for 15 min at 20°C, discard the supernatant and left the bottom sediments. And then lymphocyte washing buffer was added and mixed evenly. Centrifuged at 3,000 *g* for 10 min at 20°C, discard the supernatant and left the bottom sediments. One milliliter of erythrocyte lysate was added to the tube, mixed evenly. Centrifuged at 3,000 *g* for 10 min at 20°C, discard the supernatant and left the bottom precipitate. Two milliliter of lymphocyte washing buffer was added to the tube, gently puffed and centrifuged at 3,000 *g* for 10 min at 20°C. The supernatant was discarded and the bottom sediments were left. Finally, 2 mL of RPMI-1640 complete medium was added to the tube and gently puffed until the cells were suspended, at which point the cells were isolated as PBMCs. Ten microliter of the cell suspension was added to a centrifuge tube, then 10 μL of trypan blue staining solution was added to it. After gentle shaking and mixing, it was observed under an electron microscope and the number of cells was recorded, while the number of cells was adjusted to 1 to 2×10^6 with RPMI-1640 complete medium.

The transformation rate of peripheral blood lymphocytes. Under sterile conditions, 190 μL of PBMCs suspension was aspirated accurately into a 96-well plate, then 10 μL of Concanavalin A (**Con A**) solution (Lipopolysaccharides (**LPS**) solution or complete culture medium without mitogens) was added to each well. The 96-well plate was sealed with a sealing film and incubated in a cell culture incubator at 37°C for 68 h. Ten microliter of 5 mg/mL Thiazolyl Blue (**MTT**) solution was added to each well and incubated for 3 h. After removal, 100 μL of 10% SDS-0.04M HCl solution was added to each well and incubated for 30 min in a cell culture incubator protected from light. The OD value at 570 nm was measured using an enzyme marker. The proliferation rate of lymphocytes in the peripheral blood is usually expressed as a stimulation index (**SI**), which is calculated as shown below:

SI = Mean of OD values for each well stimulated by Con A or LPS/Mean of OD values of control wells

The phagocytic activity of macrophages. In this study, the phagocytic activity of macrophages was determined by the neutral red uptake assay. Hundred microliter of PBMCs suspension was accurately aspirated into a 96-well plate and incubated in a cell culture incubator containing 5% CO_2 at 37°C for 2 h. Each well was rinsed with lymphocyte washing buffer several times, and the supernatant was discarded after the macrophages were attached to each wall. One hundred fifty microliter of 0.1% (W:V) neutral red solution was added to each well. The 96-well plate was placed in a cell culture incubator and incubated for 20 min. And then the liquid in the wells was discarded. Rinsed with saline and 100 μL of cell lysis solutions (anhydrous ethanol: acetic acid: distilled water = 50:1:49) was added to the wells. The cells were left to stand for 12 h at 4°C, protected from light, and after complete lysis, their OD values at 540 nm were measured using an enzyme marker.

Serum Levels of Alpha-acid Glycoprotein (a-AGP)

Serum levels of a-AGP were determined using the a-AGP Chicken ELISA kit (Shanghai Guduo Biological Technology Co., Ltd., Shanghai, China).

Intestinal Secretory Immunoglobulin A (sIgA) Levels

The mucosa samples were weighed accurately to 0.1 g in a centrifuge tube, added with 0.9 mL of saline, vortex-shaken and well pulverized in a high-throughput tissue grinder (SCIENTZ-48, Ningbo Xinzhi Biotechnology Co., Ltd., Ningbo, China). Centrifuged at 4,000 *g* for 10 min at 4°C and the supernatant was taken for the assay. The concentration of sIgA was determined using a chicken sIgA ELISA kit (Shanghai Guduo Biological Technology Co., Ltd., Shanghai, China). The concentration of protein in the supernatant was determined using a protein quantification kit (Nanjing Jiancheng Bioengineering Institution, Nanjing, China). All of the above were carried out according to the instructions for each kit. Values were expressed as the levels of sIgA per g of protein.

The Numbers of Goblet Cells in the Intestine

Paraffin-embedded fixed jejunal and ileal tissues were used to make paraffin sections, stained with AB-PAS and observed under a Leica microscope (Wetzlar, Germany, ModelDMi8). It was quantified by counting the numbers of goblet cells stained per 100 μm length of intestinal villi and expressed as the average number of goblet cells per 8 villi.

16SrRNA Sequencing of Microorganisms in the Cecum

DNA Extraction and High-Throughput Sequencing. In this test, microbial DNA was extracted from the contents of the cecum using the fecal microbial DNA extraction kit (QIAamp Fast DNA Stool Mini Kit; QIAGEN, Germany). The concentration of DNA was determined by a NanoDrop 2000 microspectrophotometer and the purity of DNA was checked by 1% agarose gel electrophoresis. The common primers 515F (5′-GTG CCA GCM GCC GCG GTA A-3′) and 806R (5′GGA CTA CHV GGG TWT CTA AT-3′) of the V4 region of the 16SrRNA gene were used to amplify the bacterial DNA. According to the concentration of PCR products, the equal amounts of samples were mixed well. After amplification, PCR products run on a 2% agarose gel and were purified using a QIAquick Gel Extraction Kit (Qiagen, Germany). The library was constructed using TruSeq DNA PCR-Free Sample Preparation Kit. The constructed library was quantified by Qubit and qPCR. After the library was qualified, HiSeq2500-PE250 was used for sequencing. The sequencing analysis was performed by Novogene Biotech Co., Ltd. (Beijing, China).

Sequence Processing and Bioinformatics Analysis. Flash software (Version 1.2.7) was used to stitch the Reads of the samples to obtain Raw Tags data. The Raw Tags data were processed by referring to the QIIME (Version 1.9.1) Tag quality control process, and the sequences of the processed Tags were compared with the database by the UCHIME algorithm. And the chimeric sequences were detected and removed to obtain Effective Tags. Effective Tags were clustered using UPARSE software (UPARSE V7.0.1001). Then the sequences were clustered into operational taxonomic units (**OTUs**) with 97% consistency and representative sequences were selected. Based on the principles of the algorithm, the sequence with the highest frequency of occurrence was used as the representative sequence of OTUs, and the representative sequence was annotated with species. SSU rRNA databases from Mothur and SILVA were used for species annotation analysis (threshold = 0.8−1) to obtain taxonomic information and community composition at each taxonomic level. MUSCLE software (Version 3.8.31) was used for rapid multiple sequence alignment to obtain phylogenetic relationships for all OTUs representative sequences, and the data of each sample was also homogenized. The sample with the least amount of data was taken as the standard and the subsequent analysis of diversity was based on the homogenized data. QIIME software was used to calculate the Unifrac distance of each sample to construct UPGMA clustering trees. Linear discriminant analysis effect size (**LEfSe**) software was used to find biomarkers that were statistically different between groups (classes of bacteria with significant differences in relative content) based on linear discriminant analysis (LDA) values. The R software (Version 2.15.3) was used to draw Venn diagram, dilution curve diagram, coverage index diagram, principal component analysis (**PCA**) diagram, and principal coordinate analysis (**PCoA**) diagram, together with ANOSIM analysis. The t-tests between groups were performed for genera with relative abundance > 0.001 using R software. PICRUSt software was used to predict and analyze metagenomic function.

Metagenomics Sequencing of Ileum and Cecum

The contents of the ileum and cecum of healthy broilers at 42 d were mixed together in equal masses and the genomic DNA of the microbiota was extracted from the contents. Quality control and quantification of DNA were performed by agarose gel electrophoresis and Qubit. Covaris ultrasound was used to fragment of tested DNA samples into fragments of approximately 350 bp in length. Terminal repair, addition of sequencing connectors and A-tails were then performed. After purification and PCR amplification, sequencing library was prepared. Library was quantified and diluted by Qubit 2.0. The Agilent 2100 was used to conduct quality inspection on the insert size of the library to ensure that the sequencing library was of acceptable quality. Illumina PE150 was used to sequence the library, and the analysis of the sequencing data was entrusted to Novogene Biotech Co., Ltd. (Beijing, China). During the sequencing analysis, Clean Data was obtained from Raw date by quality control and host filtration. Metagenome assembly, gene prediction and species annotation were then performed, and finally, the relative abundance of different species was analyzed based on OTU. Functional annotation was performed based on metabolic pathways (KEGG), etc.

Statistical Analysis

The data from each treatment group were statistically analyzed using SPSS 22.0 software. Statistical analyses were conducted using a ANOVA process, and differences between treatments were analyzed using Duncan's multiple comparisons, with results expressed as mean ± standard error (SEM). All statements of significant differences were based on $P < 0.05$. When $P < 0.05$, the difference was considered significant, and the P values between 0.05 and 0.10 were classified as trends.

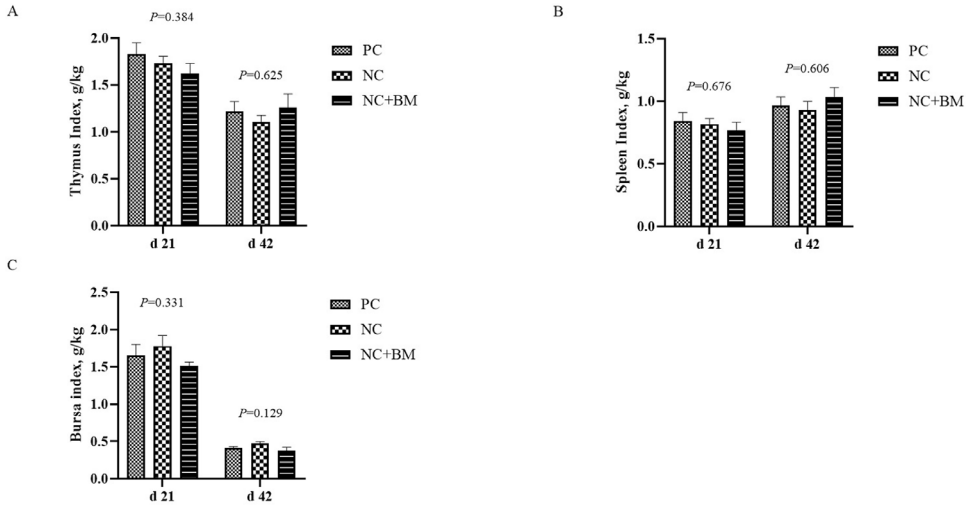

Figure 1. Effect of the addition of BM to the low-energy diet on the immune organ index in broilers. Note: All plots are expressed as mean ± SEM and P values are marked above each plot. Lower case letters (abc) in the bar graphs indicate significant differences ($P < 0.05$). The PC group is the basal diet positive control group, the NC group is the low-energy negative control group and the NC + BM group is the low-energy negative control group + 100 mg/kg BM.

RESULTS

The Immune Organ Index

As shown in Figure 1, the addition of BM to the low-energy diet had no significant effect on the immune organ index of broiler chickens compared to the NC group. There were no significant differences in the immune organ index among the groups ($P > 0.05$).

Immune Function of Peripheral Blood

As shown in Figure 2, a reduction in dietary energy level had a tendency to reduce ($P = 0.067$) the LPS SI in the peripheral blood of broilers at 42 d compared to the basal diet. Dietary supplementation of BM significantly increased ($P < 0.05$) the Con A SI and phagocytic activity of macrophages in the peripheral blood of broiler chickens at 42 d compared to the low-energy diet.

Serum Levels of a-AGP

As shown in Figure 3, there were no significant differences in serum α-AGP levels among the groups ($P > 0.05$).

Intestinal sIgA levels

As shown in Figure 4, the addition of BM to the low-energy diet had no significant effect on the intestinal sIgA of broiler chickens ($P > 0.05$).

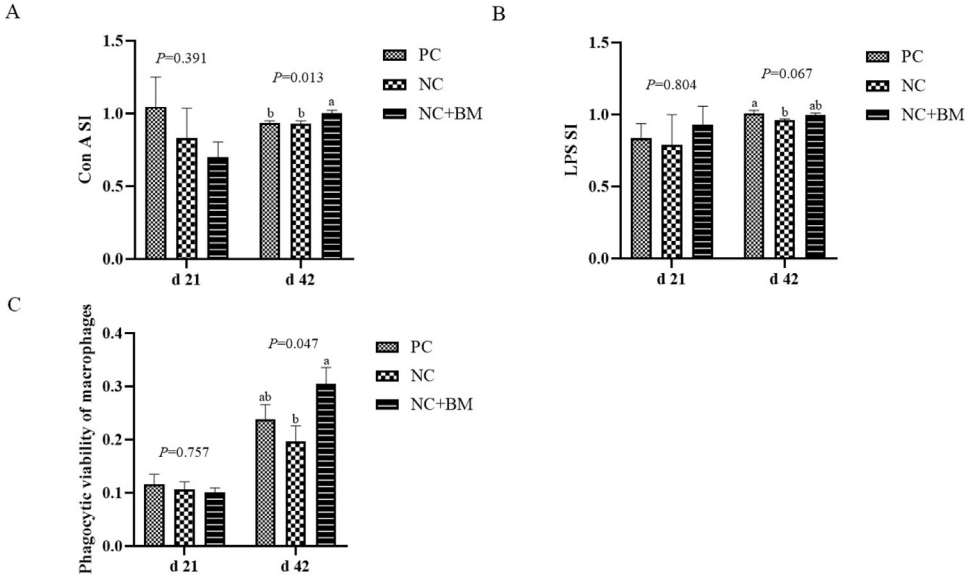

Figure 2. Effect of the addition of BM to the low-energy diet on the immune function of peripheral blood in broilers. Note: All plots are expressed as mean ± SEM and P values are marked above each plot. Lower case letters (abc) in the bar graphs indicate significant differences ($P < 0.05$). The PC group is the basal diet positive control group, the NC group is the low-energy negative control group and the NC + BM group is the low-energy negative control group + 100 mg/kg BM.

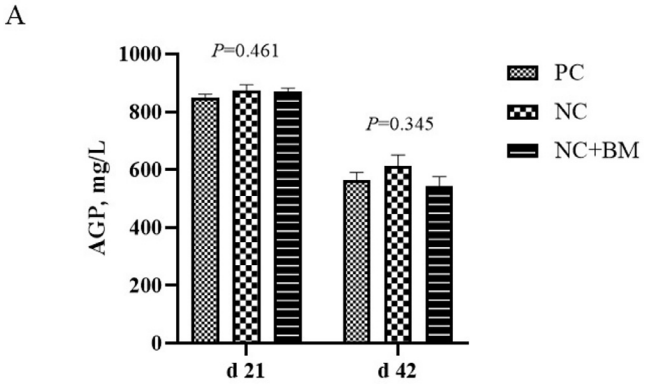

Figure 3. Effect of the addition of BM to the low-energy diet on serum α-AGP levels in broilers. Note: All plots are expressed as mean ± SEM and P values are marked above each plot. Lower case letters (abc) in the bar graphs indicate significant differences ($P < 0.05$). The PC group is the basal diet positive control group, the NC group is the low-energy negative control group and the NC + BM group is the low-energy negative control group +100 mg/kg BM.

The Numbers of Goblet Cells in the Intestine

As shown in Figure 5, when the dietary energy level was reduced, the numbers of goblet cells in the jejunum and ileum of broiler chickens were significantly lower ($P < 0.01$) compared to the basal diet. The addition of BM significantly increased ($P < 0.01$) the numbers of goblet cells in the jejunum and ileum of broilers compared to the low-energy diet.

16SrRNA Sequencing of Microorganisms in the Cecum

Statistical analysis of OTUs. As shown in Figure 6A, among the microorganisms in the cecum of broilers at 21 d, the number of OTUs shared by the PC, NC and NC + BM groups was 782, of which the number of OTUs specific to the PC group was 4185, the number of OTUs specific to the NC group was 849. And when BM was added to the low-energy diet, the number of OTUs specific to it was 1,076. Among the microorganisms in the cecum of broilers at 42 d, the number of OTUs shared by the PC, NC and NC + BM groups was 659, with 930 OTUs specific to the PC group and 2,985 OTUs specific to the NC group. When BM was added to the low-energy diet, the number of OTUs specific to it was 589.

Analysis of alpha diversity. As shown in Figure 6B −6F, compared to the basal diet, the reduction in energy level of the diet significantly reduced ($P < 0.01$) the observed_species index of the cecum microbiota of broilers at 21 d, and significantly reduced ($P < 0.05$) the Chao1 and ACE index. There were no significant

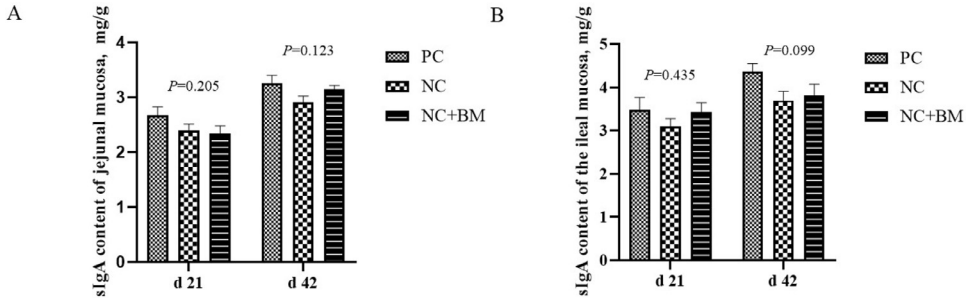

Figure 4. Effect of the addition of BM to the low-energy diet on the content of sIgA in intestine of broilers. Note: All plots are expressed as mean ± SEM and P values are marked above each plot. Lower case letters (abc) in the bar graphs indicate significant differences ($P < 0.05$). The PC group is the basal diet positive control group, the NC group is the low-energy negative control group and the NC + BM group is the low-energy negative control group + 100 mg/kg BM.

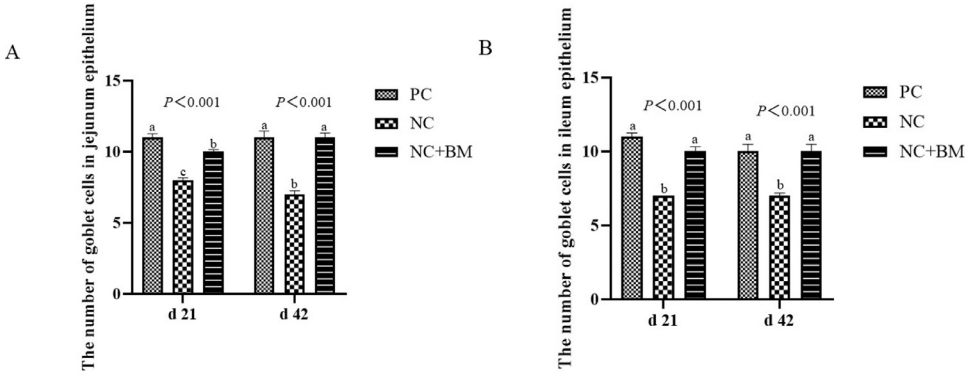

Figure 5. Effect of the addition of BM to the low-energy diet on the numbers of intestinal goblet cells in broilers. All bar charts are expressed as mean ± SEM and P values are marked above each plot. Lower case letters (abc) in the bar graphs indicate significant differences ($P < 0.05$). The PC group is the basal diet positive control group, the NC group is the low-energy negative control group and the NC + BM group is the low-energy negative control group + 100 mg/kg BM.

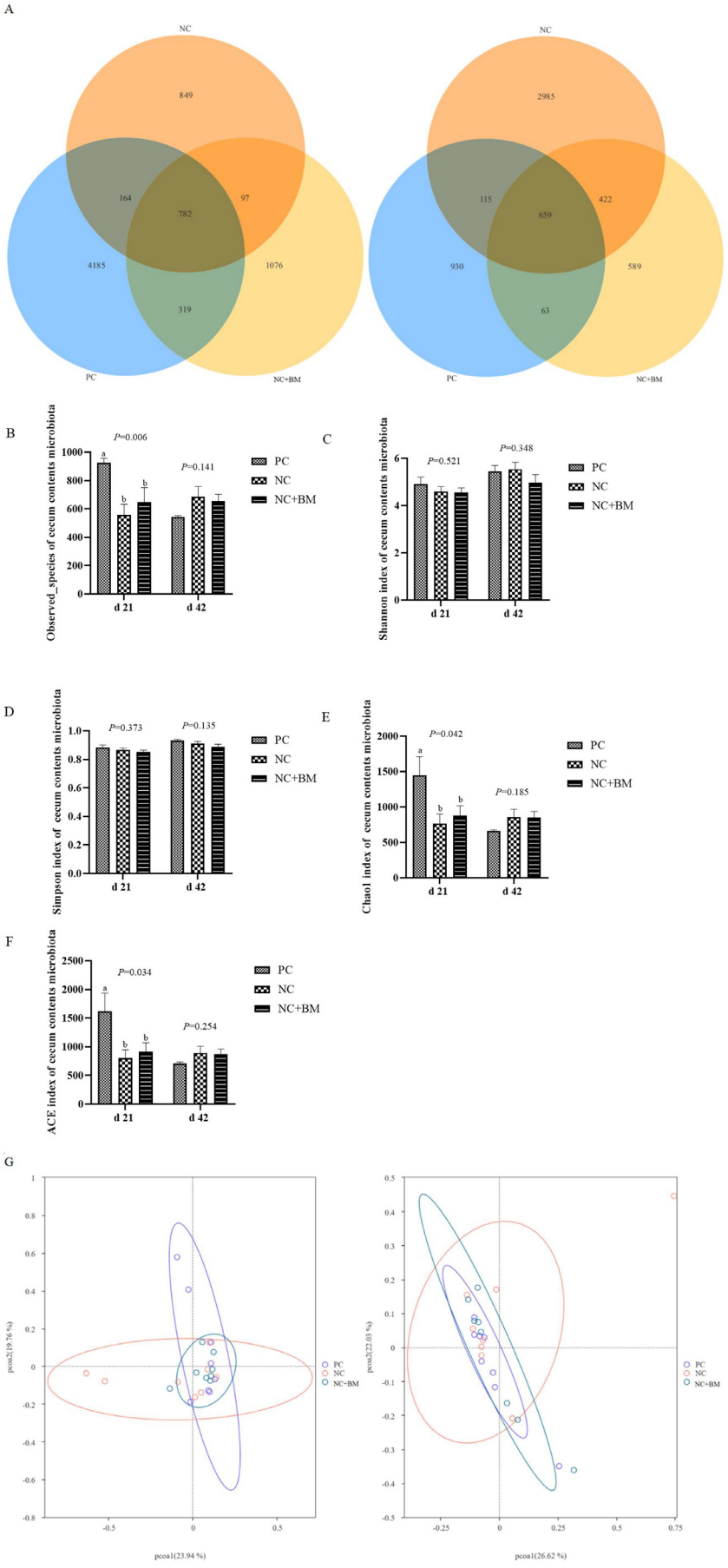

Figure 6. Effect of the addition of BM to the low-energy diet on the diversity of cecum microbiota in broilers. Note: Venn diagram (A) was used to represent the distribution of microbial OTU in the cecum of broilers at 21 d and 42 d, respectively. The α-diversity of the cecum microbiota in broiler chickens was analyzed using observed_species (B), shannon index (C), simpson index (D), Chao1 index (E) and ACE index (F). The

differences in the α-diversity among the NC group and NC + BM group ($P > 0.05$).

Analysis of beta diversity. As shown in Figure 6G, the addition of BM to the low-energy diet had no significant effect on the β-diversity of microorganisms in the cecum of broiler chickens ($P > 0.05$).

The top 5 microorganisms in relative abundance at the phylum level. As shown in Figure 7A, the top 5 microorganisms in relative abundance at the phylum level in the cecum of broiler chickens at 21 d were: Firmicutes, Proteobacteria, Bacteroidota, unidentified_Bacteria and Acidobacteriota. The top 5 microorganisms in relative abundance in the cecum of broilers at 42 d were Firmicutes, Bacteroidota, Proteobacteria, unidentified_Bacteria and Desulfobacterota.

The top 10 microorganisms in relative abundance at the genus level. As can be seen from Figure 7B, the top 10 microorganisms in relative abundance in cecum chyme of broilers at 21 d were, in order of abundance, *Pseudomonas, Alistipes, Faecalibacterium, Parabacteroides, Streptococcus, [Ruminococcus]_torques_group, Escherichia-Shigella, Enterococcus, CHKCI001* and *Ligilactobacillus*. The top 10 microorganisms in relative abundance in the cecum chyme of broilers at 42 d were, in order of abundance, *Streptococcus, Faecalibacterium, Parabacteroides, Enterococcus, Bacteroides, Alistipes, Prevotella_9, [Ruminococcus]_torques_group, CHKCI001* and *Escherichia-Shigella*.

Species abundance clustering heat map. Compared to the basal diet, the low-energy diet significantly reduced ($P < 0.01$) the relative abundance of *Tyzzerella* and *Candidatus_Arthromitus* in the cecum of broiler chickens at 21 d. In addition, the low-energy diet also significantly reduced ($P < 0.05$) the relative abundance of *CHKCI001* and *Erysipelatoclostridium* in broilers at 42 d, and also significantly increased ($P < 0.05$) the relative abundance of *Monoglobus* (Figure 7C).

Predominant microorganisms at the phylum level. Figure 7D shows the ternary phase diagrams of the dominant microorganisms at the phylum level in cecum chyme of broilers at 21 d and 42 d, respectively. As shown in the figure, the relative abundance of Firmicutes and Bacteroidota in the microbiota of the cecum chyme of broiler chickens at 21 d and 42 d were higher among the 3 groups and their distribution were more even among the 3 groups.

Analysis of Significant Differences. As shown in Figure 7E, among the microorganisms in the cecum of broilers at 21 d, Ruminococcaceae, *Candidatus_Arthromitus*, Burkholderiaceae, *Tyzzerella,* and *Clostridiaceae* were dominant in the basal diet group. The dominant flora in the low-energy diet group were *Pseudomonas, Pseudomonas_azotoformans* and Christensenellaceae, while the addition of BM to the low-energy diet significantly increased the relative abundance of *Lachnospiraceae_bacterium_615* and *Parabacteroides_johnsonii*, etc. in the cecum of broilers at 21 d. Among the microorganisms in the cecum of broilers at 42 d, the dominant microorganisms in the basal diet group mainly included *Clostridium_spiroforme, Butyricoccus_pullicaecorum*, Erysipelatoclostridiaceae and Lachnospiraceae. The dominant microorganisms in the low-energy diet group were Erysipelotrichaceae and *Lactobacillus_johnsonii*, while the addition of BM to the low-energy diet significantly increased the relative abundance of Gammaproteobacteria.

Spearman's correlation analysis. As shown in Figure 7F, ConA SI in peripheral blood of broiler chickens at 21 d was significantly negatively correlated with *Faecalibacterium* and ConA SI in peripheral blood of broiler chickens at 42 d was significantly negatively correlated with *Ligilactobacillus* ($P < 0.05$).

Metagenomic Sequencing Analysis of Ileum And Cecum

OTU statistical analysis. As can be seen from Figure 8 A, the number of OTUs shared by the PC, NC and NC + BM groups was 18,89,040, of which 27,054 OTUs were specific to the PC group, 38,096 OTUs were specific to the NC group and 28,446 OTUs were specific to the NC + BM group.

Krona analysis. As shown in Figure 8B−8D, a representative sample from each group was selected for Krona analysis. As can be seen from the Figure 8B, the relative abundance of *Lactobacillus* in the basal diet was 17%, with *Lactobacillus_salivarius* accounting for 4% and *Lactobacillus_johnsonii* accounting for 1% of the relative abundance. In addition, the relative abundance of *Butyricicoccus* and *Blautia_sp._CAG:257* each accounted for 1%. When the energy level of the diet was reduced, the relative abundance of *Streptococcus* accounted for 15% and *Enterococcus_cecorum* for 5% of the microorganisms in broilers at 21 d. And the low-energy diet resulted in an increase in the relative abundance of *Clostridium* (Figure 8C). And the relative abundance of *Lactobacillus_salivarius, Eubacterium_sp._CAG:180,* and *Blautia_sp._CAG:257* each accounted for 1% after the addition of BM to the low-energy diet (Figure 8D).

Species/functional annotations with comparison and analysis of differences. Venn diagram was used to represent the OTU distribution of microorganisms in intestine of broilers at 21 d together with Krona analysis of the species and composition of microorganisms. As can be seen from Figure 8E, the top 10 species in relative abundance at the genus level in the intestine

β-diversity (G) of the cecum microbiota in broiler chickens at 21 and 42 d was analyzed using PCoA, respectively. All bar charts are expressed as mean ± SEM and P values are marked above each plot. Lower case letters (abc) in the bar graphs indicate significant differences ($P < 0.05$). The PC group is the basal diet positive control group, the NC group is the low-energy negative control group and the NC + BM group is the low-energy negative control group + 100 mg/kg BM.

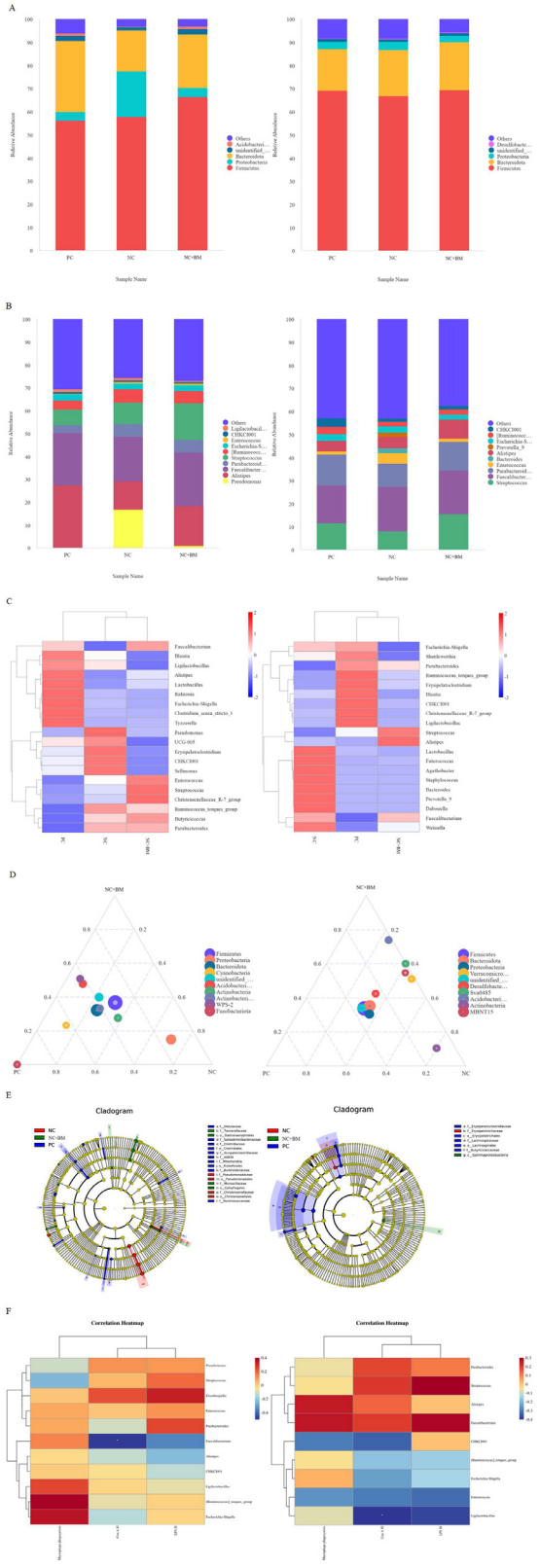

Figure 7. Effect of the addition of BM to the low-energy diet on the species of cecum microbiota in broilers. Note: The top 5 microbial groups at the phylum level (A) and the top 10 microbial groups at the genus level (B) were determined in the cecum of broiler chickens at 21 and 42 d, respectively, and a species abundance clustering heat map (C) and a ternary phase diagram (D) of the dominant microorganisms at the phylum level were established. The LEfSe (E) and Spearman's correlation analysis (F) were used to analyze the differential flora of the cecum. The PC group is the basal diet positive control group, the NC group is the low-energy negative control group and the NC + BM group is the low-energy negative control group + 100 mg/kg BM.

of broilers at 21 d were, in order, *Streptococcus, Alistipes, Lactobacillus, Enterococcus, Blautia, Faecalibacterium, Clostridium, Parabacteroides, Subdoligranulum* and *Lachnoclostridium*. The top 10 ENZYMEs in relative abundance based on the analysis of KEGG-ec level were, in order: 2.7.7.7, 3.1.-. -, 3.6.4.12, 3.6.3.-, 3.4.24.-, 3.1.21.3, 2.1.1.72, 5.99.1.2, 2.7.13.3 and 3.4.16.4 (Figure 8F). As can be seen from Figure 8G and 8H, there were no significant differences in the β-diversity of microorganisms in the intestinal chyme of broiler chickens at the genus level at 21 d. And the results of the analysis based on the KEGG-ec level were also not significantly different. In addition, the circos plot of the CARD database was used to represent the ARGs of microbiota in intestine of broilers at 21 d and the microorganisms were compared and analyzed for KEGG metabolic pathways. Among the ARGs, the highest abundance in the basal diet group was *tetW/N/W*, followed by *tetO* and *tetM*, and the lowest abundance was *ANT6-Ia*. When the energy level of the diet was reduced, the highest abundance in the ARGs taxa was *tetW/N/W*, followed by *tetO* and *tetM*, and the lowest abundance was *lnuB*. The addition of BM to the low-energy diet resulted in the highest abundance in the ARGs taxa of *tetW/N/W*, followed by *tetO* and *tetM*, and the lowest abundance was *ErmT* and *lnuB* (Figure 8I). As shown in Figure 8J, the analysis of enriched KEGG pathways showed that compared to the low-energy diet, the addition of BM inhibited broilers' microbial Fatty acid degradation (map00071) by decreasing the activity of glutaryl-CoA dehydrogenase [EC:1.3.8.6].

DISCUSSION

Immune system is mainly composed of immune organs, tissues and cells, all of which interact with each other to perform immune functions. (Dekruyff et al., 1975; Gordon and Manley, 2011). The thymus, spleen and bursa of Fabricius are important immune organs in poultry. The immune organ index is commonly used to assess the immunomodulatory effects of feed additives (Naukkarinen and Hippelainen, 1989; Nakamura et al., 2012). In this study, the addition of BM to the low-energy diet did not significantly affect the immune organ index in broilers. It showed that the immune organ development in broiler chickens was consistent among the groups. In this study, the type of BM, the amount of addition, the factors affecting the action effects and the sex of broilers may lead to the above results. The lymphocytes in peripheral blood are involved in the development of the immune response and their proliferation and differentiation can directly determine the level of immunity in the organism (Song et al., 2022). It has been shown that BM can promote the proliferation and transformation of T cells (Zou et al., 2006). In this experiment, cell-mediated immunity was assessed by measuring the proliferative response of lymphocytes stimulated by the mitogen Con A of T cells and the mitogen LPS of B cells. And the phagocytic activity of

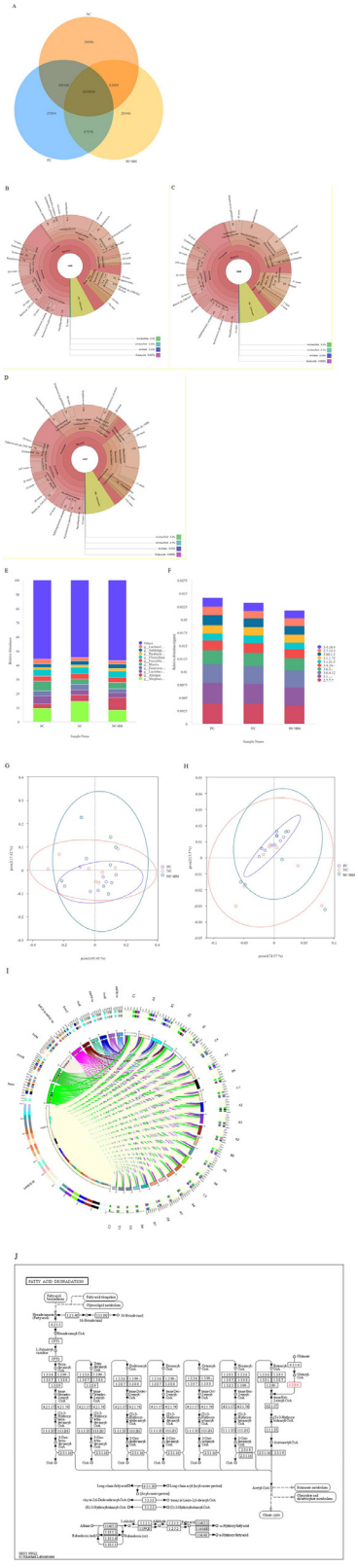

Figure 8. Effect of the addition of BM to the low-energy diet on the structure and function of the gut microbiota of broilers. Note: Venn diagram (A) was used to represent the OTU distribution of microorganisms in intestine of broilers at 21 d together with Krona analysis of the species and composition of microorganisms (B–D). The relative abundance of the flora of broilers at 21 d at the genus level was analyzed based on metagenomic sequencing (E) and the microorganisms were analyzed at the KEGG-ec level (F). The β-diversity of the microbiota in intestine of broilers at 21 d was analyzed using PCoA (G) and the microorganisms were also analyzed at the KEGG-ec level (H). The circos plot (I) of the CARD database was used to represent the ARGs of microbiota in intestine of broilers at 21 d and the microorganisms were compared and analyzed for KEGG metabolic pathways (J). The PC group is the basal diet positive control group, the NC group is the low-energy negative control group and the NC + BM group is the low-energy negative control group + 100 mg/kg BM.

macrophages in the peripheral blood of broiler chickens also was evaluated. It was found that the reduction in the energy level of the diet affected the cellular immune function in the peripheral blood of broiler chickens, while the addition of BM enhanced the cellular immune function by increasing the Con A SI and the phagocytic activity of macrophages in peripheral blood of broilers at 42 d.

Serum levels of the acute phase protein (**APP**) can be used as an important physiological indicator of stress due to reduced nutritional levels of feed (Najafi et al., 2016). AGP is an important immunomodulator that affects the function of T cells (Murata et al., 2004). SIgA is the primary immune barrier that prevents pathogens in the intestinal lumen from settling in the intestinal mucosa and maintains the homeostasis of the commensal flora (Lammers et al., 2003). Studies have shown that the extent of the increase in sIgA depends mainly on the number of bacteria (Papp et al., 2013). None of the present studies found a significant effect of BM on AGP and sIgA in broiler chickens. Furthermore, in this trial, the low-energy diet significantly reduced the numbers of intestinal goblet cells in broilers, whereas the addition of BM significantly increased the numbers of goblet cells, which was beneficial for maintaining the integrity of the intestinal mucosa. The cells of the intestinal mucosal epithelium are interspersed with a large number of goblet cells which secrete mucus and prevent bacteria from binding to the intestinal epithelium, thus maintaining the normal function of the intestinal barrier (van der Flier and Clevers, 2009).

The intestinal flora forms a multi-layered intestinal microbial barrier, which is involved in the body's nutrition and intestinal defense, and regulates the immune function of the host (Stanley et al., 2014; de Vos et al., 2022; Erttmann et al., 2022). Under normal conditions, the distribution of intestinal microorganisms is relatively stable and the microbiota is relatively balanced (Kogut et al., 2020). The diversity of the gut microbiota is closely related to the health of the host, and it has been reported that a decrease in the diversity of the gut microbiota can be an important sign of a dysbiosis (Clarke et al., 2014). In addition, it has been shown that mannan in feed can lead to disturbances in the intestinal flora of broiler chickens and that the addition of BM can mitigate this adverse effect (Gutierrez et al., 2008). Therefore, this trial further explored the effect of adding BM to the low-energy diet on the intestinal flora of broiler chickens. It was found that the reduction in dietary energy level reduced the abundance and diversity of microbial species in the cecum of broilers, which was not significantly affected by BM.

Phascolarctobacterium produces short-chain fatty acids (**SCFAs**) such as acetate and propionate, which

provide energy and nutrients to the host and maintain intestinal health (Deldot, 1993; Patterson and Burkholder, 2003; Holmstrom, 2004; Wu et al., 2017). *Anaerostipes* produces butyrate, which provides energy to the host and has a potentially beneficial role in promoting host's intestinal health (Schwiertz et al., 2002; Eeckhaut et al., 2010; Bui et al., 2021). Lachnospiraceae, a central part of the gut microbiota, can influence the host's intestinal health by producing SCFAs, etc. (Antonissen et al., 2016; Vacca et al., 2020; Cao et al., 2021). Studies have shown that *Lachnospiraceae_ UCG-006*, a common probiotic, can strengthen the intestinal barrier and slow down the inflammatory response in broiler chickens (Cao-Sy, 2019; Liu et al., 2020; Ji et al., 2022). *Pseudomonas* is a genus of ubiquitous bacteria that can cause lesions in the organism and affect intestinal health (Argudín, 2017; Ruiz-Roldan et al., 2020). In line with previous findings, we observed that the low-energy diet significantly reduced the relative abundance of beneficial bacteria such as *Phascolarctobacterium* and *Anaerostipes* in the chyme of broilers, while the addition of BM significantly increased the abundance of *Lachnospiraceae_ UCG-006* and *Lachnospiraceae_ UCG-001*, and decreased the relative abundance of *Pseudomonas*, regulating the structure of the intestinal flora. The above results are consistent with previous findings that the addition of BM to diets improves the intestinal flora structure of broilers (Dawood and Shi, 2022).

To further investigate the effects of the addition of BM to the low-energy diet on the microbial structure and function of broilers, this study performed macrogenome sequencing of gut flora. It was found that the relative abundance of *Clostridium* in the microbiota of the broilers' gut increased when the energy level of the diet was reduced. The addition of BM to the low-energy diet increased the relative abundance of *Lactobacillus_ salivarius* and *Blautia_ sp._ CAG:257* in the microbiota of broilers at 21 d, inhibited broilers' microbial Fatty acid degradation (map00071) by decreasing the activity of glutaryl-CoA dehydrogenase [EC:1.3.8.6]. BM improved the immune function and intestinal flora of broilers due to reduced energy level in the diet. In particular, BM could improve the intestinal health of broilers by enhancing cellular immunity function and regulating the structure of the intestinal flora.

CONCLUSION

The addition of BM into the low-energy diet enhanced immune function by increasing the Con A SI of peripheral blood and the phagocytic activity of macrophages in broilers. In addition, BM could promote the proliferation of probiotic bacteria such as Lachnospiraceae, inhibit the colonization of *Pseudomonas* and others in the intestine, and inhibit Fatty acid degradation of microorganisms by decreasing the activity of glutaryl-CoA dehydrogenase. The results obtained in this study provide a scientific basis for understanding the role of BM in modulating immune function and regulating the intestinal flora structure of broilers fed a low-energy diet of corn-soybean meal type.

CREDIT AUTHORSHIP CONTRIBUTION STATEMENT

Xiaodan Zhang: Conceptualization, Data curation, Methodology. **Yanhong Chen:** Software, Formal analysis. **Zengpeng Lv:** Project administration. **Liangjuan Zhou:** Funding acquisition. **Yuming Guo:** Writing – review & editing.

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DISCLOSURES

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in the present study.

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