The replacement of bacitracin methylene disalicylate with *Bacillus subtilis* PB6 in the diet of male Cherry Valley Ducks reduces the feed conversion ratio by improving intestinal health and modulating gut microbiota

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ABSTRACT In this study, we compared the impacts of *Bacillus subtilis* PB6 (**BS**) and bacitracin methylene disalicylate (**BMD**) on the growth performance, intestinal morphology, expression of tight connection protein, and cecal microbiota community of male ducks through a 42-d trial. Three-hundred and sixty male Cherry Valley meat-type ducklings (1-day-old) were distributed into 3 groups of 6 replicates: CON group (control, basal diet), BMD group (basal diet + 45 mg/kg BMD, active ingredient dose in the feed), and BS group (basal diet + 2×10^7 CFU/kg BS in the feed). Results showed that supplementing the diet with BS reduced the average daily feed intake (**ADFI**) during d 15 to 42 and d 1 to 42 compared with the CON group ($P = 0.032$). It also reduced feed conversion ratio (**FCR**) during d 15 to 42 and d 1 to 42 ($P < 0.05$) relative to the other groups. The ileal villus height (**VH**) and villus height /crypt depth ratio (**V/C**) were increased ($P < 0.05$) in both the BS and BMD groups, and the jejunal VH and V/C ratio were increased in the BS group ($P < 0.05$). Relative to the CON, BS supplementation was associated with numerical augmentation of goblet cells in the jejunal mucosa and upregulation of jejunal zonula occludens (***ZO-1***) and ileal *mucin2* ($P < 0.05$) mRNA levels. Analysis showed a negative correlation between FCR (d 0 −42) and VH, V/C, and the number of goblet cells in the jejunum ($P < 0.05$). Additionally, BMD or BS supplementation altered the alpha diversity of colonic microbiota ($P < 0.05$). Correlation analysis revealed that *Butyricimonas, Enterobacteriaceae, Clostridiaceae*, and *Tannerellaceae* were positively associated with the acetic acid and butyrate concentrations ($P < 0.05$). Taken together, the supplementation of BS in the diet of male ducks was conducive to reducing FCR by meliorating intestinal morphology, upregulating *ZO-1* and *mucin2* mRNA levels, regulating the abundance of microbiota, and metabolites, and having a greater effect than BMD supplementation.

Key words: feed conversion ratio, fight junction protein, intestinal morphology, cecal microflora, male Cherry Valley Duck

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INTRODUCTION

Meat ducks are an economically important waterfowl in Asia due to their short growth period and meat with higher in protein and lower saturated fatty acids (Farrell, 2013; Bai et al., 2020). Admittedly, the growth performance of meat ducks is closely associated with stressors, gut health, and gastrointestinal microbiome (He et al., 2019; Yang et al., 2020) and is also significantly affected by intestinal mucosal damage triggered by mycotoxins and pathogens inevitably carried in feed ingredients (www.biomin.net). Additionally, although intensive stocking density can bring high incomes, it can also drastically decrease feed conversion ratio.

An effective strategy for reducing mortality and promoting growth during high-density breeding is the use of antibiotics (Islam et al., 2014). One of the commonly used antibiotics is bacitracin methylene disalicylate (**BMD**), which acts by inhibiting the formation of cell walls, thus interfering with the protein synthesis of Gram-positive bacteria, and helping to maintain intestinal balance (Rivera-Pérez et al., 2021).However, the use of antibiotics can be problematic as the residues

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exist not only in the meat but also in the excreta of animals, which, in turn, increases the risk of antibiotic resistance genes being exchanged amongst microorganisms in the soil (Lin et al., 2017). In recent years, probiotics have gradually replaced antibiotics in livestock for combatting bacterial diseases and serving as growth promoters (Kenny et al., 2011; Abd El-hack et al., 2020).

The genus *Bacillus* spp. is extensively used for food and agriculture due to its proven safety and efficacy. The species *B. subtilis* PB6 (**BS**) belonging to *B. subtilis* (ATCC PTA-6737), is isolated from the gut tract of healthy chickens. As a spore-forming facultative anaerobe, it can withstand a temperature of 121°C for 20 min and tolerate acid and bile salts (Liu et al., 2021). These characteristics allow spores to reach the gastrointestinal tract after high-temperature granulation. *B. subtilis* is mostly dormant spores, they germinate only when the temperature is suitable and water is present, such as in the gastrointestinal tract (Leser et al., 2008). Subsequently, digestive enzymes, like alpha-amylase and beta-amylase, and antimicrobial peptides will be released once they wake up (Teo and Tan, 2005). More importantly, the protective function of BS on the intestinal tract is reflected in the improvement of intestinal morphology (Al-Fataftah and Abdelqader, 2014) and the upregulation of tight junction protein (Liu et al., 2021), both of which contribute to the effective absorption of nutrients. Additionally, the bacteriocin produced by BS fights against *Clostridium, Streptococcus*, and *Campylobacter* in vitro and in vivo (Liu et al., 2021), which is important in the context of raising livestock.

In the current literature, various doses of dietary BS supplement have been shown to prompt the growth of chickens (EFSA, 2009; EFSA, 2013; Liu et al., 2021), nevertheless, the influences of BS on the gut microbes of male Cherry Valley ducks have yet to be explored. Being able to control the cecal microorganisms of ducks can lead to better management of the use of probiotics, such as the doses and feeding stages. In our study, we regarded the Cherry Valley male ducks as a research model to explore the impacts of BS and BMD on the growth, intestinal morphology, tight junction protein expression, and cecal microflora (including microflora metabolites).

MATERIALS AND METHODS

This experiment was conducted at the experimental base in Ya'an (Sichuan, China) and all operating procedures involving animals complied with the Animal Management Regulations of Sichuan Agricultural University, Sichuan, China (approval number: 20190624). The BS was provided by Kemin Technology Co., Ltd. (Zhuhai, China) and contained 2×10^{11} CFU/kg of product. The BMD (15% w/w purity) was purchased from Hengxintong Pharmaceutical Technology Co., Ltd. (Shenzhen, China).

Animals, Feed, and Management

A total of 360 male Cherry Valley meat-type ducklings hatched on d 1 were purchased from Shehong Food Co., LTD (Shehong, China). All the ducklings had a similar body weight (**BW**) of 53 ± 0.16 g. They were allocated based on a randomized block design into 3 treatment groups (120 ducklings/ treatment) with 6 replicate cages, resulting in 20 ducklings per repeat cage (2.0 m × 1.6 m × 0.6 m). The basal diet included starter (d 1−14) and grower-finisher (d 15−42) periods, shown in Table 1, and the nutrient level met or exceeded the recommendations of the National Research Council (1994). The three groups were treated as follows: CON group, basal diet; BMD group, basal diet + 45 mg/kg BMD in the diet (based on active ingredient), and BS group, basal diet + 2×10^7 CFU/kg BS in the diet. Feed ingredients were crushed and then mixed with BS and BMD, respectively. After that, they were made into granules where the steam pressure when entering the conditioning cylinder is 3.5 kg and the outlet temperature of the conditioning cylinder is 85°C. Prepared feeds were kept in a cold storage at 4°C. Starter feed was manufactured 2 d prior to trial while grower-finisher feed

Table 1. Ingredients and nutritional value of the basal diet.

Items	Starter (day 1−14)	Grower-Finisher (day 15−42)
Ingredients (%, as-fed basis)		
Corn	52.50	59.40
Wheat bran	1.00	2.00
Soybean meal (43%)	34.00	14.00
Fish meal (67%)	3.00	-
Rapeseed meal	3.40	8.60
Corn distiller grains	-	6.00
Soybean oil	3.00	5.00
Bentonite	-	1.57
Calcium carbonate	1.00	1.50
Dicalcium phosphate	1.26	0.90
L-lysine (78.5%)	-	0.24
DL-methionine (98.5%)	0.16	0.11
Choline chloride (50 %)	0.15	0.15
Baking soda	0.10	0.10
Sodium chloride	0.20	0.20
Vitamin premix[1]	0.03	0.03
Mineral premix[2]	0.20	0.20
Total	100.00	100.00
Calculated Nutrient content		
Metabolizable energy (Kcal/kg)	2,901	3,002
Crude protein, %	22.06	16.05
Crude fat	5.73	8.17
Ca, %	1.08	0.89
Total phosphorus, %	0.74	0.55
Available phosphorus	0.32	0.30
Lysine, %	1.21	0.90
Methionine, %	0.53	0.35
Measured values, %		
CP	22.18	16.16
Total phosphorus, %	0.78	0.54
Ca	1.11	0.91

[1]Vitamin premix provides the following substances per kilogram of the diet: Vitamin A, 2,500 IU; Vitamin E, 10 IU; Vitamin D_3,400 IU; Vitamin K_3, 0.5 mg; Vitamin B_1, 1.8 mg; Vitamin B_2, 4 mg; Vitamins B_6, 2.5 mg; Vitamin B_{12}, 0.01 mg; Niacin, 55 mg; Pantothenic acid 11 mg; Biotin, 0.15 mg; Folic acid 0.55 mg.

[2]Mineral premix: copper ($CuSO_4 \bullet 5H_2O$), 8 mg; iron ($FeSO_4 \bullet 7H_2O$, 80 mg; zinc ($ZnSO_4 \bullet 7H_2O$), 90 mg; manganese ($MnSO_4 \cdot H_2O$), 70 mg; selenium ($NaSeO_3$), 0.3 mg; I (KI), 0.4 mg.

was processed on d 13 of this experiment. Feed samples (basal diet) consisted of starter and grower-finisher period were dried at 105°C for 24 h, then they were ground to pass through a 1 mm screen. Grind samples were determined for crude protein (method 968.06) using N-Kjeldahl × 6.25, calcium (method 984.01), and total phosphorus (method 965.17) with the standard of AOAC (2000). The measured calculations were present in Table 1.

The ducklings were raised in two-tier stainless-steel cages with selfsame size 20 neighboring cages on each, and the temperature was controlled by infrared lamps. In the first week, the temperature was maintained at 33 ± 1°C, and then reduced by 2°C every 7 d, finally reaching 24 ± 1°C on d 42. Interior air humidity within 55 to 65% was controlled over the course of 42 d. Throughout the feeding phase, water and feed were provided ad libitum via 5 nipple drinkers and 2 tube feeders. Replicate cages of different treatment groups were evenly distributed in the room including up-to-down and front-to-back positions in order to decline impacts caused by the cage level.

Determination of B. subtilis *PB6 Spores in Feed by Streak Plate Method*

Feed samples of CON diet, BS diet, and BMD diet were equably collected from all feed bags (500 g, respectively) and sent to the lab to detect the BS spores. Ten g of feed sample from 500 g pooled feed was diluted with 90 mL sterilized phosphate buffered saline (0.01 M phosphate, 0.137 M NaCl, pH 7.3) ranging from 10^1 to 10^8. Three dilution tubes of 4×10^7, 8×10^7, and 1×10^8, were placed in a water bath at 80°C for 10 min. Bacterial suspensions (0.1 mL) were streaked onto sterilized TSAYE agar plates (Merck Co. Ltd, Chengdu, China) in quadruplicate, then were inverted and incubated in an incubator at 37°C for 48 h. These agar plates with 30 to 300 colonies were selected to count colonies. The morphological characteristics of BS colony were short rod and off-white. All procedures were conducted in sterile operating table. The dilution level (C) and the average number of colonies under the same dilution level (B) were used to calculate the colony numbers (A, CFU/g) as follows: A = (B × C)/duplication. Results showed that there were 2.01×10^{11} CFU/kg (starter), and 2.00×10^{11} CFU/kg (grower-finisher) *B. subtilis* PB6 spores in the BS diet and both 0 in CON and BMD diet.

Sample Collection

The BW (after 6 h of starvation) and total input and surplus of feed of each cage were recorded on d 14, 28, and 42 to calculate the average daily gain (**ADG**), average daily feed intake (**ADFI**), and feed conversion ratio (**FCR**). The BW of dead ducks was registered each day, and the growth performance indices revised accordingly.

On the morning of d 42, blood (10 mL) was sampled into dipotassium ethylenediaminetetraacetic acid (EDTA-K2) vacuum tubes from the jugular vein of 1 randomly selected duck (fasted for 6 h) per cage. Serum was harvested by centrifuging blood at 3,500 × *g* for 15 min and then frozen at −20°C until analysis. After blood sampling, all ducks were provided experimental diets. On d 43, 1 duck with BW closest to the average BW of cage was euthanized by cervical dislocation. The guts were dissected from the mesentery and separated quickly on the ice. Sections (about 2 cm) from the middle of individual duodenum, jejunum, and ileum were cut and placed into 4% (v/v) paraformaldehyde solution for histomorphometry measurement. The remaining jejunal and ileal tissues were cut longitudinally and washed in normal saline, and the mucosa was scraped using a sterilized slide that was stored at −80°C until gene analysis.

Digesta collected from jejunal and ileal were put into 2 mL sterile tubes and frozen at −80°C for subsequent measurement of digestive enzyme activity. Cecal digesta was aseptically collected into RNAse-and DNAse-free tubes and frozen at −80°C until microbial pyrosequencing and short-chain fatty acid (**SCFA**) analysis.

Detection of Intestinal Morphology

The fixed jejunal segments were rinsed with running water for 30 min and dehydrated with absolute ethanol at varying concentrations. The tissues were cleared with xylene, embedded in wax, and sliced into 5-μm-thick slices using a Leica RM2235 microtome (**Leica** Biosystems, **Germany**). Finally, these slices were dewaxed and stained with hematoxylin-eosin. For each well-oriented villus, 10 measurements were recorded for both villus height (**VH**) and crypt depth (**CD**) using Image Pro Plus 6.0. The average of these10 measurements was taken to represent the VH and CD for each tissue. The V/C ratio was obtained by dividing the VH by the CD. As described by Święch et al. (2019), for assay goblet cells, in brief, these splices were deparaffinized and then dyed again by Alcian blue-periodic acid-Schiff (**PAS-AB**). The number of goblet cells (in the jejunum and ileum) was detected by 12 well-oriented villi from each tissue, and represented by goblet cells /100 um.

Determination of Enzyme Activity in Digestive Species

Frozen digesta was weighed and diluted (1:9, w/v) with phosphate buffer before cells were disrupted using an ultrasonic homogenizer (Scientz-48L, Ningbo, China). The supernatant was collected after centrifugation (3,500 × *g*, 10 min, 4°C) and used for detecting the activity of lipase, trypsin, and amylase using the corresponding kits with the catalog numbers of A054-2-1, A045-2-2, and C016-1-1 (Jiancheng Bioengineering Institute, Nanjing, China). Parallel determination was carried out for individual indices. Coefficients of inter- and intrasample variations were all controlled within 12%.

Serum Profiles Assay

The serum level of triglycerides, urea nitrogen, and glucose were assayed using Hitachi 7600 (Japan) in duplication.

Assay SCFA in Cecal Digesta

The detection of acetic acid, propionic acid, and butyric acid concentrations was carried out referring to the method of Mou et al. (2020). Concisely, 0.7 g of digesta was diluted into 1.5 mL ultra-pure water and centrifuged (15,000 × *g*, 15 min, 4°C). The supernatant was collected (1 mL) and mixed with 200 μL (25% w/v) metaphosphoric acid solution and 23.3 μL of 210 mmol/L crotonic acid solution, and again centrifuged (15,000 × *g*, 10 min, 4°C). The supernatant was again collected (300 μL) and homogenized with 900 μL carbinol, then filtered through a 0.22-μm PTFE syringe filter. The filtrate (10 μL) was finally analyzed by gas chromatography (CP-3800 Varian, Inc., Palo Alto, USA) for acetate, propionate, and butyrate concentrations with a minimum limitation of 0.1 mmol/L.

Total RNAs Extraction and Quantitative Real-Time PCR

The total RNAs of the jejunal and ileum mucosa (about 0.1 g) were extracted by Trizol (TaKaRa Biotechnology Co., Ltd, Dalian, China) following the manufacturer's instructions. The extracted RNA was evaluated by a NanoDrop 2000 (Thermo Scientific, Wilmington, DE) with the standard of OD_{260}/OD_{280} at the range of 1.8 to 2.0, and quality was assessed via gel electrophoresis (1% w/v agarose). After dilution, 1 μg RNA was reverse transcribed using HiScript Ⅲ RT SuperMix (+gDNA wiper, Vazyme Biotech Co., Ltd. Nanjing, China). The primers, shown in Table S1, of *mucin2, zonula occludens-1* (*ZO-1*), *occludin, claudin1*, and *β-actin* were designed via Prime Premier 6.0 and synthesized in Sangon Biotech Co., Ltd (Chengdu, China). A real-time quantitative PCR was carried out using SYBR qPCR Master Mix (Vazyme Biotech Co., Ltd) and an ABI 7500 real-time PCR instrument QuantStudio 6 (Thermo) with a reaction volume of 20 μL. For real-time PCR, samples underwent denaturation at 95°C for 30 s, followed by 40 cycling at 95°C for 10 s, and 30 s at 60°C. Individual samples were assayed in triplicate. Relative expression levels of the target gene were calculated using the $2^{-\triangle\triangle Ct}$ method (Livak and Schmittgen, 2001) by normalization to *β-actin* (reference gene).

Microbial Sequencing

Fecal microbiota DNA from cecal digesta samples ($n = 6$ in each treatment) was extracted using the E.Z.N.A. Stool DNA Kit (Omega BioTek Inc., Norcross, GA, USA). Before sequencing, the integrity of extracted DNA fragments was confirmed via electrophoresis (2% w/v agarose). Determination of DNA concentrations was carried out by a NanoDrop 1000 Spectrophotometer (Thermo Scientific), and the primer sequencing and bioinformatics analysis were performed by LC-Bio Technology Co., Ltd. (Hang Zhou, China) on NovaSeq PE250 platform, using the paired-end sequence. The augmentation of the V3−V4 region using 341F and 805R primers referred to the method of Zhang et al. (2020a). Raw tags of 200−500 bp were obtained from the spliced sequences by removing the primer and barcode sequences via FLASH software (version 1.2.8). Quality filtering of the raw rags was performed according to the fqtrim software (version 0.9.4), and effective tags were then generated through the removal of chimeric sequences using Vsearch software (version 2.3.4). After dereplication of the effective tags with the divisive amplicon denoising algorithm (DADA2), amplicon sequence variants were used for constructing Operational Taxonomic Units (**OUT**) and obtaining the feature table and feature sequences. Sequence alignment via the Basic Local Alignment Search Tool was executed, and the feature sequences were annotated using the SILVA database (https://www.arb-silva.de/search/) for each specific species.

Data Analysis

ADG, ADFI, and FCR were analyzed by one-way ANOVA using the general linear model via SAS 9.4 (SAS Inst, Inc., Cary, NC) with an individual repeat cage taken as a statistical unit. For analysis of serum metabolites, intestinal morphology, enzyme activity, and SCFA concentrations, an individual duck was taken as an experimental unit. Tukey test was used to compare the difference among indices, and all microbiota data were calculated via QIIME2 (https://docs.qiime2.org/2019.7/tutorials/overview/) with the Kruskal−Wallis tests. Graphs including alpha and beta diversity were drawn using the vegan and ggcor packages of R 4.0.5. Correlation analyses were carried out using Pearson's correlation of SAS and visualized by GraphPad Prism 9. The accepted significant difference was $P \leq 0.05$, and a trend was $0.05 \leq P < 0.1$.

RESULTS

Growth Performance and Serum Profiles

Over the course of 42 d, no significance in the BW and ADG was seen between the 3 treatment groups. However, BS supplementation reduced ADFI during d 15 to 42 and d 1 to d 42 ($P < 0.05$) in comparison to the CON, and it also reduced the FCR ($P = 0.01$) during d 15 to d 42 and d 15 to 42 compared with the other 2 treatment groups (Table 2). As shown in Table 3, there was a reduce ($P = 0.038$) in glucose level in the BS group compared with the CON group.

Table 2. The growth performance of male meat ducks.

Items	CON	BMD	BS	SEM[1]	*P*-value
BW, g					
Day 1	53.6	53.6	53.7	0.1	0.685
Day 14	712.0	703.7	711.3	8.2	0.735
Day 42	3,037.0	3,018.7	3,090.2	33.1	0.326
ADG, g/d					
Day 1−14	47.0	46.5	47.0	0.6	0.739
Day 15−42	83.0	82.7	85.0	1.2	0.377
Day 1−42	71.0	70.6	72.3	0.8	0.329
ADFI, g/d					
Day 1−14	59.8	58.5	59.3	0.8	0.593
Day 15−42	188.2[a]	184.5[ab]	175.6[b]	2.9	0.031
Day 1−42	145.4[a]	142.8[ab]	136.8[b]	2.0	0.033
FCR					
Day 1−14	1.271	1.261	1.261	0.008	0.667
Day 15−42	2.267[b]	2.237[b]	2.069[a]	0.028	0.001
Day 1−42	2.045[b]	2.023[b]	1.893[a]	0.021	0.001

Abbreviations: ADG, average daily gain; ADFI, average daily feed intake; BMD, basal diet+ 45 mg/kg bacitracin methylene disalicylate; BS, basal diet + 2 × 10^7 CFU/kg *Bacillus subtilis* PB6; BW, body weight; CON, basal diet without any additives; FCR, feed conversion ratio.

[a,b]Indicate significant differences.

[1]SEM, pooled standard error ($n = 6$).

Table 3. The serum profiles of male meat ducks.

Items	CON	BMD	BS	SEM[1]	*P*-value
Triglycerides, mmol/L	0.463	0.425	0.388	0.058	0.661
Urea nitrogen, mmol/L	6.188	6.050	6.163	0.550	0.982
Glucose, mmol/L	7.050[ab]	7.150[a]	6.175[b]	0.263	0.038

Abbreviations: BMD, basal diet+ 45 mg/kg bacitracin methylene disalicylate; BS, basal diet + 2 × 10^7 CFU/kg *Bacillus subtilis* PB6; CON, basal diet without any additives.

[a, b]Indicate significant differences.

[1]SEM, pooled standard error ($n = 6$).

Morphological Characteristics and SCFA

As shown in Figure 1A, the jejunal VH and V/C ratio were elevated in the BS group (both, $P < 0.001$). The ileal VH and V/C ratio (both, $P < 0.05$) were elevated in both the BS and BMD groups (Figure 1B). Shown in Figure 1C, when considering the number of goblet cells in the jejunal mucosa, ducks in the BS group had more than those in the CON group ($P < 0.05$). Figure 1D shows the comparison of acetic acid concentrations in cecal digesta, with levels in the BS group higher than in the BMD group, and in the CON, significantly lower ($P < 0.010$). On the other hand, the butyric acid concentrations were the highest in the BS group relative to the other 2 groups ($P < 0.001$). In looking at the relationship between FCR (d 1−42) and other characteristics (Figure 1E), a negative correlation was found between goblet cell numbers in jejunum (R = - 0.557, $P = 0.016$), jejunal VH (R = −0.739, $P < 0.001$), jejunal V/C (R = -0.797, $P < 0.001$).

Intestinal Enzyme Activity

As shown in Table 4, lipase activity in the jejunal digesta of ducks fed a diet supplemented with BS showed an increasing trend ($P = 0.065$). However, no significance was seen in the activity of trypsin and lipase for all 3 treatment groups in this study.

Intestinal Gene Expression

Relative to the CON group, supplementation of BS and BMD appeared to significantly upregulate the jejunal mucosa *ZO-1* mRNA levels ($P < 0.001$), and the BMD diet tended to increase jejunal *mucin2* mRNA levels ($P = 0.056$; Figure 2A). A significant increase in ileal *mucin2* mRNA levels was seen in the BS group relative to the other 2 groups ($P< 0.001$), while mRNA levels of ileal *occludin* in the BMD group showed an increasing trend ($P = 0.086$; Figure 2B). As shown in Figure 2C, the elevated mRNA level of *mucin2* in the jejunum mucosa showed a positive correlation ($R= 0.578$, $P = 0.012$) with the number of jejunum goblet cells.

Microbiological Indices

A total of 1,381,021 raw tags were recorded from 18 colonic digesta samples, with an average of 81,344 tags in the CON group, 58,075 tags in the BMD group, and 67,516 in the BS group (Figure 3A). The number of feature sequences in the CON, BMD, and BS groups was 44,156, 28,653, and 35,591, respectively. The microbial richness and biodiversity were characterized by the observed-OTUs, Goods_ coverage, Chao 1, Shannon index, and Simpson index (Figures 3B−3F). The number of observed_ OTUs was lower ($P = 0.017$) in ducks fed BMD when compared to controls, and the Goods_ coverage indices were elevated ($P = 0.004$) in both BMD- and BS-treated ducks compared to controls. Chao 1 was largest in the BS group, and lowest in the BMD group ($P = 0.009$). However, no significance ($P > 0.05$) in Shannon or Simpson was found among the 3 groups. An unweighted UniFrac PCoA plot visually confirmed a distinct separation of microbial communities between the CON group and the other 2 groups ($P = 0.002$, Figure 3G), suggesting that the addition of BMD and BS altered the composition of microorganisms.

An analysis of the relative abundance of cecal microbes to identify the top 10 phyla (Figure 4A) shows the primary bacterium belonging to Firmicutes (51.17%), followed by Bacteroidetes (29.57%), Proteobacteria (10.31%), Fusobacteria (3.54%), Verrucomicrobia (1.42%), and Cyanobacteria (1.07%). In comparing the treatment groups, the relative proportion of Verrucomicrobia was higher in both BMD- and BS-treated ducks than in controls ($P = 0.009$). As shown in Table 5, the relative abundance of the top 34 genera was ≥0.1% in any individual samples. *Lachnospiraceae _ unclassified* and *Muribaculaceae _ unclassified* were the most abundant ($P < 0.05$) in the BS group, while *Gastranaerophilales _ unclassified, Ruminococcus_ torques_ group*, and *Shuttleworthia* was the richest ($P < 0.05$) in the BMD group. According to genus diff-abundance (Figure 4B), the relative proportions of *Odoribacter*

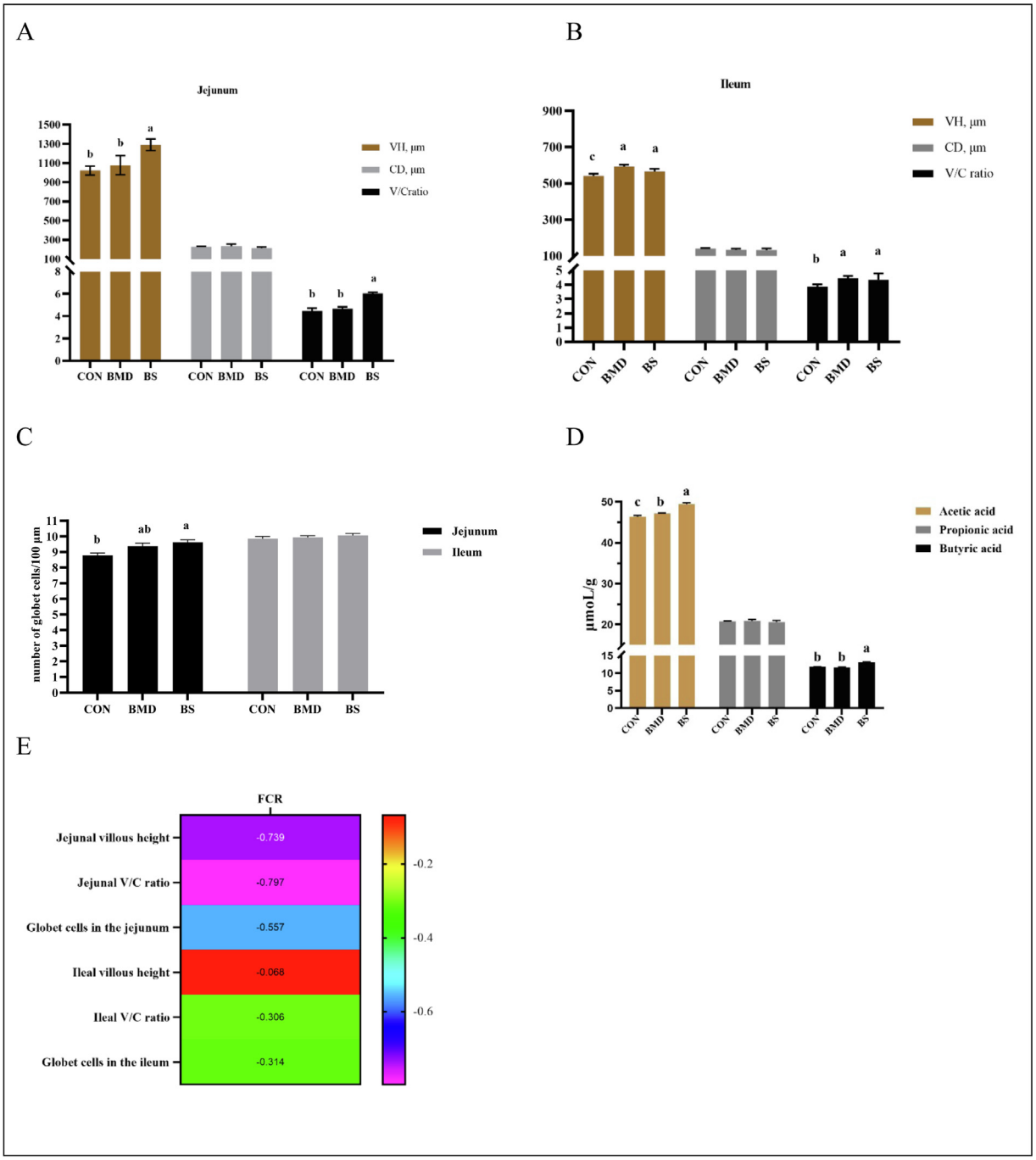

Figure 1. The intestinal morphology, goblet cells, and SCFA concentrations in the cecum of male meat ducks. (A) Morphology indices in jejunum. (B) Morphology indices in ileum. (C) The number of goblet cells. (D) The SCFA concentrations in cecal digesta. (E) Pearson correlation analysis of FCR (d 0−42) and intestinal morphology indices. Abbreviations: BMD, basal diet + 45 mg/kg bacitracin methylene disalicylate; BS, basal diet + 2×10^7 CFU/kg *Bacillus subtilis* PB6; CD, crypt depth; FCR, feed conversion ratio; CON, basal diet without any additives; SCFA, short-chain fatty acids; VH, villus height; V/C ratio, villus height/ crypt depth The data is shown as mean ± SEM, $n = 6$. [a, b, c] Indicate significant differences.

were lowest ($P = 0.034$) in the BMD group, whereas that of *Gorbachella* was the highest relative to the CON and BS group ($P < 0.001$). The most abundant genus in the BS group was *Butyricimonas* ($P = 0.005$), and *Holdemania* was enriched in the CON and BS groups. In family diff-abundance (Figure 4C), *Prevotellaceae* levels were lower ($P = 0.035$) in the BMD when compared with controls, while levels of *Tannerellaceae* were higher ($P = 0.014$) in the BS and CON groups.

As shown in Figure 4D, linear discriminant analysis (**LDA** score >3.0) showed the 13, 5, and 4 taxa to be enriched in the CON, BMD, and BS groups. *Bacteroides_ barnesiae, Prevotellaceae, Barnesiellaceae_ unclassified,* and *Prevotellaceae_ unclassified,* were significantly enriched ($P < 0.05$) in the CON group, while there were abundant ($P < 0.05$) *Alistipes _ unclassified, Gorbaachella, Bachella,* and *Shuttleworthia* in the BMD group. The ducks in the BS group showed an abundance

Table 4. Digestive enzyme activity in intestinal digesta of male meat ducks (U mg /protein).

Items	CON	BMD	BS	SEM[1]	*P*-value
Jejunum					
Amylase	0.95	0.87	0.95	0.04	0.205
Trypsin	144.83	149.17	149.83	1.90	0.181
Lipase	3.07	3.38	4.06	0.26	0.065
Ileum					
Amylase	1.12	1.08	1.15	0.02	0.152
Trypsin	43.33	42.50	43.67	1.65	0.878
Lipase	3.20	3.33	3.81	0.28	0.299

Abbreviations: BMD, basal diet+ 45 mg/kg bacitracin methylene disalicylate; BS, basal diet + 2×10^7 CFU/kg *Bacillus subtilis* PB6; CON, basal diet without any additives.

[1]SEM, pooled standard error ($n = 6$).

($P < 0.05$) of *Parabacteroides, Tannerellaceae, Parabacteroides*, and *Clostridiaceae.*

As shown in Table 6, the Pearson correlation coefficient identified that acetic acid production was positively related to levels of *Butyricimonas, Enterobacteriaceae, Clostridiaceae, Marinifilaceae*, and *Tannerellaceae* ($P < 0.05$). Under the joint action of *Butyricimonas, Enterobacteriaceae, Clostridiaceae, Tannerellaceae*, butyric acid concentrations significantly increased ($P < 0.05$).

DISCUSSION

B. subtilis PB6 has been reported to effectively improve reproduction and growth performance in mammals, broilers, and laying hens (Zhang et al., 2020a; Darsi et al., 2021). However, the effect on meat ducks has not yet been explored. Additionally, Guo et al. (2020) verified that BS was more effective in males than females in improving the growth performance and tibia strength of broiler chicken. Therefore, we chose only male Cherry Valley ducks in this study to explore the potential effects of BS. The dose was selected according to earlier findings in broilers that utilized 1×10^7 CFU /kg, 2×10^7 CFU /kg, 5×10^7 CFU /kg, and 6×10^7 CFU /kg of BS (EFSA, 2009; Darsi et al., 2021; Melegy et al., 2021). Based on this literature, we chose to use 2×10^7 CFU /kg of BS for our experiment. Subsequently, BMD as a growth promoter is widely used for broilers at the active ingredient doses of 27.5, 50, and 55 mg/kg (Fritt and Waldroup, 2003; Pedroso et al., 2006; Manafi et al., 2017; Adewole and Akinyemi, 2021). Considering the abovementioned doses and company suggestions, we used 45 mg /kg BMD in the diet in this study.

Although BMD has been utilized as a growth promoter for more than 50 yr, including poultry, our findings in Cherry Valley male ducks showed no effect on growth when eating 45 mg/kg BMD. Research indicated that the growth-promoting effects of BMD are inconsistent in different doses on broilers (Sim et al., 2004; Rivera-Pérez, 2021; Adewole and Akinyemi, 2021). Taking into account the differences in the physical structure of the gastrointestinal tract between broiler and waterfowl (Kluth and Rodehutscord, 2006), the various concentrations of BMD in these studies may explain the lack of growth promotion response of BMD in our experiment.

Regarding BS, current results showed that dietary supplementation reduced ADFI and FCR of male ducks. This differs from the previous literature on other animals in that no effect was seen in fattening turkeys (EFSA, 2013), and an improved BW, ADG, and reduced FCR was seen in broilers (Melegy et al., 2021) when fed a dose of 1×10^7 CFU/kg of BS supplementation. Concerning the reduced ADFI and FCR, there was no doubt that intestinal indicators led us to further analyze for correlation.

In this study, lipase activity tended to increase in the cecal digesta of BS-treated ducks. To the best of our knowledge, digestive enzyme activity plays a vital role in the digestion of nutrients (Jung and Ahn, 2006). Reportedly, molecular method proved that *B. subtilis* starts germinating after 18 h in the small intestine, then they can colonize in the gut (Casula and Cutting, 2002). After that, *B. subtilis* starts working. It is known that *B. subtilis* produces enzymes as well as stimulates the host to secrete digestive enzymes (Sanders et al., 2003), both of which are conducive to nutrient digestion and consistent with the increase in lipase activity. The reduced glucose level in the BS -treated ducks may response to the rapid absorption of nutrients.

The small intestine is the primary organ for the absorption of nutrients. The VH, CD, and structure of intestinal epithelial cells are all related to nutrient intussusception (Uni, 2006; Tsukahara et al., 2003). In the research of Guo et al. (2020), *B. subtilis* at 1×10^8 CFU/kg of feed improved duodenal and ileal VH and decreased duodenal CD of broilers. This was consistent with our findings in which BS-treated ducks had higher VH and larger V/C of jejunum and ileum. The function of epithelial cells that are adhered to villi is nutrient absorption, so it follows that a larger ratio of villi relates to a higher number of epithelial cells and more efficient absorption (Uni, 2006). Notably, the increased V/C in response to BS is a result of improved absorptive capacity (Liu et al., 2020). Abd El−Hack et al. (2020) pointed out that *B. subtilis* could prompt wave-like villus arrays that increases the absorption area and prolongs residence time in the digestive tract. Moreover, supplementation of BS contributes to a larger number of goblet cells, consistent with the effects seen of *B. subtilis* B10 on broilers (Rajput et al., 2013). Interestingly, goblet cells produced via mitosis of pluripotent stem cells or differentiated cells in the crypt region are responsible for producing mucin and thus can prevent bacteria from attaching to the mucosal surface (Uni, 2006). Along with other mucins secreted by goblet cells, *mucin2* acts as the first line of defense for barrier protection, preventing invasion of foreign antigens and pathogenic bacteria (Chen et al., 2020). The intestinal barrier includes the intestinal tight junction proteins in addition to the mucus gel layer (Pu et al., 2020). A powerful intestinal mucosal barrier is formed by tight junctions (consisting of occludin, claudins, and junctional adhesion molecules

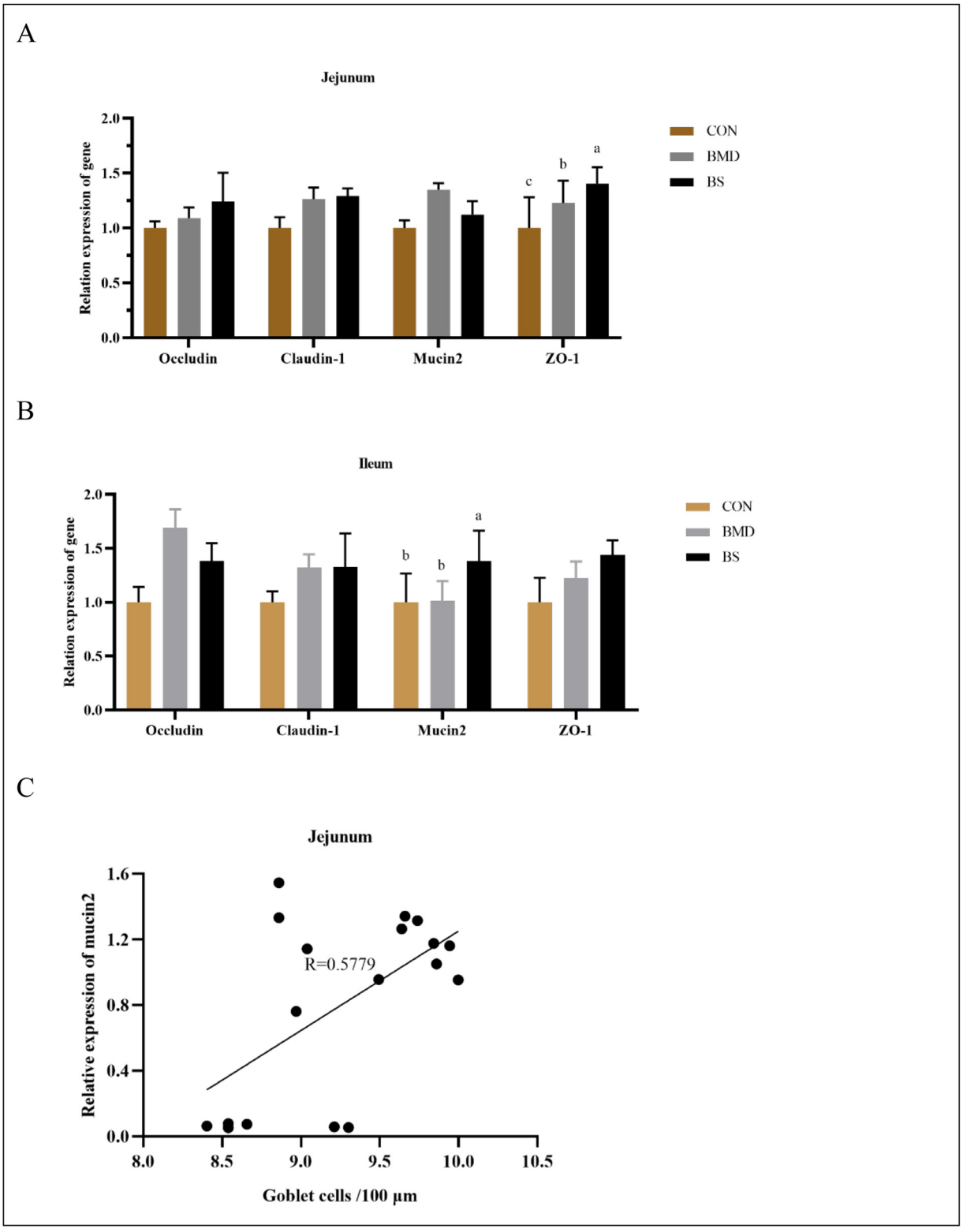

Figure 2. The mRNA levels of tight junction protein in the jejunum and ileum and the correlation between goblet cells and *mucin2* mRNA levels. (A) The mRNA levels of tight junction protein in the jejunal mucosa. (B) The mRNA levels of tight junction protein in the ileal mucosa. (C) Pearson correlation analysis between goblet cells and expression of *mucin2*. Abbreviations: BMD, basal diet+ 45 mg/kg bacitracin methylene disalicylate; BS, basal diet + 2×10^7 CFU/kg *Bacillus subtilis* PB6; CON, basal diet without any additives; ZO-1, zonula occludens 1. The data is shown as mean ± SEM, $n = 6$. [a, b] Indicate significant differences.

such as *ZO* and cingulin) that form cytoskeleton, which guarantees structural integrity and regulates paracellular permeability (Pu et al., 2020). It follows then, that an elevation of intestinal tight junction proteins *ZO-1* (jejunum) and *mucin2* (ileum) in response to BS supplementation is evidence of an improvement in the intestinal barrier, and this has been reported in ducks by Bilal et al. (2021). According to Pearson correlation analysis, the reduced FCR was positively correlated with the improvement of jejunal morphology and goblet

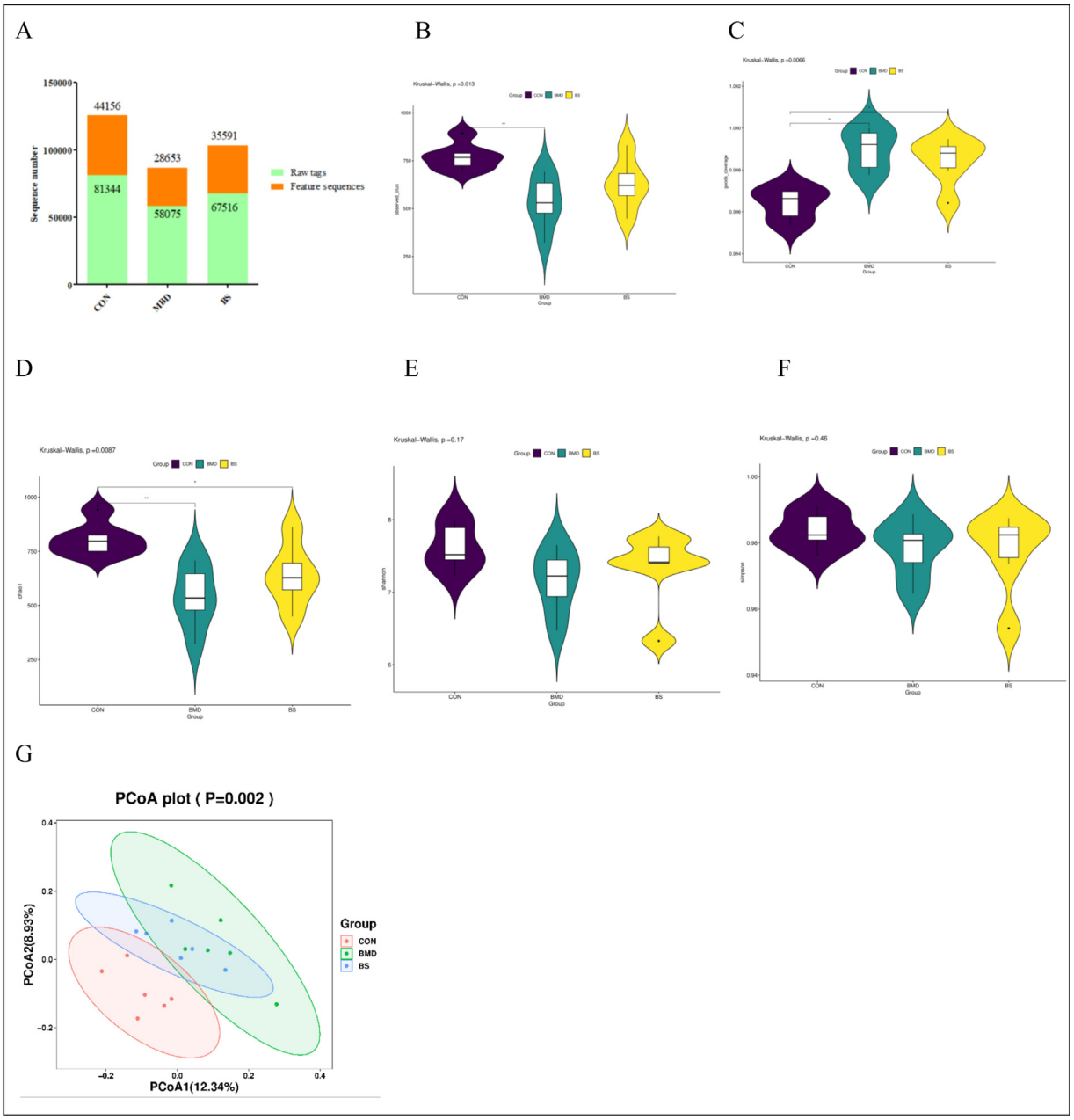

Figure 3. The sequence number, alpha diversity, and beta-diversity among the three groups. (A) The total raw tags and feature sequences in the three groups. (B–F) the comparison of alpha indices in cecum digesta among the three groups. (G) Principal Co-ordinates Analysis (PCoA) at unweighted unifrac. Abbreviations: BMD, basal diet + 45 mg/kg bacitracin methylene disalicylate; BS, basal diet + 2×10^7 CFU/kg *Bacillus subtilis* PB6; CON, basal diet without any additives.The data is shown as mean ± SEM, $n = 6$, $^*P < 0.05$, $^{**}P < 0.01$.

cells, indicating that intestinal health in the jejunum is vital for growth in this study.

The addition of 45 mg/kg BMD to the diet of ducks in this study facilitated villus growth and led to a higher V/C ratio in the ileum. Before this research, the reported effects of BMD on villi varied with a greater height, bigger V/C evident in rabbits at a dose of 100 and 50 mg/kg, and a lower height evident in broilers at a dose of 50 mg/kg (Samanta et al., 2010; Chen et al., 2021; Rivera-Pérez, 2021). Additionally, the increasing trend in jejunal *mucin2* and ileal *ZO-1* was also found in the BMD group of our study. These elevated indices suggest that the administration of BMD changed the morphology of the ileum and improved the absorptive ability of the organ. Nevertheless, no effect was seen on growth.

In poultry, the majority of microorganisms are found in the cecum, where the low oxygen and bile salt concentrations contribute to a favorable environment for survival (Best et al., 2016). Intestinal microbes are known to play a role in immunity, digestion, inflammatory bowel disease, and obesity (Delzenne et al., 2011). And the composition of microbes is easily influenced by dietary energy level, ingredients, and powder or granular form. Accordingly, oral administration of probiotics has become a common approach for regulating the delicate balance between host and microbes (Zhang et al., 2020b).

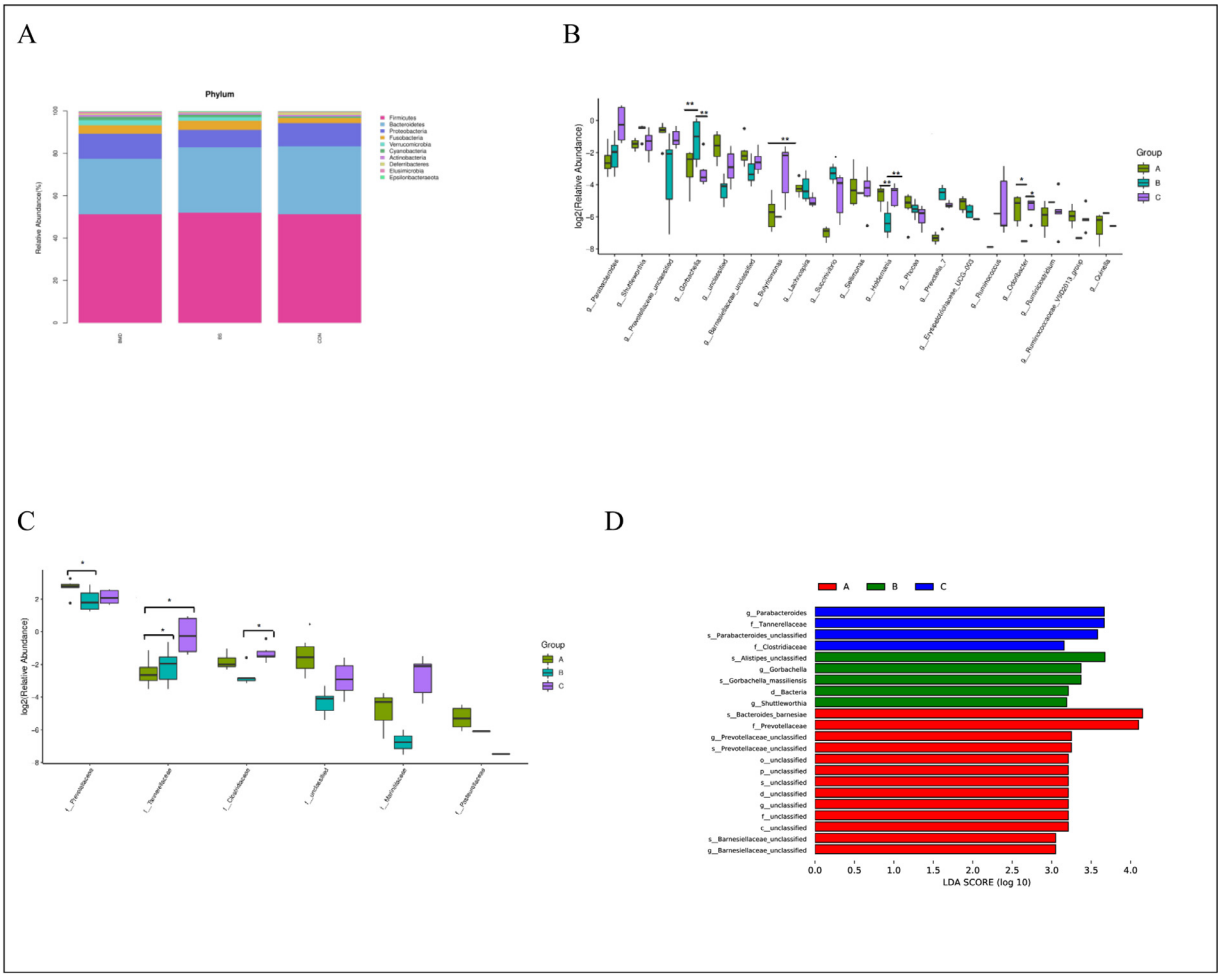

Figure 4. The microbiota structure at phylum level, diff-abundance in genus and family level, and linear discriminant analysis (LDA, significant biomarker). (A) The composition of 10 principal phyla in cecal digesta. (B) Diff-abundance in genus level. (C) Diff-abundance in family level. (D) The significant biomarker among the three groups based on LDA analysis. Abbreviations: BMD, basal diet + 45 mg/kg bacitracin methylene disalicylate; BS, basal diet + 2×10^7 CFU/kg *Bacillus subtilis* PB6; CON, basal diet without any additives.The data is shown as mean ± SEM, $n = 6$. $^*P < 0.05$, $^{**}P < 0.01$.

Based on the NovaSeq PE250 platform, alpha diversity is a description of the biodiversity (Shannon and Simpson) and the richness (Chao1 and observed _ OTUs) in a sample. In our study, BMD reduced the taxonomic richness and diversity of intestinal microorganisms, including both beneficial and harmful bacteria (Adewole and Akinyemi, 2021). This is because BMD is a narrow general antibiotic that works against Gram-positive bacteria (Proctor and Phillips, 2019). The higher Goods _ coverage indices presented in both the BMD- and BS-treated groups indicate that sequencing depth covered all species. Of note, we found the addition of both BMD and BS to the diet of male ducks led to a higher proportion of phyla Verrucomicrobia. This is consistent with the results of Dubourg et al. (2013), who found that patients receiving antibiotic treatment had a higher proportion of Verrucomicrobia in fecal samples. The advantages of intestinal Verrucomicrobia include its ability to fight intestinal inflammation and obesity, as found by Hedlund (2010) and Png et al. (2020).

At the genus level, *Ruminococcus_ torques_* group, *Gastranaerophilales_ unclassified, Shuttleworthia*, and *Gorbachella* were abundant in the BMD group. This was in line with the results from Chen et al. (2021), which showed that BMD improved the abundance of *Ruminococcus* in the cecum digesta of rabbits. *Ruminococcus* has been shown to prompt goblet cells to secrete mucin protein in calves, thus preventing invasion of harmful bacteria (Adewole and Akinyemi, 2021), leading us to hypothesize that the increase in ileal *mucin2* mRNA levels in this BMD group may be a response to elevated levels of *Ruminococcus*. But it should be noted that Zhao et al. (2019) has previously pointed out that the *Shuttleworthia* genus acts pathogenically in the cecum of ducks (Lee et al., 2017). In the BS group, an increment in *Lachnospiraceae* was the main reason for production of butyric acid (by exploiting lactate and acetate via the butyryl- or acetyl-CoA transferase or butyrate kinase pathways). Apart from this, *Lachnospiraceae* also prompts vitamin B12 synthesis (Best et al., 2016; Zhang et al., 2019). Interestingly, bacteria utilize not only the ingredients in feed but also mucins produced by the host (Rehman et al., 2007) and the sugar in intestinal mucus as nourishment to grow (Pereira et al., 2020). *Lachnospiraceae_ unclassified,* of which there were elevated levels in our BS group, is the

Table 5. The relative abundance of the top 34 genera in cecal digesta of male meat ducks[1] (%).

Items	CON	BMD	BS	*P*-value
Bacteroides	19.44 ± 1.76	18.03 ± 3.08	20.65 ± 0.88	0.614
Desulfovibrio	7.27 ± 0.89	7.21 ± 1.07	5.04 ± 0.76	0.149
Intestinimonas	5.02 ± 0.65	4.07 ± 0.45	3.09 ± 0.72	0.094
Streptococcus	6.08 ± 1.73	3.70 ± 1.22	8.04 ± 2.53	0.252
Megamonas	3.33 ± 0.47	5.71 ± 1.72	2.85 ± 1.53	0.093
Fusobacterium	2.38 ± 0.43	3.96 ± 1.94	4.28 ± 2.47	0.573
Ruminococcaceae_ UCG-014	2.35 ± 0.40	2.23 ± 0.42	3.00 ± 0.45	0.410
Lachnospiraceae_ unclassised	0.35 ± 0.06	0.57 ± 0.08	0.38 ± 0.04	0.050
Faecalibacterium	0.45 ± 0.08	0.64 ± 0.12	0.54 ± 0.17	0.464
Eubacterium_ coprostanoligenes_ group	0.57 ± 0.14	0.66 ± 0.15	0.39 ± 0.12	0.406
Ruminococcaceae_ UCG-005	0.50 ± 0.12	0.54 ± 0.07	0.74 ± 0.05	0.160
Prevotellaceae_ Ga6A1_ group	0.48 ± 0.07	0.65 ± 0.11	0.52 ± 0.03	0.288
Ruminiclostridium_ 9	0.47 ± 0.06	0.39 ± 0.07	0.42 ± 0.10	0.747
Ruminococcaceae_ unclassified	0.50 ± 0.08	0.81 ± 0.15	0.56 ± 0.11	0.165
Alistipes	1.82 ± 0.24	2.51 ± 0.55	1.66 ± 0.30	0.197
Firmicutes_ unclassified	1.51 ± 0.08	1.84 ± 0.16	1.64 ± 0.20	0.294
Prevotellaceae_ UCG-001	1.66 ± 0.31	1.15 ± 0.40	1.60 ± 0.24	0.645
Muribaculaceae_ unclassified	0.90 ± 0.16[b]	0.32 ± 0.04[b]	1.65 ± 0.16[a]	0.002
Fournierella	1.02 ± 0.18	0.64 ± 0.20	0.60 ± 0.14	0.244
Clostridiales_ vadinBB60_ group_ unclassified	1.10 ± 0.28	1.60 ± 0.69	1.13 ± 0.23	0.504
Anaerobiospirillum	1.63 ± 0.47	2.28 ± 0.81	0.73 ± 0.25	0.108
Anaerofilum	0.87 ± 0.16	0.57 ± 0.18	1.04 ± 0.36	0.369
Oscillospira	0.63 ± 0.20	0.50 ± 0.03	0.94 ± 0.13	0.128
Clostridiales_ unclassified	0.58 ± 0.13	0.91 ± 0.16	0.62 ± 0.05	0.118
Subdoligranulum	0.25 ± 0.07	0.86 ± 0.53	0.70 ± 0.06	0.061
Ruminococcaceae_ NK4A214_ group	0.74 ± 0.27	0.95 ± 0.19	0.86 ± 0.06	0.739
Gastranaerophilales_ unclassified	0.37 ± 0.15[b]	2.36 ± 1.01[a]	0.99 ± 0.33[b]	0.021
Ruminococcus_ torques_ group	0.35 ± 0.06	0.57 ± 0.08	0.38 ± 0.04	0.050
Erysipelatoclostridium	0.45 ± 0.08	0.64 ± 0.12	0.54 ± 0.17	0.464
Prevotellaceae_ NK3B31_ group	0.57 ± 0.14	0.66 ± 0.28	0.39 ± 0.12	0.406
GCA-900066575	0.50 ± 0.12	0.54 ± 0.07	0.74 ± 0.05	0.160
Butyricicoccus	0.48 ± 0.07	0.65 ± 0.11	0.52 ± 0.03	0.289
GCA-900066225	0.47 ± 0.06	0.39 ± 0.07	0.42 ± 0.10	0.747
Shuttleworthia	0.37 ± 0.03[b]	0.42 ± 0.09[b]	0.68 ± 0.06[a]	0.013

Abbreviations: BMD, basal diet + 45 mg/kg bacitracin methylene disalicylate; BS, basal diet + 2×10^7 CFU/kg *Bacillus subtilis* PB6; CON, basal diet without any additives.

[1]Values are means ± SEM ($n = 6$).

[a,b]Indicate significant differences.

Table 6. Pearson correlation coefficient between significant cecal microbiota and affected short-chain fatty acids concentrations.

Items	Acetic acid	Butyric acid
g__ Lachnospiraceae_ unclassified	P 0.366	P 0.114
	R 0.226	R 0.385
g__ Butyricimonas	P 0.038	P 0.002
	R 0.492	R 0.673
g__ Odoribacter	P 0.517	P 0.584
	R -0.163	R 0.138
g__ Gorbachella	P 0.517	P 0.114
	R -0.163	R -0.385
g__ Holdemania	P 0.769	P 0.215
	R 0.074	R 0.307
g-Shuttleworthia	P 0.924	P 0.337
	R -0.108	R -0.240
g-Muribaculaceae_ unclassified	P 0.670	P 0.774
	R -0.108	R 0.073
g-Gastranaerophilales_ unclassified	P 0.915	P 0.668
	R 0.027	R -0.109
*g-Ruminococcus_ torques_* group	P 0.637	P 0.273
	R -0.119	R -0.273
f__ Prevotellaceae	P 0.127	P 0.324
	R -0.373	R -0.246
f__ Muribaculaceae	P 0.676	P 0.766
	R -0.106	R 0.075
f__ Enterobacteriaceae	P 0.022	P 0.011
	R 0.537	R 0.787
f__ Clostridiaceae	P 0.041	P 0.002
	R 0.493	R 0.688
f_ Tannerellaceae	P 0.002	P < 0.001
	R 0.669	R 0.751

main absorber of mucin monosaccharides. We guessed the elevated *Lachnospiraceae_ unclassified* occupied the same mucin monosaccharides to squeeze the living space of pathogenic bacteria (Pereira et al., 2020). Most of the microbes in the cecum are anaerobes, such as *Holdemania,* which is known to ferment carbohydrate polysaccharides and fibers to produce acetic acid, but showed no effect on the growth or gut health of pigs (Buzoianu et al., 2012); *Butyricimonas* and *Odoribacter,* both of which have anti-inflammatory effects and can cause carbohydrate fermentation to produce butyrate (Bobin-Dubigeon et al., 2021); *Clostridiaceae,* which has been shown to consume saccharides from mucus and plants (Zeibich et al., 2019). *Tannerellaceae* abundance is negatively related to the mRNA levels of occludin1 and MyD88 in the colon (Zhao et al., 2020), while it is a propionate and butyric-producing bacteria in mice (Fu et al., 2019; Van den Abbeele et al., 2020). Levels of each of these anaerobic microbes were increased in the BS group of our study, with *Tannerellaceae* also being enriched in the BMD group.

Because of the short residence time in the small intestine, most polysaccharides, fibers, and amino acids are aseptically fermented in the cecum. The most abundant metabolic products of gut microbiota are SCFA. In this study, we found that acetic acid and butyric

acid concentrations were influenced by dietary supplementation with BMD and BS. As reported by Fu et al. (2019), the major butyric producing bacteria included *f_ Clostridiaceae, f_ _ Enterobacteriaceae, g_ Butyricimonas,* and *f_ Tannerellaceae,* all of which can be found clustered in the cecum of ducks treated with BS. We hypothesized that these bacteria were also responsible for producing the acetic acid detected in our study. As microbial metabolites, SCFA participates in the epigenetic regulation of inflammation and promotes an inflammatory response in the gut, in addition to maintaining glucose homeostasis induced by insulin sensitivity in liver and gut (Beam et al., 2021). Additionally, acetic also can be a source for synthesizing butyrate. Butyrate is regarded as the dominant energy source for colonocytes and is responsible for consolidating the integrity of epithelial cells, resisting inflammatory factors such as IL-17, and reducing oxidant stress by inhibiting pathogens (Fu et al., 2019; Akhtar et al., 2021).

CONCLUSIONS

In this study, our results showed that the supplementation of BS was more effective than supplementation of BMD in improving the intestinal health of male Cherry Valley Ducks. This is evident in the reduced ADFI and FCR resulting from an increase in VH, V/C ratio, and jejunal goblet cells. We found that the *mucin2* expression in the jejunum was positively correlated with the increased number of goblet cells in the jejunum, and that BS changed both the composition of the microbiota and metabolites fermented by microbiota. From these results, we suggest that dietary supplementation of 2×10^7 CFU/kg BS could be a suitable alternative for 45 mg/kg BMD in Cherry Valley male ducks.

DISCLOSURES

All authors declare that there is no conflict of interest.

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