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Effects of supplementation with *Bacillus subtilis* peptide on growth performance, intestinal barrier and caecal microbiota metabolism in broilers

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ABSTRACT

This study assessed *Bacillus subtilis (B. subtilis)* peptide as broiler feed additives. A total of 240 one-day-old male broilers were assigned to four groups (six replicates of 10 broilers) for 42 days. The control group was administered a basic diet, whereas the treatment groups got the basal food augmented with *B. subtilis* peptide at doses of 100, 200, or 300 mg/kg (low, medium, or high dose). The growth performance, intestinal mucosal morphology, immunity, and microbiota were measured. Results confirmed that the average feed intake of broilers in low-dose *B. subtilis* peptide group was increased at 1 ∼ 42 days old, compared to the control group ($p < 0.05$). And dietary supplementation with low-dose *B. subtilis* demonstrated an improving trend in average daily gain and a reduction in feed-to-weight ratio of broilers during the 22 to 42–day period. All doses elevated the concentrations of acetic, propionic, butyric, valeric and 2–methylbutyric acid in the caecum ($p < 0.05$). In terms of intestinal health, high-dose *B. subtilis* peptide upregulated ileal and jejunal Claudin-1 and Occludin-1 mRNA levels. At the phylum level, low-dose treatment significantly increased *Cyanobacteria* abundance ($p < 0.05$). At the genus level of community composition, all *B. subtilis* peptide treatments reduced the abundance of *Bacteroides* in broiler caecal contents. Furthermore, high-dose *B. subtilis* peptide significantly decreased caecal *Lactobacillus* abundance ($p < 0.05$). In conclusion, the addition of *B. subtilis* peptide to the diet might help to improve and maintain the intestinal barrier while modulate the metabolism of intestinal flora.

HIGHTLIGHTS

1. The low-dose *B. subtilis* peptide selectively increased caecal valeric acid production, while restructuring gut microbiota.
2. The high-dose peptide strengthened intestinal barrier function via upregulation of mRNA of Claudin-1 and Occludin-1 levels, though it reduced caecal *Lactobacillus* abundance at the genus level.

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B. subtilis peptide; broilers; growth performance; immune response; intestinal microbiota

Introduction

The abuse of antibiotics has always been an extremely sensitive topic in livestock and poultry production, potentially leading to drug resistance and compromised meat quality. Consequently, numerous nations prohibited antibiotics (EFSA et al. 2019). And consumers' simultaneous demands for product quality and safety continued to drive the advancement of antibiotic-free feeding practices. Commonly used antibiotics in breeding operations—including amoxicillin, norfloxacin, ofloxacin, ceftriaxone, and oxytetracycline (Chowdhury et al. 2009)—had raised public health concerns due to antimicrobial resistance. In response, stricter regulations on antibiotic use prompted researchers to seek sustainable, residue-free alternatives that were both environmentally friendly and effective. Moreover, it was hoped that these substitutes could improve the production performance and immunity of livestock, thus improving economic benefits. Nowadays, antimicrobial peptides (AMPs, especially metabolites produced by bacterial or fungal fermentation, were used to produce small-molecule antimicrobial peptides) were used as feed additives for mass production applications (Alagawany et al. 2019; Yasmin et al. 2020;

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Silveira et al. 2021; Zhu et al. 2022). AMPs were a class of divergent small molecule proteins with strong cationicity and heat resistance. Furthermore, AMPs exhibited significant diversity in structure, which corresponded to a broad spectrum of biological activities. Consequently, the selection of appropriate antimicrobial peptide types and optimal dosages was critical for effective application in livestock and poultry production, yet it posed a significant practical challenge that necessitated resolution.

Lipopeptide AMPs were low molecular weight molecules, which belonged to a diverse and rich biomolecular group. These peptides, produced across diverse animal and plant cells, exhibited potent antimicrobial activity against bacteria, viruses, and fungi. They constituted evolutionarily conserved components of innate immunity, providing analogous pathogen defense mechanisms in both kingdoms. AMPs commonly displayed a defining feature: the existence of paired cysteine residues linked by disulphide bonds, which provided considerable durability (Haag et al. 2012). Their structural features including amphiphilicity, cationic nature, helical conformation, and compact size, facilitated membrane insertion, inducing microbial cell death. *Salmonella* represented the primary challenge in poultry production due to its zoonotic threat to public health (Hazards et al. 2019; Thames and Sukumaran 2020). The AMP Microcin J25 demonstrated efficacy against pathogens such as *Salmonella* and *Escherichia coli*. It exhibited antibacterial activity, enhanced animal growth performance, thereby improving faecal flora composition and intestinal structure, and stimulated an effective immune response (Wang et al. 2020). In recent years, growing concerns over antibiotic abuse and food safety have spurred increased interest in AMPs as promising biological alternatives (León Madrazo and Segura Campos 2020; Silveira et al. 2021). However, the application of AMPs derived from *Bacillus subtilis* (*B. subtilis*) in the feed industry has not yet been widely reported.

The spores of *B. subtilis* exhibited advantages in heat resistance and tolerance to processing, demonstrating superior stability compared to other probiotics (Ramlucken et al. 2020). Moreover, *B. subtilis* could modulate the gut microbiota and enhance intestinal barrier integrity, mucosal immunity, digestive enzyme activity, and nutrient metabolism (Ningsih et al. 2023). We hypothesised that *B. subtilis*-derived AMPs acted as a growth promoter for broilers, but the doses of *B. subtilis* peptide during the feeding period might have varying degrees of effect. In this research, by adding *B. subtilis* peptide to the diet, the effects of *B. subtilis* peptide on growth performance, immune performance, intestinal barrier and microorganisms of broilers were investigated, which provided reference for the development and utilisation of *B. subtilis* peptide and contributed to the cause of resistance.

Materials and methods

Ethical statement

All procedures employed in the study received approval from the animal care and use committee of the Institute of Animal Science, Chinese Academy of Agricultural Sciences.

Experimental design and diets

The experiment was designed by a single factor. Two hundred forty 1–day-old male Arbour Acres broilers were obtained from the experiment farm of Guang'an Co. Ltd (Tianjin, China). Broilers were randomly allocated to four equal groups comprising six replicates with 10 broilers per replicate, and the trial duration was 42 days. The trial utilised a maize-soybean meal basal diet, administered in two phases: starter (1 ~ 21 d) and grower (22 ~ 42 d). The control (Ctrl) group received a basal diet devoid of antibiotics, additional probiotics, and enzymes, while the experimental groups were administered the basal diet augmented with 100 mg/kg (low dose lipopeptide group, LL), 200 (medium dose lipopeptide group, ML) or 300 (high dose lipopeptide group, HL) mg/kg of *B. subtilis* peptide, respectively. The basal diets were formulated to meet the feeding standard of broilers (NY/T 33–2004) recommendation, the composition and nutrient levels are shown in Table 1. Broilers were allowed ad libitum access to feed and water throughout the experimental period. The diet was granulated by a small cold granulator, and the granulation temperature was lower than 75 °C.

B. subtilis peptide was produced by Beijing Enhalor Biotechnology Co., Ltd. (Beijing, China), and was expressed and fermented by *B. subtilis* with a content of 6%. Its main function was to regulate the immune function of animals, promote growth, and inhibit Gram-positive pathogens. The product exhibited thermal stability, gastric acid tolerance, and resistance to enzymatic digestion. In addition, it exhibited both hydrophilic and lipophilic properties. As an antimicrobial peptide, surfactin's amphipathic nature enabled it to dissolve effectively in the aqueous environment of the intestinal contents, thereby enhancing its dispersion and absorption along the digestive tract. Furthermore, this amphipathic nature facilitated

Table 1. Composition and nutrition level of basal diet.

Items, %	1-21d	22-42d
Maize	51.83	53.09
Soybean meal	36.50	33.00
Flour	3.00	5.00
Soybean oil	4.00	5.50
CaHPO4	1.90	1.65
Limestone	1.20	0.90
NaCl	0.30	0.30
L-Lys 55%	0.57	0.15
DL-Met 99%	0.18	0.10
Threonine 98%	0.11	0.00
Multimineral premix[a]	0.20	0.10
Antioxidant[b]	0.03	0.03
50% choline chloride	0.15	0.15
Multivitamin premix[c]	0.035	0.035
Total	100.00	100.00
Calculated nutritive value (dry matter basis)		
Metabolizable energy, Mcal/kg	3.025	3.151
Crude protein, %	21.42	19.86
Calcium, %	0.99	0.81
Available phosphorus, %	0.45	0.40
Lysine, %	1.42	1.11
Methionine, %	0.51	0.41
Threonine, %	0.93	0.78
Arginine, %	1.46	1.36
Valine, %	0.95	0.90
Methionine + Cystine, %	0.68	0.65

[a]The multimineral premix provided the following per kg of diets : Fe, 40 mg; Cu, 16 mg; Zn, 100 mg; Mn, 120 mg; I, 1.25 mg; Se, 0.30 mg.
[b]The antioxidant utilised was a complex molecule comprising butylated hydroxytoluene, butylated hydroxyanisole, and rosemary extract.
[c] The multivitamin premix provided the following per kg of diets : VA, 12000 IU; VD_3, 4500 IU; VE, 24 IU; VK_3, 3 mg; VB_1, 3 mg; VB_2, 9.6 mg; VB_6, 3 mg; VB_{12}, 0.018 mg; pantothenic acid, 15 mg; nicotinic acid, 39 mg; folic acid, 1.5 mg; biotin, 0.15 mg.

interactions with membrane components or receptors on host immune cells, such as macrophages and dendritic cells. Collectively, the amphipathic structure of antimicrobial peptides underpinned their potent and multifunctional bioactivity making them highly valuable for applications such as reducing antibiotic use, preventing and controlling livestock and poultry diseases, enhancing immune function, improving gut health, and ultimately increasing production efficiency (Lei et al. 2019; Han et al. 2024; Wang et al. 2024a).

Growth performance index

Body weight and feed intake were recorded per replicate initially and finally during phases 1 ~ 21 d and 22 ~ 42 d. Growth performance (average daily gain (ADG), average daily feed intake (ADFI), and feed/weight ratio (F/W)) was calculated to assess *B. subtilis* peptide effects.

Antioxidant and immune indicators

At the end of the experiment, approximately 5 mL of blood was extracted from broilers using the wing vein blood collection method. Following a 30 min incubation at room temperature, the blood was centrifuged at 1698 × g for 10 min to isolate the serum, which was subsequently stored in a −20 °C freezer for the assessment of serum antioxidant and immunological markers. The antioxidant indexes (complement 3 (C3), catalase (CAT), glutathione peroxidase (Gpx), malondialdehyde (MDA) and superoxide dismutase (SOD)) and immune indexes (Immunoglobulin M (IgM), immunoglobulin A (IgA), endotoxin (ET), diamine oxidase (DAO), SIgA (secretory immunoglobulin A) and total antioxidant capacity (T-AOC)) in serum were determined using kits from Shanghai Enzyme-linked Biotechnology Co., Ltd (Songjiang, Shanghai, China). The experiment operation was strictly in accordance with the instructions. Serum samples were diluted 5-fold for proteinanalysis, with the exception of the T-AOCassay, for which undiluted serum (a 1-folddilution) was used.

Observation of intestinal tissue morphology

After the broilers were slaughtered and dissected, 1 cm segments from the mid-jejunum, mid-ileum, and mid-caecum were collected, fixed in 4% paraformaldehyde for more than 24 h, and sectioned. The Mingmei microscopic digital measurement analysis system (Guangzhou Mingmei Technology Co., Ltd, Guangzhou, China) was used to measure villus height, crypt depth and calculate the villi/crypt ratio (V/C).

Determination of short-chain fatty acids in caecal contents

Approximately 20 mg caecal content was measured, then combined with 1 mL of 0.5% phosphoric acid solution and a steel bead. Samples underwent two grinding cycles (20 Hz, 10 s each), followed by 10 min vortexing and 5 min ice-bath sonication, followed by centrifugation (10,600 × g, 10 min). Subsequently, 0.1 mL of supernatant was combined with 0.5 mL of MTBE, vortex-mixed for 3 min, and centrifuged at 10,600 × g for 10 min at 4 °C. Short-chain fatty acids (SCFAs) in 200 μL of the resulting supernatant were quantified by gas chromatography. The specific operation method of the gas chromatography in this test was carried out with reference to the literature description (Feng et al. 2022).

RNA extraction and RT-qPCR detection

Approximately 100 mg aliquots of jejunal and ileal tissue or mucosa were processed with 1 mL of TRIzol reagent *via* homogenisation (Beijing Solarbio Biotechnology Co., Ltd, Beijing, China), and the supernatant was separated after the nucleic acid protein complex was completely separated. Following chloroform addition (0.2 mL), samples were vortex-mixed

Table 2. Primer sequences for real-time PCR.

Genes	GenBank	Primer sequences(5′-3′)	Fragment length(bp)
Claudin-1	NM_001013611.2	F: CAT ACT CCT GGG TCT GGT TGG T R: GAC AGC CAT CCG CAT CTT CT	100
ZO-1	XM_040706827.2	F: CTT CAG GTG TTT CTC TTC CTC CTC R: CTG TGG TTT CATGGCTGGATC	131
Occludin-1	NM_205128.1	F: GCA GAT GTC CAG CGG TTA CT R: ATG ACG ATG AGG AAC CCA CA	150
GAPDH	NM_204305.2	F: GCA CGC CAT CAC TAT CTT R: GGA CTC CAC AAC ATA CTC AG	82

and centrifuged at 10,600 × g for 5 min, and 500 μL of the aqueous phase was aspirated. RNA was precipitated by adding an equal volume of cold isopropanol, washed three times with 75% ethanol, and air-dried at room temperature.

After verifying RNA purity and integrity, cDNA was synthesised using the HiScript® II All-in-one RT SuperMix Kit (Vazyme-innovation in enzyme technology, Nanjing, China) and then cDNA was stored at −80 °C for subsequent gene expression detection. The Tap Pro Universal SYBR qPCR Master Mix kits were then used to complete the mixing of cDNA templates and primers in the fluorescence quantitation experiment according to the instructions of Vazyme-innovation in enzyme technology, including PCR amplifications and cycling. The relative gene expression (Claudin-1, ZO-1 and Occludin-1) was calculated by the $2^{-\Delta\Delta Ct}$ method, and GAPDH was used as an internal reference. The qPCR amplification conditions were as follows, with aninitial denaturation at 95°C for 5 min, followed by 40 cycles ofdenaturation at 95°C for 10 sand annealing at 60°C for 30 s.Following amplification, a melt curve was generated byheating to 95°C for 15 s, cooling to 60°C for 60 s, and thenslowly ramping to 95°C. Primer sequences were presented in Table 2.

DNA extraction and PCR amplification

Microbial DNA was extracted from caecal contents using the E.Z.N.A. DNA kits (Omega Bio-tek, USA). Integrity was assessed and concentration quantified spectrophotometrically (NanoDrop® ND-2000, Thermo Scientific) before storage at −80 °C. The V3-V4 hypervariable region of 16S rRNA was amplified using 338 F (5′-ACT CCT ACG GGA GGC AGC AG-3′)/806R (5′-GGA CTA CHV GGG TWT CTA AT-3′) primers by an ABI GeneAmp® 9700 PCR thermocycler (ABI, CA, USA). PCR amplifications (20 μL) contained 2 μL of 2.5 mM dNTPs, 4 μL of 5× Fast Pfu Buffer, 0.8 μL of each primer, 0.4 μL of Fast Pfu polymerase, 10 ng template DNA, and ddH_2O to volume. The PCR cycling conditions consisted of an initial denaturation at 95 °C for 3 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 45 s. A final extension was performed at 72 °C for 10 min, with a hold at 4 °C. Reactions were performed in triplicate. Products were gel-extracted (2% agarose) and purified (AxyPrep DNA Gel Extraction Kit; Axygen Bio), then quantified (Quantus™ Fluorometer; Promega) following manufacturer protocols.

Illumina MiSeq sequencing

The purified amplicons were pooled equimolarly and subjected to paired-end sequencing (PE300) on an Illumina MiSeq platform (San Diego, USA) following Majorbio Bio-Pharm protocols (Shanghai, China).

Data processing

Raw FASTQ files were demultiplexed using custom Perl scripts. Quality control was performed with fastp (v0.19.6) applying (i) truncation at sliding windows (50–bp width, $Q < 20$) with removal of reads <50 bp or containing ambiguous bases and (ii) read merging *via* FLASH (v1.2.7) requiring > 10 bp overlaps with ≤ 0.2 mismatch tolerance. Demultiplexing allowed ≤ 2 primer mismatches.

Statistical analysis

SPSS 19.0 (IBM SPSS, Armonk, NY, USA) was selected for statistical analysis of test results. All results were expressed as mean ± mean squared error (SEM). These experiments were performed in triplicate. One-way ANOVA was used to statistically compare more than two groups of broilers and to perform multiple comparisons using Tukey-Kramer correction. Statistical significance was defined as $p < 0.05$ unless stated otherwise. The linear and quadratic impacts of *B. subtilis* peptide doses were checked by orthogonal polynomial contrasts. $p < 0.05$ indicated a significant difference. And the Majorbio Cloud platform (https://cloud.majorbio.com) was used to perform bioinformatics analysis of the gut microbiota.

Table 3. Effects of different treatments on feed intake, daily gain and feed to gain ratio of broilers.

Item	Ctrl	LL	ML	HL	SEM	*P*-Value	Linear	Quadratic
1 day old/g	40.32	40.28	40.18	40.48	0.278	0.286	0.473	0.130
1 ~ 21 days old								
Average daily feed intake, g	76.54^{ab}	78.85^{a}	76.44^{ab}	72.49^{b}	1.103	0.025	0.265	0.261
Final weight/g	1092.88	1073.44	1085.50	1054.25	24.99	0.410	0.186	0.736
ADG , g	50.18	49.15	49.90	48.17	1.188	0.317	0.159	0.671
F/W	1.53	1.60	1.53	1.51	0.046	0.336	0.321	0.485
22 ~ 42 days old								
ADFI, g	136.20^{c}	150.89^{a}	141.62^{bc}	144.79^{b}	2.570	0.001	0.064	0.561
Final weight, kg	2916.63	3010.29	2907.48	2856.82	140.14	0.743	0.533	0.477
ADG, g	84.49	93.39	84.92	89.68	3.517	0.628	0.773	0.715
F/W	1.63^{b}	1.62^{b}	1.69^{b}	1.64^{a}	0.117	0.958	0.667	0.498
1 ~ 42 days old								
ADFI, g	110.01^{b}	114.87^{a}	109.14^{b}	109.33^{b}	4.263	0.001	0.608	0.217
ADG, g	69.77	70.80	70.81	65.07	2.432	0.103	0.531	0.639
F/W	1.57^{b}	1.59^{ab}	1.53^{b}	1.69^{a}	0.077	0.038	0.304	0.898

$^{a}P < 0.05$ compared with the Ctrl, ML or HL group.
$^{b}P < 0.05$ compared with the Ctrl, LL or HL group.
$^{c}P < 0.05$ compared with the LL, ML or HL group. Average daily gain, ADG; average daily feed intake, ADFI; feed/weight ratio, F/W. Low dose lipopeptide group; medium dose lipopeptide group, ML; high dose lipopeptide group, HL.

Results

Effects of different doses of B. subtilis peptide on growth performance of broilers

Growth performance indicators were the most intuitive indicators to evaluate the effect of lipopeptides on broilers. Our findings indicated that the ADFI in the HL group was decreased at 1 ~ 21 days of age compared with that in the LL group ($p < 0.05$), and no linear or quadratic trends were seen (Table 3). Compared with the Ctrl group, the ADFI was increased in the LL and HL groups at 22 ~ 42 days of age, and the effect of LL was better ($p < 0.05$), and no significant linear or quadratic trends noted. Compared with the Ctrl group, the LL group exhibited significantly higher ADFI versus the Ctrl, ML, or HL groups at 1 ~ 42 days of age ($p < 0.05$), and neither linear nor quadratic trends were identified (Table 3). No significant variation in ADG or final weight was seen among broilers across several treatment groups at different growth stages ($p > 0.05$), with significant linear trends and no quadratic trends identified. In addition, compared with the Ctrl group, the F/W of broilers was increased in the HL group ($p < 0.05$) at 1 ~ 42 days of age (Table 3). In comparison to the Ctrl group, broilers administered low-dose *B. subtilis* from days 22 to 42 exhibited numerical enhancements in ADG (93.39 vs. 84.49 g/broiler/day) and a reduction in feed-to-weight ratio (F/W) (1.62 vs. 1.63), although no statistically significant differences were detected. Growth performance indexes revealed that low-dose *B. subtilis* peptide might enhance broiler growth.

B. subtilis peptide treatments enhanced the immune performance of broilers

Immune performance (including C3, CAT, GPx, MDA, SOD, IgG, IgM, IgA, ET, DAO, SIgA and T-AOC indexes) of broilers was detected (Figures 1A–L). Upon stimulation of the immunocompetent cells on the intestinal mucosal epithelium by bacterial adhesion, SIgA was synthesised at the adhesion site to inhibit the assault of intestinal microbes and their toxic compounds on the intestinal mucosa. No notable changes in C3 concentration were detected across the groups (Figure 1A), and neither linear nor quadratic trends were identified. A notable quadratic trend was detected in CAT concentration ($p < 0.05$), although no linear trend was found. In comparison to the Ctrl group, the ML group exhibited a significant elevation in serum CAT levels in broilers at 42 days ($p < 0.05$) (Figure 1B). The Gpx concentration was markedly elevated in the HL group relative to the Ctrl group ($p < 0.05$), although no significant differences were noted in the LL or ML groups. A notable linear trend was seen ($p < 0.05$), whereas no quadratic trend was identified (Figure 1C). No notable variations were seen among the groups for the amounts of MDA (Figure 1D), SOD (Figure 1E), or IgA (Figure 1H) ($p > 0.05$). The ML group exhibited a substantial reduction in IgG concentration compared to the Ctrl group ($p < 0.05$), although no alterations were noted in the LL or HL groups ($p > 0.05$) (Figure 1F). All *B. subtilis* treatments significantly diminished blood IgM levels in broilers at 42 days ($p < 0.05$, Figure 1G). The concentrations of ET and DAO were considerably elevated in the HL group relative to the Ctrl group ($p < 0.05$; Figures 1I and 1J). Furthermore, ML and LL treatments reduced the SIgA levels in the intestinal mucosa of broilers ($p < 0.05$; Figure 1K). No significant variations in T-AOC concentration were discovered among groups according to one-way ANOVA, and no significant linear trend was identified. A notable quadratic trend was observed ($p < 0.05$), indicating a non-linear association between T-AOC

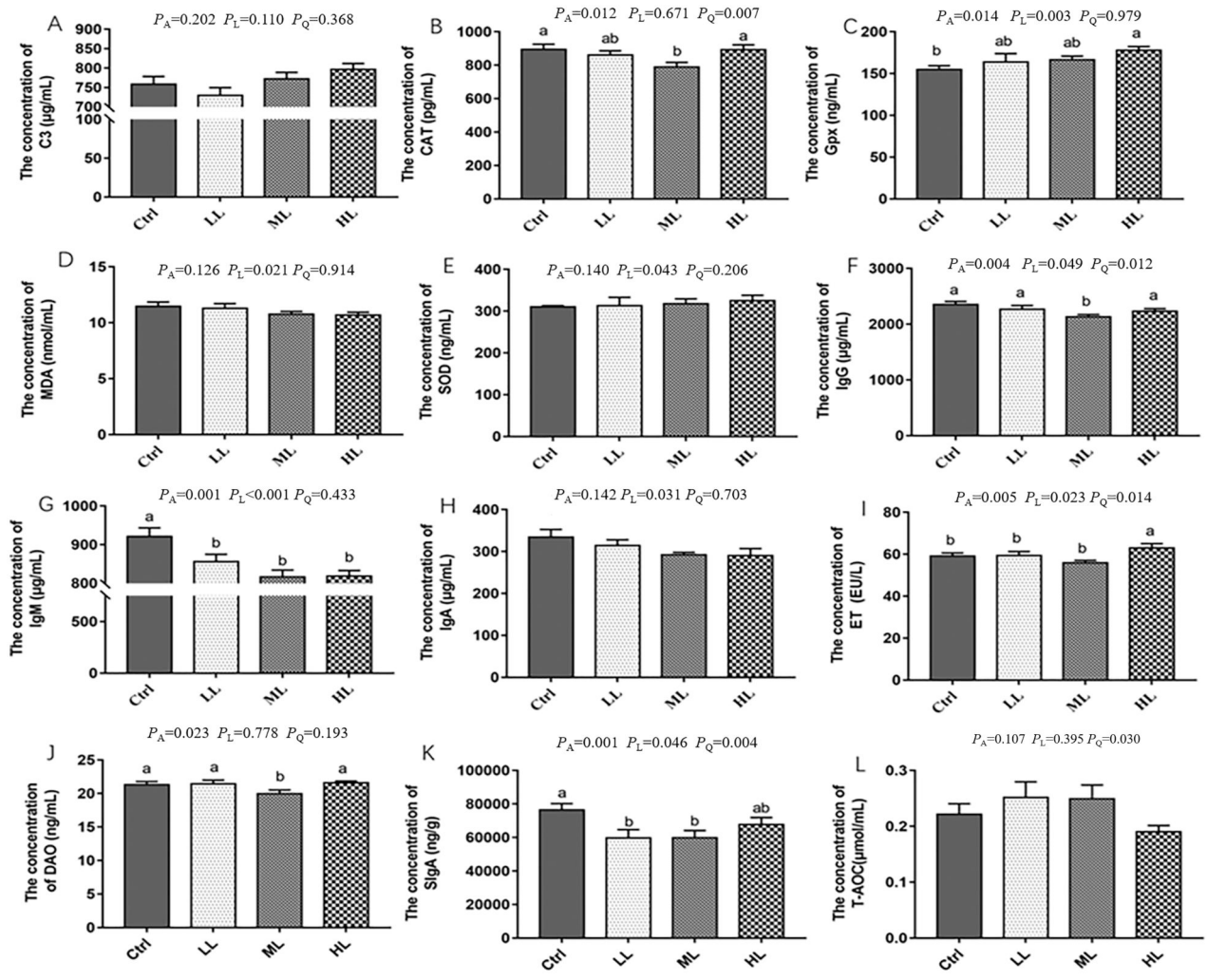

Figure 1. Effects of different treatments on immune performance of broilers. Antioxidant indexes (A-E) and immune indexes (F-L) in broiler serum were determined using ELISA kits. [a]$P < 0.05$ compared with the low dose lipopeptide group (LL), medium dose lipopeptide group (ML) or high dose lipopeptide group (HL); [b]$P < 0.05$ compared with the Ctrl, LL, ML or HL group.

concentration and the experimental groups (Figure 1L). These results suggested that *B. subtilis* peptide in the LL group might activate the immune function of broilers, resulting in a decrease in jejunal SIgA secretion and a decrease in serum IgM and CAT contents in broilers in a short period of time.

Medium or low doses of B. subtilis peptides were more likely to promote intestinal development in broilers

The degree of development of the small intestine could reflect the ability of broilers to absorb the affected substances. Compared to the Ctrl group, ileal, jejunal, and caecal villus morphology remained treatment-independent. However, jejunal villus height demonstrated a quadratic dose-response to lipopeptide supplementation ($p < 0.05$). The HL dose significantly reduced the ileal and jejunal V/C ratio versus the Ctrl group ($p < 0.05$). Ileal V/C decreased linearly with increasing *B. subtilis* peptide dose ($p < 0.05$), while overall V/C exhibited a quadratic dose-response (increase followed by decrease; $p < 0.05$; Table 4). However, these different treatments had no significant effect on the development of caecal papillae. In comparison to the LL group, both the ML and HL groups markedly diminished ileal villus height and the V/C ratio ($p < 0.05$). However, no significant alterations were noted in relation to the Ctrl group. The height of ileal villi demonstrated a linear decline with escalating dosage ($p < 0.05$), lacking a quadratic relationship. The alterations in ileal crypt depth exhibited solely a quadratic correlation. The ileal V/C ratio exhibited a linear decline and a quadratic relationship ($p < 0.05$). Morphological study of the jejunum indicated that, relative to the Ctrl group, both the LL and ML treatments considerably enhanced jejunal villus height, while the HL treatment greatly reduced it ($p < 0.05$).

Table 4. Effects of different treatments on intestinal villus height and crypt depth of broilers.

Item	Ctrl	LL	ML	HL	SEM	*P-value*	Linear	Quadratic
Ileum, μm								
Villus height	417.05^{ab}	464.59^{a}	404.94^{b}	387.04^{b}	19.250	0.036	0.047	0.368
Crypt depth	126.10	117.66	115.71	128.08	5.710	0.140	0.840	0.023
V/C	3.57^{ab}	3.99^{a}	3.46^{bc}	3.06^{c}	0.227	0.005	0.008	0.016
Jejunum, μm								
Villus height	386.13^{c}	567.54^{b}	752.39^{a}	242.99^{d}	12.711	<0.001	<0.001	<0.001
Crypt depth	151.81^{a}	141.75^{a}	139.30^{a}	121.28^{b}	6.690	0.012	0.002	0.426
V/C	2.54^{c}	4.02^{b}	5.29^{a}	2.02^{d}	0.228	<0.001	0.680	<0.001
Caecum, μm								
Villus height	94.22^{a}	90.29^{ab}	83.57^{b}	68.84^{c}	3.998	0.001	<0.001	0.098
Crypt depth	57.76^{bc}	78.17^{a}	63.78^{b}	53.57^{c}	3.010	<0.001	0.037	<0.001
V/C	1.56^{a}	1.21^{b}	1.30^{b}	1.28^{b}	0.054	0.006	0.014	0.013

$^{a}P < 0.05$ compared with the Ctrl or HL group.
$^{b}P < 0.05$ compared with the LL or HL group.
$^{c}P < 0.05$ compared with the LL, ML or HL group.
$^{d}P < 0.05$ compared with the Ctrl, LL or ML group. Villi/crypt ratio, V/C. Low dose lipopeptide group, LL ; medium dose lipopeptide group, ML ; high dose lipopeptide group, HL.

Table 5. Effects of different treatments on the content of volatile fatty acids in caecum content of broilers.

Item	Ctrl	LL	ML	HL	SEM	*P-value*	Linear	Quadratic
acetic acid, μg/g	2050.47^{c}	3160.84^{a}	2543.23 ^{b}	2343.08 ^{b}	114.886	<0.001	0.779	<0.001
propionic acid, μg/g	1280.75^{c}	2378.61^{a}	2078.95 ^{b}	2141.89 ^{b}	60.991	<0.001	<0.001	<0.001
butyric acid, μg/g	640.60^{c}	1263.69^{a}	1087.99 ^{b}	1202.27^{a}	34.416	<0.001	<0.001	<0.001
isobutyric acid, μg/g	81.46^{b}	89.86^{b}	84.56^{b}	115.00^{a}	4.463	<0.001	<0.001	0.008
valeric acid, μg/g	86.75^{d}	342.90^{a}	276.58 ^{b}	152.27 ^{c}	4.307	<0.001	<0.001	<0.001
3–methylbutyric acid, μg/g	107.26^{c}	179.37^{a}	77.06^{d}	124.20^{b}	6.668	<0.001	0.048	0.035
2–methylbutyric acid, μg/g	51.44^{c}	57.65^{b}	57.58^{b}	63.81^{a}	1.285	<0.001	<0.001	0.993

$^{a}P < 0.05$ compared with the Ctrl, ML or HL group.
$^{b}P < 0.05$ compared with the Ctrl, LL or HL group.
$^{c}P < 0.05$ compared with the LL, ML or HL group.
$^{d}P < 0.05$ compared with the Ctrl, LL, ML or HL group. Low dose lipopeptide group, LL; medium dose lipopeptide group, ML; high dose lipopeptide group, HL.

The height of jejunal villi exhibited both a linear reduction and a quadratic trend in relation to dosage ($p < 0.05$). No significant variations in jejunal crypt depth were seen between the LL and ML groups compared to the Ctrl group. However, the HL group dramatically decreased crypt depth ($p < 0.05$), which had a linear declining trend ($p < 0.05$) without any quadratic association ($p > 0.05$). The jejunal V/C ratio dramatically increased in the LL and ML groups ($p < 0.05$), while it significantly dropped in the HL group ($p < 0.05$), exhibiting a quadratic rather than a linear change pattern. In the caecum, the ML group had a significant reduction in caecal villus height compared to the Ctrl group ($p < 0.05$), demonstrating a linear declining trend ($p < 0.05$) without a quadratic association. The LL group markedly enhanced caecal crypt depth ($p < 0.05$), whilst the other two groups exhibited no significant effects. Moreover, caecal crypt depth exhibited both a linear decline and a quadratic relationship ($p < 0.05$). All three treatment groups dramatically reduced the caecal V/C ratio in comparison to the Ctrl group, with decreases demonstrating both linear and quadratic characteristics ($p < 0.05$). The results suggested that *B. subtilis* peptide in the LL groups may improve nutrient digestion and absorption in the jejunum and ileum of broilers by augmenting villus height and expanding the interface between the villi and nutrients.

B. subtilis peptide treatments promoted the increase of SCFAs in the caecal contents of broilers

The production of SCFAs in the intestine played an important role in improving the nutrient metabolism of broilers. The administration of dietary *B. subtilis* lipopeptide markedly elevated caecal concentrations of acetate, propionate, butyrate, valerate, 3–methylbutyric acid, and 2–methylbutyric acid in LL broilers compared to the control group ($p < 0.05$; Table 5). The concentrations of caecal acetate, propionate, butyrate, valerate, and 2–methylbutyric acid in LL broilers were significantly lower than those in the LL group ($p < 0.05$; Table 5). In LL broilers, butyrate, isobutyric acid, 3–methylbutyric acid, and 2–methylbutyric acid concentrations were elevated in comparison to the ML

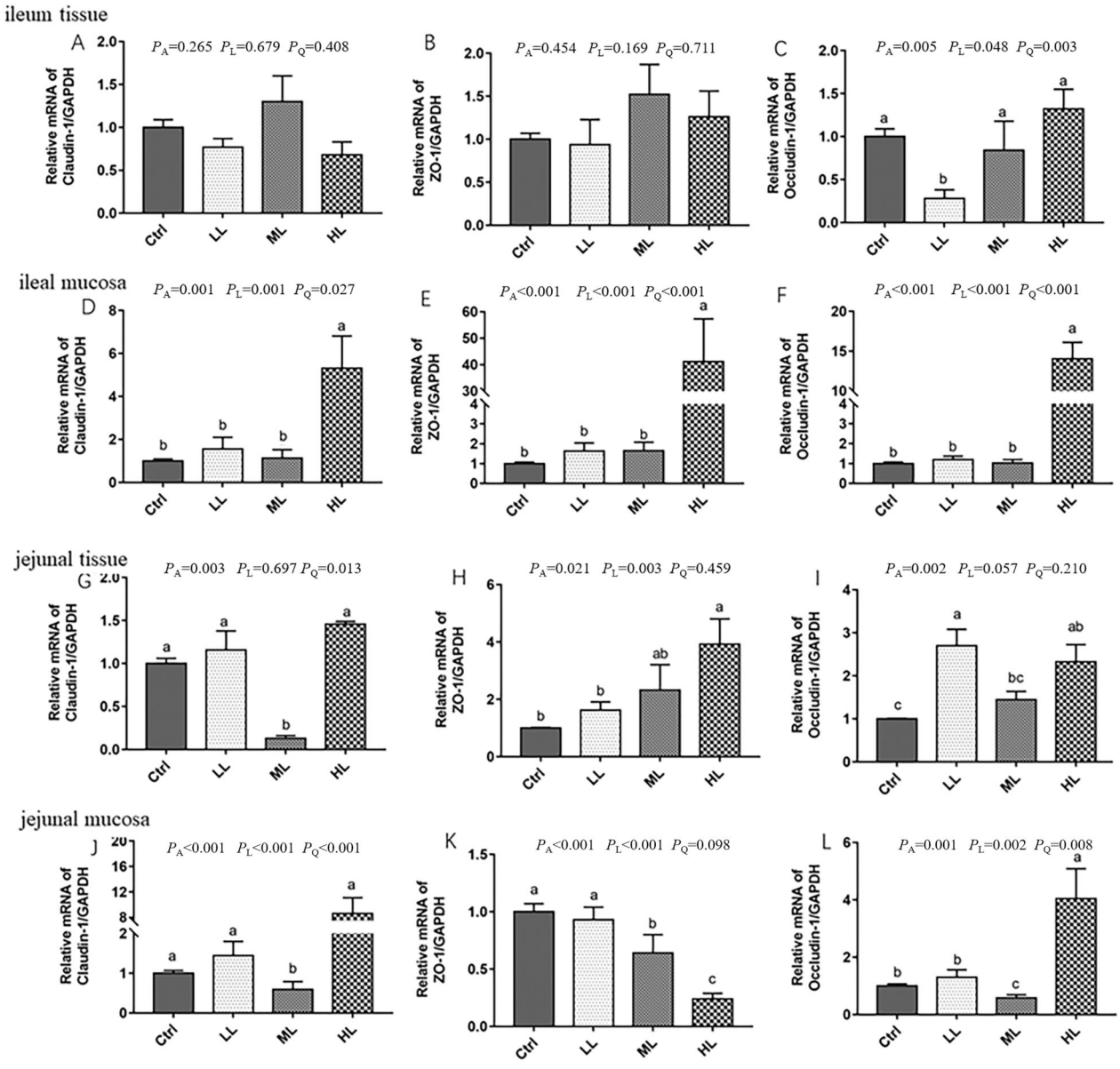

Figure 2. Effects of different doses of *B. subtilis* peptides on mRNA levels of tight junction proteins in intestinal tissues and mucosa. The mRNA levels of tight junction proteins (Claudin-1, ZO-1, occludin-1) in intestinal tissues and mucosa were measured in ileum tissues (A-C), ileal mucosa (D-F), jejunal tissues (G-I) or jejunal mucosa(J-L).
[a]$P < 0.05$ compared with the Ctrl, ML or HL group; [b]$P < 0.05$ compared with the Ctrl, LL, ML or HL group; [c]$P < 0.05$ compared with the Ctrl, low dose lipopeptide group (LL), medium dose lipopeptide group (ML) or high dose lipopeptide group (HL).

group ($p < 0.05$; Table 5). The analysis of linear and quadratic data revealed that acetic acid demonstrated solely a quadratic relationship ($p < 0.05$), whereas 2–methylbutyric acid presented a linear increasing tendency with dosage alone ($p < 0.05$). All other indicators exhibited both linear and quadratic associations ($p < 0.05$). These shifts suggested lipopeptide supplementation enhances SCFA production, potentially acidifying the caecal environment to inhibit pathogens and support intestinal health.

B. subtilis peptide treatments upregulated the mRNA level of intestinal tight junction protein gene to maintain normal intestinal permeability

The role of tight junctions was to limit the movement of substances through the space adjacent to the cell. Claudins were the main components of tight junction complexes that regulated epithelial permeability. ZO-1 was considered to regulate the actin cytoskeleton in epithelial cells, and occludin-1 contributed to tight junction stability and barrier function. The mRNA

levels of Claudin-1, ZO-1, and Occludin-1 were quantified in the ileum, jejunum, and intestinal mucosa (Figure 2A–L). In comparison to the Ctrl group, the mRNA expression levels of Claudin-1 and ZO-1 in the ileum did not change following peptide treatments (Figures 2A and B), and no significant linear or quadratic trends were observed. In the ileum, Occludin-1 expression was reduced in the LL group relative to the Ctrl group (Figure 2C), with both significant linear and quadratic trends noted ($p < 0.05$). The administration of *B. subtilis* peptide at the high dose markedly increased the expression of all three target genes in the ileal mucosa (Figures 2D–F, $p < 0.05$), exhibiting substantial linear and quadratic trends ($p < 0.05$). In contrast, the ML treatment in the jejunum reduced Claudin-1 mRNA expression in both tissue and mucosa (Figures 2G and J, $p < 0.05$), exhibiting a significant quadratic trend, but a consistent linear trend was absent. Furthermore, the HL peptide markedly enhanced the expression of ZO-1 and Occludin-1 in jejunal regions. Notably, ZO-1 expression was elevated in HL jejunal tissue but diminished in HL jejunal mucosa relative to the Ctrl group (Figures 2H and K, $p < 0.05$). A notable linear trend was identified ($p < 0.05$), however no quadratic trend was seen. Occludin-1 expression in jejunal tissue diminished in the LL group and augmented in the HL group compared to the Ctrl group. No linear or quadratic trends were observed (Figure 2L). In the jejunal mucosa, Occluding-1 expression was significantly increased in both the LL and HL groups relative to the Ctrl group, exhibiting notable linear and quadratic trends ($p < 0.05$). These results suggested that a high dose of *B. subtilis* peptide treatment helped maintain the normal permeability of the intestinal barrier and protect intestinal health.

B. subtilis peptide treatments did not affect α-diversity of caecal contents in broilers

The α-diversity in broiler caecal contents could reflect species similarity. Dietary supplementation with different doses of *B. subtilis* peptides had no effect on OTU levels (including Sob, Ace, and Chao levels) in broiler caecal contents compared to Ctrl (Figures 3A–C), with no significant linear or quadratic trends detected. The Venn diagram results indicated that there were 633 shared OUT numbers across all treatment groups, while the number of discrepancies in the Ctrl, LL, ML, and HL groups was 6, 14, 24, and 29, respectively (Figure 3D). Furthermore, the PCoA results at the OTU level corroborated that the quantity of OTUs among treatment groups was comparable, with the disparity being quite minor (Figure 3E). These results showed that different doses of *B. subtilis* peptides had little effect on the α-diversity of caecal contents of broilers, and the species similarity was high.

B. subtilis peptide treatments affected β-diversity of caecal contents on phylum and genus level in broilers

The β-diversity shifted significantly in broiler caecal microbiota. At the phylum level, *Bacteroidetes* dominated in both the Ctrl group and *B. subtilis* peptide-treated groups. Compared with the Ctrl group, the *Firmicutes* abundance was not changed in the LL, ML or HL group (Figures 4A and B). A significant linear trend was detected ($p < 0.05$), but no quadratic trend was observed. The LL peptide dose significantly increased *Cyanobacteria* abundance compared to Ctrl group ($p < 0.05$; Figures 4C). A quadratic trend was observed ($p < 0.05$), but no significant linear trend was detected ($p > 0.05$). At the genus level, *Bacteroides*, *Lactobacillus*, *Ruminococcus*, and *Firmicutes* were predominant in the Ctrl group and treated groups. All *B. subtilis* peptide treatments reduced caecal *Bacteroides* versus the Ctrl group (Figures 4D and E, $p < 0.05$), and both significant linear and quadratic trends were observed ($p < 0.05$). Furthermore, the HL peptide dose decreased *Lactobacillus* abundance ($p < 0.05$; Figures 4F). A significant linear trend was identified ($p < 0.05$), but no quadratic trend was observed . The results suggested that several *B. subtilis* peptide treatments could alter the microbiota in the caecal contents of broilers.

Analysis of community composition at genus level in caecal contents of broilers changed by different B. subtilis peptide treatments

Biocommunity data could reflect the differences in various biological populations clustered in the same area or environment at the same time. The top five bacterial genera in the caecal contents of broilers across all treatment groups were *Bacteroides, Alistipes, Lactobacillus, Christensenellaceae_R-7_group*, *Ruminococcus_torques_group* and *unclassified_f_Lachnospiraceae* (Figure 5A). At the genus level, broilers administered high, medium, or low doses of *B. subtilis* peptide (HL, ML, and LL groups) exhibited a significantly decreased abundance of *Bacteroides* compared to the Ctrl group (Figure 5B, $p < 0.05$), with both notable linear and quadratic trends ($p < 0.05$). The prevalence of *Alistipes* was significantly

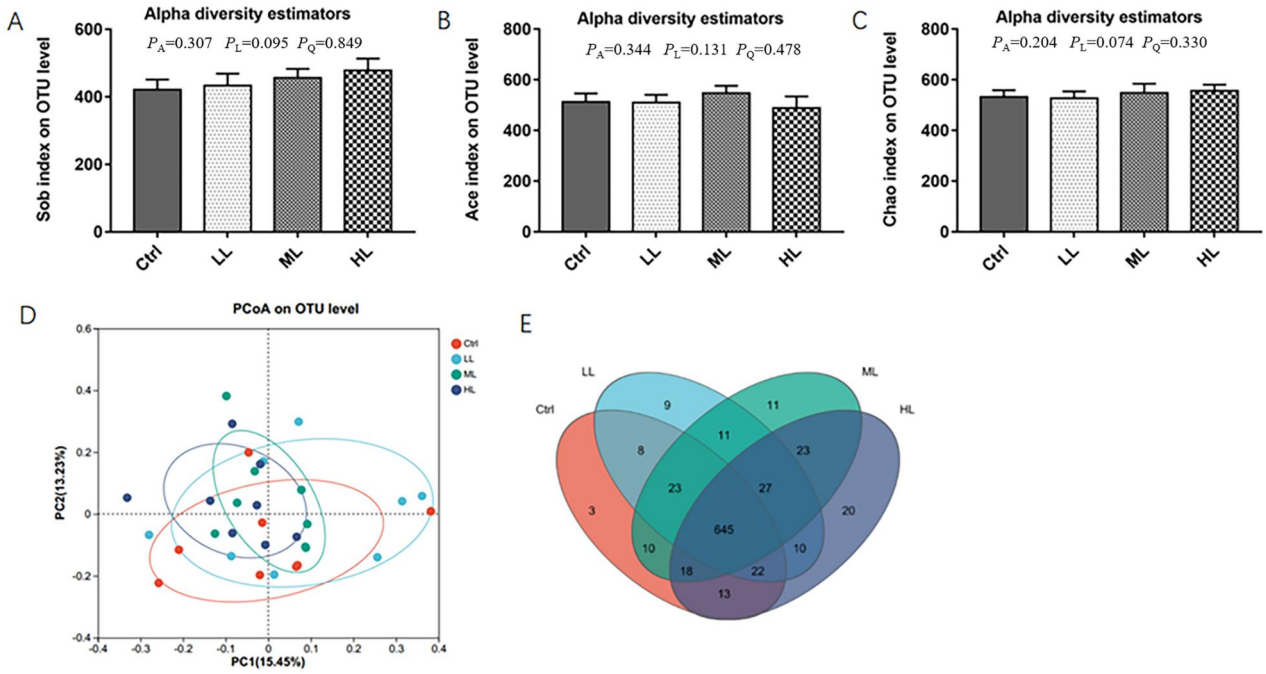

Figure 3. Effects of different treatments on α-diversity of caecal contents in broilers. Three key indicators of α-diversity (Sob, Ace and chao) were tested to assess the differences between different treatment groups (A-C). A visual comparison of data similarities or differences at the out level in caecal contents of broilers from different treatment groups and the number of OTUs shared or unique among the different treatment groups was also analysed (D-E).

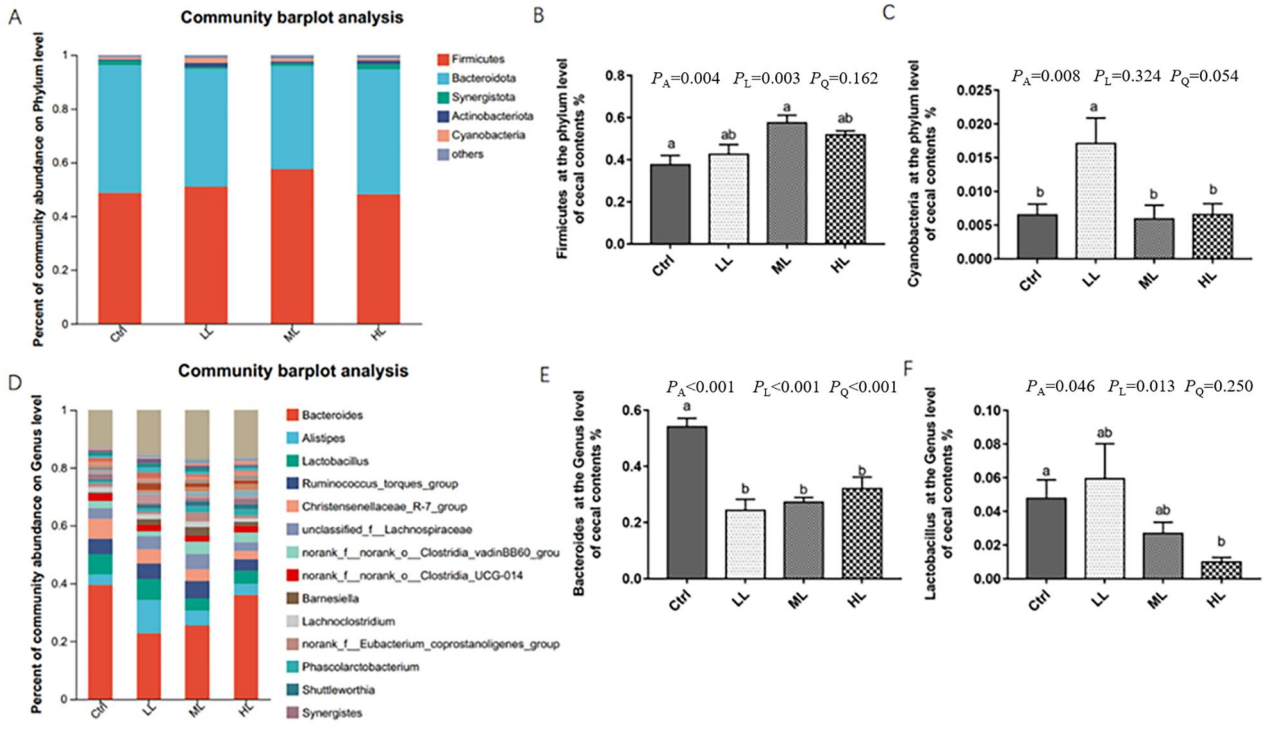

Figure 4. β-diversity and species similarity of caecal contents in broilers. The β-diversity and species similarity of the caecal content microbiota of broilers were analysed. Histograms A-C represent the differences and similarities of β-diversity at the phylum level, and histograms D-F represent the differences and similarities of β-diversity at the genus level. $^{a}P < 0.05$ compared with the Ctrl, low dose lipopeptide group (LL), medium dose lipopeptide group (ML) or high dose lipopeptide group (HL); $^{b}P < 0.05$ compared with the Ctrl, LL or ML group.

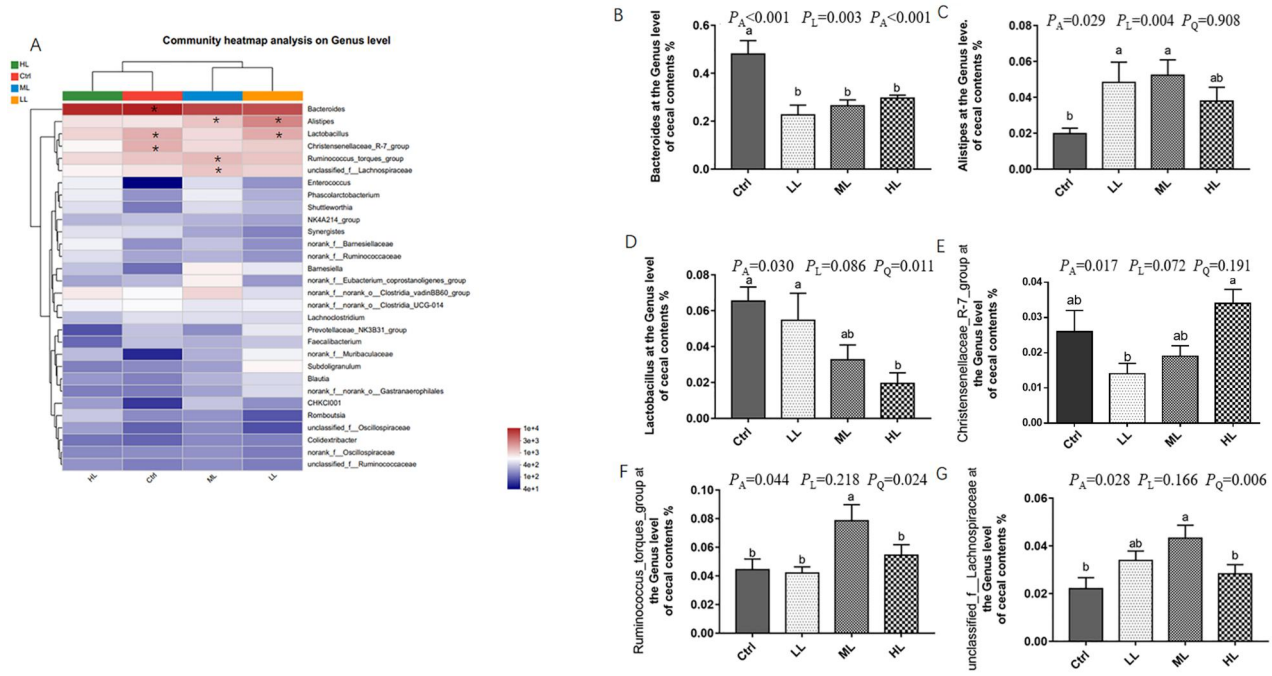

Figure 5. Analysis of community composition at the genus level in caecal contents of broilers. The similarities and differences of the genus-level community composition (top 30 species) in the caecal contents of broilers from different treatment groups were analysed (a). Histograms B-G represent six relatively significantly different flora at the genus level, respectively. [a]$P < 0.05$ compared with the Ctrl, low dose lipopeptide group (LL), medium dose lipopeptide group (ML) or high dose lipopeptide group (HL); [b]$P < 0.05$ compared with the Ctrl, LL or ML group.

increased in the LL and ML groups ($p < 0.05$, Figure 5C), exhibiting a notable linear trend ($p < 0.05$) but without a quadratic trend. The prevalence of *Lactobacillus* was markedly diminished in all treatment groups (HL, ML, LL) relative to the Ctrl group (Figure 5D, $p < 0.05$), exhibiting a substantial quadratic trend ($p < 0.05$) and an absence of linear trend. A notable decrease in the prevalence of *Christensenellaceae_R-7_group* was recorded ($p < 0.05$, Figure 5E), however no significant linear or quadratic trends were seen. Moreover, the ML treatment augmented the prevalence of *Ruminococcus_torques_group* and *unclassified_f_Lachnospiraceae* ($p < 0.05$, Figures 5F and G), exhibiting a strong quadratic trend ($p < 0.05$) without a linear trend. These results revealed that genus-level caecal microbiota composition varied significantly with *B. subtilis* peptide dosage in broilers.

Discussion

This research demonstrated that dietary supplementation with low-dose *B. subtilis* peptide in broilers enhanced the ADFI from 1 to 42 days of age, suggesting a prolonged impact of *B. subtilis* peptide on feed consumption, with effects becoming more pronounced over time. In fact, low levels of *B. subtilis* peptide also tended to increase the ADFI of broilers during the brooding and growing stages, but their effect was not as significant as that of the entire long experimental period. In view of this situation, the addition level of *B. subtilis* peptides could be appropriately reduced to prolong the experimental period of broilers so as to enhance the effect of *B. subtilis* peptides on broilers.

Research on the growth-promoting effects of *B. subtilis* in broilers had been extensively documented (Molnár et al. 2011; Gao et al. 2017). A study suggested that supplementation at levels of 1×10^8 or 10^9 CFU/kg of *B. subtilis* could enhance broiler production performance, potentially attributable to the secretion of nutrients and extracellular digestive enzymes by the bacterium (Wang et al. 2023). In recent years, some research had increasingly focused on the impact of *B. subtilis* on broilers under stress conditions, such as high stocking density or infection with *Eimeria maxima*. One finding indicated that under high stocking density (20 broilers/m^2), dietary supplementation with *B. subtilis* (500 mg/kg feed) reversed the reduction in ADG and the increase in feed conversion ratio observed in 0– to 35–day-old broilers (Elbaz et al. 2024). Furthermore, supplementation with *B. subtilis* 747 (1.5×10^5 CFU/g feed) in broilers infected with

Eimeria maxima significantly increased the total weight gain of 1– to 28–day-old broilers (Park et al. 2020). A study indicated that nutritional supplementation of 300 mg/kg *B. subtilis* in heat-stressed broilers did not significantly impair growth performance, nor did it successfully mitigate the growth inhibition caused by heat stress (Wang et al. 2024b). A study revealed that high-dose probiotics ($\geq 10^9$ CFU/kg) adversely affected the growth performance of broilers. Specifically, the high-dose group P3 (10^{10} CFU/kg) showed a significant reduction in body weight, with a final weight of 2,167 g at 42 days compared to 2,293 g in the low-dose group P1 (10^8 CFU/kg). Feed conversion ratio was also impaired in the P3 group, registering 1.92, which was significantly higher than the 1.80 in P1 and comparable to the 1.89 in the control group. Furthermore, nutrient digestibility remained unimproved in the high-dose groups (P2 and P3), with no significant enhancement in the digestion of dry matter, organic matter, ash, or ether extract. In fact, digestibility values for most nutrients were lower in these groups than in the P1 group (Mountzouris et al. 2010). These findings were consistent with our research results, which indicated that high-dose antimicrobial peptides did not favour increasing the ADG of broilers throughout the entire trial period. The reason for this might be that when the probiotic inclusion level was excessively high, the proliferation of large numbers of probiotic bacteria consumed nutrients that could otherwise be utilised for broiler growth ('host-microbe nutrient competition'), resulting in reduced nutrient digestibility. This ultimately manifested as decreased weight gain and impaired feed conversion efficiency.

Evidence indicated that *B. subtilis* enhanced broiler immunity, thereby promoting growth (Lutfullina et al. 2020; Yu et al. 2021). Whether the *B.* subtilis peptides in this study had similar efficacy remains to be confirmed. We tested the effects of *B. subtilis* on disease resistance in broilers from both antioxidant and immunological aspects. The results of immune performance showed that adding different levels of *B. subtilis* peptide to the broiler diet reduced the IgM content in the serum of 42–day-old broilers, which was contrary to most studies (Mohamed et al. 2022; Ogbuewu et al. 2022). In addition, other immune indicators, such as IgG, also revealed that the addition of antimicrobial peptides had a tendency to reduce IgG in the serum of broilers administered 200 mg/kg of *B. subtilis* peptides. SIgA index showed that the addition of medium and low levels of antimicrobial peptides down-regulated serum SIgA in broilers. The reason may be due to the high breeding density of broilers, or a series of inflammatory reactions caused by heat stress, which affect the immune performance of broilers (Li et al. 2019; Zaglool et al. 2019). The results of antioxidant indicators revealed that the medium and low doses of *B. subtilis* peptides had an upward trend in T-AOC levels in the serum of broilers, indicating that the antioxidant capacity of broilers was effectively improved.

There were a large number of microorganisms in the digestive tract of poultry, which might ferment carbohydrates in the intestine and produce SCFAs (Moran and Bedford 2022; Li et al. 2023). It could provide energy, promote the proliferation of intestinal epithelial cells and stimulate intestinal mucosal reaction, and thus protect intestinal health. As the main product of intestinal bacterial fermentation, SCFAs could regulate the differentiation of intestinal microorganisms into host intestinal immune cells, maintain the secretion function of goblet cells and the integrity of intestinal epithelial cells (Wellington et al. 2020). SCFAs could also reduce pro-inflammatory factors and protect mucosal immunity. Different doses of *B. subtilis* peptide increased the contents of acetic acid, propionic acid, butyric acid, valeric acid and 2–methylbutyric acid in broiler diets, suggesting that the addition of *B. subtilis* peptide in broiler diets helps to promote energy metabolism and prevent inflammatory diseases in broilers.

Wang et al. (2023) supplemented 100 mg/kg of AMP Gal-13, which significantly increased body weight and ADG in 7–day-old Ross 308 broilers. Supplementation of 100 mg/kg or 200 mg/kg of AMP Gal-13 increased glutathione peroxidase activity in liver and serum. Dietary supplementation with 6 mg/kg antimicrobial peptides (Zhu et al. 2022) enhanced inflammatory gene profiles, immune organ indices, villus growth, and intestinal integrity in broilers. Our results revealed that dietary B. subtilis peptide (100, 200 or 300 mg/kg) enhanced immune function and intestinal barrier integrity in broilers, with the 100 mg/kg dose optimising growth performance. Future studies should assess lower B. subtilis peptide doses to determine optimum dietary levels. In addition, we might determine the immune performance index in the liver of broilers to comprehensively evaluate the effect of B. subtilis peptide on the immune performance of broilers.

Acetate modulated bifidobacteria-pathogen competition to promote gut microbial balance (Hertli and Zimmermann 2022). Notably, SCFAs-induced changes in broiler intestinal microbiota were associated with

reduced inflammatory responses, as SCFAs can reduce LPS levels - the main component of gram-negative bacteria - the stimulation of inflammatory responses (Liu et al. 2021; Yue et al. 2022). SCFAs modulated gut barrier function through enhanced mucin and antimicrobial peptide secretion (Ali et al. 2022). Our results confirmed that *B. subtilis* peptide significantly elevated SCFA levels in the caecum of broilers, potentially acidifying the environment to inhibit pathogens and maintain intestinal health.

Intestinal flora has always been considered to be the key to affecting intestinal health, and gut microbiota modulates intestinal architecture, immune function, nutrient metabolism, and host health (Zhou et al. 2020). Having established that *B. subtilis* peptide positively influences SCFA levels, we further investigated whether alterations in SCFA directly affect the diversity of gut flora. The *Firmicutes* and *Bacteroidetes* constituted >90% of caecal microbiota. Subsequent β-diversity analysis of caecal contents of broilers revealed structural differences at phylum and genus levels. At the phylum level, broilers in the low-dose *B. subtilis* peptide group up-regulated the proportion of *Cyanobacteria* in caecal contents of broilers. However, a previous report showed that the addition of *Bacillus* licheniformis to broiler diets reduced the proportion of *Cyanobacteria* in caecal contents (Gao et al. 2017). A study reported that in broilers infected with *E. acervulina oocysts*, the relative abundance of *Cyanobacteria* at the phylum level significantly increased in duodenal chyme. Whereas, oral gavage of *B. subtilis* substantially reduced the *Cyanobacteria* abundance (Wickramasuriya et al. 2023). The difference in our results might be due to the inappropriate proportion of low-dose *B. subtilis* peptide, or broiler breeding may be due to heat stress or density. At the genus level, each *B. subtilis* peptide treatment group increased the abundance of a distinct bacterial branch. This shift may contribute to the alleviation of inflammation in broilers and help maintain normal intestinal barrier function (Parker et al. 2020). Ren et al. found that the addition of 250 and 500 mg/kg *B. subtilis* to the basal diet did not change the abundance of *Bacteroides* in the caecal contents of broilers (Ren et al. 2023). One study demonstrated that dietary supplementation with fermented *B. subtilis* product at either 1 g/kg or 3 g/kg did not affect the relative abundance of *Bacteroides* at the genus level in caecal content of lipopolysaccharide-induced broilers, compared to either the Ctrl group or the LPS-induced group without supplementation (Chen and Yu 2021). Our findings indicated that dietary supplementation with varying doses of *B. subtilis*-derived antimicrobial peptides consistently reduced the relative abundance of *Bacteroides* at the genus level in broiler caecal content. This suppression may be ascribed to either (1) direct inhibition of *Bacteroides* by antimicrobial peptides released by *B. subtilis* or (2) modification of the intestinal microenvironment (e.g. pH reduction) *via* metabolic byproducts of *B. subtilis*, thereby establishing adverse conditions for *Bacteroides* proliferation.

Conclusion

In summary, dietary supplementation with *B. subtilis* peptide enhanced the ADFI, promoted the production of SCFAs in caecal content, and upregulated the mRNA expression of intestinal tight junction proteins in broilers. It also modulated the caecal microbiota composition at both the phylum and genus levels. Under the experimental conditions of this study, the recommended dosage of *B. subtilis* peptide was 100 mg/kg.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Availability of data

All datasets generated and analysed are included in this manuscript.

Data availability statement

The datasets generated during this study were available from the corresponding author on reasonable request. The 16S rDNA gene sequence data had been deposited in the NCBI Bioproject database (URL: https://www.ncbi.nlm.nih.gov/bioproject/) (accessed on 13 August 2025), registration number PRJNA1305354.

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